JCI The Journal of Clinical Investigation

Hemoglobin Messenger RNA from Human Bone Marrow ISOLATION AND TRANSLATION IN HOMOZYGOUS AND HETEROZYGOUS β-THALASSEMIA

A. W. Nienhuis, … , P. H. Canfield, W. F. Anderson

J Clin Invest. 1973;52(7):1735-1745. https://doi.org/10.1172/JCI107355.

Research Article

A method for isolating human hemoglobin messenger RNA (mRNA) from bone marrow cells was developed to investigate the molecular basis for the defect in globin synthesis in beta thalassemia. Active mRNA was isolated from the bone marrow cells and peripheral reticulocytes of patients with homozygous beta thalassemia, heterozygous beta thalassemia, sickle cell trait, double heterozygosity for beta thalassemia and sickle cell trait, as well as from a patient with normal hemoglobin synthesis but with an elevated reticulocyte count secondary to hereditary spherocytosis. The mRNA was prepared for assay in an mRNA-dependent rabbit reticulocyte cell-free system and the amount of alpha and beta globin chains synthesized was determined by carboxymethylcellulose column chromatography. The relative synthesis of alpha to beta chains in response to normal hemoglobin mRNA was found to be a function of the amount of mRNA added to the assay system: increasing the amount of mRNA led to a decrease in the alpha-to-beta-chain synthetic ratio. Therefore, assays were carried out at limiting concentrations of mRNA.

The molecular defect in homozygous beta thalassemia was shown to be carried in the mRNA of bone marrow cells as well as in the mRNA from peripheral reticulocytes, because much less beta than alpha globin was produced in the cell-free system in response to mRNA from either type of cell. In patients doubly heterozygous for […]

Find the latest version:

https://jci.me/107355/pdf

Hemoglobin Messenger RNA from Human Bone Marrow

ISOLATION AND TRANSLATION IN HOMOZYGOUS AND HETEROZYGOUS β-THALASSEMIA

A. W. Nienhuis, P. H. Canfield, and W. F. Anderson

From the Molecular Hematology Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Abstract A method for isolating human hemoglobin messenger RNA (mRNA) from bone marrow cells was developed to investigate the molecular basis for the defect in globin synthesis in beta thalassemia. Active mRNA was isolated from the bone marrow cells and peripheral reticulocytes of patients with homozygous beta thalassemia, heterozygous beta thalassemia, sickle cell trait, double heterozygosity for beta thalassemia and sickle cell trait, as well as from a patient with normal hemoglobin synthesis but with an elevated reticulocyte count secondary to hereditary spherocytosis. The mRNA was prepared for assay in an mRNA-dependent rabbit reticulocyte cell-free system and the amount of alpha and beta globin chains synthesized was determined by carboxymethylcellulose column chromatography. The relative synthesis of alpha to beta chains in response to normal hemoglobin mRNA was found to be a function of the amount of mRNA added to the assay system: increasing the amount of mRNA led to a decrease in the alpha-to-beta-chain synthetic ratio. Therefore, assays were carried out at limiting concentrations of mRNA.

The molecular defect in homozygous beta thalassemia was shown to be carried in the mRNA of bone marrow cells as well as in the mRNA from peripheral reticulocytes, because much less beta than alpha globin was produced in the cell-free system in response to mRNA from either type of cell. In patients doubly heterozygous for beta thalassemia and sickle cell trait, little or no synthesis of beta^A globin occurred in the bone marrow cells or the peripheral reticulocytes. The alpha to beta^S synthetic ratio of the intact bone marrow cells was approximately 1, while the same ratio in the peripheral reticulocytes was between 1.5 and 2. The virtual absence of translatable beta globin mRNA in the mRNA prepared from the cells of these doubly heterozygous patients further demonstrates that the molecular defect produced by the beta thalassemia gene is in the beta globin mRNA.

Received for publication 26 December 1972 and in revised form 7 March 1973.

INTRODUCTION

Beta thalassemia is an hereditary hemolytic anemia characterized by a deficiency or absence of synthesis of beta globin (1–5), one of the polypeptide subunits of the hemoglobin molecule. This defect in beta globin synthesis occurs in the bone marrow erythroid cells as well as in the peripheral reticulocytes of patients homozygous for beta thalassemia (6), although the deficiency in beta chain synthesis appears to be less marked in the bone marrow cells as compared to the peripheral blood reticulocytes. Hemoglobin synthesis in peripheral reticulocytes of patients heterozygous for beta thalassemia is characterized by a 35–50% reduction in beta chain relative to alpha chain synthesis (3), but in the bone marrow cells of these patients an alpha/beta chain ratio of close to 1 is observed (7, 8). The molecular mechanism responsible for the apparent decrease in the relative rate of synthesis of beta globin during maturation of the beta thalassemia erythroid cells has not yet been defined.

We have previously reported isolation of hemoglobin messenger RNA (mRNA)[1] from reticulocytes of patients with homozygous beta thalassemia and described its translation into protein in a cell-free system capable of synthesizing complete alpha and beta globin chains (9). A marked deficiency in beta chain synthesis occurs in the cell-free system, thus demonstrating that there is a reduction in the amount of functional beta globin mRNA

[1] *Abbreviations used in this paper:* Hb, hemoglobin; MEM, minimal essential Eagle's medium; mRNA, messenger RNA; tRNA, transfer RNA; S100, post-ribosomal supernatant.

TABLE I
Clinical Data for Selected Patients

	Patient	Age	Hemoglobin	Hematocrit	MCV	MCH	Reticulocyte count	Hemoglobin electrophoresis	Hgb F	Hgb A_2
			mg/100 ml	%	μ^3	*pg*			%	%
Normal values										
male			16.0±2	47±5	88±5	29±2	0.5–1.5	A	<2	<2.9
female			14.0±2	42±5						
Normal (hereditary spherocylosis)	F. N.	29	12.3	44.4	90	31.9	13.7	A	ND	ND
Thal. major	J. L.	16	6.8	27.0	72	21	4.5	AF	17.8	2.0
	N. L.	20	6.4	28.0	73	19	10.2	AF	33.0	2.5
Thal. minor	F. K.	42	13.2	40.8	65	20.7	1.6	A	1.3	3.1
Sickle trait	A. K.	41	12.6	38	84	28	0.6	AS	ND	ND
	J. P.	27	12.7	39	84	27	1.1	AS	ND	ND
Sickle-thal.	J. K.	12	6.9	23	69	20.5	22	S	4.4	0.9
	N. K.	14	9.2	29.2	65	20	12	S	6.8	1.3

ND; not determined.

in homozygous beta thalassemia at the reticulocyte stage of erythroid cell maturation. Similar results were obtained by Benz and Forget (10), However, most cellular hemoglobin is synthesized in nucleated erythroid cells of bone marrow before the reticulocyte stage (11). Furthermore, as pointed out above, there is a disparity in the relative amount of beta chain synthesized in bone marrow cells compared to peripheral reticulocytes, particularly in heterozygous beta thalassemia. Therefore, we have developed means for recovering globin mRNA from human bone marrow cells and have studied the capacity of the bone marrow mRNA to direct protein synthesis in a cell-free system derived from rabbit reticulocytes (9, 12). mRNA has been obtained from bone marrow cells and peripheral reticulocytes of patients homozygous for beta thalassemia, doubly heterozygous for sickle cell trait and beta thalassemia, heterozygous for sickle cell trait or beta thalassemia, and from a patient without a defect in hemoglobin synthesis. Thus the effect of the beta thalassemia gene on functional beta globin mRNA was assessed both in heterozygous and homozygous patients. In doubly heterozygous patients who synthesize

TABLE II
Globin Chain Ratios for Selected Patients

Condition	Patient	Ratio	Retic. whole cell	Reticulocyle mRNA	Bone marrow whole cell	Bone marrow mRNA
Normal (hereditary spherocylosis)	F. N.	α/β	1.01	1.03 (8%) 0.37 (96%) 0.11 (310%)	0.93	0.74 (25%) 0.56 (50%) 0.21 (125%)
Thal major	J. L.	α/β	6.4	6.8 (16%) 5.6 (40%)	5.3	7.5 (27%) 7.6 (53%)
	N. L.	α/β	7.3	5.6 (80%)	4.6	6.7 (27%) 5.6 (56%)
Thal. minor	F. K.	α/β	2.05	—	1.14	1.21 (20%) 1.02 (50%)
Sickle trait	A. K.	$\alpha/\beta^A + \beta^S$	1.3	—	—	—
	J. P.	$\alpha/\beta^A + \beta^S$	—	—	0.95	0.80 (21%)
		$\alpha/\beta^A + \beta^S$	—	—	—	0.54 (53%)
Sickle-thal.	J. K.	α/β^S	1.61	2.3 (33%)	1.13	1.59 (16%) 1.32 (40%)
	N. K.	α/β^S	1.71	2.6 (39%)	1.08	1.65 (23%) 1.11 (58%)

Numbers in parentheses represent the percent saturation of the mRNA concentration used (see Methods).

little or no beta^{A} globin mRNA, the relative amounts of functional alpha and beta^{S} mRNA were compared during erythroid cell maturation.

METHODS

Patients studied. The hematological characteristics of the patients studied are given in Table I. N. L. and J. L. are siblings of Greek descent with homozygous beta thalassemia requiring transfusion at 6-wk intervals. Each had been splenectomized at an early age. N. K. and J. K. are siblings who are double heterozygotes for beta thalassemia and sickle cell trait. They have Hb S but no Hb A on electrophoresis, hypochromic microcytic anemia, marked splenomegaly, and unbalanced synthesis of alpha and beta^{S} globin chains in their peripheral reticulocytes. Neither had received transfusions for several years. F. K., the father of N. K. and J. K., has the hematological and biochemical characteristics of thalassemia minor. A. K., the mother of N. K. and J. K., has sickle trait and no evidence of thalassemia. J. P. is an unrelated patient with sickle trait. F. N. is a patient with hereditary spherocytosis who, before splenectomy, served as a source of control peripheral reticulocytes and bone marrow cells.

Methods. Hematological parameters were determined by standard methods (13). Percent Hb A_2 was determined by DEAE-cellulose chromatography (14), and percent Hb F by alkali denaturation (15). Bone marrow was obtained from the posterior iliac spine of each patient. Three or four aspirates yielded 6–8 cc of marrow containing a total of $2\text{–}10 \times 10^{8}$ nucleated cells. The percentage of the total nucleated cells that were erythroid precursors varied from 20 to 75% depending on the degree of marrow erythroid hyperplasia. There were 25–40% as many reticulocytes as total nucleated erythroid cells. The marrow from each aspirate was collected in a syringe containing 10 ml of minimal essential Eagle's media (MEM) (16), with 400 U of heparin/ml. The aspirates were pooled, mixed, and portions retained for nucleated cell counts and whole cell incubations. The remainder was used for the extraction of globin mRNA.

Extraction of globin mRNA from human bone marrow cells. $2\text{–}9 \times 10^{8}$ nucleated bone marrow cells suspended in approximately 40 ml of heparinized MEM were added in drops to a rapidly stirred phenol-buffer mixture at room temperature. This mixture consisted of 40 ml of acetate buffer (0.5 M sodium acetate (pH 5.0), 0.1 M NaCl, 0.01 M EDTA, and 0.5% sodium dodecyl sulfate) and 80 ml of reagent grade phenol previously saturated with the acetate buffer. Recovery of the aqueous phase, reextraction of the phenol phase at 56°C with acetate buffer, and recovery of RNA by ethanol precipitation was performed exactly as described previously for the preparation of globin RNA from sheep and goat bone marrow cells (17), except that chloroform was omitted. The RNA was fractionated by sucrose gradient centrifugation, and a 9–10S fraction collected, concentrated by ethanol precipitation, and prepared for assay in the cell-free system as previously described (9, 12, 17).

Extraction of globin mRNA from peripheral reticulocytes. Preparation of membrane-free lysates from peripheral blood (18, 19), phenol extraction of total cellular RNA, sucrose gradient centrifugation of RNA, and preparation of the mRNA (9–10 S) fraction for assay in the cell-free system have been previously described (9, 12).

Cell-free protein synthesis in the mRNA-dependent assay system. All biological components of the assay system except for mRNA were prepared from reticulocytes (18) obtained from rabbits injected with phenylhydrazine. Ribosomes were freed of rabbit globin mRNA by mild ribonuclease treatment (9, 12). The methods for preparation of (*a*) the ribosomal wash fraction containing the protein synthesis initiation factors, (*b*) the S-100 supernatant fraction containing the aminoacyl transfer RNA (tRNA) synthetases and the protein synthesis elongation factors, and (*c*) the unfractionated reticulocyte tRNA have been previously described (12, 18). Assay of the mRNA fractions was performed in a standard 0.10-ml reaction mixture containing 20 mM Tris-HCl (pH 7.5), 3.75 mM $MgCl_2$, 90 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM guanosine 5'-triphosphate, 3 mM phosphoenolpyruvate, 0.05 IU pyruvate kinase, 0.04 mM [^{14}C]leucine, 0.08 mM [^{12}C]-amino acids except leucine, 0.16 A_{260} U rabbit reticulocyte tRNA, 150 μg of the ribosomal wash fraction, 100 μg of the S-100 supernatant fraction, and 0.12 A_{260} U of ribonuclease-treated ribosomes. Each mRNA preparation was assayed at several different concentrations (range 0.01–0.50 A_{260} U/0.10 ml reaction mixture) in order to determine the amount of RNA that produced maximal activity (satu-

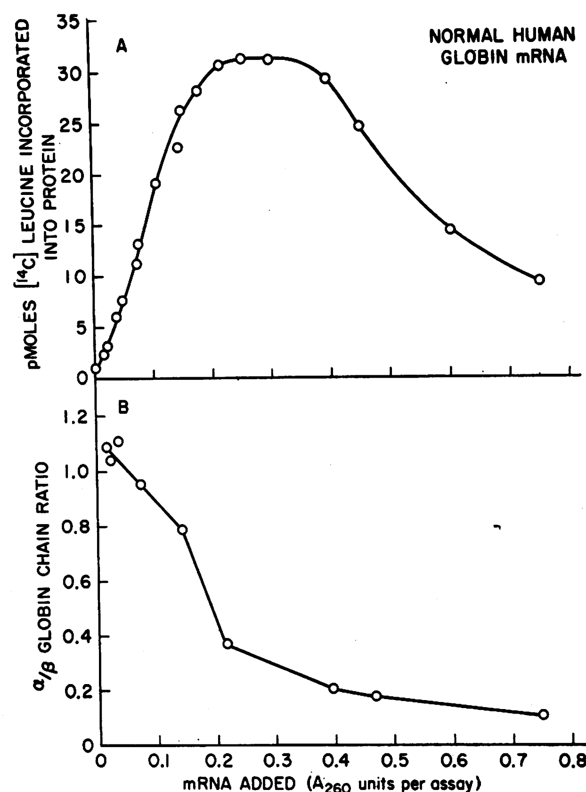
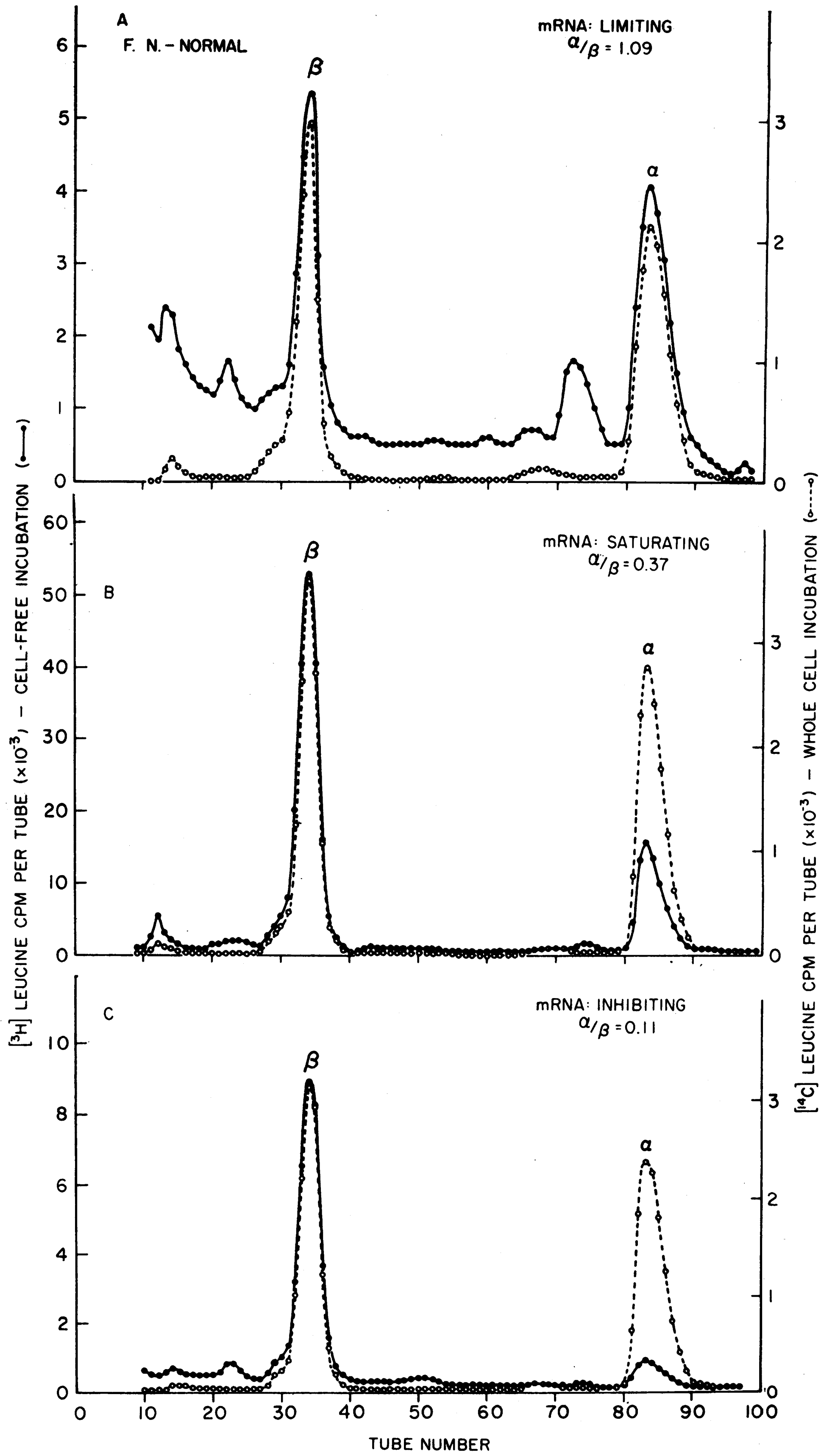


FIGURE 1 The effect of normal human globin mRNA concentration used in the cell-free assay system on the relative amounts of alpha and beta globin chains synthesized. (A) Total activity. Assays were performed in a standard 0.1-ml incubation mixture as described in Methods. (B) Alpha/beta globin chain ratio synthesized at various mRNA concentrations. The human globin product was chromatographed on a carboxymethylcellulose column and the ratio of total radioactivity incorporated into alpha and beta chains was calculated (See Methods).


A
F. N. - NORMAL
mRNA: LIMITING
α/β = 1.09
β
α
B
mRNA: SATURATING
α/β = 0.37
β
α
C
mRNA: INHIBITING
α/β = 0.11
β
α
[3H] LEUCINE CPM PER TUBE (×10−3) - CELL-FREE INCUBATION
[14C] LEUCINE CPM PER TUBE (×10−3) - WHOLE CELL INCUBATION
TUBE NUMBER

ration). Incubation was for 30 min at 37°C. After incubation, protein was precipitated by addition of 2 ml of 10% trichloroacetic acid. The reaction mixture was heated to 90°C for 15 min and cooled at 4°C for 10 min. The precipitated protein was collected by filtration on a nitrocellulose filter; the filter was dried and counted in Liquifluor-toluene (New England Nuclear, Boston, Mass.) at an efficiency of 80% for ^{14}C.

Labeling of globin in bone marrow whole cell incubations. Intact bone marrow cells were incubated with 330 μCi of [^{3}H]leucine (Schwarz/Mann, Div., Becton, Dickinson & Co., Orangeburg, N. Y., 12 Ci/mmole) or 16 μCi of [^{14}C]-leucine (Amersham/Searle Corp., Arlington Heights, Ill., 331 mCi/mmole) in MEM, containing 15% fetal bovine serum, 2 mM glutamine, 0.18% (wt/vol) $NaHCO_3$, 150 U/ml penicillin, and 150 μg/ml streptomycin. 2×10^7 nucleated bone marrow cells were incubated for 6 h; the cells were collected by centrifugation, washed twice with normal saline, and lysed in 1.5 ml of distilled water with a glass ball homogenizer. The lysate was centrifuged at 30,000 g for 30 min to remove membranes and debris; the supernatant fraction, containing labeled globin, was stored in aliquots in liquid nitrogen. Labeling of globin in reticulocyte incubations was performed as previously described (18, 19).

Product analysis of globin chains synthesized in the cell-free assay system. To analyze the products of the cell-free assay system, the size of the reaction mixture was increased 3–15 fold. The concentration of all reactants was unchanged. [^{3}H]leucine (specific activity 51 Ci/mmole) was substituted for [^{14}C]leucine. The incubations for product analysis were run at rate-limiting amounts of mRNA. The amount of mRNA tested and the percentage this amount represented of that required to give maximum protein synthesis (percent saturation) is given in Figs. 3 and 4 and in Table II. Each column was run as a double-label experiment comparing the products of the cell-free assay system (labeled with ^{3}H) to the corresponding products of a whole cell incubation (labeled with ^{14}C). The uniformly labeled globin chains obtained from whole cell incubations provided an internal control for calculating alpha/beta globin chain ratios (Table II). The globin chains were separated by carboxymethyl-cellulose column chromatography in 8 M urea-phosphate buffer after preparation of the globin by acid-acetone extraction of heme (12, 19, 20). The conditions of chromatography permitted separation of all the radioactive rabbit globin from the human alpha peak (12). The radioactivity in the column fractions was quantitated by standard double-label counting techniques. 0.75 ml of each fraction, 0.75 ml of water, and 15 ml of Triton X-100 in Liquifluor were mixed in a scintillation vial and counted directly. The efficiency for ^{3}H was 16% and for ^{14}C was 56%. The alpha/beta globin chain ratios were calculated by summing the total counts in each peak after correcting the ^{3}H counts per minute for the ^{14}C contribution.

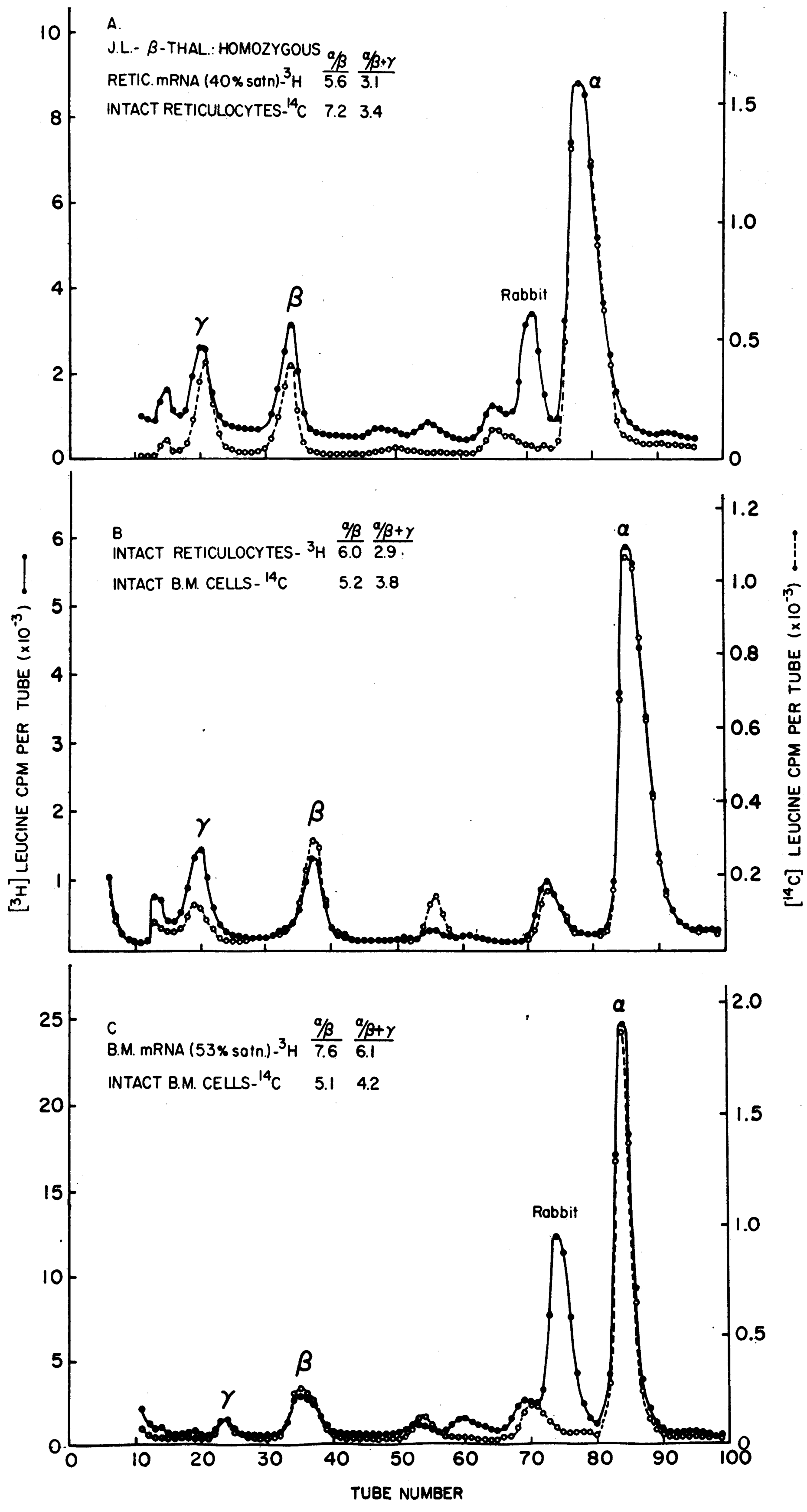
RESULTS

Globin mRNA was recovered from both total bone marrow cells and peripheral reticulocytes and tested in the cell-free assay system (see Methods). The amount of protein synthesized in the cell-free system increased with increasing amounts of either bone marrow or peripheral reticulocyte mRNA until saturation was achieved. The data for normal reticulocyte mRNA are shown in Fig. 1A. A similar concentration curve was obtained with each reticulocyte and bone marrow mRNA preparation. The maximal activity, expressed as picomoles [^{14}C]leucine incorporated into protein per A_{260} unit of RNA, ranged from 40 to 100 for the reticulocyte preparations and from 12 to 30 for the bone marrow RNA preparations. There was no correlation between disease state and maximal activity of mRNA. The most active preparations were obtained from the peripheral blood samples having highest reticulocyte counts and from bone marrow samples in which most of the cells were erythroid precursors.

The relationship between the alpha/beta synthetic ratio and the concentration of normal reticulocyte mRNA added to the cell-free assay system was determined. At limiting mRNA, the alpha/beta ratio is close to 1 (Fig. 2A); at saturating mRNA it is about 0.3–0.4 (Fig. 2B); and at inhibiting concentrations of mRNA, the ratio drops to 0.1 (Fig. 2C). The full range of alpha/beta ratios is plotted in Fig. 1B. Similar data were obtained with normal bone marrow mRNA (Table II) and had previously been found with rabbit globin mRNA. Thus, for the alpha/beta globin chain ratio in the cell-free assay system to reflect the ratio found in the intact normal cell, the concentration of mRNA assayed in the cell-free system must be below 30% saturation. The data obtained with mRNA preparations extracted from abnormal cells and assayed at various concentrations also demonstrate these observations (Table II). The percentage saturation of mRNA in each experiment is given in Table II and in the legends of Figs. 3 and 4.

The alpha/beta globin chain ratio was measured in each patient (Table I), both in intact cells by whole cell incubation and in the cell-free system in response to globin mRNA isolated from the bone marrow cells and peripheral reticulocytes (see Table II). In the two patients with homozygous beta thalassemia, a marked decrease in beta chain synthesis was evident in both marrow cells and peripheral reticulocytes. mRNA extracted from each of these cell populations reflected the same alpha/beta globin chain imbalance (Fig. 3). Little or no beta^{A} globin synthesis was demonstrated

FIGURE 2 Chromatography of the globin products produced at various concentrations of normal human reticulocyte mRNA. The cell-free product, labeled with [^{3}H]leucine (●—●), in each experiment was co-chromatographed with globin labeled by incubation of intact reticulocytes with [^{14}C]leucine (○—○) (See Methods for experimental details). (A) Rate-limiting mRNA (0.014 OD_{260}/assay). (B) Saturating mRNA (0.22 OD_{260}/assay). (C) Inhibiting mRNA (0.76 OD_{260}/assay). Compare with Fig. 1. BM, bone marrow.


A.
J.L.- β-THAL.: HOMOZYGOUS
α/β α/β+γ
RETIC. mRNA (40% satn)-³H 5.6 3.1
INTACT RETICULOCYTES-¹⁴C 7.2 3.4
γ
β
Rabbit
α
B
α/β α/β+γ
INTACT RETICULOCYTES-³H 6.0 2.9
INTACT B.M. CELLS-¹⁴C 5.2 3.8
γ
β
α
C
α/β α/β+γ
B.M. mRNA (53% satn.)-³H 7.6 6.1
INTACT B.M. CELLS-¹⁴C 5.1 4.2
γ
β
Rabbit
α
[³H] LEUCINE CPM PER TUBE (x10⁻³)
[¹⁴C] LEUCINE CPM PER TUBE (x10⁻³)
TUBE NUMBER

by whole cell incubations or in the cell-free system in response to mRNA isolated from the cells of the two patients with sickle-thalassemia (Fig. 4). However, the alpha/beta^S ratios were different. Intact cells from the bone marrow produced equal amounts of alpha and beta^S chains; in the cell-free system at rate-limiting concentrations of bone marrow mRNA, an excess of alpha globin was produced. The peripheral reticulocytes of these patients had an alpha/beta^S synthetic ratio of 1.6–1.7 in the whole cell incubation, and 2.3–2.6 in the cell-free system in response to reticulocyte globin mRNA, while the corresponding ratio for bone marrow cells was close to 1 (Table II). This disparity in synthesis of globin by intact cells of the bone marrow and peripheral reticulocytes is similar to that seen in the beta thalassemia heterozygote: the alpha/beta synthetic ratio of bone marrow was nearly 1, but that of reticulocytes was approximately 2.0 (Fig. 5). The alpha/beta ratios in the cell-free system at rate-limiting concentration of this patient's bone marrow mRNA were greater than 1 and were considerably higher than those obtained with normal bone marrow mRNA at equivalent concentrations (Table II).

DISCUSSION

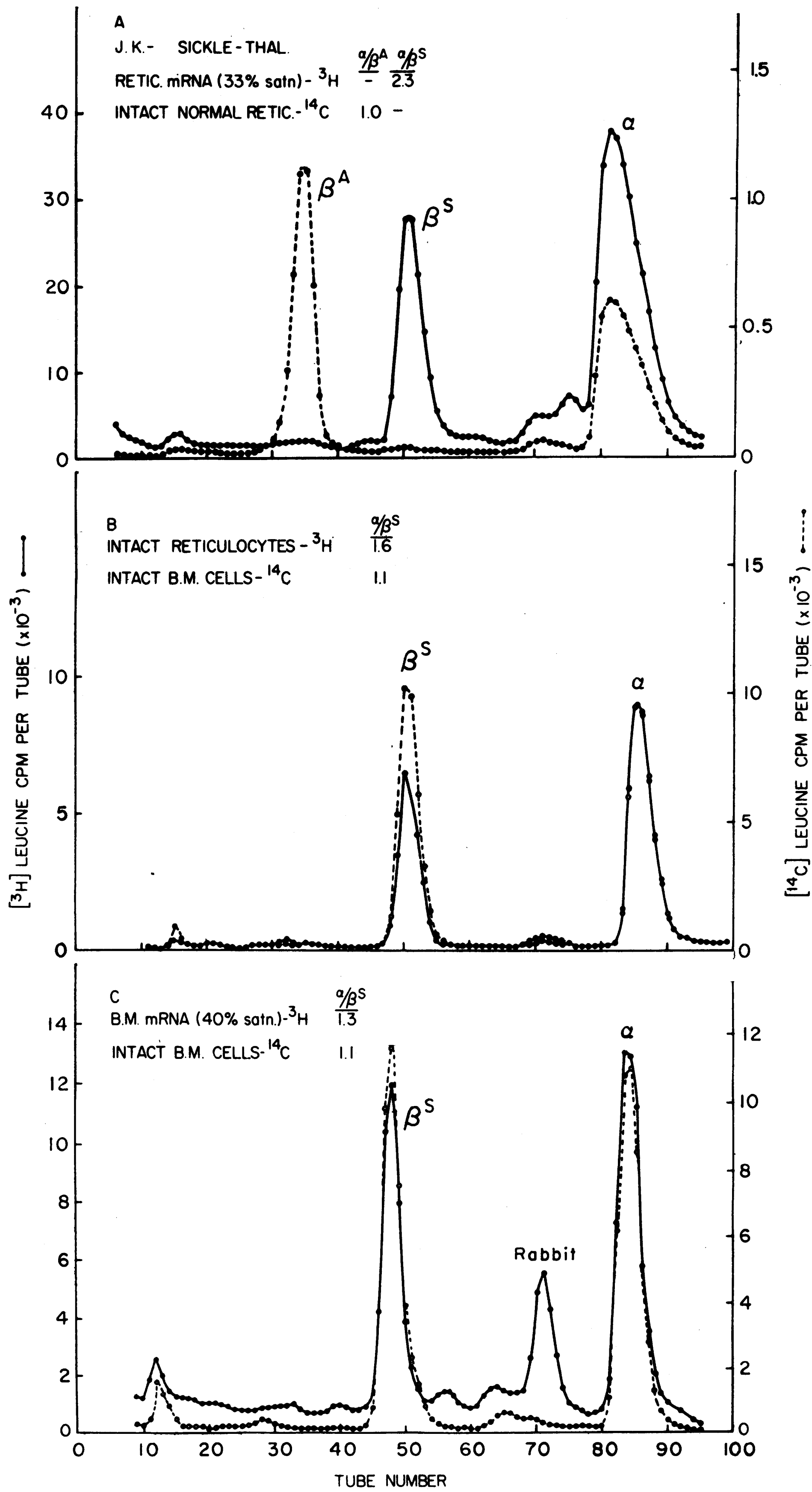
Globin mRNA, extracted from the bone marrow erythroid cells of patients with homozygous beta thalassemia, directed the synthesis of a large excess of alpha compared with beta globin in the cell-free system. This disparity in alpha/beta chain synthesis was comparable to the disparity seen during whole cell incubation. Since 75–90% of total red cell hemoglobin is produced in the nucleated marrow erythroid cells (11), this observation was necessary to confirm the interpretation, based on studies with reticulocyte globin mRNA, that the molecular defect in homozygous beta thalassemia is a reduction in the amount of functional beta globin mRNA (9, 10).

Two interesting observations have been obtained from the study of double heterozygotes with sickle-thalassemia. Little or no beta^A globin synthesis was detected in the intact erythroid cells of these patients, and little or no translatable beta^A globin mRNA was found after extraction of the RNA from these cells. This observation suggests that in the type of beta thalassemia characterized by very low or absent beta^A synthesis, the molecular defect is also carried by the beta globin mRNA. Conconi, Rowley, Del Senno, and Pontremoli, however, have obtained data using a cell-free system of low activity which they interpret to suggest that patients homozygous for thalassemia with zero beta^A production (Ferrara type) have normal beta^A globin mRNA but are missing a factor present in normal human reticulocyte lysates (21). Similar experiments performed by Rowley and Rosciolek in studies of patients with homozygous beta thalassemia who produce some beta chain have shown no alteration in the alpha/beta ratio in the cell-free system (22), confirming our previous results obtained in a highly active cell-free system derived from rabbit reticulocytes (19). It would be of interest to examine directly the bone marrow globin mRNA of patients with Ferrara-type homozygous beta thalassemia in an active cell-free system to see if a normal amount of translatable beta^A globin mRNA is present despite the lack of beta^A globin synthesis by the intact cells.

A second observation derived from the study of the sickle-thalassemia patients bears on the apparent disparity between the alpha/beta synthetic ratio in bone marrow cells and peripheral reticulocytes. This disparity would not seem to be a direct result of the beta thalassemia gene since beta^A globin synthesis is essentially absent in both marrow cells and peripheral reticulocytes, and yet the alpha/beta^S ratio is much closer to 1 in the intact bone marrow cells of these doubly heterozygous patients than in their peripheral reticulocytes. An apparently balanced synthesis of globin occurs at the expense of total reduction of hemoglobin synthesis, because the cells of these patients are hypochromic and microcytic (Table I). Recently, Gill, Atwater, and Schwartz have shown that a similar phenomenon exists in patients heterozygous for Hb Lepore: alpha/beta globin chain synthesis is close to 1 in the bone marrow of these patients while unbalanced synthesis occurs in the peripheral reticulocytes even though there is very little Hb Lepore produced in either cell type (23).

Detection of a small pool of excess alpha chains in the marrow of heterozygous beta thalassemia patients indicates that globin synthesis is not completely balanced (24). In addition, the presence of an unknown marrow protein eluting with the beta globin chain from carboxymethylcellulose columns may further complicate the picture (24). Nevertheless, the evidence indicates that bone marrow cells can achieve more closely balanced globin synthesis than occurs in the

FIGURE 3 Homozygous beta thalassemia: globin produced in rabbit reticulocyte cell-free system in response to bone marrow cell and peripheral reticulocyte mRNA compared to globin synthesized in intact cells. (A) Reticulocyte mRNA: 40% saturation ([^{3}H]leucine, ●—●) vs. intact reticulocytes ([^{14}C]leucine, ○—○). (B) Intact reticulocytes ([^{3}H]leucine, ●—●) vs. intact bone marrow cells ([^{14}C]leucine, ○—○). (C) Bone marrow cell mRNA: 53% saturation ([^{3}H]leucine, ●—●) vs. intact bone marrow cells ([^{14}C]leucine, ○—○). See Methods for experimental details.



A
J.K.- SICKLE-THAL.
RETIC. mRNA (33% satn)- 3H
INTACT NORMAL RETIC.-14C
α/βA α/βS
- 2.3
1.0 -
βA
βS
α
B
INTACT RETICULOCYTES - 3H
INTACT B.M. CELLS- 14C
α/βS
1.6
1.1
βS
α
C
B.M. mRNA (40% satn.)-3H
INTACT B.M. CELLS-14C
α/βS
1.3
1.1
βS
Rabbit
α
[3H] LEUCINE CPM PER TUBE (x10-3)
[14C] LEUCINE CPM PER TUBE (x10-3)
TUBE NUMBER

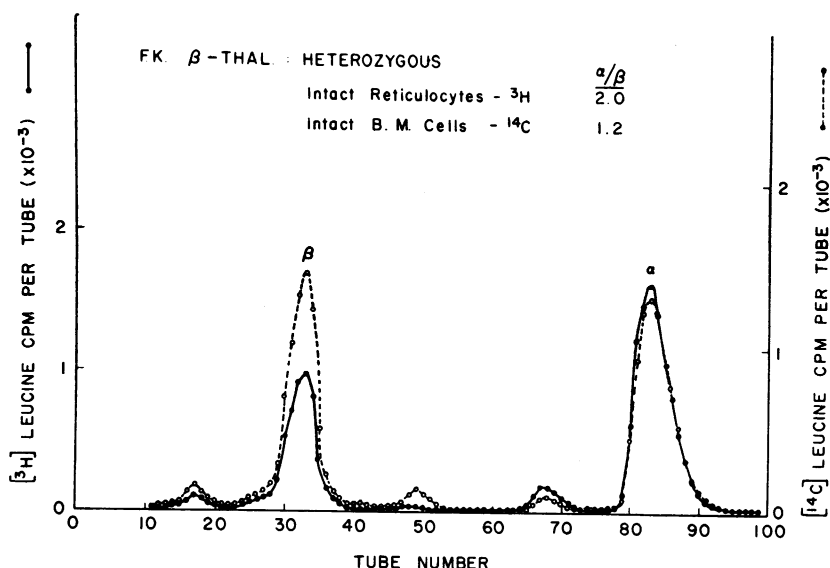


FIGURE 5 Heterozygous beta thalassemia: comparison of alpha/beta globin chain ratio in intact peripheral reticulocytes ([^{3}H]leucine, ●—●) vs. intact bone marrow cells ([^{14}C]leucine, ○—○). See Methods for experimental details.

peripheral reticulocytes despite the presence of a thalassemia gene that does not produce functional beta globin mRNA. Balanced synthesis in the bone marrow occurs at the expense of substantial reduction in hemoglobin synthesis, because the resulting erythrocytes are hypochromic and microcytic (Table I). Kan, Nathan, and Lodish (8) have calculated that in thalassemia heterozygotes there is reduction of hemoglobin synthesis to 70% of normal. Each of our patients heterozygous for beta thalassemia had approximately 20 pg of hemoglobin/cell rather than the normal 29 pg (Table I), implying a similar reduction of hemoglobin synthesis to 70% of normal. Thus, to achieve balanced synthesis there must be reduction of alpha chain production although there may also be some compensatory increase in beta chain production from the nonthalassemia gene. Thus multiple factors may account for the apparent balanced synthesis of globin in bone marrow cells of patients with heterozygous thalassemia. At the end stages of hemoglobin production as it occurs in the peripheral reticulocyte, the ability to compensate for the decrease or lack of functional beta^A globin mRNA is no longer present.

We have attempted to determine whether there is an excess of alpha mRNA in the cells of thalassemia heterozygotes. Several observations are pertinent. Reference to Table II shows that both the patient with normal hemoglobin synthesis and the patient with sickle trait have alpha/beta synthetic ratios of nearly 1 in their intact bone marrow cells. The mRNA extracted from these cells gave alpha/beta ratio of somewhat less than 1, even at the lowest concentrations tested. The whole cell bone marrow alpha/beta synthetic ratios of the three patients heterozygous for beta thalassemia are all only slightly greater than 1. The bone marrow mRNA extracted from the cells of these patients gave an alpha/beta synthetic ratio greater than 1, particularly when examined at low and rate-limiting concentrations. Thus at 15–25% saturating concentrations of mRNA, the two nonthalassemic patients had alpha/beta ratios in response to bone marrow mRNA of 0.74 and 0.80, while the thalassemic heterozygotes had values of 1.21, 1.59, and 1.65. These results are taken to suggest that there may be an excess of alpha mRNA in the bone marrow cells of the thalassemic heterozygotes. This conclusion must be regarded as tentative, however. A similar comparison can be made between the reticulocyte mRNA of the patient with

FIGURE 4 Double heterozygosity for beta-thalassemia and sickle cell anemia: globin produced in rabbit reticulocyte cell-free system in response to bone marrow cell and peripheral reticulocyte mRNA compared to globin synthesized in intact cells. (A) Reticulocyte mRNA: 33% saturation ([^{3}H]leucine, ●—●) vs. intact normal reticulocytes ([^{14}C]leucine, ○—○). (B) Intact reticulocytes ([^{3}H]leucine, ●—●) vs. intact bone marrow cells ([^{14}C]leucine, ○—○). (C) Bone marrow cell mRNA: 40% saturation ([^{3}H]leucine, ●—●) vs. intact bone marrow cells ([^{14}C]leucine, ○—○). See Methods for experimental details.

normal hemoglobin synthesis and that of the two patients doubly heterozygous for beta thalassemia and sickle cell trait. The alpha/beta ratio of the normal mRNA is equal to or less than that of the intact reticulocyte, while the alpha/beta ratio of the double heterozygote mRNA exceeds that of the corresponding intact cell. This may imply again a relative excess of alpha mRNA in these cells, the translation of which is retarded. Similar comparisons between the intact cells and mRNA preparations of the patients with homozygous thalassemia do not show any consistent differences, perhaps because of the very small amount of functional beta mRNA present. The difference in alpha/beta ratio between bone marrow cells and reticulocytes of our homozygous patients was less than the average found by Braverman and Bank (6), but there was a considerable range in this parameter among the patients they studied.

Several factors influencing the relative amount of alpha and beta globin chain synthesis have been identified in cell-free systems (18, 25–28), but whether any of these is operative in intact erythroid cells is unknown. Such knowledge might be of value, however, because deliberate reduction of alpha chain synthesis, producing more nearly balanced synthesis of globin chains, might reduce the severity of the disease in the beta thalassemia homozygote (29).

ACKNOWLEDGMENTS

We wish to thank Drs. Frederick Whiting, James Whiteside, and Robert Levy for referring patients for this study, Dr. David Weatherall for allowing us to read his manuscript before publication, and Ms. Patricia Turner for excellent technical assistance.

REFERENCES

1. Heywood, J. D., M. Karon, and S. Weissman. 1964. Amino acids: incorporation into α- and β-chains of hemoglobin by normal and thalassemic reticulocytes. *Science* (*Wash. D. C.*). **146**: 530.
2. Weatherall, D. J., J. B. Clegg, and M. A. Naughton. 1965. Globin synthesis in thalassemia: an *in vitro* study. *Nature* (*Lond.*). **208**: 1061.
3. Bank, A., and P. A. Marks. 1966. Excess α-chain synthesis relative to β-chain synthesis in thalassemia major and minor. *Nature* (*Lond.*). **212**: 1198.
4. Conconi, F., A. Bargellisi, S. Pontremoli, V. Vigi, S. Volpato, and D. Gaburro. 1968. Absence of β-globin synthesis and excess of α-globin synthesis in homozygous β-thalassemia subjects from the Ferrara region. *Nature* (*Lond.*). **217**: 259.
5. Bank, A., A. S. Braverman, J. V. O'Donnell, and P. A. Marks. 1968. Absolute rates of globin chain synthesis in thalassemia. *Blood*. **31**: 226.
6. Braverman, A. S., and A. Bank. 1969. Changing rates of globin chain synthesis during erythroid cell maturation in thalassemia. *J. Mol. Biol.* **42**: 57.
7. Schwartz, E. 1970. Heterozygous beta thalassemia: balanced globin synthesis in bone marrow cells. *Science* (*Wash. D. C.*). **167**: 1513.
8. Kan, Y. W., D. G. Nathan, and H. F. Lodish. 1972. Equal synthesis of α- and β-globin chains in erythroid precursors in heterozygous β-thalassemia. *J. Clin. Invest.* **51**: 1906.
9. Nienhuis, A. W., and W. F. Anderson. 1971. Isolation and translation of hemoglobin messenger RNA from thalassemia, sickle cell anemia, and normal human reticulocytes. *J. Clin. Invest.* **50**: 2458.
10. Benz, E. J. Jr., and B. G. Forget. 1971. Defect in messenger RNA for human hemoglobin synthesis in beta thalassemia. *J. Clin. Invest.* **50**: 2755.
11. Borsook, H., J. B. Lingrel, J. L. Scaro, and R. L. Millette. 1962. Synthesis of haemoglobin in relation to the maturation of erythroid cells. *Nature* (*Lond.*). **196**: 347.
12. Crystal, R. G., A. W. Nienhuis, N. A. Elson, and W. F. Anderson. 1972. Initiation of globin synthesis: preparation and use of reticulocyte ribosomes retaining initiation region messenger RNA fragments. *J. Biol. Chem.* **247**: 5357.
13. Cartwright, G. E. 1963. Diagnostic Laboratory Hematology. Grune & Stratton Inc., New York. 3rd edition.
14. Bernini, L. S. 1969. Rapid estimation of hemoglobin A_2 by DEAE chromatography. *Biochem. Genet.* **2**: 305.
15. Chermoff, A. I., and L. L. Horton. 1958. Determination of fetal hemoglobin in whole blood using a modified alkali denaturation test. *Amer. J. Clin. Path.* **30**: 204.
16. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* (*Wash. D. C.*). **130**: 432.
17. Nienhuis, A. W., and W. F. Anderson. 1972. Hemoglobin switching in sheep and goats: change in functional globin messenger RNA in reticulocytes and bone marrow cells. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 2184.
18. Gilbert, J. M., and W. F. Anderson. 1970. Cell-free hemoglobin synthesis: II. Characteristics of the transfer ribonucleic acid-dependent assay system. *J. Biol. Chem.* **245**: 2343.
19. Gilbert, J. M, A. G. Thornton, A. W. Nienhuis, and W. F. Anderson. 1970. Cell-free hemoglobin synthesis in beta-thalassemia. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 1854.
20. Clegg, J. B., M. A. Naughton, and D. J. Weatherall. 1966. Abnormal human hemoglobins. Separation and characterization of the α- and β-chains by chromatography, and the determination of two new variants, Hb Chesapeake and Hb J (Bangkok). *J. Mol. Biol.* **19**: 91.
21. Conconi, F., P. T. Rowley, L. Del Senno, and S. Pontremoli. 1972. Induction of β-globin synthesis in β-thalassemia of Ferrara. *Nature New Biol.* **238**: 83.
22. Rowley, P. T., and B. Kosciolek. 1972. Distinction between two types of beta thalassemia by inducibility of the cell-free synthesis of beta chains by nonthalassemic soluble fraction. *Nat. New Biol.* **239**: 234.
23. Gill, F., J. Atwater, and E. Schwartz. 1972. Hemoglobin Lepore trait: globin synthesis in bone marrow and peripheral blood. *Science* (*Wash. D. C.*). **178**: 623.
24. Clegg, J. B., and D. H. Weatherall. 1972. Hemoglobin synthesis during erythroid maturation in β-thalassemia. *Nat. New Biol.* **240**: 190.

25. Lodish, H. F. 1971. Alpha and beta globin messenger ribonucleic acid: different amounts and rates of initiation of translation. *J. Biol. Chem.* **246:** 7131.
26. Lodish, J. G., and D. G. Nathan. 1972. Regulation of hemaglobin synthesis: preferential inhibition of α- and β-globin synthesis. *J. Biol. Chem.* **247:** 7822.
27. Fuhr, J., C. Natta, P. A Marks, and A. Bank. 1969. Protein synthesis in cell-free system from reticulocytes of thalassemic patients. *Nature (Lond.).* **224:** 1305.
28. Fuhr, J. E., and C. Natta. 1972. Translational control of β- and α-globin chain synthesis. *Nat. New Biol.* **240:** 274.
29. Kan, Y. W., and D. G. Nathan. 1970. Mild thalassemia: the result of interactions of alpha and beta thalassemia genes. *J. Clin. Invest.* **49:** 635.