Diagnosis of Thalassemia Using cDNA Amplification of Circulating Erythroid Cell mRNA With the Polymerase Chain Reaction

By Shu-zhen Huang, Griffin P. Rodgers, Fan-yi Zeng, Yi-tao Zeng, and Alan N. Schechter

We have developed a technique to diagnose the α- and β-thalassemia (thal) syndromes using the polymerase chain reaction to amplify cDNA copies of circulating erythroid cell messenger RNA (mRNA) so as to quantitate the relative amounts of α-, β-, and γ-globin mRNA contained therein. Quantitation, performed by scintillation counting of [32P]-dCTP incorporated into specific globin cDNA bands, showed ratios of α/β-globin mRNA greater than 10-fold and greater than fivefold increased in patients with β- and β'-thal, respectively, as well as a relative increase in γ-globin mRNA levels. Conversely, patients with α-thalassemia showed a decreased ratio of α/β-globin mRNA proportional to the number of α-globin genes deleted. This methodology of ascertaining ratios of globin mRNA species provides a new, simplified approach toward the diagnosis of thalassemia syndromes, and may be of value in other studies of globin gene expression at the transcription level.

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THE THALASSEMA syndromes are autosomal recessive diseases due to absent or diminished synthesis of one or more globin chains.1 β-thalassemia (β-thal) is characterized by absent (β0) or decreased (β+) synthesis of the β-globin chain; α-thal is due to deletion or mutation of the α-globin chains. The thalassemia syndromes have been reported from almost all population and ethnic groups around the world. It has been estimated that about 100,000 people are born each year who are homozygous for the thalassemia syndromes. Recently, a large survey of 1 million people was performed in China, the most populous country, and β-thalassemia incidence was calculated as 0.66% and α-thal was estimated as 2.64%.2 Indeed, the thalassemia syndromes are among the most common genetic diseases in the world.

Traditional detection of β-thal has relied on the hematologic features, such as a low mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), and increased levels of hemoglobin (Hb) A2 and/or Hb F.3 Unfortunately, the differential diagnosis between iron deficiency anemia and mild β-thal can be difficult to make in practice, if there are not reciprocal increases in Hb A2 and/or Hb F. In these cases, the demonstration of a reduced β-globin synthetic rate (compared with α-globin), generally using 1H-leucine to analyze globin chain synthesis in reticulocytes, is required for a conclusive diagnosis.4 This procedure is cumbersome and not well-suited for large-scale investigations. Within the last 10 to 15 years, with improvements in techniques in molecular biology, β-thal can be diagnosed prenatally and postnatally based on DNA analyses.5 However, the current techniques, such as restriction fragment length polymorphism (RFLP) linkage analysis,6 oligonucleotide hybridization,7 and polymerase chain reaction (PCR) amplification of mutant DNA sequences8-17 are time-consuming and complicated and, frequently, require information about the specific mutations to be examined. Thus, they are not well-suited for large-scale studies, especially in developing countries. The diagnosis of α-thal similarly is complex whether performed by clinical or molecular techniques.1,8-20

One strategy for the diagnosis of thalassemia is the demonstration of decreased or absent amounts of globin messenger RNA (mRNA) from early erythroid cells. This approach exploits the common denominator that all forms of thalassemia, despite the heterogeneity of molecular mutations and deletions, have a reduction of either the amounts or functional capacity of the mRNA that codes for globin chains. The availability of the enzyme reverse transcriptase (RT) and polymerase chain methodology21,22 now makes this feasible. This report describes this approach, ie, cDNA amplification and quantitation with PCR, to the diagnosis of thalassemia syndromes. It is widely recognized that PCR analysis may be difficult to quantitate because of the exponential nature of the amplification and other complexities. Thus, several strategies for quantitation, including using synthetic RNA as an internal standard23 or competition with a closely related DNA fragment,24 have been recently developed. Our method, based on quantitation of the ratios of the several globin mRNA species in circulating erythroid cells, has turned out to be simple, rapid, and reliable for the diagnosis of the thalassemic syndromes. It may also prove useful for the detection of other genetic diseases as well as for studies of gene regulation and in monitoring the effects of therapeutic approaches based on pharmacologically altering gene expression.

MATERIALS AND METHODS

Preparation of RNA from K562 cell line and from human whole blood. RNA was extracted by the vanadyl ribonucleoside complex (VRC)25,26 method from hemin-induced K562 cells and from anticoagulated peripheral blood (PB) after separation by centrifugation or lymphocyte separation medium (Organon Technika Corp, Durham, NC) collected from 10 normal individuals, eight patients with β-thal major (five with β0-thal and three with β+thal), three patients with α-thal-1 (-ααα), two patients with β-thal major (five with β0-thal and three with β+thal), three patients with α-thal-1 (-ααα), and one fetus aborted for another genetic disease (Duchene's muscular dystrophy) were also used for analysis.

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**cDNA synthesis by RT reaction.** K562 cell RNA (0.1 ng to 1.5 μg) or 0.1 to 0.5 μg of human nucleated RNA was transferred to each Eppendorf tube and 3 μL of RT buffer (250 mmol/L Tris-HCl, pH 8.8, 375 mmol/L KCl, 15 mmol/L MgCl₂, and 50 mmol/L dithiothreitol), 1 μL of 10 mmol/L dNTP mix, 0.2 μL of oligo(dT) (0.5 mg/mL; BRL, Rockville, MD) and 0.5 μL of Moloney leukemia virus (m-MLV) RT (200 U/μL; BRL) were added. DEPC-H₂O was used to make a total volume of 15 μL that was incubated at 37°C for 30 minutes, followed by heating at 95°C for 5 minutes, was put on ice immediately, and cooled to 4°C.

**Coamplification of cDNA with PCR.** For PCR amplification, four sets of oligonucleotide primers were designed: A, B(a), B(b), and G (Table 1). All primers were chosen from sequences within the globin gene exons for cDNA amplification. They were used in three groups. Group I consisted of A and B(b) primers; the length of PCR products should be 372 bp (α) and 489 bp (β). Group II consisted of A and G primers, with lengths of 372 bp (α) and 489 bp (γ). Group III consisted of A, B(a), and G primers with lengths of 372 bp (α), 422 bp (β), and 489 bp (γ). All the oligonucleotide primers were synthesized in an Applied Biosystems 380B DNA Synthesizer (Foster City, CA) and gel-purified.

Seven microliters of each cDNA sample was mixed with group I, group II, or group III primers, and 2.5 μL of buffer (100 mmol/L Tris-HCl, pH 8.3, 15 mmol/L MgCl₂, 0.1% gelatin), 2 μL of 10 mmol/L dNTP, 0.5 μL of (α-³²P) dCTP (800 Ci/μmol; Amersham, Arlington Heights, IL), 0.5 μL of Taq polymerase (5 U/μL; Perkin-Elmer Cetus, Norwalk, CT) were added. The final volume for PCR was adjusted to 25 μL with H₂O. The reaction mixture was covered with 3% NuSieve GTG agarose (FMC No. 50082, Rockland, ME) in Tris-borate-EDTA (TBE) buffer, pH 8.3, 80 V for 1 hour. After staining with ethidium bromide, both positive and negative pictures were taken with Polaroid No. 55 film (Cambridge, MA). The DNA bands were excised from the gel and radioactivity was determined by scintillation counting.

**Analysis of PCR products.** Eight microliters of PCR products from each cDNA sample were subjected to gel electrophoresis in 1% agarose gels. Because the primers are from separate exons, any amplification of the DNA of the globin genes leads to bands of much greater molecular weight and are not seen on these gels. Figure 1 shows the results of this experiment. Note that the results are approximately linear with input RNA amounts over two logs of RNA concentration.

**RESULTS**

Nucleated cells, including reticulocytes, were isolated from the PB of normal individuals and patients with β-thal. mRNA was isolated from these cells by the VRC method. The quantity and purity of the RNA preparation were confirmed by electrophoresis in 1% agarose gels that showed predominant 18S and 28S band (data not shown). cDNA copies of the mRNA were prepared with RT and were amplified through 30 cycles of the PCR method.

To determine the linearity of this method for quantitating mRNA, as well as to determine the efficiency of the method when two target mRNA sequences were to be measured, we examined the effects of increasing input RNA obtained from hemin-induced K562 cells on the level of specific α- and γ-globin mRNA. This cell line had been previously shown to express α- and γ-globin mRNA in both a constitutive and hemin-inducible fashion. Thus, the linearity of the method was assessed after the RT reaction by coamplification using the α- and γ-globin primers (group II) and the resultant DNA bands were separated on 3% NuSieve agarose gels. Because the primers are from separate exons, any amplification of the DNA of the globin genes leads to bands of much greater molecular weight and are not seen on these gels. Figure 1 shows the results of this experiment. Note that the results are approximately linear with input RNA amounts over two logs of RNA concentra-

<table>
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<th>Table 1. Summary of Globin cDNA Amplification Primer Sets</th>
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<tr>
<td>Primer</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>II GCCTCCCTGAGCAATACTTCG</td>
</tr>
<tr>
<td>B (a)</td>
</tr>
<tr>
<td>II CTACCCCAACAGCAGGCT</td>
</tr>
<tr>
<td>B (b)</td>
</tr>
<tr>
<td>II CGTTCTTGTGTCCTCAATT</td>
</tr>
<tr>
<td>G</td>
</tr>
<tr>
<td>II AGTGGCTTCTGCCAGATAC</td>
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*Shown is the sequence in 5’-3’ orientation for the PCR primers.†The size and the kind of the amplified fragment obtained with each primer set.
tions, and that the ratio of \( \gamma/\alpha \) mRNA remains constant (2.03 ± 0.39) over most of the RNA concentrations studied. The standard deviation of four replicate counts was less than 2% (the size of the symbols in the graph).

Figure 2 shows the pattern of PCR products for 15 of these 18 individuals with the \( \alpha \)- and \( \beta \)-globin primers (group I). The intensity of \( \alpha \) bands (372 bp) and \( \beta \) bands (490 bp) for the 10 normal individuals (lanes 1 through 10) is very similar, in contrast to the absence of \( \beta \) bands in the \( \beta^- \)-thal patients (lanes 11 through 14) or the marked reduction of the \( \beta \) band in the \( \beta^- \)-thal patient (lane 15). When the group III primers for \( \gamma \) and \( \beta \) were used together, faint 489-bp bands corresponding to \( \gamma \)-mRNA were visualized as well as the 422-bp \( \beta \) bands (data not shown).

Figure 3 shows the results of coamplification using a mixture of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-primers (group III) on blood samples from patients with \( \beta^- \)-thal (lane 1), \( \beta^- \)-thalassemia (lane 2), \( \alpha \)-thal-1 (\( \alpha^-/\alpha^- \)) (lane 4), and liver from a patient with Hydrops fetalis (\( \alpha^-/\alpha^- \)) (lane 5). The \( \alpha/\beta \) ratio in the \( \beta \)-thal was increased, whereas the converse was the case in the individuals with \( \alpha \)-thal (see Table 2). It will be noted that in the fetal liver the band corresponding to the \( \gamma \)-mRNA is visible and is elevated in proportion to \( \alpha \)-mRNA in the fetus with Hydrops fetalis.

**DISCUSSION**

The characteristic imbalance of globin-chain synthesis in thalassemic erythroid cells was first shown using radiolabeled amino acids and column chromatography. These observations made it possible to confirm the specific diagnosis of either \( \alpha \)- or \( \beta \)-thal from isolated peripheral reticulocytes. Subsequent studies using globin mRNA isolated from thalassemic erythroid cells in conjunction with a heterologous cell-free translation system showed an unequivocal functional deficiency of mRNA for \( \alpha \) or \( \beta \) chains in \( \alpha \)- and \( \beta \)-thalassemia, respectively. Molecular hybridization studies using purified cDNA to quantitate the level of human globin mRNA in various erythroid cells showed that individuals with \( \beta \)-thal had a marked decreased (\( \beta^- \)) to absent (\( \beta^0 \)) level of \( \beta \)-globin mRNA present in circulating reticulocytes. Similar quantitative results were obtained in individuals with \( \alpha \)-thal of various degrees of severity. These data have been confirmed by other techniques subsequently used to measure the abundance of globin mRNA. Thus, by analyzing the ratio of \( \alpha/\beta \)-globin mRNA species isolated from reticulocytes and nucleated red blood cells, one can unambiguously diagnose and differentiate \( \alpha \)- and \( \beta \)-thal.

Many methods for identifying specific mutations (RFLPs, oligonucleotide hybridization, and polymerase chain amplification of mutant DNA) have been recently developed. However, these methods are not easily applicable to rapid diagnosis in the clinical setting or to screening populations because of the many mutations and deletions that cause thalassemia. In addition, several mutations that result in either mutant \( \beta \)-globin chain (ie, Hb E, Hb Knossos) or unstable Hbs as well as others can result in decreased...
β-globin mRNA levels. An analogous situation occurs in α-thal, but, because of the presence of four functional α-globin genes, clinically significant disorders are less likely to be recognized. Thus, measurement of the amounts of mRNA can be used for diagnosis and differentiation of various types of thalassemia.

However, this approach has rarely applied in the clinical practice, owing to many substantial difficulties. One of the major problems is that the mRNA is rather unstable and liable to degradation. The second major problem has been the quantitation of mRNA with sufficient precision and reproducibility. The procedures outlined in this report appear to overcome these technical problems in a way that allows this approach to be used for simple, rapid diagnosis of these clinically significant syndromes.

The VRC method has been used to stabilize RNA of nucleated cells against degradation by ribonucleases for analysis by Northern blots. In our studies using this method to study the nucleated cells from PB we find that almost all of the RNA moves at 18S and 28S, indicating very little degradation. Although quantitation of mRNA has previously been attempted by Northern blots and, more recently, by PCR, the results have been generally unsatisfactory because of the nonlinearity of the methods. Methods using internal RNA controls and competitors have been introduced in an attempt to solve this problem for the PCR technique. In our case, because the α- and β-mRNAs are closely comparable in amount, the α-mRNA serves adequately as an internal control for the amount of β-mRNA.

In addition, we have used purified RNA obtained from hemin-induced K562 cells to validate the linearity and efficiency of coamplification over a range in input RNA concentration of several orders of magnitude.

Thus, we find that diagnosis of β-thal, and even β'-thal, can be reproducibly accomplished even with the uncertainties of the amplification methods. We also find significant differences in the relative ratio of α/β-mRNA in samples obtained from patients with 2 or 0 functioning α-globin genes, suggesting that this methodology may be applicable for the diagnosis and differentiation of the clinically-significant α-thal phenotypes. Analogously, because in normals there is a very little γ-mRNA as compared with α- or β-mRNA, the significant increases in γ-mRNA that occur in the β-thal syndromes may easily be detected by these methods (see Table 2). It will be noted that these methods are valid for thalassemic syndromes due to decreased mRNA transcription or stability. In the rare cases in which differences in translation appear to cause the thalassemic phenotype, they may fail to establish the relevant diagnosis. However, in most of these instances, specific mRNA levels also tend to be decreased due to altered intranuclear mRNA stability or altered nuclear to cytoplasm transport of mRNA. On the other hand, we anticipate that this approach will be useful for diagnosis of the majority of cases of the prevalent α- and β-thal syndromes.

Further, this approach has potential utility in the context of a number of other genetic disorders in which knowledge of relative mRNA levels would be of diagnostic or investigative interest. In more recent work, we have found this approach to be applicable to the detection of β-thal carriers. We have also found this technique useful in monitoring the effects of hydroxyurea on γ-gene expression in sickle cell anemia patients being treated so as to elevate Hb F levels.

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