Interplay of Methionine tRNAs with Translation Elongation Factor Tu and Translation Initiation Factor 2 in Escherichia coli*

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Jean-Michel Guillou‡, Senta Heiss‡, Julie Soutourinaï, Yves Mechulam‡, Soumaya Laalami§‡, Marianne Grunberg-Manago§, and Sylvain Blanquet‡‡

From the ‡Laboratoire de Biochimie, URA CNRS 1970, Ecole Polytechnique, F91128 Palaiseau cedex, France and the §Institut de Biologie Physico-Chimique, URA CNRS 1139, 13 rue Pierre et Marie Curie, F75005 Paris, France

According to their role in translation, tRNAs specifically interact either with elongation factor Tu (EFTu) or with initiation factor 2 (IF2). We here describe the effects of overproducing EFTu and IF2 on the elongator versus initiator activities of various mutant tRNA\text{Met}\ species \textit{in vivo}. The data obtained indicate that the selection of a tRNA through one or the other pathway of translation depends on the relative amounts of the translational factors. A moderate overexpression of EFTu is enough to lead to a misappropriation of initiator tRNA in the elongation process, whereas overproduced IF2 allows the initiation of translation to occur with unformylated tRNA species. In addition, we report that a strain devoid of formylase activity can be cured by the overproduction of tRNA\text{Met}. The present study brings additional evidence for the importance of formylation in defining tRNA\text{Met} initiator identity, as well as a possible explanation for the residual growth of bacterial strains lacking a functional formylase gene such as observed in Guillou, J. M., Mechulam, Y., Schmitter, J.-M., Blanquet, S., and Fayat, G. (1992) \textit{J. Bacteriol.} 174, 4294–4301.

In all studied cells, initiation of translation involves a specialized tRNA\text{Met} species that bears identity determinants that allow it to interact with the components of the initiation apparatus and prevent it to play with the elongation factors. In eubacteria, the initiator tRNA is called tRNA\text{fMet} because the normal pathway of initiation begins with the addition of a formyl group to the methionylated tRNA (1, 2). The importance of the formylation reaction for the efficiency of the translational process has been early recognized (3, 4). Recently, the disruption of the \textit{fnt} gene encoding the formylating enzyme has brought direct evidence that formylation of the initiator tRNA is needed for ensuring the rapid growth of \textit{Escherichia coli} (5).

The nucleotides of \textit{E. coli} initiator tRNA determining its function have been studied by identity switching experiments; species of elongator tRNAs (tRNA\text{Met} and tRNA\text{His}) have been converted into initiator ones after receiving the acceptor stem and the anticodon stem and loop of tRNA\text{Met} (6, 7). These regions are respectively known to be important for (i) formylation of the aminoacyl-tRNA (8, 9) and (ii) interaction with initiator factor IF3\textsuperscript{1} and the ribosomal P site (10–12). In particular, the absence of pairing between bases 1 and 72 of the acceptor stem, a unique feature of initiator tRNA in prokarocytes (13), governs both the recognition of the aminoacyl-tRNA by methionyl-ARNt\text{Met} formyltransferase and its low affinity for the elongation factor EFTu (8, 14, 15).

Reciprocally, several variants of tRNA\text{Met} have been shown to suppress an amber mutation inside a reporter gene and therefore to be active in elongation (6, 8, 16–18). In addition to an amber anticodon (CUA), most of these tRNAs bear a mutation that restores a Watson-Crick base pair, U-A or C-G, at positions 1–72. However, some of the elongator variants of tRNA\text{Met} keep their C\text{A\textsuperscript{1}}\textsuperscript{72} pair unchanged (6, 8, 9, 16). Consequently, the lack of a base pairing at positions 1–72 does not appear sufficient \textit{per se} to prevent the initiator tRNA from entering the elongation pathway. Moreover, a tRNA\text{Met} with only the amber anticodon can become active in elongation provided that cells are rendered partially deficient in formyltransferase activity (6). This latter result has led us to consider that formylation of methionyl-tRNA\text{Met} could be important also in preventing its misappropriation by the elongation apparatus.

In combination with IF3, IF2 favors the specific binding of the inhibitor tRNA species to the 30 S mRNA complex (19). N-Acylation of the aminoacylated tRNA is required to observe the formation of a binary complex with IF2 (20–22). However, IF2 stimulates the binding of unformylated tRNAs to the 30 S ribosomal subunit \textit{in vitro} (23). Because the 30 S subunit displays a high affinity binding site for IF2 (24), IF2 is thought to act while bound to the 30 S subunit rather than free like a tRNA carrier (23, 25). Therefore, the precise mechanism of action of IF2 remains unclear.

To address the questions concerning with the interaction of tRNA\text{Met} with EFTu or IF2 \textit{in vivo}, we have overproduced each of these two factors in the cell and studied the consequences on the fates of various tRNAs. In addition we have examined the effect of the overproduction of tRNA\text{Met} and of several derived tRNA\text{Met} variants on the growth rate of an fnt\textsuperscript{−} strain.

MATERIALS AND METHODS

Oligonucleotides were synthesized on a Pharmacia gene assembler and purified by anion-exchange chromatography (Mono Q, Pharmacia Biotech Inc.).

The EcoRV-PstI fragment from the pUB1 plasmid (Ref. 26, kindly provided by Dr. M. Springer) bearing the \textit{tufB} gene was inserted between the \textit{SacI} and \textit{PstI} restriction sites of pUC18 to give pUCtuf. The insertion of the \textit{SacI}-\textit{ HindIII} fragment from pUCtuf between the corresponding sites of the pAC\text{Met} plasmid (6) gave pACtuf. The insertion of the XbaI-HindI fragment from pBSM547WA461AV451QP213DA449 (27) between the XbaI and \textit{ HindIII} sites of pACtuf gave pAC\textit{ MT S\textsuperscript{−} tuf}, which also expressed a mutant form (MTS\textsuperscript{−}) of \textit{E. coli} methionyl-tRNA

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† Present address: Inst. de Biologie Moleculaire et d’Ingénierie Genetique. Faculte de Poitiers, 40 Ave. du Recteur Pineau, F86022 Poitiers, France.

‡ To whom correspondence should be addressed. Tel.: 33-1-69-33-41-81; Fax: 33-169-33-30-13; E-mail: labo@coli.polytechnique.fr.

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synthetase. The factor of overexpression of EFTu in cells harboring either the pACMTS* or the pACTuf plasmids was quantitated by Western blot and found equal to 1.3 ± 0.1-fold.

The amplified fragment was then digested by the polymerase chain reaction using the oligonucleotides 5'-CATTTCGCAGATTGGCTAGCGGTCAACA-3' and 5'-CGGCTTTCGACGTGATCAGTAA-3'. The resulting fragment was re-directed with NheI and XhoI and then inserted into the SacI and XhoI sites of plasmid PACFatg (6). A fragment containing the infB gene was created by digestion of the BglII fragment (28) with BglII and HindIII and then inserted into the corresponding sites of the polylinker yielding pACin/B. To obtain the pACin/Bplasmid, a DNA fragment containing the fns-fmt operon was amplified from B5906 plasmid (5) by polymerase chain reaction with the use of the following primers: 5'-CTTGGCAATGGCTAGCGGTCAACA-3' and 5'-CTTGTCAGCGGCCGCTTTCGAGC-3'. The resulting fragment was re-directed with NheI and XhoI and then inserted into the SacI and XhoI sites of pACin/B. To obtain the pACfmt plasmid, the infB gene was deleted from pACin/B plasmid using HindIII and BglII and Klenow polymerase fill-in. The factor of overexpression of IF2 in cells harboring either the pACfmt or the pACin/B plasmids was quantitated by Western blot and found equal to 5 ± 2.5-fold. The factor of overexpression of formyltransferase in cells harboring either the pACin/B or the pACfmt plasmid was assayed as described (5) and found equal to 27 ± 4-fold.

The genomic library from PAL13Tr was constructed using pHCT7 (29) essentially as described previously (30). The motZ region was amplified by polymerase chain reaction using the oligonucleotides 5'-CATGCGAATTCCGGGATGATGAATATATGAAA-3' and 5'-CGCTTTTTT-TGTGTCAGCGGCCGCTTTCGAGC-3'. The amplified fragment was then cleaved with EcoRI and SstI and inserted into the corresponding sites of pACin/B plasmid, yielding pACinfZ.

Both RNA extracts were prepared according to Meinnel and Blanquet (31). The amounts of formyl-methionine and lysine accepting species in such extracts were measured according to Refs. 8 and 32, respectively. The relative amount of tRNA\textsuperscript{Met} in such extracts was calculated as an average value with the associated deviation given in the table. For each combination of plasmids, it was verified that their presence did not impair bacterial growth of the indicator strains PAL125R and UF121R, thus excluding an indirect effect on the overall protein synthesis rate.

\section*{RESULTS}

The Relative Involvement of Various tRNA\textsuperscript{Met} Amber Variants in Either the Elongation or the Initiation Step of Translation Depends on the Concentration of EFTu—The initiator and elongator activities of various tRNAs bearing an amber anticodon (CUA) could be previously evaluated through the suppression of an amber mutation located either inside or at the first position of a reporter gene (6). Here, we further measure and compare both the initiator and the elongator activities of these tRNAs as a function of the amount of EFTu present in the host cell. The suppression of an internal amber codon in the lacI-lacZ gene fusion was followed in the UF121R indicator strain (Table I; 32). In a second assay, the reporter lacZ\textsubscript{TAG1} gene, starting on an amber codon (Fig. 1), was introduced into PAL125R (an F\textsuperscript{−} strain derived from UF121R) through transformation with pRSlacZtRNA\textsubscript{Met} amber plasmid, carrying both the lacZ\textsubscript{TAG1} and the tRNA\textsubscript{Met} amber genes. The three tRNA\textsubscript{Met} amber variants (U\textsuperscript{7}, G\textsuperscript{72}, and G\textsuperscript{73}), which can participate in both the elongation and the initiation of protein synthesis (6), were studied. The formylability and interaction with EFTu of these tRNAs are affected to different extents (8, 9, 15).

UF121R cells were transformed by the five pRSinB plasmids (encoding tRNA\textsubscript{Met} amber, tRNA\textsubscript{Met} amber, or the G\textsuperscript{72}, G\textsuperscript{73}, and U\textsuperscript{7} variants of the latter). PAL125R cells were transformed by the corresponding set of pRSlacZtRNA plasmids (Fig. 1). In the case of each studied tRNA\textsubscript{Met} amber, levels of β-galactosidase activity were measured in the above UF121R and PAL125R derivatives, overexpressing or not EFTu. In order to distinguish the fate of the tRNAs actually aminoacylated in vivo with methionine, plasmids expressing either a methionyl-tRNA synthetase variant (MTS\textsuperscript{−}) capable of aminoacylating a tRNA\textsubscript{Met} bearing the amber anticodon (pACMTS\textsuperscript{−}) or both MTS\textsuperscript{+} and EFTu (pACMTS\textsuperscript{+}tuf) were used. In this case, cells were transformed with either the pACMTS\textsuperscript{+}tuf or plasmid, bearing the tufB gene, or the pACMTS\textsuperscript{+} vector as a control.

The overproduction of EFTu had minor effects on the rate of suppression in initiation obtained with tRNA\textsubscript{Met} amber or on that in elongation obtained with tRNA\textsubscript{Met} amber (Table II). In contrast, the overproduction of EFTu resulted in (i) a significant suppression with tRNA\textsubscript{Met} amber in elongation, (ii) a 2-fold increase of the suppression in elongation with the U\textsuperscript{7} and G\textsuperscript{73} variants, and (iii) a decrease of the suppression in initiation with the U\textsuperscript{7}, G\textsuperscript{72}, and G\textsuperscript{73} variants by factors ranging from 1.5 to 3-fold.

From these results, it could be concluded that the amount of intracellular EFTu influenced the selection of an amber tRNA by either the initiation or the elongation apparatus. However, in vivo, tRNA\textsubscript{Met} amber variants behave as substrates of glutaminyl-tRNA synthetase (17). Consequently, in the case of the G\textsuperscript{73} species, a poor substrate of E. coli methionyl-tRNA synthetase, the measured suppression had to be attributed to the sole glutaminylated tRNA (6, 34). With the other tRNAs, the efficiencies of suppression could be compared in the presence or the absence of MTS\textsuperscript{+} by transforming the cells with either pACYC184 or pACMTS\textsuperscript{+} and with either pACTuf or pACTufMTS\textsuperscript{−}. In the case of tRNA\textsubscript{Met} amber, the lack of effect...
of MTS suggested that suppression in elongation should be attributed to a glutaminylated tRNA species. In all other cases, the relative extents of suppression were lower in the absence of MTS, thus indicating that the suppression was actually mediated for the most part by a methionyl-tRNA species.

The Involvement of Variants of tRNA\textsuperscript{Met} Amber in Either the Elongation or the Initiation Step of Translation Depends on the Cellular Concentration of IF2—IF2 was overproduced from the pAC\textsubscript{in}B plasmid and the effect on the initiator activity of several tRNA\textsuperscript{Met} amber variants was assayed in the PAL125R indicator strain (Table III). In agreement with the data recently reported by Mangroo and RajBhandary (35), an important positive effect of IF2 on initiator activity was observed in the case of tRNA\textsuperscript{Met} amber and of its U\textsuperscript{a} and G\textsuperscript{73} variants. To know whether this effect of overproduced IF2 involved formylated tRNA species, initiation activities were also compared in the presence of an excess of the formylase enzyme overexpressed from the lacZ plasmid, whose translation initiates with a UAG start codon. Hence, it could be concluded that the increase of growth rate conferred by each plasmid varied in parallel with the degree of overproduction of the initiator tRNA.

To find precisely which determinants of initiator tRNA are the most important to sustain cell growth in a fmt strain context, we transformed the PAL13TrxFatg strain (5) with a set of pB-StRNA\textsuperscript{Met} plasmids. Cells streaked from LB plates containing 12.5 mg/ml ampicillin and 1 mg isopropyl-1-thio-β-D-galactopyranoside were further examined for growth at 37 °C on LB plates containing 0.2% glucose, conditions in which formylase activity can no more be detected in cell extracts and in which the recipient strain does not grow (5). The assayed tRNAs were tRNA\textsuperscript{Met} C\textsubscript{1}, tRNA\textsuperscript{Met} U\textsubscript{1}, tRNA\textsuperscript{Met} G\textsubscript{72}, and tRNA\textsuperscript{Met} G\textsubscript{3} on the one hand and tRNA\textsuperscript{Met} C\textsubscript{1}, tRNA\textsuperscript{Met} C\textsubscript{2}, tRNA\textsuperscript{Met} C\textsubscript{3}, tRNA\textsuperscript{Met} C\textsubscript{4}, tRNA\textsuperscript{Met} C\textsubscript{5}, tRNA\textsuperscript{Met} C\textsubscript{6}, tRNA\textsuperscript{Met} C\textsubscript{7}, tRNA\textsuperscript{Met} C\textsubscript{8} on the other hand (8). All tRNAs had a positive effect on the growth of PAL13TrxFatg, except tRNA\textsuperscript{Met} G\textsubscript{72} and tRNA\textsuperscript{Met} G\textsubscript{3} as a derivative of tRNA\textsuperscript{Met} with the acceptor stem of tRNA\textsuperscript{Met} (8), in which cases colonies could not be detected after 48 h. Nevertheless, the anticodon stems of the two latter tRNA species lack the G\textsuperscript{2}G\textsuperscript{31}C\textsuperscript{40}C\textsuperscript{40}C\textsuperscript{40} motif required for IF3 recognition. Authentic tRNA\textsuperscript{Met} and the tRNA\textsuperscript{Met} C\textsubscript{1}G\textsubscript{2}G\textsubscript{3}G\textsubscript{30}C\textsubscript{40}C\textsubscript{40}C\textsubscript{40} variant were the most efficient in supporting the growth of PAL13TrxFatg. Noticeably, nonformylatable species like tRNA\textsuperscript{Met} G\textsubscript{72} and tRNA\textsuperscript{Met} G\textsubscript{3} also had a positive effect on the growth rate of the indicator strain.
Interaction of EFTu and IF2 with Methionine tRNAs

Effect of an overproduction of EFTu on the suppression during elongation or initiation by tRNA\textsuperscript{Met} variants

| tRNA\textsuperscript{Met} amber | \textsuperscript{β}-Galactosidase activity in extracts of the following strain carrying the indicated plasmid |
|-------------------------------|---------------------------------------------|
|                               | UF121R (elongation) | PAL125R (initiation) |
|                               | pACMTS\textsuperscript*a | pACMTS\textsuperscript{tu}uf | pACMTS\textsuperscript*a | pACMTS\textsuperscript{tu}uf |
| f                             | <0.02 | 0.08 ± 0.01 | 33.1 ± 2.5 | 49.1 ± 4.0 |
| fU\textsuperscript{1}         | 4.5 ± 0.3 | 8.0 ± 0.4 | 34.4 ± 1.7 | 23.8 ± 1.0 |
| \textsuperscript{fg}G\textsuperscript{72} | 28.0 ± 0.5 | 29.5 ± 0.4 | 6.2 ± 0.3 | 1.7 ± 0.1 |
| \textsuperscript{fg}G\textsuperscript{73} | 1.4 ± 0.1\textsuperscript{a} | 2.5 ± 0.1\textsuperscript{a} | 2.1 ± 0.1\textsuperscript{a} | <0.02 |
| m                             | 71.7 ± 1.6 | 65.2 ± 1.7 | <0.02 |

\textsuperscript{a} Values are the same as those obtained in the absence of MTs\textsuperscript{a} with either pACYC184 instead of pACMTS\textsuperscript{a} or pAC\textsuperscript{tu}uf instead of pAC\textsuperscript{Ch}MTs\textsuperscript{a}.

Effect of an overproduction of IF2 on the suppression during initiation by tRNA\textsuperscript{Met} variants

| tRNA\textsuperscript{Met} amber | \textsuperscript{β}-Galactosidase activity in extracts of PAL125R carrying the indicated plasmid |
|-------------------------------|---------------------------------------------|
|                               | pAC\textsuperscript{Ch}MTS \textsuperscript{a} | pAC\textsuperscript{Ch}MTS \textsuperscript{ufmt} |
| f                             | 2.3 ± 0.1 | 4.0 ± 0.5 | 4.2 ± 0.3 | 3.5 ± 0.2 |
| fU\textsuperscript{1}         | 0.2 ± 0.1 | 0.8 ± 0.1 | 0.9 ± 0.1 | 0.8 ± 0.1 |
| \textsuperscript{fg}G\textsuperscript{72} | <0.02 | <0.02 | 0.06 ± 0.01 | 0.06 ± 0.01 |
| \textsuperscript{fg}G\textsuperscript{73} | 1.7 ± 0.1 | 4.3 ± 1.0 | 12.5 ± 0.2 | 11.4 ± 0.6 |

Effect of an overproduction of IF2 on the suppression during elongation by tRNA\textsuperscript{Met} variants

| tRNA\textsuperscript{Met} amber | \textsuperscript{β}-Galactosidase activity in extracts of UF121R carrying the indicated plasmid |
|-------------------------------|---------------------------------------------|
|                               | pAC\textsuperscript{Ch}MTS \textsuperscript{a} | pAC\textsuperscript{Ch}MTS \textsuperscript{ufmt} |
| fU\textsuperscript{1}         | 5.7 ± 0.1 | 4.2 ± 0.1 | 0.16 ± 0.01 | 0.12 ± 0.01 |
| \textsuperscript{fg}G\textsuperscript{72} | 12.1 ± 1.0 | 9.8 ± 0.2 | 11.8 ± 0.9 | 8.2 ± 0.2 |
| \textsuperscript{fg}G\textsuperscript{73} | 3.3 ± 0.3 | 2.8 ± 0.1 | 0.32 ± 0.01 | 0.25 ± 0.02 |
| m                             | 32.4 ± 3.7 | 31.6 ± 1.0 | not determined | not determined |

EFTu Is Able to Decrease the Initiator Activity of a tRNA by Misappropriation—In the present study, we show that in a fmt\textsuperscript{1} strain, a moderate overproduction of elongation factor Tu, is enough to change the fate of tRNA\textsuperscript{Met} amber variants modified or not in the acceptor stem. The 1.5-fold increase in EFTu cellular concentration both improves the activity of such tRNAs in the elongation of translation and precludes their participation in the initiation process. This behavior may reflect a competition between EFTu and the formylase for the taking over of an aminoacyl-tRNA in the cell. In particular, the overproduction of EFTu is sufficient to allow the glutaminylated tRNA\textsuperscript{Met} amber to acquire an elongator activity. However, our results show that in the fmt\textsuperscript{1} context, methionyl-tRNA\textsuperscript{Met} amber does not participate in the elongation. This behavior probably reflects the favored stronger interaction of methionylated tRNAs with the initiation apparatus, in particular with the formylase (36). In the absence of formyltransferase activity, one can imagine that part of the nonformylated methionyl-tRNA\textsuperscript{Met} would become complexed with EFTu, thus lowering the amount of tRNA available for translation initiation.

IF2 Is Able to Form a Binary Complex with tRNAs in Vivo—The question of whether IF2 forms a complex with initiator fMet-tRNA in solution has long remained unclear. \textit{In vitro}, the formation of a binary complex was reported to require the absence of Mg\textsuperscript{2+} (22, 37), whereas Petersen \textit{et al.} reported a protective effect of IF2 on the spontaneous deacylation or digestion of fMet-tRNA\textsuperscript{Met} by ribonucleases in the presence of magnesium ions (20, 21). From calculations of the intracellular concentrations of IF2 and of the 30 S ribosomal subunits and the measurement of their affinity constant (24, 38), it was proposed that most of the IF2 molecules and small ribosomal subunits are associated together in vivo (19, 39). In this context, an overproduction of IF2 such as that obtained in the present study is expected to increase the fraction of free IF2. Consequently, the observed effects on the initiator or elongator activity of a tRNA may be supposed to reflect the interaction of this tRNA with free IF2 and to indirectly establish that a productive binary complex between these macromolecules occurs in the cell. In wild-type conditions, however, the formation of such a binary complex might not be the major pathway leading to IF2-tRNA recognition.

DISCUSSION

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Translation Initiation in an fmt\textsuperscript{1} Cell Is Likely to Occur through the Residual Recognition of Unformylated Methionyl-tRNAs—Formylation of initiator tRNA is not detectable in the fmt\textsuperscript{1} PAL13Tr strain (5). This result raises the question to know how the initiation of translation can occur in such a strain. The capacity of IF2 to engage nonformylated aminoacyl-tRNA\textsuperscript{Met} in the initiation of translation has to be considered to account for the residual growing of PAL13Tr. A decreased affinity of IF2 for unformylated methionyl-tRNA\textsuperscript{Met} would explain the leaky character of the growth. In agreement with this idea, the growth rate of PAL13Tr in Exfatg is decreased by an overproduction of EFTu, as measured in liquid medium (result not shown). A straightforward interpretation is that sequestration of unformylated methionyl-tRNA\textsuperscript{Met} by the excess of EFTu decreases the concentration of free tRNA available for complexation with IF2. Surprisingly, however, an overproduction of IF2 also inhibits the growth of the PAL13Tr in Exfatg (result not shown). This unexpected result may be related to the previous report that the growth of an fmt\textsuperscript{1} strain is impaired by IF2
overproduction (40). IF2 in excess would start binding aminoacylated tRNAs other than the initiator species and the resulting IF2-aminoacyl-tRNA interaction, lacking the identity features for recognition by IF3, might behave as a poison of the 30 S ribosomal subunit.

In a fmt "Context, the Amount of Free Aminoacyl-tRNA^Met Available for Initiation Limits the Growth Rate—The stimulation of the growth of the fmt " strain PAL13Tr by overproduction of tRNA^Met sustains the above idea that in the absence of formylation, the free concentration of this tRNA limits the efficiency of translation initiation. Accordingly, tRNA^Met overproduction may compensate for both a misappropriation of the initiator tRNA by EFTu on one hand and the low affinity of IF2 for unformylated Met-tRNA^f on the other hand.

The positive effect on the growth of PAL13TrAfmt of the overproduction of tRNA^Met allowed us to detail the features of this tRNA, which are important for initiation in the absence of formylation. The accessory character of the formylability of tRNA was particularly clear in the case of the G72 and G1C72 species, which are not substrates of the formylase. On the contrary, the occurrence of a G29G30G31C39C40C41 motif in the anticodon stem of the overproduced tRNAs was needed to score a positive effect on growth. This tends to further establish that in a fmt " context, IF3 remains crucial to select the initiator tRNA whether it is formylated or not.

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