[RNA Biology 1:1, 66-73; May/June 2004]; ©2004 Landes Bioscience

Research Paper

Molecular Mimicry in Translational Regulation

The Case of Ribosomal Protein S15

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Received 03/19/04; Accepted 05/05/04

Previously published online as a *RNA Biology* E-publication:
http://www.landesbioscience.com/journals/rnabiology/abstract.php?id=958

KEY WORDS

translational regulation, ribosomal protein S15, RNA-protein interactions, molecular mimicry, evolutionary constraints.

ACKNOWLEDGEMENTS

This work was supported by the CNRS and a NIH grant CA46778 to D.P.

ABSTRACT

Ribosomal protein S15 is highly conserved among prokaryotes. It plays a pivotal role in the assembly of the central domain of the small ribosomal subunit and regulates its own expression by a feedback mechanism at the translational level. The protein recognizes two RNA targets (rRNA and mRNA) that share only partial similarity. Its interaction with 16S rRNA has been fully characterized, while mRNA interactions and regulatory mechanisms have been extensively studied in *E. coli* and in *T. thermophilus*. Recently, we have characterized which aminoacids are involved in *E. coli* mRNA recognition, using an in vivo assay allowing to identify S15 mutations affecting the S15-mRNA interactions without altering 30S subunit assembly. Here, we address the following questions: Are common determinants used by S15 to recognize its rRNA and mRNA targets? What is the extent of molecular mimicry? Is the regulatory mechanism conserved? Our results indicate that specific recognition of mRNA and rRNA relies on both mimicry and site differentiation. They also highlight the high plasticity of RNA to adapt to evolutionary constraints.

INTRODUCTION

Translational regulation is used by bacteria and phages to rapidly adapt protein expression to their need as a function of growth and environmental variations. Initiation of translation is frequently challenged by RNA-binding proteins that recognize specific targets on mRNAs. This type of control is facilitated by the fact that transcription and translation are tightly coupled, rendering nascent mRNA transcripts easily accessible to ribosomes and regulatory proteins, and allowing a high flexibility in the control process at a minimal energetic cost. In this line, the synthesis of ribosomal components is tightly coordinated. Transcription of rRNAs is controlled and adapted to growth rate,[1] while the ribosomal protein operons are regulated by a feedback mechanism allowing to adjust and coordinate the synthesis of ribosomal proteins to the rate of rRNA transcription.[2,3] The similarities observed between mRNA and rRNA targets for a number of *E. coli* ribosomal proteins, such as S7, S8, L1 (reviewed in ref. 3) and L20,[4] sustain the mechanism based on mimicry and competition previously proposed by Nomura.[2] However in other cases, i.e., *E. coli* proteins S4[5,6] and L4,[7,8] analogies between both target RNAs could not be obviously detected, at least at sequence and secondary structure levels. This was the case for *E. coli* ribosomal protein S15 (EcS15), which was shown to regulate the expression of its own mRNA by a feedback mechanism at the translational level.[9] This protein recognizes two RNA targets (rRNA and mRNA) that share only partial similarity.

Ribosomal protein S15 is highly conserved among prokaryotes. It plays a pivotal role in the assembly of the central domain of the small ribosomal subunit[10,11] and its interaction with 16S rRNA has been fully characterized (see below). Besides, S15-mRNA interactions and regulatory mechanisms have been extensively studied in *E. coli*,[9,12-18] and more recently in *T. thermophilus*.[19] Here, we will address the following questions: Are common determinants used by S15 to recognize its two rRNA and mRNA targets? What is the extent of molecular mimicry? Is the regulatory mechanism conserved?

BINDING OF S15 TO CONSERVED 16S rRNA ELEMENTS

After its localization in the central domain of 16S rRNA,[20-22] the 16S rRNA binding site of *E. coli* S15 (EcS15) has been characterized by footprinting techniques,[23-25] and a minimum binding site was defined in closely related species.[26,27] The interaction of S15 with 16S rRNA has then been characterized at high resolution, thank to the crystal structure of isolated complexes containing *T. thermophilus* S15 (TtS15),[28,29] and of the small

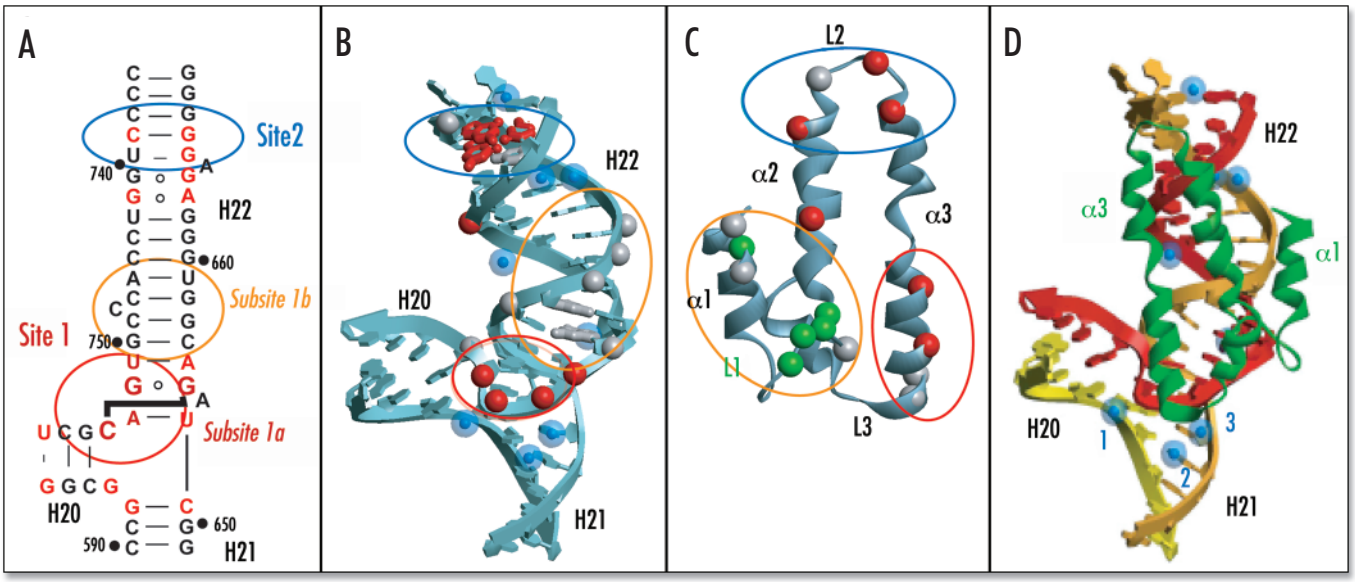


Figure 1. The 16S rRNA-TtS15 interaction. (A) Secondary structure of the16S rRNA site. Only nucleotides from the minimum binding site defined by Serganov et al[27] are shown. Bases in red are > 95 % conserved in more than ~ 6000 procaryotic sequences. The rRNA sites and subsites discussed in the text are indicated. (B) Structure of the rRNA in the TtS15/6S-rRNA complex with interacting sites indicated, adapted from Nikulin et al.[29] The ribose-phosphate skeleton is shown as a ribbon and magnesium ions as blue spheres. Contacting nucleotides are indicated. Highly conserved nucleotides are shown in red, as (A). Ribose-phosphate interactions are denoted by spheres and bases specifically interacting are highlighted. (C) Structure of TtS15 in the TtS15/6S-rRNA complex with interacting sites indicated, adapted from Nikulin et al.[29] Contacting aminoacids are shown by spheres. Aminoacids conserved among bacterial sequences are denoted by red spheres and those additionally conserved in plastids, Archaea and Eukarya are in green. Nonconserved aminoacids are shown as grey spheres. (D) The crystal structure of TtS15/6S-rRNA complex, from Nikulin et al.[29]

ribosomal subunit.[30,31] The *E. coli* complex, which is closely related to the *T. thermophilus* one, could be modeled.[32] It turns out that S15 recognizes two distinct sites on 16S rRNA. Site 1 can be divided into two subsites (subsite 1a and 1b, Fig. 1A). Subsite 1a is part of a three-way junction between helices H20, H21 and H22, constrained by an invariant C-G•G base triple.[28,29,32] Strikingly, crystallographic data unambiguously indicated that the nucleotides that built the three-way junction do not provide specific sequence determinants, but rather trigger a unique tertiary fold of the RNA backbone that is recognized by S15. The particular bent conformation of the backbone between nucleotides 752 to 755 is recognized by side chain aminoacids located in helix α3 (subsite 1a, Fig. 1B). Subsite 1b in Figure 1B, which corresponds to the shallow groove of helix H22 (655-657/749-751), is recognized by aminoacids located in loop 1 between helices α1 and α2, and the N-terminal part of α2 (Fig. 1). Mutagenesis studies indicated that any mutation in the C-G•G base triple (in subsite 1a) is deleterious for S15 binding, while mutations in subsite 1b are tolerated.[32] The second site (site 2), located one helical turn from the three-way junction in helix H22, consists of a conserved G•U/G-C motif[26,27,32] (Fig. 1). Binding at site 2, which relies on sequence specific recognition of a conserved G-U/G-C motif, only provides a minor contribution to binding and depends on proper binding at site 1.[32] Binding at both sites triggers conformational adjustments that are required for subsequent binding of protein S6 and S18.[11,28,32] Thus, the role of site 1 is to anchor S15 to the rRNA, while binding at site 2 is aimed to induce a cascade of events required for subunit assembly.

S15 TRANSLATIONAL REGULATION IN *E. COLI*

EcS15 Recognizes a Pseudoknot Motif. EcS15 was shown to regulate the expression of its own mRNA by a feedback mechanism at the translational level.[9] The translational operator overlaps the ribosome binding site and folds into two mutually exclusive structures, one consisting of two stem-loops and the other one forming a pseudoknot[12] stabilized by binding of EcS15. The pseudoknot is made of two helices coaxially stacked (S1 and S2) that are bridged by a single adenine crossing the major groove (L1). The large connecting loop 2 (L2) contains the Shine-Dalgarno sequence and the AUG codon (Fig. 2).

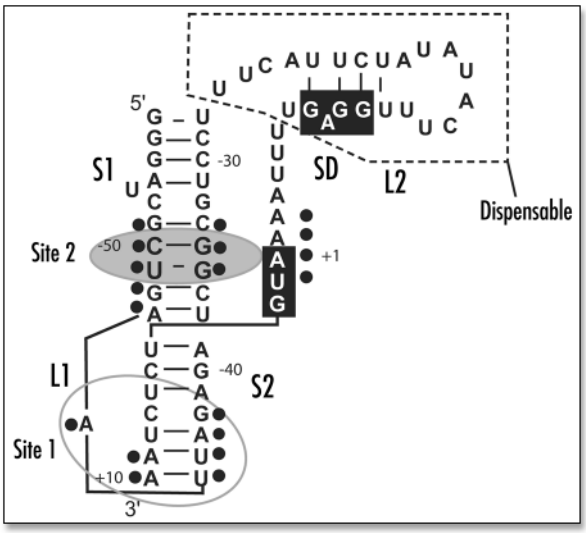


Figure 2. The pseudoknot structure of the *E. coli* mRNA recognized by EcS15. The two sites are indicated. The Shine-Dalgarno sequence (SD) and the AUG initiation codon are shown in the black boxes. Nucleotides are numbered with the A of the AUG codon as +1. Positions protected from hydroxyl radical footprinting[18] are indicated by black dots. Assays to minimize the mRNA binding site showed that the loop 2 region boxed in dotted line is dispensable for EcS15 binding.[18]

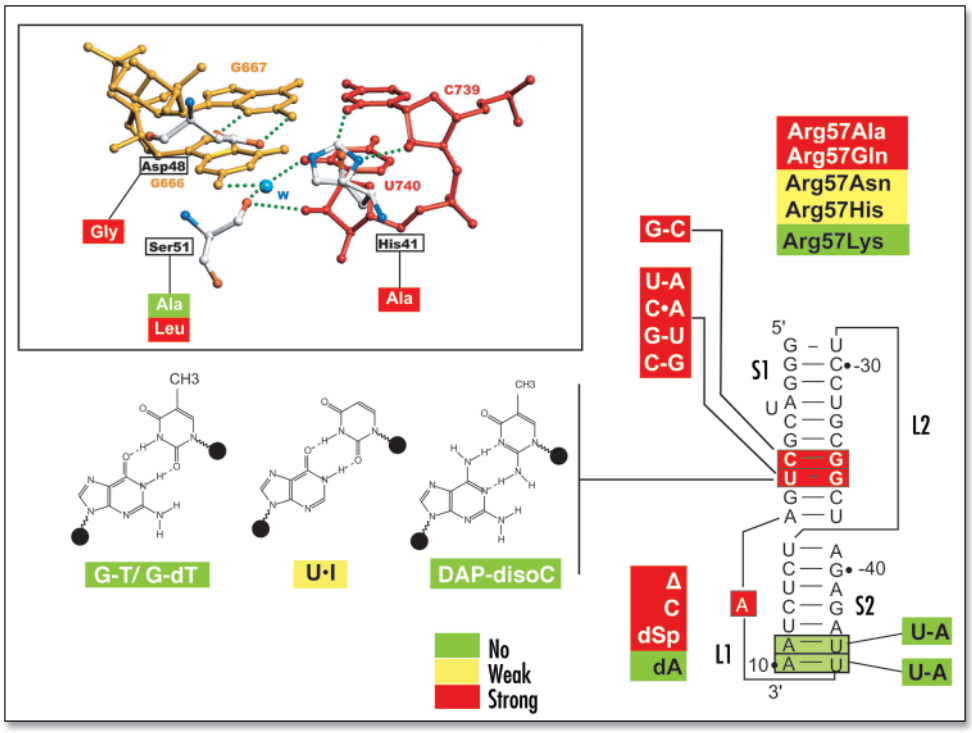
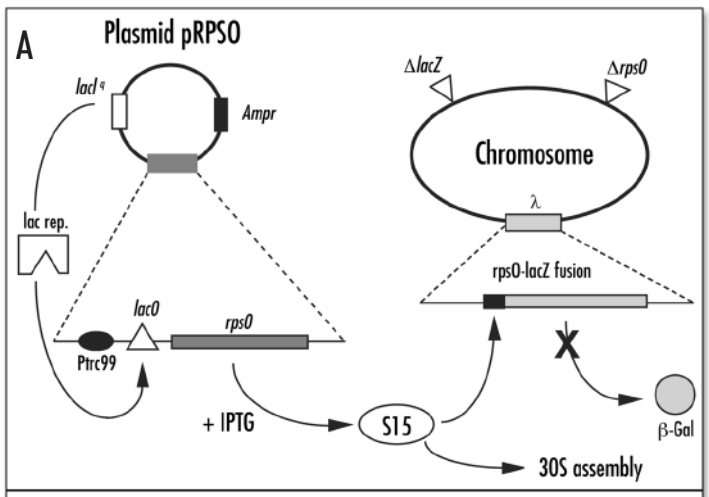


Figure 3 (Above). Effect of mutation and analogue substitution on EcS15 binding, and effect of aminoacid changes on in vivo autocontrol. The base-specific recognition of the G-U/G-G motif in helix 22 of 16S rRNA by aminoacids in loop L2 of S15, taken from the crystal structure of the S15-rRNA complex,[29] is shown in the insert. Nucleotide changes are shown on the minimal secondary structure of the pseudoknot, and the negative effect on S15 binding is indicated as follows: no effect (green), weak effect (yellow), strong effect (red). A schematic representation of the different variants of the G-U pair is indicated (G-T, G-dT, I•U, DAP-d*iso*C). Results are from Serganov et al.[18] The effects of mutation on autocontrol of aminoacids potentially involved in the recognition of the G-U/G-C motif and A(-46) are indicated, using the same color code as above. Results are from Mathy et al.[33]

The requirements for EcS15 recognition and in vivo control were investigated by extensive site-directed mutagenesis. These studies revealed that the pseudoknot stem S2 is absolutely required, without detectable base specificity.[16,18] Otherwise, the G-U/G-C motif present in stem S1 (Fig. 2) was also shown to be essential, and site-directed mutagenesis suggested that it should represent a base specific determinant.[16-18] These results correlate with hydroxyl radical footprinting experiments that suggested the presence of two contacts areas, one comprising the distal part of stem S2 and the bridging A(-46), and the second covering the G-U/G-C motif in stem S1 (Fig. 2).[15,18] No important determinants appeared to be present in loop L2 that bridges the two stems of the pseudoknot through the minor groove, since the size of the loop could be reduced up to 9 nucleotides.[18] The sole apparent similarity between the rRNA and mRNA targets is thus the presence of two distinct binding sites, one of which includes a G-U/G-C motif. However, EcS15 recognizes its mRNA operator with an affinity two orders of magnitude weaker than its rRNA target and the two sites are both required for binding,[18] while only subsite 1a is essential for rRNA binding.

Identification of mRNA and Protein Specific Determinants. Crystallographic data showed that S15 specifically recognizes the minor groove bases of nucleotides in the G•U/G-C motif of the 16S rRNA via three conserved amino acids[28,29]. The side chains of His41 and Asp48 form hydrogen bonds with the functional groups of the G667-C739 pair, while Ser51 contacts the G666-U740 pair via a water molecule, making H-bonds with the exocyclic amino group of G666 and O2 of U740 (insert, Fig. 3). The fact that these amino



B

Transformation \ Phenotype	Cellular growth	rpsO-lacZ fusion expression
+ pTrc99A +pRPSO - IPTG	40°C: + 30°C: –	40°C: 10 000 U 30°C: –
+ pRPSO + IPTG	40°C: + 30°C: +	40°C: 46 U (R = 198) 30°C: 26 U (R = 384)

Figure 4 (Right). Mutagenesis of EcS15 and effect on autocontrol. (A) Strategy used, devised by Mathy et al.[33] The *rpsO* gene is expressed from a plasmid (pRPSO, derived from pTrc99A) carrying the ampicillin resistance gene (Amp^R) and the *lacI*^{q} gene, encoding the *lac* repressor (lac rep). EcS15 is overproduced in the presence of IPTG. EcS15 preferentially binds 16S rRNA and the protein in excess binds the translational operator, located upstream of a *rpsO-lacZ* translational fusion, which is inserted into the chromosome of strain CPFΔS15. Interaction of EcS15 with mRNA prevents translation initiation, resulting in poor translation of the fusion detected by a β-galactosidase assay. The *lacZ* and *rpsO* deletions in the chromosome are indicated. The integrated λ phage carrying the translational fusion is indicated by a grey box and the corresponding chimerical messenger RNA transcribed from the *rpsO* promoter is shown enlarged. (B) Phenotype of the transformed cells. In the presence of pRPSO and absence of IPTG, the synthesis of EcS15 is repressed and cells only grow at 40°C. In the absence of EcS15, β-galactosidase is produced (~10 000 U). The same result is obtained with the empty vector (pTrc99A). In the presence of pRPSO and IPTG, EcS15 is expressed and cells grow at both 30 and 40°C. The synthesis of β-galactosidase is inhibited. The repression rate (R) is the ratio between β-galactosidase values in the absence (pTrc99A) and the presence of S15 (pRPSO).

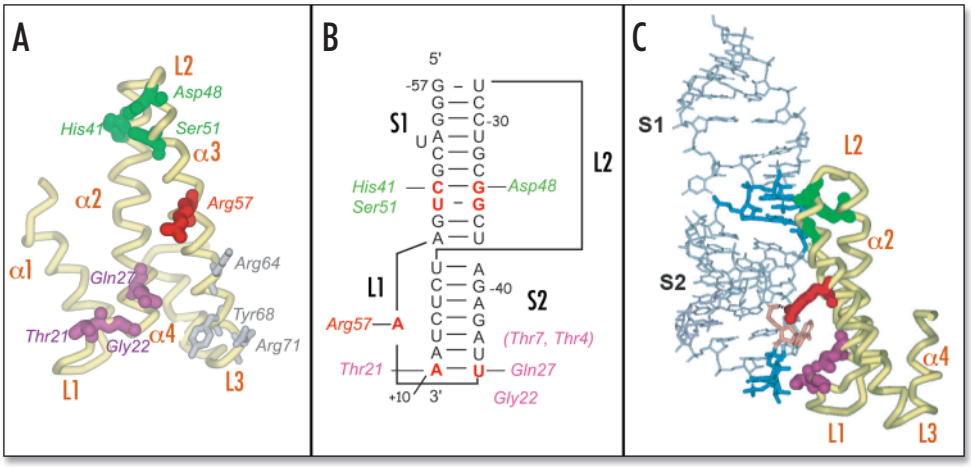


Figure 5. Specificity determinants in EcS15 and its target mRNA (adapted from Mathy et al[33]). (A) EcS15 protein was derived from the crystallographic structure of the *T. thermophilus* complex,[29] with adapted aminoacid changes.[32] The protein backbone is shown by a tube. The lateral chains of amino acids essential for autocontrol are shown in color: green for conserved amino acids that recognize the G•U/G-C motif in both 16S rRNA and mRNA, violet for conserved amino acids that interact with stem S2 of the pseudoknot and helix H22 of 16S rRNA (subsite 1b), and red for Arg57 that interacts specifically with mRNA and not 16S rRNA. Those amino acids that interact with the 16S rRNA three-way junction (subsite 1a) but not with mRNA are shown in grey. (B) mRNA pseudoknot: The nucleotides interacting with EcS15 are shown in red. Loop L2, carrying the initiation codon and the Shine-Dalgarno sequence, is schematized by a line. Amino acids interacting with mRNA are indicated, with the same color code as in (A). (C) Structure model of the EcS15-mRNA complex, from Mathy et al.[33] mRNA is shown in grey, with stems S1 and S2 coaxially stacked (loop L2 being omitted). The nucleotides interacting with S15 through amino acids also recognized by 16S rRNA are highlighted in blue. The bulged A(-46), specifically recognized by EcS15, is shown in red. The stacking of Arg57 on the adenine base ring is visible and the possible contact with the phosphate group indicated by a dashed line. The protein is represented as in (A) with the same color code used for amino acids.

acids are conserved in EcS15 suggested a common mode of recognition of the G-U/G-C motif (site 2) in both mRNA and rRNA. On the other hand, mRNA site 1 did not display any obvious resemblance with 16S rRNA. To determine the extent of mimicry between the two RNA targets, we further characterized the precise nature of specific determinants on both mRNA and protein. This was investigated by using two complementary approaches.

The first one used base analogues substitution of critical nucleotides to test critical functional groups.[18] Analogues were introduced by chemical synthesis into the determined minimal 45-nucleotide fragment (mRNA45). This short RNA preferentially folds into a pseudoknot, and binds EcS15 without loss of affinity.

The second was based on an in vivo assay allowing the identification of EcS15 mutations affecting the S15-mRNA interactions, without altering 30S subunit assembly.[33] Ec-S15 amino acids critical for mRNA binding in vivo, were characterized by site-directed and random mutagenesis of the *rpsO* gene. Experiments were based on the premise that preventing S15 to bind to its mRNA would result in a loss of autocontrol, leading to S15 overexpression.[9,13] The effect of the S15 mutations was followed by inserting a *rpsO-lacZ* translational fusion into the chromosome of a Δ*lac* strain[9] and measuring the level of β-galactosidase after transformation of the strain with a plasmid carrying the mutagenized *rpsO* gene (Fig. 4A). To avoid competition between the wild-type S15 produced by the chromosomal copy of *rpsO* gene and S15 mutants expressed from the plasmid, a Δ*rpsO* deletion was introduced into the Δ*lac* strain carrying the *rpsO-lacZ* translational fusion. This strain, named CPFΔS15, was cold sensitive at 30°C, but grew at 42°C, with a generation time of around 100 min. Thus, active ribosomes can be synthesized in the absence of S15 at 42°C, consistent with previous observations.[34,35] The cold sensitivity phenotype could be complemented by transformation with any plasmid expressing an active S15 protein. The pRPSO plasmid carried the *rpsO* gene under the control of p*Trc*, an IPTG-inducible promoter (Fig. 4A). Without inducer, transcription of the *rpsO* gene was strongly repressed by the Lac repressor synthesized from the *lacI*^{q} gene, while addition of IPTG triggered S15 expression and allowed bacteria to grow at 30°C (Fig. 4B).

The main results are summarized in Figure 3. The deduced potential contacts between EcS15 and the mRNA pseudoknot are shown in Figure 5A and B). The results obtained from protein mutagenesis allowed to improve the structure model previously proposed[33] (Fig. 5C).

The G•U/G-C Motif, a Common Site in rRNA and mRNA? Replacement of His41 and Asp48 by amino acids with shorter side chains (Ala and Gly, respectively) induced a total loss of autocontrol (Fig. 3). This is consistent with the fact that inversion or substitution of the G(-35)-C(-50) pair in the *rpsO*mRNA, the potential analogue of the G667-C739 pair in 16S rRNA, resulted in a loss of S15 binding and autocontrol[17,18] (Fig. 3). Therefore, the recognition of the G-C pair of the G•U/G-C motif by His41 and Asp48 is probably the same in both rRNA and mRNA. Unexpectedly, the replacement of Ser51 by Ala did not affect regulation. This change, resulting in the substitution of a hydroxyl group by a hydrogen atom, should have been sufficient to disrupt interactions of Ser51 with both U(-49) and G(-36), if the same interactions existed in the mRNA (Fig. 3). On the other hand, the loss of regulation caused by replacement of Ser51 with Leu suggests that Ser51 is indeed in close proximity to G(-49)-U(-36). The bulky side chain of Leu most likely causes a steric clash with the neighboring RNA. Remarkably, this effect is consistent with the fact that substitution of G(-36) by inosine (an analogue of G lacking the exocyclic N2 group) and of U(-49) by dT, had only a minor effect on the binding affinity of the mRNA pseudoknot for *Ec*-S15, again indicating that there is no direct contact with the G•U pair[18] (Fig. 4). Otherwise, the replacement of the G•U pair by the isosteric DAP•d*iso*C pair did not affect binding (Fig. 3). Taken together, these observations indicate that the G•U wobble pair in mRNA is recognized by *Ec*-S15 in a similar, but not strictly identical manner to 16S rRNA. Since it cannot be replaced by any other Watson-Crick or non canonical interaction,[16-18] the G•U pair is most likely involved in building a precise conformation of the RNA, essential for binding. Consistent with this observation, the structural environment of this motif is different in both RNAs. In the mRNA, it is part of a standard A helix, while in 16S rRNA it is adjacent to G•A pairs and a bulging A residue.[28,29]

The mRNA Site 1, a Specific mRNA Site? Previous mutagenesis experiments suggested that the bridging A(-46) was important for in

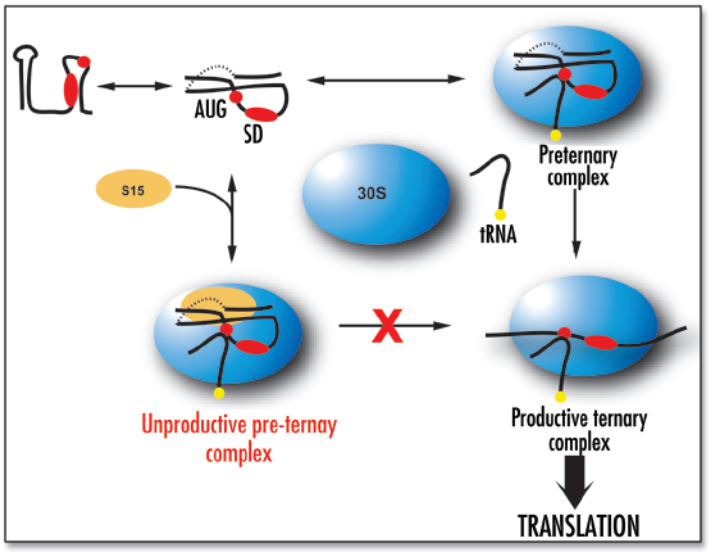


Figure 6. The entrapment model used by EcS15 for autoregulation. In the absence of EcS15, the transient preternary complex is rapidly converted into the ternary complex and translation starts. In the presence of an excess of EcS15, the preternary complex is stabilized, preventing its conversion into the ternary complex, and thus inhibiting translation.[13]

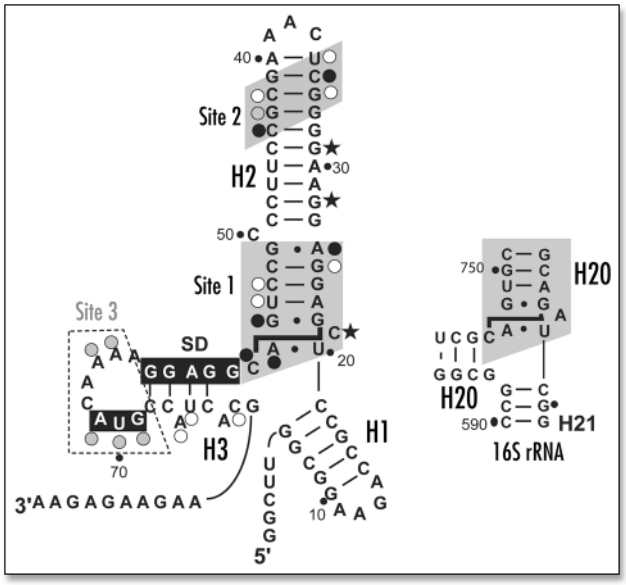


Figure 7. Secondary structure of the ThS15 mRNA target (adapted from Serganov et al[19]). The Shine-Dalgarno sequence and the AUG initiation codon are shown in black boxes. Nucleotides protected from hydroxyl radical induced cleavage are denoted by filled circles (black, grey and white for strong, medium and weak protection, respectively). Increased reactivity is indicated by star. The two sites (1 and 2) required for TtS15 binding are in grey boxes. Site 3, which can be deleted without decreasing the affinity for TtS15 is shown in dotted line. The RNA is thought to adopt a three-way junction like conformation mimicking subsite 1a of 16S rRNA, shown as an insert. The nucleotides building the three-way junction are conserved in both RNAs and their mutation induced similar effects.[19]

vivo regulation,[15,16] but it was not clear whether it was directly recognized by EcS15 or involved in building a precise conformation of the pseudoknot. Then, we showed that the A to C change abolished binding without altering pseudoknot formation.[18] In addition, substitution of dA for A(-46) indicated that the 2'OH of A(-46) is not essential. However, its deletion or its replacement by a deoxyribose phosphate spacer missing the base ring (dSp for deoxy-spacer) abolished binding[18] (Fig. 3). Inasmuch as A(-46) is most likely bulging out, as indicated by its reactivity to chemical probes in the free RNA, and is protected upon EcS15 binding,[15,18] our results were in favor of direct recognition of the base by S15. Based on the proposed model of the EcS15-mRNA interaction,[18] we mutated aminoacids in the vicinity of A(-46).[33] We found that the Arg57Ala and Arg57Asn mutations induced a strong derepression of fusion expression (Fig. 3). Arg57Gln and Arg57His sustained a weak repression, while a high level of repression was restored by Arg57Lys (Fig. 3), suggesting that a basic residue is required at position 57. Interestingly, Arg57 does not make any contact with 16S rRNA, indicating that this aminoacid is involved in a contact specific to mRNA. The bridging A(-46), which has no obvious equivalent in 16S rRNA, appears as a very likely candidate for interacting with Arg57. According to the proposed model (Fig. 5C), the lateral chain of Arg57 may stack on the adenine ring of A(-46) and make a hydrogen bond with its phosphate group.[33]

The Three-way Junction, a Specific rRNA Site? Mutations of helix α3 aminoacids involved in the specific recognition of the particular bent of the rRNA three-way junction (Arg71, Arg64, Tyr68) had no effect on S15 regulation. This indicates that the mRNA does not contain any structural equivalent of the rRNA three-way junction (site 1a). In addition to the specific recognition of the geometry of the three-way junction backbone, contacts were also observed between S15 and the poorly conserved part of the rRNA helix H22 (site 1b). Mutation of Thr21, Gly22 and Gln27, which interact with functional groups of C656-G750/G657-C749 in *T. thermophilus*,[29] and G656-C750/U657-A749 in *E. coli*,[32] all resulted in a strong derepression. These observations suggest that a subset of amino acids, which recognize the minor groove of helix H22 in 16S rRNA (site 1b), are involved in the recognition of a corresponding helical portion of the mRNA pseudoknot. Despite the absence of sequence similarity, this region presumably corresponds to the part of stem S2 of the pseudoknot, which is protected from hydroxyl radical footprinting (Fig. 2). The model indicates that Gln27 and Thr21 can potentially contact the U(-45)-A(+10) base pair in the distal part of stem S2 (Fig. 5B-C).

rRNA and mRNA Recognition Relies on Both Mimicry and Site Differentiation. This study provides a way to correlate results obtained on both S15 RNA targets and to define the extent of mimicry between these two partners. Furthermore, it was possible to distinguish amino acids that are involved in both rRNA and mRNA binding from amino acids that specifically contact rRNA but not mRNA, and conversely, to identify amino acids that specifically recognize mRNA. A remarkable correlation was found between results obtained on both mRNA[18] and protein[33] sides, concerning the recognition of the G•U/G-C motif (site 2) by amino acids His41, Asp48 and Ser51. Taken together, our results indicate that the recognition of this motif is similar but not identical in mRNA and 16S rRNA. Otherwise, site S1 that was suspected from footprinting experiments and mutagenesis data, could be clearly defined. Our results revealed an unsuspected potential mimicry that extends beyond the G•U/G-C recognition, between rRNA subsite 1b and mRNA site 1. Although not sequence specific, this type of mimicry takes advantage of a common topology: a regular helical portion located at a correct distance from the specific G•U/G-C motif. Another important result is the existence of specific contacts between EcS15 and mRNA that do not exist in the S15/16S rRNA complex. This likely corresponds to an interaction between Arg57

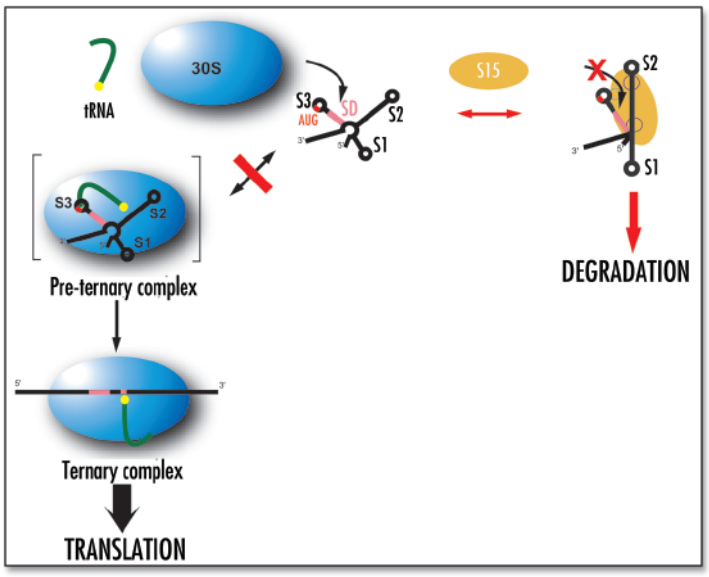


Figure 8. The displacement model used by TtS15 for autoregulation. The model assumes that TtS15 induces a conformational change of the mRNA that masks the ribosome entry site.[19] The mRNA is either bound to TtS15 and degraded, or bound to the 30S subunit and committed to the formation of a productive ternary complex.

and A(-46), which occupies a unique position in the pseudoknot.

EcS15 Inhibits Its Translation Through an "Entrapment" Mechanism. In procaryotes, translation is initiated by binding of 30S subunit and Met-tRNA^{fMet} in a random order to form a transient "preternary" initiation complex (Fig. 6), which is subsequently converted into an irreversible ternary complex.[36] When EcS15 is synthesized in excess over its rRNA target, the free protein is able to bind to its mRNA regulatory site to negatively control its translation. Binding of EcS15 to its mRNA target does not prevent 30S subunit binding but traps the subunit into an incompetent translation initiation complex.[13,14] As a result, the transition from the preternary complex to the productive ternary complex is inhibited (Fig. 6). Repression can be alleviated by 16S rRNA, which is able to displace the bound S15, thus allowing translation to proceed.[37] This mechanism, referred as "entrapment" mechanism, is an alternative to the "displacement" mechanism, in which binding of the repressor competes with ribosome binding.[38] A similar mechanism has also been proposed for the feedback control of the α operon by protein S4.[5] Notably, the location of the Shine-Dalgarno sequence and the AUG codon in loop L2 that is not recognized by EcS15 accounts for the fact that both EcS15 and 30S subunit are able to bind to the mRNA without mutual competition.

S15 TRANSLATIONAL REGULATION IN *T. THERMOPHILUS*

TtS15 Triggers a Three-way Junction Like Conformation in Its mRNA. The S15 gene promotor is one of the strongest in *T. thermophilus*.[39] Recently, we showed that TtS15 specifically binds to its mRNA with high affinity and represses its own synthesis at the translational level.[19] The TtS15 mRNA binding site was characterized by footprinting experiments, deletion analysis, and site directed mutagenesis.[19] It was mapped within the first 81 nucleotides, mainly in the 5' UTR.

Strikingly, the *T. thermophilus* mRNA does not share any resemblance with the pseudoknot structure formed by the *E. coli* mRNA, but consists of an "open" three-way junction (Fig. 7). We found that TtS15 protected three distinct sites from hydroxyl radical cleavage and induced a major conformational change of the mRNA, as revealed by dramatic accessibility changes to RNase hydrolysis.[19]

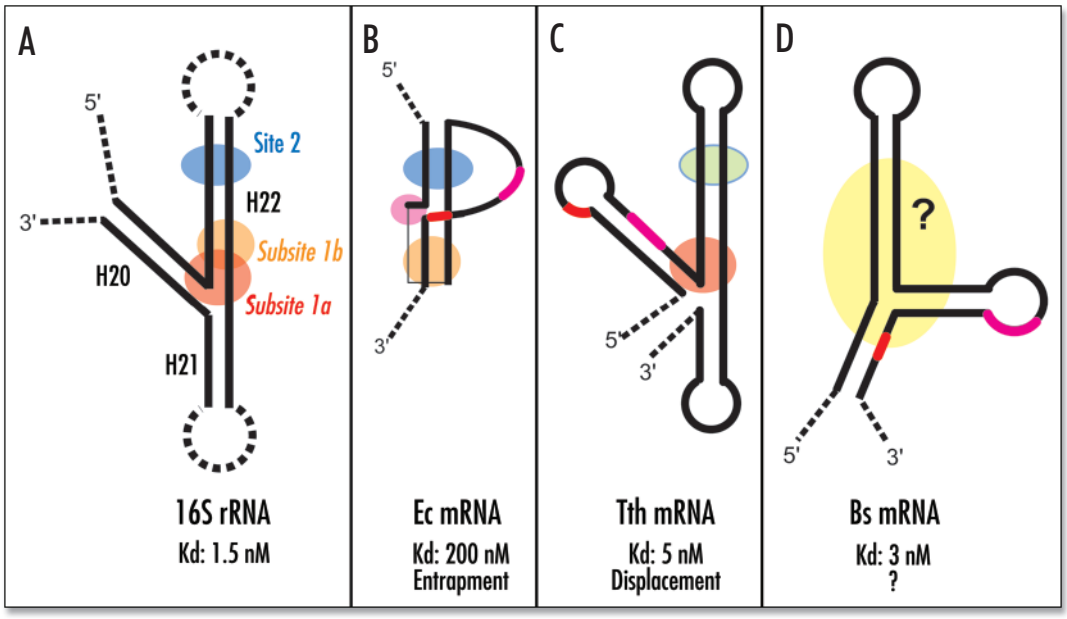


Figure 9. Recognition of rRNA and mRNA targets by S15. The different RNAs are schematized by schematic secondary structure drawings, and the affinity for their cognate S15 is indicated. (A) The *T. thermophilus* 16S rRNA target. The two protein binding sites are indicated: site 1 with subsites 1a (red) and 1b (orange), and site 2 (blue). (B) Proposed pseudoknot structure from the *E. coli* S15 mRNA specifically binding EcS15, with identified protein binding sites.[1,15-18,33] (C) Proposed secondary structure for the *T. thermophilus* S15 mRNA interacting with the TtS15 protein with identified binding sites.[19] (D) Proposed secondary structure of the *S. stearothermophilus* mRNA, adapted from Scott & Williamson.[44] The Shine-Dalgarno sequence and AUG codon are highlighted in purple and red, respectively. The sites (or subsites) that share similarities with rRNA sites are shown by the same color code. The mRNA specific sites that have no homology with rRNA are indicated in different colors. The mode of autocontrol is indicated when known.

Notably, site 1 folds into a 16S rRNA-like three-way junction upon TtS15 binding, and is recognized by the protein as the corresponding rRNA site. Results are supported by striking analogies in nucleotide composition (Fig. 7), and effect of site-directed mutagenesis. In particular, results accounted for the existence of a C57-G22•G55 base triple, analogous to its C754-G654•G752 counterpart in 16S rRNA.[32] By analogy with the rRNA three-way junction, it was assumed that helices H1 and H2 (corresponding to H21 and H22 in rRNA) could coaxially stack, with helix H3 (the mRNA equivalent of H20) making an acute angle with helix H2. However, unlike the rRNA three-way junction, mRNA site 1 is not sufficient by itself to insure stable binding that also requires site 2. This most likely reflects the fact that mRNA helices H1 to H3 form an "open" junction-like junction that is less stable than the rRNA closed junction. Otherwise, site 2 does not share any analogy with the rRNA site 2 and the mimicry is thus restricted to site 1. Unexpectedly, binding at site 3 that overlaps the ribosome loading site does not contribute to the stability of the mRNA complex.[19] Thus, contacts with this site are not required for primary interaction but should result from the S15-induced conformational change and a movement of helix H3 towards helix H2.

Our results suggest that the free mRNA is highly flexible and probably adopts multiple conformations. Thus, it has to undergo adaptive transition(s) in order to bind TtS15. Accordingly, mRNA binds TtS15 at a slower rate than rRNA and the complex cannot form at low temperature.[19] Such an induced-fit mechanism appears to be a common theme in RNA-protein recognition.[40,41]

TtS15 Inhibits Its Translation Through a "Displacement" Mechanism. We also investigated the mechanism of translation inhibition by toeprinting experiments.[19] These experiments indicated that TtS15 and 30S ribosomal subunit compete for mRNA binding. A model was proposed in which binding of TtS15 to its regulatory site should mask the ribosome entry site and prevent ribosome binding (Fig. 8). This model assumes that helices H1 to H3 are not structurally constrained in the absence of TtS15. When TtS15 is in excess over its rRNA target, it binds to its regulatory mRNA site and triggers formation of the rRNA-like three-way junction that renders the ribosome entry site inaccessible. The nontranslated mRNA might be immediately degraded upon forming a complex with the repressor, as shown for the *E. coli* S15 mRNA.[42]

Thus, it turns out that *T. thermophilus* and *E. coli* do not only use different mRNA structures and S15 recognition modes, but also different repression mechanisms (Fig. 9). Moreover, the affinity of TtS15 for its mRNA target is much higher than that of EcS15. This is related to the fact that the entrapment mechanism only needs to stabilize an unproductive initiation complex, and does not require high affinity.[43] By contrast, in the displacement mechanism, the repressor should bind mRNA with an affinity much higher than the 30S subunit, for efficient competition.

S15 TRANSLATIONAL REGULATION— BETWEEN EVOLUTIONARY CONSTRAINS AND PLASTICITY

The case of protein S15 offers an unprecedented opportunity to understand how a highly phylogenetically conserved protein recognizes different RNA ligands. The detailed description of EcS15 and TtS15 binding to their rRNA and mRNA target sites revealed an astonishing diversity at the level of their mRNA regulatory sites, while rRNA recognition is highly conserved (Fig. 9). Remarkably, the two mRNAs contain a bipartite site, while utilization of mimicry with the 16S rRNA target is limited and versatile. Sites 1 and 2 of the *E. coli* mRNA mimic the helical subsite 1b and the G-U/G-C motif of 16S rRNA (site 2, Fig. 9), respectively, while site 1 of the *T. thermophilus* mRNA mimics the rRNA three-way junction (site 1a, Fig. 9). These differences are probably related to the regulation mechanism used, since the three-way junction is expected to provide a higher affinity than the G-U/G-C motif and is therefore more adapted to the displacement mechanism. Notably, the mRNA target for *B. stearothermophilus* S15 (BsS15) was also identified.[44] This RNA binds BsS15 with a high affinity, and contains a closed three-way junction, which shares no similarity with the 16S rRNA target (Fig. 9D). Up to now, the precise RNA binding site(s) and the mechanism of regulation remain to be determined. Thus, it appears that substantial differences should exist in the recognition mode and the related regulatory mechanism.

The use of RNA mimicry in regulatory mechanisms is not restricted to the case of S15. Clear evidence has been provided in several other cases of feedback control (i.e., ribosomal proteins L1 and L4,[45-47] threonyl-tRNA synthetase[48,49]). Our results stress the extraordinary plasticity of mRNAs to fulfill their regulatory functions. This diversity contrasts with the extreme conservation of the rRNA site, which most likely reflects a strong evolutionary pressure, imposed by ribosome assembly constraints. This also suggests that mechanisms responsible for optimizing ribosomal components expression most likely reflect evolutionary constraints and environmental conditions.

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