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tRNA-like structures and gene regulation at the translational level: a case of molecular mimicry in *Escherichia coli*

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***Escherichia coli* threonyl-tRNA synthetase regulates the translation of its own mRNA by binding to it in a region, called the operator, located in front of the ribosomal binding site. The primary and secondary structures of the operator resemble those of the anticodon arm of several $tRNA^{Thr}$ isoacceptor species. We reasoned that if the interaction between the synthetase and its two partially analogous ligands, the tRNA and the mRNA, had some common features, single mutations in the enzyme should affect both interactions in a very similar way. We thus isolated synthetase mutants (called super-repressors) that repress the translation of their mRNA in *trans* to an extreme level, and other mutants that are completely unable to perform any repression. The super-repressors, which are suspected to bind their mRNA with high affinity, are shown to bind the tRNA with an increased affinity. The non-repressing mutants, which are suspected to have lost their capacity to bind the mRNA, are shown to bind their tRNA with less affinity. The binding properties of the mutant enzymes for the other substrates, ATP and threonine, are unchanged. The observed correlation between regulatory and aminoacylation defects strongly suggests that the synthetase recognizes the similar parts of its two RNA ligands—the anticodon-like arm of the mRNA and the true anticodon arm of the tRNA—in an analogous way.**

Key words: operator–repressor interaction/mRNA structure/suppression

Introduction

Several years ago, Ames *et al.* (1983) proposed that tRNA-like structures arose from tRNA gene duplications and subsequent divergence to permit a specific role in gene regulation. More recently, however, it has been suggested, in the case of such structures at the 3′ end of the RNA viral genomes, that they are very ancient remnants of an RNA world and that they were used for tagging RNA genomes to identify them as substrates for replicases and to specify the replication initiation site (Weiner and Maizels, 1987). The present functional tRNAs would have appeared later by removal of these genomic tags.

Among the many macromolecules interacting with tRNA and suspected to be able to do so with tRNA-like structures are the aminoacyl-tRNA synthetases. Besides the well-documented case of the 3′ end of viral RNAs, it has recently been shown that two synthetases are involved in mitochondrial splicing in *Neurospora crassa* (Akins and Lambowitz, 1987) and probably in *Saccharomyces cerevisiae* (Herbert *et al.*, 1988) and it has been proposed that recognition of tRNA-like structures in introns by the synthetases could be responsible for this phenomenon. The case of the *Escherichia coli* threonyl-tRNA synthetase gene (*thrS*) is interesting in that respect since it carries a $tRNA^{Thr}$ anticodon-like arm on its mRNA in front of the ribosomal binding site (Springer *et al.*, 1986; Moine *et al.*, 1988). This anticodon-like structure is known to be an essential *cis* acting site for the regulation of the expression of that gene (Springer *et al.*, 1986). The threonyl-tRNA synthetase binds to the mRNA at and around this site and competes with the 30S ribosomal subunit which binds at the adjacent translation initiation site (H.Moine and C. and B.Ehresmann, unpublished observations). Thus, when the cellular concentration of free synthetase is high, the enzyme binds to the *thrS* mRNA and competes with and excludes ribosome binding. On the contrary, if the synthetase concentration is low, the ribosome competes effectively with the synthetase and the *thrS* mRNA is efficiently translated (Springer *et al.*, 1985). Toeprinting experiments (H.Moine and C. and B.Ehresmann, unpublished) show that the regulation can be reproduced *in vitro* with only four components: the *thrS* mRNA, the initiator $tRNA_f^{Met}$, the synthetase and the ribosome, which are thus suspected to be the main protagonists of the regulatory loop *in vivo*.

The existence of some similarity between the natural substrate of the enzyme (the $tRNA_f^{Thr}$) and its translational operator on the mRNA is not sufficient *per se* to prove that there is a true functional resemblance between the way the synthetase binds to its tRNA and its mRNA. The present work identifies genetically two regions within the synthetase that are essential to the autoregulation. Furthermore, these two regions are shown to be involved also in aminoacylation. The similarity between the control and aminoacylation defects of the different isolated mutants in these two regions strongly indicates that the structural similarity between the anticodon stem of the tRNA and the operator site on the mRNA corresponds to a true functional similarity between the RNA–protein interactions that take place during regulation and aminoacylation.

Results

Isolation of trans-acting regulatory mutants of the thrS gene

As the expression of the *thrS* gene is regulated by the binding of its product to its own mRNA, one should be able to find threonyl-tRNA synthetase structural mutants that are affected in this process. We described, in the present work, the isolation of *trans*-acting *thrS* regulatory mutants falling into two categories. The first category comprises mutants that compensate for defects in the operator (where the $tRNA^{Thr}$ anticodon-like arm is located) and the second consists of

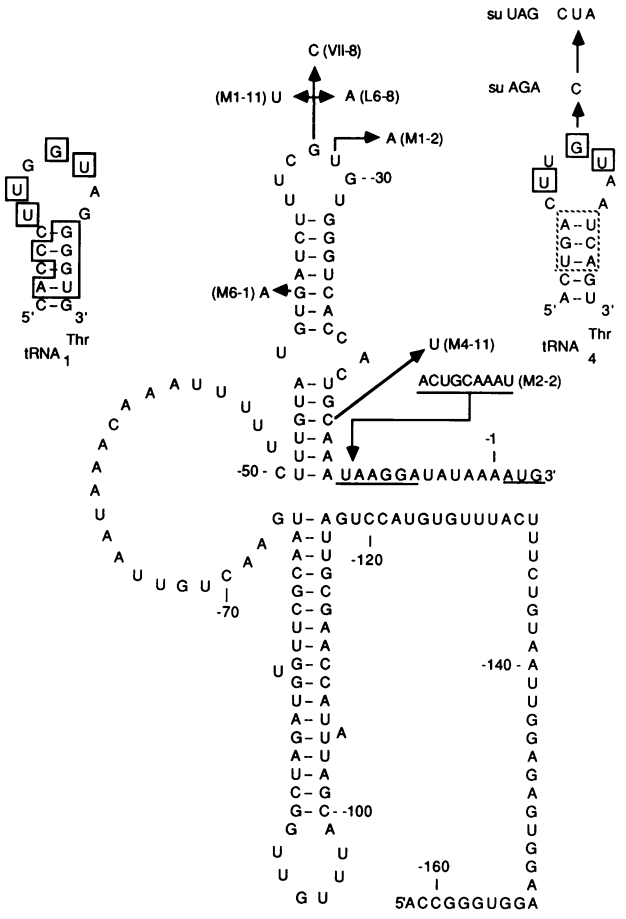

Fig. 1. Secondary structure of the 5′ end of the *thrS* mRNA and of the anticodon arm of $tRNA_1^{Thr}$ and $tRNA_4^{Thr}$. The Shine–Dalgarno sequence and the translation initiation codon are underlined. The sequences of the different tRNAs also found, at equivalent places, in the anticodon-like structure are squared with full lines. In the case of $tRNA_4^{Thr}$, the stem is squared with hyphenated lines to indicate that the identical 3 bp are placed in the mRNA between −23 to −25 and −39 to −41 (over the bulge of the anticodon-like structure).

mutants that were unable to repress the translation of the wild-type (WT) *thrS* mRNA in *trans*. We isolated the regulatory mutants on the multicopy plasmid pUB4 which carries *thrS* without its operator and causes an overproduction of ~50-fold of threonyl-tRNA synthetase. We used previously isolated *thrS–lacZ* protein fusions (Springer *et al.*, 1986) cloned in λ and integrated at *att*λ in the *E.coli* chromosome (carrying a WT copy of *thrS*) for all our selections.

The first category of mutants were isolated as being able to repress operator constitutive mutants of *thrS*, i.e. as mutants of the synthetase able to compensate in *trans* a *cis*-acting defect in the operator of a *thrS–lacZ* fusion. The starting operator constitutive mutations M6-1 and M4-11 affect the stem of the operator (Figure 1). The amount of β-galactosidase synthesized from fusions carrying these mutations is not sensitive to cellular WT threonyl-tRNA synthetase concentration. This is shown in Table I, lines 2 and 3, since in the presence of pUC8 (vector) or pUB4 (carrying *thrS*), the synthesis of β-galactosidase from these fusions is the same. The M6-1 and M4-11 fusions confer a strong Lac^+ phenotype in the presence of pUB4. To find the compensatory mutations, we mutagenized the plasmid pUB4 (see Materials and methods), transformed the two

Table I. Effect of WT and mutant *thrS* alleles (on multicopy plasmids) on β-galactosidase synthesized from WT or mutant *thrS–lacZ* fusions

Operator allele type	Operator allele name	β-galactosidase synthesis			Repression factors	
		pUC8	pUB4	pSS4-11-8	pUC8/ pUB4	pUC8/ pSS4-11-8
WT	WT1	394 (±8)	21 (±2)	0.12 (±0.02)	18	3280
Stem	M6-1	1058 (±74)	900 (±97)	341 (±24)	1.17	3.1
–	M4-11	1980 (±171)	2059 (±377)	69 (±14)	0.96	28.7
Loop	L6-8	2145 (±400)	1991 (±291)	2130 (±320)	1.07	1.0
–	M1-2	1918 (±311)	1557 (±272)	1350 (±159)	1.2	1.4
Operator–RBS insertion	M2-2	2334 (±340)	2195 (±140)	1206 (±162)	1.06	1.9
WT	WT2	638 (±50)	82 (±3)	2 (±0.17)	7.8	319
Loop	M1-11	4673 (±520)	4238 (±362)	4890 (±886)	1.1	0.95
WT	WT3	1214 (±171)	61 (±5)	2.3 (±0.06)	19	527
Loop	VII-8	2859 (±140)	2745 (±98)	2846 (±191)	1.0	1.0

The numbers are given in Miller units/A_{650} units of bacteria (Miller, 1972). The values correspond to an average of at least four measurements at A_{650} between 0.2 and 0.5. The standard deviations are given in parentheses. The *thrS–lacZ* fusions carrying the WT or mutant operators are all on λ bacteriophages integrated as monolysogens at *att*λ in the chromosome of IBPC5421 or IBPC5311 for WT3 and VII-8. WT1 and its derivatives (M6-1, M4-11, L6-8, M1-2 and M2-2) are carried by λMBXΔ20-28 and its corresponding derivatives. WT2 and M1-11 are carried by λMBX1 and λMBXM1-11 respectively. WT3 and VII-8 are carried by λMΔ20-10 and λMΔ20-10VII-8 respectively. The phage λMΔ20-10 has the same structure as λMBXΔ20-28 but with the *Hin*dIII linker located 1 bp upstream in *thrS* and used as a boundary with *lacZ* sequences. The lysogens were grown at 30°C in Mops/glucose medium supplementd with all aminoacylations (Neidhardt *et al.*, 1977) and ampicillin was added at a concentration of 100 μg/ml every 2 h during growth.

operator *thrS–lacZ* mutants and screened for colonies with a less marked Lac^+ phenotype, i.e. colonies where pUB4 is able to repress β-galactosidase synthesis from these fusions. We found 13 such mutants among 12 100 screened colonies rising from independent mutagenesis events. In none of the 13 cases could the phenotype be explained by an increased synthesis from the plasmid of threonyl-tRNA synthetase by the criteria of SDS–PAGE or Western blots (data not shown). We were unable, despite repeated attempts (see Discussion), to isolate pUB4 mutants that would compensate for any defect in the loop of the operator.

In the second screening, we looked for pUB4 derivatives that were unable to repress in *trans* the synthesis of β-galactosidase from the WT1 *thrS–lacZ* fusion. The data in Table I, line 1, show that pUB4 normally represses β-galactosidase synthesis from the WT1 fusion. After mutagenesis of pUB4 and transformation of a strain carrying the WT1 fusion, we looked for colonies that have the same Lac phenotype as the non-transformed strain. These colonies carry plasmids that are no longer able to repress β-galactosidase synthesis from the WT1 fusion. We found 69

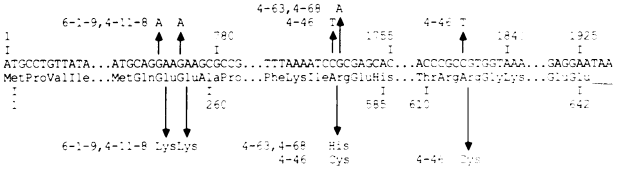

Fig. 2. Mutational changes in the two compensatory (6-1-9 and 4-11-8) and the three non-repressing (4-46, 4-63 and 4-68) derivatives of threonyl-tRNA synthetase. The nucleotide coordinates and changes are given over the sequence. The amino acid coordinates and changes are given under the sequence.

such mutant derivatives of pUB4 among 2235 screened colonies, 19 of which still overproduced threonyl-tRNA synthetase normally. Among these, 11 still complemented a chromosomal *thrS* mutant. The majority (eight) of the extracts of 11 clones carrying the complementing derivatives of pUB4 had absolutely no $tRNA^{Thr}$-charging activity (under standard conditions) over the background level due to the chromosomal WT copy of *thrS*. These mutant derivatives of pUB4 probably overproduce a synthetase that is functionally very unstable in extracts. The extracts of the other three clones showed low charging activity over the background level. We were unable to find non-repressing derivatives of pUB4 that still charged $tRNA^{Thr}$ normally at ~200 nM concentration of $tRNA^{Thr}$ (standard assay). However, the defect is reversed at much higher $tRNA^{Thr}$ concentrations, as shown later.

Genetic characterization of the selected trans-acting regulatory mutants

We selected the two compensatory mutant (out of 13) derivatives of pUB4 (pBM4-11-8 and pBM6-1-9) which had the strongest phenotype for further study. We also chose, among the non-repressing derivatives of pUB4, the three that had a residual charging activity (pUB4-46, pUB4-63 and pUB4-68) so as to be able to analyse the catalytic alterations of the corresponding threonyl-tRNA synthetases.

The five mutants were mapped by *in vitro* reconstitution of pUB4 from mutant and WT restriction fragments and subsequent phenotypic analysis (data not shown). The compensatory mutations were in both cases assigned to a 305 bp long *Pst*I–*Sma*I fragment in the middle of the *thrS* gene. The fragments were sequenced and both independent isolates showed the same double G_{772} to A and G_{775} to A changes (the A of the initiation codon of *thrS* is base 1) which correspond to changes of Glu_{258} and Glu_{259} to lysines (Figure 2). We do not know if the two mutations are essential to the phenotype but would suppose this to be so because the same two changes were found twice independently.

The non-repressing mutants were assigned to a 291 bp fragment between an *Ssp*I site and the end of *thrS*. The mutant fragments were sequenced and the defects in pUB4-63 and pUB4-68 were both associated with the change Arg_{583} to His (Figure 2). In pUB4-46 a double change of Arg_{583} to Cys and Arg_{612} to Cys was detected. We do not know if the two changes are essential for the phenotype.

All the characterizations described next were performed with plasmids reconstituted from WT and the sequenced mutant fragments. For the reconstituted plasmids, the name of the different *thrS* alleles is kept but pUB is replaced by pSS (e.g. pUB4-11-8 is replaced by pSS4-11-8). This reconstitution was done to be sure that the properties of the mutant synthetases are associated with the sequenced defects.

Table II. Suppression pattern of *thrU*(SuUAG) and *thrU*(SuAGA) in the presence of pUB4 and pSS4-11-8

Suppressor	Mutation	Growth on plates		
		pUC8	pUB4	pSS4-11-8
thrU(SuUAG)	*trpA*(UAG211)	−	+	++
thrU(SuUAG)	*argE*(UAG)	−	+	++
thrU(SuUAG)	*trpA*(UAG234)	−	−	−
thrU(SuAGA)	*trpA*(AGA211)	−	−	−
tyrT(SuUAG)	*trpA*(UAG211)	−	−	−
None	*trpA*(UAG211)	−	−	−
None	*argE*(UAG)	−	−	−

The suppressors are on the corresponding λ (Table V) with the exception of *tyrT*(SuUAG) which is on Φ80. The mutation *trpA*(UAG211) is carried by FTP3687, *trpA*(AGA211) by FTP3691, *trpA*(UAG234) by FTP3689, *argE*(UAG) by 121. The lysogens corresponding to the different combinations between suppressors and target genes shown in the table were transformed and grown on minimal A plates (Miller, 1970) supplemented with ampicillin at 50 μg/ml. Growth was tested by comparing permissive (+ Arg for 121, + Met + indole for the FTP strains) or non-permissive medium (with no complement for 121 and + Met for the FTP strains) by colony purification.
−, No growth; +, very slow growth; ++, slow growth; +++, fast growth. All the lysogens grow equally fast (+++) on plates under permissive conditions.

Physiological characterization of the trans-acting regulatory mutants

As the mutants were isolated on plasmids, we were able to analyse the effect of the *trans*-acting regulatory mutants on a set of operator mutants or on a WT operator of *thrS* simply by transformation. The effect of plasmid pSS4-11-8 (carrying the double Glu_{258} and Glu_{259} change) on different *thrS*–*lacZ* fusions is shown in Table I. Depending on the WT fusions (WT1, WT2 or WT3) the plasmid represses β-galactosidase synthesis from ~300- to >1000-fold (the 3280-fold of Table I is only an approximation since the 0.12 of the corresponding β-galactosidase value is too low to be measured precisely), i.e. between 40 and ~100 times more than the same plasmid carrying the WT *thrS* gene. In other words, this mutant behaves as a super-repressor with a WT operator. Mutations in the stem of the $tRNA^{Thr}$ anticodon-like arm of the operator are compensated (i.e. pSS4-11-8 acts as an active repressor) to various extents depending on the mutation in the operator (M4-11 or M6-1). This is an expected result since these mutants were screened for that property. In contrast, none of the mutants in the loop of the $tRNA^{Thr}$ anticodon-like arm are compensated (Table I and Figure 1). Another category of operator constitutive mutations, illustrated by the M2-2 mutation, increases the distance between the $tRNA^{Thr}$ anticodon-like structure and the ribosomal binding site of *thrS*. These mutations are either marginally or not compensated by pSS4-11-8. These results indicate that pSS4-11-8 does not compensate for the operator constitutive mutations in a systematic manner but rather in a way depending on the nature of the operator mutation. This result is discussed later. On the contrary, the non-repressing derivatives of pUB4 show no allele specificity: they completely lose the property of repressing *thrS* expression in *trans* from any WT or mutant operator, i.e. β-galactosidase levels from fusions carrying WT or mutant operators are the same with the vector (pUC8) and the non-repressing derivatives of pUB4 (data not shown).

Functional analysis of the mutant threonyl-tRNA synthetases in vivo

Threonyl-tRNA synthetase, besides being the translational repressor of its own mRNA, is also the enzyme which accomplishes the essential task of aminoacylating the threonine-specific tRNAs. The next experiments deal with aminoacylation function of our *trans*-acting regulatory mutants of the synthetase. We chose to look at aminoacylation of $tRNA^{Thr}$ *in vivo* using suppression. For this purpose, two suppressing derivatives of *thrU* (the structural gene for $tRNA_4^{Thr}$) were constructed. Although $tRNA_4^{Thr}$ differs more from the operator than the other isoacceptors, we chose that gene for practical reasons, reasoning that the main features of the interactions between the synthetase and its tRNAs should be conserved between the isoacceptors. The first derivative, *thrU*(SuUAG), is an amber suppressor (Figure 1) that on a multicopy plasmid confers a WT phenotype to a strain carrying the *trpA*(UAG211) mutation [at residue 211 Thr is known to be functional (see Murgola, 1985)] or to another strain carrying an *argE*(UAG) mutation at a site where every amino acid replacement seems to lead to an active protein. The second *thrU*(SuAGA) is a missense suppressor (also shown in Figure 1), that on a multicopy plasmid confers a Trp^+ phenotype to a strain carrying the *trpA*(AGA211) allele. Both suppressor tRNAs are charged by Thr to a level lower than 20% of WT $tRNA_4^{Thr}$. This was shown by comparing Thr aminoacylation activity and level of $tRNA_4^{Thr}$ in RNA extracted from cells carrying *thrU* or its suppressor derivatives on multicopy plasmids. Although $tRNA_4^{Thr}$ in the extracts is overproduced from the different plasmids at an equivalent level as judged on gels, the increase over background of 7- to 10-fold in aminoacylation activity is seen only with the WT gene (data not shown). The gene *thrU*(SuUAG) is a poor suppressor in that, as a single copy on λ, it is unable to suppress the *trpA*(UAG211) or *argE*(UAG) mutations that were suppressed with the suppressor on a multicopy plasmid (Table II, lines 1 and 2, first column). Some suppression is detected in both cases, however, when an excess of threonyl-tRNA synthetase is synthesized in *trans* from pUB4 (Table II, lines 1 and 2, second column). This strongly suggests that the nonsense suppressor derivative of $tRNA_4^{Thr}$ is charged with Thr at least partially. The gene *thrU* (SuAGA), a good suppressor *trpA*(AGA211) when carried on a multicopy plasmid, does not suppress the same mutation as a single copy in λ (Table II, line 4). This time, however, the suppression is insensitive to cellular concentration of threonyl-tRNA synthetase and the corresponding tRNA is therefore probably not charged by threonyl-tRNA synthetase. Interestingly, in the presence of pSS4-11-8 which carries our compensatory mutant of *thrS*, the suppression of UAG nonsense mutations in both *trpA* and *argE* is increased when compared with pUB4, carrying the WT enzyme (Table II, lines 1 and 2, last column). The suppression of the missense *trpA*(AGA211) mutation is not affected by the presence of the 4-11-8 *thrS* allele on the plasmid. It thus seems that the synthetase of the *trans*-acting regulatory mutant has an increased ability to charge the nonsense but not the missense derivative of $tRNA_4^{Thr}$. This again indicates allele specificity: the *thrS*4-11-8 allele enhances suppression (and thus charging) of *thrU* (SuUAG) but not of *thrU*(SuAGA), although the latter carries only a single change when compared with WT (Figure 1). The fact that in Table II, line 3, no suppression of *trpA*(UAG234) is seen in the presence of *thrU*(SuUAG), indicates that the suppressor tRNA is not charged by Ala or Gly since these two amino acids are the only ones to be active at 234 where Thr is known to be inactive (Murgola, 1985). The finding that *tyrT*(SuUAG) cannot replace *thrU* (SuUAG) indicates (Tables II, line 5) that the increase of suppression observed with the latter suppressor in the presence of pSS4-11-8 is dependent on the nature of the nonsense suppressor.

Table III. Suppression of an amber codon at position 181 of a *lacI−lacZ* hybrid gene in the presence of different *thrS* and/or *thrU* alleles on the same multicopy plasmid

Plasmid name	Alleles on pBR322 derivative		β-Galactosidase synthesis	Suppression (in %)
	thrS	*thrU*		
pBRUB4SuUAG	WT	SuUAG	18 (±2)	5.6
pBRUB4-11-8SuUAG	4-11-8	SuUAG	59 (±4)	18.6
pBRUB4-46SuUAG	4-46	SuUAG	7 (±0.5)	2.2
pBRUB4-63SuUAG	4-63	SuUAG	7.3 (±0.5)	2.3
pBRDAR1	−	SuUAG	4.7 (±0.5)	1.4
pBRUB4SuAGA	WT	SuAGA	0.15 (±0.06)	<0.1
pBRUB4-11-8SuAGA	4-11-8	SuAGA	0.2 (±0.1)	<0.1

The strain 121R, a *recA* derivative of 121, was transformed with the different plasmids and grown as indicated in the footnote of Table I. The β-galactosidase synthesis is given as Miller units/A_{650} of bacteria and are the average of at least eight measurements at A_{650} between 0.2 and 0.5. The numbers in parentheses are the standard deviations for each value. The suppression efficiency is the ratio of the given β-galactosidase value to 317 (±9) which is the β-galactosidase level synthesized by UF261, which carries a WT *lacI−lacZ* hybrid gene. The latter strain synthesizes the same β-galactosidase level irrespective of the plasmid present in the cell.

More quantitative data concerning suppression of an amber codon at position 181 of *lacI−lacZ* hybrid are shown in Table III. In this case, both the suppressor and the synthetase alleles are on the same multicopy plasmid. Essentially, the same phenomenon is observed, namely suppression is increased in the presence of an excess of the threonyl-tRNA synthetase from a level below background to 5%. The compensatory allele (designated as 4-11-8) increases suppression to ~20%.

In all these experiments, the non-repressing alleles of the synthetase have an effect on suppression that is lower than the WT enzyme and behave in these tests as mutants which have unspecifically lost a function. It is only in the following experiments that the specificity of these mutants will be shown.

Biochemical properties of the mutated threonyl-tRNA synthetases

An advantage in studying a translational repressor, which is also an enzyme, lies in the possibility, using enzyme kinetics, to compare the way the WT and mutant threonyl-tRNA synthetases interact with their different substrates. These measurements were carried out in crude extracts from strains carrying a WT chromosomal copy of *thrS* besides pUB4 or its mutated derivatives. Under these conditions, <5% of the synthetase is coded by the chromosomal gene if the multicopy plasmids carry the non-repressing mutants. If the multicopy plasmid carries the compensatory mutant, chromosomal enzyme levels are negligible because the

Table IV. Kinetic parameters of the WT, compensatory (4-11-8) and non-repressing (4-46 and 4-63) threonyl-tRNA synthetases

thrS allele on plasmid	Exchange reaction			Aminoacylation reaction	
	K_M for ATP (μM)	K_M for Thr (μM)	V_{max} (relative to WT)	K_M for $tRNA^{Thr}$ (nM)	V_{max} (relative to WT)
WT	235	230	1	115	1
4-46	208	135	0.8	ND	ND
4-63	260	220	0.8	1660	1
4-11-8	330	285	0.38	25	0.38

The V_{max} for the WT (pUB4) enzyme is 3.7 pmol/ng/min in the exchange reaction and 0.008 pmol/ng/min in the aminoacylation reaction.
ND, not done.

expression of the chromosomal copy of *thrS* is super-repressed (Table I).

Most of the aminoacyl-tRNA synthetases (including threonyl-tRNA synthetase) charge their respective tRNAs in two steps: first the amino acid is activated to form an enzyme-bound aminoacyl−adenylate complex (eqn 1) and then it is transferred to tRNA (eqn 2) thus

$$E + aa + ATP \rightleftharpoons (E\text{-}aa\text{-}AMP) + PPi \quad (1)$$
$$(E\text{-}aa\text{-}AMP) + tRNA \rightarrow aatRNA + AMP \quad (2)$$

Experimentally, the kinetic parameters of the first step are given by the so-called exchange reaction where one measures the incorporation of radioactive label from pyrophosphate into ATP (reaction 1 from right to left). The data in Table IV clearly show that the K_M values for threonine or ATP are not changed in a major way in the mutants when compared with the WT enzyme. The only significant and reproducible difference is the V_{max} for the compensatory enzyme (4-11-8), which is ~40% of that of the WT. The data thus show that the mutant enzymes behave almost normally with two out of three of their substrates.

In the aminoacylation reaction (right part of Table IV), the V_{max} values for the non-repressing mutants are the same as WT and that for the compensatory enzyme is again ~40% of WT. The major differences concern the K_M values for the tRNA: the compensatory enzyme has a 5-fold lower and the non-repressing enzyme a 15-fold higher K_M than the WT enzyme. The K_M value of 25 nM given for the compensatory enzyme is imprecise since it is the lower limit of the range of tRNA concentration that could be used in the assays. In all other cases, K_M values were measured with substrate concentrations that bracket the K_M.

The biochemical data thus lead us to conclude that our regulatory *thrS* mutants are also affected in aminoacylation. The observed defects are quite specific and concern the interaction with the tRNA. The compensatory mutant is shown to have an increased affinity for its natural substrate, the $tRNA^{Thr}$; in contrast, the non-repressing mutants are shown to bind $tRNA^{Thr}$ with a clearly reduced affinity.

Discussion

The present work describes the isolation of two classes of regulatory *trans*-acting mutations of *thrS*: the members of the first class were isolated as repressor mutations compensating operator defects and the members of the second class as repressor mutations that are unable to perform any regulation. The study of an example of the first class showed it to behave as a super-repressor for a WT operator which means that in the presence of this *thrS* allele on a multicopy plasmid, chromosomal threonyl-tRNA synthetase is completely repressed (similar to the *thrS−lacZ* fusions in Table I), i.e. the synthetase activity (which is essential for cellular growth) must come from the plasmid. The examples of the second class chosen for further study were those having the highest aminoacylation activity. Despite these constraints for optimal activity, the regulatory mutants we isolated are affected in aminoacylation. Furthermore, a functional relationship seems to exist between the alterations in regulation and in aminoacylation since the mutants that super-repress the translation of *thrS* mRNA, probably by binding to the operator with high affinity, display an increased binding to $tRNA^{Thr}$ as their main defect. The mutant synthetases that are unable to repress in *trans* the translation of the *thrS* mRNA, most probably because they have lost the possibility of binding to the operator, are shown to have a decreased affinity for the $tRNA^{Thr}$. In the latter case the defect is extremely specific since only the K_M for the tRNA (and not even the aminoacylation V_{max}) is affected. The *in vivo* suppression data show that the compensatory mutant has an increased capacity to charge a mutated form of $tRNA^{Thr}$, in perfect accordance with the biochemical results. The increased affinity for its RNA ligands of the compensatory mutant does not appear to be gained at the expense of specificity; this is indicated by the partially allele-specific way it interacts with its ligands: the mRNA and the tRNA. Interacting with mRNA, the synthetase mutant compensates for operator mutations in the stem of the anticodon-like arm to various extents depending on the mutation, but not for mutants in the loop region. When charging tRNA, allele specificity is also observed in the case of the compensatory mutant in its interaction with the different suppressor derivatives of $tRNA_4^{Thr}$: the nonsense suppressor is recognized by the mutated enzyme but the missense is not.

The fact that synthetase mutants compensating for loop operator mutants were not found in spite of our intensive search for them deserves some comment. We know from protection experiments that the synthetase interacts directly with the loop of the operator (H.Moine, C. and B.Ehresmann, unpublished results) which means that, *a priori*, synthetase mutants compensating for loop mutants should be found. The simplest explanation for our failure would be that such mutants are super-repressors inactive in aminoacylation. Such mutants would repress the chromosomal copy of *thrS* to the extent that the cell would be unable to charge its $tRNA^{Thr}$ at a rate necessary for growth. This explanation is unconvincing since our selection did not work even when threonyl-tRNA synthetase was provided by a multicopy plasmid carrying *thrS* without its operator (super-repression would not be effective in such a case). We rather believe that forcing the synthetase to recognize a wrong anticodon-like loop somehow changes the 'identity' of the synthetase. Such a synthetase would not only be unable to charge its own tRNA but might charge with threonine another tRNA sufficiently to impair bacterial growth. If the synthetase is forced to recognize a mutated anticodon-like loop in the operator and if the same domain of the synthetase

Table V. *E.coli* strains, plasmids and bacteriophage used

Name	Genetic markers/comments	Reference/origin
Plasmids		
pBR322	*Amp, Tet* vector	Bolivar *et al.* (1977)
pUC8	*Amp* vector	Messing (1983)
pBS$^+$	*Amp*, Bluescribe+	Vector Cloning Systems, Inc., USA
pUB4	*thrS* without its operator, *Amp*	Springer *et al.* (1985)
pBM4-11-8	*thrS4-11-8* derived from pUB4	This work
pBM6-1-9	*thrS6-1-9* derived from pUB4	This work
pUB4-46	*thrS4-46* derived from pUB4	This work
pUB4-63	*thrS4-63* derived from pUB4	This work
pUB4-68	*thrS4-68* derived from pUB4	This work
pSS4-11-8	*thrS4-11-8* reconstituted	This work
pSS4-46	*thrS4-46* reconstituted	This work
pSS4-63	*thrS4-63* reconstituted	This work
pSS4-68	*thrS4-68* reconstituted	This work
pTUB1	*tufB* operon	Miyajima *et al.* (1979)
pBSAR1	*Amp, thrU*$^+$	This work
pBSAR1SuAGA	*Amp, thrU*(SuAGA)	This work
pBSAR1SuUAG	*Amp, thrU*(SuUAG)	This work
pBRAR1	*Tet, thrU*$^+$	This work
pBRAR1SuAGA	*Tet, thrU*(SuAGA)	This work
pBRAR1SuUAG	*Tet, thrU*(SuUAG)	This work
pD4	*Amp, Tet*, f1 Ori	Dotto *et al.* (1981)
pBRDAR1	*Tet, thrU*$^+$, f1 Ori	This work
pBRDAR1SuAGA	*Tet, thrU*(SuAGA), f1 Ori	This work
pBRDAR1SuUAG	*Tet, thrU*(SuUAG), f1 Ori	This work
pBRUB4SuAGA	*Tet, thrU*(SuAGA), f1 Ori, *thrS*$^+$	This work
pBRUB4-11-8SuAGA	*Tet, thrU*(SuAGA), f1 Ori, *thrS4-11-8*	This work
pBRUB4SuUAG	*Tet, thrU*(SuUAG), f1 Ori, *thrS*$^+$	This work
pBRUB4-11-8SuUAG	*Tet, thrU*(SuUAG), f1 Ori, *thrS4-11-8*	This work
pBRUB4-46SuUAG	*Tet, thrU*(SuUAG), f1 Ori, *thrS4-46*	This work
pBRUB4-63SuUAG	*Tet, thrU*(SuUAG), f1 Ori, *thrS4-63*	This work
***E.coli* strains**		
IBPC5421	F$^-$*thi*-1, *argE3, galK2*?, *mtl-1, xyl-5, tsx-29*?, *supE44*?, *rpsL, recA1, lacZ* (non polar)	Springer *et al.* (1986)
IBPC5311	As IBPC5421 but Δ*lacX74*	Springer *et al.* (1985)
IBPC5403	As IBPC5311 but *thrS1029*	Springer *et al.* (1985)
121	F′ [*pro, lacI−lacZ*(UAG181)] *ara, argE*(UAG), Δ*lacpro, nalA, rpoB, thi*	L.Isaksson [Miller and Albertini (1983)]
121R	As 121 but *recA*	This work
UF261	As 121 but *lacI−lacZ*$^+$	L.Isaksson
FTP3687	F′ [*trpA*(UAG211)] *metB, glyV55,* Δ*(tonB, trpAB)17*	E.J.Murgola
FTP3689	As FTP2687 but *trpA*(UAG234)	E.J.Murgola
FTP3691	As FTP2687 but *trpA*(AGA211)	E.J.Murgola
EC3	*mutD5, thr, leu, his, trpA11, argE*	Degnen and Cox (1974)
Bacteriophages		
λ$^+$		Ph.Kourilsky
λnav8-5	*imm21 cI*$^+$, *ninR, pheST-lac*	Springer *et al.* (1985)
λMBXΔ20-28	*cI857, nin5, thrS-lacZ*	Springer *et al.* (1986)
λMBXΔ20-28M6-1	*cI857, nin5, thrS-lacZM6-1*	Springer *et al.* (1986)
λMBXΔ20-28M4-11	*cI857, nin5, thrS-lacZM4-11*	Springer *et al.* (1986)
λMBXΔ20-28L6-8	*cI857, nin5, thrS-lacZL6-8*	Springer *et al.* (1986)
λMBXΔ20-28M1-2	*cI857, nin5, thrS-lacZM1-2*	Springer *et al.* (1986)
λMBXΔ20-28M2-2	*cI857, nin5, thrS-lacZM2-2*	Springer *et al.* (1986)
λMXB1	*cI857, nin5, thrS-lacZ*	Springer *et al.* (1986)
λMBXM1-11	*cI857, nin5, thrS-lacZM1-11*	Springer *et al.* (1986)
λMBXΔ20-10	*cI857, nin5, thrS-lacZ*	This work
λMBXΔ20-10VII-8	*cI857, nin5, thrS-lacZVII-8*	This work
λ*thrU*(SuUAG)	*imm21 cI*$^+$, *thrU*(SuUAG)	This work
λ*thrU*(SuAGA)	*imm21 cI*$^+$, *thrU*(SuAGA)	This work
Φ80*supF*	*tyrT*(SuUAG)	M.Uzan

is responsible for the recognition of both the true anticodon and the anticodon-like structure, one might, in some cases, speculate that tRNAs with anticodon sequences corresponding to the mutated operator loop would be recognized. This hypothesis implies that the 'identity' of both $tRNA^{Thr}$ and the wrongly charged tRNA(s) is at least partially defined by the anticodon. This was recently shown to be true for several tRNAs (see Yarus, 1988). In the case of $tRNA^{Thr}$, the fact that mutational changes in the anticodon lead to a strong decrease of aminoacylation activity, and in the case of *thrU*(SuAGA) also to a change in aminoacylation specificity indicates that the anticodon contributes to the 'identity' of the $tRNA^{Thr}$.

The amino acid changes in the mutated threonyl-tRNA synthetases described in this work affect the interaction with both the mRNA and the tRNA in a similar manner. This indicates either that the binding domains of the two RNA ligands on the synthetase are overlapping or that the mutations are located in a region which, if changed, affects the two, possibly distinct, domains that bind the RNA ligands. We favour the first possibility, i.e. the mRNA and the tRNA having partially overlapping recognition domains, for the following reasons. Firstly, both the compensatory and non-repressing mutants show parallel changes in binding to the two ligands; this is less easy to explain in terms of a mutation changing the conformation of two separated domains of the protein interacting each with a separate RNA ligand. Secondly, the kind of change that would affect separated domains of a protein would be anticipated to fall in a hydrophobic (internal) part of the protein; in contrast, the two identified loci around Glu_{258} and Arg_{583} are clearly located in hydrophilic regions. This is indicated by several computer programs (Eisenberg *et al.*, 1984; Gaboriaud *et al.*, 1987) and the fact that the mutational changes always cause a charge change obviously related to the way the altered protein interacts with the negatively charged RNAs: a double Glu_{258}-Glu_{259} to Lys-Lys mutation (a very strong local charge change from negative to positive) apparently increases the affinity for the ligands and an Arg_{583} to His of Cys mutation (a local positive to less positive charge change) apparently decreases the affinity for the ligands. These charge changes are expected to have the described consequences if they occur at the surface and not in an internal part of the protein which would not be in contact with the RNA ligands.

Another reason to suspect the existence of a unique domain of the synthetase recognizing both the $tRNA^{Thr}$ anticodon arm and the arm of the operator between −10 and −50 is the structural and functional analogy between these two regions. Besides the primary sequence analogy, which is striking for the $tRNA_1^{Thr}$ and $tRNA_3^{Thr}$ isoacceptors and somewhat different for the $tRNA_4^{Thr}$ isoacceptor (Figure 1), recent experimental data (Moine *et al.*, 1988) actually show that the operator has the secondary structure drawn in Figure 1. Moreover, protection experiments show that both the anticodon and the anticodon-like loops are in contact with the synthetase (Theobald *et al.*, 1988; H.Moine and B. and C.Ehreshmann, unpublished observations). Finally, equivalent changes in the two loops have equivalent effects, e.g. a G to C change in the middle anticodon base (Figure 1) causes operator constitutivity and completely eliminates the aminoacylation of the $tRNA_4^{Thr}$ by the threonyl-tRNA synthetase. Recent results from our laboratory indicate that the anticodon-like loop of the operator has to be seven nucleotides long (the length of the normal anticodon loop) to be functional since an insertion or a deletion of a single nucleotide causes operator constitutivity.

This analogy between substrate and operator in the case of threonyl-tRNA synthetase may be related to the observation that almost all translationally autoregulated genes are thought to control their expression in an analogous manner, i.e. by their product binding to their own mRNA at a site that shares some homology to their natural nucleic acid substrates. This hypothesis has been called molecular mimicry (Campbell *et al.*, 1983) and implies that there is a common site on the protein that recognizes both nucleic acid ligands. This is a simple strategy for adding a regulatory role to a protein involved in nucleic acid binding without having the necessity for a separate regulatory domain. This kind of hypothesis has been advanced to explain regulation in several phage systems (reviewed in Stormo, 1987), in particular for gene 32 of bacteriophage T4 (McPheeters *et al.*, 1988), but also in relation to ribosomal proteins in *E.coli* (reviewed in Lindahl and Zengel, 1986). The present work reinforces the mimicry hypothesis in showing that these sequence analogies between the RNA binding sites in autoregulated systems, at least in the case of the threonyl-tRNA, correspond to a functional similarity.

Materials and methods

Strains, plasmids and bacteriophage

The *E.coli* strains, plasmids and bacteriophage are described in Table V.

Screening of the mutant derivatives of pUB4

The DNA of plasmid pUB4 was prepared by the alkaline lysis method (Birnboim and Doly, 1979) and mutagenized at 65°C for 3 h in 0.8 M hydroxylamine, 45 mM NaOH (6.5 < pH < 7). The DNA concentration was ~10 μg/ml. After mutagenesis the DNA was dialysed against TE buffer pH 8 (Maniatis *et al.*, 1982). The mutagenesis reduces the transformation efficiency between 10^2 and 10^3 times. The mutagenized plasmid was used to transform IBPC5421λMBX1 for the selection of non-repressing derivatives of pUB4 and IBPC5421λMBXΔ20-28M6-1, M4-11, L6-8 or IBPC5421λMBXM1-2, M1-11 for the selection of compensatory mutants. The phenotypes were screened on McConkey lactose plates (Miller, 1972) supplemented with ampicillin at 100 μg/ml. The candidates were analysed for the overproduction of threonyl-tRNA synthetase using SDS−PAGE (Laemmli, 1970) and/or quantitative immunoblotting (Butler *et al.*, 1986). Complementation was looked at using IBPC5403, a *thrS* mutant conferring a Thr^- phenotype. Threonyl-tRNA activity was tested in extracts as described later.

Plasmid and bacteriophage constructions

A 242 bp *Hgi*AI−*Ava*I fragment (filled-in on the *Hgi*AI side with the Klenow fragment of DNA polymerase) containing only *thrU* and its promoter was cloned from pTUB1 between the *Hin*cII and the *Ava*I site of pBS^+. The resulting plasmid, pBSAR1, was mutagenized by transformation and growth in the *mutD5* strain EC3 and retransformed in the *trpA* (AGA211) strain FTP3691. The plasmid pBSAR1SuAGA was selected as permitting FTP3691 to grow in the absence of tryptophan. Several such plasmids were sequenced and shown to carry the only G to C change in the middle of the anticodon (Figure 1). The plasmid pBSAR1SuUAG was isolated using oligonucleotide site-directed mutagenesis (Kunkel *et al.*, 1987) and carries a TGT to CTA change of the anticodon (Figure 1). The *Pst*I−*Eco*RI fragment of pBSAR1 and its mutant derivatives were cloned between the equivalent sites of pBR322 to give pBRAR1 and its mutant derivatives. An *Eco*RI fragment carrying the phage f1 replication origin was cloned from plasmid pD4 into the *Eco*RI site of the pBRAR1 plasmids in the orientation where the *Cla*I site of the *Eco*RI fragment is nearer to the *Pst*I site of pBR322 to give the

pBRARD1 plasmids. The *Eco*RI−*Hin*dIII fragment of pUB4 containing *thrS* was cloned between the same sites of pBRAR1SuUAG/SuAGA to give pBRUB4SuUAG/AGA. The same clonings with the mutant alleles of *thrS* gave pBRUB$_4$-11-8SuAGA/UAG, pBRUB4-46SuAGA/UAG and pBRUB4-63SuAGA/UAG. The *Sst*I−*Hin*dIII fragments containing the *thrU* mutant alleles were cloned between the left arm of λ^+ (up to the *Sst*I site at 24.77 kb) and the right arm of λnav8-5 starting from the unique *Hin*dIII site at 25.12 kb. The resulting phages are called λ*thrU* SuAGA/UAG.

Enzymic tests

IBPC5421λMBXΔ20-28 transformed with pUB4 or its mutated derivatives were grown overnight in LB medium (Miller, 1972) supplemented with ampicillin at 500 μg/ml. Bacteria were pelleted in aliquots of 1 A_{650} and kept at −80°C. Bacteria were resuspended in 400 μl 20 mM Tris−HCl, pH 7.5, 5% glycerol and sonicated (4 times 15 s at 100 W with an MSE sonicator, with 15 s at 0°C between the pulses). The sonicate was centrifuged at 4°C for 5 min in small polypropylene tubes and the supernatant used for testing. The threonyl-tRNA synthetase concentration in the extracts was measured by quantitative immunoblotting (Butler *et al.*, 1986) using pure enzyme as standard. The conditions for the exchange reaction were as previously described (Hirsh, 1968) except that incubation was at 25°C in 100 μl at 7 mM $MgCl_2$ and 4 mM sodium ^{32}PPi (5−7 c.p.m./pmol). The K_M for Thr was determined from a Lineweaver−Burk plot made of four points at Thr concentrations between 25 and 400 μM; ATP was at 2.5 mM. Each point of the plot corresponds to incorporation measurements at 1, 2, 3 and 4 min after addition of the extracts (at a concentration of 1.5−5.5 μg/ml of threonyl-tRNA synthetase depending on the extracts). The K_M for ATP was determined in the same way by varying the ATP concentration from 0.1 to 1 mM; Thr was at 2 mM. The aminoacylation reaction was performed in 100-μl assays at 30°C in the presence of 50 mM Tris−HCl, pH 8, 10 mM $MgCl_2$, 10 mM KCl, 7 mM β-mercaptoethanol, 1 mM ATP, 20 μM [^{14}C]Thr (100 c.p.m./pmol). After incubation, the samples were treated as previously described (Hirsh, 1968). The K_M for tRNA was determined from Lineweaver−Burk plots made from four points at $tRNA^{Thr}$ concentrations varying between 25 and 150 nM for extracts from strains transformed with pUB4, between 22 and 136 nM with pSS4-11-8 and between 422 and 1760 nM with pSS4-63. Each point of the plot corresponds to incorporation measurements at different times (20, 40 and 60 s for pUB4 and pSS4-11-8; 0.5 and 1 min for pSS4-63) and different threonyl-tRNA synthetase concentrations (between 0.3 and 1.2 μg/ml for pUB4 and pSS4-11-8; 1 and 1.5 μg/ml for pSS4-63). The tRNA fraction used in the assays is made of RNA extracted from FTP3691pBSAR1 using published procedures (Stern and Littauer, 1971). Because of the plasmid pBSAR1 that carries *thrU*, the RNA is enriched in $tRNA_4^{Thr}$ and charges at 160 pmol of threonine/A_{260}. For the experiments with tRNA concentrations up to 150 nM crude tRNA preparations from other strains were also used without any effect on the K_M values. The V_{max} values were determined from the same plots.

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