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Cell-Free Hemoglobin Synthesis in Beta-Thalassemia

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Abstract. Human ribosomes obtained from the reticulocytes of patients having either homozygous beta-thalassemia (thalassemia ribosomes) or a hematological disorder unrelated to thalassemia ("normal" ribosomes) have been utilized in a cell-free system highly active in the synthesis of intact human globin chains. This system is dependent on the addition of a ribosomal wash fraction from reticulocytes that contains factors necessary for chain initiation. In response to the ribosomal wash fraction, isolated from either thalassemia, normal human, or rabbit reticulocytes, normal human ribosomes synthesize equal amounts of alpha and beta chains. In contrast, in response to all three types of ribosomal wash fractions, thalassemia ribosomes synthesize 8-times more α than β chains, a ratio similar to that produced in the intact cells of these patients. The molecular defect in beta-thalassemia, therefore, does not appear to be associated with initiation factors.

The term thalassemia applies to a group of hereditary anemias characterized by a deficiency in synthesis of one or another of the polypeptide chains normally found in hemoglobin molecules.[1] In homozygous β-thalassemia, α chains are synthesized in excess of β chains,[2–4] due to an absolute decrease in β-chain synthesis rather than an increase in α-chain synthesis.[5] The rate of translation of mRNA for β chains in the thalassemia cell has been reported to be similar to that found in the normal reticulocyte.[6] Bank and Marks have described a cell-free system using thalassemia polysomes that is less active than with human polysomes from nonthalassemics, and which is not significantly stimulated by supernatant fractions from either nonthalassemic human or rabbit reticulocytes.[7] They have suggested that the defect in β-thalassemia is present in the ribosome–mRNA complex,[7] either as a quantitative reduction in the amount of β-chain mRNA or as a reduced initiation of translation of the β-chain mRNA.[8]

In 1968, Miller and Schweet reported a cell-free system from rabbit reticulocytes capable of making uniformly-labeled rabbit hemoglobin and dependent on the addition of a crude fraction from salt-washed ribosomes.[9] Studies from our laboratory have confirmed this observation, and have shown that the ribosomal wash fraction contains at least three factors, M_1, M_2, and M_3, apparently necessary for initiation of hemoglobin polypeptide chains.[10–13] It could be postulated, from knowledge regarding globin chain synthesis by the thalassemia cell,[2–8] that a defective or missing initiation factor is responsible for the β-thalassemia defect. To investigate this possibility, the following studies were

performed. Human ribosomes from thalassemics and nonthalassemics (normal) were freed of endogenous initiation factors and tested in a highly active cell-free system for their ability to synthesize α and β (human) globin chains in response to initiation factors isolated from thalassemia, normal human, or rabbit reticulocytes. The results demonstrate that the defect in β-thalassemia is not due to an abnormal initiation factor but must reside in the β-chain–mRNA–ribosome complex itself.

Methods. Preparation of cell-free components: Blood was obtained from patients diagnosed as either homozygous β-thalassemia (N. L. and J. L.; originally studied by Heywood *et al.*[2]), autoimmune hemolytic anemia (AHA), or sickle cell anemia. Preparation of unwashed and washed ribosomes, ribosomal wash fractions, and supernatant (S-100) fractions were performed as was previously described for rabbit reticulocyte components.[13,14] The isolation of unfractionated rabbit reticulocyte tRNA and the preparation of aminoacyl-tRNA have been described.[13,14]

Cell-free protein synthesis: Assay conditions, using either free amino acids or aminoacyl-tRNA as substrate, are similar to those reported for rabbit systems[11,13,14] and are described in the figure legends.

Uniformly labeled globin: Uniformly-labeled human globin was obtained by incubating AHA cells or thalassemia cells in the presence of L[^{14}C]leucine (344 Ci/mol) according to the method of Borsook, as described by Schapira *et al.*[15]

Product analysis: After incubation, the cell-free reaction mixture, containing [^{3}H]leucine, was cooled to 0°C. [^{14}C]leucine-labeled hemoglobin was added (in the form of lysate at 0°C) and the solution was immediately dialyzed for 2 hr against four changes of cold deionized water. After an acid–acetone procedure, the globin chains were chromatographed on a carboxymethylcellulose column with 8 M urea–phosphate buffer as described by Clegg *et al.*,[16] as modified by Nathan and co-workers.[17] A nonlinear gradient was produced with a 4-chamber gradient maker with 60 cm^3 of buffer in each chamber. The Na^+ concentration in each chamber was as follows: (*1*) 10 mM; (*2*) 17.5 mM; (*3*) 27.5 mM; and (*4*) 60 mM. 3-ml fractions were collected and counted by adding trichloroacetic acid to a final concentration of 10%. After standing for 10 min at 0°C, the precipitate was collected and washed on a Millipore filter. The filter was dissolved in Bray's solution, and counted in a liquid scintillation counter at (double-label) efficiencies of 26% for ^{3}H and 59% for ^{14}C.

Results. Characterization of the cell-free system from human reticulocytes: In preliminary experiments it was not possible to obtain a highly active, cell-free, protein synthesizing system from human reticulocytes (thalassemia or normal) using only endogenous human initiation factors. Removal of the endogenous factors by a 0.5 M KCl wash of thalassemic ribosomes, followed by replacement with factors isolated from rabbit reticulocyte ribosomes, produced a marked stimulation of hemoglobin synthesis (Fig. 1). Aminoacyl-tRNA was used as substrate in order to make the system independent of aminoacyl-tRNA synthetase activity. Rabbit supernatant protein, which did not contain the initiation factors, was ineffective in stimulating the system. Similar data were obtained with normal (AHA) ribosomes, as well as with ribosomes (sickle cell anemia) containing a β-chain hemoglobinopathy. At optimal concentrations of added components and a saturating amount of acylated tRNA, protein synthesis was linear for 5 min and linear with increasing ribosome concentration up to 0.9 A_{260} units per reaction mixture (Fig. 2).

Washed thalassemia ribosomes stimulated by rabbit initiation factors have a sharp optimal magnesium concentration at 2.5 mM (Fig. 3). Similar data were

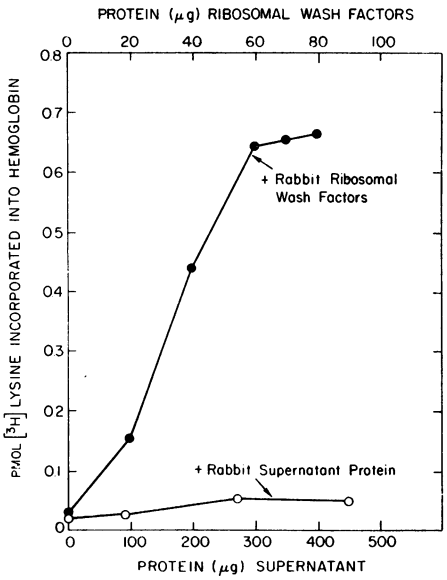

FIG. 1. [^{3}H]lysine incorporation into hemoglobin by washed thalassemic ribosomes with either rabbit ribosomal wash factors or rabbit supernatant protein added to the reaction. Each 50-μl reaction mixture contained: Tris·HCl (pH 7.5) 20 mM; KCl, 80 mM (supplied in the rabbit reticulocyte ribosomal wash); $MgCl_2$, 2.5 mM; ATP, 1.0 mM; GTP, 0.2 mM; phosphoenolpyruvate, 3.0 mM; pyruvate kinase, 0.3 international units; dithiothreitol, 1.0 mM; 0.28 A_{260} units of washed thalassemia ribosomes and 0.14 A_{260} units of acylated rabbit reticulocyte tRNA ([^{3}H]lysine, 7500 Ci/mole, plus 19 [^{12}C]amino acids). Incubations were for 4 min at 37°C. Hot trichloroacetic acid-precipitable radioactivity was determined as described.[14]

obtained with washed AHA ribosomes and with washed rabbit ribosomes. When thalassemia or AHA initiation factors were used with any of the three types of washed ribosomes, activity was less, but magnesium optima at about 3 mM Mg^{++} were obtained (data not shown). Washed thalassemic ribosomes in the absence of initiation factors had barely detectable activity, which peaked at 5 mM Mg^{++}, while unwashed ribosomes have a broad Mg^{++} optimum from 4–6 mM (Fig. 3).

Comparison of human and rabbit cell-free protein synthesis: The protein synthesizing activities of washed ribosomes from rabbit, AHA, and thalassemia reticulocytes were compared. The assay for this and subsequent experiments used free amino acids, rather than aminoacyl-tRNA, in order to obtain a greater amount of product for subsequent chromatography. Initiation factor dependency, magnesium concentration optimum, and linear ribosome dependency

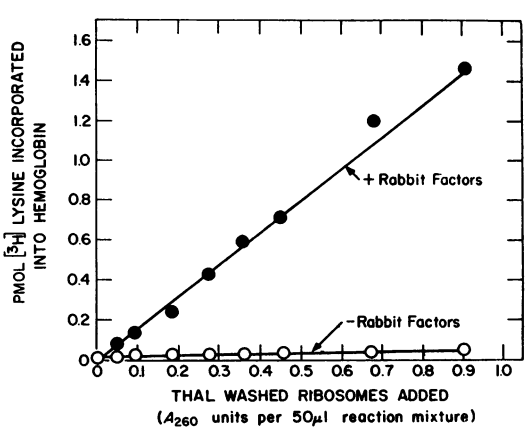

FIG. 2. [^{3}H]lysine incorporation into hemoglobin as a function of increasing amounts of washed thalassemia ribosomes added, with and without rabbit ribosomal wash factors. Reaction mixture components as in Fig. 1. In those reactions with rabbit factors, 80 μg (8 μl at 10 mg/ml protein in 0.5 M KCl) of rabbit ribosomal wash were added. Incubations were for 3 min at 37°C. A zero time blank of 0.03 pmol was subtracted from all values.

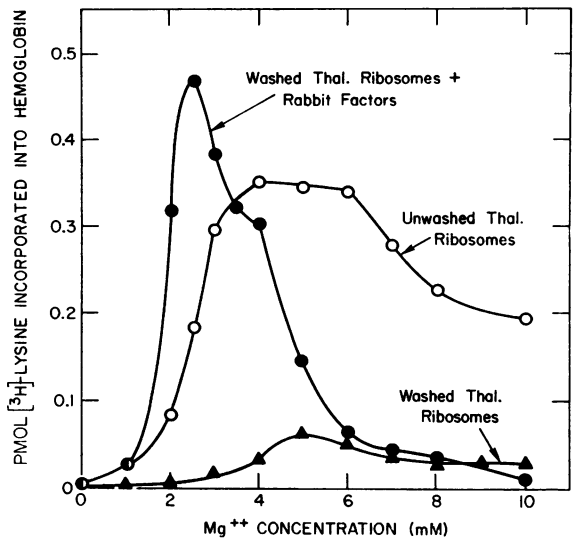

FIG. 3. [3H]lysine incorporation into hemoglobin by thalassemia ribosomes as a function of Mg^{++} concentration. Components for the reaction mixtures are listed in the legend to Fig. 1 except as indicated below. Those reactions labeled "Washed Thal. Ribosomes + Rabbit Factors" (*closed circles*) contained 0.23 A_{260} units of washed thalassemia ribosomes and 80 μg of rabbit ribosomal wash factors per 50 μl reaction mixture. Incubations were for 3.5 min at 37°C. Those reactions labeled "Washed Thal. Ribosomes" (*closed triangles*) contained 0.68 A_{260} units of washed thalassemia ribosomes per 50-μl reaction mixture. Incubation was for 4 min at 37°C. The reactions labeled "Unwashed Thal. Ribosomes" contained 0.71 A_{260} units of unwashed thalassemia ribosomes per 50-μl reaction mixture. Incubation was for 3 min at 37°C. A zero time control of between 0.02 and 0.03 pmol was subtracted from each value.

remained unchanged from the previous assay. The data obtained when rabbit initiation factors were used with each type of ribosome are shown in Fig. 4. While ribosomes of human origin were less active than those from the rabbit, all were in a comparable range (see also Table 1). Part of the difference in activity between thalassemia and AHA ribosomes can be attributed to the deficiency in beta chain synthesis by the former.

Product analysis of the human cell-free system: [14C]leucine-(uniformly) labeled hemoglobin from intact AHA reticulocytes was cochromatographed on a carboxymethyl-cellulose column with [3H]leucine-labeled globin produced by AHA ribosomes stimulated by rabbit initiation factors in the cell-free system. α and β chains were produced in equal quantities by both the intact cell and the cell-free system (Fig. 5). Fingerprint analysis of tryptic digests of the labeled globin synthesized in the human cell-free system gave human globin peptides. Labeled rabbit globin, if present, could be easily distinguished from the human chains, eluting from the column just ahead of the human α peak. Thus, normal human ribosomes stimulated by rabbit initiation factors produced human globin with an equal amount of α and β chains. An α/β ratio of 1 was also obtained when thalassemia or AHA initiation factors were used (Table 1). Furthermore,

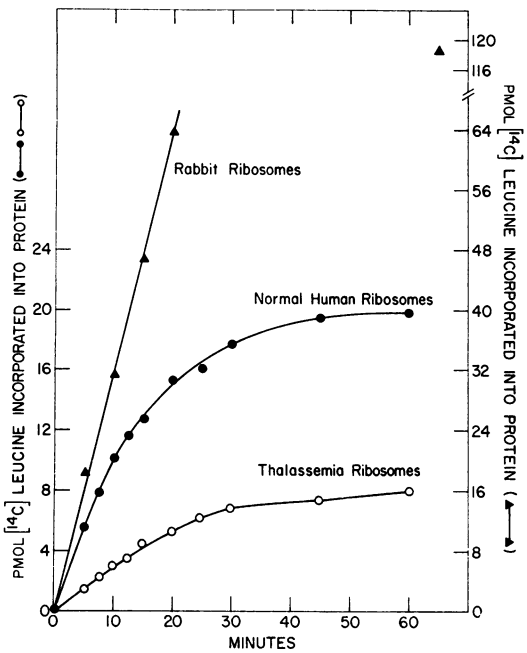

FIG. 4. [14C]leucine incorporation into hemoglobin by rabbit, normal human, and thalassemia ribosomes in response to rabbit ribosomal wash factors. Three 600-μl reaction mixtures were incubated at 37°C. Each contained 5.6 A_{260} units of washed reticulocyte ribosomes (either rabbit, AHA, or thalassemia, as indicated), 960 μg of rabbit ribosomal wash fraction, 0.78 A_{260} units of unfractionated rabbit reticulocyte tRNA and either 24 μl of rabbit S-100 (for the rabbit ribosomes) or 36 μl of AHA S-100 (for both types of human ribosomes). The concentration of reaction mixture components are similar to those listed in Fig. 1, except that the Mg^{++} concentration was 3 mM and the aminoacyl-tRNA was deleted and free amino acids were added in the concentration of 50 μM for L[14C]leucine (344 Ci/mol) and 100 μM for each of the 19 [12C]amino acids. At the specified time intervals, 50 μl of each reaction mixture was removed and added to 2 ml of cold 10% trichloroacetic acid. Hot trichloroacetic acid-precipitable radioactivity was determined as described.[14]

an α/β (rabbit globin) ratio of 1 was produced when thalassemia initiation factors were used with washed rabbit ribosomes.

Similar chromatographic analysis was performed with globin synthesized by the intact thalassemia cell and by washed thalassemia ribosomes stimulated by rabbit initiation factors in the cell-free system. The cell-free system faithfully reproduced the α/β chain ratio of 8/1 that is found in this patient's intact cells (Fig. 6). An α/β ratio of 8/1 was also obtained when thalassemia or AHA initiation factors were used (Table 1).

Discussion. The objective of the present studies was to develop an active, cell-free, protein-synthesizing system from human reticulocytes in order to determine if a defect in the polypeptide chain initiation factors was responsible for the deficiency in β-chain synthesis seen in β-thalassemia. Since normal human ribosomes synthesize equal numbers of α and β chains in response to thalassemic, normal human, or rabbit initiation factors, while thalassemic ribosomes synthesize 8-times more α chains than β chains in response to any of the three sources of initiation factors, the molecular defect in β-thalassemia in the patients studied does not appear to be the result of an initiation factor defect.

Initiation factors isolated from human sources (thalassemia and AHA) are equally active when utilized with all types of washed reticulocyte ribosomes, but are considerably less active than rabbit factors. The supernatant (S-100) fractions from thalassemia and from AHA cells are equally active for short time periods using a homologous or heterologous human system; but, reaction

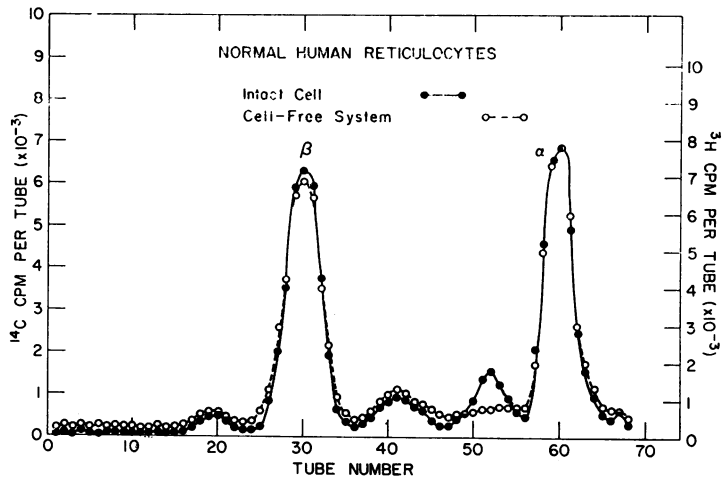

FIG. 5. Double-label carboxymethylcellulose chromatography of human globin chains produced in intact AHA reticulocytes ([^{14}C-]leucine) and by normal (AHA) human ribosomes in the cell-free system in response to rabbit initiation factors ([^{3}H]leucine). Globin was prepared in the cell-free system by incubating for 20 min at 37°C a 400 μl reaction mixture containing 1.25 A_{260} units of washed AHA ribosomes, 640 μg of rabbit ribosomal wash fraction (treated with DEAE-cellulose to remove nucleic acids as described previously[14]), 24 μl of AHA S-100, 0.52 A_{260} units of unfractionated rabbit reticulocyte tRNA, 50 μM [^{3}H]leucine (9900 Ci/mol) and 100 μM in each of the other 19[^{12}C]amino acids. The concentration of other reactants is listed in the legend to Fig. 1. The procedures used for preparing human globin uniformly labeled with [^{14}C]leucine (344 Ci/mol) and for chromatographing the double-label mixture of globins were as described in *Methods*.

TABLE 1. *Protein synthetic activity and product analysis of various ribosome and ribosomal wash fraction combinations.*

Source of ribosomes	Source of ribosomal wash fraction	[^{14}C]leucine incorporated into protein (pmol)	Alpha/beta globin chain ratio
Thalassemia	Thalassemia	0.35 ± 0.05	7.9 ± 1.8
Thalassemia	AHA	0.35 ± 0.04	8.3
Thalassemia	Rabbit	3.13 ± 0.10	7.3 ± 0.9
AHA	Thalassemia	3.4 ± 0.1	1.4
AHA	AHA	3.2 ± 0.1	1.0 ± 0.1
AHA	Rabbit	17.5 ± 1.5	1.2 ± 0.2
Rabbit	Thalassemia	9.7 ± 1.0	1.05
Rabbit	AHA	10.4 ± 0.3	—
Rabbit	Rabbit	46.0 ± 5.4	1.01

Reaction mixtures (50 μl) were incubated exactly as described in the legend to Fig. 4. Protein synthesis is calculated for 0.25 A_{260} units of ribosomes for 10 min incubation. Average deviation for four identical assays are shown. Since the ribosomes and ribosomal-wash fractions used for these studies were different from those examined in Fig. 4, absolute values are not the same between the two sets of data. 8 μl of the indicated ribosomal wash fraction (in 0.5 M KCl) was added. Each reaction was run under conditions that were linear with respect to time, at rate-limiting concentration of ribosomes, and at optimal or saturating levels of the other components (as determined in preliminary experiments). Chain ratios are the actual ratio: total cpm under the α peak/total cpm under the β peak. When several experiments were performed, the average value with the average deviation is indicated. AHA, autoimmune hemolytic anemia.

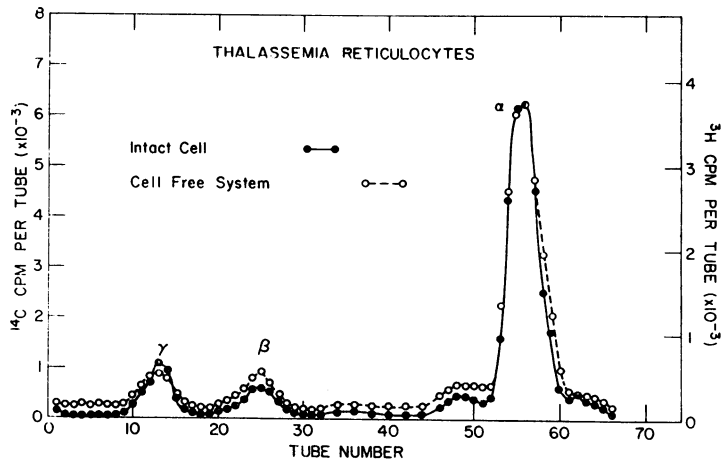

FIG. 6. Double-label carboxymethylcellulose chromatography of human globin chains produced in intact thalassemia reticulocytes ([^{14}C]-leucine) and by thalassemia ribosomes in the cell-free system in response to rabbit initiation factors ([^{3}H]leucine). Globin was prepared in the cell-free system by incubating for 10 min at 37°C a 600 μl reaction mixture containing 4.5 A_{260} units of washed thalassemia ribosomes, 960 μg of rabbit ribosomal wash fraction (treated with DEAE-cellulose to remove nucleic acids), 36 μl of AHA S-100, 0.78 A_{260} units of unfractionated rabbit reticulocyte tRNA, and [^{3}H]leucine and other [^{12}C]-amino acids as in Fig. 5. The concentration of other reactants is listed in the legend to Fig. 1.

mixtures containing S-100 from AHA remain linear in protein synthesis for a longer period of time. An explanation for these observations is not clear.

Marks and co-workers have reported an ultrafiltrable factor in cell-free extracts of β-thalassemic cells that inhibits protein synthesis in their cell-free system from nonthalassemic humans.[8] A recent communication reports that this substance inhibits α-chain synthesis and stimulates β-chain synthesis.[18] An exact role for this substance in the thalassemic cell has not yet been determined. Since our studies indicate that the β-thalassemia defect is present in systems in which only the washed ribosomes are of thalassemic origin (supporting the original suggestion of Bank and Marks that the defect is in the mRNA-ribosome complex[7]) it is unlikely that any soluble substance would be responsible for the β-thalassemia cell's deficiency in β-chain synthesis.

The most likely possibilities for the molecular basis of β-thalassemia would appear to be (*1*) an absolute decrease in the amount of β-chain mRNA; (*2*) an abnormality in the initiation process for the β-chain mRNA relating either to a different mechanism for the initiation of the β chain, to an abnormality in the codon signals that precede the initiation codeword, or to a ribosome structural defect; (*3*) or to the presence of a "regulatory" codon (i.e., a codon recognized only by a tRNA present in rate-limiting amounts[19]) in the thalassemia β-chain mRNA that is not in the mRNA of the normal cell.[20]

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Abbreviation: AHA, autoimmune hemolytic anemia.

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