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Ribosomal protein S15 from *Escherichia coli* modulates its own translation by trapping the ribosome on the mRNA initiation loading site

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ABSTRACT **From genetic and biochemical evidence, we previously proposed that S15 inhibits its own translation by binding to its mRNA in a region overlapping the ribosome loading site. This binding was postulated to stabilize a pseudoknot structure that exists in equilibrium with two stem–loops. Here, we use "toeprint" experiments with Moloney murine leukemia virus reverse transcriptase to analyze the effect of S15 on the formation of the ternary mRNA–30S–$tRNA_f^{Met}$ complex. We show that the binding of the 30S subunit on the mRNA stops reverse transcriptase near position +10, corresponding to the 3′ terminus of the pseudoknot, most likely by stabilizing the pseudoknot conformation. Furthermore, S15 is found to stabilize the binary 30S–mRNA complex. When the ternary 30S–mRNA–$tRNA_f^{Met}$ complex is formed, a toeprint is observed at position +17. This toeprint progressively disappears when the ternary complex is formed in the presence of increasing concentrations of S15, while a shift from position +17 to position +10 is observed. Beside, RNase T1 footprinting experiments reveal the simultaneous binding of S15 and 30S subunit on the mRNA. Otherwise, we show by filter binding assays that initiator tRNA remains bound to the 30S subunit even in the presence of S15. Our results indicate that S15 prevents the formation of a functional ternary 30S–mRNA–$tRNA_f^{Met}$ complex, the ribosome being trapped in a preternary 30S–mRNA–$tRNA_f^{Met}$ complex.**

A number of prokaryotic and phage RNA-binding proteins are controlled by a translational feedback mechanism that allows modulation of the protein synthesis rate with respect to the intracellular concentration of its substrate RNA (for reviews, see refs. 1–4). It is commonly assumed that the regulatory mechanism proceeds through the binding of the repressor protein to the mRNA, in a target region generally near or overlapping the ribosome loading site. Up to now, regulation was believed to proceed through a simple mechanism of competition between the repressor and the ribosome. Such a mechanism has been experimentally supported for *Escherichia coli* threonyl-tRNA synthetase, which was shown to prevent the formation of the ternary 30S–mRNA–$tRNA_f^{Met}$ complex (5) and the binary 30S–mRNA complex (P. Romby, personal communication). An alternative repression mechanism has been postulated by Draper (3) in which the repressor traps the ribosome on its initiation site and prevents further elongation steps. However, no direct evidence has been provided yet for the existence of such a mechanism.

The expression of *E. coli* ribosomal protein S15 was shown to be negatively controlled at the translational level by a feedback mechanism and the regulatory site was located in the leader of the mRNA overlapping the ribosome loading site and the first codons (6). We have shown (7) that the regulatory region folds into three domains (Fig. 1). The first and second domains in the 5′ part of the mRNA leader correspond to very stable stem–loop structures. The third domain can fold into two alternative conformations. One corresponds to a stem–loop structure (with the Shine–Dalgarno sequence engaged in the stem). The second results in a pseudoknot structure, involving base pairing of nucleotides from domain III and nucleotides in the loop of domain II. Both genetic and biochemical experiments suggest that these two conformations are in a dynamic equilibrium and that the binding of S15 stabilizes the pseudoknot form (7, 8). The pseudoknot is assumed to be the essential element of the regulatory mechanism, since mutations preventing its formation abolish the autocontrol (6–8). In contrast, a mutation leading to the stabilization of the pseudoknot (replacing C^{15} with G) does not alter the control. In the present work, we use "toeprint" and footprint experiments to investigate how the binding of S15 to its target site affects the formation of the translational initiation complex and demonstrate a mechanism of regulation in which the protein blocks the ribosome in a preinitiation complex.

MATERIALS AND METHODS

Preparation of the Biological Material. The wild-type and CFP5517 mutant RNAs were transcribed from a construction resulting from the fusion between the *rpsO* and *lacZ* genes that slightly differ from the previously described fusions (6). The present CFP5517 mutant corresponds to CFP5516 (7) except that it is in a fusion that contains the first 16 codons of *rpsO* and lacks the additional 5′ sequence that was present in the previous fusion. Briefly, a *Hpa* I site was created in the 5′ end of the S15 mRNA by replacing C^{-101} with G. A *Sal* I site was also introduced after the 16th codon by replacing TG at positions +51 and +52 with AC. The *Hpa* I–*Sal* I fragment was cloned into *Sma* I–*Sal* I sites of Bluescribe. After elimination of the short fragment located between the *Eco*RI and *Hpa* I sites, DNA was purified by CsCl equilibrium centrifugation. In this construction, 7 nt at the 5′ end of S15 mRNA were replaced by 11 nt from the Bluescribe vector. The plasmid was linearized by *Hin*dIII and transcribed by T7 RNA polymerase as described (7). The RNA transcripts were purified by filtration on a Bio Sil TSK 250 HPLC gel column followed by ethanol precipitation. The mRNA precipitate was resuspended in buffer TP (20 mM Tris acetate, pH 7.5/60 mM NH_4Cl/10 mM magnesium acetate/3 mM 2-mercaptoethanol) and renatured prior use by incubation at 42°C for 10 min in the appropriate buffer before cooling on ice. Protein S15 was fractionated as described (9). *E. coli* 30S subunits

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Abbreviation: MMLV, Moloney murine leukemia virus.

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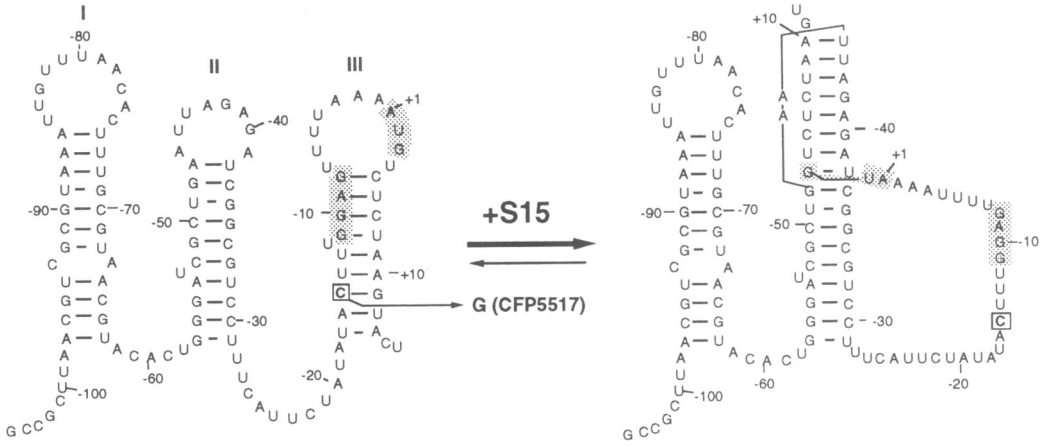

FIG. 1. Postulated equilibrium between the two alternative secondary structures adopted by the wild-type mRNA. The equilibrium is shifted to the pseudoknot conformation as a consequence of S15 binding. The structure is from Philippe *et al.* (7). In CFP5517, a weak helix may pair AUCUUA^{-25} to UGAGGUU^{-13}, with A^{-9} bulging out (7). The Shine–Dalgarno sequence and the initiation codon are shadowed in both structures. The CFP5517 mutations is boxed.

were prepared by a procedure adapted from ref. 10 and incubated for 15 min at 37°C prior use. Initiator tRNA$_f^{Met}$ was 5′-labeled as described (11).

Extension Inhibition (Toeprint) Assays. Toeprint experiments were adapted from Hartz *et al.* (12). In a standard experiment, mRNA (24 nM), the labeled primer (complementary to nt +47 to +59), 30S subunits (200 nM), all four dNTPs (each at 50 μM), and S15 (at the specified concentration) were added to 10 μl of buffer TP and incubated for 10 min at 20°C and then for 5 min at 37°C. When specified, nonaminoacylated tRNA$_f^{Met}$ was added at a final concentration of 2 μM, and the mixture was incubated for 5 min at 37°C. Primer extension was conducted with 1 unit of Moloney murine leukemia virus (MMLV) reverse transcriptase (Bethesda Research Laboratories) for 15 min at 37°C. The reactions were stopped by the addition of 10 μl of loading buffer (deionized formamide/1% xylene cyanol/1% bromophenol blue) and then heated to 90°C for 2 min. The mixture was loaded on a 10% polyacrylamide/8 M urea gel and electrophoresed at 1200 V for 2 h.

Filter Binding Assays. The binding of the labeled tRNA$_f^{Met}$ (10 nM and 80,000 cpm) to the 30S–mRNA complex was measured in 50 μl of buffer TP2 (TP/0.02% bovine serum albumin) containing mRNA (0.3 μM) and 30S subunit (0.4 μM). The concentration of the repressor protein was varied from 0 to 3 μM. Each sample was filtered through a nitrocellulose membrane (Millipore GS; 0.22 μm, pore size). The filters were washed with 1 ml of TP/80 mM KCl and dried, and radioactivity was measured.

Footprint Experiments. RNase T1 footprint analysis was conducted on mRNA in the following stages: (*i*) in its naked form, (*ii*) in the presence of S15, and (*iii*) in the presence of 30S subunit and tRNA$_f^{Met}$ (ternary complex) with or without S15. For a standard incubation: mRNA was 30 nM, 30S subunit was 0.1 μM, initiator tRNA was 0.5 μM, S15 was 0.3 μM, and incubation was in 20 μl of buffer TP2. The various components were first incubated for 20 min at 20°C and then at 37°C for 5 min. The incubation was continued for 10 min at 37°C in the presence of initiator tRNA. Hydrolysis with RNase T1 (5×10^{-4} unit) was for 5 and 8 min at 37°C. The reaction was stopped by phenol extraction and the RNA was precipitated with ethanol. Cleavage was detected by primer extension with avian myeloblastosis virus reverse transcriptase as described (7).

RESULTS

Protein S15 Increases the Formation of the Binary 30S–mRNA Complex. The formation of the ternary 30S–mRNA–tRNA$_f^{Met}$ initiation complex was shown to proceed through the transitory formation of preinitiation complexes, such as binary 30S–mRNA complexes (12, 13). Toeprint experiments using MMLV reverse transcriptase under suboptimal conditions (low temperature or concentration) allow detection of the transient binary complexes and ternary complexes (12). We investigated the formation of the binary 30S–mRNA complex with MMLV reverse transcriptase at 7°C and high concentration (20 units; results not shown) or at 37°C and low concentration (1 unit; Fig. 2*a*). A strong toeprint signal was observed at position +10 (with adenine of the AUG initiation codon at position +1), accompanied with weak variable signals at positions 9, 11, 13, and 14. These toeprint positions differ from those reported by Hartz *et al.* (12). The major stop (position +10) coincided with the 3′ terminus of the pseudoknot structure, suggesting that the pseudoknot was stabilized by the 30S subunit and prevents reverse transcriptase elongation. When the concentration of reverse transcriptase was further increased (20–200 units at 7°C), the toeprint at position +10 decreased and new toeprints appeared close to the Shine–Dalgarno sequence (results not shown) in positions similar to that described by Hartz *et al.* (12). Strong stops were not observed on the naked wild-type or CFP5517 mRNAs, even if the latest was stabilized in the pseudoknot conformation (Fig. 2 *a* and *c*), suggesting that the pseudoknot by itself failed to stop reverse transcriptase under the conditions used.

Strikingly, the toeprint signal was not reduced by the addition of increasing concentrations of S15 but was rather increased (Fig. 2*a*). Note that a strong stop was present below the full-length transcript of the wild-type RNA, and at a lesser extent in CFP5517. This band mapped at C^{93} near the junction of the 11 nt from the Bluescribe vector added at the 5′ end of the RNA and seemed to be slightly increased in the presence of S15. We assume that it represents an experimental artifact, since the full-length product decreased with S15 concentration, as expected. Our result strongly suggests that S15 does not prevent the formation of the binary complex but increases its formation.

We also checked whether S15 itself was able to induce stops around position +10 by stabilizing the pseudoknot conformation. Indeed, the addition of increasing concentrations of S15 to the mRNA in the absence of 30S subunit induced the appearance of a cluster of stops at positions +10, +11, +13, and +14 that increased with S15 concentration, but to a lower extent than in the presence of the 30S subunit. This was observed with both wild-type mRNA and CFP5517 (for CFP5517, see Fig. 2*c*). Thus, these observations indicate that both S15 and 30S subunit stabilize the pseudoknot

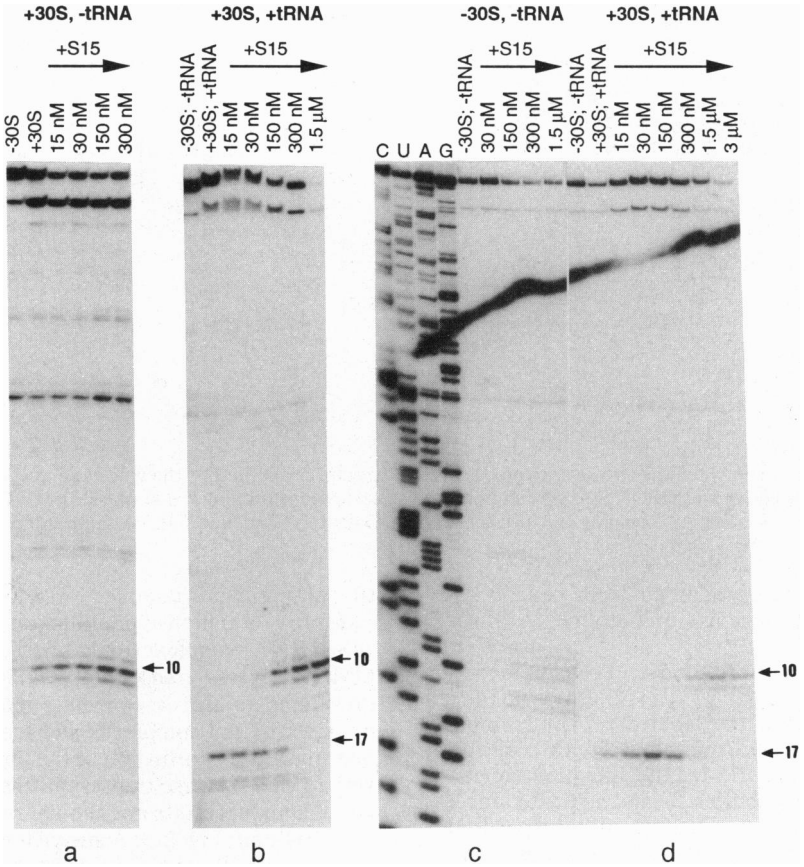

FIG. 2. Effect of the addition of protein S15 on the formation of the binary and ternary complexes. Toeprint experiments were conducted with MMLV reverse transcriptase at 37°C; the stops at position +17 (ternary complex) and at position +10 (binary complex) are indicated by arrows. (*a*) Formation of the wild-type mRNA–30S binary complex, with increasing concentrations of S15 as indicated. (*b*) Formation of the wild-type mRNA–30S–$tRNA_f^{Met}$ ternary complex. Effect of addition of increasing concentrations of S15 on MMLV reverse transcriptase elongation of free CFP5517 mRNA (*c*) and on the formation of the CFP5517 mRNA–30S–$tRNA_f^{Met}$ ternary complex (*d*). The concentrations of S15 are indicated. C, U, A, and G are sequencing lanes. In *c* the apparent increase of the toeprint at position +17 at low S15 concentration is an experimental artifact due to unequal amounts of material in the various lanes. Indeed, the intensity of the toeprint relative to the full-length transcript progressively decreases in all experiments.

conformation and have cumulative effects when added together.

Protein S15 Entraps the Ribosome in a Preternary 30S–mRNA–tRNA Complex. The ternary complex stopped MMLV reverse transcriptase at nt +17. When the ternary complex was formed in the presence of increasing concentrations of protein S15, the stop at nt +17 progressively disappeared, indicating that S15 inhibits the formation of the ternary complex (Fig. 2*b*). However, the disappearance of the ternary-complex-specific toeprint was accompanied by the simultaneous appearance of new bands that coincided with the same toeprint signals induced by both 30S subunit and S15. This observation and the fact that S15 seemed to stabilize the binary complex highly suggest that S15 does not occlude 30S binding but traps the 30S subunit on its mRNA in an intermediary stage and prevents the formation of the active ternary complex.

RNase T1 footprint experiments were further used to directly confirm the simultaneous presence of the 30S subunit and S15 on the mRNA in a preinitiation complex. As shown in Fig. 3, binding of S15 enhanced the cleavage of G^8, G^{10}, and G^{11} in the Shine–Dalgarno sequence, whereas cleavage of G^{40} and G^{42} was strongly reduced. As expected, the formation of the ternary complex inhibited the cleavage in the Shine–Dalgarno sequence. More surprising was the enhancement of cleavage at G^{40} and G^{42} and the appearance of new cleavage sites at G^{35} and G^{36} that probably reflect conformational rearrangements of the mRNA. When the ternary complex was formed in the presence of S15, the signature of S15 (no cleavage at G^{40} and G^{42}) was observed with the signature of the 30S subunit (no cleavage in the Shine–Dalgarno sequence). However, the cleavage at G^{35} and G^{36} induced by the 30S subunit was not observed. It should be noted that the addition of S15 alone was found to increase the intensity of pauses and nonspecific cleavage (e.g., U^4, C^{26}, U^{29}, and U^{49}), whereas this effect was not observed in the presence of 30S subunit. The reason for this behavior is not clear. However, the results concerning specific RNase T1 cuts were unambiguous and we conclude from these footprint experiments that S15 and 30S subunit simultaneously bind to the same mRNA molecule.

The next question was whether the initiator tRNA was able to form a preternary complex in which it would bind to the ribosome without base pairing with the initiation codon, as postulated by Gualerzi and Pon (13, 14) and Hartz *et al.* (12). Since such a preternary complex is indistinguishable from the binary complex in toeprint experiments, we used a nitrocellulose filter retention to test the possible binding of 5′-labeled $tRNA_f^{Met}$ to the 30S–mRNA–S15 complex (Fig. 4*a*). In these experiments, tRNA was kept in limiting amount and the filters were washed in the presence of KCl to avoid nonspecific retention. When mRNA was omitted, ≈5% of the tRNA input was retained on the filter, in the presence or in the absence of S15. When mRNA was added, tRNA retention was increased to 15% of the input, reflecting the formation of the ternary complex. The presence of increasing concentrations of protein S15 up to 5 μM did not reduce tRNA binding.

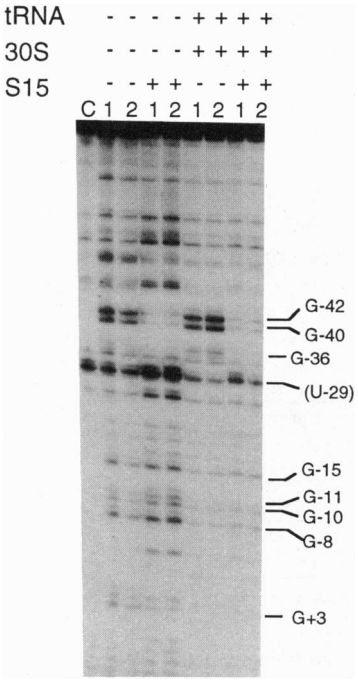

FIG. 3. RNase T1 footprint experiments on CFP5517 mRNA. Hydrolysis with RNase T1 was for 5 (lanes 1) or 8 (lanes 2) min. Lane C corresponds to the incubation control. Note that lane 1 of free mRNA (-tRNA, -30S, -S15) contains a little more radioactive material than the other ones, thus accounting for the general increased intensity of bands in this lane. The strong stop at U^{-29} corresponds to nonspecific cleavage.

This experiment provides evidence for the presence of $tRNA_f^{Met}$ on the 30S–mRNA–S15 complex. A negative control was provided by conducting the same experiment with threonyl-tRNA synthetase and its own regulatory mRNA region (P. Romby, personal communication). Since threonyl-tRNA synthetase is known to inhibit its own translation by competing with the ribosome (5), the retention of initiator tRNA was expected to decrease upon addition of the enzyme. Indeed, the binding of tRNA did decrease with the addition of threonyl-tRNA synthetase (Fig. 4*b*). Therefore, our results indicate that the ribosome is trapped in the presence of S15, not only at the 30S–mRNA binary complex stage but also in a 30S–mRNA–$tRNA_f^{Met}$ preternary complex.

It was postulated (12, 14) that the 30S–mRNA–tRNA preternary to ternary complex transition requires a conformational rearrangement. When we tested whether S15 was able to bind to the preformed ternary complex, we observed that S15 was unable to bind to the preformed ternary complex (results not shown). Since the formation of the ternary complex is almost irreversible (15, 16), this experiment suggests that the mRNA in the ternary complex is not recognized by S15 (probably due to the disruption of the pseudoknot, as supported by footprint experiments). Therefore, S15 should play its inhibitory effect in an early stage of the formation of the initiation complex, before the formation of the active ternary complex.

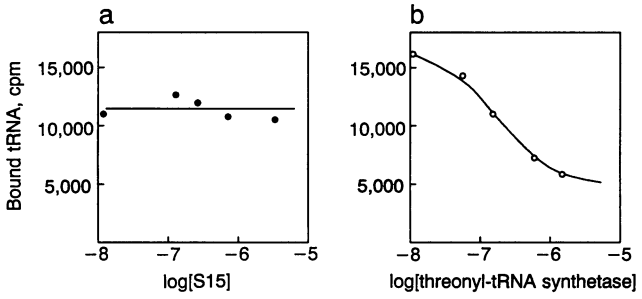

FIG. 4. Effect of the addition of protein S15 on the binding of $tRNA_f^{Met}$ to the 30S–mRNA binary complex. 30S subunits (0.4 μM), wild-type mRNA (0.3 μM), and 5′-^{32}P-labeled initiator tRNA (10 nM) were incubated in buffer TP2 in the presence of increasing amounts of S15. The retention of labeled tRNA was measured. The bound tRNA (cpm) is plotted as a function of the concentration of S15 (*a*). A similar experiment is shown as a negative control, using the ThrS mRNA translational operator (5) and increasing concentrations of threonyl-tRNA synthetase in identical conditions (*b*).

DISCUSSION

In the present work, we provide evidence that ribosomal protein S15 does not regulate its own translation by preventing the ribosome from binding to its mRNA loading site, as described and proposed for many other translational repressors (5, 19–21). Previous experiments strongly suggested that the mRNA operator can alternatively adopt a stem–loop structure in equilibrium with a pseudoknot structure and that S15 stabilizes the pseudoknot (7). Toeprint experiments indicate that the 30S subunit and S15 stabilize the pseudoknot. Indeed, mRNA-bound 30S subunit stops MMLV reverse transcriptase on the 3′ side of the pseudoknot (position +10), instead of on the 3′ side of the Shine–Dalgarno sequence, as described by Hartz *et al.* (12). This can be compared to the α operon mRNA, which was shown to adopt a complex pseudoknot structure (17, 18) that is also stabilized by the 30S subunit (15). In the latter case, MMLV reverse transcriptase pauses over a space of 7 bases near the 3′ terminus of the pseudoknot 30 nt downstream from position +1.

Furthermore, our experimental data indicate that S15 and 30S subunit bind simultaneously to the mRNA operator and that the initiator tRNA is able to join this complex. However, the resulting complex does not yield the toeprint specific for the ternary complex but still gives a toeprint at position +10. Therefore, repression should occur at one early step of the translation initiation process by preventing the formation of a functional ternary 30S–mRNA–$tRNA_f^{Met}$ complex, the ribosome being trapped in a 30S–mRNA–initiator tRNA preternary complex. This is experimental evidence for the existence of such an intermediary complex that was suggested by Gualerzi and Pon (13, 14) and Hartz *et al.* (12). Interestingly, our experiments clearly show that the pseudoknot conformation displays the unexpected property of providing determinants for both repressor and ribosome recognition. This is fundamentally different from another possible regulation mechanism also proceeding through dynamic changes in mRNA structure, in which the repressor recognizes one conformer and the ribosome recognizes another one (19).

A model of regulation that accounts for the different experimental observations is summarized in Fig. 5. The two alternative conformations are presented schematically, the pseudoknot being stabilized by the binding of S15. The model implies that the pseudoknot binds to the 30S subunit and is even stabilized. It should be stressed that in the stem–loop structure, the Shine–Dalgarno sequence is sequestered in base pairing, while it is not involved in a stable interaction in the pseudoknot structure. Furthermore, pairing between the Shine–Dalgarno sequence and the 3′-terminal sequence of 16S rRNA should occur without disrupting the pseudoknot structure. Consistently, the CFP5731 mutant lacking nt −54 to −32, in which nt −17 to +13 are stabilized in the stem–loop conformation (7), binds the 30S subunit with a highly reduced association rate (results not shown). The intermediary preternary complex is indicated. The pseudoknot structure is assumed to persist and the codon–anticodon interaction probably does not take place. In the absence of S15, mRNA

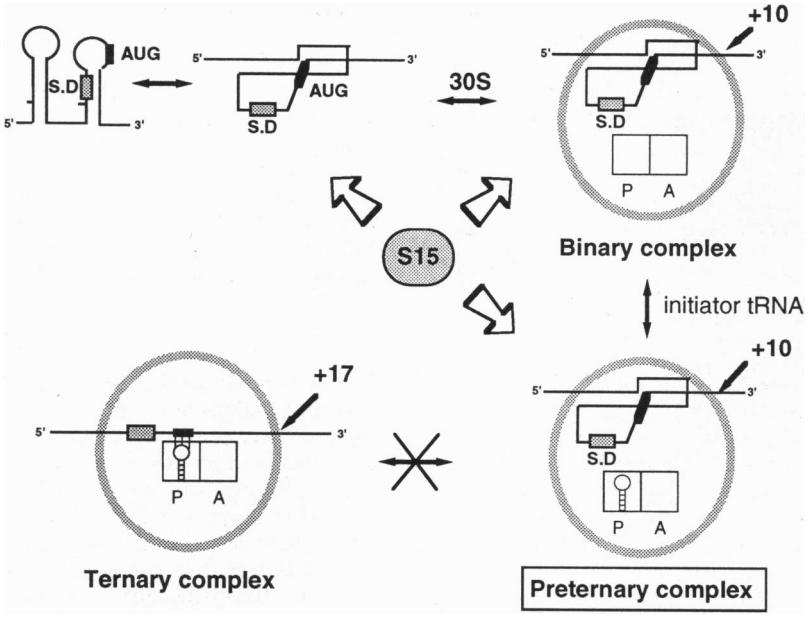

FIG. 5. Model for the regulatory mechanism of translational regulation of protein S15. The stem–loop and pseudoknot conformations are shown, the Shine–Dalgarno sequence (S.D.) and AUG initiation codon are indicated; the ribosomal A and P sites are indicated by boxes, and the initiator tRNA is shown by a stem–loop. The position of the toeprint is indicated by arrows.

rearrangement occurs, allowing formation of the codon–anticodon interaction and leading to the active ternary complex. In the presence of S15, the preternary complex is stabilized, preventing the formation of the ternary complex.

We could identify the precise step of the translational initiation process that is blocked by the binding of the repressor protein. The reason why the preternary to ternary complex transition cannot occur remains unclear. One possible cause of this inactivation would be the inability of the initiator tRNA to form the codon–anticodon interaction, as the result of the proximity of the pseudoknot or of an incorrect position of the mRNA on the subunit. As an alternative possibility, the codon–anticodon interaction can form but the rearrangement of the mRNA is prevented. It should also be pointed out that these experiments were done in simplified *in vitro* systems in the absence of initiation factors, whereas in the cell the translational initiation steps are kinetically controlled by initiation factors (14).

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