THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 42, Issue of October 19, pp. 38494–38501, 2001
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Translational Repression of the *Escherichia coli* α Operon mRNA

IMPORTANCE OF AN mRNA CONFORMATIONAL SWITCH AND A TERNARY ENTRAPMENT COMPLEX*

Received for publication, July 23, 2001
Published, JBC Papers in Press, August 14, 2001, DOI 10.1074/jbc.M106934200

Paula J. Schlax‡, K. Asish Xavier§, Thomas C. Gluick¶, and David E. Draper‖

From the Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Ribosomal protein S4 represses synthesis of the four ribosomal proteins (including itself) in the *Escherichia coli* α operon by binding to a nested pseudoknot structure that spans the ribosome binding site. A model for the repression mechanism previously proposed two unusual features: (i) the mRNA switches between conformations that are "active" or "inactive" in translation, with S4 as an allosteric effector of the inactive form, and (ii) S4 holds the 30 S subunit in an unproductive complex on the mRNA ("entrapment"), in contrast to direct competition between repressor and ribosome binding ("displacement"). These two key points have been experimentally tested. First, it is found that the mRNA pseudoknot exists in an equilibrium between two conformers with different electrophoretic mobilities. S4 selectively binds to one form of the RNA, as predicted for an allosteric effector; binding of ribosomal 30 S subunits is nearly equal in the two forms. Second, we have used S4 labeled at a unique cysteine with either of two fluorophores to characterize its interactions with mRNA and 30 S subunits. Equilibrium experiments detect the formation of a specific ternary complex of S4, mRNA pseudoknot, and 30 S subunits. The existence of this ternary complex is unambiguous evidence for translational repression of the α operon by an entrapment mechanism.

Synthesis of nearly all ribosomal proteins in *Escherichia coli* is regulated by an autogenous feedback mechanism: translation of each ribosomal protein operon is repressed by one of its encoded r-proteins, which invariably also has a strong affinity for rRNA (1). In the case of the α operon, the translational repressor, S4, recognizes a nested pseudoknot structure (Fig. 1*A*) surrounding the ribosome binding site of the first gene of the operon (S13) (2–4). An unusual repression mechanism has been proposed for this operon (5, 6) based on (i) a conformational switch between two folded states of the mRNA pseudoknot structure, and (ii) an inactive "entrapment" complex formed among S4, mRNA, and initiating ribosomes (Fig. 1*B*). The present work presents evidence in support of these two key aspects of the proposed mechanism and shows that the RNA is an active participant in repression.

Translation initiation in bacteria has two essential steps (for a review, see Ref. 7). First, $tRNA_f^{Met}$, mRNA, and the 30 S subunit form a preinitiation complex stabilized by base pair formation between the mRNA Shine-Dalgarno sequence and its complement on the 3′-end of the 16 S rRNA (8). This complex is in rapid equilibrium with its components. The second step is a slow, essentially irreversible conformational change that allows the initiation codon to base pair to the $tRNA_f^{Met}$ anticodon and form a stable initiation complex. Translation rates are frequently regulated at the first step, where proteins that stabilize RNA structure encompassing the Shine-Dalgarno sequence may prevent binding of initiating ribosomes and reduce translational efficiency. This kind of competition between a repressor protein and initiating ribosomes has been termed a "displacement" repression mechanism (9).

An entirely different regulation mechanism has been proposed for regulation of α operon ribosomal proteins (Fig. 1*B*). Kinetic studies of initiation complex formation suggested that the mRNA adopted two conformations, both capable of binding 30 S subunits, but only the "active" conformation supported formation of the ternary initiation complex (10). In further studies of S4 binding kinetics, it appeared that the repressor bound only the "inactive" conformation and trapped 30 S subunits in a dead-end complex unable to bind $tRNA_f^{Met}$ (5). This repression strategy has two novel aspects. First, S4 functions as an allosteric effector in that it is proposed to shift an equilibrium between two RNA conformational states. Second, S4 acts at the second, irreversible initiation step to stabilize an inactive 30 S subunit·mRNA complex, a mechanism termed *entrapment* (9).

Experiments strongly supporting both of these unusual repression strategies, an mRNA-based allosteric switch and formation of an S4·mRNA·ribosome entrapment complex, are presented in this work. We first show that two electrophoretically distinguishable mRNA conformers have properties expected for the postulated active and inactive mRNA states. We then use S4 modified with a fluorescent tag to observe formation of the postulated entrapment complex.

MATERIALS AND METHODS

Plasmids for RNA Transcription—Plasmids for transcription of an RNA fragment corresponding to nucleotides 16–127 of the transcript from the α operon promoter, called 127 RNA, and for transcription of L11-Cys76 mRNA have been described (11, 12). A *Pvu*II-*Hin*dIII DNA restriction fragment containing a T7 RNA polymerase promoter sequence followed by α operon sequence −16 to +139 was prepared from a previously described pT7-1 derivative (4) and inserted into pUC19 cut with *Hin*dIII and *Sma*I. This created an in-frame fusion of the first 139 nucleotides of α mRNA with the pUC19 *lacZ* fragment which could be transcribed from a T7 promoter. An *Sph*I site was used to linearize the

This is an open access article under the CC BY license.

* This work was supported by National Institutes of Health Grant GM56968 and a National Research Service Award postdoctoral fellowship (to P. J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Chemistry, Bates College, Lewiston, ME 04240.

§ Present address: Message Pharmaceuticals, Inc., 30 Spring Mill Dr., Malvern, PA 19382.

¶ Present address: Dept. of Chemistry and Biochemistry, University of Texas at Arlington, Arlington, TX 76019.

‖ To whom correspondence should be addressed: Dept. of Chemistry, The Johns Hopkins University, 3400 North Charles St., Baltimore MD 21218. Tel.: 410-516-7448; Fax: 410-516-8420; E-mail: draper@jhunix.hcf.jhu.edu.

This paper is available on line at http://www.jbc.org

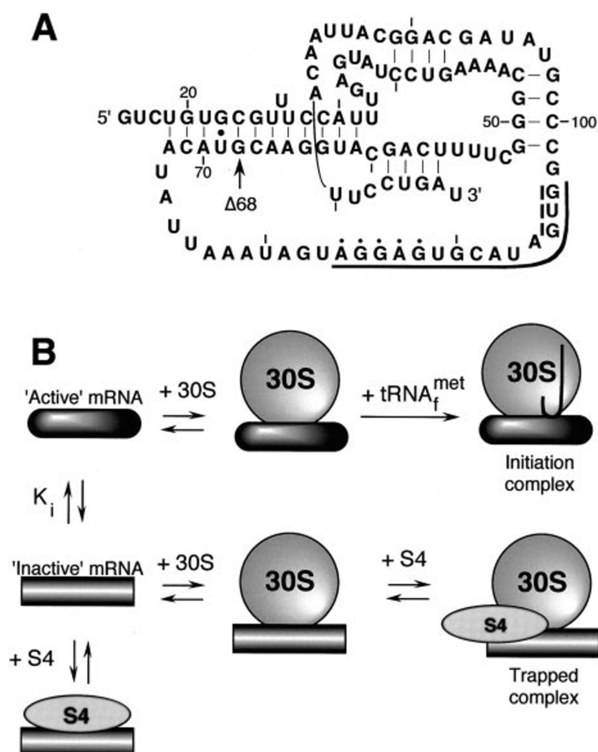

FIG. 1. **Translational repression in the α operon.** *Panel A*, nested pseudoknot structure surrounding the ribosome binding site of the first gene of the operon. *Dots* indicate the Shine-Dalgarno sequence, the *dashed line* is the GUG initiation codon, the *underline* is the complementarity of a 14-nucleotide anti-Shine-Dalgarno DNA, and the *arrow* is the 5′-end of Δ68. *Tic marks* are located every 10 nucleotides, and *numbering* is from the first base of the α operon promoter (24). *Panel B*, proposed model for translational repression by S4 protein (5). For an explanation, see the "Introduction."

plasmid to transcribe RNA for 30 S subunit binding studies (α RNA), and an *Ssp*I site was used to transcribe RNA for *in vitro* translation. Site-directed mutagenesis Cys[67] → Ala then created a unique *Bsr*GI site within the α mRNA pseudoknot sequence in this plasmid. Synthetic DNA fragments inserted between the *Bsr*GI site and the downstream *Sph*I site created a derivative with the Shine-Dalgarno sequence (A84-G88) changed to its Watson-Crick complement. A DNA fragment inserted between *Bsr*GI and an upstream *Kpn*I site created a deletion in which transcription starts with a 12-nucleotide sequence (GGGC-CCAATCTT) followed by G68. Transcripts from these plasmids were termed ASD RNA and Δ68 RNA, respectively.

Ribosome Preparation—Ribosomes were purified approximately as described (10) from MRE600 cells grown to an absorbance of 1 A_{600} in Luria broth. Frozen cells were thawed on ice, resuspended in buffer A (20 mM Tris-Cl (pH 7.5 at 4 °C), 100 mM NH_4Cl, 10.5 mM $Mg(OAc)_2$, 0.5 mM EDTA, 3 mM 2-mercaptoethanol; 2 ml/g cells), and lysed by passage through a prechilled French press (Amicon; 12,000 p.s.i.) one or two times. Lysed cells were centrifuged immediately at 30,000 × *g* for 30 min, and the supernatant was retained. The fraction precipitating between 35 and 70% ammonium sulfate (first addition, 19.6 g of ammonium sulfate/100 ml of supernatant; second addition, 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 Ti55.2 rotor) for 140 min, and the pellet material was resuspended overnight at 0 °C in buffer A. Ribosomes were salt washed once by centrifugation through an equal volume of buffer A plus 15% (w/v) sucrose and 500 mM NH_4Cl; resuspended ribosomes (buffer A or buffer A with 1.1 mM $Mg(OAc)_2$) were stored frozen at −70 °C. 30 S subunits were prepared by sucrose gradient sedimentation of ribosomes in buffer A with 1.1 mM $Mg(OAc)_2$, as described (10).

RNA Transcription—Linear plasmid DNA was transcribed using procedures outlined by Gurevich (13) or described previously (14). Radiolabeled RNA used in nitrocellulose filter binding assays was purified using Nensorb reverse phase columns (14). RNA used in gel mobility experiments was excised from denaturing polyacrylamide gels and extracted by either electroelution (Schleicher & Schuell Elutrap) or soaking crushed gel in buffer.

α mRNA fragments and their derivatives were renatured for 8 min at 65 °C in buffer containing 350 mM KCl, 10 mM MOPS[1] (pH 7.0) followed by 22 min at 42 °C in the same buffer with or without 40 mM $MgCl_2$ (11). RNA encoding L11-C76 was renatured in buffer A at room temperature, as the RNA precipitated when renaturation conditions for α mRNA were used. After renaturation, RNA was placed on ice, and buffer and salt concentrations were adjusted as needed for binding or translation assays.

Purification of S4 Protein—*E. coli* ribosomal protein S4 was purified from a T7 overexpression system by a described protocol with slight modifications (15). Briefly, cells were grown in 1 liter of LB containing 100 μg/ml carbenicillin, induced at an A_{600} of ~0.6 with 1 mM isopropyl β-D-thiogalactopyranoside (final concentration), and harvested after another 3 h of growth. Cells were lysed twice using a French press, and the final sodium chloride concentration was adjusted to 0.7 M. The cell debris was removed by centrifugation at 5,000 rpm, and the supernatant was dialyzed against 2 liters of 20 mM potassium phosphate, 6 M urea, and 1 mM dithiothreitol (pH 5.6). Protease inhibitors phenylmethylsulfonyl fluoride and benzamidine were added at this stage to a final concentration of 0.1 mM. The dialyzed material was centrifuged at 10,000 rpm to remove aggregated material and then filtered through a 0.22-μm filter (Millipore). This material was loaded on a Bio-Gel TSK-SP-5-PW column (Bio-Rad). S4 eluted at about 450 mM potassium chloride and was estimated to be >95% pure by Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis.

Labeling and Characterization of S4 Protein—Pyrene and IAEDANS were used for labeling the single cysteine of S4 using maleimide chemistry (Molecular Probes). These dyes were chosen as the two fluorophores for studying translational repression in the S4 system because of their relatively long excited lifetimes. Labeling was done in the presence of 6 M urea at pH 7.5 to avoid labeling residues other than the cysteine. The appropriate dye in dimethyl formamide was added slowly to the S4 solution with gentle stirring. The dye to protein ratio was 10:1, and the final concentration of dimethyl formamide was kept lower than 1% by volume. For labeling with pyrene the reactants were incubated at room temperature for 3 h, whereas with IAEDANS the incubation was for 90 min at 37 °C. Both reactions were quenched by adding dithiothreitol to a final concentration of 1 mM and letting the reaction proceed at room temperature for 30 min.

The labeled material was dialyzed extensively, any precipitated material was discarded, and the labeled protein was purified as described above at pH 7.5. Both pyrene- and IAEDANS-labeled S4 eluted as two distinct peaks in the range of 450–500 mM potassium chloride; both contained labeled protein. For both pyrene-S4 and IAEDANS-S4, fractions with the highest extent of protein labeling were used. The purified protein was refolded by dialysis against 10 mM MOPS, 350 mM KCl, and 1 mM dithiothreitol (pH 7.2) at 4 °C and stored at −70 °C. On an 8 M urea (pH 4.5) polyacrylamide gel, nonlabeled S4 and the two fractions of pyrene- and IAEDANS-labeled S4 ran similarly, indicating that they had the same net charge. The concentration of labeled protein was determined using an absorbance at 280 nm and calculated extinction coefficient (15) after correcting for absorbance of the label. Using dual wavelength analysis, pyrene-S4 and IAEDANS-S4 were ~27 and 60% labeled, respectively. S4 was renatured before an experiment by warming at 37 °C for 30 min, slowly cooling, and then placing it on ice.

Electrophoretic mobility shift assays performed using ^{35}S-labeled 127 RNA at ~8 °C showed that S4, pyrene-S4, and IAEDANS-S4 were about 65, 64, and 33% active, respectively. These fractional activities agreed with experiments using methylene blue-stained gels and were used in calculations of association constants and stoichiometry of interaction(s). Unlabeled S4 and pyrene- and IAEDANS-labeled S4 were subjected to MALDI-TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometry. The respective calculated and observed molecular weights were S4, 23,339 and 23,350; pyrene-S4, 23,635 and 23,645; IAEDANS-S4, 23,646 and 23,660. The results suggest that both label-S4 derivatives are singly labeled. No evidence of a doubly labeled specie was seen in either case.

Nitrocellulose Filter Binding Assays—Nitrocellulose filters from Schleicher & Schuell were wetted and stored in buffer A prior to binding experiments. Filters were maintained at room temperature during the assays. Varying concentrations of 30 S subunits (0–5 μM) were incubated with a fixed amount of renatured RNA in buffer A at 0 °C for equilibrium constant determinations; incubation times from 20 to 90

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid.

min did not affect the final results. Stoichiometric assays were performed using 0.1–0.2 μM unlabeled RNA; titrations for determination of equilibrium constants used trace amounts of RNA. For equilibrium competition assays, fixed concentrations of subunits (50–300 nM) and radiolabeled α mRNA were incubated with varying concentrations (0–10 μM) of unlabeled competitor RNA for 40 min at 0 °C. In all assays, 25–35 μl of each titration point was filtered on a prewashed filter, and the filter was washed immediately with 200 μl of ice-cold buffer A. Filters were dried before quantitation by scintillation counting.

Filter titration data were fit to single site binding isotherms as described by Draper *et al*. (16); errors in the reported binding constants are typically ± 25%. In experiments that competed labeled and unlabeled RNAs for 30 S subunit binding, the concentration of 30 S subunits bound to labeled RNA was considered negligible, and the total subunit concentration therefore is given by

$$[30\ \mathrm{S}]_0 = [30\ \mathrm{S}] + \frac{K_c[30\ \mathrm{S}]}{1 + K_c[30\ \mathrm{S}]}[\mathrm{C}]_0 \qquad \text{(Eq. 1)}$$

where $[30\ \mathrm{S}]_0$ and $[\mathrm{C}]_0$ are the total concentrations of subunits and competitor RNA, respectively, and K_c is the affinity of the competitor RNA for subunits. This equation was solved for [30 S], which was then used to predict the extent of subunit binding to labeled RNA. Kaleidagraph (Synergy Software) was used for least squares fitting of both standard and competition binding curves.

Native Gels of mRNA Conformers—The fast and slow conformers of 127 RNA were resolved by electrophoresis as described (11), using 8% polyacrylamide gels containing 30 mM Tris (pH 7.4), 100 mM potassium acetate, and 2 mM $MgCl_2$; the electrophoresis apparatus was thermostatted at 8 °C with buffer recirculation. For band shift experiments, S4 protein was first renatured at 37 °C for 30 min in 30 mM MOPS (pH 7.0), 350 mM KCl, 2.5 mM dithiothreitol; RNA was renatured as described above (RNA transcription), only using 10 mM $MgCl_2$ in the buffer. After mixing protein and RNA (50 pmol) in 10 μl at 0 °C the final buffer was adjusted to 30 mM MOPS, 350 mM KCl, 10 mM $MgCl_2$, and electrophoresis was carried out. Binding of ^{32}P-labeled 5′-DNA to 127 RNA was studied in a similar way except that 20 mM $MgCl_2$ was present during RNA renaturation. DNA oligomer was added to the RNA at 0 °C, incubated for 10 min, run on a gel, and the bands quantitated on a PhosphorImager (Molecular Dynamics).

In Vitro Translation—Commercial S30 extracts (Promega) were used to translate purified, renatured RNAs. Reactions were run according to the manufacturer's instructions (using [^{35}S]methionine to label the translated proteins) with the exception that 0.25–0.5 mg/ml rifampicin was added to reduce background expression in the system (17). Reactions were a 30-μl total volume with 4 pmol of added RNA and were incubated for 20–60 min. Aliquots were treated immediately with ice-cold acetone to precipitate protein and were centrifuged. The pelleted protein was resuspended in SDS-gel loading buffer and boiled for 5 min. Equal counts of radioactivity were loaded on each lane of standard SDS gels (19% acrylamide). Gels were dried, and the amounts of radioactivity in individual protein bands were quantitated using a PhosphorImager.

Fluorescence Polarization Spectroscopy—An SLM 48000 fluorometer was used in L-format for emission spectroscopy and in T-format with appropriate cut-on filters and polarizers for steady-state polarization measurements. Both dyes were excited at 345 nm with a 2 nm bandpass; 380 nm and 450 nm cut-on filters were used on the emission side for pyrene and IAEDANS, respectively. A manual shutter after the excitation monochromator was used to allow exposure of the samples to light only during the course of a measurement. Loss of fluorescence with time was reduced further by treating the cuvette (Starna Cells) with Gel Slick (J. T. Baker), coating it with 0.3 mg/ml hen egg lysozyme (Sigma), and then rinsing it thoroughly. This procedure decreases the fluorescence loss to below 7% over the typical time span of an experiment. Based on changes in protein tryptophan fluorescence over the duration of an experiment, most of the decrease in fluorescence could be attributed to photobleaching of the fluorophore rather than adsorption of protein to the cuvette. Control experiments showed that the anisotropy was stable to within ± 0.002. All experiments were done in 10 mM MOPS, 350 mM KCl, and 5 mM $MgCl_2$ (pH 7.2) at 15 ± 0.5 °C to "freeze" the conformations of 127 RNA during the course of the experiment. The contents of the cuvette were allowed to equilibrate for 10 min after each addition of titrant, and appropriate correction was made for dilution in the data analysis. The solution was mixed using a microsubmersible magnetic stirrer, and its temperature was maintained using a circulating water bath.

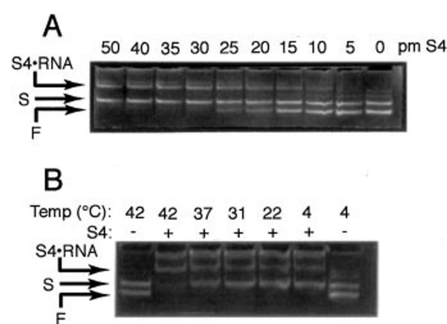

FIG. 2. **S4 binds one conformer of the α mRNA pseudoknot.** Native gel electrophoresis of the α mRNA leader sequence (127 RNA) was carried out as described under "Materials and Methods." The gels resolve two conformers, S (slow) and F (fast) as indicated by *arrows*. In the presence of S4 protein, a slower migrating S4·RNA complex is observed. *Panel A*, increasing amounts of S4 incubated with 50 pM RNA at 0 °C. Densitometer scans of the gel (stained with methylene blue) show a linear decrease in the intensity of band S and corresponding increase in S4·RNA, with about 35 pmol S4 necessary to titrate band S completely. *Panel B*, S4·RNA complexes (50 pmol each) were formed at 0 °C, shifted to the indicated temperature for 10 min, cooled to 5 °C, and run on the gel. Controls without protein were subjected to the same temperature shifts.

RESULTS

S4 Binds One of Two Electrophoretically Distinguishable Forms of the α mRNA Pseudoknot—A fragment of the α operon mRNA leader sequence containing the pseudoknot regulatory structure migrates in gels as two distinct bands under nondenaturing conditions. We have shown previously that these electrophoretically "fast" and "slow" species represent alternative RNA conformations (11). At temperatures above ~30 °C, the two conformers interconvert readily, and the position of the equilibrium is sensitive to Mg^{2+} concentration and pH; at low temperatures, the interconversion is extremely slow, and the conformers are kinetically trapped. RNA samples can be prepared in predominantly fast or slow forms by renaturation in buffer with or without 40 mM Mg^{2+} at 42 °C, respectively, followed by rapid chilling.

Electrophoretic mobility shift assays were used to determine whether the two conformers differ in their affinity for S4 repressor. S4 protein was incubated with renatured 127 RNA (nucleotides 16–127 of the α mRNA transcript; see Fig. 1*A* and "Materials and Methods") at 0 °C, where interconversion of the two RNA conformations is extremely slow, and reactions were run on a nondenaturing gel at cold temperatures. The fast form is titrated stoichiometrically (1:1) by the protein, and the complex appears as a slower moving band (Fig. 2*A*). This shifted band stains with both Coomassie Blue and methylene blue, indicating that it contains both RNA and protein. The slow form is unaffected by high S4 concentrations (up to 10 μM). When protein is incubated with RNA at a temperature that allows rapid equilibration of the two RNA conformers (42 °C), S4 is able to shift all of the RNA into a binary complex (Fig. 2*B*). We conclude that S4 specifically recognizes only one of the two RNA conformations and is therefore able to drive the equilibrium between the conformations entirely to the fast form.

Accessibility of the Shine-Dalgarno Sequence in the Two mRNA Conformers—In the proposed repression model, 30 S subunits bind both mRNA conformations. To see whether ribosomes may bind the slow and fast mRNA forms differently, we first used a gel assay to determine if the Shine-Dalgarno sequence is accessible in each form. As seen in Fig. 3, a ^{32}P-labeled DNA 14-mer that complements the mRNA from the Shine-Dalgarno sequence through the initiation codon (see Fig. 1*A*) appears to bind equally well to both mRNA forms. The calculated affinities are 27 μM^{-1} for fast form RNA and 33 μM^{-1}

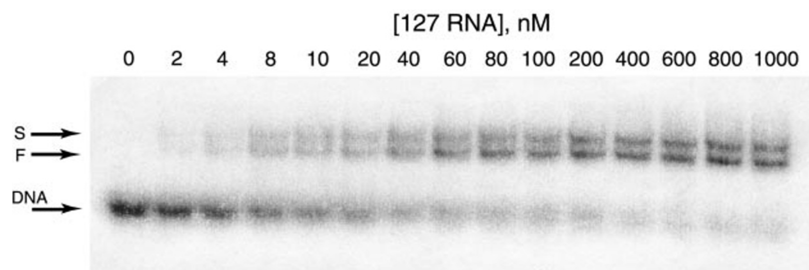

FIG. 3. **An anti-Shine-Dalgarno DNA oligomer binds equally well to the fast and slow forms of 127 RNA.** ^{32}P-Labeled DNA oligomer (14 nucleotides; complementary to the Shine-Dalgarno and initiation codons shown in Fig. 1*A*) was incubated with increasing concentrations of 127 RNA, as indicated in the figure, the complex run on a nondenaturing gel as in Fig. 2, and a PhosphorImager was used to quantitate radioactivity. The two *upper bands* correspond to the slow (*S*) and fast (*F*) conformers of the RNA and appear to bind the DNA with approximately equal affinity.

for slow form RNA. Essentially the same result was obtained with an 11-mer that did not include nucleotides complementary to the initiation codon. Gels with this oligomer showed broader bands, and it was more difficult to resolve the two binding constants, but the average binding was 28 μM^{-1}, and there was no suggestion that the two RNA conformers bound the oligomer differently. That the two oligomers bound the expected position on the mRNA was confirmed by digestion of the DNA-RNA hybrids with RNase H; only two RNA fragments (of the expected sizes) were observed on denaturing gels. We conclude that the Shine-Dalgarno region has nearly the same accessibility for hybridization in the two mRNA conformers.

30 S Subunit Binding to the Two mRNA Conformers—We measured the affinity of the two mRNA conformers for 30 S subunits directly using nitrocellulose filter binding assays at low temperatures where the renatured RNA is trapped predominantly in the fast or slow form. In some titrations, 0.1–0.2 μM unlabeled mRNA was added to each titration point, which increased the amount of 30 S subunits needed to reach a given level of saturation compared with titrations using only trace concentrations of unlabeled RNA. From the resulting shifts in the binding curves, we estimated that the 30 S subunit preparations used in these studies were >90% active in mRNA binding. Fast and slow form mRNAs bind 30 S subunits with nearly the same affinity at 0 °C (Fig. 4*A* and Table I). Binding constants were slightly stronger when measured at 22 °C but still not significantly different between forms (slow form, 26 μM^{-1}; fast form, 36 μM^{-1}).

To find out whether 30 S subunit binding to the mRNA fragments depends on the presence of the Shine-Dalgarno sequence, an "anti-Shine-Dalgarno" RNA was prepared in which the AGGAG sequence was changed to its Watson-Crick complement (ASD RNA). Binding of this RNA to 30 S subunits could not be detected in a filter binding assay; either the complex forms only weakly, or the complex is not efficiently retained on the filter. To distinguish these possibilities, we competed unlabeled ASD RNA and labeled wild type RNA for 30 S subunit binding in the filter assay. The ASD RNA is much less efficient than unlabeled wild type RNA at reducing the extent of labeled α RNA binding (Fig. 4*B*). From these data, we estimate that ASD RNA binds 7-fold more weakly than α RNA, although the data systematically deviate from the calculated curve. Relevant to this discrepancy, calculations of the relative affinity assumed only one binding site for 30 S subunits on ASD RNA; because the binding is so weak, we could not measure the stoichiometry directly. If there is more than one potential subunit binding site on the mRNA (a likely possibility for weak, nonspecific binding), then the calculation will have correspondingly overestimated the binding strength. Calogero *et al*. (8) also found about an order of magnitude difference in 30 S subunit binding affinity between RNAs with and without a Shine-Dalgarno sequence.

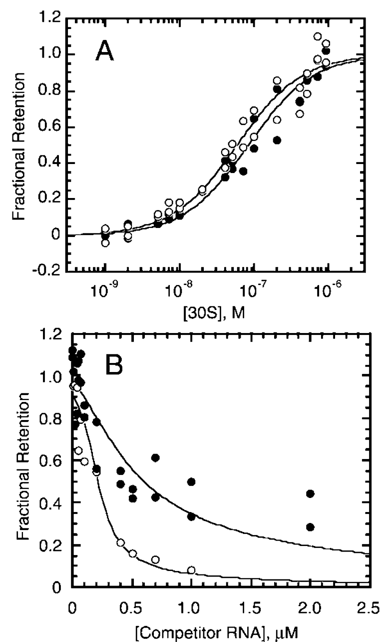

FIG. 4. **Affinities of 30 S subunits for α mRNA and other ribosome binding site sequences.** *Panel A*, comparison of fast and slow α mRNA conformations. α mRNA was renatured in the presence of 40 mM Mg^{2+} (○, ~65% fast form) or the absence of Mg^{2+} (●, >90% slow form). Binding curves are best fits to the data with association constants of 17 μM^{-1} (fast form) or 12 μM^{-1} (slow form). *Panel B*, competition of ^{35}S-labeled α RNA with unlabeled ASD RNA. The competition curves were calculated as described under "Materials and Methods," using the 30 S subunit·α RNA affinity listed in Table I. The best fit gave an ASD RNA affinity of 9.3 μM^{-1} and an α RNA affinity of 71 μM^{-1}; it is not known why the latter affinity is somewhat higher than measured in direct titration experiments.

It is also noteworthy that ASD RNA is resolved into two bands on nondenaturing gels, but both migrate slightly faster than the wild type sequence. Binding of S4 to ASD RNA could not be detected in filter binding assays by either titration of labeled ASD RNA with S4 or competition of unlabeled ASD RNA for labeled α RNA ($K < 10^5$ M^{-1}). Thus mutation of the Shine-Dalgarno region has a major effect on the folding of the pseudoknot structure.

30 S subunit affinities for two other mRNA ribosome binding sites were also measured for comparison with the wild type α mRNA (Table I); both measurements suggest that the α mRNA pseudoknot only weakly restricts ribosome access to the Shine-Dalgarno sequence. One RNA is the α mRNA leader from which an upstream sequence forming the pseudoknot has been deleted (Δ68 RNA; Fig. 1*A*). Δ68 RNA binds less than 2-fold better than the wild type RNA (Table I). There is the possibility of a weak secondary structure forming between the Shine-Dalgarno and downstream sequences (83–90 with 118–125;

TABLE I
30 S subunit binding and translational efficiency of mRNAs

Binding affinities were determined at 0 °C as described under "Materials and Methods"; note that α RNA and its derivatives used for binding studies terminated at position 139 of the α mRNA, whereas *in vitro* translation used longer transcripts. The affinity of ASD RNA is based on competition experiments and is an upper limit based on the assumption of one potential subunit site/RNA. Translational efficiencies are calculated from densitometer scans of SDS-gels using ^{35}S-labeled protein and are reported relative to the translation level of the slow α mRNA conformer run in a parallel reaction and analyzed on the same gel. Each value is the result of an individual experiment; each RNA was seen to give qualitatively similar results in other experiments which were not quantitated. ND, not determined.

RNA	K_{obs}	Relative K_{obs}	Translational efficiency
	μM^{-1}		
α RNA (slow)	12.6 (± 3.0)	1.00	1.00
α RNA (fast)	15.5 (± 4.8)	1.2	0.85, 1.07
Δ68 RNA	23.8 (± 6.0)	1.9	0.55, 0.71
ASD RNA	3.0 (± 2.3)	<0.24	ND
L11-C76 RNA	34 (± 17)	2.7	33

$\Delta G°$ (37 °C) ≈ -4.2 kcal/m as calculated by "Turner rules"), so the restrictive effect of the pseudoknot may be marginally greater than indicated by the small increase in subunit binding affinity. L11-C76 is a ribosomal protein fragment expressed from a pET11 protein expression plasmid; its mRNA has a six-nucleotide anti-Shine-Dalgarno sequence (AAGGAG) in a region with no potential for stable secondary structure. This mRNA binds 30 S subunits about 2.7-fold more strongly than the α mRNA fragment, under similar conditions (Table I). L11-C76 mRNA is translated at very high levels (see below), and its affinity for 30 S subunits is probably near the upper end of the range of natural mRNAs. This measurement also suggests that the pseudoknot has not drastically restricted ribosome access to the Shine-Dalgarno region.

Effect of Pseudoknot Structure on Translational Efficiency of α Operon mRNA—To see if the pseudoknot structure modulates the overall translational efficiency of α mRNA, equimolar amounts of different purified and renatured RNA transcripts were added to an *in vitro* translation system. The concentrations of mRNA (~0.1 μM) and ribosomes (~5 μM) were such that all of the added mRNA should be saturated with initiating ribosomes. Any variation in translational efficiency is therefore an indication of the different efficiencies with which an initiation complex begins translation and not the affinity of the ribosome for the mRNA. Relative translation levels are listed in Table I. The wild type α mRNA (fused to a fragment of β-galactosidase; see "Materials and Methods") is translated at a level about 30-fold lower than the L11 protein fragment. Renaturation of α mRNAs under conditions favoring slow or fast forms did not alter the level of translation, but no effect was expected because the temperature required for *in vitro* translation also promotes interchange of the conformations. Δ68 RNA, lacking the pseudoknot structure, is translated at a lower level than wild type (Table I). The α mRNA sequence is thus a surprisingly inefficient translational initiation site, and the pseudoknot structure seems to enhance translational efficiency by a small factor.

S4 Preference for Fast mRNA Measured by Fluorescence—The existence of a ternary S4·mRNA·30 S subunit entrapment complex distinguishes the Fig. 1*B* repression mechanism from the more common displacement mechanism, in which repressor and 30 S subunits compete directly for mRNA binding. To obtain direct evidence of this complex, we prepared pyrene and IAEDANS fluorescent derivatives of S4 (see "Materials and Methods") so that fluorescence could be used to detect S4 binding to mRNA in the presence or absence of ribosomes. The

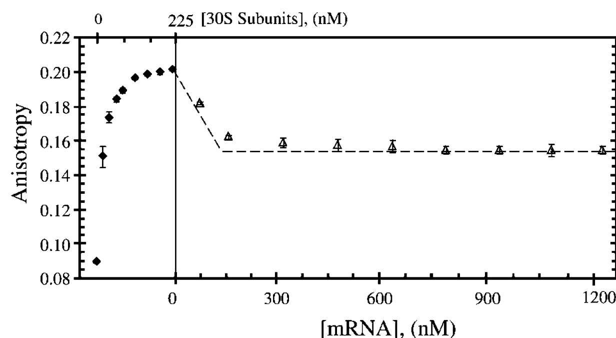

FIG. 5. **Nonspecific interaction between pyrene-S4 and 30 S subunits.** When 30 S subunits were added to 130 nM pyrene-S4 (♦), there was a significant increase in anisotropy. Addition of fast form 127 RNA (△) to this nonspecific complex reduced the anisotropy to the level expected for formation of specific ternary complex. The *dashed line* is the expected curve if 127 mRNA titrated pyrene-S4 stoichiometrically.

fluorescent tags modify Cys^{31}, which is in an unstructured region of the protein which is not essential for mRNA recognition (15).

We first measured binding of the two derivatized S4 proteins to 127 RNA trapped in predominantly fast or slow conformations, using either fluorescence intensity or anisotropy to follow the binding reaction. For IAEDANS-S4, fluorescence is quenched by about 40% upon binding 127 RNA, and affinities of 13 (±8) μM^{-1} (fast form) or 2.5 (±0.5) μM^{-1} (slow form) were calculated (data not shown). Filter binding experiments with S4 and the mRNA leader sequence under similar buffer conditions established the association constant for the mRNA fast form to be ~10 μM^{-1} (15). Thus the IAEDANS label does not significantly perturb the S4·mRNA complex. In addition, emission spectra and polarization experiments showed that IAEDANS by itself does not interact with the mRNA.

Titrations of 127 RNA with pyrene-S4 using fluorescence intensity or anisotropy as an indicator of binding showed biphasic behavior (data not shown). Competition of the pyrene-S4·mRNA complex with unlabeled S4 indicated the existence of two populations of labeled S4, of which one binds with about the same affinity as unlabeled S4, and the other (~15%) is not dissociated from the RNA. Therefore a small fraction of pyrene-labeled S4 binds much more tightly to 127 RNA than unlabeled protein; it may be an incorrectly renatured fraction of the protein.

S4–30 S Subunit Interactions—Both labeled proteins unexpectedly bound 30 S subunits alone (shown for pyrene-S4 in Fig. 5; similar results were obtained for IAEDANS-S4). A similar interaction was observed with the 70 S particles used for preparing the 30 S subunits. Several lines of evidence indicate that this complex is nonspecific and dependent on the presence of the fluorophore. (i) Less than stoichiometric amounts of 30 S subunits were needed to saturate the S4 protein. (ii) Unlabeled S4 did not compete significantly with either of the label-S4·30 S subunit complexes (data not shown). (iii) IAEDANS itself has a weak affinity for 30 S subunits at submicromolar concentrations. (iv) The fluorescence of both label-S4 proteins was enhanced upon binding 30 S subunits, in contrast to the quenching observed with mRNA. It appears that the fluorescent tags are binding in hydrophobic pocket(s) on the 30 S subunit and are largely responsible for the observed binding affinity.

Formation of Ternary Complex—On adding 127 RNA to either of the labeled S4 proteins, the fluorescence anisotropy increased, indicating the expected formation of the S4·mRNA complex. When 30 S subunits were added stoichiometrically to the S4·mRNA complex, the anisotropy increased further (Fig. 6). Although the increase in anisotropy was small, 0.077

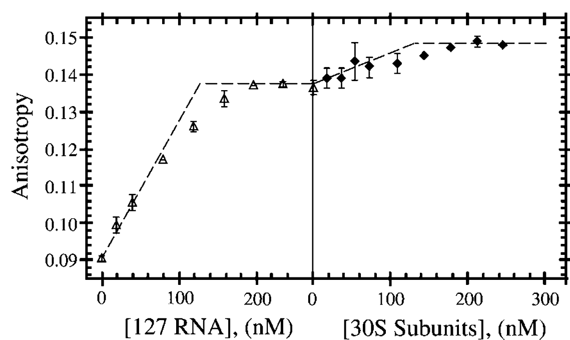

FIG. 6. **Formation of ternary complex as followed by fluorescence anisotropy.** 127 RNA (renatured in the fast form) was added to 130 nM pyrene-S4 (△), forming a 1:1 protein·RNA complex. The complex was then titrated with 30 S subunits (♦), and a further increase in anisotropy was observed. *Dashed lines* show the expected curves for stoichiometric titrations.

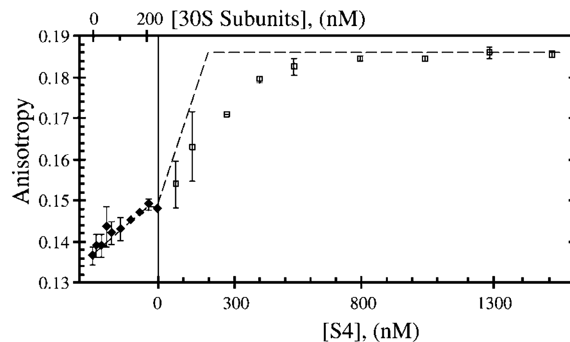

FIG. 7. **Competition of unlabeled S4 for pyrene-S4 in the ternary complex.** A 1:1 complex of 127 RNA and pyrene S4 (200 nM) was first titrated with 30 S subunits (♦) to form the ternary complex and then titrated further with unlabeled S4 (□). *Dashed lines* are expected curves for stoichiometric titrations.

(±0.002) → 0.083 (±0.001) for the IAEDANS-S4·mRNA complex and 0.138 (±0.002) → 0.150 (±0.002) for the pyrene-S4·mRNA complex, it was significant compared with the error involved in the measurement. Also, when 127 RNA was added to the nonspecific complex of S4 and 30 S subunits, the anisotropy decreased to about the same value (0.111 (±0.001) → 0.084 (±0.001) and 0.202 (±0.001) → 0.154 (±0.002) for IAEDANS-S4 and pyrene-S4, respectively), and an amount of mRNA stoichiometric with respect to S4 was required (Fig. 5). Formation of this complex requires the S4-binding form of 127 RNA, because 127 RNA that had been renatured in the absence of Mg^{2+} did not affect the anisotropy of the label-S4·30 S subunit complex (data not shown). These titrations suggest that the final complex in both cases is the same ternary complex of S4, 127 RNA, and 30 S subunits and that the ternary complex is stoichiometric in each of the components. The anisotropy changes in the different complexes are consistent with the location of the fluorophore in a flexible protein tail that is not directly involved in RNA binding.

When 5-fold molar excess of 127 mRNA is added to the label-S4·mRNA·30 S complex, there is a small decrease in the anisotropy (0.083 (±0.001) → 0.080 (±0.001) and 0.150 (±0.002) → 0.147(±0.002) for ternary complexes with IAEDANS-S4 and pyrene-S4, respectively), although the decrease is roughly a third of that expected if S4 and 30 S subunits bind independently to the mRNA. The addition of the same volume of buffer verified that dilution was not responsible for the observed decrease in anisotropy, and neither a noncognate RNA (a 58-nucleotide RNA fragment from 23 S rRNA) nor 127 RNA renatured in the absence of Mg^{2+} changed the anisotropy of the ternary complex. Thus only the S4-binding form of 127 RNA competes for formation of the ternary complex, and we suppose that cooperativity between S4 and 30 S subunits is responsible for the large excess of mRNA needed to favor the binary S4·RNA complex over the ternary complex.

Experiments competing unlabeled S4 for label-S4 in the ternary complex have also been carried out (Fig. 7). A significant increase in anisotropy is observed, as unlabeled S4 replaces label-S4 in the ternary complex, and label-S4 then binds nonspecifically to 30 S subunits. (Anisotropy changes are 0.083 (±0.001) → 0.105 (±0.001) and 0.150 (±0.002) → 0.186 (±0.001) for ternary complexes with IAEDANS-S4 and pyrene-S4 respectively.) Because the nonspecific affinity of pyrene-S4 for 30 S subunits and the possible cooperativity between S4 and 30 S subunits in binding mRNA are both unknown quantities, we have not attempted to fit the competition curve to obtain relative affinities of pyrene-S4 and unlabeled S4 for the 30 S·mRNA complex. However, the result does qualitatitvely confirm that label-S4 binding in the ternary complex does not depend strongly on the presence of the fluorescent tag.

DISCUSSION

Experiments reported in the present work support the translational repression scheme of Fig. 1*B* by providing direct evidence for the existence of the alternative mRNA conformations and the ternary repression complex; all forms and complexes of the α mRNA proposed in Fig. 1 have now been observed directly. Repression by S4 thus takes advantage of both *allostery* (S4 is an effector that binds one of two mRNA conformations) and *entrapment* (S4 stabilizes an inactive 30 S·mRNA complex). In addition, the present work shows that the α mRNA is a relatively inefficient initiation site and traces this to the initiation sequence itself rather than the pseudoknot structure of the α mRNA. These aspects of α mRNA translation are discussed in the following sections.

Active and Inactive Forms of the mRNA—In previous work, we have shown that the α mRNA pseudoknot folds into two electrophoretically distinct conformations (or sets of conformations), termed slow and fast (11). They are detected *in vitro* over a wide range of salt and temperature conditions, and they are detected at very short times after transcription with T7 RNA polymerase;[2] we therefore conclude that the conformers are not artifacts of the RNA preparation or renaturation procedures and are potentially relevant to the translational repression mechanism. In the present work, we show that S4 protein preferentially binds to the fast form, by both an electrophoretic mobility shift assay and titrations with fluorophore-tagged S4. As predicted by this preference, S4 is able to drive the fast-slow form equilibrium entirely to the fast form (Fig. 2). In contrast, 30 S subunits bind both forms with roughly the same affinity, and the crucial Shine-Dalgarno sequence is almost equally accessible in the two conformations (Figs. 2 and 3; Table I). Thus initiating ribosomes and S4 recognize different features of the mRNA.

An important feature of the proposed repression mechanism is that S4 behaves as an allosteric repressor by driving an equilibrium between active and inactive mRNA conformations toward the inactive form. Experiments first suggesting that the α mRNA exists in two forms were based on a toeprint assay (5, 10), in which a 30 S subunit· $tRNA_f^{Met}$·mRNA initiation complex stops reverse transcription at G110 (Fig. 1*A*), and a 30 S subunit·S4·mRNA complex induces transcriptase termination at the downstream edge of the pseudoknot, U120-G125. When either S4 or $tRNA_f^{Met}$ was added to 30 S subunit·mRNA com-

[2] T. C. Gluick, unpublished observations.

plexes, there was a rapid burst of ternary complex formation followed by a slower accumulation of complexes. The fraction of initiation complexes in the initial burst increased with temperature in the range 20–45 °C, whereas 30 S subunit·S4·mRNA complexes showed the opposite behavior. These and other similar experiments led to the conclusion that two forms of the 30 S·mRNA must exist: one binding $tRNA_f^{Met}$ rapidly (an active complex in translation) and the other competent to bind S4 but not $tRNA_f^{Met}$ (therefore inactive in translation), as illustrated in Fig. 1*B*. The fast and slow electrophoretic conformers investigated in the present work have properties expected of these active and inactive complexes, *i.e.* (i) S4 recognizes only of the two conformers (the fast conformer, corresponding to the inactive form), and (ii) 30 S subunits are able to bind both conformers. In addition, experiments with fluorophore-tagged S4 show that the fast conformer is able to form a ternary complex with 30 S subunits and S4 as predicted from the repression model, further supporting identification of the fast conformation as the inactive form of α operon mRNA.

One feature of the active-inactive α mRNA switch which is not reproduced by the characteristics of the slow-fast conformational change is its strong temperature dependence. Using the toeprint assay, more than 90% of the RNA was in the inactive (S4-binding) form at 20 °C, but the situation was reversed at 45 °C; the apparent van't Hoff enthalpy for the switch was estimated as ~40 kcal/mol (5). The fast → slow conformational equilibrium is only weakly temperature-dependent and has an apparent ΔH of ~12 kcal/mol (11) (although note that the kinetics of interchange are strongly temperature-dependent). However, toeprint experiments detecting the active and inactive mRNA fractions were all done in the presence of saturating levels of 30 S subunits, which may alter the temperature dependence of the equilibrium between the two mRNA forms.

Evidence for an Entrapment Mechanism of Translational Repression—As described in the Introduction, a crucial question is whether S4 competes with 30 S subunits for binding mRNA (a more common displacement mechanism) or whether S4 and 30 S subunits bind mRNA simultaneously as proposed in Fig. 1*B* (entrapment). Fluorescence experiments were designed to detect formation of the putative ternary S4·mRNA·30 S subunit complex. Pyrene or IAEDANS tags were attached to Cys^{31}, which is within a 46-residue N-terminal tail that is known from protease digestion (18, 19) and NMR (20) studies to be completely disordered. Binding studies with protein fragments have shown that deletion of the tail has very modest effects on mRNA recognition (15).[3] Three different complexes of label-S4 with RNA and ribosome subunits were observed, and very similar results were obtained with either fluorescent tag. We first consider the S4·mRNA complex. The label does not seem to perturb S4 binding to the mRNA; label-S4 binds 127 RNA with approximately the same affinity as unlabeled S4, it selectively binds the same fast electrophoretic conformer of the RNA as unlabeled S4, and competition between label-S4 and unlabeled S4 shows no effect of the label (with the exception of a small fraction of the pyrene-S4, which appears to bind RNA irreversibly). A second complex, label-S4 with 30 S subunits, was unexpected. However, competition experiments and stoichiometries of the complexes strongly argue that these complexes are dependent on the presence of the fluorophore and do not reflect a specific interaction of S4 with 30 S subunits.

The third complex is observed only when label-S4, 127 RNA, and 30 S subunits are present in approximately stoichiometric quantities. The specificity of the complex is demonstrated by its formation only with the S4-binding form of 127 RNA and is further supported by its dissociation in the presence of excess unlabeled S4 or fast form 127 RNA; a ribosomal RNA fragment or slow form 127 RNA does not compete for its formation. Some cooperativity between S4 and 30 S subunits in binding the mRNA was suggested by the relatively weak competition of 127 RNA for the complex. Competition between 30 S subunits and S4 for binding mRNA, or label-dependent binding of S4 to a 30 S·RNA complex is ruled out by the various competition experiments. We conclude that a specific complex forms between the fast mRNA conformation and both S4 and 30 S subunits, in support of an entrapment mechanism for repression.

A repressor acting by a displacement mechanism must bind mRNA with a much higher affinity than 30 S subunits (>10 μM^{-1}) or be present in much higher concentrations than free 30 S subunits to compete directly with ribosomes for mRNA binding. A repressor that traps the translational initiation complex in an inactive state need only bind tightly enough to stabilize an unproductive conformation of the complex. In other words, it is energetically much less costly to hold the ribosome in place on the mRNA than to prevent ribosomes from binding. S4 affinities for rRNA or mRNA are on the order of 10 μM^{-1} (3, 21); thus its use of an entrapment repression mechanism may have been dictated by its modest binding affinity.

Translational Efficiency of the α mRNA Pseudoknot—Expression of α mRNA-β-galactosidase fusions *in vivo* gave surprisingly low levels of expression (22), although those experiments did not distinguish whether transcription or translation was responsible. The *in vitro* translation experiments reported here use purified, equimolar amounts of mRNA to compare uncoupled translation levels of α and other mRNAs. The results confirm that the α mRNA leader sequence is a weak translation initiation site, at least 30-fold less active than the ribosome binding site from an overexpression plasmid (Table I). Our experiments narrow the possible explanations for this phenomenon.

As mentioned in the Introduction, translation initiation can be divided into two steps: a first equilibrium formation of a preinitiation complex, whose stability is determined largely by the extent and accessibility of the Shine-Dalgarno complementarity, followed by a second unimolecular rearrangement of the complex in which $tRNA_f^{Met}$ is paired with the initiation codon (7). Our studies eliminate the first equilibrium binding step as a cause of the low translation levels. First, the 30 S subunit·α mRNA binding constant is only 2-fold weaker than the same ribosome binding site lacking the pseudoknot (Δ68 RNA) and only ~3-fold weaker than an mRNA with a 6-base pair Shine-Dalgarno complementarity (L11-C76 mRNA); thus the accessibility of the Shine-Dalgarno sequence is only weakly affected by the presence of the pseudoknot structure. Second, the *in vitro* translation experiments were done with a relatively high concentration of ribosomes that should have saturated the mRNA ribosome binding sites; thus the large difference in translation rates in these experiments must be a consequence of differences in the second, unimolecular rearrangement, and not in the equilibrium binding step.

Factors that are thought to affect the second step of initiation are (i) spacing between the 3′-end of 16 S rRNA (determined by the Shine-Dalgarno sequence) and the initiation codon, and (ii) the initiation codon (AUG, GUG, or UUG) (7, 23). The Shine-Dalgarno to initiation codon spacing for the α mRNA is 7 nucleotides, within an optimum range of 6–9 nucleotides. However, the GUG initiation codon should decrease translation substantially. Ringquist *et al.* (23) compared a series of mRNAs differing in Shine-Dalgarno spacing and initiation codon; for a 7-nucleotide spacing, substitution of GUG for AUG decreased

[3] R. Gerstner and D. E. Draper, unpublished observations.

in vivo expression by ~10-fold. This is probably the major factor accounting for the difference between Δ68 RNA and L11-C76 RNA translational efficiency.

If a GUG initiation codon is so poor, why has the α mRNA not switched to AUG? *In vivo* studies have shown that such a switch *decreases* expression from the α mRNA ribosome binding site by about 6-fold (22), suggesting that the rules for translational initiation in the context of the α mRNA pseudoknot are different than in the context of an unstructured RNA. It seems likely that the unusual characteristics of the second step of initiation in α mRNA (*i.e.* it is slow and favors a GUG codon) are related to the repression mechanism, which depends on the ability of an mRNA conformational change to affect $tRNA_f^{Met}$ binding. The structural details of how this is accomplished remain an interesting question.

Acknowledgments—We thank Drs. Ludwig Brand, Dmitri Toptygin, and Michael E. Rodgers for help with fluorescence experiments and Resi Gerstner for helpful discussions.

REFERENCES

1. Nomura, M., Gourse, R., and Baughman, G. (1984) *Annu. Rev. Biochem.* **53,** 75–117
2. Thomas, M., Bedwell, D. M., and Nomura, M. (1987) *J. Mol. Biol.* **196,** 333–345
3. Deckman, I. C., Draper, D. E., and Thomas, M. S. (1987) *J. Mol. Biol.* **196,** 313–322
4. Tang, C. K., and Draper, D. E. (1989) *Cell* **57,** 531–536
5. Spedding, G. S., and Draper, D. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4399–4403
6. Draper, D. E., Gluick, T. C., and Schlax, P. J. (1998) in *RNA Structure and Function* (Simons, R. W., and Grunberg-Manago, M., eds) pp. 415–436, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7. Draper, D. E. (1996) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., ed) pp. 902–908, American Society for Microbiology, Washington, D. C.
8. Calogero, R. A., Pon, C. L., Canonaco, M. A., and Gualerzi, C. O. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6427–6431
9. Draper, D. E. (1987) in *Translational Regulation of Gene Expression* (Ilan, J., ed) pp. 1–26, Plenum Press, New York
10. Spedding, G. S., Gluick, T. C., and Draper, D. E. (1993) *J. Mol. Biol.* **229,** 609–622
11. Gluick, T. C., Gerstner, R. G., and Draper, D. E. (1997) *J. Mol. Biol.* **270,** 451–463
12. Xing, Y., and Draper, D. E. (1996) *Biochemistry* **35,** 1581–1588
13. Gurevich, V. V. (1996) *Methods Enzymol.* **275,** 382–397
14. Draper, D. E., White, S. A., and Kean, J. M. (1988) *Methods Enzymol.* **164,** 221–237
15. Baker, A. M., and Draper, D. E. (1995) *J. Biol. Chem.* **270,** 22939–22945
16. Draper, D. E., Deckman, I. C., and Vartikar, J. V. (1988) *Methods Enzymol.* **164,** 203–220
17. Kohrer, C., Mayer, C., Grobner, P., and Piendl, W. (1996) *Eur. J. Biochem.* **236,** 234–239
18. Changchien, L. M., and Craven, G. R. (1976) *J. Mol. Biol.* **108,** 381–401
19. Newberry, V., Yaguchi, M., and Garrett, R. A. (1977) *Eur. J. Biochem.* **76,** 51–61
20. Sayers, E. W., Gerstner, R. B., Draper, D. E., and Torchia, D. A. (2000) *Biochemistry* **39,** 13602–13613
21. Vartikar, J. V., and Draper, D. E. (1989) *J. Mol. Biol.* **209,** 221–234
22. Tang, C. K., and Draper, D. E. (1990) *Biochemistry* **29,** 4434–4439
23. Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J., Stormo, G. D., and Gold, L. (1992) *Mol. Microbiol.* **6,** 1219–1229
24. Bedwell, D., Davis, G., Gosink, M., Post, L., Nomura, M., Kestler, H., Zengel, J. M., and Lindahl, L. (1985) *Nucleic Acids Res.* **13,** 3891–3903