Initiator Methionine Transfer Ribonucleic Acid from Wheat Embryo

PURIFICATION, PROPERTIES, AND PARTIAL NUCLEOTIDE SEQUENCES*

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SUMMARY

Initiator methionine tRNA (tRNA\textsubscript{Met}) species from wheat embryo has been purified by fractionation on a benzoylated DEAE-cellulose column followed by chromatography on a DEAE-Sephadex column. Transfer RNA\textsubscript{Met} can be aminoacylated by the homologous enzyme as well as by Escherichia coli aminoacyl-tRNA synthetase. Unlike other eukaryotic cytoplasmic initiator tRNAs, the wheat embryo initiator tRNA cannot be formylated by E. coli transformylase. Amino acid incorporation studies in vitro using a wheat embryo ribosomal system and poly[r(A-U-G)] as messenger show that at low Mg\textsuperscript{2+} concentration polymethionine synthesis from Met-tRNA\textsubscript{Met} (chain propagator species) is dependent on the presence of Met-tRNA\textsubscript{Met}. Met-tRNA\textsubscript{Met} cannot insert methionine internally in the homologous system but in a heterologous E. coli or rabbit reticulocyte ribosomal system Met-tRNA\textsubscript{Met} appears to donate methionine into internal positions of the polypeptide chain.

Analysis of oligonucleotides produced by digestion of tRNA\textsubscript{Met} with ribonuclease T1 shows that the 5'- and 3'-terminal nucleotide sequences are 5'-U-C-A-G- and A-U-A-C-A-A-OH, respectively. As in other eukaryotic cytoplasmic initiator tRNAs the tetranucleotide sequence T-C-A-G(A) always present in loop IV of other tRNAs is absent. The tentative sequence of loop IV in the case of wheat embryo tRNA\textsubscript{Met} is 5'-A-U-C-G-uA-A-A. Except for the presence of U* instead of U, this sequence is identical with those found in yeast, mouse myeloma, rabbit liver, and sheep mammary gland initiator tRNAs and different from that of E. coli initiator tRNA.

Initiation of protein synthesis in bacteria and eukaryotic organelles, namely mitochondria and chloroplasts, involves a formylatable species of methionine tRNA (1, 2). Only one of the two major species of methionine tRNA found in eukaryotic sources such as yeast or mammalian can be both aminoacylated and formylated by the Escherichia coli enzymes (2, 3). Studies involving both in vivo and in vitro protein synthesis in eukaryotic systems show that this formylatable species of methionine tRNA, tRNA\textsubscript{Met}

1 The abbreviations used are: tRNA\textsubscript{Met}, formylatable Escherichia coli initiator methionine tRNA; tRNA\textsubscript{Met}*, initiator methionine tRNA from yeast or mammalian tissues which can be formylated by E. coli formylase; tRNA\textsubscript{Met}, nonformylatable and noninitiator methionine tRNA from E. coli, yeast, or mammalian tissues; tRNA\textsubscript{Met}, initiator methionine tRNA from wheat embryo which cannot be formylated by E. coli formylase; tRNA\textsubscript{Met}, noninitiator and nonformylatable methionine tRNA from wheat embryo; poly[d(A-T-G)-d(C-A-T)], double-stranded deoxyribonucleotide polymer which contains alternating units of adenylate, thymidylate, and guanylate in one strand and cytidylate, adeny late, and thymidylate in the complementary strand; poly[r(A-U-G)], single-stranded ribonucleotide polymer which contains alternating units of adeny late, uridy late, and guanylate; BD-cellulose, benzoylated DEAE-cellulose; BMV RNA, single-stranded RNA isolated from the plant virus, bromegrass mosaic virus; RPC-5, reverse phase chromatographic system 5; EF, elongation factor; AUG, ribonucleotide ApUpG; GUC, ribonucleotide OpUpG; EF-1 and EF-2, elongation factors 1 and 2, respectively.

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elongation factors and GTP (9), although this ternary complex is unstable and does not perform the usual initiation methionyl-tRNA to the ribosomes (10). Several groups have, however, reported on internal incorporations of methionine from initiator tRNA<sup>Met</sup> species in both homologous and heterologous ribosomal systems (11-13).

Elucidation of the primary sequence of yeast initiator tRNA<sup>Met</sup> (14) revealed two unique features which distinguished that RNA from all other tRNAs, including the E. coli initiator tRNA. These were: (a) absence of the sequence G-T-Ψ-C-G(A), present in loop IV of all tRNAs active in protein synthesis, and its replacement by G-A-U-C-G; and (b) the presence of A as the last nucleotide. Subsequent studies have shown that these features are common also to mammalian cytoplasmic initiator tRNAs, and that the sequence of loop IV in all these tRNAs is A-U-C-G-m'A-A-A (15-18).

In an effort to understand further the relationship between the structure and the function of eukaryotic initiator tRNAs, we have been studying wheat embryo initiator tRNA<sup>Met</sup> species. Here we report the purification, properties and partial sequences of wheat embryo tRNA<sup>Met</sup>.

**Materials and Methods**

Uniformly labeled L-<sup>3</sup>H-methionine (specific activity, 5 x 8 x 3 Ci per mmole) and L-<sup>35</sup>S-methionine (specific activity, 12 to 20.3 Ci per mmole) were supplied by Amersham-Searle. All radioactive compounds were checked by paper chromatography before use. N-Formylmethionine was obtained from Sigma. Methionine sulfoxide and N-formylmethionine sulfoxide were prepared as described earlier (19). Counting and scanning of radioactivity, paper chromatography, and paper electrophoresis were performed as described earlier (20). Unfractionated wheat embryo tRNA was prepared from commercial wheat embryo (21).

**Column Chromatography**—Chromatography of tRNA samples on BD-cellulose was as described (20). DEAE-Sephadex A-50 beads (Pharmacia) were processed according to Nishimura et al. (22). The following solvent systems were used for both paper and thin layer chromatography: Solvent A, n-propyl alcohol-concentrated NH<sub>4</sub>OH-water, 70:15:15 (v/v); Solvent B, n-butanol-acetic acid-concentrated HCl-water, 70:15:15 (v/v); and Solvent C, isobutyric acid-concentrated NH<sub>4</sub>OH-water 66:1:33 (v/v) at pH 4.3. Solvent D, isobutyric acid-concentrated NH<sub>4</sub>OH-water 2:1 (v/v) at pH 3.7. Paper chromatography was on Whatman No. 1 paper. The following solvents were used: Solvent E, isopropanol-concentrated NH<sub>4</sub>OH-water, 7:1:2 (v/v); Solvent F, isobutyric acid-concentrated NH<sub>4</sub>OH-water, 7:1:2 (v/v). Two-dimensional thin layer chromatography was carried out on glass plates (Brinkmann Instruments, Inc., Celplate-22) coated with cellulose. Two different solvent systems were used for this purpose. These were: (a) Solvent A in the first dimension followed by Solvent B in the second dimension or (b) Solvent C in the first dimension followed by Solvent D in the second dimension.

**5'-<sup>32</sup>P Labeling of tRNA Fragments**—Wheat embryo tRNA<sup>Met</sup> was treated with a mixture of T<sub>r</sub>-RNase and E. coli alkaline phosphatase or pancreatic RNase and E. coli alkaline phosphatase. The mixture of enzymes used was subsequently labeled with <sup>32</sup>P at the 5' end using T<sub>r</sub> polyribonucleotide kinase and [γ-<sup>32</sup>P]ATP. The procedure used was identical with that described previously (15, 18) except that the amount of E. coli alkaline phosphatase present was 0.01 unit and not 5 units. The <sup>32</sup>P-labeled fragments were then separated by two-dimensional electrophoresis (24).

Individual oligonucleotides isolated from column fractions and further purified by paper chromatography were labeled with <sup>31</sup>P at the 5' end as above except that the first step of incubation contained only E. coli alkaline phosphatase and no T<sub>r</sub>-RNase or pancreatic RNase. The 5'-<sup>31</sup>P-labeled oligonucleotides were then separated by electrophoresis on DEAE-cellulose paper in 7% formic acid at 8 volts per cm for 15 to 20 hours.

**End Group and Sequence Analysis of 5'-<sup>31</sup>P-Labeled Oligonucleotides**—End group analysis was carried out as described before using T<sub>r</sub>-RNase (15). The sequence of oligonucleotides was determined by partial digestion with snake venom phosphodiesterase followed by electrophoresis on DEAE-cellulose paper at pH 3.5 and by comparing the M values of the various partial digestion products (25). The M values alone cannot be used for unambiguously establishing the sequence of those oligonucleotides which migrate faster than the blue dye marker. In such cases, parallel partial snake venom phosphodiesterase digests carried out on 5'-<sup>31</sup>P-labeled oligonucleotides of known sequence provided the necessary markers for comparing the electrophoretic mobilities.

**Oligo- and Polynucleotides**—Ribonucleotide AcPuG was synthesized as described by Miles. Poly[d(A-T-G)] was obtained from the reaction of DNA-dependent RNA polymerase with poly[d(A-T-G)]-poly[d(C-A-T)] as templates, as described earlier (20). RNA from the plant virus, bromegrass mosaic virus, was isolated by the procedure of Stubbs and Kaesberg (29).

**Enzymes**—Pancreatic DNase, electrophoretically pure and free of RNase, pancreatic RNase, snake venom phosphodiesterase, and alkaline phosphatase were obtained from Worthington; T<sub>r</sub>-RNase and T<sub>r</sub>-RNase (Sankyo) were from Calbiochem and rabbit muscle pyruvate kinase was from either Sigma or Calbiochem. A partially purified wheat embryo aminoacyl-tRNA synthetase free from tRNA was prepared according to Nishimura et al. (22). Sources of other enzymes used in this work have been described previously (15, 18).

Crude wheat embryo synthetase was prepared as described earlier (20). This enzyme preparation contained very little tRNA. Alternatively, the dialyzed fraction was applied on a DEAE-cellulose column which had been equilibrated with 0.05 m Tris-HCl (pH 7.5), 50 mM MgCl<sub>2</sub> and 0.01 M mercaptoethanol. The mixture of synthetases was eluted with 0.05 m Tris-HCl, pH 7.5, containing 0.01 M 2-mercaptoethanol and 0.25 M KCl. Proteins were concentrated by precipitation with 75% saturation of ammonium sulfate and dialyzed as described earlier (20). This preparation was stored in liquid nitrogen.

**Preparation of Wheat Embryo Methionyl-tRNA**—The incubation mixture contained 0.1 m Tris-HCl (pH 7.1), 5 mM MgCl<sub>2</sub>, 4 mM ATP, 0.2 mM CTP, 10 mM 2-mercaptoethanol, 20 mM KCl, 1 mM EDTA, 200 μg of bovine serum albumin, 0.02 n mole of [L-<sup>35</sup>S]methionine (specific activity, 2000 to 5000 mCi per mmole), 2 to 4 A<sub>260</sub> units of purified wheat embryo tRNA<sup>Met</sup>, and 2 to 3 mg of wheat embryo aminoacyl-tRNA synthetase, which have been passed through a BD-cellulose column, in 1 ml of reaction mixture. After incubation at 37°C for 15 min, the radioactive aminoacyl-tRNA was recovered as described above (20).

**Assay for Amino Acid Acceptor Activity**—The reaction mixture was identical with that described under preparation of aminoacyl-tRNA except that the final volume was 0.1 ml and after 10 min at 37°C, a 0.05 ml aliquot was withdrawn. Acid-insoluble radioactivity was measured on filter paper as described previously (20).

When E. coli aminoacyl-tRNA synthetase was used as the enzyme the reaction mixture was the same as described for wheat embryo enzyme except that 0.1 m sodium cacodylate (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM ATP, and 0.5 mg of E. coli enzyme protein per ml were present. Ribosomes and Supersupernatant Factors—A ribosomal system for protein synthesis from wheat embryo was obtained as described earlier (20). Ribosomes, supernatant factors, and initiation factors were isolated from E. coli MRE 600 by procedures described previously (20). Previously incubated reticulocyte ribosomes were prepared as described by Gupta et al. (27).

**Assay for In Vitro Amino Acid Incorporation**—As in previous work (20) a two-step procedure was used. Stage I involved the synthesis of a polynucleotides by DNA like polymers as template for RNA polymerase. DNA dependent RNA polymerase with poly[d(A-T-G)]-poly[d(C-A-T)] was used without isolation for polypeptide synthesis. In Stage II, the final reaction mixture contained 0.05 m Tris-Cl (pH 7.5), 0.05 mM KCl, 0.5 mM diithiothreitol, 2 mM ATP, 20 μM CTP, 5 mM phosphoenolpyruvate, 0.3 mM MnCl<sub>2</sub>, 4 mM 2-mercaptoethanol, 1.25 mM MgCl<sub>2</sub>, necessary amounts of magnesium acetate to make up the final Mg<sup>2+</sup> concentration as indicated, 30 to 40 A<sub>260</sub> units of wheat
embryo ribosomes per ml. 0.6 mg of wheat embryo supernatant fraction per ml, radioactive methionyl-tRNAs as described, the ribopolyenucleotide template synthesized, the DNA template, ribonucleotide 5'-triphosphates, and RNA polymerase as introduced from Stage I. The reaction was carried out at 30\(^\circ\) and aliquots (0.02 to 0.05 ml) were withdrawn at the indicated interval. The polypeptide formed was assayed as hot 5% trichloroacetic acid-insoluble product on filter paper discs as described earlier (20). The conditions for the Stage II reaction using \(E. coli\) system (23) and reticulocyte ribosomes (27) have been described.

**Assay of Aminoacyl-tRNA Binding to Ribosomes**—The general procedure of Nirenberg and Leder (28) was followed. The incubation mixture (0.05 ml) contained 0.05 M Tris-HCl (pH 7.1), 0.1 M NH\(_4\)Cl, 0.01 M dithiothreitol, 1.4 to 2.0 \(A_{260}\) units of wheat embryo ribosomes, 0.05 \(A_{260}\) unit of AUG, 0.002 to 0.03 M magnesium acetate, and \(14\)S]methionyl-tRNAs as indicated. Incubation was at 25\(^\circ\) for 20 min. The reaction mixture was diluted with cold 0.05 M NH\(_4\)Cl and 0.002 to 0.03 M magnesium acetate and immediately filtered through nitrocellulose filter paper (Millipore, type HA, pore size 0.45 \(\mu\)m), washed, dried, and counted (20).

**NH\(_2\)-Terminal Amino Acid Determination of Polypeptide Synthesized**—A microscale Edman degradation method described by Gray and Hartley (29) was used. Radioactive polymethionine was synthesized, isolated, oxidized with performic acid, and the phenylthiohydantoin derivative of NH\(_2\)-terminal methionine was isolated by the procedure described earlier by Ghosh et al. (19).

**RESULTS**

**Separation and Properties of Two Methionine tRNA Species from Wheat Embryo**

Crude wheat embryo tRNA was fractionated on a column of benzoylated DEAE-cellulose. The results are shown in Fig. 1. Two clearly separated peaks of methionine acceptor activity were observed when the fractions were assayed with wheat embryo aminocetyl-tRNA synthetase. The two major peaks were designated as tRNA\(_{\text{Met}}\) (Peak I) and tRNA\(_{\text{Met}2}\) (Peak II) in order of their emergence from the column. The fractions were also tested for aminoacylation with methionine using \(E. coli\) aminocetyl-

**tRNA synthetase, only Peak I (tRNA\(_{\text{Met}}\)) could be aminoacylated.

Previous reports showed that wheat embryo tRNA\(_{\text{Met}}\) could not be formylated by \(E. coli\) transformylase (4-6). Attempts were made to formylate tRNA\(_{\text{Met}}\) under various conditions of pH, Mg\(^{2+}\) ion concentrations, KCl concentrations and in the presence of up to 25% of dimethylsulfoxide. In none of these cases was any formylation or Met-tRNA observed. Transfer RNA\(_{\text{Met}}\), thus, appeared to be different from similar species of eukaryotic methionine tRNA (tRNA\(_{\text{Met}}\)) which can be both charged and formylated with \(E. coli\) enzymes (2, 3).

Fractionation of crude tRNA on DEAE-Sephadex column also separated two methionine tRNAs. However, the order of elution of the two tRNAs in this case was reversed (not shown).

**Further Purification of tRNA\(_{\text{Met}}\)**

Partially purified tRNA\(_{\text{Met}}\) obtained from the BD-cellulose column was further purified on a DEAE-Sephadex column (Fig. 2). The methionine acceptor activity was separated from the major ultraviolet-absorbing peak. The specific activities of the methionine tRNA fractions were fairly constant, 1700 to 1800 pmoles per \(A_{260}\) unit of tRNA in the peak tubes. These were pooled and the tRNA was recovered by precipitation. The recovered tRNA\(_{\text{Met}}\) samples had a maximum specific activity of 1810 pmoles per \(A_{260}\) unit. The purified tRNA\(_{\text{Met}}\) probably consisted of a single isoacceptor species. Thus, upon further chromatography on a BD-cellulose column in the presence of 1 mM EDTA or a reverse phase column (RPC-5) only one methionine acceptor peak which coincided with the ultraviolet absorbing peak was obtained.

**Role of tRNA\(_{\text{Met}}\) and tRNA\(_{\text{Met}2}\) in Protein Synthesis**

**Ribosomal Binding Specificity of tRNA\(_{\text{Met}}\) and tRNA\(_{\text{Met}2}\)**—The bacterial initiator tRNA\(_{\text{Met}}\) can bind to ribosomes at a lower Mg\(^{2+}\) ion concentration in the presence of the triplets AUG, GUG, or RNA from bacteriophage f2 (30, 31). The binding of Met-tRNA\(_{\text{Met}}\) and Met-tRNA\(_{\text{Met}2}\) to wheat embryo ribosomes in the presence of AUG triplet and at different concentrations of Mg\(^{2+}\) ions is shown in Fig. 3. It can be seen that 70% of input
Met-tRNA\textsubscript{Met} can bind to ribosome-AUG complex at 4 mM Mg\textsuperscript{2+} concentration. This binding at 4 mM Mg\textsuperscript{2+} is absolutely dependent upon the presence of AUG. At 10 mM Mg\textsuperscript{2+}, 90% of the input Met-tRNA\textsubscript{Met} can be bound, although at this high concentration of Mg\textsuperscript{2+}, substantial binding is observed even in the absence of AUG. Methionyl-tRNA\textsubscript{Met}, in contrast, binds to the ribosome-AUG complex only at a higher Mg\textsuperscript{2+} ion concentration. Thus at Mg\textsuperscript{2+} ion concentration of 10 mM only 20% of the added Met-tRNA\textsubscript{Met} is bound. The amount of binding increases with increasing Mg\textsuperscript{2+} ion concentrations. Addition of wheat embryo ribosomal extracts or a partially purified fraction containing EF-1 and EF-2 does not stimulate the binding of any of the methionyl-tRNAs (not shown). The observed preferential binding of Met-tRNA\textsubscript{Met} to wheat ribosomes in presence of AUG and at a lower rate Mg\textsuperscript{2+} ion concentration is in agreement with the previous reports of Monasterio et al. (32).

Further evidence that tRNA\textsubscript{Met} represents the initiator species is also derived from its selective binding to wheat embryo ribosomes directed by the BMV RNA at 10 mM Mg\textsuperscript{2+} (Table I). It is now well established that the eukaryotic methionyl-tRNA species chargeable and formylatable by \textit{E. coli} extracts (tRNA\textsubscript{Met}) can initiate protein synthesis in a cell-free eukaryotic system (2, 3). The methionine residue is transferred from Met-tRNA\textsubscript{Met} or Met-tRNA\textsubscript{Met} into polymethionine as directed by poly[r(A-U-G)]. The reaction mixture in the first and second stages was as described under "Materials and Methods." The amount of poly[r(A-U-G)] present was 67 nmoles per ml, that of [\textsuperscript{35}S]Met-tRNA\textsubscript{Met} or [\textsuperscript{35}S]Met-tRNA\textsubscript{Met} was 306 pmoles per ml or 292 pmoles per ml, respectively. The control tube (poly AUG) contained either [\textsuperscript{35}S]Met-tRNA\textsubscript{Met} or [\textsuperscript{35}S]Met-tRNA\textsubscript{Met}, but contained no DNA template in the first stage. The two Mg\textsuperscript{2+} concentrations used (11 and 16 mM) are indicated at the top of the figures. The reaction mixture (0.1 ml) was incubated at 30° and aliquots (0.05 ml) were withdrawn to assay the hot trichloroacetic acid-insoluble material.

**Incorporation of Methionine from tRNA\textsubscript{Met} and tRNA\textsubscript{Met}\textsuperscript{35}S.** It is now well established that the eukaryotic methionyl-tRNA species chargeable and formylatable by \textit{E. coli} extracts (tRNA\textsubscript{Met}) can initiate protein synthesis in a cell-free eukaryotic system (2, 3). The methionine residue is transferred from Met-tRNA\textsubscript{Met} or Met-tRNA\textsubscript{Met} into polymethionine as directed by poly[r(A-U-G)]. The reaction mixture in the first and second stages was as described under "Materials and Methods." The amount of poly[r(A-U-G)] present was 67 nmoles per ml, that of [\textsuperscript{35}S]Met-tRNA\textsubscript{Met} or [\textsuperscript{35}S]Met-tRNA\textsubscript{Met} was 306 pmoles per ml or 292 pmoles per ml, respectively. The control tube (poly AUG) contained either [\textsuperscript{35}S]Met-tRNA\textsubscript{Met} or [\textsuperscript{35}S]Met-tRNA\textsubscript{Met}, but contained no DNA template in the first stage. The two Mg\textsuperscript{2+} concentrations used (11 and 16 mM) are indicated at the top of the figures. The reaction mixture (0.1 ml) was incubated at 30° and aliquots (0.05 ml) were withdrawn to assay the hot trichloroacetic acid-insoluble material.

**Table I.**

<table>
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<th>Conditions</th>
<th>[\textsuperscript{35}S]Met-tRNA Bound</th>
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<td>Met-tRNA\textsubscript{Met}</td>
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<tr>
<td>BMV RNA</td>
<td>1025</td>
</tr>
<tr>
<td>BMV RNA (12 A\textsubscript{260} units/ml)</td>
<td>6405</td>
</tr>
<tr>
<td>BMV RNA (24 A\textsubscript{260} units/ml)</td>
<td>9653</td>
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</table>

Met-tRNA\textsubscript{Met} can bind to ribosome-AUG complex at 4 mM Mg\textsuperscript{2+} concentration. This binding at 4 mM Mg\textsuperscript{2+} is absolutely dependent upon the presence of AUG. At 10 mM Mg\textsuperscript{2+}, 90% of the input Met-tRNA\textsubscript{Met} can be bound, although at this high concentration of Mg\textsuperscript{2+}, substantial binding is observed even in the absence of AUG. Methionyl-tRNA\textsubscript{Met}, in contrast, binds to the ribosome-AUG complex only at a higher Mg\textsuperscript{2+} ion concentration. Thus at Mg\textsuperscript{2+} ion concentration of 10 mM only 20% of the added Met-tRNA\textsubscript{Met} is bound. The amount of binding increases with increasing Mg\textsuperscript{2+} ion concentrations. Addition of wheat embryo ribosomal extracts or a partially purified fraction containing EF-1 and EF-2 does not stimulate the binding of any of the methionyl-tRNAs (not shown). The observed preferential binding of Met-tRNA\textsubscript{Met} to wheat ribosomes in presence of AUG and at a lower rate Mg\textsuperscript{2+} ion concentration is in agreement with the previous reports of Monasterio et al. (32).

Further evidence that tRNA\textsubscript{Met} represents the initiator species is also derived from its selective binding to wheat embryo ribosomes directed by the BMV RNA at 10 mM Mg\textsuperscript{2+} (Table I). It is seen that BMV RNA stimulates the binding of Met-tRNA\textsubscript{Met} by 6- to 9-fold. No binding of Met-tRNA\textsubscript{Met} was observed under identical conditions. The results are essentially similar to the binding of fMet-tRNA\textsubscript{Met} and Met-tRNA\textsubscript{Met} to \textit{E. coli} ribosomes directed by AUG or f2 RNA (30, 31).
**protein synthesizing system.** Fig. 6 shows the results of a study which compares the kinetics of polymethionine synthesis from figures. 

| Conditions of polymethionine synthesis at 4 mM Mg\(^{2+}\) concentration in the presence of both [\(^{35}\)S]Met-tRNA\(_{\text{Met}}\) and [\(^{3}H\)Met-tRNA\(_{\text{Met}}\) as directed by poly[r(A-U-G)] and using cell-free protein synthesizing systems derived from E. coli, rabbit reticulocytes, or wheat embryo. It is seen that approximately 20% of the input methionine is polymerized in the bacterial system. Several lines of evidence suggest that this incorporation in the bacterial system utilizes the Met-tRNA\(_{\text{Met}}\) directly. Thus (a) addition of an excess of nonradioactive methionine does not decrease the incorporation of [\(^{35}\)S]methionine indicating that there is no transfer of [\(^{35}\)S]methionine from tRNA\(_{\text{Met}}\) to contaminating tRNA\(_{\text{Met}}\), which is subsequently incorporated internally. (b) [\(^{3}H\)Methionine added to the reaction mixture is not incorporated into polymethionine. This suggests that the E. coli system used is free of endogenous tRNA.

In the rabbit reticulocyte system, however, only about 4% of the input methionine from Met-tRNA\(_{\text{Met}}\) is polymerized. The rabbit reticulocyte system used could contain endogenous tRNA and, therefore, the observed internal incorporation was possibly transfer of methionine to contaminating reticulocyte tRNA\(_{\text{Met}}\).

Evidence that the observed polymethionine synthesis in E. coli system using wheat embryo Met-tRNA\(_{\text{Met}}\) is not an artifact caused solely by the use of high Mg\(^{2+}\) concentration is provided on Fig. 7. It can be seen that substantial incorporation occurs even at 6 mM Mg\(^{2+}\). Optimum incorporation at 4 or 6 mM Mg\(^{2+}\) concentration is obtained in the presence of both initiation factors and fMet-tRNA\(_{\text{Met}}\). Presence of initiation factors alone, however, shows significant incorporation. No stimulation of polymethionine synthesis by fMet-tRNA\(_{\text{Met}}\) is observed in the absence of initiation factors (not shown).

**Effect of Formylation of Wheat Embryo tRNA\(_{\text{Met}}\) and tRNA\(_{\text{Met}}\) on Protein Synthesis**

Formylation of initiator tRNA\(_{\text{Met}}\) is obligatory for efficient recognition by initiation factors in an E. coli system (33). The inability of E. coli formylase to formylate Met-tRNA\(_{\text{Met}}\) and
The conditions of the reactions were the same as described in Fig. 3 except that the incubation mixture contained 1.9 A260 units of E. coli ribosomes (washed 4 times) and 20 ug of unfracionated E. coli initiation factors free from nucleic acids. The amounts of fMet-tRNA^Met, Met-tRNA^Met, fMet-tRNA^I Met and fMet-tRNA^z Met present were 21,900, 27,400, 8,200, and 10,150 cpm, respectively.

**TABLE III**

**Recognition of formylated methionyl-tRNAs by Escherichia coli initiation factors**

The conditions of the reactions were the same as described in Fig. 3 except that the incubation mixture contained 1.9 A260 units of E. coli ribosomes (washed 4 times) and 20 ug of unfracionated E. coli initiation factors free from nucleic acids. The amounts of fMet-tRNA^Met, Met-tRNA^Met, fMet-tRNA^I Met and fMet-tRNA^z Met present were 21,900, 27,400, 8,200, and 10,150 cpm, respectively.

![Graph](http://www.jbc.org/)

**Fig. 7.** Polymethionine synthesis from Met-tRNA^Met in an *Escherichia coli* system at different Mg^2+ concentrations. Conditions were the same as described in Fig. 6. ●— ●, —poly-

![Graph](http://www.jbc.org/)

**Fig. 8.** Effect of formylated methionyl-tRNAs on the incorporation of methionine from Met-tRNA^Met of *Escherichia coli* system at 4 mM Mg^2+ concentration. Conditions were the same as described in Fig. 7 except that 2.5 X 10^6 cpm of [3H]Met-tRNA^Met and as indicated 3.5 X 10^6 cpm of [3H]Met-tRNA^Met or 3.8 X 10^6 cpm of [3H]Met-tRNA^Met or 4.1 X 10^6 cpm of [3H]Met-tRNA^Met were present in 0.1 ml of reaction mixture, and 0.02-ml aliquots were withdrawn to determine the [3H]methionine polymerized.

the internal recognition of tRNA^Met in the E. coli system thus raises the question of recognition of tRNA^Met by E. coli initiation factors. We have earlier shown that tRNA^Met and tRNA^Met are not recognized by E. coli initiation factors (6). We, therefore, formylated both the wheat embryo methionyl-tRNA^Met samples by a mild chemical formylation procedure using N-hydroxysuccinimide (33). The chemically formylated Met-tRNA samples were then tested for AUG-directed binding to E. coli ribosomes in the presence of initiation factors. Results presented in Table III showed that a wheat embryo Met-tRNA^Met is also similar to that of the other eukaryotic cytoplasmic initiator tRNAs studied to date (14–18) lack the common sequence T-Ψ-C-G(A)- present in loop IV of other tRNAs active in protein synthesis. In the yeast initiator tRNA, whose total sequence is known T-Ψ-C-G- is replaced by A-U-C-G. (14). Since T1-RNase digests of the other eukaryotic initiator tRNAs also yielded A-U-C-G and no T-Ψ-C-G, it was suggested that replacement of T-Ψ-C-G by A-U-C-G or a closely related sequence might be a phenomenon common to all eukaryotic cytoplasmic initiator tRNAs (15). This has been subsequently confirmed, and it has, in fact, been shown that the entire sequence of loop IV of the four eukaryotic initiator tRNAs is identical and is A-U-C-G-m'A-A-A-A. (17, 18). From the results to be described below, we suggest that the sequence of loop IV of wheat embryo tRNA^Met is also similar to that of the other eukaryotic cytoplasmic initiator tRNAs except that it contains U* (a modified uridine) in place of U. Thus (a) fingerprints of T1-RNase digests of this tRNA (see “Materials and Methods”) show that the spot corresponding to [3H]T1-RNase-C-G is almost totally absent in this tRNA (Fig. 9). The presence instead of a spot (Spot 8) previously characterized to be [3H]A-U-C-G (15), along with the finding that other eukaryotic initiator tRNAs contain A-U-C-G in place of T-Ψ-C-G, suggests that A-U-C-G is located in loop IV of the wheat embryo tRNA^Met. Experiments to establish this conclusively are now under way. (b) Fingerprints of pancreatic RNase digests of this tRNA (Fig. 10) yielded a spot (Spot 7) which is also present in similar digests of all the eukaryotic cytoplasmic initiator tRNAs (15, 35, 36). This spot was characterized to be [3H]G-m'A-A-A-C. Since the
Fig. 9 (upper left). Autoradiogram of 5'-32P-labeled oligonucleotides obtained upon T1-RNase digestion of wheat embryo initiator tRNA (see "Materials and Methods"). B, blue dye marker.

Fig. 10 (right). Autoradiogram of 5'-32P-labeled oligonucleotides obtained upon pancreatic RNase digestion of wheat embryo initiator tRNA (see "Materials and Methods"). B, blue dye marker.

Fig. 11 (lower left). Autoradiogram of partial snake venom phosphodiesterase digests on [32P]Gm'A-A-A-C obtained from wheat embryo initiator tRNA (right) and rabbit liver cytoplasmic initiator tRNA (left). Electrophoresis was on DEAE-cellulose paper in 0.05 M pyridine acetate, pH 3.5, and at 14 volts per cm for 16 hours. Band a contains pG as one of the components as shown by the identical mobility of an ultraviolet absorbance marker of pG added to each of these digests. B, blue dye marker. O, origin; the numbers at the origin represent minutes of incubation at room temperature with snake venom phosphodiesterase.
modified nucleoside m^1A, when present in a tRNA, is located almost exclusively in loop IV of a tRNA (37), this suggests that the [7-P]G-m^1A-A-C is present as part of loop IV of wheat embryo tRNA^Met.

The sequence of [7-P]G-m^1A-A-C obtained above was established as follows. (a) Digestion with T1-RNase followed by thin layer chromatography in Solvent B showed that all the [7-P] radioactivity was present in [7-P]Gp (15). (b) The pattern of radioactive activity (Fig. 11) obtained upon DEAE-paper electrophoresis of a partial snake venom phosphodiesterase digest was identical with that produced by the corresponding compound obtained from rabbit liver cytoplasmic initiator tRNA. Both oligonucleotides produced only four bands (Fig. 11, Bands a to d), rather than five as expected from a pentanucleotide. This is explained by the finding that under the electrophoretic conditions used, [7-P]G and [7-P]G-m^1A do not separate and are both present in Band a, as shown by subsequent analysis by paper chromatography in Solvent A of material eluted from Band a. Fig. 11 shows the sequence of the rest of the radioactive bands, the relative mobilities of which are in agreement with the expected M values.

**Terminal Sequence of Wheat Embryo tRNA^Met**

**5'-Terminal Sequence is pA-U-C-A-G** —Although not unique to euukaryotic initiator tRNAs, another interesting observation is that all of the four euukaryotic cytoplasmic initiator tRNAs studied to date (14, 35, 36) contain pA at the 5' end. The following lines of evidence show that wheat embryo tRNA^Met also contains pA at the 5' end and in the sequence pA-U-C-A-G (Peak 13 of Fig. 12).

1. Fig. 12 shows the pattern obtained upon chromatography of a T1-RNase digest of wheat embryo tRNA^Met. Material in Peak 13 was further purified by paper chromatography in Solvent A. Digestion with T1-RNase followed by two-dimensional thin layer chromatography showed that the products were pAp, Up, and Gp. The presence of pAp in such a digest shows that this oligonucleotide is derived from the 5' terminus of the tRNA.

2. Presence of a 5'-terminal phosphate in this oligonucleotide is further supported by the observation that phosphorylation of this oligonucleotide at the 5' end with 32P (see "Materials and Methods") required prior treatment with bacterial alkaline phosphatase.

3. Finally, the sequence of this oligonucleotide was established as follows. pA-U-C-A-G was dephosphorylated with E. coli alkaline phosphatase and then labeled with 32P at the 5' end. The 32P-labeled material was purified by electrophoresis on DEAE-cellulose paper in 7% formic acid and was found identical with Spot 13 of Fig. 9. Thin layer chromatography with non-radioactive markers of C-G-, C-A-G-, C-C-A-G-, and C-U-C-A-G- in Solvent A showed that this material behaved like a pentanucleotide (15). Partial digestion with snake venom phosphodiesterase and subsequent electrophoresis yielded a total of five bands (a to e) (Fig. 13). The sequences of Bands a to c were established from their identical mobility with markers of pA, pA-U, and pA-U-C (not shown) and those of Bands d and e by their M values (M_1 = 3.2, M_2 = 2.2) corresponding to the removal successively of G and A from the 3' end.

**3'-Terminal Sequence Is A-U-A-C-C-Aox** —The following lines of evidence establish the 3'-terminal sequences A-U-A-C-C-Aox.

1. This oligonucleotide is located in Peak 9 of Fig. 12 and was further purified by paper chromatography in Solvent A. Digestion with T1-RNase followed by two-dimensional thin layer chromatography yielded Ap, Cp, Up, and A. The presence of A and the absence of Gp in such a digest shows that this oligonucleotide is derived from the 3' terminus of wheat embryo tRNA^Met.

2. The absence of a phosphomonoester group was further confirmed by thin layer chromatography in Solvent A before and after treatment of this oligonucleotide with bacterial alkaline phosphatase. No change in mobility was observed.

3. The oligonucleotide was phosphorylated with 32P at the 5' end and the 32P-labeled material was isolated by DEAE-paper electrophoresis in 7% formic acid. The labeled material (identical with Spot 11 of Fig. 9) was then subjected to partial digestion with snake venom phosphodiesterase. Fig. 14 shows the pattern of radioactive spots present in such a digest, along with a parallel digest carried out on 32P-A-U-A-G. A total of six radioactive bands (Bands a to f) were obtained; the sequences of Bands a to e were established by their identical mobility with markers of [32P]A, [32P]A-U, and [32P]A-U-A, respectively, and those of Bands d to f by their M values (M_1 = 2.3, M_2 = 0.9, M_3 = 0.62) corresponding to the removal successively of A, C, and G from the 3' end.

**DISCUSSION**

In an attempt to elucidate the role of methionine tRNAs in euukaryotic protein synthesis we have purified the initiator methionine tRNA from wheat embryo, studied its properties, and determined the partial nucleotide sequence of tRNA^Met.

**Purification of tRNA^Met** —This was achieved in two simple

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Fig. 12. DEAE-cellulose chromatography of fragments obtained by RNase T1 digestions of tRNA^Met; 32A_{16s} units of wheat embryo tRNA^Met were incubated with 50 units of RNase T1 (Sankyo) in 0.06 M Tris (pH 7.5) for 16 hours. The digest was chromatographed on a DEAE-cellulose column (1 × 42 cm) previously equilibrated with 0.02 M Tris (pH 7.5)-1 M urea. The oligonucleotides were eluted with a linear gradient of 0 → 0.45 M NaCl in 0.2 M Tris (pH 7.5)-7 M urea (total volume of gradient, 1 liter), 3-ml fractions were collected every 12 min. The absorbance was monitored with a continuous recorder.
steps. Fractionation of crude wheat embryo tRNA on a BD-cellulose column separated two methionine tRNAs. A maximum purification of 8- to 10-fold and about 4-fold, respectively, of Peak I (tRNA\textsubscript{iMet}) and Peak II (tRNA\textsubscript{aMet}) was achieved. The two tRNA\textsubscript{iMet} peaks were present in equal amounts. Fractionation of the partially purified tRNA\textsubscript{iMet} on a DEAE-Sephadex column resulted in a further 3- to 4-fold purification and tRNA\textsubscript{iMet} accepting 1780 to 1810 pmoles of methionine per A\textsubscript{260} unit was isolated with a final yield of 25% of the unfraccionated methionine tRNA.

Properties of tRNA\textsubscript{iMet}—Several lines of evidence show that the tRNA\textsubscript{iMet} represents the initiator tRNA in wheat embryo. Thus, (a) in the presence of AUG, the binding of Met-tRNA\textsubscript{iMet} to wheat embryo ribosomes occurs at low Mg\textsuperscript{2+} concentration, whereas the binding of Met-tRNA\textsubscript{aMet} occurs maximally at high Mg\textsuperscript{2+} concentration (Fig. 3); (b) the plant viral RNA, BMV RNA, stimulates specifically the binding of Met-tRNA\textsubscript{iMet} and not Met-tRNA\textsubscript{aMet} to wheat embryo ribosomes (Table I); (c) in response to poly[r(A-U-G)], the wheat embryo tRNA\textsubscript{iMet} initiates protein synthesis in vitro as did the initiator tRNA species from yeast or mammalian sources (Fig. 8 and Table II) (6, 34, 38).

A major difference between the wheat embryo tRNA\textsubscript{iMet} and mammalian or yeast tRNA\textsubscript{iMet} lies in the fact that the wheat embryo initiator tRNA is not formylated by the E. coli methionyl-tRNA formylase. Yarwood et al. (39) have reported similar findings for the initiator tRNA from bean seeds. It would thus appear that the inability of wheat embryo and bean seed initiator tRNAs to be enzymatically formylated by E. coli methionyl-tRNA formylase might be a phenomenon common to all plant initiator tRNAs.

Our results with chemically formylated initiator and noninitiator methionyl-tRNA species from wheat embryo showed that E. coli initiation factors recognize not only the formyl group but some structural aspect unique to the initiator tRNA species (Table III and Fig. 8). This result is essentially similar to that observed by Rudland et al. (33) and different from a recent report of Drews et al. (40).

Our present studies and reports from other laboratories (6, 39, 41) show that in a wheat embryo ribosomal system, the homologous initiator tRNA cannot transfer methionine into internal positions. In a heterologous system, however, such as in E. coli cell-free extracts or, to a limited extent, in reticulocyte cell-free extracts, the same initiator methionyl-tRNA is active in transferring methionine internally. This latter finding is in agreement with that of several other groups who have shown that initiator tRNA from bacterial or mammalian sources are active in transfer of methionine into internal peptidyl linkages even in
the respective homologous systems (11–13), the only known exception being the yeast initiator tRNA (13, 27, 42). The significance of these observations and the mechanism by which the various protein-synthesizing systems discriminate in vivo, if at all, initiator methionine tRNA from the noninitiator tRNA is not clear at the moment.

Partial Sequence Studies—In attempts to correlate the structure of the wheat embryo tRNA$^{\text{Met}}$ to its function, our initial sequence studies have included those regions of the molecule in which the other eukaryotic initiator tRNAs possess a unique sequence. These studies have shown that this tRNA, similar to other eukaryotic cytoplasmic initiator tRNAs, also lacks the common sequence T-Ψ-C-G-. It is further shown that the sequence of loop IV of this tRNA is probably A-U* C-G- and identical with that of other eukaryotic cytoplasmic initiator tRNAs, except for the presence of U* instead of U. Thus, the two unique features noted originally in loop IV of yeast cytoplasmic initiator tRNA (14) and shown to be present also in mammalian cytoplasmic initiator tRNA (18, 35, 36) are (a) replacement of T by A and (b) presence of A as the last nucleoside of this loop instead of a pyrimidine nucleoside, probably present in a plant initiator tRNA. A further similarity between initiator tRNA of wheat embryo with those of yeast and mammalian sources is the presence of a 5′A′3′U base pair as the first base pair of the acceptor stem. None of these unique features are present in E. coli initiator tRNA. To what extent these features account for the properties of eukaryotic cytoplasmic initiator tRNAs are areas of further investigation.

An interesting finding from the sequence studies on mammalian cytoplasmic initiator tRNAs was that the total sequence of rabbit liver, mouse myeloma, and sheep mammary gland initiator tRNAs were identical (35, 36). Comparison of fingerprints of T1-RNase (Fig. 9) and pancreatic RNase (Fig. 10) digests of wheat embryo tRNA$^{\text{Met}}$ with similar digests of yeast (15) and mammalian initiator tRNAs (35, 36) suggests that the sequence of wheat embryo tRNA$^{\text{Met}}$ is not identical with that of either the yeast or the mammalian initiator tRNAs. If the evolution of eukaryotic cytoplasmic tRNAs correlates with biological evolution in general, it would be expected that between the yeast and wheat embryo initiator tRNA the latter would be more similar to mammalian initiator tRNA. Further studies on the sequence of wheat embryo initiator tRNA are clearly necessary to establish this, and such studies are now in progress.

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