Decreased $\alpha$ Globin mRNA in Nucleated Red Cell Precursors in $\alpha$ Thalassemia

By Clayton L. Natta, Francesco Ramirez, James A. Wolff, and Arthur Bank

The $\alpha$ thalassemias are associated with a decrease in $\alpha$ chain synthesis. Hemoglobin H (HbH) disease is a moderately severe form of $\alpha$ thalassemia characterized by the production of 5%-20% of HbH, while $\alpha$ thalassemia trait is a milder form of $\alpha$ thalassemia. In two patients with HbH disease, the ratio of $\alpha$ chain synthesis to $\beta$ chain synthesis ($\alpha/\beta$ ratio) was decreased in both bone marrow cells and reticulocytes. When isolated mRNA from bone marrow cells and reticulocytes was translated in a heterologous cell-free system, the $\alpha/\beta$ ratios were lower than the intact cell ratios. These findings were confirmed by hybridization of the mRNA of both marrow cells and reticulocytes using purified $\alpha$ and $\beta$ cDNA probes. In the intact cells of two patients with $\alpha$ thalassemia trait, the $\alpha/\beta$ ratios were also decreased and were similar in marrow cells and reticulocytes. Cell-free studies of translatable mRNA also demonstrated decreased $\alpha/\beta$ ratios, but, unlike the HbH studies, the cell-free $\alpha/\beta$ ratios were similar to the intact cell ratios. One hybridization study utilizing peripheral blood mRNA had an $\alpha/\beta$ ratio consistent with the cell-free ratios. These results indicated that, in both HbH disease and $\alpha$ thalassemia trait, there was decreased $\alpha$ globin mRNA present in both nucleated red cell precursors and reticulocytes. In addition, the data suggested that there may be translational mechanisms that operate in intact HbH cells which attempt to balance globin chain production. In $\alpha$ thalassemia trait cells, no such controls appeared to be active and globin chain synthesis was directly proportional to the amount of $\alpha$ and $\beta$ globin mRNA in the cells.

THE $\alpha$ THALASSEMIA SYNDROMES are characterized by decreased production of $\alpha$ chains affecting the synthesis of hemoglobins A, A$, and F.$^1$ The genetics of $\alpha$ thalassemia are not completely understood. It is currently believed that there are two $\alpha$ globin genes per haploid chromosome set. Two types of $\alpha$ thalassemia genes have been postulated: an $\alpha$-thal-1 gene is associated with complete inhibition of $\alpha$ chain synthesis and is due to either deletion or inactivity of two $\alpha$ globin genes. Individuals with no detectable $\alpha$ globin synthesis have hydrops fetalis and are homozygous for $\alpha$-thal-1 genes. These individuals have been shown to have a deletion of their $\alpha$ globin genes.$^2$ The $\alpha$-thal-2 gene causes a reduced rate of $\alpha$ chain production$^3$ and is associated with the deletion of one $\alpha$ globin gene in at least some patients. Hemoglobin H disease results from a double heterozygosity of $\alpha$-thal-1 and $\alpha$-thal-2 genes, and it is an intermediate form of $\alpha$ thalassemia associated with the production of $5\alpha, 20\alpha$ HbH, mild anemia and hypochromia and microcytosis on...
smear. The α thalassemia trait is due to the presence of one α-thal-1 gene or two
α-thal-2 genes and is characterized by morphologic abnormalities of the red
cells and decreased α globin synthesis. Previous studies have shown that the
decreased α globin chain synthesis present in intact cells of patients with HbH
disease can be reproduced when reticulocyte mRNA from these patients is
added to cell-free systems.7,8

The present study was undertaken to determine if the decreased α globin
synthesis in α thalassemia was reflected in decreased α chain mRNA in ery-
throid precursor cells in bone marrow, and if there was a difference in the rela-
tive amounts of α and β globin mRNA and globin synthesis as bone marrow
cells mature in patients with the α thalassemia syndromes.

MATERIALS AND METHODS

Hemoglobin levels and red cell indices were obtained with a Model S Coulter Counter (Coulter
Electronics, Inc., Hialeah, Fla.). Cellulose acetate electrophoresis using Tris- EDTA-borate buffer
at pH 8.6 was performed in each case. The level of HbF was measured by alkali denaturation.9
Starch gel electrophoresis was used to measure HbA2, and to identify HbH.10

Patient Data

In the course of routine screening of peripheral blood samples, four patients were found to have
low red cell mean corpuscular volumes (MCV) by Coulter Counter indices; they all had normal
serum iron and iron-binding capacity (IBC), and normal HbA2 and F levels. Two of the blood
samples contained HbH on starch gel electrophoresis.

The first patient (I) was a young woman of Mediterranean origin, in her late 20s. Her physical
examination was normal except for mild splenomegaly. She had a hemoglobin value of 10.4 g/100
ml, reticulocyte count of 12%, and an MCV of 58 cu μ. She was found to have 6% - 10% HbH and
was diagnosed as having HbH disease.

The second patient (II) was a 24-yr-old woman from Guyana, South America. She was of
Chinese ancestry and has been mildly anemic all her life. Her physical examination was negative,
except for mild conjunctival pallor. Her Hb was 10 g/100 ml and hematocrit was in the low 30s.
Her reticulocyte count was 20%. Her smear was hypochromic and microcytic. The MCV was
53 cu μ. Starch gel electrophoresis revealed a HbH level of approximately 6%. She was diagnosed
as having HbH disease.

The third patient (III) was a 32-yr-old black woman who was being followed because of long-
standing mild anemia. Her physical examination was within normal limits. Her hematocrit was
31.9%, Hb 10.3 g/100 ml, and MCV 68. Her reticulocyte count was normal. Her smear showed
moderate anisocytosis, microcytosis, and moderate hypochromia. Her HbA2 level was 2.2%, and
HbF level was 0.4. Her serum iron and IBC were normal. Her bone marrow revealed adequate
iron stores. She was thought to have α thalassemia trait.

The fourth patient (IV) was a woman of African extraction in her 50s seen because of chronic
anemia manifested by hypochromia, microcytosis, and polychromasia. Physical examination re-
vealed a thin pale woman. The spleen was not palpable. Repeated starch gel determinations failed
to reveal HbH. Her HbA2 level was 1.0%, and her HbF level was 2.6. Her diagnosis was α thalas-
semia trait.

Measurement of Globin Synthesis and Globin mRNA

Samples of heparinized bone marrow were obtained from the posterior iliac crests of the four
patients, and from patients with autoimmune hemolytic anemias. The samples were washed twice
with minimal essential medium (minus glutamine), and then once with phosphate-buffered saline.
Packed red cells were washed thrice with isotonic saline. To determine the rates of synthesis of
α and β globin by bone marrow (BM) cells and reticulocytes, 1-ml aliquots were incubated with
an equal volume of Krebs–Ringer–bicarbonate (KRB) buffer minus leucine but with 100 μCi in
0.1 ml of 3H-leucine (39 Ci/mmmole) (New England Nuclear, Boston, Mass.) at pH 7.5 for
GLOBIN mRNA

I hr, as previously described. After incubation, the cells were lysed with four volumes of distilled water, and the radioactivity incorporated into purified α and β globin determined. Globin mRNA was extracted from reticulocytes and BM cells as described previously. The 6-16S fractions were isolated by two successive sucrose density gradients. The mRNA was assayed for its biologic activity using a cell-free system prepared from fungus ascites cells supplemented by rabbit ribosomal wash as previously described. The mRNA was tested in concentrations ranging from 0.2 to 2 µg per 0.1 ml of total reaction mixture.

The α and β globin mRNA content was measured by molecular hybridization to purified α and β globin cDNA. Highly radioactive normal cDNA was prepared from normal human reticulocyte mRNA using reverse transcriptase. α and β cDNAs were separated from total radioactively labeled cDNA using mRNA purified from the liver of a patient with hydrops fetalis.

Bone marrow and reticulocyte mRNA were hybridized to purified α and β cDNA to measure α and β globin mRNA content. The cDNA was prepared from 1H-dCTP, of specific activity 24.8 Ci/mole from New England Nuclear. Between 2000 and 2500 cpm of cDNA were used in each hybridization. The relative amounts of α and β mRNA were calculated from the relative C0t (C0t halfway between initial and final hybridization plateaus) using α and β cDNA.

RESULTS

All four patients had decreased α compared to β globin synthesis (α/β ratio) in both reticulocytes and bone marrow cells (Table 1). There was a significant variation in the α/β ratios of intact cells in the two HbH patients. As shown in Fig. 1, globin synthesis by intact cells of a HbH patient (II) showed diminished α chain synthesis with the α/β ratio being 0.47 in bone marrow and 0.21 in reticulocytes. In patient I, the α/β ratio of intact bone marrow cells was similar to that of patient II, the ratio being 0.40. However, the α/β ratio of peripheral blood was higher than that of patient II, 0.55. In the two patients with α thalassemia trait (III, IV) the intact cell ratios were generally higher than those of the

<table>
<thead>
<tr>
<th>Patients</th>
<th>Intact Cells*</th>
<th>Cell Free</th>
<th>Hybridization†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM (8)**</td>
<td>1.0 ± 0.2 (SD)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>PB (11)**</td>
<td>1.0 ± 0.2 (SD)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>HbH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I BM</td>
<td>0.40</td>
<td>0.23</td>
<td>0.30</td>
</tr>
<tr>
<td>PB</td>
<td>0.55</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>II BM</td>
<td>0.47</td>
<td>0.34</td>
<td>0.37</td>
</tr>
<tr>
<td>PB</td>
<td>0.21</td>
<td>0.19</td>
<td>0.30</td>
</tr>
<tr>
<td>α thal trait</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III BM</td>
<td>0.54</td>
<td>0.54</td>
<td>—</td>
</tr>
<tr>
<td>PB</td>
<td>0.42</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>IV BM</td>
<td>0.65</td>
<td>0.80</td>
<td>—</td>
</tr>
<tr>
<td>PB</td>
<td>0.75</td>
<td>0.80</td>
<td>—</td>
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</table>

*The ratio of α/β globin synthesis by intact cells determined by column chromatography. Globin was prepared from cell-free assay mixtures to which 3H-leucine had been added and which contained mRNA isolated from BM and PB. Globin labeled with 14C-leucine and prepared from intact reticulocytes from normal patients was added as carrier. Fractions (5 ml) of the column eluate were collected. The α/β ratios were calculated as the ratio of 3H cpm to 14C cpm in the peaks.

†The relative amount of α mRNA/β mRNA calculated from C0t½ β cDNA/C0t½ α cDNA. (C0t½ is C0 halfway between initial and final hybridization plateaus.)

§The numbers in parentheses are the number of patients studied; BM, bone marrow; PB, peripheral blood.
HbH patients although the values for patient III overlapped those of patient I with HbH disease (Table 1). In the four patients studied, there was no consistent change in α/β ratios between those obtained using bone marrow cells and reticulocytes.

Globin synthesis was measured using the mRNA obtained from bone marrow and reticulocytes in a Krebs ascites tumor lysate system (Table 1, Fig. 2). The α/β ratios using mRNA from reticulocytes of the two HbH patients in peripheral blood were 0.17 and 0.19, while using bone marrow mRNA the ratios were 0.23 and 0.34. In both patients, the α/β ratios obtained using isolated globin mRNA were lower than those of the intact cells (Table 1). By contrast, in the two patients with α thalassemia trait, there was no change in the α/β ratios in either bone marrow or reticulocytes when globin synthesis by intact cells and isolated mRNA were compared.

As shown in Fig. 3A, when bone marrow mRNA from patient II was hybridized to purified α and β 3H-labeled cDNAs, the C₀t ½ was threefold greater with α cDNA than with β cDNA indicating a threefold excess of β over α mRNA. The α/β mRNA ratios by hybridization in the two HbH patients were similar using both reticulocyte and bone marrow RNA, the ratio being 0.30. In one α thalassemia trait patient (III), the α/β ratio in reticulocyte mRNA by
Fig. 2. Characterization of human globin product of cell-free incubations with mRNA from bone marrow (A) and reticulocytes (B) of patient II. Globin was prepared from the cell-free assay mixture to which $^3$H-leucine had been added and which contained mRNA isolated from bone marrow and peripheral blood. (---), cpm $^3$H; (-----), OD$_{280}$ nm.

hybridization was 0.52 (Fig. 3B), which was similar to the ratio obtained by cell-free assay. mRNA prepared from the reticulocytes of a nonthalassemia patient (Fig. 3C) gave a ratio of $\alpha/\beta$ mRNA of 1.0 by hybridization. Table 2 shows the range of the ratios of $\alpha/\beta$ mRNA in different nonthalassemia mRNA preparations. In four of six samples, the $\alpha/\beta$ mRNA ratio was 1.0, while in two others, the ratios were 1.4 and 0.8. By contrast, in two separate hybridizations using mRNA isolated from peripheral blood of patient III with $\alpha$ thalassemia trait, the $\alpha/\beta$ mRNA ratios were 0.5 and 0.6. These results indicated that the relative decrease in $\alpha$ mRNA content in this latter patient was reproducible and differed significantly from values in nonthalassemia patients.

DISCUSSION

Reticulocytes of patients with HbH disease generally synthesize 30%–60% as much $\alpha$ chain as $\beta$ chain. In studies of $\alpha$ thalassemia, Schwartz et al. found $\alpha/\beta$ ratios averaging 0.44 in HbH disease, and 0.77 in $\alpha$ thalassemia trait. Patients with the silent carrier state had higher $\alpha/\beta$ ratios. In another report of intact cell synthetic ratios of a HbH patient, the $\alpha/\beta$ ratio was 0.49. In general, the $\alpha/\beta$ ratios of patients with $\alpha$ thalassemia trait have been 0.70 and
The α/β ratios in intact reticulocytes of our patients were comparable to those previously described and showed significant variation. The α/β ratio in intact marrow cells of our two patients was also variable. In one patient it was higher than in reticulocytes and in the other, not significantly different. In a previously reported patient, the α/β ratio in BM was higher than in reticulocytes. It should be noted that different ethnic populations with α thalassemia may have different genetic expression. Hydrops fetalis appears to be more common in Asiatic populations than in individuals of Italian or Spanish extraction. Gene deletion in α thalassemia has only been demonstrated to date in Asiatic subjects. Thus, the heterogeneity of α/β ratios in HbH patients may reflect differing genetic defects and perhaps different numbers of α globin genes. For
Table 2. Comparison of Relative Amounts of α and β Globin mRNA by Hybridization

<table>
<thead>
<tr>
<th>Patients</th>
<th>Cαt Values</th>
<th>αβ*</th>
<th>α</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I PB</td>
<td>4.0 x 10^-3</td>
<td>1.0</td>
<td>4.0 x 10^-3</td>
<td>1.0</td>
</tr>
<tr>
<td>II BM</td>
<td>10^-1</td>
<td>1.0</td>
<td>10^-1</td>
<td>1.0</td>
</tr>
<tr>
<td>III PB</td>
<td>1.1 x 10^-2</td>
<td>0.8</td>
<td>8.5 x 10^-3</td>
<td>1.4</td>
</tr>
<tr>
<td>IV PB</td>
<td>2.2 x 10^-3</td>
<td>1.4</td>
<td>3.0 x 10^-3</td>
<td>1.4</td>
</tr>
<tr>
<td>V PB</td>
<td>1.8 x 10^-3</td>
<td>1.0</td>
<td>1.8 x 10^-3</td>
<td>1.0</td>
</tr>
<tr>
<td>VI PB</td>
<td>4.2 x 10^-3</td>
<td>1.0</td>
<td>4.2 x 10^-3</td>
<td>1.0</td>
</tr>
<tr>
<td>Thal trait (patient III in text)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I PB</td>
<td>2.1 x 10^-1</td>
<td>0.5</td>
<td>1.1 x 10^-1</td>
<td>0.5</td>
</tr>
<tr>
<td>I PB</td>
<td>2.1 x 10^-1</td>
<td>0.6</td>
<td>1.2 x 10^-1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The relative amount of α mRNA/β mRNA calculated from Cα+β cDNA/Cα+β α cDNA.
†PB denotes mRNA isolated from peripheral blood; BM denotes mRNA isolated from bone marrow.

example, the decrease in the α/β ratio in peripheral blood in our Chinese patient (II) was more marked than in our patient of Italian extraction (I).

This is the first study to date of the biological activity of mRNA obtained from the bone marrow of patients with HbH disease and in cells of patients with α thalassemia trait. The α/β ratios obtained using HbH mRNA are lower than those in intact cells both in bone marrow and reticulocytes in HbH disease. In the α thalassemia traits (III, IV) the α/β ratios are the same using isolated mRNA as in the intact cells. The hybridization experiments, using the purified α and β cDNA probes, indicate a decrease in α mRNA content in both bone marrow cells and reticulocytes of patients with HbH disease and the reticulocytes of a patient with α thalassemia trait. The α/β mRNA content by hybridization agrees closely with the relative amount of biologically active α and β mRNA obtained by assay in the cell-free system.

In β thalassemia trait, it has been shown that balanced globin synthesis in intact bone marrow cells is associated with decreased β mRNA by cell-free assay and hybridization. These results have suggested that control mechanisms exist in intact bone marrow cells which attempt to balance α and β globin synthesis. The two most likely mechanisms are either more active α globin proteolysis in bone marrow cells or feedback inhibition by the pool of excess α chains on α mRNA translation in these cells. In HbH disease, β chain excess might also result in feedback inhibition of β globin mRNA translation and lead to more balanced α/β ratios in intact cells than that of isolated mRNA (Table 1). However, this effect may be less pronounced because of the greater tendency of free β chains to form stable β tetramers (HbH) as compared to free α chains. The finding that in α thalassemia trait the α/β ratios in intact cells and of isolated mRNA are similar (Table 1) would be consistent with the lack of sufficient numbers of free β chains to inhibit β globin synthesis in these cells. It should be emphasized that all of the available data on translational control of globin synthesis in human cells are indirect, and it has not yet been shown convincingly that the presence of free globin chains can directly influence their own biosynthesis.

In summary, we have demonstrated by both hybridization and cell-free as-
says that there is less $\alpha$ mRNA relative to $\beta$ mRNA in reticulocytes and bone marrow cells in two patients with HbH disease, and in the reticulocytes of two patients with $\alpha$ thalassemia trait. The results indicate that, as in $\beta$ thalassemia, the major defect in globin synthesis in $\alpha$ thalassemia cells is associated with reduced production of chain specific globin mRNA. The basic genetic defect in at least some $\alpha$ thalassemia cells appears to be due to a deletion of $\alpha$ globin genes\textsuperscript{2,4} which results in decreased $\alpha$ globin mRNA in both bone marrow cells and reticulocytes. The amount of $\alpha$ and $\beta$ globin synthesis is then roughly proportional to the amount of $\alpha$ and $\beta$ mRNA present. Post-transcriptional controls of globin synthesis in $\alpha$ thalassemia trait cells appear to be much less active than in $\beta$ thalassemia heterozygotes although more balanced $\alpha/\beta$ synthesis in intact HbH cells than by isolated mRNA suggests that translational controls may exist in this latter disorder.

ACKNOWLEDGMENT

We wish to thank Joyce V. O'Donnell, Judy Banks, Deborah Starkman, and Yolanda Ashby for their expert technical assistance.

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