Cell-free Hemoglobin Synthesis

II. CHARACTERISTICS OF THE TRANSFER RIBONUCLEIC ACID-DEPENDENT ASSAY SYSTEM*

JEFFREY M. GILBERT AND W. FRENCH ANDERSON

From the Section on Human Biochemistry, National Heart and Lung Institute, National Institutes of Health,
Bethesda, Maryland 20014

SUMMARY

A cell-free protein-synthesizing system derived from rabbit reticulocytes is described which is dependent on the addition of transfer RNA for the translation of endogenous hemoglobin messenger RNA. Product analysis indicates that the system is active in the initiation of new chains. When hemoglobin is synthesized in the presence of a limiting amount of tRNA, there is a 50% decrease in α chain production relative to β chain production. Deacylated rabbit liver tRNA cannot, but deacylated Escherichia coli tRNA can, be substituted for transfer RNA for the translation of endogenous hemoglobin.

Although many of the steps involved in Escherichia coli protein synthesis have been determined (1), gaps remain in understanding the mechanism and regulation of protein synthesis in higher organisms. The rabbit reticulocyte cell-free hemoglobin-synthesizing system appears to be well suited for studying mammalian protein synthesis. Reticulocytes, obtainable in large quantity from phenylhydrazine-treated rabbits (2), have lost their nucleus and appear to contain only the apparatus necessary for translation of previously synthesized messenger RNA (3). In addition, 85 to 95% of the protein synthesized is one product, hemoglobin (4). Procedures for characterizing hemoglobin are well established (5).

Allen and Schweet (6) described a cell-free system capable of completing the synthesis of nascent hemoglobin chains on rabbit reticulocyte polysomes. Miller and Schweet (7) reported a similar system which was capable of initiating new hemoglobin chains and which depended for its activity on the addition of a ribosomal salt wash fraction. Our work further extends these reports. The present paper describes a cell-free system derived from rabbit reticulocytes which is dependent on added transfer RNA for translating endogenous mRNA. This system, which maintains the capacity to initiate new chains, is being utilized to study the functions of tRNA in hemoglobin biosynthesis (8, 9) and to analyze the biological activity of individual tRNA species. Isolation of active factors from the ribosomal wash (enzyme fraction) is being reported separately (10). tRNA-dependent cell-free systems derived from E. coli and rabbit reticulocytes which translate artificial mRNA templates have been described (11, 12).

EXPERIMENTAL PROCEDURE

Materials—Uniformly labeled L-[14C]-Valine, uniformly labeled L-[15N]-leucine, and L-[4,5-2H]-leucine were obtained from Amersham-Searle and L-[2,3-2H]-valine from Schwarz BioResearch. The isotopes were either used directly or diluted with unlabeled amino acid; the specific activity used in each experiment is listed in the appropriate legend. Isotopes for the data in Table IV were obtained from Amersham-Searle, Schwarz BioResearch, or Tracerlab. In some cases, two sources of isotope were used in separate experiments to verify results. Dithiothreitol, phosphoenolpyruvate, phosphoenolpyruvate kinase, 3C-l-amino acids, ninhydrin, heparin, and DEAE-cellulose (Cellex D, Bio-Rad) were obtained from Calbiochem; ATP and GTP from Schwarz BioResearch; vitamin B12, folic acid, and phenylhydrazine hydrochloride from Sigma; 1-fluoro-2,4-dinitrobenzene from Eastman; chromatography paper (No. 3MM) and carboxymethyl cellulose (CM-52) from Whatman; rabbit liver tRNA and E. coli B tRNA from General Biochemicals; and trypsin from Worthington.

Preparation of Rabbit Reticulocyte Lysate—Lysate made from rabbit blood having a high reticulocyte count was used as the starting material for the preparation of polysomes, enzyme fraction, and tRNA. New Zealand white rabbits less than 8 weeks of age and weighing less than 2.5 kg were injected subcutaneously with a 2.5% (w/v) phenylhydrazine solution at a dosage of 0.25 ml per kg per day for 6 consecutive days. On the 1st day of injections each rabbit was given also an intramuscular injection of 10 mg of cyanocobalamin and 100 mg of folic acid in 100 ml of 0.9% NaCl. No phenylhydrazine injection was given on the 7th day, and the rabbits were bled via direct cardiac puncture on the 8th day with a heparinized needle and syringe. The isotopes were either used directly or diluted with unlabeled amino acid; the specific activity used in each experiment is listed in the appropriate legend. Isotopes for the data in Table IV were obtained from Amersham-Searle, Schwarz BioResearch, or Tracerlab. In some cases, two sources of isotope were used in separate experiments to verify results. Dithiothreitol, phosphoenolpyruvate, phosphoenolpyruvate kinase, 3C-l-amino acids, ninhydrin, heparin, and DEAE-cellulose (Cellex D, Bio-Rad) were obtained from Calbiochem; ATP and GTP from Schwarz BioResearch; vitamin B12, folic acid, and phenylhydrazine hydrochloride from Sigma; 1-fluoro-2,4-dinitrobenzene from Eastman; chromatography paper (No. 3MM) and carboxymethyl cellulose (CM-52) from Whatman; rabbit liver tRNA and E. coli B tRNA from General Biochemicals; and trypsin from Worthington.

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TABLE I

Dependence of protein synthesis on various components in complete system

| Contents of reaction mixture | Activity a | Inhibition % |
|-----------------------------|------------|-------------|
| Complete                    | 21.0       | 0           |
| Minus Tris-HCl              | 21.0       | 0           |
| Minus MgCl2                 | 0.2        | 99          |
| Minus dithiothreitol        | 22.0       | 0           |
| Minus ATP                   | 1.0        | 95          |
| Minus GTP                   | 19.5       | 7           |
| Minus phosphoenolpyruvate, phosphoenolpyruvate kinase | 5.0 | 76 |
| Minus 14C-amino acids       | 4.0        | 81          |
| Minus enzyme fraction       | 0.0        | 100         |
| Minus high-low polysomes   | 0.0        | 100         |
| Minus tRNA                  | 0.8        | 96          |

* Activity is expressed as micromicromoles of 14C-valine incorporated into protein in 30 min.

Preparation of tRNA-free Polysomes (High-Low Polysomes)—The lysate preparation was centrifuged at 78,000 × g for 90 min. The supernatant fraction was removed and used for preparation of tRNA (see below). The upper surface of the polysome pellets was washed with standard sucrose (0.25 M sucrose, 1 mm dithiothreitol, and 0.1 mm EDTA, neutralized to pH 7 with NaOH) in order to remove amorphous material. The pellets were then suspended in sufficient standard sucrose to give an absorbance at 260 nm of between 275 and 300 units per ml (measured in water). The polysome suspension was treated with high salt as described by Miller and Schweet (7). KCl (4.0 M) was added slowly over a 2-min period with stirring to the polysomes until a final KCl concentration of 0.30 M. Six milliliters of the diluted solution were mixed with 3.5 ml of washed packed DEAE-cellulose. Complete stability was achieved after 2 weeks and loses approximately 10% activity after 1 month.

Preparation of tRNA-free Enzyme Fraction—The tRNA-free enzyme fraction was prepared from the high salt polysome wash fraction. The tRNA-free enzyme fraction was prepared by treatment with DEAE-cellulose. Cellulose D anion exchange cellulose (0.91 meq per g of exchange capacity) was cycled according to the procedure of Peterson and Sober (13), equilibrated with 0.02 M Tris-HCl (pH 7), and stored at 2° in the same buffer. Complete stability was achieved after 2 months. The yield is approximately 2 A260 units of high-low polysomes per ml of lysate.

Preparation of Unfractionated Rabbit Reticulocyte tRNA—An equal volume of water-saturated redistilled phenol (but no potassium acetate) was added to the high speed (105,000 × g) supernatant from the lysate centrifugation (see above), and the mixture was shaken vigorously for 5 min at room temperature. The phases were separated by centrifugation at 10,000 × g for 10 min, and the aequous phase was removed. The phenol phase was re-extracted with 0.5 volume of 2% potassium acetate (pH 5.5)-1 mm β-mercaptoethanol. The aequous phases were pooled, and the nucleic acid was precipitated by the addition of 2 volumes of -20°C 95% ethanol. The mixture was allowed to remain at -20°C for 12 to 16 hours, and the RNA was collected by centrifugation at 10,000 × g for 15 minutes at -20°C. The RNA was dissolved in 0.01 M Tris HCl (pH 7), 0.01 M KCl, 0.01 M Mg++, concentration. Each 50-μl reaction mixture contained 0.16 A260 unit of high-low polysomes, 0.057 A260 unit of unfractionated rabbit reticulocyte tRNA, 15 μl (0.15 mg) of enzyme fraction, 14C-valine (specific activity, 87 mCi per mmole), and other components as described under “Experimental Procedure.” Incubation was for 30 min at 37°.

The yield is approximately 2 A260 units of high-low polysomes per ml of lysate.

Preparation of Unfractionated Rabbit Reticulocyte tRNA—An equal volume of water-saturated redistilled phenol (but no potassium acetate) was added to the high speed (105,000 × g) supernatant from the lysate centrifugation (see above), and the mixture was shaken vigorously for 5 min at room temperature. The phases were separated by centrifugation at 10,000 × g for 10 min, and the aequous phase was removed. The phenol phase was re-extracted with 0.5 volume of 2% potassium acetate (pH 5.5)-1 mm β-mercaptoethanol. The aequous phases were pooled, and the nucleic acid was precipitated by the addition of 2 volumes of -20°C 95% ethanol. The mixture was allowed to remain at -20°C for 12 to 16 hours, and the RNA was collected by centrifugation at 10,000 × g for 15 minutes at -20°C. The RNA was dissolved in 0.01 M Tris HCl (pH 7), 0.01 M KCl, 0.01 M Mg++. The yield is approximately 2 A260 units of high-low polysomes per ml of lysate.

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Cell-free Hemoglobin Synthesis. II

ATP CONCENTRATION (mM)

FIG. 2. 14C-Valine incorporation into protein as a function of ATP concentration. Each 50-μl reaction mixture contained 0.31 A260 unit of high-low polysomes, 0.051 A260 unit of unfraccionated rabbit reticulocyte tRNA, 15 μl (0.16 mg) of enzyme fraction, 14C-Valine (specific activity, 65 mCi per mmole) and other components as described under “Experimental Procedure.” Incubation was for 30 min at 37°C.

PEP:ONCEN~RATION (mM)

FIG. 3. 14C-Valine incorporation into protein as a function of phosphoenolpyruvate and phosphoenolpyruvate kinase concentrations. Each 50-μl reaction mixture contained 0.41 A260 unit of high-low polysomes, 0.051 A260 unit of unfraccionated rabbit reticulocyte tRNA, 15 μl (0.19 mg) of enzyme fraction, 14C-Valine (specific activity, 87 mCi per mmole) and other components as described under “Experimental Procedure.” Incubation was for 30 min at 37°C. Other experiments have shown similar values for 0.2 and 0.3 i.u. of phosphoenolpyruvate kinase per reaction mixture and the latter value has been used routinely as the standard amount.

MgCl2, 1 mM β-mercaptoethanol, and 0.1 mM sodium EDTA. Sephadex G-200 gel filtration was performed according to the procedure of Delhais and Stachelin (14) as modified by Anderson (15). The second A260 peak contained the tRNA, which was pooled and then concentrated by ethanol precipitation as described above. The tRNA was dissolved in 0.1 mM MgCl2 and 1 mM β-mercaptoethanol, dialyzed against the same solution, lyophilized, and stored at -20°C. The yield is about 1 A260 unit per ml of lysate (measured in 1 mM MgCl2).

FIG. 4. 14C-Valine incorporation into protein as a function of the amino acid concentration. Equimolar amounts of 14C-L-valine (specific activity, 87 mCi per mmole) and each of the 19 14C-amino acids were mixed together. One to three dilutions (with water) of this mixture were assayed and plotted on a logarithmic scale. Each 50-μl reaction mixture contained 0.41 A260 unit of high-low polysomes, 0.051 A260 unit of unfraccionated rabbit reticulocyte tRNA, 15 μl (0.19 mg) of enzyme fraction, and other components as described under “Experimental Procedure.” Incubation was for 20 min at 37°C.

FIG. 5. 14C-Valine incorporation into protein as a function of enzyme fraction added to the reaction. Each 50-μl reaction mixture contained 0.16 A260 unit of high-low polysomes, 0.057 A260 unit of unfraccionated rabbit reticulocyte tRNA, 14C-L-valine (specific activity, 87 mCi per mmole) and other components as described under “Experimental Procedure.” Incubation was for 30 min at 37°C.

Preparation of Rabbit Liver and E. coli B tRNA—E. coli and rabbit liver tRNA obtained commercially was further purified by Sephadex G-200 gel filtration and stored as described in the preceding section.

Protein Synthesis Assay Conditions—Each 50-μl assay mixture contained the following: Tris-HCl (pH 7.5), 20 mM; KCl, 80
Fig. 6. $^{14}$C-Valine incorporation into protein as a function of high-low polysomes added to the reaction. Each 50-μl reaction mixture contained 15 μl of enzyme fraction (0.19 mg), $^{14}$C-L-valine (specific activity, 90 mCi per mmole), and other components as described under "Experimental Procedure." Those reactions labeled with tRNA also contained 0.051 A$_{260}$ unit of unfractionated rabbit reticulocyte tRNA per tube. Incubation was for 20 min at 37°C.

Fig. 7. $^{14}$C-Valine incorporation into protein as a function of time of incubation at 37°C. A 50-μl aliquot was removed at each time point from a 500-μl reaction mixture which contained 2.2 A$_{260}$ units of high-low polysomes, 0.51 A$_{260}$ unit of unfractionated rabbit reticulocyte tRNA, 150 μl (1.6 mg) of enzyme fraction, $^{14}$C-L-valine (specific activity, 90 mCi per mmole), and other components as described under "Experimental Procedure."

Fig. 8. $^{14}$C-Valine incorporation into protein as a function of unfractionated rabbit reticulocyte tRNA added to the reaction. Each 50-μl reaction mixture contained 0.164 A$_{260}$ unit of high-low polysomes, 15 μl (0.15 mg) of enzyme fraction, $^{14}$C-L-valine (specific activity, 87 mCi per mmole), and other components as described under "Experimental Procedure." Incubation was for 30 min at 37°C. Values for $^{14}$C-valine incorporation are not exactly comparable among Figs. 1 to 8 since several different polysome and enzyme fraction preparations were utilized for the studies.

mm (partially supplied by the enzyme fraction); MgCl$_2$, 3.0 mm; ATP (neutralized to pH 7 with KOH before use), 1.0 mm; GTP (neutralized to pH 7 with KOH before use), 0.2 mm; phosphoenolpyruvate (neutralized to pH 7 with KOH before use), 3.0 mm; phosphoenolpyruvate kinase, 0.3 i.u.; dithiothreitol, 1.0 mm; uniformly labeled $^{14}$C-valine, 0.08 mM; $^{15}$N-amino acids, 0.08 mM each; high-low polysomes, 0.1 to 0.5 A$_{260}$ unit; enzyme fraction (10 to 15 mg of protein per ml in 0.3 M KCl), 15 μl; unfractionated rabbit reticulocyte tRNA, 0.02 to 0.05 A$_{260}$ unit. Incubation was carried out for 20 or 30 min at 37°C and the reactions were stopped by the addition of 2 μl of 10% trichloracetic acid. The mixtures were heated at 90-95°C for 10 min and then cooled in an ice bath for 10 min. The trichloracetic acid-precipitable material was collected on nitrocellulose filters (Millipore, type HA, 0.45 μm pore size, 25-mm diameter). Each filter was washed with cold 5% trichloracetic acid, dried, and counted in 10 ml of Liquifluor in toluene in a liquid scintillation counter at an efficiency of 82% for $^{14}$C.

Chromatography of Globin Chains on Carboxymethyl Cellulose—Separation of the α and β chains was carried out by the method of Dintzis (4).

Preparation of Uniformly Labeled Hemoglobin—Preparation of rabbit hemoglobin uniformly labeled with radioactive L-valine or L-leucine for use as standards was carried out by the method of Borsook as described by Schapira et al. (16).

Two-Dimensional Chromatography of Tryptic Peptides (Fingerprinting)—Globin was prepared from the salt-free hemoglobin sample by an acid-acetone precipitation and was subjected to tryptic digestion (16). The tryptic peptides were separated by the method of Dintzis (4). Fingerprint analysis of separate α
overnight enzyme fraction, W-L-leucine (specific activity, 312 mCi per mmole). The p4 peptide was lost when it ran off the paper after chromatography was performed in order to verify the origin of each peptide. 

The reaction mixture (1.0 ml) on which analysis was carried out contained 5.96 A_280 units of high-low polysomes, 0.95 A_280 unit of rabbit reticulocyte unfraccionated tRNA, 0.3 ml (3.0 mg) of enzyme fraction, ^14C-L-leucine (specific activity, 312 mCi per mmole), and other components as described under "Experimental Procedure." Incubation was for 40 min at 37°C. Hemoglobin, 1.5 mg, uniformly labeled with ^3H-L-leucine (specific activity, 1000 mCi per mmole) was added and the mixture was dialyzed overnight against 4 liters of toluene-saturated water at 2°C. Fingerprint analysis was carried out as listed under "Experimental Procedure." Total ^14C counts per min were normalized to total ^3H counts per min and the ratio of ^14C to ^3H counts per min was then calculated for each peptide.

Hemoglobin, 3.0 mg, uniformly labeled with ^14C-L-leucine mixed and fingerprinted with 1.5 mg of hemoglobin uniformly labeled with ^3H-L-leucine. The p4 peptide was lost when it ran off the paper during chromatography.

Table II

| Peptide | Ratio of ^14C-L-leucine to ^3H-L-leucine | A | B |
|---------|------------------------------------------|---|---|
| α1      | 1.05                                     | 1.02 | 0.96 |
| α5      | 1.07                                     | 1.07 | 0.94 |
| α8,9    | 1.07                                     | 1.07 | 0.96 |
| α9      | 1.11                                     | 1.04 | 0.97 |
| β1      | 0.96                                     | 0.92 | 0.97 |
| β2      | 0.92                                     | 0.96 | 0.97 |
| β3      | 0.92                                     | 0.97 | 0.97 |
| β4      | 0.93                                     | 0.97 | 0.97 |
| β10     | 0.90                                     | 1.02 | 0.97 |

* Peptide nomenclature of Dintzis (4).

† ^14C-L-leucine-labeled reaction mixture product mixed with ^3H-L-leucine uniformly labeled hemoglobin. The reaction mixture (1.0 ml) contained 1.0 A_280 units of high-low polysomes, 1.0 A_280 unit of rabbit reticulocyte unfraccionated tRNA, 300 μl (3.0 mg) of enzyme fraction, ^14C-L-leucine (specific activity, 312 mCi per mmole), and other components as described under "Experimental Procedure." Incubation was for 40 min at 37°C. Hemoglobin, 1.5 mg, uniformly labeled with ^3H-L-leucine (specific activity, 1000 mCi per mmole) was added and the mixture was dialyzed overnight against 4 liters of toluene-saturated water at 2°C. Finger- print analysis was carried out as listed under "Experimental Procedure." Total ^14C counts per min were normalized to total ^3H counts per min and the ratio of ^14C to ^3H counts per min was then calculated for each peptide.

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and α chains obtained from carboxymethyl cellulose chromatography was performed in order to verify the origin of each peptide. Peptides were eluted from the chromatography paper with 5% acetic acid and counted in Bray's solution in a liquid scintillation counter at single label efficiencies of 74% for ^14C and 40% for ^3H, and double label efficiencies of 56% for ^14C and 24% for ^3H.

NH_2-Terminal Valine Analysis—NH_2-Terminal analysis of hemoglobin was carried out as described by Bishop, Leahy, and Schweit (17). Derivatives of the NH_2-terminal amino acid were made with 1-fluoro-2,4-dinitrobenzene. The derivative is extracted into the ether phase after acid hydrolysis while unsubstituted amino acids remain in the aqueous phase. Aliquots of each phase were counted in Triton X-100 (1 liter of Triton X-100, 2 liters of toluene, 165 ml of Liquifluor) with an efficiency of 79% for ^14C and 44% for ^3H.

RESULTS

Optimization of System—The optimum concentrations of components are listed under "Experimental Procedure." As can be seen in Table I, the system is dependent on Mg++, ATP, an ATP-generating system, amino acids, enzyme fraction, polysomes, and tRNA. The nondependence on dithiothreitol is probably because an adequate amount of this component is already present in the enzyme and polysome fractions. Since the enzyme fraction is in 0.3 M KCl, monovalent cation dependence was not attempted. An optimum for KCl was found at 80 to 90 mM. Optimum concentrations for Mg++, ATP, phosphoenolpyruvate, phosphoenolpyruvate kinase, and amino acids were determined from the data shown in Figs. 1 to 4. Although the use of creatine phosphate and creatine phosphokinase has been found to result in greater activity in the lysate system (18), we have found these reagents to be less effective than phosphoenolpyruvate and phosphoenolpyruvate kinase in this fractionated system. Hemin has no effect in this system, and the addition of DPN+ results in a small, but not reproducible, increase in the extent of protein synthesis.

For maximum activity it is essential that a correct ratio of polyamines to enzyme fraction be used. The enzyme fraction contains, in addition to T1, T2, and aminocyl-tRNA synthetases, other factors required for protein synthesis de novo (7, 10). The concentration curve for this multifactor component is sigmoidal, not linear, as can be seen in Fig. 5. If a lesser amount of polyamines is used the enzyme fraction curve is shifted to the left, resulting in a more obvious saturation plateau; if a greater amount of polyamines is used the curve is shifted to the right, resulting in an inability to saturate the system for this particular enzyme fraction preparation within a KCl concentration of 90 mM. In the presence of saturating concentrations of enzyme fraction and tRNA, the polyamines are rate-limiting as shown in Fig. 6. Under these conditions, incorporation of amino acids into hemoglobin is linear for 45 min, as shown in Fig. 7. The system has a 25- to 30-fold dependence on added tRNA as shown in Fig. 8 and Table I.

Characterisation of Products—To confirm that the products of the tRNA-dependent assay system are complete new chains of hemoglobin the following studies were performed. (a) The ^14C-

Table III

| Sample analyzed | NH_2-Terminal valine[^a] | NH_2-Terminal valine[^b] |
|----------------|-------------------------|-------------------------|
| Hemoglobin product labeled with ^14C-valine... | 7.0 | 0.48 |
| Hemoglobin product labeled with ^14C-valine... | 7.25 | 0.43 |
| Hemoglobin uniformly labeled with ^14C-valine... | 7.14 | 0.0 |
| Theoretical values for uniformly labeled hemoglobin... | 7.14 | 0.0 |

[^a]: Calculated by dividing counts per min in the ether phase by counts per min in the ether plus water phases and multiplying by 100 (see "Experimental Procedure").

[^b]: The reaction mixture (1.0 ml) on which analysis was carried out contained 5.96 A_280 units of high-low polysomes, 0.95 A_280 unit of rabbit reticulocyte unfraccionated tRNA, 0.3 ml (3.0 mg) of enzyme fraction, ^14C-L-valine (specific activity, 260 mCi per mmole), and other components as described under "Experimental Procedure." Incubation was for 45 min at 37°C. After the incubation, carrier rabbit hemoglobin (0.4 mg) was added to the reaction mixture and NH_2-terminal analysis was carried out as described under "Experimental Procedure."

[^c]: Same reaction conditions as described in b, except that the radioactive amino acid was ^14C-L-leucine (specific activity, 312 mCi per mmole).

[^d]: ^3H-L-Valine was at a specific activity of 6000 mCi per mole.

[^e]: ^5H-L-Leucine was at a specific activity of 6000 mCi per mole.
FIG. 9. Carboxymethyl cellulose chromatography of the $\alpha$ and $\beta$ chains synthesized in the presence of excess and of limiting unfractionated rabbit reticulocyte tRNA. See text for details.

The $^3$H and $^{14}$C scales were adjusted to normalize the $\beta$ peaks. The $\alpha$ values have not been adjusted to account for the one less leucine in the $\alpha$ chain compared with the $\beta$ chain. Absolute micromicromoles of labeled leucine obtained by integrating under the peaks (and confirmed by measuring radioactivity in the pooled peaks) are: $^3$H-$\alpha$ chain, 286; $^3$H-$\beta$ chain, 323; $^{14}$C-$\alpha$ chain, 63.2. Efficiencies for double label counting were: $^3$H, 25%; $^{14}$C, 58%.

valine-labeled reaction mixture was chromatographed on a carboxymethyl cellulose column in the presence of carrier rabbit hemoglobin. The radioactive products exactly cochromatograph with the absorbance peaks of rabbit $\alpha$ and $\beta$ chains. (b) The $^{14}$C-valine-labeled reaction mixture was combined with $^3$H-valine uniformly labeled rabbit hemoglobin and the pooled sample was fingerprinted, the peptides were eluted, and the ratio of $^{14}$C to $^3$H counts per min in each peptide was determined. The data, given in Table II, show uniform labeling of the $^{14}$C peptides; i.e. as much label was found in the NH$_2$-terminal Peptides $\alpha_1$ and $\beta_1$ as in the internal peptides, indicating new chain synthesis. This result was the same whether or not the ribosomes were removed from the reaction mixture after the incubation. (c)

FIG. 10. Effect of addition of unfractionated rabbit reticulocyte, rabbit liver, or E. coli tRNA on the rate of hemoglobin synthesis. Each 50-μl assay mixture contained 0.16 A$_{260}$ unit of high-low polysomes, 15 μl (0.15 mg) of enzyme fraction, $^{14}$C-L-valine (specific activity, 57 mCi per mmole), and other components as described under “Experimental Procedure.” Incubation was for 30 min at 37°C.

TABLE IV

| Amino acid tested | E. coli$^a$ | Rabbit liver$^a$ | Rabbit reticulocyte$^a$ |
|-------------------|------------|-----------------|---------------------|
|                   | $\lambda^b$ | $\rho^a$        | $\lambda^b$         |
|                   | $\lambda^b$ | $\rho^a$        | $\lambda^b$         |
| Ala               | 77         | 80              | 73                  |
| Arg               | 112        | 107             | 106                 |
| Asn               | 3          | 6               | 32                  |
| Asp               | 2          | 7               | 86                  |
| Cys               | 28         | 21              | 28                  |
| Glu               | 5          | 0               | 65                  |
| Gly               | 7          | 2               | 79                  |
| His               | 3          | 3               | 21                  |
| Ile               | 52         | 52              | 47                  |
| Leu               | 58         | 58              | 107                 |
| Lys               | 45         | 49              | 47                  |
| Met               | 16         | 39              | 49                  |
| Phe               | 0          | 0               | 30                  |
| Pro               | 1          | 0               | 43                  |
| Ser               | 34         | 32              | 82                  |
| Thr               | 11         | 14              | 67                  |
| Trp               | 6          | 9               | 19                  |
| Tyr               | 14         | 11              | 32                  |
| Val               | 7          | 4               | 62                  |
| Totals            | 481        | 494             | 1158                |

$^a$ Results are expressed as micromicromoles of amino acid per A$_{260}$ unit of unfractionated tRNA. The total percentage acylation shown at the bottom of the table is based on 1870 μmole per A$_{260}$ unit of tRNA (measured in 1 mM MgCl$_2$).

$^b$ Acylation was carried out under standard acylating (A) conditions (15). Each 25-μl reaction contained: potassium phosphate (pH 6.8), 0.1 M; MgCl$_2$, 5 mM; ATP, 2 mM; $^{14}$C-amino acid, 0.1 mM; 19 $^{14}$C-amino acids, 0.1 mM; 0.1 to 0.4 A$_{260}$ unit of tRNA, and 90 μg of rabbit reticulocyte unfractionated synthetase protein. Preparation of the synthetase protein and method of assay were described previously (15). Incubation was for 15 min at 37°C. Three or more tRNA concentrations were assayed for each point. For every amino acid, all three tRNA sources were assayed under both conditions A and P during the same experiment.

$^c$ Acylation was carried out under protein synthesis (P) conditions as listed under “Experimental Procedure,” but with the deletion of polysomes and enzyme fraction and the addition of 90 μg of synthetase protein. Extent of acylation under protein synthesis conditions is as good or better than that obtained under standard conditions. Unfractionated E. coli tRNA falls into three classes: (a) species not acylated by rabbit reticulocyte enzyme under protein synthesis conditions—Gln, Cys, Pha, Pro; (b) species acylated equally well—Ala, Arg, Cys, Ile, Lys; and (c) species only partially acylated—remainder.
Effect of Limiting the tRNA Concentration In order to determine the effect on the rates of synthesis of the α and β hemoglobin chains that is produced by an alteration in the concentration of unfractionated tRNA in the reaction mixture, the following experiment was performed. Two reaction mixtures were incubated simultaneously: one (0.5 ml) contained 3H-L-leucine (1000 mCi per mmole) and 0.05 A₄₀₀ unit of rabbit reticulocyte tRNA per 50 μl ("excess" tRNA); the other (1.0 ml) was identical except for 14C-L-leucine (311 mCi per mmole) and only 0.002 A₄₀₀ unit tRNA per 50 μl ("limiting" tRNA). The two mixtures were pooled after a 45-min incubation at 37°, the hem was removed by acid-acetone precipitation, and the globin chains were separated by carboxymethyl cellulose column chromatography. As shown in Fig. 9, although the synthesis of both chains was depressed under conditions of "limiting" tRNA, α chain synthesis was reduced to 50% of the production of β chains.

Effect of Adding tRNA from Heterologous Sources—Rabbit liver tRNA appears to stimulate the rate of hemoglobin synthesis to an equal extent as rabbit reticulocyte tRNA at limiting tRNA concentrations, as shown in Fig. 10. However, a higher rate of synthesis is achieved in the presence of saturating reticulocyte tRNA than liver tRNA. E. coli tRNA is unable to stimulate the synthesis of hemoglobin, as shown in Fig. 10. A partial explanation for this result is seen in Table IV. Rabbit reticulocyte aminocyl-tRNA synthetases are unable to acylate some species of E. coli tRNA. If unfractionated E. coli tRNA is first acylated with E. coli aminocyl-tRNA synthetases, purified, and then added to the rabbit reticulocyte reaction mixture, stimulation of hemoglobin synthesis does occur.1

DISCUSSION

The cell-free protein-synthesizing system described in this paper is unique in that it is the first nonbacterial assay system reported that is dependent on added tRNA to translate natural messenger RNA. The products of the reaction are primarily complete α- and β-globin chains; i.e. initiation of new chains is active. This assay system is being utilized to study the role of tRNA in hemoglobin biosynthesis, the biological activity of individual tRNA species, and the mechanism of initiation in the cell-free synthesis of hemoglobin.

A role of tRNA in regulating the rate of translation of hemoglobin mRNA was first postulated by Itano (19). A theoretical foundation for such a role was shown when Weisblum et al. (20) demonstrated that leucine tRNA can be separated into two fractions which appear to insert leucine into different positions in the hemoglobin chains. This work has been extended to show that a similar phenomenon appears to exist for tRNAs carrying serine (21), arginine (22), or glutamic acid (23). Since different species of tRNA might be responsible for inserting an amino acid into different positions in the hemoglobin chains, the presence of a limiting amount of one species might produce a reduced rate of mRNA translation at a given point. Winslow and Ingram (24) have shown, in fact, that the rate of assembly of hemoglobin chains in human bone marrow cells may contain a "control point" in the synthesis of each chain beyond which the growth of the polypeptide chain is reduced. Clegg et al. (25), however, were unable to detect a control point during the assembly of β chains in either normal or thalassemic reticulocytes.

In previous studies with cell-free E. coli systems, we have examined the effect of tRNA species on the rate of translation of artificial mRNA templates (11). 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 determine 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 was examined in the tRNA-dependent rabbit reticulocyte cell-free system. When only a limiting amount of tRNA is available for hemoglobin synthesis, the data of Fig. 9 indicate that α chain production is reduced to 50% of the production of β chains. When a saturating amount of unfractionated tRNA is used to supplement the system, approximately equal α and β chain synthesis was produced (8). It therefore appears that one (or more) species of tRNA, which is used to a greater extent for α than for β chain synthesis, has become rate-limiting. In previous studies a similar result was obtained when cell-free hemoglobin synthesis was carried out in the presence of saturating levels of tRNA, but where several tRNA species were deleted (8). The fractionated tRNA was produced by combining portions from a Freon reversed phase chromatography of unfractionated tRNA.

Unequal chain synthesis with fractionated tRNA or limiting concentrations of unfractionated tRNA lends support to the hypothesis that a different combination of tRNA species is utilized in translating α- and β-hemoglobin mRNA molecules in vivo. Even though the α and β chains of rabbit hemoglobin both contain all 20 amino acids, the α chain must contain at least one codon which is used either less often or not at all in β chain synthesis. This possibility exists because most amino acids are recognized by more than one codeword (26). In fact, evidence exists which suggests that the leucine codon UUG might be utilized only once in the rabbit α chain and not at all in the β chain (20, 27). Preliminary evidence suggests that the tRNA responsible for the inhibition observed in our studies, however, might be a species of tRNA specific for glycine. This conclusion is based on the observation that the synthesis of 14C-valine from aminocyl-tRNA into hemoglobin, produced by supplementing a tRNA-limiting system with additional tRNA plus aminocyl-tRNA synthetases, is lost if the one amino acid, glycine, is omitted from the reaction mixture.

The present experiments do not examine control in vivo of normal rabbit reticulocyte hemoglobin synthesis. However, since the rates of translation of the hemoglobin mRNAs can be differentially slowed in vitro either by reducing the total amount of tRNA present or by deleting a tRNA fraction, it is not unreasonable to postulate that similar effects might take place within the intact cell either as normal control mechanisms or as a result of mutations.

The experiments examining hemoglobin synthesis in the presence of unfractionated tRNA prepared from heterologous sources are not easy to interpret. It is possible that liver and reticulocyte tRNA contain the identical types and amounts of tRNA species in vivo, but not after purification. Certainly, the purification of rabbit liver tRNA requires several different steps from those described under "Experimental Procedure" for the purification of reticulocyte tRNA since the former must be separated from active nucleases, membranes, nuclear contents, etc. It is also possible, however, that liver cells and reticulocytes each

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contain their own spectrum of tRNA species and that these species reflect the synthetic requirements of that cell type.

The data of Fig. 10 and Table 1 indicate that at least some E. coli tRNA species are distinctly different from rabbit species. Previous work from this laboratory examining heterologous acylation reactions between E. coli, rabbit liver, and human spleen sources is consistent with the present data (15). Under investigation is the possibility that at least some E. coli tRNA species cannot be directly utilized in cell-free mammalian protein synthesis, but might participate indirectly by transferring their amino acids to reticulocyte tRNA species.*

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REFERENCES

1. Leitgely, P., and Soll, D., Bacteriol. Rev., 33, 263 (1969).
2. London, I. M., Sheinin, D., and Rittenberg, D., J. Biol. Chem., 183, 749 (1950).
3. Marks, P. A., Burek, E. R., and Schlesinger, D., Proc. Nat. Acad. Sci. U. S. A., 48, 2163 (1962).
4. Dintzis, H. M., Proc. Nat. Acad. Sci. U. S. A., 47, 247 (1961).
5. Braunitzer, G., Hilse, R., Rudloff, V., and Hilschmann, N., Advan. Protein Chem., 19, 1 (1964).
6. Allen, E. H., and Schweitz, R. S., J. Biol. Chem., 237, 700 (1962).
7. Miller, R. L., and Schweitz, R., Arch. Biochem. Biophys., 139, 632 (1968).
8. Anderson, W. F., and Gilbert, J. M., Biochem. Biophys. Res. Commun., 36, 456 (1969).
9. Anderson, W. F., and Gilbert, J. M., Cold Spring Harbor Symp. Quant. Biol., 34, 585 (1969).
10. Shafritz, D. A., Prichard, P. M., Gilbert, J. M., and Anderson, W. F., Biochem. Biophys. Res. Commun., 36, 721 (1970).
11. Anderson, W. F., Proc. Nut. Acad. Sci. U. S. A., 62, 500 (1969).
12. Gupta, N. K., J. Biol. Chem., 243, 4595 (1968).
13. Peterson, E. A., and Sober, H. A., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. V, Academic Press, New York, 1962, pp. 3-27.
14. Delius, N., and Stahlhelin, M., Biochim. Biophys. Acta, 119, 385 (1966).
15. Anderson, W. F., Biochemistry, 8, 3887 (1969).
16. Schafritz, G., Rosa, J., Malekina, N., and Padiev, P., in L. Grossman and K. Moldave (Editors), Methods in enzymology, Vol. XII, Part R, Academic Press, New York, 1968, pp. 747-769.
17. Bishop, J., Leahy, J., and Schweitz, R., Proc. Nat. Acad. Sci. U. S. A., 46, 1030 (1966).
18. Adamson, S. D., Herbert, E., and Godchaux, W., III, Arch. Biochem. Biophys., 125, 671 (1968).
19. Itano, H. A., in J. H. F. Jones (Editor), Abnormal hemoglobin in Africa, Blackwell Scientific Publications, Oxford, 1965, pp. 3-16.
20. Weissblum, B., Gonano, F., Von Ehrenstein, G., and Benzer, S., Proc. Nat. Acad. Sci. U. S. A., 53, 328 (1965).
21. Gonano, F., Biochemistry, 6, 977 (1967).
22. Weissblum, B., Chervil, J. D., Bock, R. M., and Soll, D., J. Mol. Biol., 26, 275 (1967).
23. Seifiya, T., Takahashi, K., and Uryu, T., Biochim. Biophys. Acta, 182, 811 (1969).
24. Winslow, R. M., and Ingram, V. M., J. Biol. Chem., 241, 1144 (1966).
25. Clegg, J. B., Weatherall, D. J., Na-Nakorn, S., and Wasi, P., Nature, 220, 664 (1968).
26. Nirenberg, M., Caskey, T., Marshall, R., Brimacome, R., Kellogg, D., Doctor, B., Hatfield, D., Levin, J., Rottman, F., Pestka, S., Wilcox, M., and Anderson, F., Cold Spring Harbor Symp. Quant. Biol., 31, 11 (1966).
27. Von Ehrenstein, G., Cold Spring Harbor Symp. Quant. Biol., 21, 705 (1956).
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