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Translation of Rabbit Hemoglobin Messenger RNA *In Vitro* with Purified and Partially Purified Components from Brain or Liver of Different Species

(guinea pig/mouse/ribosomal subunits/initiation factors)

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ABSTRACT **Two mammalian systems have been developed for efficient *in vitro* translation of exogenous messenger RNA (rabbit globin mRNA). One system is completely derived from guinea pig brain, and the other is from liver of different species. Both systems consist of purified 60S and 40S ribosomal sununits, unseparated initiation factors partially purified by ammonium sulfate precipitation and DEAE-cellulose chromatography, and pH 5 enzyme fractions as sources of elongation and termination factors, aminoacyl-tRNA synthetases, and transfer RNA. Translation depends completely upon exogenous mRNA and initiation factors. Extraction of initiation factors from microsomes or ribosomes has been improved for these tissues by inclusion of Mg^{++} ions in the 0.5 M KCl extraction solution. Both systems synthesize complete rabbit α- and β-globin chains, but in variable ratios. The overall efficiencies of the two systems are about 60% (liver) and 40% (brain) of a comparable system with rabbit reticulocyte initiation factors.**

Polypeptide chain initiation seems the most probable step in translation where regulation could operate. Initiation of translation and its specificity in eukaryotes has been studied during the last 3 years by many investigators in various *in vitro* systems (for literature, see ref. 1). For most of these studies, unfractionated (30,000 × *g*) postmitochondrial supernatants of different cell or organ homogenates or lysates were used.

These systems may be very useful to answer the question of whether or not a certain mRNA can be translated in a heterologous system. However, for more quantitative analyses and comparisons, fractionated systems reconstituted from purified or partially purified subcomponents have several advantages. (*i*) The subcomponents are better defined and can be manipulated individually. (*ii*) They are absolutely dependent upon exogenous mRNA. (*iii*) In purified systems, one can compare the translation of different exogenous mRNAs, either separately or in direct competition, in the absence of products of already initiated endogenous mRNA.

In several cases where fractionated systems were used, the translation or binding of a heterologous mRNA depended on the presence of at least one initiation factor from the cell type from which the mRNA originated (2–5). However, these findings were in at least one instance (5) explicitly not interpreted as proof for tissue specificity. From the same laboratory, Crystal *et al.* (6) have recently reported the partial purification of the initially missing initiation factor $IF\text{-}M_3$ from rabbit liver that is required for the translation of globin mRNA.

Our independent study with a liver system confirms the work of Crystal *et al.* (6) with regard to the lack of tissue specificity for rabbit globin synthesis, and extends it to show a lack of species specificity for the same tissue. Our results with an all-brain system extend these conclusions to a tissue that is not known to be involved in hematopoiesis.

Both our liver and brain systems are totally dependent upon mRNA (rabbit globin) and initiation factors. Up to 90% of the synthesized products are complete rabbit α- and β-hemoglobin chains.

METHODS

Polysomes from mouse liver and guinea-pig brain were prepared essentially according to Falvey and Staehelin (7) [modified from Wettstein *et al.* (8)] with minor alterations. Postmitochondrial supernatant fluid from the respective organs containing microsomes, free ribosomes, and soluble cell components was made 1% in sodium deoxycholate and centrifuged for 16–18 hr in a SW27 Spinco rotor through a double layer of 7 ml of 1.8 M and 7 ml of 1 M sucrose containing 0.1 M NH_4Cl–5 mM $Mg(acetate)_2$–0.02 M Tris·HCl, (pH 7.6)–1 mM dithiothreitol. The pelleted polysomes were used for run-off in a complete protein synthesizing system (7) to produce free monoribosomes for subsequent ribosome subunit preparation in sucrose gradients. The subunits from the peak fractions of sucrose density gradients were concentrated by high-speed centrifugation rather than by ethanol precipitation (7), since we observed that ethanol-precipitated subunits, although almost as active in the poly(U) system, had a higher Mg^{++} concentration optimum for poly(phenylalanine) synthesis than did pelleted subunits. pH 5 Enzymes from rat liver or guinea-pig brain were prepared according to Falvey and Staehelin (7).

Highly Purified 9S mRNA was prepared from rabbit reticulocyte polysomes washed with 0.5 M KCl. A detailed method will be published elsewhere (Schreier, Sundkvist, and Staehelin, in preparation).

Initiation Factors from Guinea-Pig Liver and Guinea-Pig Brain were isolated as follows: All manipulations were done at 0–4°. Liver or brain was homogenized in 0.27 M sucrose, 0.03 M KCl, 5 mM $Mg(acetate)_2$, 1 mM dithiothreitol, and 0.02 M Tris·HCl (pH 7.6) (2–2.5 ml/g of tissue) in a loose-fitting Potter–Elvehjem homogenizer at 1200 rpm (10 strokes). The postmitochondrial supernatant (12,000 rpm, 10 min in a Sorvall SS34 rotor) was centrifuged 4 hr at 40,000 rpm in an IEC A 170 rotor. The microsomal and ribosomal pellet was homogenized at 1600 rpm in about three times its volume of

0.25 M sucrose and either 0 or 5 mM Mg (acetate)$_2$, 0.03 M Tris·HCl (pH 7.6), 1 mM dithiothreitol, and 0.1 mM EDTA. To this homogenate, 4 M KCl was added slowly with stirring at 0°, to a final concentration of 0.5 M. Stirring was continued for 20 min, followed by centrifugation for 2.5 hr at 60,000 rpm in an IEC A 270 or Spinco Ti 65 angle rotor. The supernatant (microsome KCl-wash fluid) was fractionated with ammonium sulfate from 25 to 60% saturation. This fraction was taken up in, and dialyzed for 12–16 hr against, 0.12 M KCl 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 0.02 M Tris·HCl, (pH 7.6). It is referred to as crude initiation factors. After clarification by low-speed centrifugation, the crude fraction was applied to a DEAE–cellulose column (Whatman DE 52) (about 2 mg of protein per ml bed volume) equilibrated with the above buffer. After the column was washed with two column volumes of the same solution, a step elution with 0.30 M KCl, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.02 M Tris·HCl (pH 7.6) was performed. The eluted fraction was dialyzed against 0.12 M KCl, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.02 M Tris·HCl (pH 7.6) and concentrated to about 5 mg of protein per ml in an Amicon pressure filtration cell with a UM 10 filter. It is referred to as partially purified initiation factors, or IF-fraction A. Both crude and A-fractions are stable for several days at 0° without significant loss of activity. Approximate yields: 200–250 g liver → 450 ml of postmitochondrial supernatant (containing 15,000–20,000 A_{260} units of polysomes, single ribosomes, and subunits) → about 400 mg of crude initiation factors (25–60% ammonium sulfate fraction) → 100–130 mg of IF-fraction A.

Cell-Free Rabbit-Globin Synthesis. Standard reaction mixtures of 0.1 ml contained 0.4 or 0.5 A_{260} units of purified ribosome subunits, in an A_{260} 60S to A_{260} 40S ratio of 2.5:1, and 2 μg of rabbit 9S mRNA, initiation factors as indicated, and the following ingredients per ml: 25 μmol of creatine phosphate, 3.75 units of creatine kinase, 1 μmol of ATP, 0.4 μmol of GTP, 70 μmol of KCl, 4.3 μmol of $MgCl_2$, 30 μmol of Tris·HCl (pH 7.6), 1 μmol of dithiothreitol, 30 nmol of each L-aminoacid, 0.75 μ Ci of [^{14}C]leucine (final specific activity = 23 Ci/mol), and 0.25 ml of pH 5 enzymes (about 6 mg of protein and 0.25 mg of tRNA). Incubation was at 30° for 40 min. The reaction was stopped by the addition of 0.5 ml of H_2O and 3 ml of 10% CCl_3COOH. After they were heated for 10 min at 90°, the samples were collected onto Whatman GF A glass-fiber filters and washed three times with 3 ml (each) of 5% CCl_3COOH. The dried filters were counted in PPO–POPOP–toluene in a Packard Tricarb scintillation spectrometer at about 76% efficiency.

Product Analysis of In Vitro Globin Synthesis. Carrier rabbit hemoglobin (40–60 mg) labeled in whole reticulocytes with [^{14}C]leucine according to Schapira *et al.* (9) was added to an incubated (*in vitro*) synthetic mixture labeled with [^{3}H]-leucine. Globin chains were isolated and chromatographed on carboxymethyl-cellulose as described by Dintzis (10), as modified by Schapira *et al.* (9), and the fractions were processed and assayed for radioactivity according to Lingrel (11).

RESULTS

Fig. 1 shows an initiation factor titration for rabbit globin synthesis in an all-liver system. Two differently extracted factor preparations, isolated and tested simultaneously, are

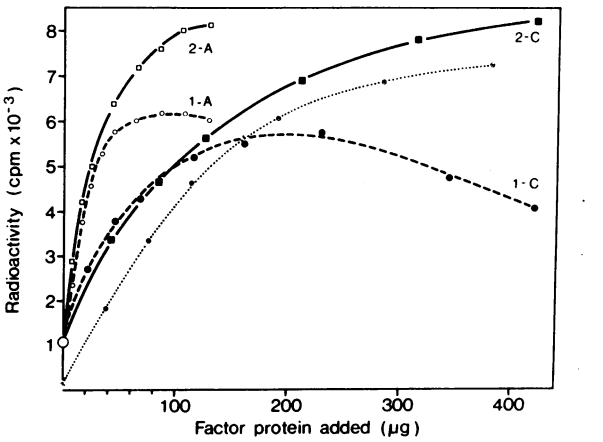

FIG. 1. Activities of crude and partially purified liver initiation factors in a complete liver-derived system. Guinea-pig liver microsomes were extracted in 0.5 M KCl, either in the absence of Mg^{++} (*1-C* and *1-A*) or in the presence of 5 mM Mg(acetate)$_2$ (*2-C* and *2-A*). 0.1-ml Reaction mixtures for rabbit globin synthesis contained 0.5 A_{260} units of mouse liver ribosome subunits, 2 μg of rabbit 9S mRNA, rat-liver pH 5 enzymes (0.5 mg of protein), all other components for protein synthesis (see *Methods*), and the amounts of initiation factor protein indicated on the *abscissa*. Incubation was at 30° for 40 min. 1000 cpm = 26 pmol of leucine. ●– – –● (*1-C*) and ○– – –○ (*1-A*) = crude fraction (25–60% ammonium sulfate precipitate) and IF-fraction A, respectively, from microsomal KCl wash extracted in the absence of Mg^{++}. ■——■ (*2-C*) and □——□ (*2-A*) = crude fraction and IF-fraction A, respectively, from microsomal KCl wash extracted at 5 mM Mg(acetate)$_2$. ⊙···⊙ = separate experiment with a crude IF preparation [extraction at 5 mM Mg(acetate)$_2$] and a pH 5 enzyme preparation devoid of native 40S ribosome subunits, which themselves carry initiation factors, causing a high background incorporation. This background is strictly dependent upon exogenous mRNA.

compared. For the first preparation, the KCl extraction of the microsomes was performed in the absence of Mg ions, i.e., in 0.25 M sucrose, 0.1 mM EDTA, 0.03 M Tris·HCl (pH 7.6), 1 mM dithiothreitol, and 0.5 M KCl; for the second preparation, 5 mM Mg acetate was included in the above extraction solution. The presence of Mg ions during the KCl extraction of microsomes resulted in a significant increase in the activity of initiation factors in both the crude and the A-fraction (Fig. 1, curves *2-C* and *2-A*). The lower activity of the initiation factors extracted in the absence of Mg ions may be due to extraction of some inhibitory component(s) as well. The inhibition becomes quite obvious at high inputs of the crude factor preparation (Fig. 1, curve *1-C*). The Mg^{++} optimum for globin synthesis was quite narrow at 4.0–4.5 mM for all the different factor preparations. Mg ions were completely removed from initiation factors by the ammonium sulfate precipitation step, followed by extensive dialysis against a Mg^{++}-free buffer containing 0.2 mM EDTA (see *Methods*). The significant background synthesis in the absence of added initiation factors (about 1100 cpm) was due to the presence of small amounts of native 40S ribosome subunits in the pH 5 enzyme preparation used in this experiment. These native 40S subunits carry initiation factors (Staehelin, Sundkvist, and Schreier, manuscript in preparation). After becoming aware of this fact, we prepared pH 5 enzymes with particular care to eliminate native 40S subunits. The initiation factor

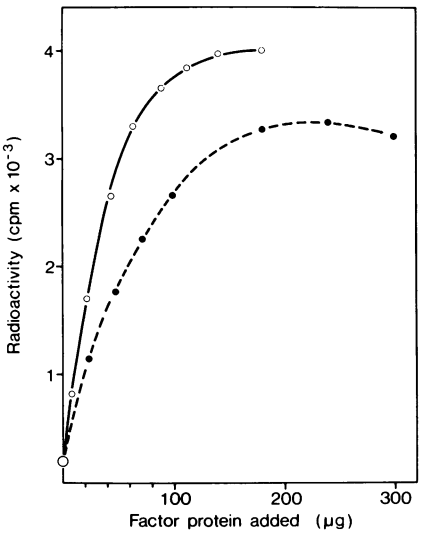

FIG. 2. Activities of crude (25–60% ammonium sulfate precipitate) and partially purified (IF-fraction A) initiation factors from guinea-pig brain in a complete brain-derived translation system. Guinea-pig brain microsomes were extracted in 0.5 M KCl in the presence of 5 mM Mg(acetate)$_2$. 0.1-ml Reaction mixtures contained 0.4 A_{260} units of guinea-pig brain ribosome subunits, 2 μg of rabbit 9S mRNA, guinea pig-brain pH 5 enzymes (0.45 mg of protein), all other components for protein synthesis (see *Methods*), and the amounts of initiation factor protein indicated on the *abscissa*. Incubation was at 30° for 40 min. 1000 cpm = 26 pmol of leucine. O– – –O = crude fraction; O——O = IF-fraction A.

dependence in an experiment using crude factors and a 40S-free pH 5 enzyme preparation is shown in the dotted curve in Fig. 1. The background was seven times lower (150 cpm), and the stimulation at saturating factor concentration was about 50-fold instead of only 7- to 8-fold. Fig. 1 also shows that the purification of initiation factors from the crude fraction to the IF-fraction A is about 3-fold, as estimated from the slopes of the protein titration curves.

Fig. 2 shows rabbit hemoglobin synthesis in an all-brain system from guinea pig consisting of purified brain 60S and 40S ribosome subunits, brain pH 5 enzymes (devoid of 40S subunits), and brain initiation factors extracted from microsomes in the presence of 5 mM Mg^{++}. The results are similar to those with the all-liver system. Considering the lower input of brain ribosome subunits per assay (0.4 A_{260} units compared to 0.5 A_{260} units of liver ribosomes) the all-brain system had about 70% of the activity of the liver system. The purification by the DEAE-cellulose step seemed somewhat less effective for brain initiation factors, i.e., the specific activity increased by only about a factor of two. Still, the activity of the IF-fraction A at saturating concentration was about 20% higher than that of the crude fraction.

Fig. 3 demonstrates the absolute dependence of protein synthesis in both the liver and the brain system upon rabbit globin mRNA. In both experiments shown in Fig. 3, 0.5 A_{260} units of liver or brain ribosome subunits, with saturating amounts of the respective IF-fraction A, were tested with the same mRNA preparation. It seems that the liver system utilized rabbit globin mRNA somewhat more efficiently than the brain system. The saturating concentration of 9S mRNA in the liver system was indeed very low in this experiment, i.e., one 9S mRNA molecule of molecular weight 220,000 per two ribosomes, as compared to about one 9S molecule per one ribosome in the brain system or in a system using the same liver ribosome subunits but reticulocyte initiation factors (1).

Another difference between the brain system and the liver system becomes apparent in the product analysis shown in Fig. 4. In both systems, about 90% of the hot CCl_3COOH-precipitable radioactivity of an incubated mixture was recovered from carboxymethyl-cellulose chromatography as complete α- and β-globin chains, coinciding rather well with the *in vivo* labeled carrier globin. However, the α- to β-globin chain ratio differed considerably between the two systems. Whereas the liver system synthesized α- and β-chains in almost equal amounts, in the brain system there was a 2-fold excess of α-chain synthesis. For both analyses the same preparation and amount of 9S mRNA was used. With reticulocyte initiation factors and liver factors we also found variable differences in α- and β-globin chain synthesis; however, the β-chain was usually synthesized in excess. The ratio of products from different mRNAs translated in a competitive situation may be influenced (*i*) by the degree of saturation of the system with mixed mRNAs, (*ii*) by the ionic conditions, and (*iii*) by the ratio of the different initiation factors in a system (*iii*, W. L. McKeehan, personal communication; for literature and discussion, see ref. 14).

DISCUSSION

In this study we developed from different organs and species two partially purified translation systems dependent on added mRNA. With rabbit reticulocyte initiation factors (IF-fraction A), between 1.5 and 2.5 hemoglobin chains are usually synthesized per ribosome (mouse liver subunits) in the cell-free system (1). In the all-liver system, up to 1.5 globin chains, and in the all-brain system up to 1 globin chain, were polymerized per ribosome present in the assay. On the average, the liver system was about 60% and the brain system about 40% as efficient as was a comparable reticulocyte system. These

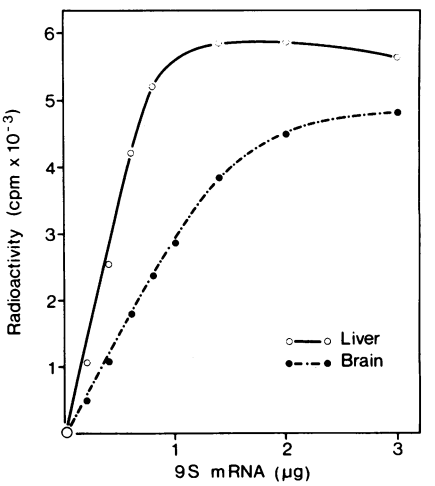

FIG. 3. Dependence of rabbit globin synthesis upon addition of purified 9S mRNA in the complete liver (O——O) and the complete guinea pig brain (O– – –O) translation systems. 0.1-ml Reaction mixtures contained 0.5 A_{260} units of ribosome subunits, pH 5 enzymes, and initiation factors, all from the respective tissues (85 μg of liver IF-fraction A and 140 μg of brain IF-fraction A, respectively) plus the other components for protein synthesis.

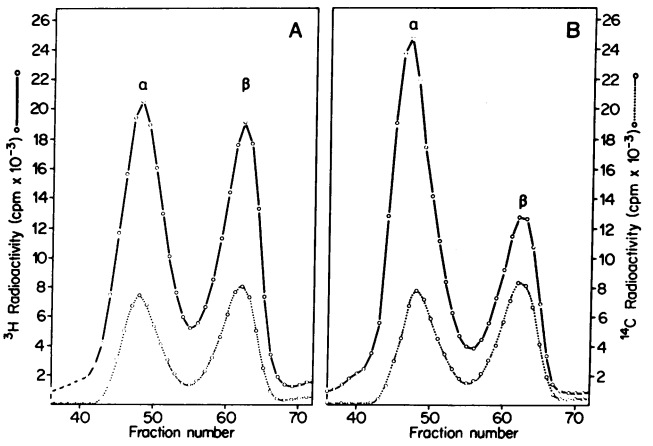

FIG. 4. Product analyses of 9S mRNA-dependent protein synthesis in the complete liver system (*A*) and the complete guinea pig brain system (*B*). 0.3-ml Reaction mixtures of each system contained 1.5 A_{260} units of the respective ribosome subunits, 6 μg of 9S mRNA, saturating amounts of the respective initiation factors and pH 5 enzymes, as well as the other components and [^{3}H]leucine (final specific activity = 1800 Ci/mol). Incubation was at 30° for 60 min. After incubation, 60 mg of carrier rabbit hemoglobin labeled with [^{14}C]leucine in whole reticulocytes was added to each mixture. The globin chains were then extracted, chromatographed on carboxymethyl-cellulose, and processed for scintillation counting. Counting efficiency for ^{3}H was 16% and for ^{14}C was 61%. ^{14}C spill-over into the ^{3}H window was 10%, and was corrected for. O···O = carrier [^{14}C]globin; O——O = *in vitro* synthesized [^{3}H]globin.

relatively small differences cannot be taken as evidence for different degrees of tissue specificity for globin mRNA. The individual initiation factors might not be present in optimal amounts in the different preparations enriched only in total initiation factor activity. The ribosomes from liver, brain, and reticulocytes had very similar activities when tested with reticulocyte initiation factors and liver pH 5 enzymes (1). The pH 5 enzymes were prepared exactly like liver pH 5 enzymes, with no attempts to optimize the procedure for brain. However, replacement of the brain pH 5 enzymes by liver pH 5 enzymes did not significantly improve the efficiency of globin synthesis with brain initiation factors and ribosomes. Therefore, we conclude that the different efficiencies of the various systems reflect mainly the different activities or qualities of the respective initiation factor preparations.

In conclusion, we show that in these two systems there is no tissue or species specificity for translation of rabbit globin mRNA. Our results from the liver system confirm the recent findings of Crystal *et al.* (6), who have separated and partially purified from rabbit liver all the factors corresponding to those that they had characterized in rabbit reticulocytes. Moreover, this conclusion was independently arrived at in two other studies, one of which used a crude liver translation system (12), the other a liver system reconstituted from a ribosome fraction and a high-speed supernatant fraction (13).

With regard to the lack of tissue specificity for globin synthesis, our results from the all-brain system are more significant than those from liver systems. The liver is a very active hematopoietic organ during embryogenesis and can revert to that function in severe anaemias even in an adult, whereas the brain is a highly differentiated organ that is never known to be involved in hematopoiesis.

Systems like those described here should be useful to study degrees of species and tissue specificities for various mRNAs by comparisons of the relative translation efficiencies of messengers, either individually or in competition, in the different systems.

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