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# The relative levels of $\alpha_2$-, $\alpha_1$-, and $\zeta$-mRNA in HB H patients with different deletional and nondeletional $\alpha$-thalassemia determinants

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**Abstract**

We have analyzed the $\alpha_2/\alpha_1$-, $\alpha/\beta$-, $\zeta/(\alpha+\zeta)$-mRNA ratios in the retic-ulocytes of 40 patients with Hb H disease. 21 patients had deletional Hb H disease ($--/-\alpha$), namely combinations of one of four types of $\alpha$-thal-1 (MED-I, MED-II, $-(\alpha)20.5$, SEA) and one of two types of $\alpha$-thal-2 ($-3.7$ or $-4.2$ kb); 13 had Hb H disease because of combinations of one of these $\alpha$-thal-1 deletions with either a 5 nt deletion at the 5′ splicing site of IVS-I, or a terminating codon mutation (Hb CS), or a poly(A) mutation, and six were homozygous for either a poly(A) mutation or the 5 nt deletion. Significant differences were observed between the deletional types ($--/-\alpha$; $\alpha_2/\alpha_1$ ratio of zero; $\alpha/\beta$ ratio of $\sim 1$) and non-deletional types ($--/\alpha^T\alpha$; $\alpha_2/\alpha_1$ ratio of 0.05–0.3 for those with $T$ = the 5 nt deletion or the terminating codon mutant, and $\sim 1.0$ for those with $T$ = a poly(A) mutation; $\alpha/\beta$ ratio in all types of $\sim 0.7$). Comparable data were found for the nondeletional $\alpha$-thal-2 homozygotes. The noted differences were highly significant and the determination of the two ratios may be diagnostically of considerable value. The low $\alpha_2/\alpha_1$-mRNA ratio in the two patients with $--/\alpha^{-5nt}\alpha$ and the one patient with $\alpha^{-5nt}\alpha/\alpha^{-5nt}\alpha$ indicates the presence of minute amounts of $\alpha_2$-mRNA; apparently splicing at the donor site is greatly impaired by this deletion but not eliminated. The high $\alpha_2/\alpha_1$-mRNA ratio in the four patients with $--/\alpha^{PA\text{-}2}\alpha$ and the five patients with $\alpha^{PA\text{-}1}\alpha/\alpha^{PA\text{-}1}\alpha$ (PA-1 and PA-2 are poly(A) mutations) is due to the presence of an elongated $\alpha_2$-mRNA which uses an alternate location as polyadenylation site. The relative levels of $\zeta$-mRNA varied considerably; the highest levels were found in patients with the $-(\alpha)20.5/-\alpha$ or $--^{SEA}/-\alpha$ deletional types but not in those with the $-(\alpha)20.5/\alpha^{PA\text{-}2}\alpha$, $-(\alpha)20.5/\alpha^{-5nt}\alpha$, or $--^{SEA}/\alpha^{CS}\alpha$ nondeletional types. No definitive explanation can be given for these differences; perhaps certain sequences that are part of some of the $\alpha$-thal-1 deletions are important for the suppression of the $\zeta$-globin gene.

*Keywords:* Anemia; Hb H; Deletion; Mutation; mRNA ratio imbalance; Hb $A_2$; $\zeta$-chain

## 1. Introduction

Hb H disease is a moderately severe hemolytic anemia that results from a significant decrease in the synthesis of $\alpha$-chains, thus preventing adequate production of normal Hb A ($\alpha_2\beta_2$) and Hb $A_2$ ($\alpha_2\delta_2$) in the adult patient and of fetal hemoglobin or Hb F ($\alpha_2\gamma_2$) in the fetus and newborn baby. The excess $\beta$- and $\gamma$-chains form tetramers of $\beta$-chains in the adult (Hb H or $\beta_4$) and of $\gamma$-chains in the newborn (Hb Bart's or $\gamma_4$). Loss of $\alpha$-chain production is most often caused by genetic abnormalities involving one or both genes directing the synthesis of $\alpha$-chains. These $\alpha$ genes are located on the short arm of chromosome #16 in the order: Telomere-$\zeta$-$\psi\zeta 1$-$\psi\alpha_2$-$\psi\alpha_1$-$\alpha_1$-$\alpha_2$-$\theta 1$-centomere [1]. Three of the listed genes ($\zeta 2$, $\alpha_2$, $\alpha_1$) are functional genes, three are pseudo genes ($\psi\zeta 1$, $\psi\alpha_2$, $\psi\alpha_1$), while the function of the $\theta 1$ gene is unknown and a

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θ1-chain has not been detected. Reciprocal recombination between specific homologous subsegments produces a chromosome with only one α-globin gene ($-\alpha^{-3.7\ kb}$; rightward deletion), while a similar mechanism between a second set of homologous subsegments also results in a chromosome with one α-globin gene but characterized by a deletion of 4.2 kb ($-\alpha^{-4.2\ kb}$; leftward deletion). These two conditions are known as α-thalassemia-2 (α-thal-2) or ($-\alpha/\alpha\alpha$). In addition, there are numerous deletions described in which both α-globin genes are completely or partially absent; chromosomes with such a deletion will direct no α-chain synthesis (α-thal-1 or $--/\alpha\alpha$).

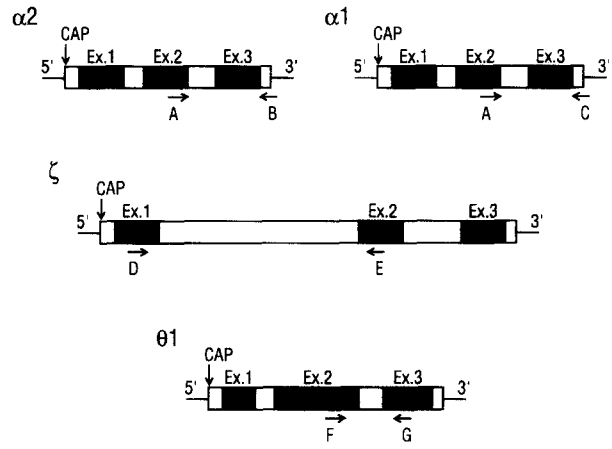
Hb H disease most frequently results from the interaction of an α-thal-2 ($-\alpha/$) determinant and an α-thal-1 ($--/$) determinant; thus, a subject with this condition carries only one functional α-globin gene ($--/-\alpha$). This type of Hb H disease is most commonly found in Southeast Asia and in Mediterranean countries. Several nondeletional α-thal determinants have also been discovered (reviewed in Ref. [1]). These concern mutations or frameshifts primarily in the $\alpha_2$-globin gene and some in the $\alpha_1$-globin gene, rendering such a gene nonfunctional ($\alpha^T\alpha$ or $\alpha\alpha^T$). Hb H disease may therefore also result from the co-inheritance of a nondeletional α-thal and a deletional α-thal-1 ($\alpha^T\alpha/--$) or even of two nondeletional α-thal alleles ($\alpha^T\alpha/\alpha^T\alpha$). Thus, the genetic basis of Hb H disease differs resulting in a considerable variability in clinical severity, which in turn correlates with the degree of α-chain deficiency.

Reverse transcription-polymerase-chain reaction (RT-PCR) procedures have become available for an assessment of the expression of each of the many globin genes. For instance, this technique has been applied to the two γ- ($^G\gamma$- and $^A\gamma$-) globin genes [2], the β- and γ-globin genes in β-thalassemia (thal) [3–5], and has been used for the determination of the $\beta^A$- and $\beta^X$-mRNA ratios in sickle cell trait [6] and in unstable hemoglobin (Hb) conditions and Hb E heterozygotes [7]. Similar data for α-chain variants have also been published [8]. In a previous publication in this journal [9] we had the opportunity to analyze the relative ratios between the various mRNAs in reticulocytes from persons with different forms of α-thal. More recently, blood samples from as many as 40 Hb H patients became available for similar analyses that included the determination of the ratio between the $\alpha_2$- and $\alpha_1$-mRNAs, between the total α-mRNA and β-mRNA, and even between the total α-mRNA and ζ-mRNA. The blood samples came from 34 patients with Hb H disease due to combinations of deletional α-thal-1 determinants ($--/$) and deletional α-thal-2 determinants ($-\alpha/$) or a few nondeletional alleles ($\alpha^T\alpha/$), while in six patients the Hb H disease was caused by a homozygosity for one of two different nondeletional α-thal-2 alleles. The data obtained in these analyses will be described here; they allow a detailed comparison between the expression of the $\alpha_2$- and $\alpha_1$-globin genes relative to that of the normal β-globin gene, and an evaluation of the expression of the single ζ-globin gene in these various deletional and nondeletional types of Hb H disease. Furthermore, some of the data, mainly the $\alpha_2/\alpha_1$- and α/β-mRNA ratios, can be correlated with the different phenotypes observed in the various patients and their determination might therefore have diagnostic significance.

## 2. Materials and methods

Blood samples from 40 patients with Hb H disease were collected in vacutainers with EDTA as anticoagulant. Those from Turkey and Northern Cyprus were transported by one of the authors or shipped by fast air mail service. The same procedure was followed for the samples from Hawaii and from Kuwait, while those collected from a few Vietnamese families living in South Carolina were transported in ice by car to the laboratory in Augusta, GA, USA. Informed consent was obtained.

Hematological data were collected with an automated cell counter. Hb $A_2$ was quantified by microcolumn chromatography [10] and the ζ-chain by reversed phase high performance liquid chromatography (HPLC) [11]. Quantitation of the unstable Hb H was not attempted because most had precipitated during transport; however, data for




| | Sequences of Primers | Location Relative to CAP Site |
|---|---|---|
| A | 5' - GGCAAGAAGGTGGCCGACG - 3' | +332 to +350 (α2;α1) |
| B | 5' - GGGAGGCCCATCGGGCAGGAGGAAC - 3' | +768 to +744 (α2) |
| C | 5' - GGGGGGAGGCCCAAGGGGCAAGAA - 3' | +778 to +755 (α1) |
| D | 5' - CAAGACTGAGAGGACCATCATTGTG - 3' | +64 to +89 (ζ) |
| E | 5' - TCAGGACAGAGGATACGACCGATAG - 3' | +1547 to +1522 (ζ) |
| F | 5' - GCTTTCCCCGCCACGAAGACC - 3' | +384 to +404 (θ1) |
| G | 5' - GCGGCCACCCACCCACAGTT - 3' | +835 to +816 (θ1) |

| Fragment Sizes (bp) | | |
|---|---|---|
| Primers A+B | DNA: 437 | cDNA: 295 |
| Primers A+C | 447 | 298 |
| Primers D+E | 1481 | 403 |
| Primers F+G | 452 | 344 |

Fig. 1. The locations and sequences of the primers used in determining the ratios of the different mRNAs (α2, α1, ζ, θ1). The sizes of the amplification products are listed at the bottom.

most patients were available from earlier publications [9,12,13]. The same papers list the types of α-thal-1 and deletional and nondeletional types of α-thal-2 present in the 40 individuals. The presence or absence of a ζ-globin gene deletion or triplication was evaluated in more than 50% of all samples with gene mapping methodology as used in previous studies [14].

RNA was isolated from 5–10 ml blood with the method of Chomczynski [15]. An RT-PCR procedure was used to determine the relative quantities of the $\alpha_2$-, $\alpha_1$-, β-, ζ-, and $\theta_1$-mRNAs. Details of the methods used for determining the $\alpha_2/\alpha_1$ and α/β ratios have been given before [7–9]. The same RT product was used for the measurements of the α/ζ, $\alpha/\theta_1$, and $\zeta/\theta_1$ ratios. Five or 10 μl of this material served as template for 30 cycles of amplification. The method used the 'hot-start' PCR procedure to avoid primer dimerization. The 1 × PCR buffer contained 2 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pM of the primer A and 25 pM of the primers B, C, D and E, or F and G (Fig. 1), and 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) in a final volume of 100 μl. Initial denaturation was for 5 min at 99°C; the enzyme was added at 'hot-start'. The amplification profile for each cycle consisted of denaturation for 1 min at 95°C, primer annealing for 1 min at 64°C, and extension for 1 min 30 s at 72°C. The sequences of the primers and their locations are shown in Fig. 1. All primer pairs were selected in such a way that an intron is located between them; absence of a larger amplification product excludes the presence of contaminating genomic DNA. PCR fragments were labeled by primer extension with γ-$^{32}$P-labeled primers A, D, and F during one cycle of PCR [3]. Three μl of the labeled PCR product were analyzed on a non-denaturing 6% polyacrylamide gel and autoradiographed for 1, 3, and 6 h to evaluate the possible error due to limited film capacity. Relative amounts were determined by densitometric scanning with a Shimadzu (Kobe, Japan) CS 9000 densitometer. The average α/β ratio for normal adults (4.2–4.5) is higher than that of 2.52 reported by Lin et al. [16] who used different sets of primer and other experimental conditions, and similar to the 4.5 reported by Chami et al. [17] who also used different sets of primers but included a synthetic internal standard. The average value for the $\alpha_2/\alpha_1$-mRNA ratio of 2.45–2.65 is comparable to that obtained by Molchanova et al. [8]. Differences in efficiency of translation of the α- and β-mRNAs will result in a balanced synthesis of α and β-chains in the normal adult and will explain the marked differences in the relative levels of α- and β-mRNAs [18].

## 3. Results

Twelve of the 40 patients with Hb H disease, aged 2 to 53 yr, were from Northern Cyprus, 17 from Ankara, Turkey, five from Kuwait, two from Hawaii, and four from Vietnamese families living in South Carolina. Twenty-one subjects had different types of deletional Hb H disease ($--/-\alpha$), namely, four types of α-thal-1 (the MED-I type with a 17.5 kb deletion [19], the MED-II type with a 26.5 kb deletion that includes the ζ-globin gene [20], the $-(\alpha)20.5$ type with the 20.5 kb deletion [21], the SEA

Table 1
The relative quantities of α2-, α1-, ζ-, and β-mRNA in reticulocytes from patients with Hb H disease [a]

| No. | Genotype [b] | n | Hb (g/dl) | Hb $A_2$ [c] (%) | ζ Chain [d] (% of α + ζ) | mRNA | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | $\alpha_2/\alpha_1$ ratio | α/β ratio | $\zeta \cdot 100/(\alpha+\zeta)$ |
| | normals (αα/αα) | many | 12–16 | 2.0–3.2 | < 0.01 | 2.45–2.65 | 4.2–4.5 | < 0.01 |
| 1 | $--^{MED-II}/-\alpha^{-3.7}$ | 4 | 10.2 ± 0.9 | 0.9 ± 0.15 | 0.27 ± 0.19 | 0 | 1.02 ± 0.03 | 0.19; 0.22; 0.59 |
| 2 | $--^{MED-I}/-\alpha^{-3.7}$ | 9 | 9.1 ± 1.1 | 1.05 ± 0.15 | 0.31 ± 0.28 | 0 | 1.01 ± 0.12 | 1.6; 1.7; 1.8; 2.2 |
| 3 | $--^{MED-I}/-\alpha^{-4.2}$ | 1 | 9.7 | 1.05 | 0.20 | 0 | 1.01 | 1.1 |
| 4 | $-(\alpha)20.5/-\alpha^{-3.7}$ | 3 | 10.8 ± 1.8 | 1.0 ± 0.05 | 0.30 ± 0.08 | 0 | 1.02 ± 0.04 | 10.5; 17.6 |
| 5 | $--^{SEA}/-\alpha^{-3.7}$ | 3 | 9.1 ± 0.6 | 0.95 ± 0.10 | 0.40;1.00 | 0 | 1.03 ± 0.04 | 15.7; 8.6; 5.1 |
| 6 | $--^{SEA}/-\alpha^{-4.2}$ | 1 | 9.0 | 0.7 | 0.30 | 0 | 1.07 | 19.3 |
| 7 | $--^{MED-I}/\alpha^{-5nt}\alpha$ | 6 | 9.5 ± 0.6 | 0.55 ± 0.05 | 0.22 ± 0.01 | 0.15 ± 0.09 | 0.71 ± 0.07 | 0.29; 0.40; 0.41 |
| 8 | $-(\alpha)20.5/\alpha^{-5nt}\alpha$ | 1 | 9.7 | 0.8 | 0.13 | 0.10 | 0.59 | 0.24 |
| 9 | $--^{MED-I}/\alpha^{PA-2}\alpha$ | 2 | 8.7;9.0 | 0.65;0.35 | 0.24;0.21 | 1.04;1.08 | 0.85; 0.89 | 0.88; 0.77 |
| 10 | $-(\alpha)20.5/\alpha^{PA-2}\alpha$ | 2 | 8.7;7.2 | 0.6;0.9 | 0.43;0.45 | 1.09; 1.06 | 0.82; 0.79 | 0.44; 0.40 |
| 11 | $--^{SEA}/\alpha^{CS}\alpha$ | 2 | | transfused | | 0.23; 0.18 | 0.53; 0.61 | 0.70; 0.43 |
| 12 | $\alpha^{PA-1}\alpha/\alpha^{PA-1}\alpha$ | 5 | 10.2 ± 1.9 | 1.8 ± 0.45 | 0 | 0.83 ± 0.14 | 1.15 ± 0.05 | 0 |
| 13 | $\alpha^{-5nt}\alpha/\alpha^{-5nt}\alpha$ | 1 | 10.5 | 0.4 | 0 | 0.14 | 0.81 | 0 |

[a] Average data with SDs are listed for groups of three patients or more; an exception is the percentage of ζ-mRNA which is listed for each individual patient tested.

[b] Deletional α-thal-1 (− −) are: MED-II = 26.5 kb deletion including the ζ gene; MED-I = 17.5 kb deletion; $-(\alpha)20.5$ = 20.5 kb deletion that includes the $\alpha_2$ and the 5′ end of $\alpha_1$; SEA = 18 kb deletion. Deletional α-thal-2 ($-\alpha$) are the 3.7 and 4.2 kb deletions. Nondeletional α-thal are: $\alpha^{-5nt}\alpha$ = deletion of TGAGG at the donor splice site of the IVS-I; $\alpha^{PA-1}\alpha$ and $\alpha^{PA-2}\alpha$ are poly(A) mutations or AATAAA → AATAA*G* and AAT*G*AA, respectively; CS = Hb Constant Spring or a terminating codon (α2 142, *T*AA → *C*AA).

[c] By microcolumn chromatography [10].

[d] By reversed phase HPLC [11].

type with an ~ 18 kb deletion that includes the θ1-globin gene [19]) and one of the two common α-thal-2 types (the 3.7 or the 4.2 kb deletion). Thirteen had Hb H disease due to a combination of one of the four listed α-thal-1 deletions with a nondeletional type of α-thal-2, namely the 5 nucleotide (nt) deletion at the donor splicing site of the first intervening sequence (IVS-I), the poly(A) mutation AATAAA → AATGAA, and the terminating codon mutation Hb Constant Spring (Hb CS) (for references, see Higgs and Weatherall [1]). The six remaining patients were either homozygous for another poly(A) mutation (AATAA*A* → AATAA*G* [22]) or for the 5 nt deletion. Table 1 provides some details that include the total Hb levels, the Hb $A_2$ values, and the levels of the ζ-chain, determined by reversed phase HPLC. Previous analyses [12] had confirmed the fact that Hb H levels are considerably higher in patients with nondeletional Hb H disease ($--/\alpha^T\alpha$) than with the deletional type ($--/-\alpha$). Differences were also apparent in the levels of Hb $A_2$ that were significantly lower in the subjects with the nondeletional Hb H disease ($--/\alpha^T\alpha$; average 0.60 with a range of 0.35–0.9) than in those with the deletional Hb H disease ($--/-\alpha$; average 0.98 with a range of 0.7–1.3). The levels of ζ-chain were low and about the same in both groups (0.34% with a range of 0.10–1.12 for $--/-\alpha$, and 0.25 with a range of 0.13–0.45 for $--/\alpha^T\alpha$). No ζ-chain was detected in the six patients with a homozygosity for a nondeletional α-thal determinant.

A summary of the mRNA analysis is provided in Table 1, while Fig. 2 illustrates some of the separations that have been obtained. As expected, the $\alpha_2/\alpha_1$ ratio was zero for all Hb H patients with deletion of three α-globin genes ($--/-\alpha$); their reticulocytes contained $\alpha_1$-mRNA only (the mRNA of the $\alpha_2\alpha_1$ hybrid gene due to the 3.7 kb deletion is determined as an $\alpha_1$-mRNA [8]). Extremely low values for the $\alpha_2/\alpha_1$-mRNA ratio were observed in patients with $-/\alpha^T\alpha$ in which the T is the 5 nt deletion at the IVS-I donor splicing site or the *T*AA → *C*AA terminating codon mutation. The values were considerably higher at ~ 1.0 for patients in whom T is a poly(A) mutation. Similar differences were seen between the five patients with a homozygosity for a poly(A) mutation (average 0.83) and the one with a homozygosity for the 5 nt deletion (0.14).

The α/β-mRNA ratio averaged ~ 1.0 for the patients with $--/-\alpha$, a decrease of nearly 80% of the normal ratio (4.2–4.5). These values were lower (~ 0.7) in the patients with $--/\alpha^T\alpha$; this decrease was independent of the type of nondeletional α-thal. The ratios were slightly higher for the five subjects with the homozygosity for a poly(A) mutation (average 1.15) but not for the patient with the homozygosity for the 5 nt deletion (0.81).

Extremely low quantities of ζ-mRNA appear to be present in reticulocytes of normal adults, and accurate ratios between this embryonic globin gene transcript and that of the α-globin genes could not be established. Increased ratios were found for all patients with Hb H disease except for the six with a homozygosity for a nondeletional α-thal (Table 1). In most instances the ζ-mRNA level was below 1.0%, but significant increases were seen in the subjects with $--^{MED-I}/-\alpha^{-3.7}$ (average 1.83%) and in the patients with the $-(\alpha)20.5/$ or

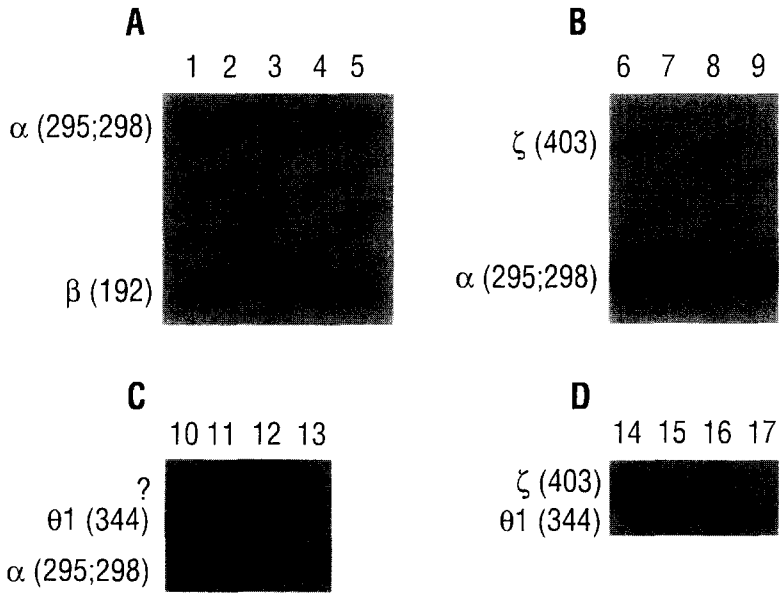



Fig. 2. Separation of the RT-PCR products on a 6% polyacrylamide gel. A. The α/β ratio. Lanes 2,3,4,5: Homozygous poly(A) patients or $\alpha^{PA-1}\alpha/\alpha^{PA-1}\alpha$; lane 1: Normal control. B. The α/ζ ratio. Lanes 6 and 7: Same sample with $--^{MED-I}/-\alpha^{-3.7}$; lane 8: Sample with $-(\alpha)20.5/-\alpha^{-3.7}$; lane 9: Sample with $--^{MED-I}/\alpha^{-5nt}\alpha$. C. The α/θ1 ratio. Lanes 10 and 11: Same sample with $--^{MED-I}/-\alpha^{-3.7}$; lanes 12 and 13: Two additional samples with $--^{MED-I}/-\alpha^{-3.7}$. The larger fraction remains unidentified. D. The ζ/θ1 ratio. Lane 14: Sample with $--^{SEA}/\alpha^{CS}\alpha$; lane 15: Normal control; lanes 16 and 17: Same sample with $--^{MED-I}/\alpha^{-5nt}\alpha$. The numbers behind the symbols indicate sizes of the fragments.

$--^{SEA}/\alpha$-thal-1 combined with a deletional α-thal-2 (either −3.7 or −4.2 kb) but not with a nondeletional α-thal-2 (either a poly(A) mutation, the 5 nt deletion, or the Hb CS terminating codon mutation). These differences were highly significant, varying from 5.1–19.3% in the patients with the deletional Hb H disease [$--^{SEA}/-\alpha$ or $-(\alpha)20.5/-\alpha$] and 0.25–0.7 in those with the nondeletional Hb H disease [$--^{SEA}/\alpha^{CS}\alpha$, $-(\alpha)20.5/\alpha^{-5nt}\alpha$, or $-(\alpha)20.5/\alpha^{PA\text{-}2}\alpha$]. None of the patients had a ζ-globin gene deletion or triplication.

The θ1-mRNA was present in all samples that were tested and its quantity varied considerably (Fig. 2). Unfortunately, an unexplained larger fragment appeared when the α- and θ1-cDNA were amplified simultaneously, thus preventing any quantitative measurements. This was not the case for the co-amplification of the ζ- and θ1-cDNAs (Fig. 2D). Considerably more θ1 transcript was present than ζ transcript except for the patients with the SEA α-thal-1 deletion that includes the θ1 gene in addition to the $\alpha_2$- and $\alpha_1$-globin genes [1].

## 4. Discussion

Thirty-four of our 40 patients with Hb H disease had combinations of one of four α-thal-1 deletions with either one of two deletional or one of three nondeletional determinants. The sizes and locations of the six deletions are shown in Fig. 3; the nondeletional α-thal-2 alleles are of interest because one concerns a 5 nt deletion at the donor splicing site of the IVS-I that affects splicing of this intron, a second, i.e. Hb CS with a *TAA* → *CAA* mutation at codon 142, modifies the terminating codon, while the third, i.e. an AATAA*A* → AATAA*G* mutation of the poly(A) site, affects the polyadenylation of the $\alpha_2$-mRNA. This considerable diversity makes an evaluation of the relative levels of the $\alpha_2$- and $\alpha_1$-mRNAs in the total α- and β-mRNAs worthwhile, as it allows a comparison between the expression of the genes of the α cistron, and may explain some of the clinical differences that have been observed between these patients. Earlier communications from this laboratory [8,9] have reported similar data, however, mainly for simple heterozygotes for some of these determinants.

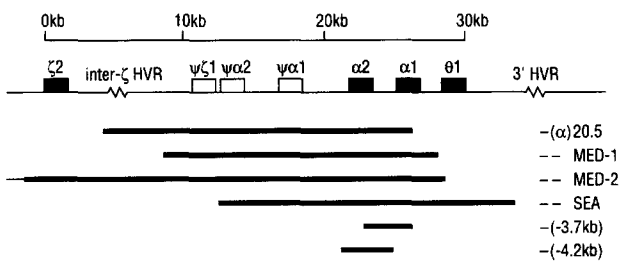



Fig. 3. The four α-thal-1 and two α-thal-2 deletions present in most of our patients with Hb H disease.

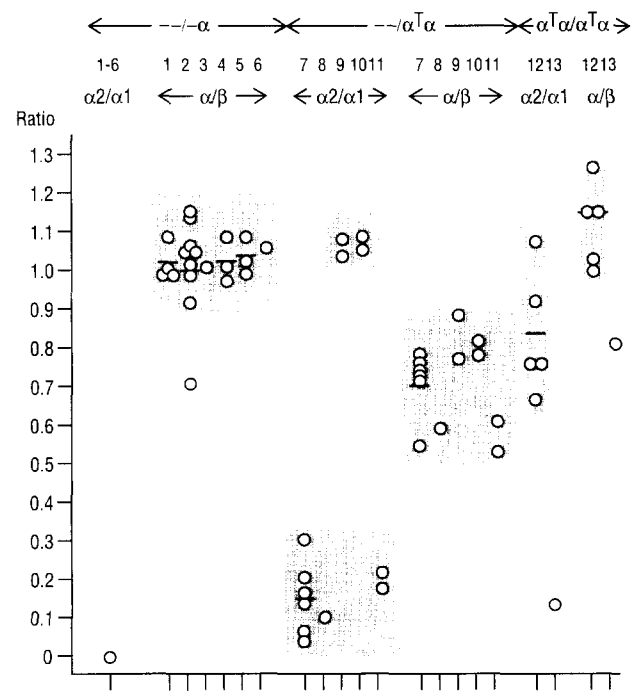



Fig. 4. The $\alpha_2/\alpha_1$- and α/β-mRNA ratios for the individual Hb H patients (total number is 40). The numbers at the top of the figure refer to the genotypes listed in Table 1.

The mRNA data, which form the basis of the summary given in Table 1, are plotted individually in Fig. 4. Patients with deletional types of Hb H disease ($--/-\alpha$) have no $\alpha_2$-mRNA and an α/β-mRNA ratio of approximately one. This value is remarkably similar for all six types; the one remaining $\alpha_1$-globin gene transcribes one-fourth of the level of α-mRNA in normal adults. Translation must be most efficient in these patients who are able to maintain 8–10 g/100 ml of α-chain-containing Hbs in their peripheral blood. The data for the 13 patients with a nondeletional type of Hb H disease ($--/\alpha^T\alpha$) are different. According to the $\alpha_2/\alpha_1$-mRNA ratio the subjects with the 5 nt deletion at the donor splicing site of IVS-I ($--/\alpha^{-5nt}\alpha$) must have a small amount of $\alpha_2$-mRNA in their reticulocytes, as does the one patient with a homozygosity for this allele. Apparently the TGAGG deletion at the donor site $AG_{\downarrow}GTGAGGCTCCC \rightarrow AG_{\downarrow}GCTCCC$ greatly decreases but does not completely eliminate the splicing. The $\alpha_2/\alpha_1$ ratio in the two subjects with the Hb CS mutation ($--/\alpha^{CS}\alpha$) is also low at about 0.2; earlier studies [23] have suggested that this type of mRNA is elongated and uses the TAA sequence in the normal AATAAA poly(A) sequence as terminating codon and the cryptic poly(A) sequence at 1048 bp past the terminating codon 142 as its poly(A) site. A similar situation exists for the four patients with the poly(A) mutation ($--/\alpha^{PA\text{-}2}\alpha$). The much larger relative quantity of $\alpha_2$-mRNA in these four subjects, as well as in the five Hb H patients with a homozygosity for the PA-1 mutation (AATAA*A* → AATAA*G*) resulting in high $\alpha_2/\alpha_1$ ratios

of 0.65–1.1, is entirely of the elongated type with a normal terminating codon and an abnormal polyadenylation sequence [23]. The low $\alpha/\beta$-mRNA ratio in the 13 patients with $--/\alpha^{T}\alpha$ Hb H disease suggests that the mutation in the $\alpha_2$-globin gene indirectly affects the transcription of the $\alpha_1$-globin gene; the mechanism remains unknown. This $\alpha/\beta$-mRNA ratio (average 0.7) is significantly lower ($P = < 0.01$) than that observed in patients with deletional Hb H disease ($--/-\alpha$; average value $\sim 1.0$) which is in accordance with the more serious clinical condition of these patients, their lower Hb level, their higher percentage of Hb H, and their lower level of Hb $A_2$ (Table 1).

The determination of the $\alpha_2/\alpha_1$- and $\alpha/\beta$-mRNA ratios is done from one amplification reaction and can be completed within 2–3 days for samples from several patients simultaneously. The differences in these two ratios are highly diagnostic and the methodology can be used to distinguish between deletional and nondeletional types of Hb H disease.

The presence of $\zeta$-globin chains in red cells from newborn babies and adults has been evaluated with sensitive immunoassays [24–26] and by reversed phase HPLC methodology [11,20,26]. Red cells from normal babies often have a minute quantity of $\zeta$ which increases with the presence of $\alpha$-globin gene deficiencies. Notable amounts are found in babies and adults with the SEA $\alpha$-thal-1 deletion and Chui et al. [24] suggest that its level can serve as a marker for this $\alpha$-thal-1 haplotype. We have detected minute quantities (0.5% of total $\alpha + \zeta$) in all patients with Hb H disease except for the six who were homozygous for one of two nondeletional $\alpha$-thal determinants (Table 1). The highest level was seen in the three patients with the $--^{SEA}/-\alpha^{-3.7\text{ or }-4.2}$ type of Hb H disease.

Albitar et al. [27] and Tang et al. [28] used a PCR technique to evaluate the presence of $\theta$1-, $\zeta$-, and $\epsilon$-globin mRNAs and indeed observed minute quantities of these mRNAs in reticulocytes of normal adults. We have made a similar observation; however, an acceptable $\zeta/\alpha$-mRNA ratio was often not obtained with our technique. Increased levels of $\zeta$-mRNA were observed in the reticulocytes of all our patients with Hb H disease except those with a homozygosity for a nondeletional $\alpha$-thal-2 determinant (Table 1). The calculated ratio (%$\zeta$ of $\alpha + \zeta$) was the lowest in the reticulocytes of the patients with the nondeletional Hb H disease ($--/\alpha^{T}\alpha$) with an average of 0.5% and no distinct differences between the types of $\alpha$-thal determinants. The values for the patients with deletional Hb H disease ($--/-\alpha$) are quite different. Except for those with the $--^{MED\text{-}II}/-\alpha^{-3.7}$ condition, where low levels were present because the MED-II deletion includes the $\zeta$-globin gene (Fig. 3), the values were increased to $\sim 1.7\%$ for $--^{MED\text{-}I}/-\alpha^{-3.7\text{ or }-4.2}$, to 14.0% for $-(\alpha)20.5/-\alpha^{-3.7}$, and to 12.2% for $--^{SEA}/-\alpha^{-3.7\text{ or }-4.2}$ (Table 1). These results are difficult to explain but may suggest that certain sequences are important for the suppression of the $\zeta$-globin gene expression. Suggested areas are sequences between the $\zeta$2- and $\psi\zeta$-globin genes (high $\zeta$-mRNA in Hb H patients with the $-(\alpha)20.5/-\alpha^{-3.7}$ type) and those involving the $\theta$1-globin gene (high $\zeta$-mRNA in Hb H patients with the $--^{SEA}/-\alpha^{-3.7\text{ or }-4.2}$ genotype). Translation of this $\zeta$-mRNA appears not to be efficient because of the low levels of $\zeta$-chains observed in the reticulocytes of these patients. Finally, the presence of the 3.7 or 4.2 kb deletion on the opposite chromosome must play a major role, and it may well be that the $\zeta$-globin gene of this chromosome contributes the major portion of the elevated level of $\zeta$-mRNA. Why else would the $\zeta$-mRNA level be below 1% in Hb H patients with the same deletional $\alpha$-thal-1 [$--^{SEA}/$; $-(\alpha)20.5/$] but in combination with a nondeletional $\alpha$-thal determinant!

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## References

[1] Higgs, D.R. and Weatherall, D.J. (eds.) (1993) The Hemoglobinopathies, Vol. 6, W.B. Saunders Company, London.
[2] Efremov, D.G., Dimovski, A.J. and Huisman, T.H.J. (1994) Blood 83, 3340–3355.
[3] Efremov, D.G., Dimovski, A.J., Sukurova, E., Schiliro, G., Zisovski, N., Efremov, G.D., Burrone, O.R. and Huisman, T.H.J. (1994) Br. J. Haematol. 88, 311–317.
[4] Hall, G. and Thein, S. (1994) Blood 83, 2031–2037.
[5] Smetanina, N.S. and Huisman, T.H.J. (1996) Acta Haematol., in press.
[6] Dimovski, A.J., Efremov, D.G., Gu, L-H. and Huisman, T.H.J. (1994) Br. J. Haematol. 87, 353–356.
[7] Smetanina, N.S. and Huisman, T.H.J. (1996) Am. J. Hematol., in press.
[8] Molchanova, T.P., Pobedimskaya, D.D. and Huisman, T.H.J. (1994) Br. J. Haematol. 88, 300–306.
[9] Smetanina, N.S., Leonova, J.Ye., Levy, N. and Huisman, T.H.J. (1996) Biochim. Biophys. Acta 1315, 188-192.
[10] Schleider, C.T.H., Mayson, S.M. and Huisman, T.H.J. (1977) Hemoglobin 1, 503–504.
[11] Kutlar, F., Fei, Y.J., Wilson, J.B., Kutlar, A. and Huisman, T.H.J. (1987) J. Chromatogr. 394, 333–343.
[12] Baysal, E., Kleanthous, M., Bozkurt, G., Kyrri, A., Kalogirou, E., Angastiniotis, M., Ioannou, P. and Huisman, T.H.J. (1995) Br. J. Haematol. 89, 496–499.
[13] Adekile, A.D., Gu, L-H., Baysal, E., Haider, M.Z., Al-Fuzae, L., Aboo-backer, K.C., Al-Rashied, A. and Huisman, T.H.J. (1994) Acta Haematol. 92, 176–181.
[14] Gu, Y.C., Landman, H. and Huisman, T.H.J. (1987) Br. J. Haematol. 66, 245–250.
[15] Chomczynski, P. (1993) BioTechniques 15, 532–537.
[16] Lin, S-F., Liu, T-C., Chen, T-P., Chiou, S-S., Liu, H-W. and Chang, J-G. (1994) Br. J. Haematol. 87, 133–138.

[17] Chami, B., Liu, B., Garel, M.C. and Galactéros, F. (1994) Br. J. Haematol. 87, 59, Suppl. 1, abst. 233.
[18] Cash, F.E., Gaskell, U.J., Shakin-Eshleman, S. and Liebhaber, S.A. (1989) Blood 74, 7a, Suppl. 1.
[19] Pressley, L., Higgs, D.R., Clegg, J.B. and Weatherall, D.J. (1980) Proc. Natl. Acad. Sci. USA 77, 3586–3589.
[20] Kutlar, F., Gonzalez-Redondo, J.M., Kutlar, A., Gurgey, A., Altay, Ç., Efremov, G.D., Kleman, K. and Huisman, T.H.J. (1989) Hum. Genet. 82, 179–186.
[21] Nicholls, R.D., Higgs, D.R., Clegg, J.B. and Weatherall, D.J. (1985) Blood 65, 1434–1438.
[22] Higgs, D.R., Goodbourn, S.E.Y., Lamb, J., Clegg, J.B. and Weatherall, D.J. (1983) Nature 306, 398.
[23] Molchanova, T.P., Smetanina, N.S. and Huisman, T.H.J. (1995) Biochem. Biophys. Res. Commun. 214, 1184–1190.
[24] Chui, D.H.K., Wong, S.C., Chung, S-W., Patterson, M., Bhargava, S. and Poon, M-C. (1986) N. Engl. J. Med. 314, 76–79.
[25] Chui, D.H.K., Mentzer, W.C., Patterson, M., Iarocci, T.A., Embury, S.H., Perrine, S.P., Mibashan, R.S. and Higgs, D.R. (1989) Blood 74, 1409–1414.
[26] Epstein, M., Fucharoen, S., Gu, L-H., Huisman, T.H.J., Moore, R., Ugarte, E. and Epstein, N. (1991) Blood 78, Suppl. 1, Abst. 782.
[27] Albitar, M., Peschle, C. and Liebhaber, S.A. (1989) Blood 74, 629–637.
[28] Tang, W., Luo, H-Y., Albitar, M., Patterson, M., Eng, B., Waye, J.S., Liebhaber, S.A., Higgs, D.R. and Chui, D.H.K. (1992) Blood 80, 517–522.