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Escherichia coli formylmethionine tRNA: Mutations in $\frac{GGG}{CCC}$ sequence conserved in anticodon stem of initiator tRNAs affect initiation of protein synthesis and conformation of anticodon loop

(tRNA gene expression/tRNA purification/initiation factor/ribosome binding/puromycin reaction)

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ABSTRACT **We have generated mutants of *Escherichia coli* formylmethionine initiator tRNA in which one, two, and all three G·C base pairs in the $\frac{GGG}{CCC}$ sequence in the anticodon stem are changed to those found in *E. coli* elongator methionine tRNA. Overproduction of the mutant tRNAs using M13 recombinants as an expression vector and development of a one-step purification scheme allowed us to purify, characterize, and analyze the function of the mutant tRNAs. After aminoacylation and formylation, the function of mutant formylmethionyl tRNAs was analyzed in an MS2 RNA-directed *in vitro* protein-synthesizing system, in AUG-dependent ribosomal P site binding, and in initiation factor binding. The mutant tRNAs show progressive loss of activity in initiation, the mutant with all three G·C base pairs substituted being the least active. The mutations affect binding to the ribosomal P site. None of the mutations affects binding to initiation factor 2. We also show that there is a progressive increase in accessibility of phosphodiester bonds in the anticodon loop of the three mutants to S1 nuclease, such that the cleavage pattern of the mutant with all three G·C base-pair changes resembles that of elongator tRNAs. These results are consistent with the notion that the contiguous G·C base pairs in the anticodon stem of initiator tRNAs impart on the anticodon loop a unique conformation, which may be important in targeting the initiator tRNA to the ribosomal P site during initiation of protein synthesis.**

Two classes of methionine tRNAs are present in all organisms. The initiator is used exclusively for initiation of protein synthesis, while the elongator is used for inserting methionine internally in a polypeptide chain (1–3). Because of their special function, initiator tRNAs exhibit unique properties, which distinguish them from elongator tRNAs: (*i*) initiator tRNAs specifically bind to initiation factor; (*ii*) initiator tRNAs bind directly to the ribosomal P site, whereas elongator tRNAs are first bound to the A site and later transferred to the P site; (*iii*) initiator tRNAs do not normally bind to the ribosomal A site and, therefore, do not insert methionine internally.

Initiator tRNAs also possess unique sequence and structural features not found in most elongator tRNAs (4). Both prokaryotic and eukaryotic initiator tRNAs contain a run of three Gs and three Cs in the anticodon stem, forming three consecutive G·C pairs. Prokaryotic initiator tRNAs share two additional unique features, the absence of a Watson–Crick base pair at the end of the acceptor stem and the presence of a purine-11–pyrimidine-24 base pair instead of a pyrimidine-11–purine-24 base pair in the dihydrouridine stem (5). The strong conservation of these features suggests that they are related to one or more of the unique properties of initiator tRNAs.

We have used oligonucleotide-directed mutagenesis (6, 7) to remove two of the features described above in *Escherichia coli* $tRNA_2^{fMet}$. First, we changed C-1 to T-1 such that the mutant initiator tRNA now has a Watson–Crick base pair at the end of the acceptor stem (Fig. 1). We then introduced upon this mutation changes in the $\frac{GGG}{CCC}$ sequence of the anticodon stem such that one, two, and all three G·C base pairs are changed to those found in *E. coli* elongator $tRNA^{Met}$. The T-1 mutation has no effect on activity of this tRNA in initiation of protein synthesis. Mutations in the anticodon stem, however, affect activity of the mutant tRNAs in initiation. These mutant tRNAs bind equally well to initiation factor 2 (IF-2), but they are defective at the step of binding to the P site on the ribosome. Using S1 nuclease as a probe, we show that these mutant tRNAs also have an altered anticodon loop structure.

MATERIALS AND METHODS

Subcloning of *E. coli* Initiator tRNA Gene. A 450-base-pair (bp) *Pst* I/*Taq* I fragment derived from plasmid pKUI (8) and containing the $tRNA_2^{fMet}$ gene was cloned into the *Pst* I/*Acc* I site of M13mp8 to give M13trnfM.

Oligonucleotide-Directed Site-Specific Mutagenesis. Mutagenic primers were synthesized on an Applied Biosystems (Foster City, CA) 380A DNA synthesizer and purified (9). The DNA template for mutagenesis was a gapped duplex obtained by hybridizing M13trnfM virion DNA (8.5 kilobases long) with a *Pst* I/*Bam*HI fragment of M13mp8 (8 kilobase pairs) in a molar ratio of 1:2 (10). Plaque lifts were screened with 5′ ^{32}P-labeled mutagenic primer. Hybridization was for 3 hr at room temperature. A single wash with 3.0 M tetramethylammonium chloride at 4°C below dissociation temperature (T_d) (50°C for 16-nucleotide-long primer and 64°C for 25-nucleotide-long primers) was used to identify plaques with the desired mutation (11).

Isolation and Purification of Mutant tRNAs. An overnight culture of JM103 cells (4 ml) was diluted to 200 ml with fresh 2YT medium (10 g of yeast extract, 16 g of tryptone, and 5 g of NaCl per liter) and infected with the appropriate M13 recombinant virus stock at a multiplicity of 10. After growth at 37°C for 6 hr in a shaker incubator (approximately middle to late logarithmic phase), the cells were harvested by centrifugation. Total tRNA was isolated from the cells by phenol extraction followed by chromatography on DEAE-cellulose. Yield of tRNA was 70–100 A_{260} units.

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Abbreviation: IF-2, initiation factor 2.

The tRNA (30–60 A_{260} units in 150–300 μl) was applied to a 5-cm-wide slot of a 12% polyacrylamide slab gel without urea (20 × 40 × 0.25 cm). The running buffer for electrophoresis was 89 mM Tris borate/89 mM boric acid/3 mM EDTA, pH 8.0 (12). The gel was run overnight, usually for 12–18 hr, at a setting of 400 V and 50 mA and at a gel temperature of <35°C, until the xylene cyanole dye had migrated 37–38 cm. The gel was wrapped in SaranWrap, placed on top of a fluorescent cellulose thin-layer plate, and tRNA bands were localized under a short-wave UV lamp. The gel strip containing the mutant tRNA band was excised and used for elution of the tRNA.

Use of S1 Nuclease as a Probe of tRNA Structure. The purified mutant tRNAs were labeled at their 5′ ends with ^{32}P (13) and used for S1 nuclease digestion (14, 15).

Isolation and Fingerprint Analysis of ^{32}P-Labeled $tRNA_2^{fMet}$. A 5-ml culture of JM103 cells in 2YT medium was infected with M13 recombinant virus carrying either wild-type or mutant $tRNA_2^{fMet}$ gene as described above. After 3 hr at 37°C, the cells were collected by centrifugation and resuspended in 1 ml of low-phosphate medium (16) containing 200 μCi of [^{32}P]phosphate (1 Ci = 37 GBq). After incubation for 1 hr at 37°C, total RNA was extracted with phenol. Mutant tRNAs were purified by gel electrophoresis as described above and characterized by fingerprint analysis (13).

Assays for Activity of Mutant fMet-tRNAs in Initiation of Protein Synthesis, Ribosome Binding, and Binding to IF-2. The incubation mixture for fMet-tRNA synthesis contained [^{35}S]methionine, formyltetrahydrofolate (fH_4folate), a purified preparation of *E. coli* Met-tRNA synthetase (a gift of P. Rosevear), and a concentrated *E. coli* extract freed of nucleic acids (17) as a source of N^{10}-fH_4folate Met-tRNA transformylase (18). The isolation of f[^{35}S]Met-tRNA was essentially as described (17) except that the extensive dialysis step used to remove ATP was omitted.

MS2 RNA-directed protein synthesis was as described by Goldman and Gibel (19) using an S30 extract made from *E. coli* MRE600 strain (20, 21). MS2 RNA was kindly provided by E. Goldman. High salt washed ribosomes and crude initiation factors were prepared according to Wahba and Miller (22) for the assay of AUG-dependent initiation factor-stimulated ribosome binding of f[^{35}S]Met-$tRNA^{fMet}$. Purified IF-2 was kindly provided by H. Weissbach.

Formation of binary complex between f[^{35}S]Met-$tRNA^{fMet}$ and IF-2 in the presence of $MgCl_2$ was assayed using the glutaraldehyde crosslinking method (23). Puromycin reactivity of ribosome-bound f[^{35}S]Met-$tRNA^{fMet}$ was assayed as described by Leder and Bursztyn (24).

RESULTS

Mutagenesis of the *E. coli* $tRNA^{fMet}$ Gene. Two different initiator tRNAs are found in *E. coli*. The minor species $tRNA_2^{fMet}$, which represents ≈20% of total initiator tRNA, differs from the major species $tRNA_1^{fMet}$ in having A-46 instead of m^7G-46 (25) (Fig. 1). The *E. coli* $tRNA_2^{fMet}$ gene, sequenced by Imamoto and co-workers (8), is the promoter proximal gene of an operon that, among others, consists of *nusA* and the initiation factor IF-2 gene *infB* (26). We subcloned a 450-bp fragment that contains the tRNA gene and its promoter into M13mp8 and used it to isolate the four mutants listed in Table 1.

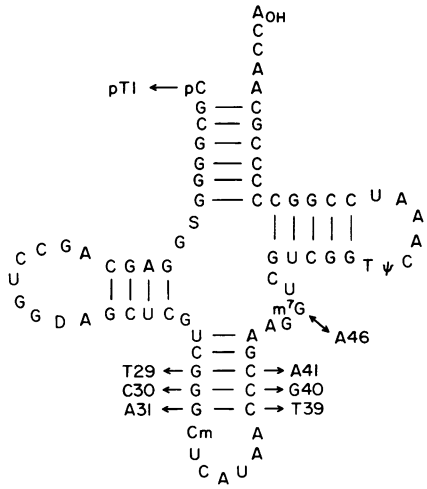

FIG. 1. Sequence of *E. coli* $tRNA^{fMet}$ in cloverleaf form. Arrows indicate mutants described in this paper. Double-headed arrow at position 46 is the site of a naturally occurring variant. $tRNA_1^{fMet}$, the major species has m^7G-46, whereas $tRNA_2^{fMet}$, has A-46. The mutants shown were all derived from $tRNA_2^{fMet}$.

For mutagenesis (6, 7, 27), we used the gapped duplex method (10). A 16-nucleotide primer (Table 1) was used to isolate the T-1 mutant. Recombinant M13 virion DNA carrying this mutation was then used as a template to obtain the anticodon stem mutants. Oligonucleotides 25 nucleotides long and carrying two, four, or six mismatches from the template sequence (Table 1) were used as primers for this purpose. [The anticodon stem mutants all carry the T-1 mutation. The reason for this is that our long-range objective is not only to investigate whether these anticodon stem mutants still act as initiators but also whether they will act as elongators. Chemical modification studies of *E. coli* $tRNA^{fMet}$ has shown that change of C-1 to U-1 enables $tRNA^{fMet}$ to bind to elongation factor Tu (28). Since binding to elongation factor Tu is a prerequisite to ribosomal A site binding, only mutants on a T-1 background can be tested for activity in elongation.]

Screening of phage plaques (6, 29) used 3.0 M tetramethylammonium chloride, where T_d depends on length of oligonucleotide and not on base composition (11). All mutants were characterized by DNA sequencing of the entire tRNA gene.

Expression of Mutant tRNA Genes. M13 recombinant phages carrying the mutant tRNA genes could be used directly as high-level expression vectors. The level of expression of the $tRNA_2^{fMet}$ mutants over endogenous $tRNA_2^{fMet}$ is 15- to 20-fold. This estimate is based on the following: (*i*) in all cases, the methionine acceptor activity of total tRNA isolated from cells infected with M13 recombinant phages

Table 1. Mutagenic primers and mutants generated

Mutagenic primer	Mutant
5′ GTTTC AGG*T*G CGGGG T 3′	T-1
5′ GCTCG TC*T*GG CTCAT AACC*A* GAAGA 3′	T-1 T-29 A-41
5′ GCTCG TC**T*CG CTCAT AAC**G*A GAAGA 3′	T-1 T-29 C-30 G-40 A-41
5′ GCTCG TC***T*A CTCAT AA***T*A GAAGA 3′	T-1 T-29 C-30 A-31 T-39 G-40 A-41

Mutations are indicated by asterisks.

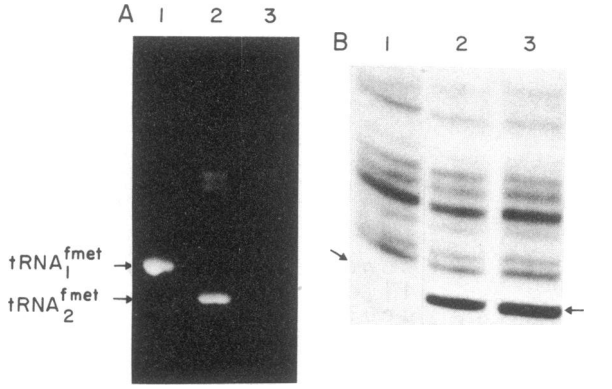

FIG. 2. (*A*) Ethidium bromide staining pattern of *E. coli* tRNA after 12% polyacrylamide gel electrophoresis under native conditions. Lanes: 1, a marker of *E. coli* $tRNA_1^{fMet}$; 2, total tRNA isolated from *E. coli* cells infected with M13trnfM phage; 3, total tRNA from uninfected *E. coli*. (*B*) Polyacrylamide gel electrophoresis of total ^{32}P-labeled tRNA isolated from *E. coli*. Lanes: 1, uninfected; 2, infected with M13trnfM T-1 mutant; 3, infected with M13trnfM T-1 T-29 C-30 A-31 T-39 G-40 A-41 mutant. Arrow indicates location of $tRNA_2^{fMet}$ species.

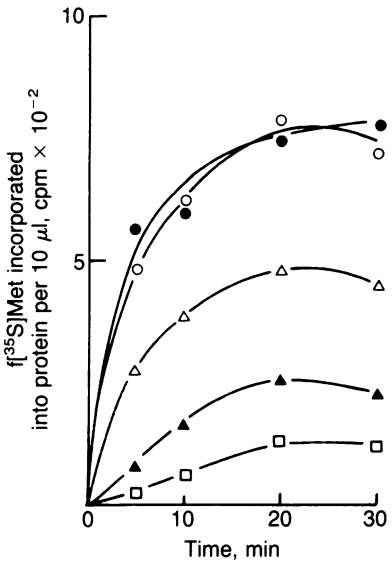

FIG. 3. Time course of incorporation of f[^{35}S]Met from the various f[^{35}S]Met-tRNAs into protein in an MS2 RNA-directed *in vitro* translation system. The amount of input f[^{35}S]Met-tRNA was 1.9×10^5 cpm (≈3 pmol). Aliquots (10 μl) were taken at the times indicated from a total reaction vol of 80 μl. ●, Wild type; ○, T-1 mutant; △, T-1 T-29 A-41 mutant; ▲, T-1 T-29 C-30 G-40 A-41 mutant; □, T-1 T-29 C-30 A-31 T-39 G-40 A-41 mutant.

carrying the $tRNA_2^{fMet}$ gene increased 3-fold (a net increase of 200%) compared to tRNA from uninfected cells, and (*ii*) the $tRNA_2^{fMet}$ species represents 10–15% of total methionine tRNA in *E. coli*.

Purification of the Mutant tRNAs. Electrophoresis on a single 12% or higher percentage (unpublished data) nondenaturing polyacrylamide gel separates $tRNA_2^{fMet}$ species from all other *E. coli* tRNAs including $tRNA_1^{fMet}$, from which it differs by a single nucleotide (Fig. 2*A*, compare lanes 1 and 2). Since there is a 15- to 20-fold overproduction of the mutants, the purity of gel-isolated $tRNA_2^{fMet}$ mutants is 93–95%, the remainder being wild-type $tRNA_2^{fMet}$. The substantial overproduction and simple purification means that a single preparative polyacrylamide gel can be used to isolate enough mutant tRNAs for all of the functional studies. Similarly, ^{32}P-labeled mutant tRNAs can be purified readily for their characterization, as shown in Fig. 2*B* for two of the mutants, T-1 (lane 2) and T-1 T-29 C-30 A-31 T-39 G-40 A-41 (lane 3).

The results in Fig. 2 *A* and *B* also provide further evidence for the overproduction of $tRNA_2^{fMet}$ in cells carrying the recombinant phage. tRNA isolated from cells infected with M13trnfM phage contains a major band (Fig. 2*A*, lane 2), which is a minor component in tRNA from uninfected cells (lane 3). Similarly, the predominant ^{32}P-labeled tRNA band in cells infected with M13trnfM mutant phages (Fig. 2*B*, lanes 2 and 3) is a minor component in tRNAs from uninfected cells (lane 1).

Characterization of Mutant tRNAs. All four of the mutant tRNAs were characterized by comparison of their T-1 fingerprints (27, 30) to those of wild-type $tRNA_2^{fMet}$ (data not shown). Modified base composition analyses of the mutant tRNAs shows that all of the mutants have the same base modifications as wild-type $tRNA_2^{fMet}$. The only exception is the T-1 T-29 C-30 A-31 T-39 G-40 A-41 mutant tRNA, which has acquired a new Ψ modification at position 39.

Function of Mutant tRNAs *in Vitro*. The mutant tRNAs were aminoacylated with [^{35}S]methionine, formylated, and the f[^{35}S]Met-tRNAs were used for studies on their function.

(*i*) *Initiation of protein synthesis.* Fig. 3 shows the time course of incorporation of f[^{35}S]Met from the various f[^{35}S]Met-tRNAs into protein in a MS2 RNA-directed *E. coli* protein synthesizing system. The T-1 mutant is as active as wild-type tRNA. With the anticodon stem mutants, however,

Table 2. AUG-dependent ribosome binding of various mutant tRNAs and puromycin reactivity of the bound fMet-tRNAs

Mutant	Ribosome binding		Puromycin reactivity	
	f[^{35}S]Met tRNA bound, cpm	%	f[^{35}S]Met tRNA reacted, cpm	%
T-1	86,100	83	48,700	75
T-1 T-29 A-41	34,900	34	15,000	24
T-1 T-29 C-30 G-40 A-41	16,300	16	7,700	12
T-1 T-29 C-30 A-31 T-39 G-40 A-41	8,800	8.5	3,500	5.5
Wild type	103,800	100	63,500	100
Wild type (-AUG)	3,300	3.2	500	0.8

Incubation mixture (100 μl) contained 1.3 A_{260} of salt-washed ribosome, 38 μg of crude initiation factor, 13 nmol of AUG and f[^{35}S]Met-tRNA, 2.3×10^5 cpm in 50 mM Tris·HCl, pH 7.4/50 mM NH_4Cl/5 mM $Mg(OAc)_2$/3 mM 2-mercaptoethanol/0.27 mM GTP. Ribosome binding assay was done according to Wahba and Miller (22). Binding plateaued within 15 min (data not shown). Puromycin reactivity of the bound f[^{35}S]Met-tRNA was measured in a separate incubation. After 20 min of incubation, puromycin was added to a final concentration of 1 mM. The f[^{35}S]Met-puromycin formed was extracted into ethylacetate (0.7 ml) and a 0.5-ml aliquot was used for counting (24).

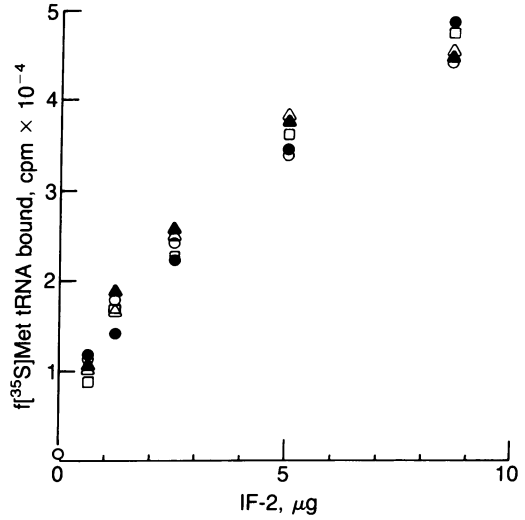

FIG. 4. IF-2 concentration dependence of binding of wild-type and f[^{35}S]Met-tRNAs to IF-2. Amount of input tRNA was 1.1×10^5 cpm (≈1 pmol). ○, Wild type; ●, T-1 mutant; △, T-1 T-29 A-41 mutant; ▲, T-1 T-29 C-30 G-40 A-41 mutant; □, T-1 T-29 C-30 A-31 T-39 G-40 A-41 mutant.

as one, two, and all three G·C base pairs are altered, there is a progressive decrease in the rate and extent of incorporation of f[^{35}S]Met. In two of a total of four such experiments, the T-1 T-29 C-30 A-31 T-39 G-40 A-41 mutant showed virtually no activity in initiation, with the residual activity being most likely due to the 5–7% of wild-type tRNA^{fMet} in the sample.

(*ii*) *Binding to ribosomal P site.* The results of AUG-dependent ribosomal P site binding (31) follow exactly the same trend as in initiation of protein synthesis (Table 2). The mutant with one G·C base-pair change binds less efficiently (34%) compared to wild-type tRNA (100%) or the T-1 mutant (83%), that with two G·C base-pair changes binds even less efficiently (16%), and that with all three G·C base-pair changes is the least efficient (8.5%). Puromycin reactivity (24) of the ribosome-bound f[^{35}S]Met-tRNAs also follows the same trend and provides further evidence that the AUG and initiation factor-dependent binding of fMet-tRNAs to the ribosome is at the P site.

(*iii*) *Binding to initiation factor IF-2.* Fig. 4 shows the effect of IF-2 concentration on the formation of a binary complex between IF-2 and the various f[^{35}S]Met-tRNAs (23). All four mutants bind to IF-2 with basically the same affinity as wild-type tRNA$_2^{fMet}$.

Structure Mapping of Mutant tRNAs Using S1 Nuclease. Wrede *et al.* (15) showed by S1 nuclease mapping that the conformation of the anticodon loop of initiator tRNAs is different from that of elongator tRNAs. They attributed this difference to the presence in initiator tRNAs of the three contiguous G·C base pairs that flank the anticodon loop. Our results with the anticodon stem mutants lacking one, two, and all three of these G·C base pairs support their hypothesis (Fig. 5). In wild-type initiator tRNA, only two of the phosphodiester bonds (nucleotides 34 and 35) in the anticodon loop are initially cleaved by S1 nuclease. With the anticodon stem mutants, however, as one, two, and all three G·C base pairs are altered, other phosphodiester bonds (nucleotides 33, 36, and 37) in the anticodon loop become accessible. The cleavage pattern for two of the three mutants (T-1 T-29 C-30 G-40 A-41 and T-1 T-29 C-30 A-31 T-39 G-40 T-41) resembles that of elongator tRNAs (15). The only difference is that in elongator tRNAs there is no cleavage at nucleotide 37. This is most likely due to the fact that nucleotide 37 in all elongator tRNAs is a modified base (5), whereas in the tRNA$_2^{fMet}$ mutants it remains unmodified, as in wild-type tRNA$_2^{fMet}$.

DISCUSSION

What makes an initiator tRNA an initiator and not an elongator? In an attempt to answer this question, we removed from *E. coli* tRNA$_2^{fMet}$ two of the features common to all prokaryotic initiator tRNAs, isolated and characterized the mutant tRNAs, and studied their function in protein synthesis *in vitro*. The T-1 mutation (Fig. 1) has no effect on initiation of protein synthesis. In contrast, the $\overset{GGG}{CCC}$ sequence conserved in the anticodon stem of both prokaryotic and eukaryotic initiator tRNAs is important for initiation. As one, two, and all three of the G·C base pairs are altered to those found in *E. coli* elongator methionine tRNA, there is, with

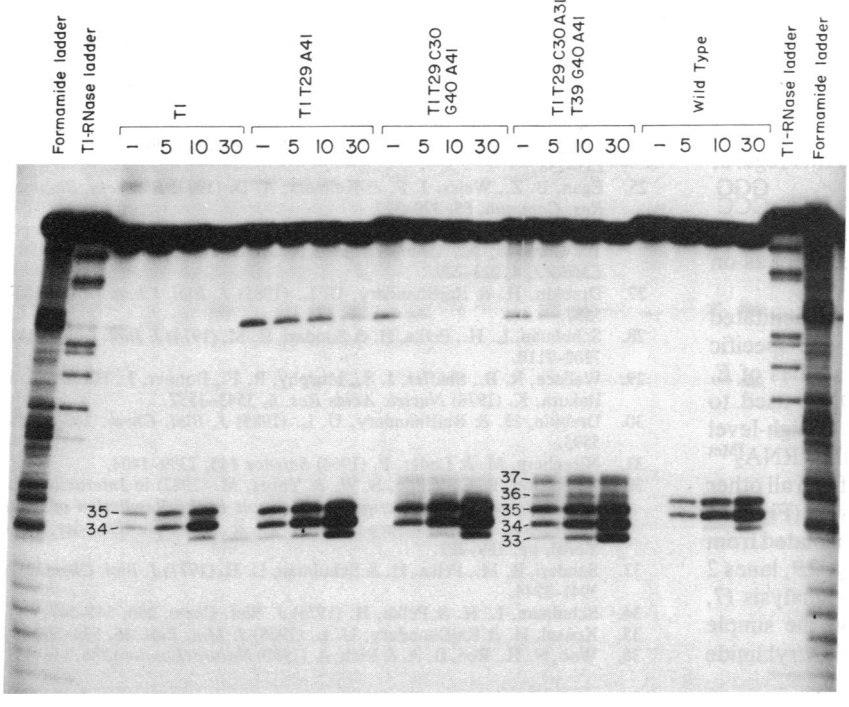

FIG. 5. Structure mapping of anticodon loop of 5′ ^{32}P-labeled tRNA^{fMet} (wild type and mutants) by S1 nuclease. Intact 5′ ^{32}P-labeled tRNA was isolated by electrophoresis on a 20% polyacrylamide gel containing 8 M urea (13). After preliminary experiments to determine the level of S1 nuclease to be used, time dependence of S1 nuclease cleavage was followed. Numbers at the top indicate time (in min) of treatment with S1 nuclease. Numbers within the gel indicate sites of cleavage in the anticodon loop. S1 nuclease digestions were carried out in 25 mM NaOAc, pH 4.5/5 mM $MgCl_2$/50 mM KCl/1 mM $ZnCl_2$ at 37°C. Reactions were stopped at indicated time intervals by taking aliquots and adding them into "stop" solution (10 mM ATP/15 mM nitrilotriacetic acid/6 μg of *E. coli* tRNA/8 M urea, and bromophenol blue and xylene cyanole dye) and put into dry ice immediately. Reactions were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea.

each mutation, a progressive and significant decrease in the activity of the mutant tRNAs in protein synthesis initiation, a mutant with all three G·C base pairs altered being the least active (Fig. 3). The effect of the mutation is at the step of initiator tRNA binding to the ribosomal P site (Table 2).

Mutations in the $\frac{GGG}{CCC}$ sequence lead to a gradual rather than a complete loss of activity in the three anticodon stem mutants. This means that while these G·C base pairs may not be absolutely essential in initiation, they are important for "maximal" function of the initiator tRNAs. Our results in this regard are somewhat similar to those of Yarus and co-workers (32) on an elongator tRNA, who have found that U-33, a highly conserved nucleotide, is not essential as such for function of a tRNA in suppression but is needed for optimal efficiency of suppression.

Our finding that the T-1 mutant is fully functional in initiation supports and extends the previous work of Schulman and co-workers (33). This finding does not, however, mean that absence of a Watson–Crick base pair at the end of the acceptor stem, which is a hallmark of all prokaryotic initiator tRNAs, is not an important feature of these tRNAs. Studies on chemically modified tRNAs have shown (28, 34) that the *E. coli* $tRNA^{fMet}$, which contains U-1 instead of C-1, differs from wild-type tRNA in two respects: (*i*) the modified Met-$tRNA^{fMet}$ can form a ternary complex with elongation factor Tu and GTP, and (*ii*) the modified fMet-$tRNA^{fMet}$ is hydrolyzed by *E. coli* peptidyl-tRNA hydrolase. Thus, the unique structural feature in the acceptor stem of *E. coli* $tRNA^{fMet}$ is important not in initiation *per se* but in (*i*) preventing the tRNA from binding to the ribosomal A site and hence from inserting methionine internally, and (*ii*) in protecting the fMet-$tRNA^{fMet}$ from enzymatic deacylation by the peptidyl-tRNA hydrolase (35), leading to its energetically wasteful turnover.

A striking result of our work is that the gradual loss in activity of the anticodon stem mutants is coupled to a parallel progressive change in anticodon loop conformation. This raises an interesting question. Is the gradual loss of activity primarily due to decremental loss of contacts between the G·C base pairs in the anticodon stem and RNA and/or protein components(s) in the ribosomal P site, or is it due to changes in conformation of the anticodon loop to one that is not easily accommodated into the ribosomal P site? We cannot answer this question until we know the exact nature of anticodon loop structure difference between initiator tRNAs and the various anticodon stem mutants. Our results are, however, consistent with the attractive notion (36) that the $\frac{GGG}{CCC}$ sequence in the anticodon stem of initiator tRNAs imparts on the anticodon loop a unique conformation, which is important in targeting this tRNA to the ribosomal P site. In this regard, since eukaryotic initiator tRNAs also have the same $\frac{GGG}{CCC}$ sequence, it would be interesting to see whether changes in this sequence of these tRNAs also has the same effect as on the *E. coli* $tRNA^{fMet}$.

Finally, several developments listed below have facilitated this work and broaden greatly the scope of site-specific mutagenesis in structure–function relationship studies of *E. coli* $tRNA^{fMet}$. First, the M13 recombinant DNA used to generate the mutants can itself be used as a high-level expression vector (Fig. 2*A*, lane 2). Second, the $tRNA_2^{fMet}$ species (wild type and mutant) can be separated from all other *E. coli* tRNAs, including $tRNA_1^{fMet}$, in a single step (Fig. 2*A*, lane 2). Third, ^{32}P-labeled mutant tRNAs can be isolated from cells infected with recombinant M13 phages (Fig. 2*B*, lanes 2 and 3) and characterized readily by fingerprint analysis (7, 16). The overproduction of mutant tRNAs and the simple purification scheme means that preparative polyacrylamide gels can be used to isolate substantial amounts of purified mutant tRNAs. P. Rosevear and his colleagues (personal communication) have used the procedure described here to isolate several milligrams of pure *E. coli* $tRNA_2^{fMet}$. This opens up the possibility of detailed solution structure and x-ray structure analysis (36) of the $tRNA_2^{fMet}$ mutants.

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