JOURNAL OF BACTERIOLOGY, July 1993, p. 4507–4514
0021-9193/93/144507-08$02.00/0
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Vol. 175, No. 14

Importance of Formylability and Anticodon Stem Sequence To Give a $tRNA^{Met}$ an Initiator Identity in *Escherichia coli*

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Received 22 February 1993/Accepted 6 May 1993

In bacteria, the free amino group of the methionylated initiator tRNA is specifically modified by the addition of a formyl group. The functional relevance of such a formylation for the initiation of translation is not yet precisely understood. Advantage was taken here of the availability of the *fmt* gene, encoding the *Escherichia coli* Met-$tRNA_f^{Met}$ formyltransferase, to measure the influence of variations in the level of formyltransferase activity on the involvement of various mutant $tRNA_f^{Met}$ and $tRNA_m^{Met}$ species in either initiation or elongation in vivo. The data obtained established that formylation plays a dual role, firstly, by dictating $tRNA_f^{Met}$ to engage in the initiation of translation, and secondly, by preventing the misappropriation of this tRNA by the elongation apparatus. The importance of formylation in the initiator identity of $tRNA_f^{Met}$ was further shown by the demonstration that elongator $tRNA_m^{Met}$ may be used in initiation and no longer in elongation, provided that it is mutated into a formylatable species and is given the three G · C base pairs characteristic of the anticodon stem of initiator tRNAs.

The recognition of initiator Met-tRNA by specific factors governs the initiation of translation (35). In addition, initiator Met-tRNA must not be recognized by the elongation apparatus, which uses a distinct $tRNA^{Met}$ for the incorporation of internal methionines.

In procaryotic cells, prior to its involvement in translation, initiator Met-$tRNA_f^{Met}$ is modified by the addition of a formyl moiety on the NH_2 group of the esterified methionine. This reaction, catalyzed by 10-formyltetrahydrofolate:L-methionyl-$tRNA_f^{Met}$ *N*-formyltransferase (FMT; EC 2.1.2.9), is enough to prevent the incorporation into elongating peptide chains of the methionine esterified to $tRNA_f^{Met}$. However, it is not yet known whether formylation is dispensable for Met-$tRNA_f^{Met}$ initiator activity. In vitro studies with natural mRNA templates and *Escherichia coli* extracts showed that the formylation of Met-$tRNA_f^{Met}$ enhanced translation rates (5, 12). In particular, the formyl group seemed to improve the efficiency of the selection of $tRNA_f^{Met}$ by initiation factor IF2 (10, 24, 34). In vivo, it was found that the initiator activity of glutaminylated $tRNA_f^{Met}$ variants was related to their formylability (37). Moreover, the characterization of an *fmt* strain pointed out the importance of $tRNA_f^{Met}$ formylation in sustaining the rapid growth of *E. coli* (6).

Besides determinants involved in its formylation, $tRNA_f^{Met}$ may carry other structural determinants involved in its functional specificity towards the initiation step. The anticodon stem and loop sequences are of importance in the selection of $tRNA_f^{Met}$ by initiation factor IF3 (9). Mutations in the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif, which is conserved in the anticodon stem of most initiator tRNAs, reduced the initiator activity of *E. coli* $tRNA_f^{Met}$ in an in vitro translational assay (28). The same mutations made in an initiator tRNA bearing the amber anticodon ($tRNA_f^{Met}$amber) reduced its ability to initiate in vivo translation from an amber start codon (14).

In the present study, the availability of the *fmt* gene was used to explore further the role of formylation. The strategy consisted of an in vivo assay in which variants of initiator or elongator tRNAs were exposed to various intracellular levels of FMT activity. The variants of $tRNA_f^{Met}$ and $tRNA_m^{Met}$ were constructed on the basis of the precise knowledge of the nucleotides governing the recognition of Met-$tRNA_f^{Met}$ by FMT (7, 14). In particular, positions 73 and 1.72 were mutated to render a tRNA formylatable or not. The conclusion reached was that the formylation of Met-$tRNA_f^{Met}$ plays an important role in its orientation towards translation initiation. In addition, a restricted set of nucleotide changes capable of switching the elongator $tRNA_m^{Met}$ into an initiator species in vivo was defined. These changes included the introduction of the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif and the mutation of the $G_1 \cdot C_{72}$ base pair to $C_1 \cdot A_{72}$, the latter substitution giving Met-$tRNA_m^{Met}$ the capacity to become a substrate of FMT. Findings in perfect agreement with ours were independently reported by Varshney et al. (36) during the review of the present paper.

MATERIALS AND METHODS

Strains and general techniques. General genetic and recombinant DNA techniques were those of Miller (21) and Sambrook et al. (25).

The inactivation of the chromosomal *fmt* gene in UF121 (22) was achieved as described previously (6, 8), the chromosomal *fmt* gene being replaced by a disrupted copy with the help of thermosensitive plasmid pMAFkan. The resulting strain (PAL22/pMAF) was rendered recombination deficient (*recA*) by conjugation with JC10240 (4), yielding PAL22R/pMAF. PAL22R/pMAF was then lysogenized with λFatg, bearing the *fmt-1* (ATG) gene (6), and the pMAF plasmid was cured at 37°C, yielding PAL22RλFatg.

Construction of synthetic genes. Oligonucleotides were synthesized on a Pharmacia gene assembler and purified by anion-exchange chromatography (MonoQ; Pharmacia). tRNA genes were obtained by assembling six different overlapping oligonucleotides as described previously (15). Thereafter, tRNA genes were ligated between the *Eco*RI and *Pst*I sites of the pBSTNAV2 expression vector (16). The

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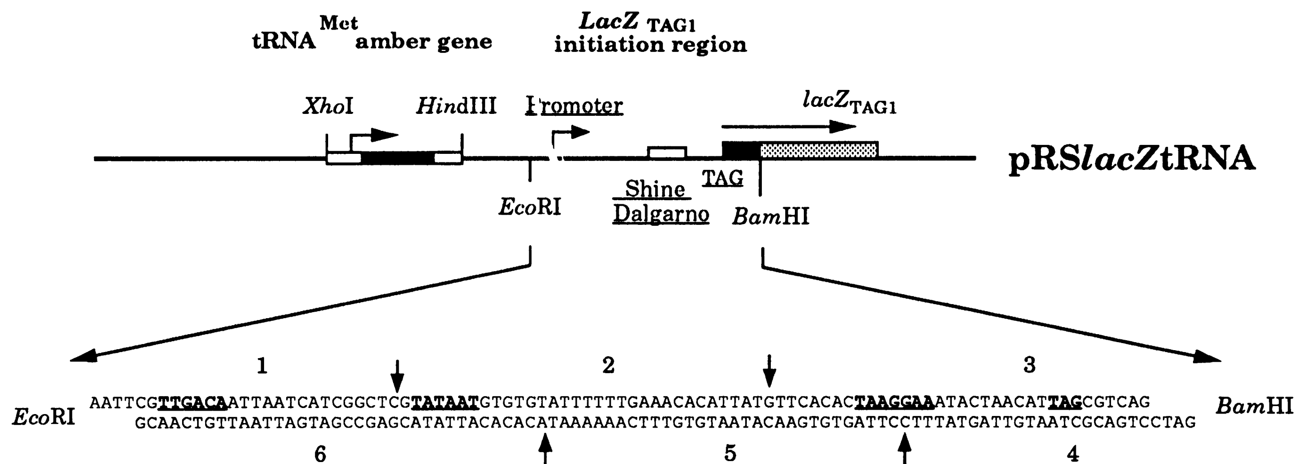

FIG. 1. Structure of the pRS*lacZ*tRNA plasmids. The synthetic part of the $lacZ_{TAG1}$ gene was inserted between the *Eco*RI and *Bam*HI restriction sites of plasmid pRS414XB to yield pRS$lacZ_{TAG1}$. The sequence of the six oligonucleotides composing the 5′ part of the $lacZ_{TAG1}$ gene is shown. The vertical arrows indicate the ends of the assembled oligonucleotides. The transcription control region of the $lacZ_{TAG1}$ gene was derived from the *tac* promoter sequence, with the elimination of the operator site for the *lacI*-encoded repressor. The translation initiation region is that of the bacteriophage λ *c*II gene, with the replacement of the ATG start codon of *lacZ* by a TAG amber codon. In addition, a TAA ochre codon was created immediately upstream from the amber start codon. The underlined sequences indicate the RNA polymerase recognition signals, the ribosome binding site and the UAG start codon, from left to right. The *Xho*I-*Hin*dIII fragment from a given pBStRNA plasmid was then inserted between the same sites of pRS$lacZ_{TAG1}$, yielding the corresponding pRS*lacZ*tRNA plasmid.

resulting plasmids, expressing various tRNAs, were named pBStRNA plasmids. tRNA species were named in accordance with the following rule: a mutated position in the sequence of a tRNA is indicated by the substituted base followed by its position, e.g., $tRNA_f^{Met}amberG_{72}$ stands for $tRNA_f^{Met}$ bearing the amber (CUA) anticodon and having the base at position 72 changed to a G.

Construction of plasmids. Plasmid pRS414XB was obtained by the insertion of a *Xho*I-*Kpn*I-*Hin*dIII-*Eco*RI-*Sma*I-*Bam*HI polylinker between the *Eco*RI and *Bam*HI sites of pRS414 (30). pRStRNA plasmids were obtained by inserting the *Xho*I-*Hin*dIII fragment from the corresponding pBStRNA plasmids. The structure of the pRS*lacZ*tRNA plasmids is detailed in Fig. 1. pACtRNA plasmids resulted from the insertion of the *Xho*I-*Hin*dIII fragment from pBStRNA plasmids between the *Sal*I and *Hin*dIII sites of pACYC184 (2). The pACFatg plasmid resulted from the insertion of the *Pvu*II-*Xho*I fragment from pBSFatg (6) between the *Ava*I and *Sal*I sites of pACYC184. The pACMTS* plasmid, expressing a mutant form (MTS*) of *E. coli* methionyl-tRNA synthetase (MetRS), resulted from the insertion of the *Xba*I-*Xho*I fragment from pBSM547WA461AV451QP213DA449 (18) between the *Xba*I and *Sal*I sites of pACYC184. The pACMTS*Fatg plasmid was obtained through the insertion of the *Eco*RI-*Ava*I fragment from pACMTS* between the *Eco*RI and *Sal*I sites of pACFatg.

Enzymatic activity measurements. β-Galactosidase activity was measured in toluene-treated cells (21) grown as follows. A single colony was inoculated into 0.3 ml of M9 minimal medium (unless otherwise indicated) containing 50 μg of ampicillin and 25 μg of chloramphenicol per ml and supplemented with 0.2% Casamino Acids and grown overnight at 37°C without shaking. Afterwards, 3 ml of the same medium was added to each of the 0.3-ml cultures. The cultures were grown further at 37°C with shaking, and aliquots were withdrawn for β-galactosidase measurements when the optical densities at 650 nm of the cultures ranged between 0.4 and 0.8 optical density unit. Two different clones from the same strain were systematically compared. The resulting average measured activities, expressed as described by Miller (21), and associated errors are given in the tables.

FMT activity was measured in the above-described culture aliquots as reported previously (6). In all cases, the levels of overproduction of FMT resulting from the presence of the pACFatg or pACMTS*Fatg plasmid ranged between 13 and 32 times the level measured in the absence of the plasmid. The same aliquots were used to measure MetRS activity as described previously (20). For each studied tRNA, the variations in MTS* expression in strain UF121R or PAL125R harboring pACMTS* or pACMTS*Fatg, respectively, were fewer than ±20%.

Michaelis-Menten parameters for the formylation reaction of derivatives of Met-$tRNA_m^{Met}$ were determined as described previously (7).

RESULTS

The orientation of variants of $tRNA_f^{Met}$amber towards either the elongation or the initiation step depends on the cellular FMT concentration. For exploration of the importance of formylation in specifying the initiator identity of $tRNA_f^{Met}$ in vivo, the initiator and elongator activities of various tRNAs bearing the amber (CUA) anticodon were evaluated through the suppression of amber mutations located either inside or at the first position of a reporter gene. This strategy was chosen because it was already known (37, 38) that the initiation of protein synthesis could occur from an amber codon, making possible the measurement of the efficiencies of suppression of $tRNA_f^{Met}$amber variants aminoacylated in vivo by glutaminyl-tRNA synthetase. Here, the availability of the *fmt* gene encoding *E. coli* methionyl-$tRNA_f^{Met}$ formyltransferase allowed us to measure and compare both the initiator and the elongator activities of the tRNAs directly as a function of the FMT activity present in the host cell.

In the first assay, the suppression of an internal amber codon in the *lacI-lacZ* gene fusion by a tRNA bearing the

TABLE 1. Description of strains and plasmids used in this study

Strain or plasmid	Description
Strains	
UF121R	*ara argE*(Am) Δ*lac-proB nalA rif thi recA56 srl*-300::Tn*10* F′ *proAB*$^+$ *lacI-lacZ* (UAG at position 181); indicator strain carrying the reporter gene in the elongation assay
PAL125R	F$^-$ derivative of UF121R; host strain in the initiation assay
PAL22RλFatg	Same as UF121R but *fmt*Δ*1*::*kan* λFatg; indicator strain in the elongation assay, expressing FMT from the *fmt-1* (ATG) gene under the control of the *lac* promoter
Plasmids	
pRStRNA	pRS414 (30) derivatives overproducing tRNAs in the elongation assay in UF121R
pRS*lacZ*tRNA	pRS414 derivatives overproducing tRNAs in the initiation assay and carrying the reporter $lacZ_{TAG1}$ gene
pACFatg	pACYC184 (2) derivative overproducing FMT
pACMTS*	pACYC184 derivative overproducing the MTS* MetRS variant
pACMTS*Fatg	pACYC184 derivative overproducing both the MTS* MetRS variant and FMT
pACYC184	Vector used for the construction of pACFatg, pACMTS*, pACMTS*Fatg, and pACtRNA, used as a control in initiation and elongation assays
pACtRNA	pACYC184 derivatives overproducing tRNAs in the elongation assay with PAL22RλFatg
pMC9	pBR322 derivative overproducing the *lac* operon repressor (1)

amber (CUA) anticodon was monitored in the UF121R indicator strain (Table 1; 32). In the second assay, the reporter $lacZ_{TAG1}$ gene, starting on an amber codon (Fig. 1), was introduced into PAL125R (an F$^-$ strain derived from UF121R) through transformation with the pRS*lacZ*tRNA$_f^{Met}$ amber plasmid, carrying both the $lacZ_{TAG1}$ and the tRNA$_f^{Met}$ amber genes (Fig. 2). As expected from previous results (38), the transformation resulted in the appearance of β-galactosidase activity. Further, the β-galactosidase production responded to the presence of the tRNA$_f^{Met}$amber gene and not to that of the tRNA$_m^{Met}$amber gene, although overexpressed tRNA$_m^{Met}$amber is also known to be aminoacylated in vivo (by lysyl-tRNA synthetase [LysRS]; 23). It could therefore be concluded that the level of β-galactosidase activity in

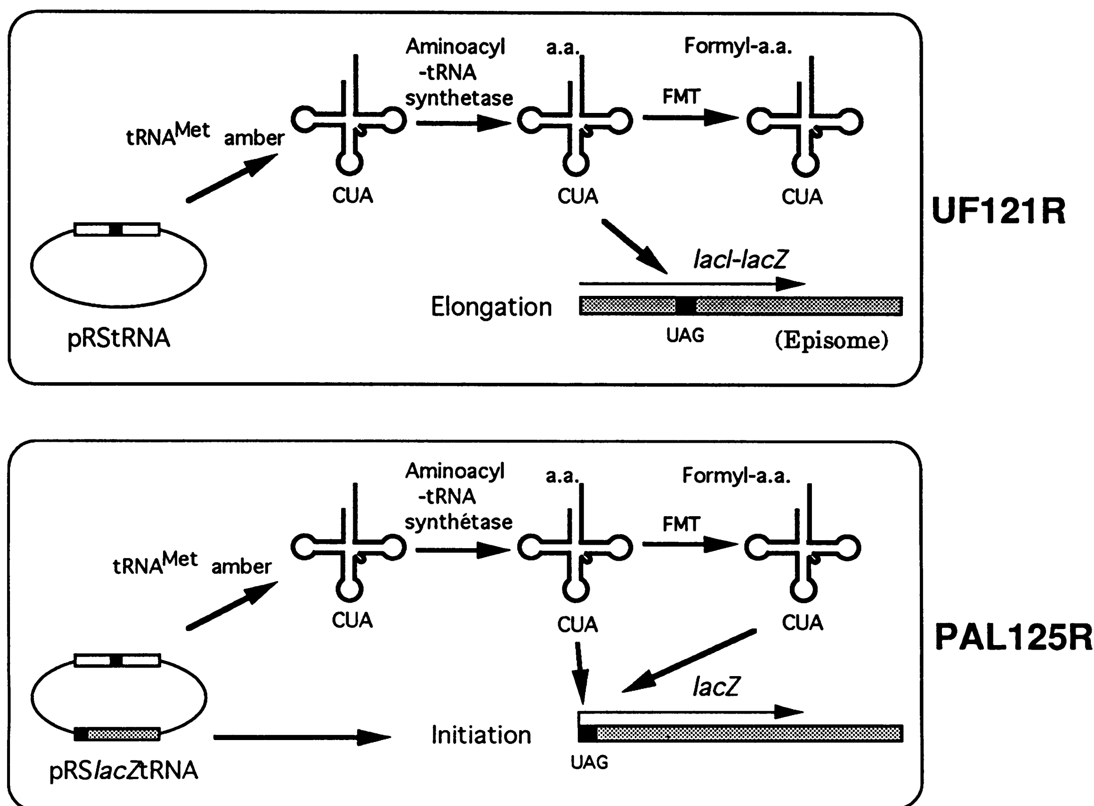

FIG. 2. In vivo assay for measuring the activity of a tRNA in initiation or in elongation. In strain UF121, a tRNA bearing the amber anticodon is produced from a pRStRNA plasmid. The elongator efficiency of this tRNA is reported by the suppression of an internal amber codon inside the episomal *lacI-lacZ* gene fusion (22). In strain PAL125R, derived from UF121 and lacking the episome, a tRNA bearing the amber anticodon is produced from a pRS*lacZ*tRNA plasmid. The initiator efficiency of this tRNA is reported by the expression of β-galactosidase from the $lacZ_{TAG1}$ gene (borne by the pRS*lacZ*tRNA plasmid), whose translation initiates on a UAG start codon. a.a., amino acid.

TABLE 2. Effect of overproduction of FMT on the suppression during elongation or initiation by $tRNA_f^{Met}$amber variants aminoacylated with glutamine

$tRNA^{Met}$amber	β-Galactosidsae activity in extracts of the following strain carrying the indicated plasmid[a]:			
	UF121R (elongation)		PAL125R (initiation)	
	pACYC184	pACFatg	pACYC184	pACFatg
f	<0.1	<0.1	2.0 ± 0.1	3.5 ± 0.3
fU_1	2.9 ± 0.3	0.1	0.3 ± 0.1	0.8 ± 0.1
fG_{72}	5.0 ± 0.4	4.7 ± 0.5	<0.1	0.1
fG_{73}	2.0 ± 0.1	0.2 ± 0.1	1.4 ± 0.1	11.1 ± 0.7
m[b]	17.5 ± 1.5	18.9 ± 0.3	<0.1	<0.1

[a] β-Galactosidase activity is reported in Miller units. Plasmid pACFatg carries the *fmt-1* (ATG) gene. pACYC184 is the control plasmid without the *fmt* gene.
[b] $tRNA_m^{Met}$amber is aminoacylated with lysine in vivo (23).

TABLE 3. Effect of overproduction of FMT on the suppression during elongation or initiation by $tRNA^{Met}$amber variants in the presence of MTS*

$tRNA^{Met}$amber	β-Galactoside activity in extracts of the following strain carrying the indicated plasmid[a]:			
	UF121R (elongation)		PAL125R (initiation)	
	pACMTS*	pACMTS*Fatg	pACMTS*	pACMTS*Fatg
f	<0.1	<0.1	12.9 ± 0.5	18.0 ± 3.0
fU_1	3.8 ± 0.2	0.3 ± 0.1	12.3 ± 0.3	15.4 ± 1.1
fG_{72}	25.2 ± 0.5	6.2 ± 1.2	2.9 ± 0.2	25.2 ± 0.3
fG_{73}	1.8 ± 0.3	0.2 ± 0.1	1.3 ± 0.1	13.4 ± 2.4
m	91.9 ± 5.0	104.1 ± 1.5	<0.1	<0.1

[a] β-Galactosidase activity is reported in Miller units. Plasmid pACMTS* carries the gene encoding the MTS* variant of MetRS. Plasmid pACMTS*Fatg carries in addition the *fmt-1* (ATG) gene.

PAL125R cells varied in relation to the capacity of the introduced $tRNA^{Met}$amber species to be used as an initiator tRNA.

The efficiencies of suppression of three $tRNA_f^{Met}$amber variants (U_1, G_{72}, and G_{73}) were then studied. The U_1, G_{72}, and G_{73} mutations are known to confer on $tRNA_f^{Met}$amber the capability to participate in the elongation of protein synthesis (7, 27). These mutations affect tRNA formylability to different extents (7, 14). In vitro, the G_{73} mutation decreases the efficiency of formylation (k_{cat}/K_m) by FMT of methionyl-$tRNA_f^{Met}$ by 1 order of magnitude. The U_1 mutation does not significantly modify the catalytic parameters of methionyl-$tRNA_f^{Met}$ formylation. The G_{72} mutation dramatically reduces, by a factor of 10^3, the catalytic efficiency of formylation.

UF121R cells were transformed by the five pRStRNA plasmids (encoding $tRNA_m^{Met}$amber, $tRNA_f^{Met}$amber, or the G_{72}, G_{73}, and U_1 variants of the latter). PAL125R cells were transformed by the corresponding set of pRS*lacZ*tRNA plasmids (Fig. 2). In the presence of each $tRNA^{Met}$amber, the levels of β-galactosidase activity were measured in the UF121R and PAL125R derivatives, overexpressing or not overexpressing FMT activity. For this purpose, the cells were transformed with either the pACFatg plasmid, bearing the *fmt-1* (ATG) gene (6), or the pACYC184 vector as a control.

In a wild-type FMT context, the three variants, U_1, G_{72}, and G_{73}, of $tRNA_f^{Met}$amber were active in elongation, as indicated by the suppression in UF121R, whereas variants U_1 and G_{73} but not variant G_{72} were also active in initiation in PAL125R (Table 2). The overproduction of FMT had a minor effect on the suppression during initiation obtained with $tRNA_f^{Met}$amber or on that during elongation obtained with $tRNA_m^{Met}$amber. On the contrary, the overproduction of FMT resulted in (i) a decrease in the extent of the suppression during elongation observed with the U_1 and G_{73} variants and (ii) an increase in the extent of the suppression during initiation observed with the same variants. Finally, the overproduction of FMT had little effect on the suppression induced by $tRNA_f^{Met}$amberG_{72} during elongation but resulted in the expression of low but significant β-galactosidase activity in the initiation assay.

On the basis of these results, it could be concluded that, for elongation-proficient variants U_1 and G_{73}, the level of intracellular FMT activity had a determining influence on their orientation towards either the elongation or the initiation of translation. However, these tRNAs, as well as variant G_{72}, are known to be glutaminylated in vivo (27). It was therefore of interest to study the fate of the same tRNAs aminoacylated in vivo with methionine. For this purpose, plasmids expressing either a MetRS variant (MTS*) capable of aminoacylating a $tRNA^{Met}$ bearing the amber anticodon (pACMTS*; see Materials and Methods; 18) or both MTS* and FMT (pACMTS*Fatg) were constructed. Strains UF121R and PAL125R containing the above-mentioned $tRNA^{Met}$amber variants were transformed in parallel by these two plasmids. The effect of MTS* expression was evaluated by comparing β-galactosidase activities in cells harboring either pACYC184 or pACMTS*. The effect of FMT overproduction was evaluated by comparing β-galactosidase activities in cells harboring either pACMTS* or pACMTS*Fatg, i.e., cells overexpressing either MTS* alone or MTS* plus FMT (Table 3).

The only $tRNA_f^{Met}$amber variant for which the associated suppression was insensitive to MTS* expression was the G_{73} variant. This result was likely to be due to the negative effect of the G_{73} mutation on tRNA methionylation, as shown in vitro (13, 17). For the other studied amber tRNAs, the expression of MTS* systematically caused an increase in suppression activity, either for initiation ($tRNA_f^{Met}$, $tRNA_f^{Met}U_1$; $tRNA_f^{Met}G_{72}$) or for elongation ($tRNA_m^{Met}$; $tRNA_f^{Met}$ U_1; $tRNA_f^{Met}$ G_{72}). These positive responses indicated that the amber tRNAs could be at least partly methionylated by MTS*.

In the presence of MTS*, the overproduction of FMT decreased the suppression during elongation induced by variants U_1 and G_{72}, whereas it increased the suppression during initiation induced by variant G_{72}. The effects of FMT in the presence of MTS* were therefore the same as those in the absence of MTS*, in which variants U_1 and G_{73} are esterified by glutamine. In particular, the overproduction of FMT switched the identity of the methionylated G_{72} variant from elongator to initiator in vivo.

$tRNA_f^{Met}$amber may acquire elongator activity in response to a decrease in the cellular FMT concentration. Because the overproduction of FMT reduced the elongator activity of $tRNA_f^{Met}$amber variants, the conclusion was reached that the observed elongator activity of these tRNAs resulted from a defect in their formylation rate in vivo. In this context, it was interesting to monitor the behavior of $tRNA_f^{Met}$amber itself under conditions in which the intracellular level of FMT was artificially decreased.

A strain (PAL22RλFatg) expressing a low level of formylase activity was derived from UF121. In strain

TABLE 4. Correlation between cellular FMT activity and the suppression during elongation by $tRNA_f^{Met}$amber

Strain	$tRNA^{Met}$amber expressed[a]	β-Galactosidase activity[b]	FMT activity (pmol s^{-1} · A_{280} $unit^{-1}$)
UF121R/pMC9	f	<0.03	6.6 ± 0.7
	m	3.9 ± 0.4	6.0 ± 0.7
	None	<0.03	5.3 ± 0.7
PAL22RλFatg/pMC9 (+IPTG)[c]	f	0.11 ± 0.03	0.33 ± 0.05
	m	2.3 ± 0.3	0.52 ± 0.05
	None	<0.03	0.37 ± 0.05
PAL22RλFatg/pMC9	f	0.9 ± 0.1	<0.07
	m	3.6 ± 0.2	<0.07
	None	<0.03	<0.07

[a] tRNAs were expressed from pACtRNA plasmids. pACYC184 is the control plasmid without the tRNA gene.

[b] Cells were cultured at 37°C in LB medium containing 50 μg of ampicillin and 25 μg of chloramphenicol per ml. β-Galactosidase activity is reported in Miller units.

[c] The expression of the *fmt-1* (ATG) gene, carried by λFatg, was induced by growing the cells in the presence of 1 mM IPTG.

PAL22RλFatg, the *fmt* gene is under the control of the *lac* promoter. For modulation of the level of FMT activity, PAL22RλFatg was transformed with plasmid pMC9, which overproduces the *lac* operon repressor (1).

Strains PAL22RλFatg/pMC9 and UF121R/pMC9 were transformed with pACtRNA$_f^{Met}$amber, a plasmid producing $tRNA_f^{Met}$amber. As controls, the same strains were transformed with pACYC184 and pACtRNA$_m^{Met}$amber (expressing $tRNA_m^{Met}$amber). After exposure of the different cells to the presence or absence of isopropyl-β-D-thiogalactopyranoside (IPTG), the β-galactosidase activities were determined (Table 4).

The absence of IPTG in the growth medium caused a lowering of FMT activity in PAL22RλFatg/pMC9. With pACtRNA$_f^{Met}$amber, this lowering was accompanied by increased suppression of the *lacI-lacZ* internal amber codon. This suppression strictly depended on the presence of $tRNA_f^{Met}$amber, as indicated by control experiments with pACYC184 and pACYCtRNA$_m^{Met}$amber. However, the appearance of β-galactosidase activity could have resulted indirectly from the low rate of growth of strain PAL22RλFatg/pMC9: the corresponding doubling time (125 min in Luria-Bertani [LB] medium without IPTG at 37°C) was higher than that of strain UF121R/pMC9 harboring pACtRNA$_f^{Met}$amber (30 min in LB medium containing ampicillin and chloramphenicol at 37°C). We therefore checked that β-galactosidase activity was not produced in strain UF121R/pMC9/pACtRNA$_f^{Met}$amber grown in minimal medium (doubling time, 200 min).

On the basis of the above-described set of data, it was deduced that a lowering of the rate of formylation of $tRNA_f^{Met}$amber was enough to allow this tRNA to enter the elongation process.

Conversion of $tRNA_m^{Met}$amber into an initiator species active in vivo. The above-described results showed that formylation played an important role in $tRNA_f^{Met}$ initiator identity by (i) favoring the orientation of this polynucleotide towards the initiation machinery and by (ii) preventing its participation in the elongation process. We wondered whether an elongator $tRNA^{Met}$ could be forced to behave as an initiator species provided that it could be formylated.

$tRNA_m^{Met}$ may be changed into a good substrate for the

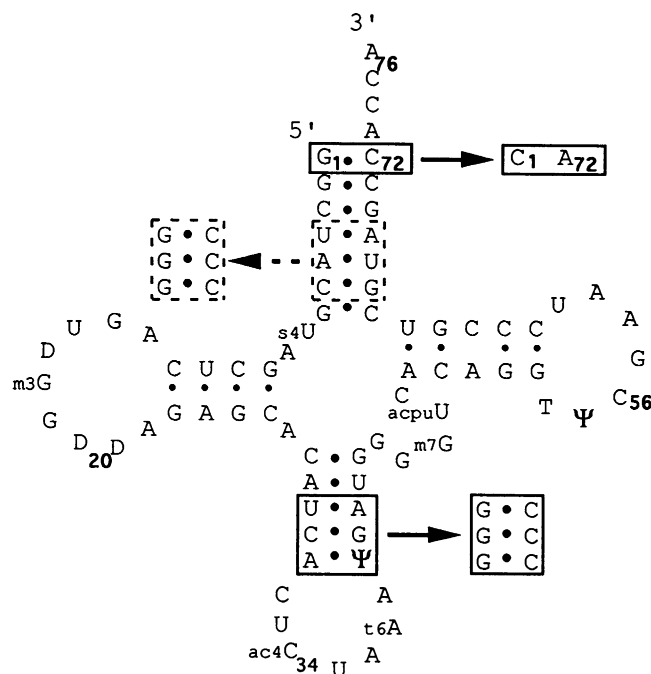

FIG. 3. Mutations converting the elongator $tRNA_m^{Met}$amber species into an initiator species in vivo. The sequence of $tRNA_m^{Met}$amber is shown in the cloverleaf representation. The abbreviations for modified bases are those described by Sprinzl et al. (33). The mutated positions in $tRNA_m^{Met}$amber and the new sequences originating from $tRNA_f^{Met}$ are boxed. The broken-line boxes indicate mutations that are dispensable for initiator identity in cells overexpressing the formylase.

FMT in vitro upon the sole substitution of its acceptor stem by the corresponding region of $tRNA_f^{Met}$, resulting in the following mutations: $C_1 \cdot A_{72}$, $G_4 \cdot C_{69}$, $G_5 \cdot C_{68}$, and $G_6 \cdot C_{67}$ (7) (Fig. 3). The activity of the amber derivative of this chimeric tRNA ($tRNA_{fasm}^{Met}$amber) in the initiation of protein synthesis in vivo was assayed. pRStRNA and pRS*lacZ*tRNA plasmids each encoding $tRNA_{fasm}^{Met}$amber were respectively introduced into UF121R and PAL125R cells expressing or not expressing the MTS* variant. It is noteworthy that $tRNA_{fasm}^{Met}$amber is likely to be methionylated by MTS* in vivo, as suggested by the high catalytic efficiency of $tRNA_{fasm}^{Met}$ aminoacylation of native MetRS in vitro (17). The measurements of suppression levels (Table 5) indicated that when $tRNA_m^{Met}$amber was changed to $tRNA_{fasm}^{Met}$amber, most of the elongator activity disappeared, while only a very small amount of initiator activity was gained. On the basis of these data, it was concluded that $tRNA_{fasm}^{Met}$amber has actually acquired the capacity to be formylated in vivo, thus preventing its participation in elongation, but that N-acylation of its esterified amino acid is not sufficient to give the tRNA the ability to engage efficiently in initiation.

The importance of the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif in $tRNA_f^{Met}$ initiator activity was previously demonstrated (14, 28). Therefore, we tested whether the introduction of the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif could by itself turn $tRNA_m^{Met}$amber into an initiator species. The suppression values in Table 5 indicated that the $tRNA_m^{Met}$amber elongator identity was not modified by the creation of $G_{29}G_{30}G_{31}$ $C_{39}C_{40}C_{41}$ mutations.

In another experiment, the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif was

TABLE 5. Elongator and initiator activities of tRNA$^{Met}_{m}$amber variants

tRNA^{Met}amber	β-Galactosidase activity in extracts of the following strain carrying the indicated plasmid[a]:			
	UF121R (elongation)		PAL125R (initiation)	
	pACYC184	pACMTS*	pACYC184	pACMTS*
m	17.5 ± 1.5	91.9 ± 5.0	<0.1	<0.1
fasm	2.7 ± 0.2	1.1 ± 0.1	<0.1	0.1
$mG_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$	7.0 ± 0.2	89.7 ± 0.8	<0.1	<0.1
$C_1 \cdot A_{72}mG_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$	2.6 ± 0.1	7.5 ± 0.2	<0.1	3.7 ± 0.3
$fasmG_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$	0.8 ± 0.1	0.5 ± 0.1	<0.1	8.9 ± 0.2
$G_1 \cdot C_{72}fasmG_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$	4.0 ± 0.3	25.2 ± 0.1	<0.1	<0.1
f	<0.1	<0.1	2.0 ± 0.1	12.9 ± 0.5

[a] β-Galactosidase activity is reported in Miller units. Plasmid pACMTS* carries the gene encoding the MTS* variant of MetRS. pACYC184 is the control plasmid.

introduced into tRNA$^{Met}_{fasm}$. The resulting tRNA$^{Met}_{fasm}G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ was still formylatable in vitro (K_m = 4.7 ± 0.8 μM; k_{cat} = 3.1 ± 0.3 s^{-1}). In a strain producing MTS*, the corresponding amber species became an initiator (Table 5). Consequently, switching of the identity of tRNA$^{Met}_{m}$amber from an elongator to an initiator in vivo required the introduction of motifs from both the acceptor and the anticodon stems of tRNA$^{Met}_{f}$ (Fig. 3).

The introduction of a $G_1 \cdot C_{72}$ base pair into tRNA$^{Met}_{f}$ abolishes its formylation in vitro (7). As expected, the creation in tRNA$^{Met}_{fasm}G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ of a $G_1 \cdot C_{72}$ base pair had the same effect on the formylation of this tRNA (k_{cat}/K_m, $<10^{-4}$ $\mu M^{-1} \cdot s^{-1}$). The in vivo initiator activity of this no-longer formylatable tRNA^{Met}amber was studied. The results in Table 5 show that the substitution of $C_1 \cdot A_{72}$ with $G_1 \cdot C_{72}$ was sufficient to abolish the acquired initiator activity of tRNA$^{Met}_{fasm}$amber $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ and to restore its elongator activity. Therefore, both formylability and the presence of the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ sequence appear necessary to give a tRNA the initiator identity of tRNA$^{Met}_{f}$.

Finally, mutation of the $G_1 \cdot C_{72}$ base pair of tRNA$^{Met}_{m}$ to $C_1 \cdot A_{72}$ is known to be enough to render this tRNA a substrate of FMT, although a much less efficient one than tRNA$^{Met}_{f}$ or tRNA$^{Met}_{fasm}$ (7). After having verified that tRNA$^{Met}_{m}C_1A_{72}G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ could be formylated in vitro (k_{cat}/K_m = 0.0038 ± 0.0003 $\mu M^{-1} \cdot s^{-1}$), we introduced the C_1A_{72} mutation into the amber derivative of tRNA$^{Met}_{m}G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$. The resulting tRNA$^{Met}_{m}$amber $C_1A_{72}G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ had lost most of its activity in elongation and had gained a small but significant amount of activity in initiation (Table 5). For ensuring a higher rate of formylation of this tRNA, FMT activity was overproduced in the recipient cells through the introduction of plasmid pACMTS*Fatg. Under these conditions, tRNA$^{Met}_{m}$amber $C_1A_{72}G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ behaved as a more efficient initiator species (0.5 ± 0.1 U of β-galactosidase in UF121R and 24.8 ± 1.0 U in PAL125R). This experiment established that for tRNA$^{Met}_{m}$ to be converted to an initiator species, it was sufficient to give it the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ sequence and the $C_1 \cdot A_{72}$ nucleotides provided that in addition FMT activity was overproduced (Fig. 3).

DISCUSSION

Varshney and RajBhandary (38) were the first to demonstrate that initiation may take place on a UAG amber start codon when tRNA$^{Met}_{f}$amber is present in the cell. Moreover, such initiation of translation on a non-AUG codon does not require that the suppressor tRNA be aminoacylated with methionine (3, 19, 38). Recently, advantage was taken of these properties to compare in vivo the initiator activity of several mutants of *E. coli* tRNA$^{Met}_{f}$ harboring a CUA anticodon. Mutant tRNAs carrying a formylmethionine were significantly more active in initiation than the same ones carrying a formylglutamine (39). Furthermore, a convincing correlation was found between the rate of suppression induced by these tRNAs and their capacity to be formylated (37, 39).

In the present study, the initiator and elongator activities of several tRNAs were evaluated in vivo in the presence of abnormally high or low cellular concentrations of FMT. In addition, the methionylation of these tRNAs, which carried a CUA anticodon, was ensured through the production in the host cells of a variant of MetRS selected for its ability to aminoacylate tRNAs^{Met}amber.

It was shown that increasing in vivo FMT activity switches the identity from elongator to initiator of Gln-tRNA$^{Met}_{f}$amberG_{73}, Gln-tRNA$^{Met}_{f}$amberU_1, and Met-tRNA$^{Met}_{f}$amberG_{72}. Since the G_{72} and G_{73} mutations reduce the catalytic efficiency of Met-tRNA$^{Met}_{f}$ formylation by FMT in vitro (7), it is reasonable to assume that the overproduction of FMT corrected the defect in the formylation of the corresponding aminoacyl-tRNA variants in vivo. Because Met-tRNA$^{Met}_{f}U_1$ behaves as a good substrate for FMT in vitro, it is surprising that the elongator activity of tRNA$^{Met}_{f}$amberU_1 was also decreased upon the overexpression of FMT. However, since the U_1 variant of Met-tRNA$^{Met}_{f}$ is known to have an increased affinity for EF-Tu · GTP (26, 29), the repression of tRNA$^{Met}_{f}$amberU_1 elongator activity upon the overproduction of the formylase may, in fact, reflect the competition between FMT and EF-Tu · GTP for taking care of this aminoacylated tRNA and its further formylation.

In contrast to the three above-described mutant tRNAs, which display activity in elongation, wild-type tRNA$^{Met}_{f}$ strictly behaves as an initiator species in the *fmt*$^+$ context. This property can be related to the absence of a Watson-Crick base pair at positions 1 and 72, a unique feature of procaryotic initiator tRNAs, proposed to account for the lack of an interaction with EF-Tu · GTP (26, 29). However, the G_{73} variant of tRNA$^{Met}_{f}$amber, although possessing unpaired $C_1 \cdot A_{72}$ bases, shows activity in elongation. Therefore, as already observed (31), the absence of a Watson-Crick base pair at positions 1 and 72 is not sufficient to prevent an aminoacyl-tRNA from being engaged in elongation. Furthermore, when the intracellular level of FMT activity is lowered, glutaminylated tRNA$^{Met}_{f}$amber begins to

participate in elongation, as indicated in this study by the suppression of a *lacI-lacZ* internal amber mutation.

Taken together, our results suggest that formylation is important in preventing the participation of $tRNA_f^{Met}$ in the elongation process. Consequently, it may be proposed that formylation is involved not only in the positive selection of initiator Met-tRNA by IF2 (10, 24, 34), but also in the rejection of initiator Met-tRNA by EF-Tu · GTP. This idea is consistent with previous results showing that Met-$tRNA_f^{Met}$ lacking the formyl modification is able to interact with EF-Tu · GTP (11).

The importance of fMet-$tRNA_f^{Met}$ nucleotide determinants in the expression of its initiator activity is emphasized by the $tRNA_m^{Met}$amber identity switching experiment. The transplantation of both the $tRNA_f^{Met}$ acceptor stem and $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ sequences in the elongator species is enough to turn it into an initiator one in vivo. The acceptor stem of $tRNA_f^{Met}$ accounts for the addition of formylability to $tRNA_m^{Met}$ (7, 14). The $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif in the anticodon stem is known to cause a unique conformation of the anticodon loop of $tRNA_f^{Met}$ (28, 40), which would be an important feature for initiator tRNA selection by IF3 (9). Since, with the exception of two posttranscriptional modifications, the two species of $tRNA^{Met}$ share the same anticodon loop sequence, it is likely that the introduction of the $G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ motif in the anticodon stem of $tRNA_m^{Met}$ gives the anticodon loop of $tRNA_m^{Met}$ a conformation identical to that of the anticodon loop of $tRNA_f^{Met}$.

Reversion of the $tRNA_{fasm}^{Met}$amber$G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ identity from initiator to elongator was ensured by the creation of a sole $G_1 \cdot C_{72}$ base pair. This result indicates the prime importance of formylation for the orientation of a tRNA towards translation initiation and, consequently, that of the nucleotides governing the formylation reaction. However, determinants in the $tRNA_f^{Met}$ acceptor stem involved in other steps in the initiation process, for example, recognition by IF2, should not be overlooked. Such determinants are likely to overlap those involved in the formylation and methionylation reactions (6, 17) and, consequently, may be difficult to recognize. However, the possibility of modulating the intracellular activity level of one or another of the involved macromolecules may be helpful at this stage. For instance, in this work, the artificial creation in the bacterium of an excess of the FMT enzyme was useful in establishing a link between the formylation of $tRNA_m^{Met}$amber$G_{29}G_{30}G_{31}C_{39}C_{40}C_{41}$ upon the sole $C_1 \cdot A_{72}$ change and its conversion into an initiator species.

ACKNOWLEDGMENTS

We thank Emmanuelle Schmitt and Thierry Meinnel for helpful advice.

This work was supported in part by the Fondation pour la Recherche Médicale.

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