tRNA-Dependent Translational Control of *In Vitro* Hemoglobin Synthesis

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Received June 18, 1969

SUMMARY

A tRNA-dependent rabbit reticulocyte cell-free system has been developed which is highly active in synthesizing hemoglobin. When unfractionated tRNA is used to supplement the system, approximately equal amounts of α and β chains are produced. When tRNA fractionated by Freon reverse phase chromatography is utilized, but with a specific portion deleted, α chain production is reduced to approximately 67% of the production of β chains.

A role of tRNA in regulating the rate of translation of hemoglobin mRNA was first postulated by Itano (1). Several workers have examined codon recognition by the tRNA species involved in the synthesis of rabbit hemoglobin (2-5). Winslow and Ingram (6) studied the rate of assembly of hemoglobin chains in human bone marrow cells and they presented evidence consistent with the presence of a "control point", beyond which the growth of the polypeptide chain is reduced, in the synthesis of each chain. They suggested as one possibility that the delay might be due to a low level of one or more species of tRNA. Clegg *et al.* (7), however, were unable to detect a control point during the assembly of β chains in either normal or β-thalassemic reticulocytes.

Previously, we have studied the effect of tRNA species on the rate of translation of artificial mRNA templates in *E. coli* extracts (8,9). It was suggested that AGA and AGG might be "regulatory" codons in *E. coli*, i.e., codewords recognized by species of tRNA present in rate-limiting amounts. In order to examine whether or not individual species of tRNA might be capable of influencing the rate of natural mRNA translation in mammalian cells, the synthesis of hemoglobin in a tRNA-dependent rabbit reticulocyte cell-free system

has been examined. Our data indicate that the concentration of one (or more) tRNA species can cause a specific alteration in the ratio of α and β chains synthesized in vitro.

METHODS

In vitro polymerization assay: Reactions, as described in the legend to Fig. 1, were incubated for 20 to 60 min at 37°C (amino acid incorporation is

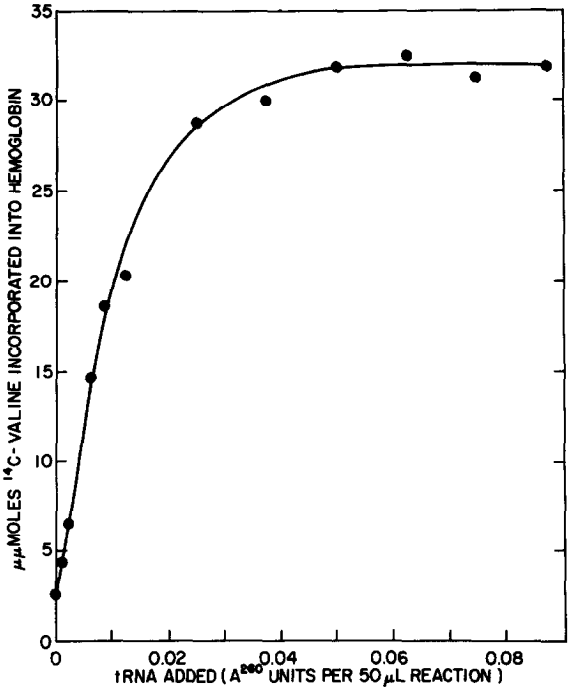

Fig. 1. ^{14}C-valine incorporation into protein as a function of concentration of unfractionated rabbit reticulocyte tRNA added to the reaction. Each 50 μl assay mixture was incubated at 37°C for 20 min and contained: 20 mM Tris Cl, pH 7.5, 80 mM KCl, 3 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 3 mM PEP, 0.3 units of pyruvic kinase, 1 mM DTT, 0.08 mM ^{14}C-L-valine (90 mC/mmole), 0.08 mM each of the other 19 unlabeled amino acids, 0.35 A^{260} units washed polysomes, 72 μg enzyme protein, and tRNA as indicated. tRNA was removed from polysomes by resuspending and pelleting the ribosome fraction once in 0.25 M sucrose, 1 mM DTT, 0.1 mM Na EDTA, 0.5 M KCl, followed by resuspending and pelleting once in 0.25 M sucrose, 1 mM DTT, and 0.1 mM Na EDTA. tRNA was removed from enzyme protein by suspending the supernatant fraction from a 0.5 M KCl polysome wash (12) in DEAE-cellulose at 0.35 M KCl followed by centrifugation to remove the DEAE particles. An incorporation of 3060 μμmoles ^{14}C-valine/hr/mg ribosome can be calculated from the plateau of the plot (using 1 mg ribosomes equal to 11.2 A^{260} units).

linear for 45-60 min), and were stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA). Radioactivity of the hot TCA precipitable material was measured on nitrocellulose filters with a liquid scintillation counter.

Product identification: The procedure of Dintzis (10) was used for separation of α and β chains by carboxymethyl (CM) cellulose chromatography and for two-dimensional chromatography of tryptic peptides (fingerprinting).

tRNA fractionation: Rabbit liver tRNA was fractionated by Freon reverse phase chromatography, as described in the legend to Fig. 2.

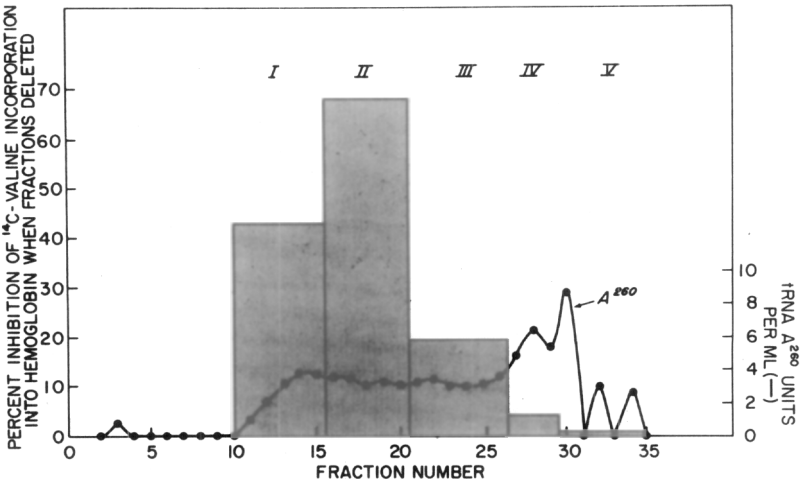

Fig. 2. Fractionation of rabbit liver tRNA by Freon reverse phase chromatography. A concave gradient from 0.20 M to 0.35 M NaCl in 0.01 M Tris-Cl, pH 7.0, 0.01 M $MgCl_2$, and 0.5 mM Na EDTA, at 37°C, was run according to the procedure of Weiss and Kelmers (11). Tubes 30, 32 and 34 are 0.40 M, 0.50 M, and 1.0 M NaCl washes, respectively. The tRNA was pooled into 5 groups as shown. Reactions, as described in the legend to Fig. 1, were incubated with each combination of 4 of the 5 groups using 0.01 A^{260} unit of tRNA from each group. The bar graphs represent the percent inhibition produced by deletion of the group under the Roman numeral as compared with ^{14}C-valine incorporation when all 5 groups are present.

RESULTS

tRNA-dependent cell-free hemoglobin synthesizing system

Although it is relatively easy to obtain a tRNA-dependent E. coli protein-synthesizing system (9), it has proven difficult to prepare a tRNA-dependent

reticulocyte cell-free system which will translate natural messenger. The procedures are described in the legend to Fig. 1. A 15-fold stimulation of ^{14}C-valine incorporation into protein can be obtained upon the addition of unfractionated rabbit reticulocyte tRNA to the reaction system, as shown in Fig. 1.

Effect of tRNA fractions on hemoglobin synthesis

Rabbit liver tRNA was fractionated by Freon reverse phase chromatography and was then pooled into 5 groups as shown in Fig. 2. The decrease in the rate of ^{14}C-valine incorporation into hemoglobin was measured when each one of the 5 groups was deleted from the reaction mixture. The bar graphs in Fig. 2 demonstrate that the deletion of group II tRNA produces the greatest inhibition.

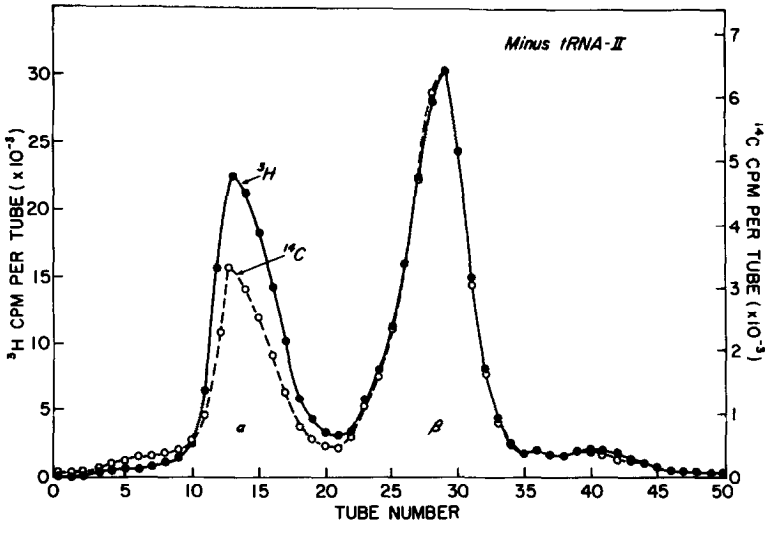

Fig. 3. CM-cellulose chromatography of the α and β chains synthesized when group II tRNA (see Fig. 2) is deleted. Two reactions were incubated which were identical except that one (0.5 ml) contained ^3H-leucine (1000 mC/mmole) plus 0.5 A^{260} unit of unfractionated rabbit reticulocyte tRNA, while the other (1.0 ml) contained ^{14}C-leucine (311 mC/mmole) plus 0.2 A^{260} unit each of the 4 fractionated rabbit liver tRNA groups I, III, IV, and V, i.e., group II tRNA was deleted. At the end of 60 min incubation at 37°C, the two reactions were pooled and then processed according to the procedure of Dintzis (10). The ^3H and ^{14}C scales were adjusted to normalize the β peaks. The α values have not been adjusted to account for the one less leucine in the α chain compared with the β chain. Absolute μmoles of labeled leucine obtained by integrating under the peaks (and confirmed by measuring radioactivity in the pooled peaks) are: ^3H-α-chain, 224; ^{14}C-α-chain, 45.2; ^3H-β-chain, 336; ^{14}C-β-chain, 95.5. Efficiencies for double label counting were: ^{14}C, 58%; ^3H, 25%.

In order to determine whether or not the rates of synthesis of the two hemoglobin chains were inhibited equally by the deletion of group II tRNA, the products of the reaction were identified by CM-cellulose chromatography and by fingerprinting. Because recovery of α and β chains is not always complete during acid-acetone precipitation and column chromatography (10), double-label experiments, as described in the legend to Fig. 3, were performed to provide an internal control. In this way ^{14}C-labeled α and β chain production could be compared between experiments by comparison with the ^{3}H values. The data of Fig. 3 indicate that α and β chains are not synthesized to the same extent in the absence of group II tRNA; rather, α chain production is reduced to 0.70 of the production of β chains. A duplicate experiment demonstrated an α to β chain ratio of 0.69. Incubation for 30 rather than 60 min produced a ratio of 0.65. This unequal synthesis of chains is eliminated by the addition of group II tRNA, as shown in Fig. 4. Using the ^{3}H-leucine in an incubation with all 5 tRNA groups and the ^{14}C-

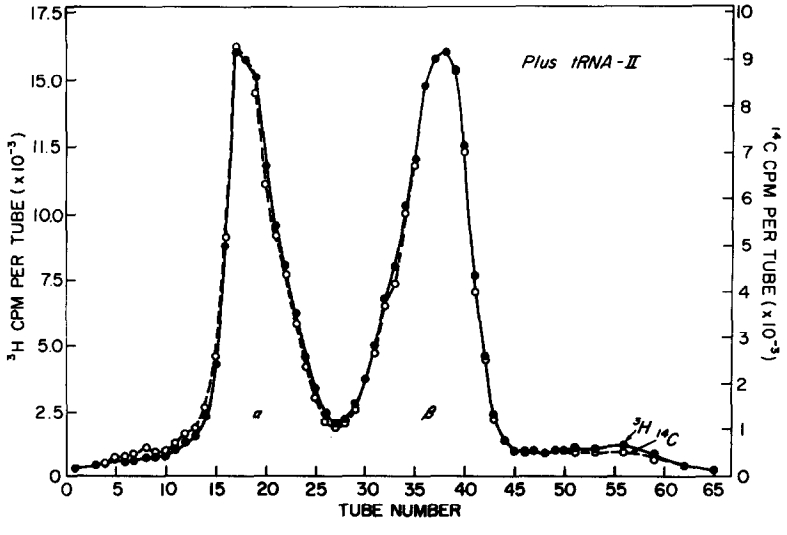

Fig. 4. CM-cellulose chromatography of the α and β chains synthesized when group II tRNA was present. Conditions were identical to those for Fig. 3 except that group II tRNA was added to the ^{14}C-leucine tube (which already contained groups I, III, IV, and V). Absolute μmoles of labeled leucine are: ^{3}H-α-chain, 204; ^{14}C-α-chain, 160; ^{3}H-β-chain, 255; ^{14}C-β-chain, 198.

leucine with group II tRNA deleted, the ratio observed was 0.70. The ratio calculated from tryptic peptides obtained by analysis of a total product digestion was 0.59. Fingerprint analysis demonstrated, in addition, uniform labeling of all peptides in the two chains, both in the presence and absence of group II tRNA. The average of all experiments showed an α to β chain ratio of 0.67, and a range from 0.59 to 0.70.

DISCUSSION

Unequal chain synthesis in the absence of a tRNA fraction indicates that different combinations of tRNA species are utilized in translating mRNA molecules. Even though the α and β chains of rabbit hemoglobin are similar and both contain all 20 amino acids (13), the α chain must contain at least one codon which is not used in the β chain (or is used much less often). This possibility exists because most amino acids are recognized by more than one codeword (14). Preliminary evidence suggests that the tRNA responsible for the inhibition observed in our studies might be a $tRNA^{Gly}$ species.

Because the present experiments have been carried out with arbitrary combinations of tRNA fractions and since rabbit liver rather than rabbit reticulocyte fractionated tRNA has been used, we are not proposing that the translational control we have demonstrated is representative of the intact reticulocyte. We do suggest, however, that since the rate of translation of a specific natural mRNA can be differentially slowed *in vitro* by the simple manipulation of deleting a tRNA fraction, then it is theoretically possible that a similar mechanism might occur in the cell by the mutation, or the turning off, of a tRNA genome.

ACKNOWLEDGEMENT

We would like to thank Mr. Moses B. Middleton for excellent technical assistance.

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