*A FACTOR PROMOTING THE INITIATION OF GLOBIN SYNTHESIS IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM**

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Communicated by David Rittenberg, January 8, 1969

Abstract.—The synthesis of globin has been studied in a reticulocyte cell-free system in which the initiation of polypeptide chain synthesis can proceed for as long as ten minutes of incubation. With this system, we have found evidence for a soluble factor in the reticulocyte that stimulates protein synthesis and appears to act by promoting initiation.

The study of protein synthesis in mammalian cells has been hampered by the inability of mammalian cell-free systems to initiate new polypeptide chains for more than a brief period of incubation.[1] With a procedure for the isolation of active reticulocyte polyribosomes described by Arnstein *et al.*,[2] we have obtained a cell-free system that is easy to prepare, active in protein synthesis, and able to initiate new globin chains for at least ten minutes of incubation at 37°C. With this system, we have found evidence for a factor that can restore the ability of this system to initiate globin chains when added after ten minutes of incubation.

Materials and Methods.—Reticulocytes were obtained from rabbits made anemic by injection of acetylphenylhydrazine.[3] The blood was allowed to coagulate. After clot retraction, the serum was removed and the clot was washed with 0.15 *M* sodium chloride. The clot was homogenized in four cell volumes of reticulocyte standard buffer[4] (0.01 *M* Tris, 0.01 *M* KCl, 0.0015 *M* $MgCl_2$) and one cell volume of sucrose KCl (1.5 *M* sucrose, 0.15 *M* KCl, 0.006 *M* mercaptoethanol) by five strokes of the tight pestle of the Dounce homogenizer. Cell debris was removed by centrifugation for 30 min at 27,000 × *g*. The pH of the supernatant solution was adjusted to 5.4 with 1 *N* acetic acid. The precipitate containing polyribosomes, tRNA, and pH 5 enzymes was harvested by centrifugation at 3,000 × *g* for 20 min. The pellet was redissolved in 0.05 *M* Tris, 0.01 *M* KCl, 0.0015 *M* $MgCl_2$, pH 7.8, so as to contain approximately 2 mg of protein per milliliter of solution (20–25 A_{260} units/ml) and was designated pH 5.4 fraction.

The supernatant was readjusted to pH 7.5 with 1 *N* NaOH, and additional protein was precipitated by the addition of ammonium sulfate to 40% saturation. The pellet was washed once with 40% ammonium sulfate and was then redissolved in a volume of standard buffer equal to the original volume of the pH 5.4 supernatant. The solution was dialyzed in the cold overnight against the standard buffer. The fraction thus obtained was designated "factor."

The ingredients of the incubation mixture are listed in Table 1. Lysine-C^{14} (248 mc/mM) and valine-C^{14} (209.5 mc/mM) were obtained from New England Nuclear Corp. Reactions were terminated by rapid chilling of the incubation flasks in an ice-water slurry. Charged tRNA molecules were discharged by adding 0.3 ml of *N* NaOH and incubating the mixture for 15 min at 37°C. After the addition of 1.3 ml of 10% trichloroacetic acid (TCA), the precipitates were adsorbed on Millipore filters, which were then washed twice with 5% TCA, glued to planchettes, and counted in a gas-flow counter.

A modified fluorodinitrobenzene method was used to isolate N-terminal valine.[7] After acid hydrolysis, the conjugated N-terminal amino acid was twice extracted from the aqueous phase with an equal volume of ethyl acetate. The extract was concentrated and the radioactivity was determined with a liquid scintillation counter.

Polyribosome profiles were obtained by layering the entire reaction mixture on a 7.5–30% linear sucrose gradient. The gradients were centrifuged and assayed as previously described.[8]

TABLE 1. *Incubation mixture.*

pH 5.4 fraction	0.20 ml (containing 5 A_{260} units*)
Reagent mixture	1.0 ml
ATP	1 μmole
GTP	0.2 μmole
Creatine phosphate	5 μmoles
Creatine phosphokinase	75 International units
Tris HCl pH 7.5	50 μmoles
$MgCl_2$	8.5 μmoles
KCl	45.0 μmoles
Sucrose	5.0 μmoles
Amino acid mixture[5]	20 μl
C^{14} lysine or valine	1 μc

* 1.0 mg ribosomes = 11.2 A_{260} units.[6]

Results.—The modified cell-free system leads to the linear incorporation of labeled amino acids into completed globin chains for 15 minutes at the initial rate (Fig. 1). The length of time during which the initial rate of synthesis could be maintained with pH 5.4 precipitates from different samples of blood varied from 5 to more than 15 minutes. During the period of linear incorporation at the initial rate, excellent polyribosomal profiles are observed in this cell-free system (Fig. 2).

The material precipitated at pH 5.4 contains ribosomes, RNA, amino acid–activating enzymes, and transfer enzymes.[9] The addition of factor to this system stimulates the incorporation of amino acids into (1) nascent chains attached to the polyribosomes (Fig. 2), (2) completed chains released from the polyribosomes (Fig. 4), and (3) the total system containing both completed and nascent chains (Fig. 3). This stimulation is not merely the result of adding more transfer enzymes, since the 40–70 per cent ammonium sulfate fraction, which is known to contain these enzymes,[10] did not possess significant stimulatory activity (Table 2). The factor is heat-labile and is inactivated by tryptic digestion. The active material could not be extracted by phenol at 25°C (Table 3). The data indicate that the factor is, at least in part, a protein; but it may be a ribonucleoprotein. The elution of active material from a

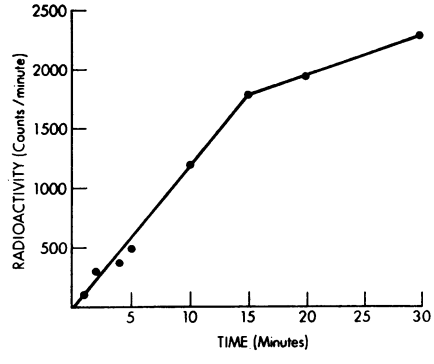

FIG. 1.—Incorporation of lysine-C^{14} into hemoglobin by the cell-free incubation mixture described in Table 1. In this experiment the ribosomes, with incomplete globin chains, were removed by centrifugation at 269,000 × *g* for 90 min at the end of the incubation before the total radioactivity was determined as described in *Materials and Methods*.

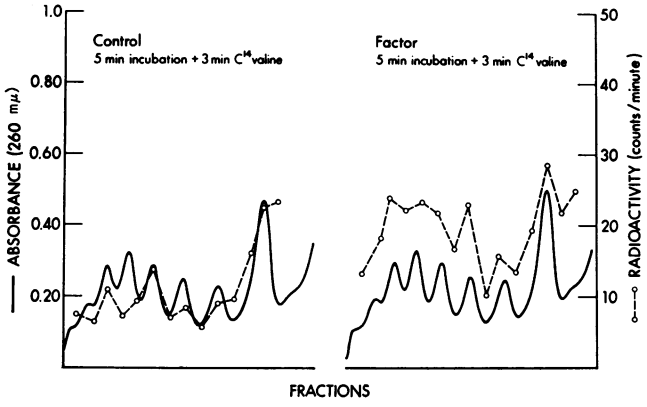

FIG. 2.—Sucrose density gradient analysis of the cell-free incubation mixture. The factor (20 μl) was added at the beginning of the incubation and the valine-C^{14} added at 5 min. At 8 min, the incubation was terminated by rapid chilling and the contents of each incubation mixture were immediately layered on the top of 30 ml of a 7.5-30% linear sucrose gradient and centrifuged at 25,000 rpm for 180 min in a SW 25.1 rotor (Spinco Division, Beckman Instruments, Inc.). The contents of each gradient were analyzed for absorbance at 260 mμ and for radioactivity as described previously.[8]

Sephadex G200 column just after the exclusion volume suggests that the factor may be of large molecular weight. The factor is stable for several days when stored in liquid nitrogen.

The relatively short period of time during which the reticulocyte cell-free system is able to sustain active globin synthesis has been ascribed to inability to initiate new globin chains.[11] Since the addition of factor prolonged the period of active protein synthesis and stimulated synthesis when added after 10 or 15 minutes of preincubation, we examined the effect of the factor on initiation of protein synthesis by measuring the incorporation of valine-C^{14} into the N-terminal position of completed and released globin chains (see Fig. 4). The lower half of Figure 4 shows the total incorporation of the valine-C^{14} into globin by the complete system (*closed circles*) in the presence of factor from zero time (*bull's-eye*) and with the factor added at ten minutes (*open circles*). Marked stimulation occurred when the factor was added after ten minutes of incubation, as well as at zero time. The upper portion of Figure 4 shows the incorporation of valine-C^{14} into the N-terminal position of the same globin chains and demonstrates the ability of the factor to restore the capacity of the system to incorporate valine into the N-terminal position when added after ten minutes of incubation.

The possible role of factor in initiation was explored in experiments on the effect of factor in the presence of sodium fluoride. Fluoride has been reported to inhibit globin synthesis by interfering with the initiation of polypeptide chains; however, in the presence of 10 mM NaF, the reticulocyte cell-free system is able to complete polypeptide chains that were begun in the intact cell.[12, 13] In these experiments (Table 4), the addition of NaF (10 mM) alone

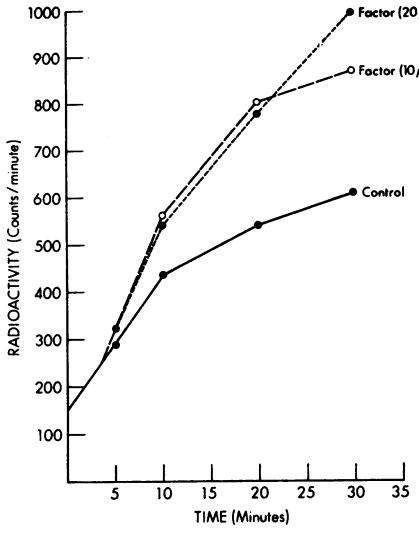

FIG. 3.—Stimulation of globin synthesis by factor added at 3 min to the cell-free incubation mixture described in Table 1. Ribosomes were not removed before the total radioactivity was determined as described in *Materials and Methods*.

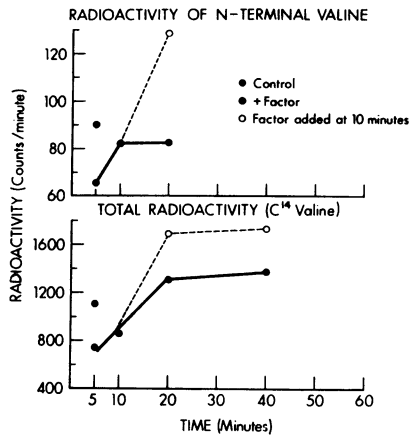

FIG. 4.—Comparison of the total and N-terminal radioactivity in the same globin samples obtained from the cell-free system incubated with C^{14} valine. Control (●—●), with factor present from time zero (⊙), and with factor added at 10 min (○--○). The ribosomes were removed by precipitation at pH 5.4 followed by centrifugation at 3000 × *g* for 20 min before globin was prepared for analysis of the total and N-terminal radioactivity.[7]

TABLE 2. *Stimulatory activity of postribosomal fractions.*

Additions	Cpm (10 min)
Complete system (Table 1)	609
+pH 5.4 supernatant (20 μl)	814
+0–40% ammonium sulfate fraction (20 μl)	871
+40–70% ammonium sulfate fraction (20 μl)	651

The pH 5.4 supernatant solution was readjusted to pH 7.5. The protein precipitated by ammonium sulfate fractionation was redissolved in reticulocyte standard buffer equal in volume to the pH 5.4 supernatant solution.

TABLE 3. *Effect of pretreatment on the stimulatory activity of factor.*

Treatment	Per Cent Stimulation by Factor (20 μl)	
	Treated	Untreated
Heat, 100°C, 5 min	+ 8	+42
Heat, 65°C, 5 min	− 7	+43
Phenol extract, 25°C*	−16	+42
Tryptic digestion†	0	+34
Dialysis, 18 hr	+47	+58

All incubations lasted for 5 min, and the radioactivity incorporated in the control incubation without added factor was 506 cpm.

* The factor, dissolved in reticulocyte standard buffer, was shaken with an equal volume of 88% phenol for 5 min at 25°C. The aqueous layer was separated by centrifugation at 1000 × *g* for 5 min at 4°C and removed. Residual phenol was removed from the aqueous layer by repeated extraction with cold ether, and the ether was eliminated by bubbling nitrogen through the solution.

† A solution containing factor was incubated with thrice-crystallized trypsin (Worthington Biochemical Corp.) in a ratio of 20 μg of trypsin per milligram of factor protein for 5 min at 25°C; and soybean trypsin inhibitor, equal in amount to the trypsin, was then added. The addition of trypsin and soybean inhibitor to the incubation mixture without added factor had no inhibitory effect.

TABLE 4. *Effect of factor in the presence of NaF.*

	Expt. 1 (cpm)	Expt. 2 (cpm)
Control	610	561
Control + NaF (10 mM)	430	296
Factor (20 μl)	950	867
Factor (20 μl) + NaF (10 mM)	595	515

The incubations were carried out for 10 min at 37°C.

resulted in diminished amino acid incorporation. When factor alone was added, amino acid incorporation was enhanced. When NaF and factor were added simultaneously, amino acid incorporation was much reduced below that observed when factor alone was added.

Discussion.—By means of the pH 5.4 precipitate cell-free system, we find that the initiation of new globin chains persists for 10 minutes at 37°, the pattern of polyribosomes is maintained for at least 8 minutes, and the initial rate of globin synthesis is maintained for about 15 minutes before changing to a slower rate of amino acid incorporation. These results are in contrast to the findings with the totally fractionated cell-free system,[10] in which the rate of incorporation of amino acids into protein per ribosome is less than 1–2 per cent of the rate in the intact cell.[1, 11] This limited synthesis is associated with the limited capacity or failure of such systems to initiate new globin chains[1] and with rapid breakdown of polyribosomes. Even in the unfractionated reticulocyte hemolysate system of Lamfrom and Knopf,[11] in which the rate of globin synthesis is 10–25 per cent of the intact cell rate, the incorporation of valine into the N-terminal position of globin chains ceases within five minutes of incubation at 37°C, and considerable breakdown of the polyribosomes is evident in that time.[11, 14]

When the initiation of globin chains in the pH 5.4 precipitate cell-free system has almost ceased, further initiation can be induced by the addition of a factor that can be precipitated from the pH 5.4 supernatant by 40 per cent ammonium sulfate. This factor appears to promote initiation and, as a result, to stimulate the synthesis of globin. The stimulation of polypeptide synthesis by the addition of supernatant factor is not merely the result of adding amino acid–activating or transfer enzymes. These enzymes are not limiting in the reaction mixture, since chain completion proceeds in the absence of added factor; furthermore, they are precipitated to a considerable extent at pH 5.4, while factor not already bound to ribosomes remains in the supernatant.

Evidence that the factor promotes the initiation of globin chains consists of the following: (1) the factor restores the ability of the system to incorporate valine into the N-terminal position when added after such incorporation has essentially ceased, and (2) the stimulatory activity of the factor is diminished in the presence of 10 mM NaF.

Miller and Schweet[15] describe a factor which can be eluted from sedimented reticulocyte ribosomes by 0.5 *M* KCl. Its characteristics of heat lability, inactivation by proteolytic enzymes, elution pattern on Sephadex G200, and ability to stimulate the incorporation of valine into the N-terminal position

of globin chains suggest that it is identical with or very similar to the factor we describe. The data of Miller and Schweet indicate that the factor is bound to ribosomes but, as shown in our experiments, it is either bound in limiting amounts or is inactivated during a short period of incubation at 37°C.

The factor described in our experiments appears to promote initiation. In *E. coli*, three initiation factors have been delineated: one is required for the binding of *E. coli* ribosomes to the messenger RNA and two others for the binding of the N-formyl-methionine-tRNA to the ribosome-mRNA complex.[16] Whether the factor we have described is required for the binding of reticulocyte ribosomes to messenger RNA or for the binding of valyl- RNA to the complex is currently under investigation.

* Supported by USPHS grant HE-02803.

† Career scientist of the Health Research Council of the City of New York (592).

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