*TRANSLATION OF THE GENETIC MESSAGE, IV. UAA AS A CHAIN TERMINATION CODON**

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The discovery that certain mutations result in the production of unfinished, amino-terminal peptide fragments of the corresponding wild-type proteins suggested that "nonsense" codons may normally signal termination of polypeptide chain synthesis.[1-3] Moreover, the finding that these "amber" mutations are suppressible, whereby the resulting polypeptide contains serine (codon, UCG) in place of glutamine (CAG) or tryptophan (UGG),[4-6] led to the prediction[2, 3] that UAG is the chain terminator of "amber" mutants. The study of other nonsense ("ochre") mutations suggested further that UAA also functions as a chain termination codon.[3] These suggestions were in accord with the fact that no amino acids are coded by UAG or UAA.[7, 8] There are indications that UAA rather than UAG may be the codon normally concerned with chain termination.[9]

In vitro experiments with synthetic polynucleotides of random base sequence lent some support to the view that UAA is involved in chain termination. The polypeptides synthesized under the direction of U- and A-containing copolymers or natural messengers consist largely of free, i.e., released peptide chains, whereas the peptide chains formed with polynucleotides which do not contain U and A residues are attached to tRNA.[10-12]

In order to determine unambiguously whether UAA is a chain-termination codon, we have studied the *in vitro* translation of a series of oligonucleotides having, along with the chain-initiating formylmethionine codon (AUG) and lysine codons (AAA), leucine (UUA), phenylalanine (UUU), or "ochre" (UAA) codons in specified sequences as follows: (*a*) AUGAAAAAAAAA...AAA ($AUGA_n$); (*b*) AUGUAA-AAAAAA...AAA ($AUGUA_n$); (*c*) AUGUUAAAAAAA...AAA ($AUGU_2A_n$); (*d*) AUGUUUAAAAAA...AAA ($AUGU_3A_n$); (*e*) AUGUUUUAAAAA...AAA ($AUGU_4A_n$).

The (*a*) polymers promoted the incorporation into acid-insoluble products of methionine and lysine; the (*c*) polymers, that of methionine, leucine, and lysine; and the (*d*) polymers, that of methionine, phenylalanine, and lysine. On the other hand, the (*b*) and (*e*) polymers promoted the incorporation of only small amounts of lysine, with little or no incorporation of methionine or phenylalanine. The inference that the (*e*) oligonucleotides directed the synthesis and release of *acid-soluble* formylmethionyl-phenylalanine was proved correct by isolation of the dipeptide. This work provides conclusive proof that UAA is a chain-termination codon. It also provides a suitable system for study of the mechanism of chain termination.

Materials and Methods.—These were as in previous work[13, 14] unless otherwise noted.

Amino acid incorporation: The low-nuclease system consisting of purified *Escherichia coli* Q13 ribosomes and *Lactobacillus arabinosus* supernatant,[15] supplemented with initiation factors,[13, 16] was used throughout. In experiment 1 of Table 1 the samples contained the following components in a final volume of 0.125 ml: Tris-HCl buffer, pH 7.8, 60 mM; ammonium chloride, 50 mM;

2-mercaptoethanol, 16 mM; magnesium acetate, 14 mM; ATP, 1.2 mM; GTP, 0.3 mM; phosphocreatine, 18 mM; creatine kinase, 9 μg; *E. coli* W tRNA, 0.60 mg; purified *E. coli* Q13 ribosomes, 7 A_{260} units; *L. arabinosus* supernatant, 0.2 mg of protein; N^{10}-formyltetrahydrofolic acid (FTHF), 8 mM; initiation factors (F_1 and F_2), 6 and 21 μg of protein, respectively; 20 amino acids (one of them with C^{14} label), each 0.2 mM; without or with oligonucleotide messenger, 15–20 μg. The specific radioactivities (μc/μmole) of the labeled amino acids were: lysine, 2; methionine, 20; leucine, 22; and phenylalanine, 40. The composition of the samples in experiment 2 of Table 1 was similar except that only three amino acids, namely, lysine (0.2 mM), methionine (0.02 mM), and phenylalanine (0.02 mM) were present, one of them having C^{14} label. Their specific radioactivities (μc/μmole) were: lysine, 10; methionine, 186; and phenylalanine, 176. After incubation at 37° the reaction was terminated by addition of KOH (final concentration, 0.5 *M*). The mixtures were kept at 37° for 3 hr, followed by neutralization, and the acid-insoluble radioactivity was determined as previously described.[16]

Polynucleotides: The methods used in this laboratory for the preparation of various kinds of oligonucleotides of specified base sequence have been described.[17] The preparation of the polymers used in the present work is described below.

$AUGA_n$ polynucleotides were prepared[17] with ADP and polynucleotide phosphorylase, using ApUpG[18] (AUG) as primer. For the other polymers, the series of primers ApUpGpU (AUGU), ApUpGpUpU ($AUGU_2$), ApUpGpUpUpU ($AUGU_3$), and ApUpGpUpUpU ($AUGU_4$) was prepared by limited addition of uridylic acid residues from UDP to ApUpG primer with polynucleotide phosphorylase and chromatographic isolation of the individual oligonucleotides. Adenylic acid residues were then added to each of these primers, by incubation with ADP and polynucleotide phosphorylase. For characterization purposes the polymers were prepared with H^3-labeled guanylic acid residues. In the following description this label will be indicated by an asterisk.

ApUpG* was prepared from ApU (lot no. B 3503, Gallard-Schlesinger) as described previously.[17] ApUpG*pU, ApUpG*pUpU, ApUpG*pUpUpU, and ApUpG*pUpUpUpU were synthesized by the addition of uridylic acid residues to ApUpG* under the following conditions. The reaction mixture (pH 8.2) contained the following components (in μmoles/ml unless otherwise indicated) in a final volume of 4.0 ml. ApUpG*, 7.3; UDP (lot no. 1116, Pabst), 40; Tris-HCl, 150; magnesium acetate, 10; EDTA, 1; sodium chloride, 500; polynucleotide phosphorylase, 300 μg of protein/ml. After incubation for 20 min at 30°, the solution was held at 100° for 5 min, cooled, diluted 50-fold, and applied to a column (1.8 × 39 cm) of DEAE-cellulose (lot no. 1675, 1 mEq/gm, Brown). Oligonucleotides of the form ApUpG* with 1, 2, 3, etc. residues of uridylic acid added to the 3′-end were separated at 25° by gradient elution with ammonium bicarbonate (0.01 *M* ammonium bicarbonate, pH 8.6, in a 2-liter mixer; 0.5 *M* ammonium bicarbonate, pH 8.6, in a reservoir) at 1 ml/min. In order to remove water and salt, the oligonucleotides were recovered by flash evaporation and lyophilization. The yields were, in moles of oligonucleotide/mole input ApUpG*: ApUpG*, 0.142; ApUpG*pU, 0.295; ApUpG*pUpU, 0.166; ApUpG*pUpUpU, 0.119; and ApUpG*pUpUpUpU, 0.072. The structure and purity of each oligonucleotide was confirmed by paper chromatography in two solvents (solvent 1, 1.0 *M* ammonium acetate, pH 7.5/95% ethanol, 6/4, v/v; solvent 2, 40 gm ammonium sulfate plus 100 ml of 0.1 *M* potassium phosphate, pH 7.0), by degradation with RNase T_1 followed by identification of ApUpG*p plus the expected length of uridylic acid residues (i.e., ApUpG*p plus UpUpU were recovered from ApUpG*pUpUpU), and by the determination of the ratio of uridine to uridylic acid following complete alkaline hydrolysis.

Adenylic acid residues were added to the 3′-ends of each of the purified oligonucleotide primers, and the resulting polymers were fractionated according to chain length and recovered as previously described.[17, 19] The structure was confirmed by the identification of the low-molecular-weight products arising from the degradation of each polymer with RNase T_1 and by pancreatic RNase. For example, ApUpG*pUpU$(pA)_n$ gave rise to ApUpG*p following RNase T_1 digestion, and to ApUp, G*pUp, and Up, in the molar ratio 1/1/1 following digestion with pancreatic RNase.

The polynucleotide phosphorylase was a preparation from *Micrococcus lysodeikticus* with an absolute requirement for primer oligonucleotides. The enzyme catalyzed the polymerization of 6.5 μmoles of ADP/min/mg protein at 37° under the following conditions (reagents in μmoles/ml):

ADP, 40; ApApA, 1; sodium chloride, 500; magnesium acetate, 10; EDTA, 1; and Tris-HCl buffer, pH 8.2, 150.

Isolation and characterization of (H³)formyl-(C¹⁴)methionyl-(C¹²)phenylalanine and (H³)formyl-(C¹²)methionyl-(C¹⁴)phenylalanine: The incubation mixtures were as described under *Amino Acid Incorporation* (expt. 1 of Table 1) with the following exceptions: H³-FTFH (formate-labeled, 2000 μc/μmole), 0.022 mM; either C¹⁴-methionine (186 μc/μmole) or C¹⁴-phenylalanine (352 μc/μmole), 0.02 mM; nonlabeled amino acids, either 19 amino acids, each 0.2 mM, or lysine (0.2 mM) and phenylalanine or methionine (0.02 mM); without or with $AUGU_4A_{\overline{10}}$, 15–20 μg. Pancreatic DNase (electrophoretically purified, Worthington), 25 μg, was also added to the samples to prevent any synthesis of endogenous messenger RNA.

Prior to adding the ribosomes, the samples were incubated for 5 min to allow time for transfer of H³-formate from H³-FTFH to met ~ $tRNA_F$.[20] The ribosomes were then added and the incubation was continued for 15 min. The short incubation was chosen to minimize enzymatic removal of labeled formyl residues from the peptides synthesized.[21] After incubation, the samples were chilled in ice and treated with 0.127 ml of 6% perchloric acid. The precipitate was removed by centrifugation and washed three times with 30-μl portions of ice-cold 3% perchloric acid. The combined supernatant and washings were neutralized with 0.168 ml of 1.0 *N* KOH. The potassium perchlorate precipitate was removed by centrifugation and washed repeatedly with 50-μl portions of cold water until the washings were free of radioactivity. The supernatant and the washings were combined and the pH of the solution was adjusted to 7.8, with Tris-HCl buffer, prior to chromatography on a Dowex-1 (Cl^-) column (0.6 × 5 cm). The column was washed with 10 ml of water, containing 1–2 mg of nonlabeled phenylalanine or methionine, and the formylated amino acids and peptides were then eluted with 8.0 ml of 0.5 *N* acetic acid adjusted to pH 2.7 with pyridine. This solution was passed through a Dowex-50 (H^+) column (0.6 × 20 cm). The formylated compounds were washed through with 8.0 ml of 0.001 *N* HCl. For separation of formylmet and formylmet-phe, the eluate was lyophilized, the residue taken up in 0.5 ml of water, and aliquots of the solution were subjected to high-voltage paper electrophoresis, along with markers of formylmet and formylmet-phe, as previously described,[14] except that the electrophoresis was run for 7 hr. For measurement of radioactivity after electrophoresis, the paper was cut into strips (1 cm long, 2.5 cm wide) which were placed in the bottom of scintillation vials. The strips were covered with solvent (4 gm of 2,5-diphenyloxazole (PPO) and 50 mg of p-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) per liter of toluene) and the radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. Under these conditions, the counting efficiency of the two isotopes on Whatman 3MM paper was about 29% for C¹⁴ and 0.9% for H³, with 12% of the C¹⁴ counts appearing in the H³ channel, and less than 0.1% of the H³ counts in the C¹⁴ channel. The total amount of formylmet-phe calculated from the electrophoretic data was corrected for small aliquots of material withdrawn, during the processing of the reaction mixtures, for determination of the acid-soluble radioactivity. This correction was never greater than 6%.

Formylmet-phe was prepared by formylation of L-methionyl-L-phenylalanine.[22] L-methionyl-L-phenylalanine was obtained from the Mann Research Laboratories, Inc., New York.

Results and Discussion.—Incorporation of amino acids into acid-insoluble products: As shown in Table 1, oligonucleotides of the series $AUGA_n$, $AUGU_2A_n$, and $AUGU_3A_n$ promote the incorporation of lysine, along with smaller amounts of methionine ($AUGU_{\overline{18}}$), methionine and leucine ($AUGU_2A_{\overline{10}}$), and methionine and phenylalanine ($AUGU_3A_{\overline{10}}$). It may further be noted that the incorporation of methionine and leucine, or methionine and phenylalanine, promoted by poly $AUGU_2A_{\overline{10}}$ or $AUGU_3A_{\overline{10}}$, respectively, is roughly equimolar. Moreover, there is negligible or no incorporation of phenylalanine with poly $AUGU_2A_{\overline{10}}$ and the same is true of leucine in the case of poly $AUGU_3A_{\overline{10}}$. This shows that these polymers are read in phase from the 5′-end.

In contrast to the above results, the incorporation of the various amino acids into acid-insoluble material is markedly diminished when oligonucleotides of the series

TABLE 1

POLYPEPTIDE SYNTHESIS WITH OLIGONUCLEOTIDE MESSENGERS OF SPECIFIED BASE SEQUENCE*

Expt. no.	Incubation time (min)	Messenger	Amino Acid Incorporation†			
			Lysine	Methionine	Leucine	Phenylalanine
1	40	None (blank)	81	43	24	18
		$AUGA_{\overline{18}}$	1261 (1521)	163 (152)	0	0
		$AUGUA_{\overline{11}}$	162 (142)	10 (10)	0	0
		$AUGU_2A_{\overline{10}}$	644 (563)	65 (58)	56 (51)	0
		$AUGU_3A_{\overline{10}}$	543 (425)	56 (47)	2	62 (50)
		$AUGU_4A_{\overline{10}}$	282 (153)	10 (10)	2	4 (9)
2	15	None (blank)	43	3	—	5
		$AUGU_3A_{\overline{10}}$	371	29	—	21

* Conditions as described under *Materials and Methods*. In expt. 2 the samples were preincubated for 5 min prior to starting the reaction by addition of the ribosomes.

† Values expressed in $\mu\mu$moles/sample. The first line for each expt. gives the blank values in the absence of added oligonucleotide. All others are net values, the blanks without polymer having been subtracted from the values with polymer. The net values given in parentheses are the combined averages of expt. 1 and three other experiments carried out at different times between August 1966 and February 1967. Duplicate samples were run in each experiment and the results averaged.

$AUGU_4A_n$ ($AUGU_4A_{\overline{10}}$), having the UAA triplet in the third position from the 5′-terminus, are used as messengers. Although there is some lysine incorporation, that of methionine and phenylalanine is very low. This incorporation is referable to slight contamination of poly $AUGU_4A_n$ with $AUGU_3A_n$. Table 1 also lists an experiment with poly $AUGUA_{\overline{11}}$, having the UAA triplet in the second position from the 5′-end. Some lysine but little if any methionine is incorporated.

Release of formylmethionyl-phenylalanine upon translation of $AUGU_4A_n$ polymers: In order to determine whether acid-soluble formylmet-phe is synthesized and released upon translation of the $AUGU_4A_n$ messengers, incubations with and without polymer were conducted with either H^3-FTHF and C^{14}-methionine or H^3-FTHF and C^{14}-phenylalanine. The nonlabeled amino acids were either 19 amino acids (no methionine or no phenylalanine) or lysine with either phenylalanine or methionine. After incubation, any labeled acid-insoluble products, including formylmet ~

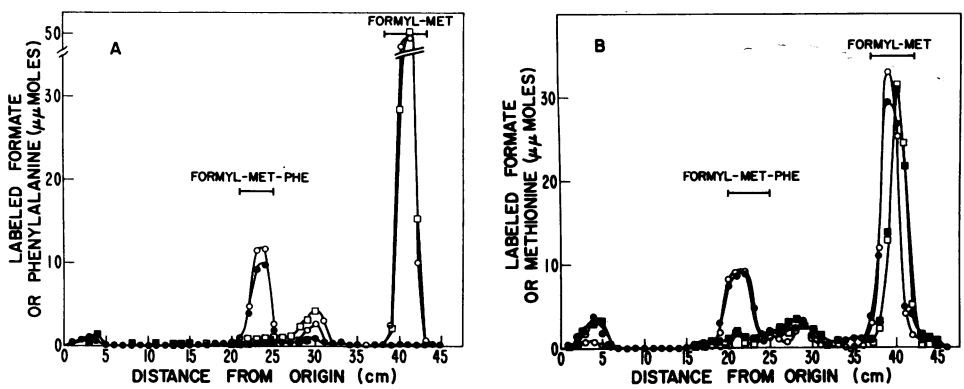

FIG. 1.—Electrophoretic patterns of labeled peptides synthesized with $AUGU_4A_{\overline{10}}$ oligonucleotide messenger. Aliquots corresponding to 10% of the sample were used for electrophoresis. The anode is to the right. The position of formylmet-phe and formylmet markers is indicated. (*A*) Incubation with H^3-FTHF and C^{14}-phenylalanine. (*B*) Incubation with H^3-FTHF and C^{14}-methionine. In this experiment the samples contained the remaining 19 C^{14}-amino acids. Two other experiments were carried out with similar results. *Squares*, blank samples (no oligonucleotide added); *circles*, samples with oligonucleotide added. *Open symbols* s(□, ○), H^3 label; *solid symbols* (■, ●), C^{14} label.

TABLE 2

H^3 AND C^{14} RADIOACTIVITY IN THE FORMYLMETHIONYL-PHENYLALANINE AREA OF THE ELECTROPHEROGRAMS OF FIGURE 1*

Expt.	Radioactive label	Blank sample† (*a*)	Sample with $AUGU_4A_{\overline{1}}$ (*b*)	Polymer-promoted (*b* − *a*)
Fig. 1*A*	H^3 (formate)	3.4	32.1	28.7
	C^{14} (phenylalanine)	2.5	26.2	23.7
Fig. 1*B*	H^3 (formate)	4.8	32.6	27.8
	C^{14} (methionine)	7.3	34.2	26.9

* Values expressed as $\mu\mu$moles of formate, phenylalanine, or methionine/sample.
† Sample with no oligonucleotide added.

$tRNA_F$, met ~ $tRNA_M$, and phe ~ tRNA, were removed by precipitation with perchloric acid and the supernatants worked up as described under *Materials and Methods*.

The electrophoretic patterns of the H^3- and C^{14}-labeled products (Fig. 1) show two main peaks. One of them is in the position of formylmet-phe, the other in the position of formylmet. The presence of formylmet would appear to reflect the occurrence of some deacylation of formylmet ~ $tRNA_F$ during the incubation. It may be noted that the blank incubations, with no added oligonucleotide, show roughly the same amount of radioactivity in the position of formylmet but little or no radioactivity in the region of the dipeptide.

The H^3 and C^{14} radioactivity under the formylmet-phe peak, expressed as $\mu\mu$-moles of product per incubation sample, is given in Table 2. Reference to experiment 2 of Table 1 shows that the formation of formylmet-phe with poly $AUGU_4A_{\overline{10}}$ is of the same order of magnitude as the incorporation of methionine and phenylalanine into acid-insoluble material with $AUGU_3A_{\overline{10}}$ messenger.

The above results indicate that the $AUGU_4A_n$ polymers are translated with the same efficiency as the others except for termination of chain growth and release once formylmet-phe is synthesized. It may be emphasized in this connection that formylmet-phe is not released as peptidyl ~ tRNA (a compound that would be acid-insoluble) but as the free dipeptide. Thus, complete chain termination is achieved. The isolation of formylmet-phe in good yields, upon translation of the 5′-end sextet AUGUUU, also provides further proof for the triplet nature of the genetic code.[23]

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[18] Shorthand writing of polynucleotides and most of the abbreviations used are standard. Other abbreviations are as follows: FTHF, formyltetrahydrofolic acid; tRNA, transfer RNA; met $\sim$ $tRNA_F$, the species of methionyl tRNA whose methionine residue can be formylated; met $\sim$ $tRNA_M$, the nonformylatable species; phe $\sim$ tRNA, phenylalanyl tRNA; formylmet, formylmethionine; formylmet-phe, formylmethionyl-phenylalanine.

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