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Methionine and Formylmethionine Specific tRNAs Coded by Bacteriophage T5

(T5 phage tRNA/RNA·DNA hybridization/reversed-phase chromatography)

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ABSTRACT **By RNA·DNA hybridization, as well as chemical and chromatographic analysis, evidence is provided that the bacteriophage T5 codes for the synthesis of two isoacceptor methionine transfer RNA species, $tRNA^{Met}$ and $tRNA_f^{Met}$. Because of the differences in chromatographic properties of T5 phage and host methionine tRNAs, the phage tRNA species are readily distinguishable.**

$tRNA_f^{Met}$ is widely distributed in nature and is found in bacteria and higher cell organelles such as chloroplasts and mitochondria (1). It is generally believed that this species of tRNA, when charged and formylated, is involved in the active initiation of protein synthesis. In the cytoplasm of eukaryotic cells, a nonformylated Met-$tRNA_f^{Met}$ species has been implicated as the initiator of protein synthesis (2).

The presence of bacteriophage-coded tRNAs in T-even phage-infected *Escherichia coli* is now well documented (3–7). Some time ago, our laboratory also reported that phage-specific tRNAs could be detected in extracts from T5 phage-infected cells (8). It was determined that T5 induces the synthesis of at least 14 different tRNA species, one of these being $tRNA^{Met}$. Since nearly a full complement of tRNA species appeared to be coded by the T5 genome, it seemed reasonable to ask whether a T5-specific $tRNA_f^{Met}$ was also represented. In this report, we wish to describe experiments showing that RNA preparations from T5-infected cells contain two new isoaccepting species of methionine tRNA, $tRNA^{Met}$ and $tRNA_f^{Met}$, both of which are coded by T5 DNA.

METHODS

E. coli F was grown in NCG medium (1 liter contained 8 g of nutrient broth, 10 g of casamino acids, 16 ml of glycerol, and 2.5 mM Tris·HCl, pH 7.8) at 37° in rotary shaker. T5 phage was prepared by infection of *E. coli* F (2.5×10^8 cells per ml) at a multiplicity of 0.1 with vigorous shaking at 37° for 3 hr. Phage was purified essentially as reported elsewhere (9). After CsCl banding and dialysis, phage was stored at 4° in MGM medium (10) supplemented with 1 mM $CaCl_2$.

For the preparation of T5 phage tRNA, 2–4 liters of *E. coli* F (2.5×10^8 cells per ml) were infected at a multiplicity of 10 and shaken for 20 min at 37°, and replication was stopped by the addition of NaN_3 (0.01 M final concentration) and chilling in ice. Cells were collected by centrifugation and stored at −80°. Infected cells for tRNA isolation were also prepared in 80-liter batches by use of a New Brunswick fermenter. Cells infected in the fermenter were prepared essentially as above, except that a multiplicity of 6 was used and a crude infected lysate (free of cell debris) served as the phage source. The tRNA from infected and uninfected cells was isolated by the phenol-extraction method and further purified by chromatography on Sephadex G-100 (3). T5 tRNA, free of host RNA, was prepared by the hybridization of infected-cell tRNA to T5 DNA filters, extensive washing of filters, and recovery of the complexed RNA as previously reported (11).

RNA·DNA hybridization was carried out on membrane filters (type B-6, Schleicher and Schuell, Inc.) impregnated with denatured T5 DNA by the procedure of Gillespie and Spiegelman (12). Radioactive Met-tRNA was annealed with the DNA filters in saline-citrate solutions containing 50% formamide, and radioactivity "fixed" to the filters was determined as reported before (3).

Aminoacyl synthetase was prepared from soluble cell extracts of *E. coli* MRE-600 by the method of Kelmers *et al.* (13). In addition, the enzyme preparation containing 20 mM potassium phosphate (pH 7.5), 2 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 10% glycerol was adsorbed onto DEAE-cellulose, washed extensively with the same buffered solution, and then eluted with a similar solution containing 0.25 M potassium phosphate (pH 6.5). Protein was concentrated by $(NH_4)_2SO_4$ precipitation (70% saturation at 0°), dialyzed against a solution containing 20 mM Tris·HCl (pH 7.5), 20 mM 2-mercaptoethanol, and 10% glycerol (v/v), adjusted to contain 40% glycerol, and stored at −20°. The final protein concentration was approximately 12 mg/ml, with an absorbance ratio of A_{280}/A_{260} of 1.7 or greater. This same enzyme preparation was used for the formylation of Met-$tRNA_f^{Met}$ and for the enzymatic deacylation of Met-tRNA, as described below.

Transfer RNAs were charged with L-[*methyl*-^{3}H]methionine, 3.3 Ci/mmol (Schwarz BioResearch), or L-[^{35}S]methionine, 100–236 Ci/mmol (New England Nuclear), as reported by Kelmers *et al.* (13). *N*-Formylmethionyl (fMet)-tRNA was formed under the same conditions, except that the reaction mixture contained, in addition to the reagents used for Met-tRNA formation, calcium formyltetrahydrofolate (Leucovorin: Lederle) at a concentration of 0.24 mg/ml. The charging mixture contained 40 μl of enzyme per ml, and incubation was at 37° for 15 min. After reaction, 0.2 volumes of 1 M ammonium acetate (pH 5.4) was added and the mixture was extracted three times with phenol. RNA was precipitated with ethanol, suspended in 0.01 M NaCl, and dialyzed in the cold against the same solution.

The deacylation of Met-tRNA was carried out in a 2-ml mixture containing 60 mM imidazole·HCl (pH 7.5), 30 mM $MgCl_2$, 10 mM AMP, 8 mM sodium pyrophosphate (pH 7.0),

Abbreviations: Met-Ado, methionyladenosine; fMet-Ado, formylmethionyladenosine; RPC, reversed-phase chromatography.

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TABLE 1. *Hybridization of [^{35}S]Met-tRNA and f[^{35}S]Met-tRNA from T5-infected cells to T5 DNA*

Source and type of tRNA	cpm added to annealing mixture ($\times 10^{-6}$)	cpm fixed to T5 DNA filters
T5 [^{35}S]Met-tRNA	4.56	2976
		3049
T5 f[^{35}S]Met-tRNA	2.00	2875
		3582
E. coli F	3.35	5
f[^{35}S]Met-tRNA		11

The labeled tRNAs were prepared as described under *Methods*. Their specific activities, in cpm/μg of RNA, were as follows: T5 [^{35}S]Met-tRNA, 8.15×10^4; T5 f[^{35}S]Met-tRNA (subjected to enzymatic deacylation), 2.03×10^4; and *E. coli* F f[^{35}S]Met-tRNA (subjected to enzymatic deacylation), 1.14×10^4. The DNA filters used for hybridization contained 40 μg of T5 DNA. The annealing mixture (0.90 ml) conditions and treatment of the filters after reaction were as described under *Methods*. All annealings were carried out in duplicate.

and 0.8 mg of enzyme (14). After incubation at 37° for 15 min, the reaction was stopped and tRNA was isolated in a manner similar to that described above. Under these conditions, Met-tRNA is deacylated and fMet-tRNA remains intact.

Analysis for Met- and fMet-tRNA was determined from the liberation of methionyladenosine (Met-Ado) and formylmethionyladenosine (fMet-Ado) after RNase hydrolysis by the procedure of Marcker and Sanger (15). Met-Ado and fMet-Ado were separated by paper electrophoresis in 0.5 M acetate–0.3% pyridine (pH 3.5) at 3000 V for 100–110 min at 4°. Strips of paper, 2-cm wide, were cut into 1-cm pieces and placed in counting vials with 0.5 ml of water; 10 ml of toluene–Triton X-100 (3:1) scintillation mixture were added, and the vials were analyzed in a Mark I Nuclear Chicago scintillation spectrometer.

Reversed-phase chromatography (RPC) was accomplished with the RPC-5 system described by Pearson *et al.* (16). Plaskon impregnated with Adogen 464 was purchased from Miles Laboratories. Chromatography of acylated tRNAs was done on Plaskon-packed columns, 0.63 × 24 cm, under 300–350 lb./inch^2 (2.1 to 2.4 MPa) of pressure at room temperature. Columns were equilibrated with a solution containing 0.4 M NaCl, 0.01 M $MgCl_2$, 0.01 M ammonium acetate (pH 4.6), and 2 mM 2-mercaptoethanol; the columns were then loaded with the charged tRNA sample (50–300 μg) and washed with 20 ml of the same equilibration solution. Acylated tRNAs were eluted with a 210-ml linear salt gradient (0.4 M NaCl to 0.7 M NaCl) containing the other constituents of the equilibration solution. The chromatography was completed by fraction number 160 (0.9–1.05 ml per fraction), which took approximately 140 min. Samples from the fractions were either analyzed for radioactivity directly or used for hybridization to T5 DNA.

RESULTS

The first experimental results suggesting that T5-infected cells might contain a phage-specific tRNA$_f^{Met}$ were from RNA·DNA hybridization analysis. Table 1 shows that when highly radioactive [^{35}S]Met-tRNA and f[^{35}S]Met-tRNA from

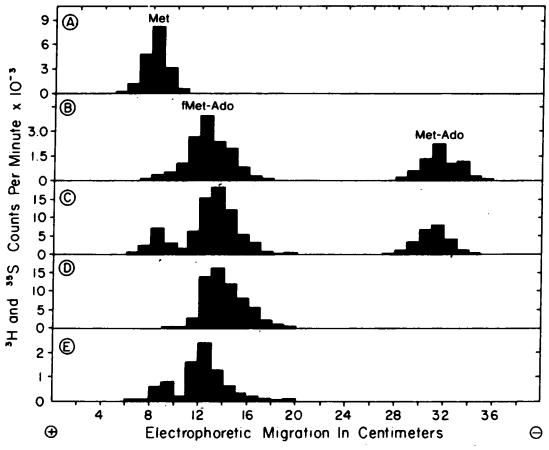

FIG. 1. Electrophoretic analysis of formylated [^{35}S]Met-tRNA taken from T5-infected cells and subjected to RNase treatment. Samples of labeled tRNA were digested with pancreatic RNase and electrophoresed for 100 min at 3000 V at 4°. The compounds were located using as markers [^{3}H]methionine and purified *E. coli* tRNA$_f^{Met}$ in the charged form [^{3}H]Met-tRNA and f[^{3}H]Met-tRNA and are referred to as: Met, methionine; Met-Ado, methionyladenosine; and fMet-Ado, formylmethionyladenosine. The radioactive materials subjected to RNase treatment and paper electrophoresis were as follows: (A) [^{3}H]methionine; (B) purified *E. coli* [^{3}H]Met-tRNA formylated as described in *Methods;* (C) T5-infected cell f[^{35}S]Met-tRNA; (D) T5-infected cell f[^{35}S]Met-tRNA subjected to enzymatic demethionylation; (E) same as (D), but annealed and recovered from T5 DNA hybrid.

T5-infected *E. coli* F were annealed with T5 DNA, both charged tRNA preparations effectively hybridized to T5 DNA. Under similar annealing conditions, f[^{35}S]Met-tRNA prepared from uninfected *E. coli* F tRNA forms no hybrid complex with T5 DNA. This information suggested the presence of a T5-coded tRNA$_f^{Met}$ species. If, however, the T5 f[^{35}S]Met-tRNA preparation (subjected to enzymatic deacylation prior to its use) contained a significant amount of unformylated [^{35}S]Met-tRNA, the meaning of the hybridization data would be ambiguous.

Pancreatic RNase treatment of Met- and fMet-tRNA produces methionyladenosine and formylmethionyladenosine, respectively, as well as other hydrolytic products. Fig. 1A and B illustrates the relative positions of free methionine, fMet-Ado and Met-Ado after paper electrophoresis as described under *Methods*. Both fMet-Ado and Met-Ado are present after RNase treatment of f[^{3}H]Met-tRNA formed from purified *E. coli* tRNA$_f^{Met}$ (a gift from G. D. Novelli), indicating that formylation of Met-tRNA$_f^{Met}$ is incomplete in our enzymatic reaction system. Fig. 1C shows that our preparation of T5-infected cell f[^{35}S]Met-tRNA also contains unformylated [^{35}S]Met-tRNA, since both radioactive fMet-Ado and Met-Ado were found after RNase digestion. However, after T5 f[^{35}S]Met-tRNA is subjected to enzymatic deacylation, only fMet-Ado, but no Met-Ado, is observed in the RNase digest (Fig. 1D). In addition, examination of the radioactive material bound to T5 DNA filters after hybridization with demethionylated T5 f[^{35}S]Met-tRNA, by the same analytical procedure, showed the presence of radioactive fMet-Ado, but not of Met-Ado (Fig. 1E).

When a mixture of [^{3}H]Met-tRNA from uninfected cells and [^{35}S]Met-tRNA from T5-infected cells was chromato-

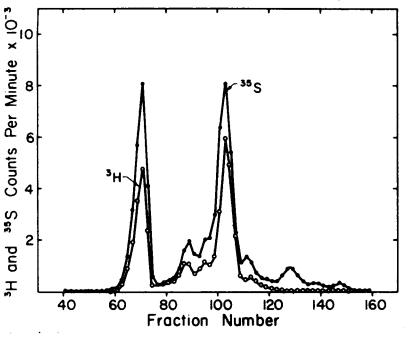

FIG. 2. Reversed-phase cochromatography of *E. coli* F and T5 radioactive Met-tRNA. A mixture of *E. coli* F [^{3}H]Met-tRNA (230,000 cpm total) and T5-infected *E. coli* F [^{35}S]Met-tRNA (241,000 cpm total) was subjected to chromatography as described in *Methods*. Fractions (1 ml each) were collected and 0.7-ml samples were analyzed on two channels of a Mark I Nuclear Chicago scintillation spectrometer, set for double counting. Appropriate corrections were made for ^{35}S spillover into the ^{3}H channel.

graphed in the RPC-5 system, the elution profiles for the two different labels were not identical (Fig. 2). The RPC-5 patterns of both Met-tRNA preparations contain two major peaks of radioactivity. The positions of both peaks are coincident for the two preparations in the early and middle portions of the gradient. However, several small ^{35}S-containing peaks appeared in the latter part of the chromatography (fractions 110–150), which were either noncoincident or unassociated with any tritium radioactivity (uninfected cells).

The reversed-phase chromatography of infected-cell [^{35}S]-Met-tRNA, alone, was repeated. The distribution of radioactivity was determined by direct counting, and selected fractions were subjected to hybridization analysis with T5 DNA. Fig. 3 shows that three peaks of radioactive material

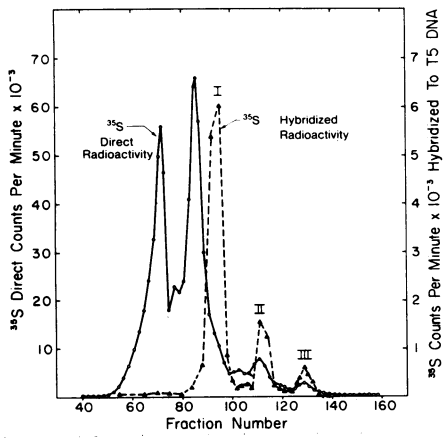

FIG. 3. Reversed-phase chromatography of T5-infected *E. coli* F Met-tRNA and detection of T5-specific isoacceptor Met-tRNAs by hybridization to T5 DNA. T5-infected *E. coli* F [^{35}S]Met-tRNA (24.4 × 10^6 cpm total) was subjected to chromatography as described in *Methods*. Aliquots (0.05 ml) were taken from fractions and analyzed directly for radioactivity; other fraction samples were annealed with T5 DNA to be tested for hybridization as described above. The annealing mixture contained 0.50 ml of fraction sample, 0.50 ml of formamide, and filters impregnated with 40 μg of T5 DNA. Conditions for annealing and treatment of the DNA filters after reaction were as described in *Methods*.

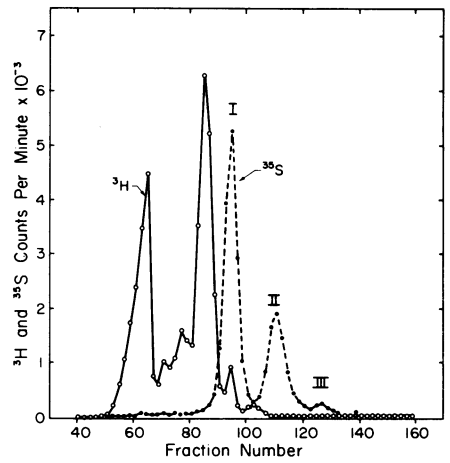

FIG. 4. Reversed-phase cochromatography of *E. coli* F Met-tRNA and purified T5 Met-tRNA. A mixture of uninfected *E. coli* F [^{3}H]Met-tRNA (115,000 cpm total) and T5 [^{35}S]Met-tRNA (105,000 cpm total) (free of host tRNA) was subjected to chromatography in the RPC-5 system. Aliquots (0.70 ml) from the fractions collected were analyzed directly for the detection of both isotopes, as described for Fig. 2.

hybridizable with T5 DNA were located. The first and largest peak of hybridizable [^{35}S]Met-tRNA overlapped slightly with the major ^{35}S region determined by direct counting, whereas the two smaller T5 DNA [^{35}S]Met-tRNA peaks appeared in higher salt fractions coincident with the direct counts.

Fig. 4 shows that when T5 tRNA, purified by hybridization to T5 DNA and free of host tRNA, was charged with [^{35}S]-methionine and cochromatographed with *E. coli* F [^{3}H]Met-tRNA, three ^{35}S peaks also appeared in a region of the gradient similar to that shown in Fig. 3 for the T5 DNA hybridizable peaks. The T5 [^{35}S]Met-tRNA fractions did not coincide with host [^{3}H]Met-tRNA. The radioactive profiles of Fig. 4 also indicate that there was no gross contamination of T5 tRNA with host tRNA, since no significant amount of labeled sulfur was found associated with the two major *E. coli* ^{3}H peaks.

The presence of multiple T5 Met-tRNA peaks, as visualized in the RPC-5 system, suggested the possibility of at least two separate methionine tRNA species coded by T5 DNA, namely, $tRNA_f^{Met}$ and $tRNA^{Met}$. Fractions from peaks I, II, and III of Fig. 4 were separately combined, dialyzed and concentrated, subjected to enzymatic formylation, and then analyzed for fMet-tRNA formation as described under *Methods*. Fig. 5B shows that after enzymatic formylation, 80% of the total radioactivity from an RNase digest of peak I material appeared as free methionine and Met-Ado, indicating that little formylation had occurred during the reaction. On the other hand, Fig. 5C and D indicates that [^{35}S]Met-tRNA from peaks II and III was extensively formylated, since 75% and 53%, respectively, of the radioactivity appeared as fMet-Ado after formylation and RNase hydrolysis.

Fig. 6 shows a comparison of chromatographic profiles of T5 Met-tRNA before and after enzymatic formylation. It may be observed that peak I remains essentially unaltered after formylation; peak II, however, is almost completely removed, while peak III is enhanced by an amount that is proportional to the reduction in peak II. It is evident, therefore, that formylation of T5 Met-tRNA alters the chromato-

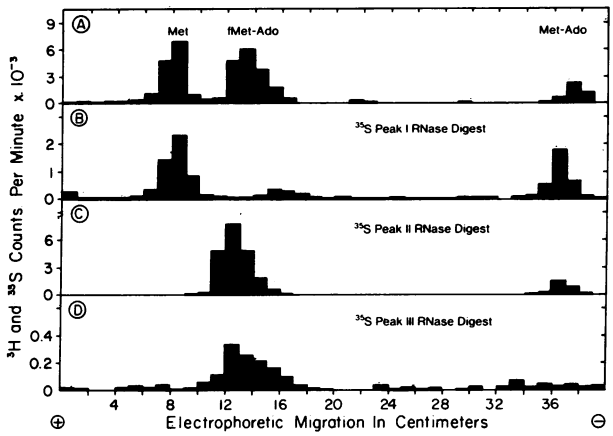

FIG. 5. Electrophoretic analysis of T5 [^{35}S]Met-tRNA in peaks I, II, and III of Fig. 4 after formylation and ribonuclease treatment. Selected fractions from peaks I, II, and III of Fig. 4 were separately combined, dialyzed, concentrated, and subjected to enzymatic formylation under conditions described in *Methods*. After formylation, the individual reaction mixtures were extracted with phenol, and the aqueous phase was dialyzed against H_2O and concentrated by lyophilization. The lyophilized residues were suspended in 0.05 ml of H_2O, incubated with 10 μg of pancreatic RNase at room temperature, and applied directly to paper for electrophoresis (3000 V, 120 min, pH 3.5). The detection of radioactivity after electrophoresis is described under *Methods*. The markers were derived as in Fig. 1 and are represented above in panel (A). Panels (B), (C), and (D) represent the RNase ^{35}S products from peak I, II, and III material, respectively. The percent of the total radioactivity recovered migrating as Met, fMet-Ado, and Met-Ado were: for (B) 49, 11, and 29; for (C) 2, 75, and 12; for (D) 6, 53, and 11.

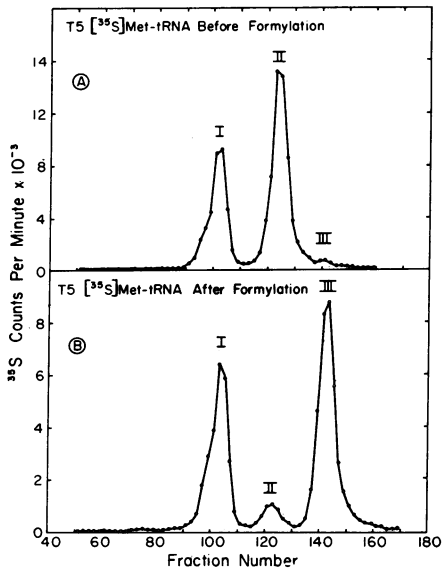

FIG. 6. Reversed phase chromatography of T5 Met-tRNA before and after enzymatic formylation. Nonformylated (A) and formylated (B) T5 [^{35}S]Met-tRNA (245,000 cpm and 99,600 cpm, respectively, free of host tRNA) were chromatographed separately as described in *Methods*. Aliquots were taken from the fractions collected for direct measurement of radioactivity. The activities shown are corrected to 1 ml.

graphic elution position for at least one of the T5 isoacceptor Met-tRNA species.

DISCUSSION

Evidence is provided in this report for the synthesis of two T5-coded methionine tRNA species in T5-infected cells, tRNA$_f^{Met}$ and tRNA^{Met}. The properties of f[^{35}S]Met-tRNA prepared from phage-infected cells and subjected to enzymatic deacylation were the following: (a) No unformylated [^{35}S]-Met-tRNA was detectable; (b) T5 f[^{35}S]Met-tRNA hybridized to T5 DNA; and (c) RNase digestion of the labeled RNA isolated from the T5·DNA hybrid complex released radioactive formylmethionyladenosine. These findings support the presence of a T5-specific tRNA$_f^{Met}$ species in T5-infected extracts.

The presence of multiple T5-specific Met-tRNA species was observed by reversed-phase chromatography. The T5 Met-tRNA isoacceptor species elute at salt concentrations higher than those for host Met-tRNA species. Chromatography of T5 Met-tRNA (free of host tRNA), followed by enzymatic formylation of the RPC-5 fractions, indicates that only two of the three Met-tRNA peaks were formylated (Fig. 4, peaks II and III), while peak I remained resistant to formylation. These results suggest that peak I represents T5 tRNA^{Met} and peaks II and III represent T5 tRNA$_f^{Met}$. This conclusion is consistent with the finding shown in Fig. 6 that chromatography of formylated T5 [^{35}S]Met-tRNA results in no relative change in the position or amount of peak I radioactivity, but produces a significant displacement of peak II ^{35}S to the peak III position. It is apparent that the gain of peak III radioactivity after formylation is almost exactly equivalent to the radioactivity lost in peak II. We as well as others (17) have observed that formylation of purified Met-tRNA$_f^{Met}$ shifts its RPC-5 elution position to a slightly higher salt concentration. The small amount of peak III radioactivity seen when unformylated T5 [^{35}S]-Met-tRNA is used is most probably fMet-tRNA. We are reasonably certain that our enzyme preparation contains trace quantities of 10-formyltetrahydrofolic acid, the active formyl donor. Hence, peak III probably derives from the enzymatic formylation of peak II Met-tRNA$_f^{Met}$ in our enzyme charging system and does not represent a separate Met-tRNA species. We, therefore, conclude that there are only two T5 isoacceptor methionine tRNA species, tRNA^{Met} (peak I) and tRNA$_f^{Met}$ (peak II).

It is quite apparent that there are large differences in the relative amounts of the two isoacceptor Met-tRNA species observed in Figs. 3 and 4 compared with that of Fig. 6. The most probable explanation for these quantitative differences resides in the type of infected cells used throughout the course of this work. In the early experiments T5-infected cells were prepared in 2- to 4-liter batches in a rotary shaker, whereas the later T5 tRNA preparations were isolated from infected cells grown in a New Brunswick 100-liter fermenter, under slightly different conditions. It is very likely that phage replication kinetics were not identical for these two different growth procedures, and that timing differences in the infectious process are reflected in the quantitative difference between the two methionine tRNA species synthesized and subsequently isolated.

The replication of T4 phage in *E. coli* B appears to be independent of T4 phage tRNA synthesis (18). Unpublished work in this laboratory also indicates that the absence of multiple tRNA genes in several different T5 phage deletion mutants does not impair replication. Nevertheless, the detection of T5-coded tRNA^{Met} and tRNA$_f^{Met}$ species, the latter

known to function as an initiator of protein synthesis, raises the question what, if any, is its function in T5 phage replication. Preliminary results indicate that T5 fMet-tRNA does recognize the initiation codon triplet, AUG; whether or not T5 $tRNA_f^{Met}$ is used in some specific manner for the synthesis of phage proteins, however, remains to be demonstrated.

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