Nucleic Acids Research, Vol. 20, No. 21 5633–5640

Molecular mimicry in translational control of *E.coli* threonyl-tRNA synthetase gene. Competitive inhibition in tRNA aminoacylation and operator-repressor recognition switch using tRNA identity rules

Pascale Romby, Christine Brunel, Joël Caillet[1], Mathias Springer[1], Marianne Grunberg-Manago[1], Eric Westhof, Chantal Ehresmann and Bernard Ehresmann

UPR du CNRS no. 9002, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg and [1]URA du CNRS no. 1139, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

Received August 19, 1992; Revised and Accepted October 12, 1992

ABSTRACT

We previously showed that: (i) *E.coli* threonyl-tRNA synthetase (ThrRS) binds to the leader of its mRNA and represses translation by preventing ribosome binding to its loading site; (ii) the translational operator shares sequence and structure similarities with $tRNA^{Thr}$; (iii) it is possible to switch the specificity of the translational control from ThrRS to methionyl-tRNA synthetase (MetRS) by changing the CGU anticodon-like sequence to CAU, the $tRNA^{Met}$ anticodon. Here, we show that the wild type (CGU) and the mutated (CAU) operators act as competitive inhibitors of $tRNA^{Thr}$ and $tRNA_f^{Met}$ for aminoacylation catalyzed by *E.coli* ThrRS and MetRS, respectively. The apparent K_d of the MetRS/CAU operator complex is one order magnitude higher than that of the ThrRS/CGU operator complex. Although ThrRS and MetRS shield the anticodon- and acceptor-like domains of their respective operators, the relative contribution of these two domains differs significantly. As in the threonine system, the interaction of MetRS with the CAU operator occludes ribosome binding to its loading site. The present data demonstrate that the anticodon-like sequence is one major determinant for the identity of the operator and the regulation specificity. It further shows that the tRNA-like operator obeys to tRNA identity rules.

INTRODUCTION

The expression of the *E.coli thrS* gene encoding for threonyl-$tRNA^{Thr}$ synthetase (ThrRS) was shown to be negatively autoregulated at the translational level *in vitro* and *in vivo* (1–3). A *cis*-acting element (the translational operator) was localized upstream from the AUG initiation codon (4). Moine and coworkers (5) showed that the binding of ThrRS to the operator inhibits its own translation by occluding ribosome binding at the translational initiation site, and that $tRNA^{Thr}$ competes with the operator for ThrRS binding. Therefore, $tRNA^{Thr}$ acts as an anti-repressor by displacing ThrRS from the mRNA, restoring the accessibility to the ribosome. Detailed probing experiments (6, 7) evidenced that the translational operator is folded in four well-defined domains (Fig. 1). Domain 1 contains the Shine and Dalgarno sequence and the translational initiation codon. Domain 2 shares sequence and structure analogies with the anticodon arm of $tRNA^{Thr}$. It contains the threonine anticodon CGU in a seven base-loop and sequence similarities with the anticodon stem. Domain 3 may serve as an articulation between domains 2 and 4. Finally, domain 4 displays similarities with the acceptor domain. It contains a ACCA sequence (with A_{-107} homologous to the 3′ terminal A of tRNA) located at an equivalent distance from the two G_{-112}-C_{-89} and U_{-115}-A_{-76} pairs analogous to the C_2-G_{71} and A_5-U_{68} pairs conserved in the four *E.coli* $tRNA^{Thr}$ isoacceptors. Domains 2 and 4 that display analogies with specific domains of $tRNA^{Thr}$ are shielded by ThrRS (6, 7). From a theoretical analysis, it was suggested that G_{34} and U_{35} in the anticodon, and base pairs C_2-G_{71} and A_5-U_{68} are involved in threonine specificity (8). A recent study of Hasewaga *et al.* (9) proved the role of the C_2-G_{71} pair as a recognition element.

Recent studies revealed that a relatively small number of nucleotides govern tRNA identity and that the specificity of a tRNA can be switched by transplanting its recognition elements in other tRNA species (for reviews see, 10–12). In the case of *E.coli* $tRNA_f^{Met}$ (13–15) and $tRNA^{Thr}$ (9, 16), the anticodon is one major determinant of aminoacylation specificity. Furthermore, changing the CAU anticodon of elongator $tRNA^{Met}$ into GGU (threonine anticodon) switches the identity from methionine to threonine (17). Recently, it was showed that by changing the CGU threonine anticodon-like sequence in the *thrS* translational operator by CAU (methionine) causes the expression of ThrRS to become regulated by the intracellular concentration of methionyl-tRNA synthetase (MetRS) (18). Altogether, these observations strongly suggest that ThrRS recognizes the operator and its cognate tRNA in a similar way.

In the present work, we used the fact that the translational repressor is also an enzyme to define the dissociation constant

of the operator/repressor complex in the wild type and switched situation when MetRS represses *thrS* expression with a CAU mutation in the anticodon-like sequence (18). In both cases, we showed that the operator acts as a competitive inhibitor of the cognate tRNA aminoacylation catalyzed by the enzyme. We measured also the relative importance of the anticodon-like and acceptor-like domains on the stability of the CGU operator/ThrRS and the CAU operator/MetRS complexes. Our data further indicate that the translational regulation mechanism of the CAU operator by MetRS is similar to that described for ThrRS. Altogether our results provide clear evidence that the *thrS* operator mimics the tRNA structure and that it is possible to specifically modulate the recognition by the corresponding aminoacyl-tRNA synthetase, using tRNA identity rules.

MATERIALS AND METHODS

Preparation of the biological material

RNA transcripts. The wild-type mRNA fragment (WT) corresponding to nucleotides −211 to +61 (+1 being the A of the *thrS* translational initiation codon) and the various RNA mutants (BS4-9, U_{-107}BS4-9, U_{-107}WT and ΔV (deletion of C-22 to G-42)) were obtained by inserting *Hinc* II-*Hind* III fragments from M13mp8Δ20-10 recombinants between the *Hinc* II-*Hind* III sites of pTZ18R (18, 19). After plasmid purification, the DNAs were linearized by *Hind* III (58 nucleotides downstream from the AUG initiation codon) and transcribed by *in vitro* transcription with T_7 RNA polymerase. M6-1 RNA was transcribed *in vitro* by SP_6 RNA polymerase from the pSP65 plasmid derivative according to (6). Prior use, the RNA fragments were renatured by incubation at 50°C for 5 min and cooled at 20°C for 20 min in the appropriate buffer.

Enzymes and tRNAs. E.coli ThrRS, purified from the overproducing strain AB 5311 λMΔ20-10 pUB4 (2) was prepared according to (20). *E.coli* MetRS was a generous gift of G. Fayat, Y. Mechulam and S. Blanquet (Palaiseau). AMV-reverse transcriptase was from Life Sciences Inc. *E.coli* $tRNA_f^{Met}$ was purchased from Boerhinger-Mannheim and *E.coli* $tRNA_3^{Lys}$ was from Subriden RNA.

Aminoacylation assays

Methionine acceptance. The reaction mixture used (55 μl) contained 20 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 2 mM ATP, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 21.5 μM [^{3}H]methionine (2500 cpm/pmol, Dositek). Initial rates of

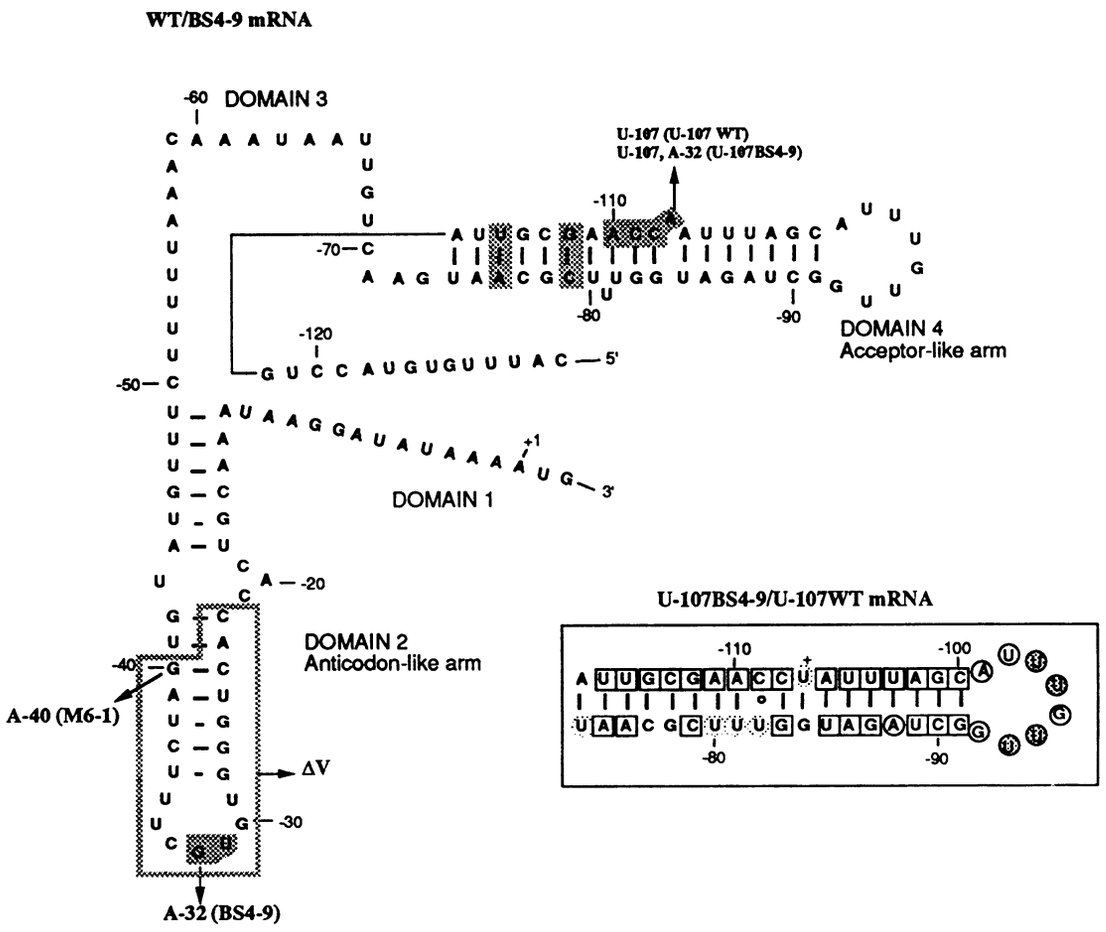

Figure 1. Secondary structure model of the wild-type *thrS* operator, as deduced from (7, 19). The model is shown in the proposed L-shape conformation and the different domains are indicated. The invariant nucleotides in the four $tRNA^{Thr}$ isoacceptors are shadowed: the anticodon sequence (9, 16, 17) and the base pair C-G (9, 19) are involved in threonine identity. The studied mutations are indicated. The deduced secondary structure of domain 4 of U_{-107}BS4-9 and U_{-107}WT is shown in the insert. The reactivity of Watson–Crick positions from DMS and CMCT modifications are indicated: nucleotides reactive under native conditions are encircled and the reactivity is proportional to the intensity of the circle (from marginal to strong); nucleotides unreactive under both native and semi-denaturing conditions are squared, (+) denotes an increased reactivity after removal of magnesium (semi-denaturing conditions). The absence of symbol means not determined.

reactions (v_i) were measured according to (15), at different $tRNA_f^{Met}$ concentrations (0.04 to 0.65 μM) in the absence and in the presence of mRNA as competitors : 20 and 40 μM for BS4-9, WT and U_{-107}BS4-9, 40 μM for ΔV. Samples were equilibrated at 25°C for 5 min before adding purified *E.coli* MetRS, and incubated from 1 to 7 min at 25°C. Aliquots of 10 μl were taken out at different time intervals and layered on Whatman 3MM papers. Unbound amino acid was removed by extensive washing with 5% TCA, then with ethanol. Bound amino acid was measured by liquid scintillation. Control experiments in the absence of RNAs or of enzyme were done in parallel.

Threonine acceptance. Reaction mixtures (130 μl) were as described in (20): 50 mM Hepes-NaOH (pH 7.5), 16 mM $MgCl_2$, 4 mM ATP, 10 mM DTE, 0,1 mg/ml bovine serum albumin, 100 μM [^{14}C]threonine (400 cpm/pmol, Dositek). The initial rates of reactions were measured at different concentrations of *E.coli* $tRNA_3^{Thr}$ (0.09 to 0.75 μM), in the absence and in the presence of competitor RNAs (0.1, 0.25 and 0.5 μM of WT, 0.25 and 0.4 μM of U_{-107}WT, 2 μM of BS4-9 and 3 μM of ΔV). Samples were equilibrated 5 min at 37°C before addition of purified *E.coli* ThrRS. Incubation was from 1 to 8 min at 37°C. Aliquots of 25 μl were taken out and treated as above.

Probing and footprinting experiments

RNA-MetRS complexes were formed by incubating the mRNA fragments (60 nM) with increasing concentrations of MetRS (from 10 nM to 5 μM) for 10 min on ice. Probing with RNase T_1 (to test single-stranded Gs), dimethylsulfate (DMS) (to test A(N-1) and C(N-3)) and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMCT) (to test G(N-1) and U(N-3)) of the mRNA either free or complexed with the enzyme were conducted according to (5). Modified bases or cleavages were identified by primer extension, as described in (21). Incubation controls were run in parallel in order to detect nicks or pauses of reverse transcription. The DNA primer used was a 5′ end labeled oligodeoxyribonucleotide complementary to nucleotides +47 to +61.

Inhibition analysis of the translational initiation complex formation (toeprinting)

The formation of the translational initiation complex and toeprinting conditions were adapted from Hartz *et al.* (22). The oligonucleotide used as a primer was the same as above. Experimental conditions are strictly identical to those described in (5), except that we used *E.coli* $tRNA^{Lys}$ (U*UU) to form the translational initiation complex instead of initiator $tRNA_f^{Met}$.

RESULTS AND DISCUSSION

Structural features of the different mRNA mutants used

The secondary structure model of the *thrS* operator and the different mutants studied are shown in Figure 1. All mutants display a total or partial loss of control of *thrS* expression by ThrRS (19, Table 1). Their conformation was monitored by a variety of chemical and enzymatic structure probes and compared to the wild-type (WT) mRNA conformation. The G to A substitution at position −32 (BS4-9) has no effect on the overall conformation of the mRNA leader (6). The G to A substitution at position −40 (M6-1) disrupts a G-C pair and results in an extensive conformational rearrangement encompassing domains 1, 2 and 3 (6). In ΔV, the anticodon-like domain (nucleotides −22 to −42) was deleted, but the stable stem-loop structure of domain 4 is still preserved as shown by chemical probing (unpublished result). In both U_{-107}WT and U_{-107}BS4-9, the bulged nucleotide A_{-107} was replaced by U. Unexpectedly, in both RNAs the mutation induces local reactivity changes in the acceptor-like domain: the uridines −80, −81, −82 and −107 become marginally reactive under native conditions, and the base pair U_{-85}-A_{-106} is stabilized (Fig. 2). This result suggests a local rearrangement of domain 4 that involves the following base pairs: U_{-80}-A_{-111}, U_{-81}-A_{-110}, G_{-83}-C_{-108} and G_{-84}-U_{-107}, leading to a continuous helical structure (Fig. 1). The two bases C_{-109} and U_{-82} are either stacked inside the helix or involved in a non canonical interaction.

The operator acts as a competitive inhibitor of tRNA aminoacylation

The ability of WT and BS4-9 mRNAs to compete with $tRNA^{Thr}$ and $tRNA^{Met}$ for aminoacylation with ThrRS and MetRS, respectively, was studied. From these experiments, the

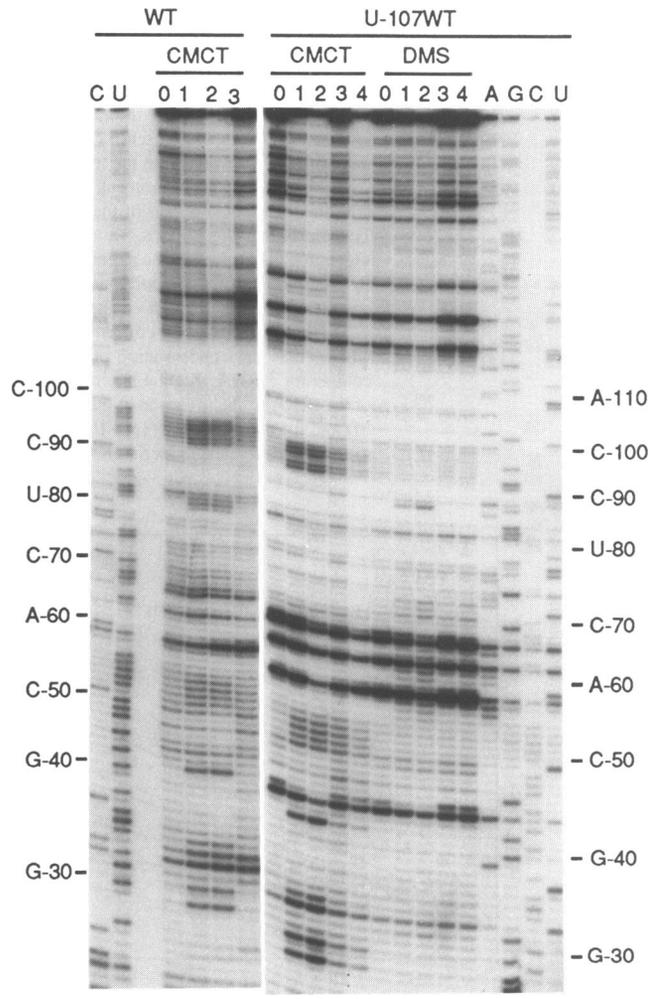

Figure 2. Probing experiments on WT and U_{-107}BS4-9 mRNAs. (a) Gel electrophoresis fractionation of products resulting from modifications with DMS (WT and U_{-107}BS4-9) and CMCT (U_{-107}BS4-9). Native conditions: (lane 0) incubation control, (lane 1) 5 min for DMS, 15 min for CMCT, (lane 2) 10 min for DMS and 30 min for CMCT; semi-denaturing conditions: (lane 3) 2 min for DMS and 5 min for CMCT, (lane 4) 5 min for DMS and 15 min for CMCT. Lanes A, C, G, U are sequencing products generated in the presence of ddTTP, ddGTP, ddCTP and ddATP, respectively.

dissociation constant of the complexes between the enzyme and the competitor mRNAs was deduced. Competition curves are shown in Figure 3 and results are summarized in Table 1. In the threonine system, the apparent K_m (K'_m) value of $tRNA^{Thr}$ increases in the presence of WT mRNA, whereas the maximal velocity (V_{max}) remains unchanged (see Fig. 3a). It clearly shows that the WT mRNA acts as a competitive inhibitor of $tRNA^{Thr}$ aminoacylation. The deduced dissociation constant of the WT/ThrRS complex (K_d = 0.1 ± 0.03 μM) is close to that of the $tRNA^{Thr}$/ThrRS complex ($K_d \cong 0.1$ μM) determined by filter binding assays (5). The finding that the inhibition is competitive provides evidence that the binding site for the operator is identical or overlaps the binding site for the $tRNA^{Thr}$. However, it cannot be totally excluded that the operator interacts at a different site and obstructs binding of the tRNA either by conformational changes of ThrRS or by steric hindrance. Interestingly, ΔV ($K_d \cong 1.55 \pm 0.3$ μM) and BS4-9 ($K_d \cong 1.75 \pm 0.3$ μM) still bind to ThrRS and also behave as competitive inhibitors, but their dissociation constant is strongly increased as compared to that of the WT/ThrRS complex (Table 1). Footprinting data indicate that ThrRS does not interact with the anticodon-like region of BS4-9 but shields only the acceptor-

Table 1. Table summarizing the apparent dissociation constant values calculated for mRNA and the effect of *thrS* mRNA leader mutations on the repression caused by ThrRS or MetRS on β-galactosidase synthesis from *thrS-lacZ* fusions.

	MetRS		ThrRS	
mRNAs	Repression (*in vivo*)	K_d (10^{-7} M)	Repression (*in vivo*)	K_d (10^{-7} M)
BS4-9	11.8	11 ± 3	1.05	17.5 ± 3
WT	2.3	33 ± 3	14.5	1.0 ± 3
U_{-107}BS4-9	nd	17.3 ± 3	nd	nd
U_{-107}WT	nd	nd	2.4	3.0 ± 3
ΔV	1.04	>100	1.05	15.5 ± 3

The repression is given by the ratio of β-galactosidase values obtained with a plasmid control to that obtained in the presence of the overproducing plasmid pUB4 (for ThrRS) and pNAV7 (for MetRS). The values for the repression induced by MetRS are from (18) and those for ThrRS are from (19). In each experiment, apparent michaelian constant values were derived from iterative non-linear least-squares fits of the theoretical equation to the experimental values (from Fig. 1). To note is that the same K_i value could be detected by using various mRNA concentrations suggesting that in the RNA concentration range used, the competitive inhibition is linear. Therefore, we assumed that the K_i corresponds to the dissociation constant (K_d) of the operator/ThrRS complex. The dissociation constants were calculated (i) from the slope of the plot of K'_m as a function of the mRNA concentration or (ii) by using the equation $K'_m = K_m [1 + (I)/K_i]$ where (I) is the concentration of the competitor mRNA, K_i the dissociation constant for the competitor mRNA, K_m the michaelian constant of the tRNA and K'_m the michaelian constant of the tRNA in the presence of the competitor mRNA. K_m values are for the $tRNA^{Thr}$ ($2.5\ 10^{-7}$ M) and for the $tRNA_f^{Met}$ ($1.15\ 10^{-7}$ M). The experimental errors have been defined from two to three independent experiments.

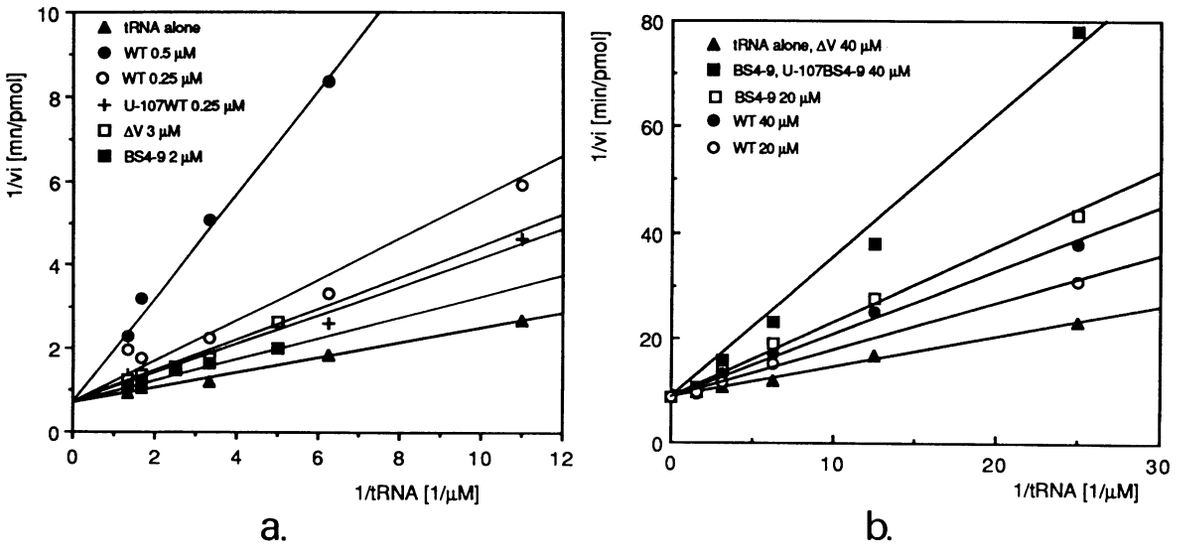

Figure 3. The (CAU) and (CGU) operators act as competitive inhibitors of $tRNA_f^{Met}$ and $tRNA^{Thr}$ aminoacylation by MetRS and ThrRS, respectively. Initial rates of reaction were measured in the absence (filled triangle) and in the presence of the WT mRNA and several mutant mRNAs (see the symbols in the figure) at the indicated concentration. (a) $tRNA^{Thr}$ aminoacylation catalyzed by *E.coli* ThrRS; (b) $tRNA_f^{Met}$ aminoacylation catalyzed by *E.coli* MetRS. The experimental conditions are described in material and methods.

like arm (5). Also the footprint of ThrRS in the acceptor-like arm of ΔV remains unchanged as compared to the WT operator (unpublished result). Therefore, the deletion of the anticodon-like domain and even the ponctual mutation in the anticodon strongly affects the binding of ThrRS, most probably by loss of specific contacts. Furthermore, U_{-107}WT which displays a continuous helix in the acceptor-like domain (Fig. 1) has an apparent K_d reproducibly higher (0.3 ± 0.03 μM) than that of the WT/ThrRS complex. The reduced binding affinity of the synthetase for this mutant probably results from the local rearrangement in the acceptor-like domain. This result also provides evidence that the acceptor-like domain contributes to the stability of the WT/ThrRS complex, consistent with *in vivo* data showing that the U_{-107} mutation reduces the efficiency of regulation (19, Table 1).

Using the same approach, we showed that BS4-9 behaves as a competitive inhibitor of $tRNA_f^{Met}$ aminoacylation catalyzed by *E.coli* MetRS (Fig. 3b, Table 1) with a dissociation constant of 1.1 ± 0.3 μM, one order of magnitude higher than that determined for the WT/ThrRS complex (Table 1). Strikingly, the WT mRNA also acts as a competitive inhibitor for $tRNA_f^{Met}$ aminoacylation (Fig. 3b, Table 1) with a dissociation constant reproducibly three times higher than that for the BS4-9/MetRS complex (K_d = 3.3 ± 0.3 μM). This result is correlated with *in vivo* data showing that the WT also reduces the efficiency of *thrS* regulation by MetRS (18, Table 1). As expected, deleting the anticodon-like domain (ΔV) does not significantly modify the Michaelian parameters of $tRNA_f$Met aminoacylation (K_m = 0.115 μM), even at high ΔV concentration (40 μM). Otherwise, the double mutant U_{-107}BS4-9 is as efficient as BS4-9 in

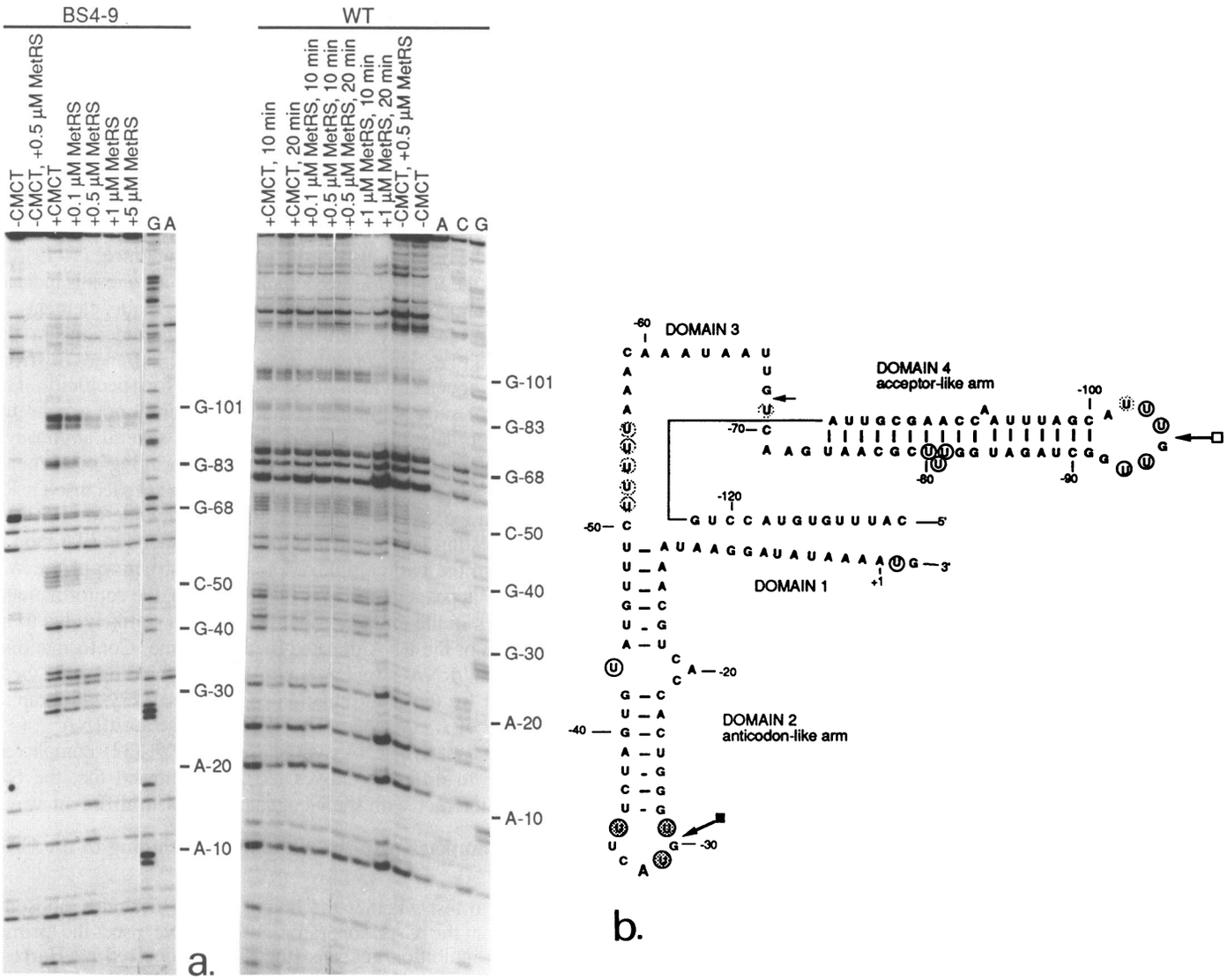

Figure 4. MetRS shields the anticodon- and acceptor-like domains of BS4-9. (a) CMCT probing of WT and BS4-9 mRNAs in the presence of increasing concentrations of MetRS, as indicated. A, C and G are sequencing lanes. (b) Secondary structure model of BS4-9 in the L-shape conformation summarizing the effect of MetRS binding. The reactivity changes induced by the enzyme at G(N1) and U(N3) are indicated: protected nucleotides are encircled and the degree of protection is proportional to the intensity of the circle (from strong to minor). RNase T_1 cuts protected by MetRS are shown by arrows: strong and medium protection are denoted by a filled square and empty square, respectively. The intensities of the reactivity changes have been determined from three different experiments in which the gels were densitometrically scanned.

competing for $tRNA_f^{Met}$ aminoacylation, in contrast to U_{-107}WT in which the same mutation reduces the competition strength (Fig. 3b, Table 1). These results indicate that the acceptor-like domain does not (or very little) contribute to the stability of the BS4-9/MetRS complex. It has to be noted that the $[Mg^{2+}]$/[ATP] ratio is different in the threonylation (r = 4) and in the methionylation (r = 2.35) assays. However, in the magnesium concentration used (from 5 to 15 mM), probing and footprinting experiments revealed no significant effect of magnesium on the conformation of the operator and on the synthetase footprint suggesting that the stability of the operator/repressor complex is not drastically affected by the magnesium changes (results not shown).

These results stress the importance of the anticodon-like sequence in the operator in defining MetRS recognition. This is corroborated by Meinnel and co-workers (15) who showed that a tRNA fragment corresponding to the anticodon arm of $tRNA_f^{Met}$ is sufficient to compete with the tRNA for MetRS binding, the formation of this complex being dependent on the presence of the cognate CAU anticodon sequence (15). Interestingly enough, the measured dissociation constant for this tRNA fragment (33 μM) is higher to that determined for the BS4-9/MetRS complex (1.1 $\pm$ 0.3 μM), suggesting the presence of additional contacts in the operator. However, it appears that MetRS recognizes BS4-9 with a lower affinity than the initiator $tRNA^{Met}$ ($K_m \cong 0.11$ μM) and than ThrRS does for the WT mRNA. One can assume that elements contributing to methionine specificity are missing in BS4-9, and/or that several nucleotides in the mRNA may negatively affect MetRS recognition. Recently, Martinis and Schimmel (23) showed that a RNA minihelix corresponding to the acceptor stem of $tRNA_f^{Met}$ is sufficient to confer aminoacylation by MetRS, indicating the importance of the conserved base pairs G_2-C_{71}, C_3-G_{70} and A_{73} for methionine specificity. To note is that the two conserved base pairs in the acceptor stem of $tRNA^{Met}$ are respectively replaced in BS4-9 by the base pairs C_{-79}-G_{-112} and G_{-78}-C_{-113} as compared to the $ACCA_{-107}$ sequence. However, it has to be noted that a duplex containing the acceptor stem of $tRNA^{Val}$ can not be aminoacylated by MetRS (23) whereas this tRNA bearing the anticodon methionine can be charged by the enzyme (14). This apparent contradiction indicates that the interaction of MetRS to the anticodon CAU is sufficient to overcome the lack of some of the recognition elements in the acceptor arm, as it is probably in the BS4-9 operator. However, there is a striking difference between the acceptor arm of the tRNA and the acceptor-like domain of the operator if we assumed that the ACCA sequence of the mRNA correspond to the 3′ terminal sequence of the tRNA. In the operator, this sequence is not free but is prolonged by a hairpin loop (Fig. 1). Both genetic and biochemical investigations are currently in progress in order to define more precisely the molecular bases of the operator-repressor recognition in the acceptor-like domain.

MetRS shields the anticodon-like arm but also the acceptor-like domain of the CAU operator

The conformation of BS4-9 was probed by the use of RNase T_1 and CMCT, in the presence of increasing concentrations of MetRS. A typical experiment is shown in Figure. 4a and the results are summarized in Figure 4b on the secondary structure model of the operator region of the mRNA. The binding of MetRS to BS4-9 causes significant reduction of reactivity in both anticodon- and acceptor-like domains. The strongest protections are observed in the anticodon-like loop (at U_{-29}, U_{-31} and U_{-35}) and at U_{-43}. Protections are also induced in the external loop (U_{-93} to U_{-97}) and near the bulged U_{-81} of the acceptor-like domain. Weak protections are observed in the connecting loop of domain 3. The protection induced by MetRS at the different sites appears to depend on MetRS concentration in a nearly similar extent. MetRS does not induce any detectable protection on WT mRNA (Fig. 4a) and on M6-1 in a concentration range of 10 nM to 1 μM. Furthermore, we showed by using RNase T_1 footprinting that $tRNA_f^{Met}$ (1 μM) is able to displace equally the anticodon- and acceptor-like domains of BS4-9 from the enzyme (results not shown).

The fact that the anticodon-like domain of BS4-9 is shielded by MetRS but not by ThrRS (5) strongly suggests the existence of specific contacts between the anticodon-like CAU sequence and MetRS. This result is well correlated with the fact that methionine acceptance can be conferred to different tRNA species by inserting a CAU anticodon (15, 24, 25), and that the enzyme tightly binds to C_{34} at the wobble position of the initiator $tRNA_f^{Met}$ (24, 26, 27). Our results show that ThrRS (5) and MetRS both shield the anticodon-like region as well as the acceptor-like domain of the wild type and switched *thrS* mRNA, respectively. Strikingly, both enzymes shield the external loop of domain 4 which has no equivalent in tRNA if we assume that the $ACCA_{-107}$ sequence is analogous to the 3′ extremity of the tRNA. In the case of the WT/ThrRS interaction, genetic studies showed that the anticodon-like domain is essential and that the acceptor-like domain also participates to the control (7, 19). In the case of the BS4-9/MetRS complex, our *in vitro* results indicate that the anticodon-like sequence is the main element of recognition. Possibly, the MetRS-induced protections in the acceptor-like domain result from steric hindrance or from additional contacts which are not essential for specificity. The existence of such additional contacts apparently not crucial for the function have already been reported in several aminoacyl-tRNA synthetase/tRNA complexes by footprinting experiments (*e.g.* 28–30). Interestingly, CMCT probing reveals that ThrRS induces an increased reactivity of G_{-30} (5), while MetRS does not, suggesting that the geometry of the anticodon-like loop is different in the two complexes. Since the structure of the WT and BS4-9 appears to be identical (6) (Fig. 1), the conformational differences in the anticodon-like loop are most likely due to an adaptation of the RNA dictated by the enzyme. Conformational changes in tRNA structure upon enzyme binding have been evidenced by the determination of the X-ray crystallographic structures of *E.coli* glutaminyl-tRNA synthetase/$tRNA^{Gln}$ (31) and yeast aspartyl-tRNA synthetase/$tRNA^{Asp}$ (32) complexes, belonging to different classes (33), and showed that the two enzymes interact with their cognate tRNAs in different ways.

MetRS competes with the ribosome for binding to the CAU operator

In order to test whether the bound MetRS occludes ribosome binding on the CAU operator (BS4-9), we used the primer extension inhibition analysis (toeprinting) deviced by Hartz *et al.* (22). This approach uses the fact that the elongation by reverse transcriptase (from a DNA primer annealed to the mRNA dowstream from the initiation codon) is blocked at position +16 by the formation of the ternary [30S/mRNA/initiator tRNA] complex. Toeprinting was already used to show that ThrRS and the ribosome compete for binding to the WT mRNA (5). Earlier work of Hartz *et al.* (34–35) showed that a toeprint could also

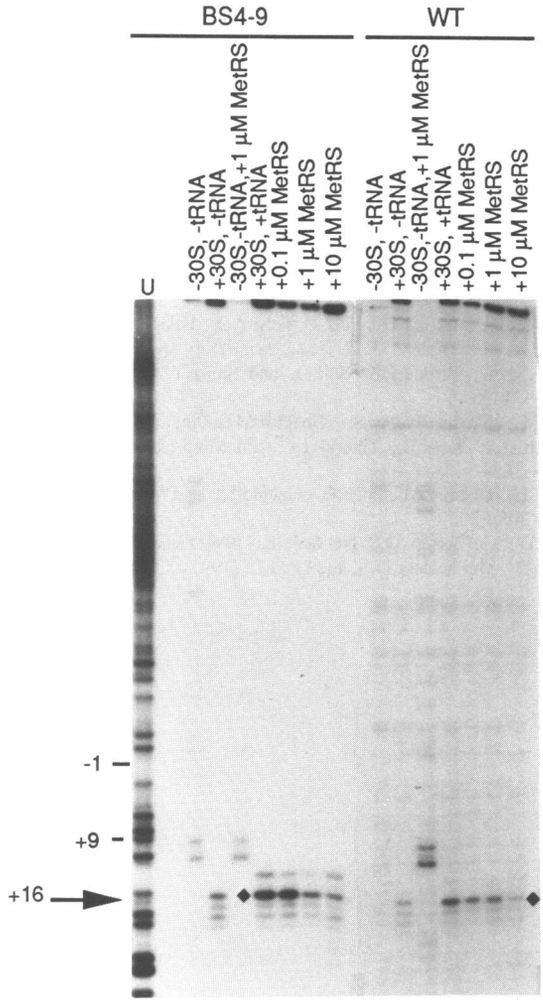

Figure 5. MetRS and 30S subunit compete for binding to BS4-9. Toeprinting experiments are conducted with BS4-9 and WT mRNAs in the presence of increasing concentrations of MetRS (as indicated). The ternary complex was formed with $tRNA_3Lys$ instead of initiator $tRNA_f^{Met}$. The toeprint +16 is indicated by an arrow. U is a sequencing lane. Two stops at position +10 and +12 are observed in the control lanes. These unspecific stops are unreproducibly observed and probably result from intrinsic fragility of the mRNA.

be obtained with elongator tRNAs 15 nucleotides downstream from the first base of the recognized codon (in the vicinity of the AUG initiation codon). Therefore, in the present experiments, the ternary complex was formed with the *E.coli* $tRNA^{Lys}$ (U*UU), instead of the initiator $tRNA_f^{Met}$, in order to avoid competition between $tRNA_f^{Met}$ and BS4-9 for MetRS binding. Unexpectedly, the 30S/$tRNA^{Lys}$/BS4-9 ternary complex still yields one major toeprint at position +16, most probably resulting from an incorrect interaction between the anticodon UUU of $tRNA^{Lys}$ and the AUG codon (Fig. 5). The toeprint at position +14 corresponding to the reading of the cognate codon (AAA_{+1}) appears to be the minor one and is almost absent with the WT mRNA (Fig. 5). An incorrect toeprint was also detected with the ternary complex formed by the 30S subunit, a transcript RNA containing the gene 32 ribosome-binding site and $tRNA^{Lys}$ (34) and with the *rpsO* mRNA/30S/$tRNA^{Lys}$ ternary complex (C. Philippe, personal communication) but to a different extent.

The presence of increasing concentrations of MetRS decreases the yield of the toeprint (between 1 and 10 μM of enzyme). This result is in reasonable agreement with the K_d value for the BS4-9/MetRS complex (1.1 ± 0.3 μM) despite the fact that the experimental conditions used for the toeprint and for the competition experiments are slightly different. On the opposite, the addition of ThrRS to the ternary 30S/$tRNA_f^{Met}$/BS4-9 complex was shown to have no effect on the yield of toeprint even at a concentration of 10 μM of enzyme (5). Control experiments shows that MetRS fails to inhibit toeprinting (results not shown) with M6-1, in which the anticodon-like arm is totally reorganized but the acceptor-like arm remains unaffected (6). This result indicates that the acceptor-like domain by itself is unable to ensure MetRS-mediated inhibition of ribosome binding, as it is for ThrRS (5). Our results show that MetRS and the 30S subunit specifically compete for the binding to BS4-9, indicating that MetRS acts as a repressor by occluding the binding of the 30S subunit to its translational initiation site. Therefore, MetRS represses the expression of the gene controlled by the CAU operator by a mechanism identical to that described for ThrRS with the wild type operator (5).

When increasing concentrations of MetRS are added to the ternary complex formed with the WT mRNA, a significant decrease of the toeprint is observed, but only at higher MetRS concentration (10 μM) (Fig. 5b). In agreement with the competition experiments, it further confirms that the WT mRNA is weakly recognized by MetRS. These results are also consistent with the *in vivo* data that evidenced a weak repression of the expression of the *thrS* mRNA by MetRS (18). This result reflects a low but specific binding of MetRS, since this effect was not observed with a mRNA leader having the anticodon region deleted or containing an arginine or a glutamine anticodon (18).

CONCLUDING REMARKS

The comparative study of the CAU operator/MetRS and the GCU (WT) operator/ThrRS complexes stresses the fact that the operator obeys to the same identity rules as their parent tRNAs and further confirms that the specific recognition of tRNAs or tRNA-like structures (here a mRNA operator) is governed by a small number of nucleotides. In both cases, the interaction of the enzyme to the anticodon-like sequence is essential to prevent the binding of the ribosome to its translational initiation site. The present work also shows that with both ThrRS and MetRS, the operator behaves as a competitive inhibitor of tRNA aminoacylation catalyzed by the cognate enzyme. In particular, with ThrRS, the dissociation constant of the CGU operator/ThrRS complex is almost identical to that of the $tRNA^{Thr}$/ThrRS complex. Furthermore, the mutant having the anticodon deleted still binds to ThrRS and also acts as a competitive inhibitor. This indicate that the acceptor-like domain contributes to the stability of the complex between the CGU operator and ThrRS while it does not (or poorly) in the complex between the CAU operator and MetRS. The possibility to switch the specificity of the control from ThrRS to MetRS and the fact that the *thrS* leader mRNA is a competitive inhibitor of tRNA aminoacylation are the first direct evidences for the existence of molecular mimicry between the translational operator and tRNA. They strongly suggest that the operator and the tRNA bind to the same or overlapping sites in the enzyme and that all the recognition elements for ThrRS binding are present in the *thrS* operator region. This is also supported by the isolation of ThrRS mutants that have an effect on the autoregulation but also on the aminoacylation of $tRNA^{Thr}$ (16). Therefore the mRNA should be able to adopt a tRNA-like

structure. Genetic data (19) and conformational studies in solution combined with graphic modeling (to be published) indicate that the loop (domain 3) connecting the anticodon-like and the acceptor-like domains help to orientate these two domains with respect to each other. This articulation confers to the mRNA a more relaxed conformation (as compared to the classical tRNA), a property which facilitates its adaptation on its cognate aminoacyl-tRNA synthetase as well as its interaction with the ribosome.

ACKNOWLEDGEMENTS

This work is dedicated to the memory of Prof. J-.P.Ebel. He was a grand Director who always gave us enthusiasm and fruitful advices. We will miss him. We are grateful to G.Fayat, Y.Mechulam, T.Meinnel and S.Blanquet for providing us *E.coli* methionyl-tRNA synthetase and for many advices and discussions. We are indebted to C.Philippe, H.Moine and D.Kern for fruitful discussions. We thank W.Studier (Brookhaven, USA) for providing us the plasmid coding for T_7 RNA polymerase. This work was supported by grants from the Centre National de la Recherche Scientifique and from the Fondation de la Recherche Médicale.

REFERENCES

1. Lestienne,P., Plumbridge,J.A., Grunberg-Manago,M. and Blanquet,S. (1984) *J. Biol. Chem.*, **259**, 5232–5237.
2. Springer,M., Plumbridge,J.A., Butler,J.S., Graffe,M., Dondon,J., Mayaux,J.F., Fayat,G., Lestienne,P., Blanquet,S. and Grunberg-Manago, M. (1985) *Proc. Natl. Acad. Sci. USA*, **83**, 4384–4388.
3. Butler,J.S., Springer,M., Dondon,J. and Grunberg-Manago,M. (1986) *J. Bacteriol.*, **165**, 198–203.
4. Springer,M., Graffe,M., Butler,J.S. and Grunberg-Manago,M. (1986) *Proc. Natl. Acad. Sci. USA.*, 83, 4384–4388.
5. Moine,H., Romby,P., Springer, M., Grunberg-Manago,M., Ebel,J.P., Ehresmann,B. and Ehresmann,C. (1990) *J. Mol. Biol.*, **216**, 299–310.
6. Moine,H., Romby,P., Springer,M., Grunberg-Manago,M., Ebel,J.P., Ehresmann,C. and Ehresmann,B. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 7892–7896.
7. Romby,P., Moine,H., Lesage,P., Graffe,M., Dondon,J., Ebel,J.P., Grunberg-Manago,M., Ehresmann,B., Ehresmann,C. and Springer,M. (1990) *Biochimie*, **72**, 485–494.
8. Mc Clain,W.H. and Nicholas,H.B. (1987) *J. Mol. Biol.*, 194, 635–642.
9. Hasewaga,T., Miyano,M., Himeno,H., Sano,Y., Kimura,K. and Shimizu,M. (1992) *Biochem. Biophys. Res. Comm.*, **184**, 478–484.
10. Normanly,J. and Abelson,J. (1989) *Annu. Rev. Biochem.*, 58, 1029–1049.
11. Lapointe,J. and Giegé,R. (1991) In Trachsel,H. (ed.), Translation in Eukaryotes, CRC Press Inc. Boca Raton, pp 35–69.
12. Schulmann,L.H. (1991) *Prog. Nucl. Acid Res. Molec. Biol.*, **41**, pp 23–87.
13. Schulmann,L.H. and Pelka,H. (1983) *Proc. Natl. Acad. Sci. U.S.A.*, 80, 6755–6759.
14. Schulmann,L.H. and Pelka,H. (1988) *Science*, **242**, 765–768.
15. Meinnel,T., Mechulam,Y., Blanquet,S. and Fayat,G. (1991) *J. Mol. Biol.*, 220, 205–208.
16. Springer,M., Graffe,M., Dondon,J. and Grunberg-Manago,M. (1989) *EMBO J.*, 78, 2417–2424.
17. Schulmann,L.H. and Pelka,H. (1990) *Nucleic Acids Res.*,**18**, 285–289.
18. Graffe,M., Dondon,J., Caillet,J., Romby,P., Ehresmann,C., Ehresmann,B. and Springer,M. (1992) *Science*, **255**, 994–996.
19. Brunel,C., Caillet,J., Lesage,P., Graffe,M., Dondon,J., Moine,H., Romby,P., Ehresmann, C., Ehresmann,B., Grunberg-Manago,M. and Springer,M. (1992) *J. Mol. Biol.*, in press.
20. Théobald,A., Springer,M., Grunberg-Manago,M., Ebel,J.P. and Giegé,R. (1988) *Eur. J. Biochem.*, 175, 511–524.
21. Mougel,M., Eyermann,F., Westhof,E., Romby,P., Expert-Bezançon,A., Ebel,J.P., Ehresmann,B. and Ehresmann,C. (1987) *J. Mol. Biol.*, 198, 91–107.
22. Hartz,D., McPheeters,D.S., Traut,R. and Gold,L. (1988) Methods Enzymol. **164**, 419–425.
23. Martinis,S.A. and Schimmel,P. (1992) *Proc. Natl. Acad. Sci. USA.*, 89, 65–69.
24. Meinnel,T., Mechulam,Y., Dardel,F., Schmitter,J.M., Hountondji,C., Brunie,S., Dessen,P., Fayat,G. and Blanquet, S. (1990) *Biochimie*, **72**, 625–632.
25. Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Nishimura,S., Miyazawa,T. and Yokohama,S. (1988) *Nature (London)* **336**, 179–181.
26. Pelka,H. and Schulmann,L.H. (1986) *Biochemistry*, **25**, 4450–4456.
27. Meinnel, T., Mechulam, Y., Le Corre, D., Panvert, M., Blanquet, S. and Fayat, G. (1991) *Proc. Natl. Acad. Sci. USA.*, 88, 291–295.
28. Park,S. J. and Schimmel,P.R (1988) *J. Biol. Chem.*, **263**, 16527–16530.
29. Schatz,D., Leberman, R. and Eckstein, F. (1991) *Proc. Natl. Acad. Sci. USA.*, 88, 6132–6136.
30. Rudinger,J., Puglisi,J.D., Pütz,J., Schatz,D., Eckstein,F., Florentz,C. and Giegé,R. (1992) *Proc. Natl. Acad. Sci. USA.*, in press.
31. Rould,M.A., Perona,J.J., Söll,D. and Steitz,T.A. (1989) *Science* **246**, 1135–1142.
32. Ruff,M., Krishnaswamy,S., Boeglin,M., Poterzman,A., Mitschler,A., Podjarny,A., Rees,B., Thierry,J.C. and Moras,D. (1991) *Science*, **252**, 1682–1689.
33. Eriani,G., Delarue,M., Poch,O., Gangloff,J. and Moras,D. (1990) *Nature*, **337**, 203–206.
34. Hartz,D., McPheeters,D.S. and Gold,L. (1989) *Genes Dev.* , **3**, 1899–1912.
35. Hartz,D., McPheeters,D.S. and Gold,L. (1991) *J. Mol. Biol.* **218**, 83–97.