We show that the structure and/or sequence of the first three base pairs at the end of the amino acid acceptor stem of Escherichia coli initiator tRNA and the discriminator base 73 are important for its formylation by E. coli methionyl-tRNA synthetase. This conclusion is based on mutagenesis of the E. coli initiator tRNA gene followed by measurement of kinetic parameters for formylation of the mutant tRNAs in vitro and function in protein synthesis in vivo. The first base pair found at the end of the amino acid acceptor stem in all other tRNAs is replaced by a C:A "mismatch" in E. coli initiator tRNA. Mutation of this C:A to U:A, a weak base pair, or U:G, a mismatch, has little effect on formylation, whereas mutation to C:G, a strong base pair, has a dramatic effect lowering $V_{\text{max}}/K_{\text{m}}$ by 495-fold. Mutation of the second base pair G2:C71 to U2:A71 lowers $V_{\text{max}}/K_{\text{m}}$ by 236-fold. Replacement of the third base-pair C3:G70 by U3:A70, A3:U70, or G3:C70 lowers $V_{\text{max}}/K_{\text{m}}$ by about 67-27%, and 30-fold, respectively. Changes in the rest of the acceptor stem, dihydrouridine stem, anticodon stem, anticodon sequence, and T:V:C stem have little or no effect on formylation.

Of the two classes of methionine tRNAs present in all organisms, the initiator is used exclusively for initiation of protein synthesis, whereas the elongator is used for inserting methionine into internal peptide linkages (1). In eubacteria, mitochondria, and chloroplasts the initiator is used for formylmethionyl-tRNA (fMet-tRNA). Following aminoacylation of the initiator tRNA (tRNA$^{\text{Met}}$), the methionyl-tRNA (Met-tRNA$^{\text{Met}}$) is formylated to fMet-tRNA$^{\text{Met}}$ by methionyl-tRNA transformylase (2). The recognition of Met-tRNA$^{\text{Met}}$ by the Met-tRNA transformylase is highly specific, the enzyme formylates only Met-tRNA$^{\text{Met}}$ but not Met-tRNA$^{\text{Met}}$, the elongator species, or any other aminoacyl-tRNA (3). The sequence and/or structural features important for this specific recognition are not known.

In previous work, we described mutagenesis of the E. coli tRNA$^{\text{Met}}$ gene followed by functional studies of the mutant tRNAs in protein synthesis in vitro and in vivo. We showed: (i) that the GGG:CCC sequence conserved in the anticodon stem of all initiator tRNAs was important in targeting the tRNA to the ribosomal P site (4) and (ii) that the C1.A72 mismatch at the end of the acceptor stem was important in preventing the tRNA from functioning in the elongation step of protein synthesis (5, 6). Here we describe the generation of several more mutants and measurement of kinetic parameters for formylation of the mutant Met-tRNAs by Met-tRNA transformylase. Our results show that the sequence and/or structural elements important for recognition of tRNA by Met-tRNA transformylase are localized mostly around the end of the acceptor stem. These include the discriminator base A73, either a weak or a disrupted base pair in the first position, a G2:C71 base pair in the second position and a C3:G70 base pair in the third position.

**MATERIALS AND METHODS**

**Oligonucleotide-directed Site-specific Mutagenesis**—Mutagenic primers were synthesized on an Applied Biosystems 380A DNA synthesizer and purified as described (7). Mutagenesis of single-stranded DNA in M13 vectors used the gapped duplex approach (8) or the phosphorothioate-based approach (9) using the kit from Amersham. The mutant tRNA genes were characterized by sequencing of the entire tRNA gene. Most of the mutant tRNAs were also characterized by fingerprint analysis of nuclease digests (10) and by modified base composition analysis (data not shown).

**Localization of Mutant tRNAs on Gels by Northern Hybridization**—Northern hybridization followed a procedure modified from that described before (11). One AZM) unit of total tRNA was electrophoresed on either 12% or 5% SDS polyacrylamide gels. Most of the mutant tRNAs could be overproduced. tRNAs were visualized by ethidium bromide staining for analytical gels or by UV shadowing for preparative gels. The location of mutant tRNAs which were not overproduced and which migrated differently from wild type tRNA were determined by Northern hybridization or hybrid selection as described below.

**Hybrid Selection of $^{32}$P-labeled Mutant tRNAs**—Total tRNAs were isolated (4) and fractionated by gel electrophoresis on either 12% or 20% nondenaturing polyacrylamide gels. Most of the mutant tRNAs were visualized by fingerprint analysis of nuclease digesta (10) and by modified base composition analysis (data not shown).
washed three times with 0.2 M tetrahydrofolic acid. Before use in formylation, an aliquot was neutralized to pH 7.5 with 1 M NaOH and incubated at room temperature for 5 min. An aliquot (16 μl) was applied on Whatman 3MM paper discs, which were then washed for 10 min with cold 10% trichloroacetic acid containing 0.1% folic acid followed by three washes for 10 min each with cold 5% trichloroacetic acid and once for 5 min with ethanol. After drying acid insoluble radioactivity was measured.

Activity of Mutant tRNAs in Protein Synthesis in Vivo—Assays for activity of mutant tRNAs in initiation and elongation steps of protein synthesis were as described in the following paper (16).

RESULTS

Mutants of E. coli Initiator tRNA

The mutants used in this work contain changes in the acceptor stem, dihydrouridine stem, anticodon stem, and TψC stem (Fig. 1). For in vivo functional studies changes in the dihydrouridine stem, anticodon stem, or the TψC stem were coupled to changes in the anticodon sequence. Mutation in the second base pair of the acceptor stem alone produced very little mutant tRNA presumably because mutant tRNA precursors were either not processed in vivo or were unstable. Therefore, mutations in the second and third base pairs were in most cases coupled to either the T1 or the G72 mutations to generate a base pair between nucleotides 1 and 72.

Incubation was at 37 °C for 5 min. An aliquot (16 μl) was applied on Whatman 3MM paper discs, which were then washed for 30 min with cold 10% trichloroacetic acid containing 0.1% folic acid followed by three washes for 10 min each with cold 5% trichloroacetic acid and once for 5 min with ethanol. After drying acid insoluble radioactivity was measured.

FIG. 2. Localization of the C11C12A13:A22G23G24 mutant on polyacylamide gel by Northern hybridization. Total tRNAs were isolated from E. coli infected with M13 containing either the wild type (lanes 1 and 3) or the C11C12A13:A22G23G24 mutant tRNA*^Met^ gene (lanes 2 and 4). The tRNAs were separated on a 12% native polyacylamide gel. Lanes 1 and 2, ethidium bromide staining patterns; lanes 3 and 4, autoradiogram of Northern hybridization.

FIG. 3. Localization of C52:G62 and A49G50A51C52: G62T63C64T65 mutant tRNAs on polyacylamide gel using hybrid selected tRNA as a marker. A, electrophoresis of hybrid selected tRNAs on a 12% denaturing polyacylamide gel. Lane 1, wild type tRNA^Met^; lane 2, the A49G50A51C52:G62T63C64T65 mutant tRNA; and lane 3, the C52:G62 mutant tRNA. B, tRNAs purified from panel (A) were renatured and then used as radioactive markers on a 12% native polyacylamide gel. Lane 1, ethidium bromide staining pattern of total tRNAs from cells infected with M13 carrying the wild type tRNA gene; lanes 2–4, autoradiograms of hybrid selected tRNAs; lane 2, wild type tRNA^Met^; lane 3, the A49G50A51C52: G62T63C64T65 mutant tRNA; lane 4, the C52:G62 mutant tRNA.
Recognition of tRNA by Met-tRNA Transformylase

## TABLE I

Kinetic parameters in formylation of methionyl-tRNAs by E. coli Met-tRNA transformylase

| Mutants        | Location         | Relative $V_{\text{max}}/K_m$ | $K_m$     | Relative $V_{\text{max}}$ |
|----------------|------------------|-------------------------------|-----------|--------------------------|
| WT tRNA$^{\text{Met}}$ |                  | 1.0                           | 1.7       | 1.0                      |
| T1             | Acceptor stem    | 1.4                           | 2.4       | 1.0                      |
| T1/G72         | Acceptor stem    | 6.3                           | 4.3       | 0.4                      |
| G72            | Acceptor stem    | 495.0                         | 67.0      | 0.08                     |
| G72/G73        | Acceptor stem    | Inactive$^a$                  |           |                          |
| T2/A71/G72     | Acceptor stem    | 296.0$^a$                     | 40.0      | 0.1                      |
| T1/T3:A70      | Acceptor stem    | 67.0$^b$                      | 80.0      | 0.7                      |
| T1/A3/T70      | Acceptor stem    | 27.0$^b$                      | 18.2      | 0.4                      |
| G3/C70         | Acceptor stem    | 30.0                          | 10.0      | 0.2                      |
| C4/G68         | Acceptor stem    | 4.7                           | 8.7       | 0.6                      |
| C5/G68         | Acceptor stem    | 2.2                           | 2.2       | 0.6                      |
| C6/G67         | Acceptor stem    | 1.3                           | 2.5       | 1.1                      |
| C7/G68         | Acceptor stem    | 1.4                           | 3.8       | 1.6                      |
| C11/G24        | Dihydrouridine stem | 8.4                          | 14.6      | 1.0                      |
| C11/C12/A13:A22/G23/G24 | Dihydrouridine stem | 0.6                          |           |                          |
| C52/G62        | TyC stem         | 1.4                           | 3.7       | 1.6                      |
| A49/G50/A51/C52/G62/T63/C64/T65 | TyC stem | 0.6                           | 5.4       | 5.3                      |
| T1/T29/C30/A31:T39/G40/A41 | Anticodon stem | 1.3                           | 2.0       | 0.9                      |
| C27/A28/T29/C30/A31:T39/G40/A41/T42/G43 | Anticodon stem | 0.9                           | 2.2       | 1.5                      |
| T35/A36        | Anticodon        | 2.5$^d$                       |           |                          |
| tRNA$^{\text{Met}}$ |                  | Inactive$^e$                  |           |                          |

*Rate was too low to be detected.
*These numbers represent the combined effect of mutations of C1 to T1 and of mutations in the second and third base pair and are used as such throughout the text.
*Based on comparison of initial rates.
*Mutant T35/A36 was aminoacylated with glutamine using GlnRS (obtained from Dr. Dieter Soll) and then formylated.

## TABLE II

Activity of the various mutant tRNA in initiation and elongation steps of protein synthesis in vivo

| tRNA             | Initiation$^*$ | Elongation$^*$ |
|------------------|----------------|----------------|
|                  | -GlnRS         | +GlnRS         | -GlnRS     | +GlnRS         |
| T35/A36/A49/G50/A51/C52/G62/T63/C64/T65 | 35.5           | 61.1           | 0.2         | 0.4           |
| C11/C12/A13:A22/G23/G24/T35/A36 | 44.3           | 56.3           | 0.7         | 5.1           |
| T1/T29/C30/A31:T39/G40/A41/T35/A36 | 0.9            | 0.8            | 21.4        | 51.4          |
| T29/C30/A31:T39/G40/A41/T42/G43 | 1.0            | 2.5            | 0.4         | 0.8           |
| T35/A36         | 100.0          | 110.0          | 0.2         | 0.7           |
| Su'2            | 100.0          | 100.0          |             |               |

*Based on chloramphenicol acetyltransferase activities (%) in extracts of E. coli CA274 transformed with CATam12.5 and various mutant tRNA genes.
*Based on β-galactosidase activities (suppression of lacZamlZ5 mutation in E. coli CA274 and expressed as % of the Su').

Isolation and Purification of Mutant tRNAs

Mutant tRNAs with changes in the acceptor stem or anticodon stem migrated to the same position on a native polyacrylamide gel as wild type tRNA$^{\text{Met}}$. These mutant tRNAs were purified using a single step of gel electrophoresis as described before (4).

Mutant tRNAs with changes in the dihydrouridine stem were expressed to a lower extent and migrated differently from wild type tRNA$^{\text{Met}}$ and tRNA$^{\text{Met}}$. Their position on 12% native polyacrylamide gels were determined by hybridization. Fig. 2 shows the results of Northern blot analysis on total tRNAs present in transformants carrying either the wild type (lane 3) or the C11/C12/A13:A22/G23/G24 mutant tRNA gene (lane 4). The probe hybridizes strongly to the desired mutant tRNA (lane 4, indicated by arrow) and weakly to the wild type tRNA$^{\text{Met}}$ and tRNA$^{\text{Met}}$ species (weak bands in lanes 3 and 4). The mutant tRNA is clearly separated from the elongator tRNA$^{\text{Met}}$ and both of the initiator species and can, therefore, be isolated free of these tRNAs.

Mutant tRNAs with changes in the TyC stem also migrated differently from wild type tRNA$^{\text{Met}}$. The positions of these tRNAs on native polyacrylamide gels were determined by using gel purified, hybrid selected 32P-labeled mutant tRNAs (Fig. 3A) as markers. Fig. 3B shows (i) that each of the hybrid selected tRNAs consists of single tRNA species (lanes 2–4) and (ii) that the C52/G62 and the A49/G50/A51/C52/G62/T63/C64/T65 mutant tRNAs (lanes 4 and 3, respectively) are clearly separated from the wild type E. coli elongator and both of the initiator methionine tRNAs.

Prior to their use as substrate for Met-tRNA transformylase, the methionine acceptance activity of the mutant tRNA species listed in Table I was determined. The mutant tRNAs
were found to be essentially pure with the exception of those carrying changes in the D stem or the T$C stem which migrated differently from wild type tRNA on native polyacrylamide gels. The purity of these tRNAs, based on methionine acceptance, were estimated to be 50% for the C11:G24 mutant, 20% for the C11C12A13:A22G23G24 mutant, 28% for the C52:G62 mutant, and 30% for the A49G50A51C52:G62T63C64T65 mutant.

Kinetic Parameters for Aminoclaylation of Mutant tRNAs with E. coli Met-tRNA Synthetase

Kinetic parameters for amination of the mutant tRNAs listed in Table I were determined using purified E. coli MetRS. Our results agree with the conclusions of Schultman and co-workers that the anticodon sequence is the major site on E. coli methionine tRNAs for MetRS recognition (17). Mutation of A73 to G73 and C3:G70 to G3:G70 had small effects in aminoclaylation by MetRS (6-7-fold decrease in $V_{max}/K_{pp}$. The rest of the mutants had basically the same $K_{m}$ and $V_{max}$ parameters as wild type tRNA$^{Met}$. 

Kinetic Parameters for Formylation of Mutant tRNAs with E. coli Met-tRNA Transformylase

Acceptor Stem Mutants—Data in Table I show that mutation of CLA72 to U1:A72 (T1 mutant), a weak base pair, has essentially no effect on formylation (18), whereas mutation to C1:G72 (G72 mutant) a strong base pair, lowers $V_{max}/K_{pp}$ by 495-fold (19). In contrast, the double mutant T1:G72 having a U1:G72 mismatch is affected only slightly, $V_{max}/K_{pp}$ being about 6-fold lower. These results suggest that either a weak base pair or a disrupted base pair at the end of acceptor stem is important for formylation. Mutation of G2:C71 base pair to U2:A71 in the T1:T2:A71 mutant lowers $V_{max}/K_{pp}$ by 236-fold whereas the T2:A71:G72 mutant is essentially inactive in formylation, indicating that G2:C71 base pair is important for formylation. Mutation of C3:G70 base pair to U3:G70 (in the T1:A70 mutant), A3:U70 (in the T1:A3:T70 mutant), or C3:G70 (in the G3:C70 mutant) base pairs lowers $V_{max}/K_{pp}$ by 67-, 27-, and 30-fold, respectively, indicating that C3:G70 is also important for formylation.

Although the C2 mutant is a poor substrate for Met-tRNA transformylase, it can be quantitatively formylated with an excess of enzyme (20). However, the G72G73 mutant is essentially not formylated. This suggests that the "discriminator" base A73 is also important for formylation.

Mutation of G4:C69 to C4:G69 has a small effect on $V_{max}/K_{pp}$ in formylation (5-fold). Mutations in the rest of the acceptor stem (C5:G68, C6:G67, and C7:G66 mutants) have little or no effect on formylation. These results show that sequences clustered around the end of acceptor stem in E. coli tRNA are important in recognition of tRNAs by E. coli Met-tRNA transformylase.

Dihydrouridine Stem Mutants—One of the unique sequence features found in all prokaryotic initiator tRNAs is the presence of a purine 11:pyrimidine 24 base pair instead of pyrimidine 11:purine 24 base pair. Mutation of A11:U24 in the E. coli tRNA to C11:G24 lowers $V_{max}/K_{pp}$ by 8-fold. This effect is primarily due to an increase in $K_{pp}$. However, a mutation which changes 3 of the 4 base pairs in the dihydrouridine stem, including the A11:U24 base pair (C11C12A13:A22G23G24 mutant), which has the same sequence in the D stem as found in E. coli tRNA and in yeast tRNA$^{Met}$, has no effect on formylation.

Anticodon Stem Mutants—Mutations of the 3 G:C base pairs unique to all initiator tRNAs to T29C30A31:T39G40A41 of all 5 base pairs in the anticodon stem to the sequence found in tRNA$^{Met}$ (227A28T29G30A31:T39G40A41T42G43 mutant) had virtually no effect on formylation. Thus, although the 3 G:C base pairs in the anticodon stem of tRNA$^{Met}$ are important for binding of the tRNA to the ribosomal P site during initiation of protein synthesis (4), they have no direct role in recognition of tRNA by E. coli Met-tRNA transformylase.

Anticodon Sequence Mutant—The T35A36 mutant in which the anticodon sequence has been changed from CAU → CUA is now aminoclaylated with glutamine (6, 21). The mutant tRNA aminoclaylated with glutamine is still a good substrate for Met-tRNA transformylase, (a 2.5-fold difference in $V_{max}/K_{pp}$). This result indicates that the anticodon sequence is not important in recognition of tRNAs by Met-tRNA transformylase.

T$C$ Stem Mutants—The G52:C62 base pair is conserved in all initiator tRNAs, prokaryotic or eukaryotic, which are substrates for E. coli Met-tRNA transformylase. Mutation of G52:C62 to G52:G62 has no effect on formylation. Mutation which changes 4 of the 5 base pairs in the T$C$ stem (A49G50A51C52:G62T63C64T65 mutant) including the G52:C62 base pair also has little effect on formylation, suggesting that sequences in the T$C$ stem are not directly involved in Met-tRNA transformylase recognition of tRNAs.

Properties of the Mutant tRNAs in Initiation of Protein Synthesis in Vivo—The above results suggest that major sequence alterations in the D stem, anticodon stem, anticodon sequence, and T$C$ stem have no effect on kinetic parameters for formylation of the mutant tRNAs. Therefore, mutant tRNAs carrying these alterations would be expected to function in initiation of protein synthesis unless the mutations affect a step in initiation subsequent to formylation of the initiator tRNA. To test for this, we coupled the multiple base pair mutations in the dihydrouridine stem, anticodon stem or the T$C$ stem, to a change in the anticodon sequence from CAU to CU A. The latter mutation enables the mutant tRNAs to potentially initiate protein synthesis from UAG in a mutant chloramphenicol acetyl transferase (CAT) gene whose initiator codon has been changed from AUG to UAG (22). Mutant tRNAs which are active in initiation in vivo should produce CAT protein in E. coli transformed with plasmids carrying the various mutant tRNA genes and the mutant CAT gene. Results of such an experiment are shown in Table II. Mutant tRNAs with alterations in 3 of the 4 base pairs in the D stem and 4 of the 5 base pairs in the T$C$ stem are quite active in initiation in vivo ~50%. This confirms that the sequences are, most likely, not directly important for Met-tRNA transformylase recognition of tRNA and also suggests that they play no direct role in activity of tRNA in initiation.

The mutant tRNAs with changes in the 3 G:C base pairs in the anticodon stem are inactive (Table II). This result is expected and supports our previous conclusion, based on in vitro studies, that the 3 G:C base pairs in the anticodon stem are important for binding of the initiator tRNA to the ribosomal P site (4). Table II also shows that the T1:T29G30A31:T39G40A41:T35A36 mutant tRNA, which is inactive in initiation, is, nevertheless, active in suppression of amber codons at the ribosomal A site (right panel). Therefore, lack of activity of this mutant in initiation in vivo is not due to defects in synthesis or in aminoclaylation of the tRNA.

**DISCUSSION**

We have shown that the sequence and/or structural features important for the highly specific recognition of E. coli
tRNA\textsuperscript{Met} by E. coli Met-tRNA transformylase are, virtually all, clustered around the end of the acceptor stem. These include a weak or disrupted base pair between positions 1 and 72 at the end of the acceptor stem, a G2:C71 base pair, a C3:G70 base pair and A73 at the discriminator position (Fig. 1). The G4:C69 base pair and the A11:U24 base pair could also play some role in the recognition process. These features are conserved in all eubacterial, mitochondrial, and chloroplast tRNAs (23). Alterations in the rest of the tRNA in the D stem, anticodon stem, anticodon sequence, T\textsuperscript{C}G stem, and in base pairs 5–7 in the acceptor stem of E. coli tRNA\textsuperscript{Met} have no effect on the kinetic parameters for formylation.

**Base Pair 1:72**—The constellation of nucleotides in the acceptor stem important for specific interaction of tRNA\textsuperscript{Met} with Met-tRNA transformylase appears, at first glance, to be similar to that of E. coli tRNA\textsuperscript{Met} with GlnRS (24). Thus, like GlnRS, Met-tRNA transformylase requires a weak or a disrupted base pair between nucleotides 1 and 72. In the crystal structure of GlnRS-tRNA\textsuperscript{AG1} complex, there are no sequence specific contacts between GlnRS and nucleotides 1 or 72 of tRNA\textsuperscript{AG1} implying that the nature of nucleotides 1 and 72 is less important than the fact that the base pair between them be disrupted (6, 24). Whether this is also the case for Met-tRNA transformylase interaction with tRNA is not known. It is possible that besides requiring that the 1:72 base pair be disrupted, the Met-tRNA transformylase also makes direct contact with one or both of the nucleotides. Some indication for this comes from the fact that while most eukaryotic cytoplasmic initiator tRNAs (yeast, human etc.) can be quantitatively formylated by the E. coli enzyme (15), the $V_{\text{max}}/K_{\text{m}}$ for these tRNAs is 50–100-fold lower than for E. coli tRNA\textsuperscript{Met}.\textsuperscript{3} These eukaryotic initiator tRNAs have all of the important nucleotides and base pairs in the acceptor stem as in E. coli tRNA\textsuperscript{Met} except for a A:U base pair between nucleotides 1 and 72. Mutants of E. coli tRNA\textsuperscript{Met}, in which nucleotides 1 and 72 are changed to each of the other three nucleotides, are being used as substrates for Met-tRNA transformylase to answer the above question.\textsuperscript{4}

**Base Pair 2:71**—Mutation of G2:C71 conserved in eubacterial, mitochondrial, and chloroplast initiator tRNAs (E. coli G24 tRNA\textsuperscript{AG1} lower $V_{\text{max}}/K_{\text{m}}$ by a factor of 236 (Table I). Thus, as in the case of E. coli GlnRS-tRNA\textsuperscript{AG1} interaction, this base pair is important for recognition of tRNA\textsuperscript{Met} by Met-tRNA transformylase. The importance of this base pair in formylation is also underscored by the fact that although the G72 mutant with a "strong" C1:G72 base pair at the end of the acceptor stem is a very poor substrate, it can be quantitatively formylated in \textit{vitro} with an excess of enzyme (20). However, coupling of the T2:A71 mutation with the G72 mutation essentially abolishes formylation. The importance of G2:C71 in formylation may also explain why plant cytoplasmic initiator tRNAs are not formylated by the \textit{E. coli} Met-tRNA transformylase (25). Of all the eukaryotic cytoplasmic initiator tRNAs, the plant tRNAs are the only ones which contain a U2:A71 base pair instead of a G2:C71 base pair in others.

**Base Pair 3:70**—Mutation of C3:G70 to any one of the other 3 base pairs reduces $V_{\text{max}}/K_{\text{m}}$ by a factor of 27– to 67-fold (Table I). Assuming that Met-tRNA transformylase makes sequence-specific contact with this base pair, the fact that mutation of C3:G70 to any of the other 3 base pairs has more or less equally detrimental effects would seem to disagree with the notion that proteins contact base-paired regions of RNAs primarily in the minor groove. While the results obtained with the T3:A70 and A3:T70 mutants would be understand-

\textsuperscript{3}C. P. Lee and U. L. RajBhandary, unpublished work.

\textsuperscript{4}M. R. Dyson and C. P. Lee, unpublished data.

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