CHAIN TERMINATION IN VITRO. STUDIES ON THE SPECIFICITY OF AMBER AND OCHRE TRIPLETS.

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The nonsense triplets UAA (ochre), UAG (amber), and possibly UGA code for polypeptide chain termination in vivo and in vitro (1-8). The mechanism by which these triplets elicit cleavage of a finished polypeptide from the terminal tRNA is unknown. Termination may occur by the specific recognition of these triplets or because there are no tRNA's for these codons. To date, the only tentative evidence that these triplets are specific is that a co-polymer of uridylic and xanthylic acid (a non-coding base) does not terminate (4,6).

In this report we examine whether termination can occur by removal of specific tRNA's or by recognition of nonsense triplets by a special tRNA. To this end, specific tRNA's were purified and used in peptide synthesis directed by non-terminating polynucleotides so that translation of some triplets would be interrupted by the absence of the appropriate tRNA. To look for a special tRNA, purified tRNA's were used in peptide synthesis directed by messengers containing terminating triplets. No chain termination could be detected when readable codons were not translated due to the absence of appropriate tRNA's or when non-coding bases were present in messenger RNA. This suggests that termination involves the active participation of amber or ochre triplets. No evidence for a specific tRNA was obtained. After completion of these experiments, Bretscher (9) also reported that purification of the specific tRNA's required to synthsize a hexapeptide with amber mutants of $f_2$phage mRNA did not result in loss of chain termination. A hexapeptidyl-tRNA accumulated

with the wild type mRNA when glutamyl-tRNA, the seventh amino acid in the phage coat protein, was removed (9).

MATERIALS AND METHODS

E. coli B or Q_{13} homogenates were prepared as described by Gottesman (10). Ribosomes were washed twice in 1.0 M NH_4Cl and 2 mM $MgCl_2$ and once in 0.5 M NH_4Cl and 10 mM $MgCl_2$. Initiation factors were prepared according to Iwasaki *et al.* (11). To remove tRNA, supernatants or ammonium sulfate fraction of initiation factors were passed through DEAE-cellulose (Cl^-) equilibrated with 0.22 M KCl buffer. Incorporation experiments and analysis of acid insoluble peptide synthesis products other than polyphenylalanine were as previously described (5,6) except that 8 mM $MgCl_2$, 30 ug of Ca^{+2} leucovorin, 150 ug salt washed ribosomes, 90 ug of initiation factors, and 100 ug of supernatant enzymes were used in 0.1 ml incubations. Polyphenylalanyl-tRNA was separated from polyphenylalanine on SDS sucrose density gradients (12) using 0.1 M lithium acetate, pH 5, and 18 hours of centrifugation at 38,000 rpm (SW-39). C^{14}-polyphenylalanine markers were prepared by treatment of incubation mixtures with RNase after addition of 20 mM EDTA. Typical separations are shown in Figure 1A. Acid soluble peptides were analyzed by paper electrophoresis (Whatman 3MM) at 20 v/cm in 0.5 M formic acid (13) followed by descending chromatography in an ethylacetate:pyridine:acetic acid:water buffer (14) before and after KOH hydrolysis. The released hexapeptide formed with amber (sus_3) RNA was purified according to Capecchi (15) before and after KOH hydrolysis of the incubations. Lysine tRNA was purified by 1,000 transfers on counter current distribution according to Goldstein *et al.* (16). Fraction 110 contained 6 and 2 fold enriched quantities of arginine and asparagine tRNA, respectively. Fraction 120 contained all amino acid tRNA's which are incorporated by poly AU(3:1) except for isoleucine tRNA. Purified phenylalanine tRNA, a generous gift of Dr. D.Novelli, did not contain amino acid tRNA's other than phenylalanine and some tyrosine tRNA. Polynucleotides were from Miles. Poly UX, AX, and AG(2:1) were synthesized according to Singer and

Guss (17). Bases were analyzed according to Lane (18), except for AX which was analyzed by chromatography in 65:35 95% ethanol:1.0 M ammonium acetate (19).

RESULTS AND DISCUSSION

Incorporation of amino acids into peptides depended upon added tRNA. Generally 100-fold stimulations were obtained. Significant levels of free peptides were released with poly AU(3:1) or (1:3) or amber (sus_3) mRNA (see Table 1, Section A). In separate experiments it was verified that release with AU copolymers occurred only under conditions needed to release with oligonucleotides of defined sequence containing UAA (20) and was not due to selective product degradation. It was also verified that the method used to purify the product formed with sus_3 RNA is specific for the hexapeptide, Fmet.ala.ser.asNH_2.phe.thr.(15). These controls indicated that chain termination was operative with messengers containing amber or ochre triplets.

It is shown in Table 1 that insertion of xanthine decreased polymerization relative to poly A and poly U as expected without increasing chain termination. The small level of release with AX was the same as that found with poly A; non-enzymic deacylation of polylysyl-tRNA was about 10%. Prolonging the time of incubation up to 30 minutes increased non-enzymic deacylation but gave no termination.

Table 1 also summarizes the incorporation and termination data obtained when synthesis was carried out with crude or purified tRNA's. Purification of tRNA's resulted in decreased polymerization which correlated with the purity of the tRNA's used (see Methods and Table 1, Section C). No significant incorporation of various amino acids other than phenylalanine could be detected with the poly UG polymers tested, using saturating levels of purified phenylalanine tRNA. This indicated that polyphenylalanine chains must have been next to "empty" triplets of U and G. Similar controls indicated that the drop in polymerization with AG and AC copolymers must be due to empty triplets of A and C and A and G.

TABLE 1

TOTAL AND FREE PEPTIDES FORMED WITH CRUDE AND PURIFIED tRNA's

Section	Polynucleotide	tRNA	Total Peptides (cpm)	% Free Peptides
A	AU(3:1)	crude	24,250	60
	sus_3	crude	1,450	91
B	AX(3:1)	crude	18,070	9
	UX(3:1)	crude	18,476	8
	A	crude	51,070	11
	U	crude	220,962	7
C	AC(7:1)	crude	6,990	20
		"lys" (110)	4,950	23
	AG(2:1)	crude	5,550	19
		"lys" (110)	3,620	16
	UG(3:1)	crude	53,350	8
		"phe"	9,450	8
	UG(1:1)	crude	114,657	9
		"phe"	16,334	18
D	AU(3:1)	crude	20,000	57
		"lys" (120)	9,100	52
	UA(3:1)	crude	356,100	36
		"phe"	34,770	41

The peptides from 0.1 ml incubations were analyzed as described in Methods. C^{14}-lysine (specific activity = 220) was used with Poly A, AX, AU(3:1), AC, and AG; C^{14}-phenylalanine (specific activity = 360) was used with all other polymers except for the sus_3 RNA products which were labelled with C^{14}-threonine (specific activity = 80). Incubations were for 10 minutes at 35°C. Details of analysis, including recoveries, were previously described (5). Small levels of total or free peptides minus messengers were subtracted.

The bulk of the acid insoluble peptides formed with AC, AG, or UG(3:1) and (1:1), remained attached to tRNA when synthesized with either crude or purified tRNA's (Table 1). The peptidyl-tRNA remained largely bound to ribosomes (Figures 1B and 1C). As previously reported (5,6) a considerable frac-

tion of the polylysyl-tRNA synthesized with poly A is released from the ribosomes. This type of "leakage" is minimal with the AC and AG polymers studied. The slightly higher levels of free peptides formed with AC and AG (Table 1) were due to a small level of proteolytic breakdown in these experiments. The apparent small increment in free peptides observed with UG(1:1) was within the error of the assay method (see Figure 1A). The possibility that acid soluble

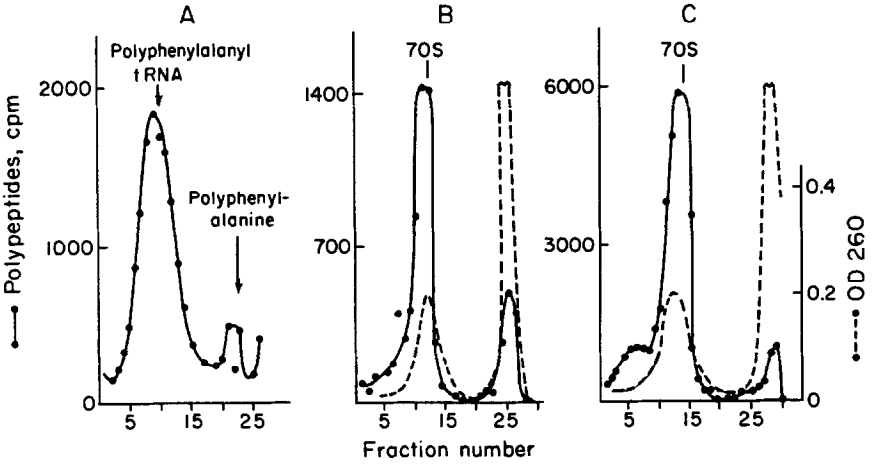

Figure 1. Sucrose density gradient analysis of products. A) SDS 5-12.5% sucrose density gradient (see Methods) of UG(1:1) products with purified phenylalanine tRNA. B) 5-20% sucrose density gradient (24) of UG(3:1) with purified phenylalanine tRNA. C) 5-20% sucrose density gradient (24) of UG(3:1) with crude tRNA. 0.1 ml incubations (see Methods) using C^{14}-phenylalanine (specific activity = 360) were layerd on the gradients. 0.1 ml fractions in A) and 0.15 ml fractions in B) and C) were collected, hydrolyzed for 15 minutes at 90°C in 15% TCA, collected and washed on Millipore filters, and counted at 50% efficiency in 5 ml of Bray's solution (25). Recoveries were 90% for A) and 82% for B) and C).

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peptides were released was examined by analyzing the products as described in Methods. No small peptide products could be detected before exposure of the incubation mixtures to KOH hydrolysis. After KOH hydrolysis, these products co-chromatographed with the respective di- and tri-peptide standards. These results show that the insertion of non-coding bases in mRNA or the mere absence of a tRNA during translation does not suffice to trigger chain termination.

In order to see if a special tRNA were needed for release with terminating systems, other amino acid tRNA's were purified. The lysine tRNA (fraction 120) contained all the amino acid tRNA's which are incorporated by poly AU(3:1) except isoleucine tRNA. The same extent of release was obtained with these purified tRNA's as with crude tRNA's (Table 1). As previously reported (**6**), scanning the entire area of the lysine peak did not reduce termination with AU(3:1). Moreover, 40% termination was observed with AU(1:3) with either crude or highly purified phenylalanine tRNA. Therefore, if there were a terminating tRNA, it should have been present in both these purified tRNA preparations. To examine this possibility, a different test was used. In amber suppressing bacterial extracts, a modified tRNA prevents termination by insertion of an amino acid (21, 22). Using sus_3 mRNA, 50 to 70% of the incorporation observed with wild type $f_2$phage mRNA was obtained when the extracts were supplemented with tRNA from *E. coli* K 55 (which contains serine suppressing tRNA). The resulting chain propagation was not reduced by crude tRNA from non-suppressing cells, as previously reported (23), nor by increasing levels of purified RNA's used in these experiments as would be expected if these RNA's recognized UAG *per se*.

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