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Biochemistry

Allosteric mechanism for translational repression in the *Escherichia coli* α operon

(ribosomes/S4 protein/translational initiation/RNA pseudoknot)

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ABSTRACT **The ribosomal protein S4 is a translational repressor that binds to a complex mRNA pseudoknot structure containing the ribosome binding site for the first gene of the α operon. Either 30S subunits or S4 protein bound to the mRNA causes Moloney murine leukemia virus reverse transcriptase to pause near the 3′ terminus of the pseudoknot. There is no competition between subunits and S4 for mRNA binding. The kinetics of forming S4–30S–mRNA complexes are biphasic, and the fraction of mRNA molecules reacting more rapidly decreases as the temperature is increased from 30°C to 40°C. The complex cannot be detected with mRNA mutants that cannot be repressed. We have previously shown similar kinetic behavior for the formation of $tRNA_f^{Met}$ initiation complexes with $tRNA_f^{Met}$, 30S subunits, and mRNA, except that the fraction reacting rapidly increases when the temperature is increased over the same 30–40°C range. Thus the two sets of experiments show that there are two forms of 30S–mRNA complexes that differ in their abilities to bind S4 and $tRNA_f^{Met}$. The results support an allosteric model for translational repression in which S4 traps the mRNA in a conformation able to bind 30S subunits but unable to form an initiation complex with $tRNA_f^{Met}$.**

A number of genes are regulated at the translational level by repressor proteins that bind mRNA and inhibit translation. The first repressor–mRNA complex observed was between the phage R17 replicase RNA and the phage coat protein (1). Since then, a number of other prokaryotic translational repressors have been identified (2–4). Among them are a set of ribosomal proteins that regulate the translation of most ribosomal protein operons in *Escherichia coli* (5), a substantial fraction of the total protein synthesis in the cell.

Ribosomal protein S4 binds to a single target site in the α operon mRNA and represses the translation of all four ribosomal proteins in the operon (6). The target structure recognized by S4 and required for translational repression is a complex pseudoknot that encompasses the ribosome binding site (7). Comparison of *in vitro* S4 binding data and *in vivo* measurements of translational repression with a series of mRNA mutants gave the unexpected result that some mutants were able to bind S4 with approximately wild-type affinities but showed substantially reduced S4-mediated repression levels (8). This result can be explained if S4 is an allosteric inhibitor of translation: the model proposes that the ribosome and S4 repressor bind to separate sites on the mRNA and that S4 binding traps the mRNA in a conformation unable to form a competent initiation complex. We have presented (9) evidence for the existence of "active" and "inactive" mRNA conformations required by this model.

In this paper we examine the effects of S4 protein on initiation complex formation and find support for the allosteric mechanism of translational repression. The protein apparently traps the mRNA in a conformation able to bind 30S subunits but unable to bind $tRNA_f^{Met}$ stably.

MATERIALS AND METHODS

Preparation of mRNAs by T7 polymerase transcription and 30S ribosomal subunits were as described by Spedding *et al.* (9). S4 protein was purified from an overproducing strain constructed in this laboratory by A. M. Baker. The final purification is identical to that previously described (10). Prolonged incubation of the protein with stoichiometric amounts of mRNA at micromolar concentrations did not give any evidence of nuclease activity (T. C. Gluick, personal communication). The protein was renatured before use as described (10). "Toeprint" assays with 10 units of Moloney murine leukemia virus (MMLV) reverse transcriptase in 10 μl for 15 min were carried out as described (9), except that S4 buffer (60 mM NH_4Cl/35 mM KCl/10 mM Tris acetate, pH 7.4/10 mM magnesium acetate/6 mM 2-mercaptoethanol/50 mM urea) was used.

The strengths of toeprint signals were quantitated from autoradiograms by densitometry (9). The "relative toeprint" is the intensity of the toeprint bands divided by the sum of the toeprint and full-length transcript intensities.

RESULTS

30S Subunit-Dependent Toeprint Signals with the α mRNA. Hartz *et al.* (11) have shown that the ternary $tRNA_f^{Met}$–30S subunit–mRNA initiation complex inhibits transcription by reverse transcriptases. The prematurely terminated transcripts, which generally end +15 or +16 nt downstream of the initiation codon, have come to be called the toeprint of the initiation complex. The relative amount of the truncated and full-length transcripts is a quantitative assay for the extent of ternary complex formation (9). We have recently shown that the initiation complex formed on the first ribosome binding site of the α mRNA transcript gives a standard toeprint signal at G^{110}, +15 nt from the GUG initiation codon, when avian myeloblastosis virus (AMV) reverse transcriptase is used, and a similar signal at G^{110} and G^{111} when MMLV transcriptase is used (9). An example toeprint with the MMLV enzyme is shown in Fig. 1*A*, lane 5. Here we refer to this reverse transcriptase stop as a tRNA–30S toeprint, to distinguish it from other transcriptase stops described below.

In the absence of $tRNA_f^{Met}$, a binary 30S–mRNA complex with T4 gene *32* mRNA was too labile to be detected by AMV reverse transcriptase but weakly inhibited the MMLV transcriptase at a position just downstream of the Shine–Dalgarno

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Abbreviations: MMLV, Moloney murine leukemia virus; AMV, avian myeloblastosis virus.

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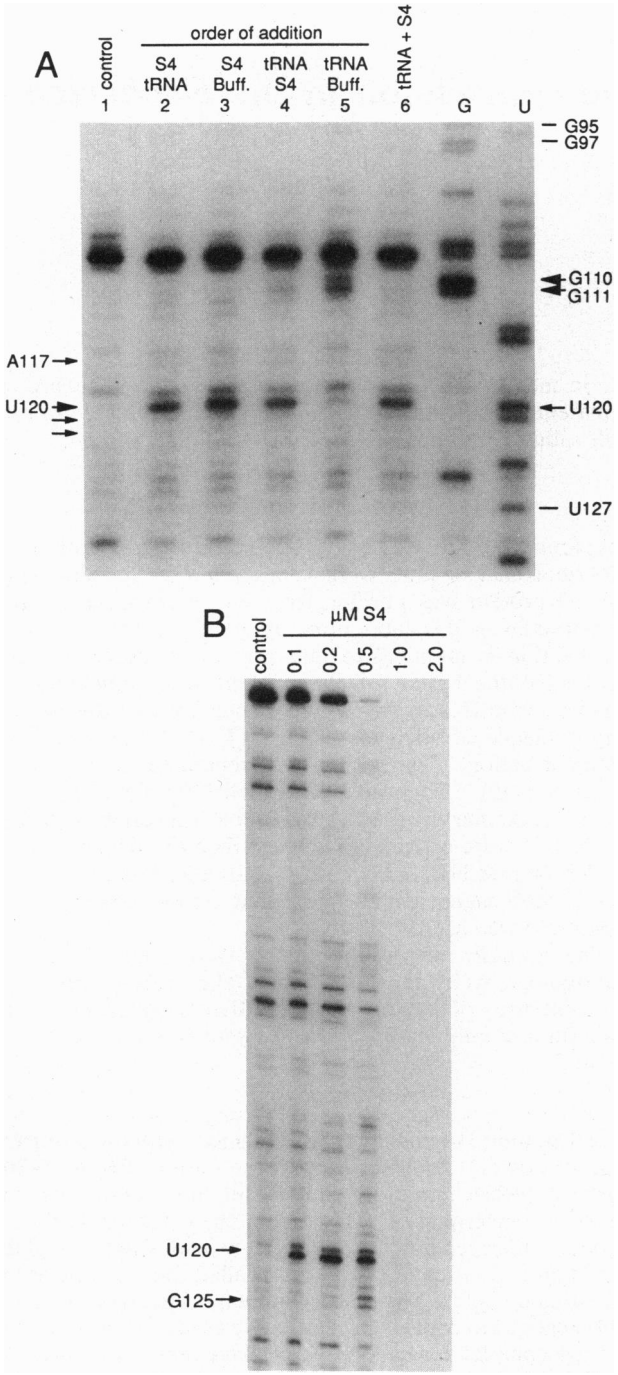

FIG. 1. Toeprint signals obtained on the α mRNA with MMLV reverse transcriptase. (*A*) Toeprints obtained in S4 buffer in the presence of 30S subunits. Lane 1 is reverse transcription of mRNA alone; lanes 2–6 also contain 1 μM 30S subunits. In lanes 2–4, either S4 (2 μM) or $tRNA_f^{Met}$ (2.5 μM) was added and incubated 10 min at 37°C, a second addition of S4, $tRNA_f^{Met}$, or buffer (Buff.) was made, and incubation continued for another 10 min before carrying out the transcriptase reaction (mRNA concentrations, ≈50 nM). Lanes G and U are sequencing reaction products with AMV transcriptase. Arrows on the left indicate S4-dependent bands, and signals on the right tRNA-dependent bands. The initiation codon sequence (G^{95}–G^{97}) and the approximate 3′ terminus of the pseudoknot (U^{127}) are also indicated. (*B*) MMLV reverse transcriptase reactions in the presence of S4. Reactions were carried out at 32°C in S4 buffer, after a 10-min incubation of protein and α mRNA. The control lane is α mRNA alone; the other lanes contain the indicated concentration of S4.

sequence (12). The α mRNA behaved quite differently. Below 30°C, 30S subunits strongly inhibited the MMLV enzyme between U^{120} and U^{127}, well into the coding region near the 3′ boundary of the pseudoknot structure (Fig. 2) (9). We attributed this 30S toeprint to stabilization of the pseudoknot structure by bound ribosomes, since MMLV transcriptase paused at an almost identical set of sites when used at low concentrations with the mRNA alone. This unusual binary complex was unable to continue to the stable $tRNA_f^{Met}$–30S subunit–mRNA initiation complex at low temperatures. Binary complexes that have been formed at higher temperatures do not give toeprints with either AMV or MMLV transcriptase (9) but do react rapidly with $tRNA_f^{Met}$ to form initiation complexes.

S4-Dependent Toeprint Signals. Fig. 1*B* shows that S4 protein alone caused pausing of MMLV transcriptase. The set of bands in this S4 toeprint contained approximately the same nucleotides as the 30S toeprint, U^{120}–U^{127}, though the relative intensities were much different. A similar pattern was observed at 20°C (data not shown). The pseudoknot secondary structure shown in Fig. 2 must be recognized by S4, since it was deduced by assaying compensatory base mutations for S4 binding affinity (7). The pattern of transcriptase stops near the 3′ terminus of the pseudoknot is consistent with the expected stabilization of the pseudoknot by S4.

At S4 concentrations >0.5 μM, the reverse transcriptase was inhibited; at 2 μM S4 protein, virtually no transcription was observed. Temperatures between 20°C and 37°C did not alter this behavior. We suspect this is a consequence of nonspecific S4 binding to the mRNA. The S4 nonspecific binding affinity was comparable to the specific affinity in the lower salt (0.1 M monovalent ion) buffers used here, and only 10- to 50-fold weaker than specific binding under the most favorable conditions (14). Thus it is likely that several S4 molecules are bound to each mRNA when the specific site is only partially saturated. A weak interaction of S4 with the transcriptase was also a possibility. Whatever the mechanism, this inhibition precluded any estimate of the S4–mRNA binding affinity by the toeprint assay, though the appearance of the S4 toeprint at 0.1 μM S4 is consistent with the binding constant of $\approx 10^7\ M^{-1}$ measured by a filter assay (14). A toeprint signal from the S4–mRNA complex was also seen when AMV transcriptase was used in place of the MMLV enzyme, though it was much weaker and the only stop detected was at U^{120}. AMV transcriptase was also inhibited by higher S4 concentrations (data not shown).

When S4 and 30S subunits were both added to the mRNA, a pattern of transcriptase stops similar, but not identical, to those obtained with S4 alone was seen; by far the largest effect was at U^{120} (Fig. 1*A*, lane 3). [At the temperature of this experiment (37°C), 30S subunits alone did not cause transcriptase stops.] With the subunits present, 2 μM S4 no longer inhibited the transcriptase as severely. 30S subunits

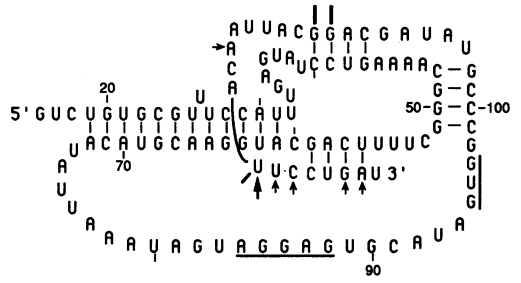

FIG. 2. Summary of MMLV transcriptase toeprint signals on the pseudoknot structure of the α mRNA (adapted from ref. 13). Numbering is from the 5′ terminus of the *in vivo* transcript, and the secondary structure is that deduced by Tang and Draper (7). Tic marks are located every 10 nt. Approximate toeprint signal intensities with 30S subunits plus either S4 protein (arrows) or $tRNA_f^{Met}$ (lines) are indicated.

alone at 1 μM reduced the MMLV transcriptase activity by 2- to 3-fold, and this inhibition was only slightly stronger when S4 was added. We think it likely that subunits, by binding the mRNA, displace nonspecifically bound S4 and thus relieve the transcription inhibition.

It appears from these data that 30S subunits and S4 are able to bind simultaneously to their respective specific sites on an mRNA. If S4 displaced 30S subunits from the mRNA, or vice versa, then the S4 toeprint should be substantially weakened by increasing concentrations of subunits. This was not the case; densitometer scans showed that the relative toeprint signals obtained at U^{120} with 0.1 and 0.2 μM S4 were about the same whether or not 2 μM 30S subunits were present (data not shown). The temperature dependence of the toeprint obtained in the presence of both S4 and 30S subunits, discussed below (see Fig. 4*B*), was also identical in the presence of 30S subunits at 0.2 or 1.0 μM. We conclude that S4 and 30S subunits did not compete for binding to the mRNA but, to a first approximation, bound independently. At the concentrations of 2 μM S4 and 1 μM 30S subunits standardly used in the experiments that follow (Figs. 3–4), both subunits and S4 were probably bound to the mRNAs. We call the U^{120} stop observed in the presence of subunits and higher S4 concentrations (that would otherwise inhibit the transcriptase) an S4–30S toeprint.

Effects of $tRNA_f^{Met}$ on the S4–30S Toeprint. We have observed (9) biphasic kinetics in the association of $tRNA_f^{Met}$ and 30S subunits with mRNA; at 37°C, about half the mRNA molecules formed a ternary initiation complex within minutes, while the remainder reacted only very slowly over a period of hours. Qualitatively similar kinetics of association were seen when S4 and 30S subunits were added to mRNA (Fig. 3). A fraction of the mRNA rapidly formed an S4–30S toeprint complex before the first time point could be assayed; this reaction was followed by a slower association of the remaining mRNA, with half-times of an hour or less. A larger fraction reacted rapidly at 32°C than at 37°C.

When $tRNA_f^{Met}$ was also present with subunits and S4, the same fraction of mRNA gave an S4-dependent toeprint signal at short times, but the remaining mRNA associated more slowly by a factor of ≈3. We conclude that $tRNA_f^{Met}$ cannot compete with S4 for binding to the rapidly associating fraction of 30S–mRNA complexes but does compete with S4 for binding to the more slowly reacting fraction. Thus S4 and $tRNA_f^{Met}$ each prefer to react with a different fraction of 30S–mRNA complexes.

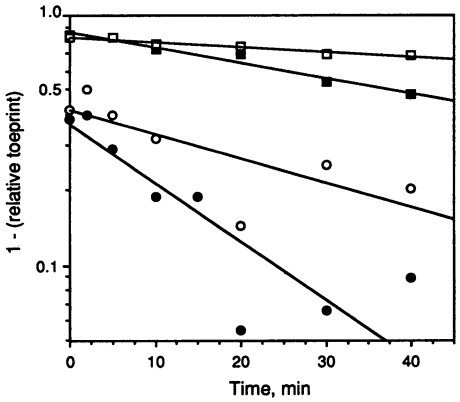

FIG. 3. Kinetics of S4 and 30S subunit association with α mRNA. Reactions of 1 μM 30S subunits and 2 μM S4 with α mRNA in S4 buffer were assayed at various times after addition of components. The graph shows the first-order disappearance of full-length transcripts for reactions at 32°C (●) or 37°C (■). Open symbols (○ and □) are for similar reactions with the addition of 2.5 μM $tRNA_f^{Met}$, also at 32°C and 37°C, respectively.

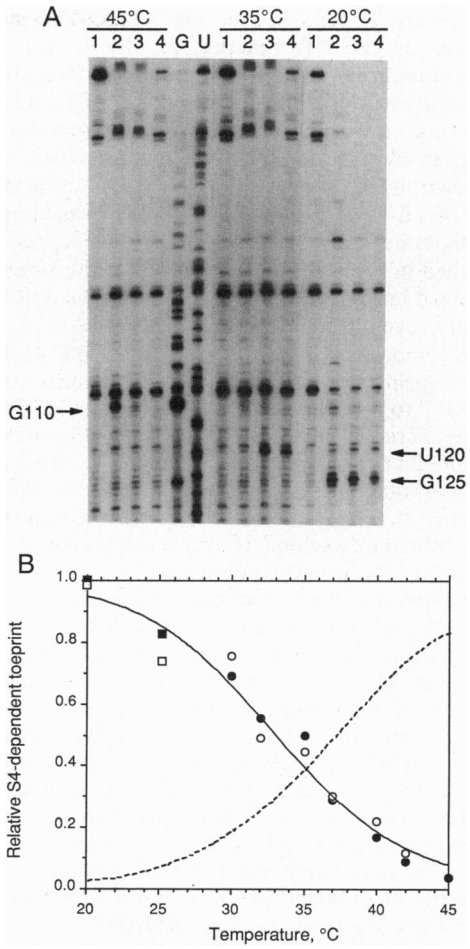

FIG. 4. Temperature dependence of the S4–30S toeprint. (*A*) MMLV transcriptase assays of α mRNA alone (lanes 1), plus 1 μM 30S subunits and 2.5 μM $tRNA_f^{Met}$ (lanes 2), plus 30S subunits, $tRNA_f^{Met}$, and 2 μM S4 (lanes 3), or plus 30S subunits and S4 (lanes 4). Sets of reactions were incubated 10 min and assayed at the indicated temperatures. Lanes G and U are sequencing lanes. (*B*) The relative strengths of the S4-dependent bands, obtained from experiments as in *A*, are plotted as a function of temperature. ○ and □, toeprint signal in the absence of $tRNA_f^{Met}$; ● and ■, toeprint signal in the presence of $tRNA_f^{Met}$. □ and ■ include intensity from bands C^{123}–U^{127}, while the other points are taken from U^{120} only. The curve is a two-state transition with a melting temperature of 33°C and enthalpy of 40 kcal/mol (1 cal = 4.184 J). The dotted line is a similar fit (melting temperature, 37°C; enthalpy, 40 kcal/mol) to the $tRNA_f^{Met}$-dependent relative toeprint data obtained by Spedding *et al.* (9).

This point is also shown by the set of experiments shown in Fig. 1*A*. Lanes 2, 4, and 6 had the same final concentrations of components, but S4 and $tRNA_f^{Met}$ were added in different orders. If the fraction of 30S–mRNA complexes that reacts rapidly with $tRNA_f^{Met}$ is the same fraction that reacts rapidly with S4, then the 10-min preincubation of $tRNA_f^{Met}$, 30S subunits, and mRNA should drastically reduce the intensity of the S4-dependent toeprint signal. [Once formed, the ternary initiation complex with $tRNA_f^{Met}$ does not dissociate during the time of this experiment (9).] Comparison of lanes 4 and 6 shows that the preincubation has no effect, as expected.

From Fig. 1*A*, lanes 4 and 5, it might appear that added S4 substantially reduces the intensity of the $tRNA_f^{Met}$–30S toeprint signal. Densitometer scans showed that the weaker G^{110} and G^{111} toeprint was an artifact of the reduced transcriptase activity in the presence of S4. The intensity of the $tRNA_f^{Met}$-dependent toeprint relative to the full-length transcript remained the same with or without S4 present.

Temperature Dependence of the S4–30S Toeprint. The fraction of the mRNA reacting rapidly to form ternary initiation complexes is a strong function of temperature (9); about half the mRNAs reacted rapidly at 37°C, whereas the fraction was very small at 32°C. This temperature dependence was evident in the tRNA–30S toeprints (bands G^{110} and G^{111}) shown in Fig. 4*A*. In contrast, the fraction of mRNA reacting rapidly with S4 and 30S subunits was largest at the lower temperatures, as shown in Fig. 4*A* (U^{120} toeprint signal) and graphed in Fig. 4*B*. As expected from the kinetic experiment shown in Fig. 3, addition of $tRNA_f^{Met}$ had no effect on the rapidly reacting fraction (Fig. 4*B*).

As the temperature was lowered from 30°C to 25°C, the S4–30S toeprint signal changed from primarily an intense band at U^{120} to a set of bands from positions 123 to 127 that was more characteristic of the toeprint of 30S subunits alone (9) (cf., the 20°C and 35°C lanes of Fig. 4*A*). It seems likely that the toeprint changed simply because higher salt or lower temperature stabilized the RNA structure within the bound complex sufficiently so that the transcriptase paused closer to the "edge" of the pseudoknot structure.

The fraction of mRNAs that was able to react rapidly with S4 and 30S subunits nearly mirrors the fraction rapidly reacting to form initiation complexes when $tRNA_f^{Met}$ was present (Fig. 4*B*); the midpoints of the two temperature-dependence curves were 34°C and 37°C, respectively. The two experiments are not exactly comparable, since the low concentration of urea present in the S4 measurements decreased the toeprint signal slightly. The qualitative observation that the two complexes had opposite temperature dependences was unaffected by this difference. We conclude that, to a first approximation, the mRNA can exist in either of two states when bound to 30S subunits, one preferentially binding S4 and the other binding $tRNA_f^{Met}$.

Effects of Mutations on the S4–30S Toeprint. A number of α mRNA mutations have been made that affect either S4 binding *in vitro* (7) or translational repression by S4 *in vivo* (8). We tested eight of these mutations to see whether the appearance of a U^{120} toeprint correlated with the measured S4 affinity and repression. The properties of these mutants in several assays are summarized in Table 1. Mutants that bind S4 weakly (affinity relative to wild type <0.15) did not give detectable S4–30S toeprints. CKT20 RNA, which binds S4 with ≈5-fold reduced affinity, showed an S4–30S toeprint that was significantly weaker than the toeprint obtained with wild-type RNA.

Two mutants bound S4 with nearly wild-type affinities but did not show the S4–30S toeprint signal. One of these was CKTΔ4, which binds S4 with the same affinity as wild type but shows severely reduced levels of repression *in vivo*. This suggests that the U^{120} toeprint is related to the ability of S4 to repress translation and not simply its ability to recognize the mRNA. The other was CKT18, which binds S4 with only ≈2-fold reduced affinity and shows only slightly reduced repression; no S4-dependent toeprint was detected. This mutant changes nt 123 and 124, which are very close to the main S4-induced stop site (U^{120}) and within the main region of 30S subunit-dependent pauses at low temperature. The stability of the RNA structure in this region may have been sufficiently weakened that the transcriptase was unaffected even in the presence of the S4 and subunits.

We also note that there was a large variation in the tRNA–30S toeprint signal intensity that does not correlate well with levels of translation observed *in vivo*. This contradicts the proposal that toeprint "strength" correlates with translational efficiency (15). As discussed by others (9, 16), the toeprint assay need not reflect the rate-limiting step in translation.

Table 1. Summary of α operon mutant data

Mutant	Mutation	Rel. K_{S4}	Rel. trans.	Re-press.	Relative toeprints: tRNA	Relative toeprints: S4
wt	—	1.0	1.0	1.97	0.16	0.30
CKT12	$G^{98} \rightarrow C$	0.12	0.006	—	≤0.02*	—
CKT16	$C^{52} \rightarrow G$	0.11	1.2	1.20	≤0.02*	—
CKT17	$A^{109}G \rightarrow UC$	0.12	5.3	1.25	0.10	—
CKT18	$C^{123}U \rightarrow GA$	0.56	40.	1.86	0.42	—
CKT17/18	CKT17 + CKT18	0.15	28.	1.01	0.34	—
CKT19	$G^{95} \rightarrow A$	1.0	0.15	1.81	≤0.02*	0.31
CKT20	$A^{106} \rightarrow U$	0.23	0.81	1.07	≤0.02*	0.10
CKTΔ4	ΔA^{79}–G^{82}	0.92	0.70	1.18	≤0.02*	—

wt, Wild type. Relative affinity constant (Rel. K_{S4}) data taken from ref. 7; the wild-type binding constant is 12 μM^{-1}. The relative translation (Rel. trans.) and repression (Repress.) data taken from ref. 8 are relative β-galactosidase translation rates in *E. coli* transformed with a plasmid containing a fusion between the first 203 bp of the α operon and the *lacZ* gene. The repression ratio is the normal rate of β-galactosidase synthesis divided by the rate after induction of S4 overproduction. Relative toeprints are the intensities of MMLV stop bands relative to full-length transcripts, taken from experiments performed at 37°C as shown in Fig. 4*A*, lanes 2 and 4. tRNA–30S toeprints are taken from the cluster of MMLV stops at G^{110} and G^{111}, and S4–30S toeprints are the U^{120} band. Dashes indicate no detectable toeprint signal.

*Detectable band but too weak to quantitate accurately.

DISCUSSION

$tRNA_f^{Met}$ and S4 Bind to Different Forms of the 30S–mRNA Complex. We propose the scheme shown in Fig. 5 to account for our S4 toeprint data; it is based in large part on previous studies of initiation complex formation on the α mRNA (9). In those studies, we observed fast and slow phases in the association kinetics of the initiation complex. These could be explained only by supposing that the mRNA is trapped in two conformations that react in parallel at widely different rates. Since temperature-shift experiments showed that mRNA alone quickly interconverts between the different conformations, we were forced to presume that 30S subunits bound and trapped the mRNA in the two forms. We termed these active and inactive complexes and were able to detect the postulated inactive 30S–mRNA complex directly using MMLV transcriptase.

The toeprint results obtained in the presence of S4 are accounted for if S4 binds to the inactive 30S–mRNA complex. As with the initiation complex, the kinetics of S4 binding are biphasic, again suggesting the existence of two slowly interconverting 30S–mRNA conformations, one of which associates with S4 rapidly. A key observation is contained in the temperature-dependence data in Fig. 4*B*: at low temperatures most of the mRNA is in the form reacting rapidly with S4, while only a small fraction reacts rapidly with $tRNA_f^{Met}$; the situation is reversed at higher temperatures. This is very strong evidence that the same mRNA conformational switch affects both S4 and $tRNA_f^{Met}$ association.

Conformational Changes in the α mRNA. The pseudoknot structure shown in Fig. 2 was deduced by assaying compensatory base changes for S4 binding affinity at 0°C (7). Since we have identified the inactive mRNA conformation as the one that is predominant at lower temperatures and preferentially binds S4, we deduce that this conformation contains the intact pseudoknot. What conformational changes might occur in going from the active to the inactive conformation? The distinctive functional difference between the two forms is their ability to bind $tRNA_f^{Met}$ in a stable initiation complex. Therefore, a reasonable speculation is that the switch to the active form renders the GUG initiation codon accessible.

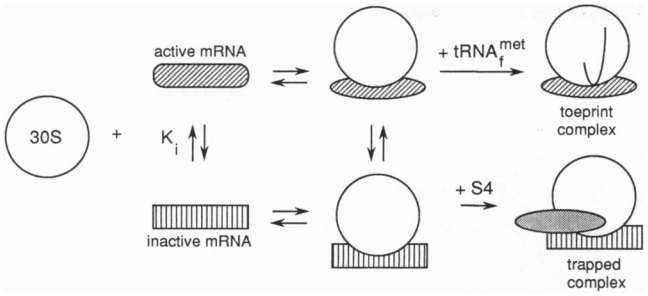

FIG. 5. Scheme for translational initiation and repression of the α mRNA by S4 protein.

Although we have no detailed structure of the ribosome binding site in either the active or inactive mRNA conformation, we have evidence that there is substantial tertiary structure in this region. Three of the mutants we have examined change nucleotides within the ribosome binding site and have properties not predicted by the known mRNA secondary structure. Both CKT19, in which $G^{95} \rightarrow A$ changes the GUG initiation codon to AUG, and CKT12 ($G^{98} \rightarrow C$) are translated with substantially reduced efficiency *in vivo* (8), and CKT19 shows a much slower rate of initiation complex formation in the toeprint assay (9). If the pseudoknot contains a tertiary structure that promotes the kinetics of codon–anticodon pairing once the ribosome is bound, then disruption of the structure would slow this process. The CKTΔ4 mutation deletes 4 nt just upstream of the Shine–Dalgarno sequence. The S4 binding affinity of this mutant is unaltered, which means that the basic pseudoknot secondary structure required for S4 recognition must be intact, but its ability to support repression is greatly diminished (8) and no S4–30S toeprint is observed. We can only argue that the deletion has disrupted some as yet unidentified structure that the bound protein uses to stabilize the inactive conformation of the ribosome binding site.

Relation of the Active–Inactive Switch to Translational Repression. For most translational repressors, it has been presumed that the repressor–mRNA interaction precludes ribosome binding. The R17 coat protein, for instance, stabilizes a hairpin containing both the Shine–Dalgarno sequence and the initiation codon (17), which must certainly prevent ribosomes from associating with the mRNA. This kind of competition clearly does not take place on the α mRNA; instead, some step in $tRNA_f^{Met}$ binding is affected. The possibility that repression could occur by "entrapment" of the 30S–mRNA complex in an unproductive conformation, rather than by "displacement" of the ribosome from the mRNA, was discussed some time ago (18), but until now had not been experimentally described.

An entrapment–repression scheme such as we show in Fig. 5 would still work if the repressor protein recognized only the 30S–mRNA complex and not the mRNA alone. Such a situation may apply for ribosomal protein S20, which represses its own translation (19) but apparently does not bind to the purified mRNA (20).

An advantage of an entrapment mechanism is that it does not demand that the repressor bind tightly enough to displace the ribosome, which has a substantial affinity for the mRNA. This is a potential advantage for ribosomal proteins, which bind RNA with affinities on the order of $10^7\ M^{-1}$, comparable to or weaker than the affinity of initiating ribosomes (18). At a growth rate of one doubling per hour, the pool size of each ribosomal protein is $\approx 0.4\ \mu M$ and the free ribosomal subunit concentration is $\approx 3\ \mu M$ (see calculations and references in ref. 18). By a displacement mechanism, repression should be very weak, but under the same growth conditions the ribosomal proteins in the α operon are translationally repressed by $\approx 50\%$ (21). These numbers are compatible with the strong toeprint signal seen at micromolar concentrations of S4 and 30S subunits and an entrapment mechanism.

In relating the *in vitro* experiments to *in vivo* conditions, we should emphasize that the interconversion of the active and inactive mRNA forms and the competition between S4 and $tRNA_f^{Met}$ for forming ternary complexes is kinetically controlled in the toeprint assay. Initiation of translation *in vivo* takes place in the presence of initiation factors, and there is ample evidence that the factors alter the kinetics of initiation (22). Initiation factor 3, for instance, substantially increases the otherwise extremely slow exchange rate of $tRNA_f^{Met}$ bound to initiation complexes (23). The exchange between active and inactive complexes, which is very slow under our conditions, therefore, may be substantially faster *in vivo*. It is not clear at this point which steps in either translational initiation or repression may actually come to equilibrium *in vivo*.

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1. Bernardi, A. & Spahr, P. F. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3033–3037.
2. Butler, J. S., Springer, M., Dondon, J., Graffe, M. & Grunberg-Manago, M. (1986) *J. Mol. Biol.* **192**, 767–780.
3. Moine, H., Romby, P., Springer, M., Grunberg-Manago, M., Ebel, J.-P., Ehresmann, B. & Ehresmann, C. (1990) *J. Mol. Biol.* **216**, 299–310.
4. Gold, L. (1988) *Annu. Rev. Biochem.* **57**, 199–233.
5. Nomura, M., Gourse, R. & Baughman, G. (1984) *Annu. Rev. Biochem.* **53**, 75–117.
6. Thomas, M., Bedwell, D. M. & Nomura, M. (1987) *J. Mol. Biol.* **196**, 333–345.
7. Tang, C. K. & Draper, D. E. (1989) *Cell* **57**, 531–536.
8. Tang, C. K. & Draper, D. E. (1990) *Biochemistry* **29**, 4434–4439.
9. Spedding, G., Gluick, T. C. & Draper, D. E. (1993) *J. Mol. Biol.* **229**, 609–622.
10. Draper, D. E., Deckman, I. C. & Vartikar, J. V. (1988) *Methods Enzymol.* **164**, 203–220.
11. Hartz, D., McPheeters, D. S. & Gold, L. (1988) *Methods Enzymol.* **164**, 419–425.
12. Hartz, D., McPheeters, D. S. & Gold, L. (1991) *J. Mol. Biol.* **218**, 99–105.
13. Draper, D. E. (1992) *Acc. Chem. Res.* **25**, 201–207.
14. Deckman, I. C., Draper, D. E. & Thomas, M. S. (1987) *J. Mol. Biol.* **196**, 313–322.
15. Hartz, D., McPheeters, D. S. & Gold, L. (1991) *J. Mol. Biol.* **218**, 83–97.
16. Ma, C. & Simons, R. W. (1990) *EMBO J.* **9**, 1267–1274.
17. Carey, J. & Uhlenbeck, O. C. (1983) *Biochemistry* **22**, 2610–2615.
18. Draper, D. E. (1987) in *Translational Regulation of Gene Expression*, ed. Ilan, J. (Plenum, New York), pp. 1–26.
19. Parsons, G. D., Donly, B. C. & Mackie, G. A. (1988) *J. Bacteriol.* **170**, 2485–2492.
20. Donly, B. C. & Mackie, G. A. (1988) *Nucleic Acids Res.* **16**, 997–1010.
21. Jinks-Robertson, S. & Nomura, M. (1981) *J. Bacteriol.* **145**, 1445–1447.
22. Gualerzi, C. & Pon, C. L. (1990) *Biochemistry* **29**, 5881–5889.
23. Risuleo, G., Gualerzi, C. & Pon, C. (1976) *Eur. J. Biochem.* **67**, 603–613.