Correlation of genotype and phenotype in Beta-thalassemia

CORRELATION OF GENOTYPE AND PHENOTYPE IN ß-THALASSEMIA

PROEFSCHRIFT

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Dedicated to Maria and Kristian

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ABBREVIATIONS

AS	sickle cell trait
ASO	allele specific oligonucleotide
BS	borate saline
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAT	chloramephenicol acetyltransferase
CD(s)	codon(s)
cpm	counts per minute
dATP	deoxy adenosine triphosphate
ddATP	dideoxy adenosine triphosphate
ddCTP	dideoxy cytosine triphosphate
ddGTP	dideoxy guanosine triphospate
ddTTP	dideoxy thymidine triphosphate
ddUTP	dideoxy uridine triphosphate
DEAE-	diethylaminoethyl-
dGTP	deoxy guanosine triphosphate
DIG	digoxigenin
DMF	dimethyl formamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxy thymidine triphosphate
EDTA	ethylene diamine tetra acetate disodium salt
FACS	fluorescence-activated cell sorting
FSC	frameshift codon
G6PD	glucose-6-phosphate dehydrogenase
Hb(s)	hemoglobin(s)
HCl	hydrochloric acid
HPFH	hereditary persistence of fetal Hb
HPLC	high performance liquid chromatography
HS	hypersensitive site
H_2O_2	hydrogen peroxide
IEF	isoelectrofocusing
IVS	intervening sequence
IVS-I	first intervening sequence
IVS-II	second intervening sequence
KCl	potassium chloride
KCN	potassium cyanide
LCR	locus control region
mAb	monoclonal antibody
MCH	mean corpuscular Hb
MCHC	mean corpuscular Hb concentration
MCV	mean corpuscular volume
MOPS	(3[N-morpholino]propanesulfonic acid)
mRNA	messenger ribonucleic acid
MW	molecular weight
O_2	oxygen
OD	optical density
nt(s)	nucleotide(s)

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR(s)	polymerase chain reaction(s)
PCV	packed cell volume
poly A	polyadenylation
PVP	polyvynilpyrolidone
RBC(s)	red blood cell(s)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcription/transcribed
SDS	sodium dodecilsuphate
SEA	Southeast Asian
SS	sickle cell anemia
SSC	sodium saline citrate buffer
SSPE	sodium saline phosphate EDTA buffer
TMAC	tetramethyl ammonium chloride
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TEA	Tris EDTA acetate buffer
TFA	trifluoroacetic acid
T_m	melting temperature
thal	thalassemia
UTR	untranslated region
UV	ultraviolet
WBC(s)	white blood cell(s)

CHAPTER 1

INTRODUCTION
AND
REVIEW OF THE LITERATURE

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR(s)	polymerase chain reaction(s)
PCV	packed cell volume
poly A	polyadenylation
PVP	polyvinylpyrrolidone
RBC(s)	red blood cell(s)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SDA	Southern's dot assay
SS	sickle cell anemia
SSC	saline sodium citrate buffer
TAPE	separation by adsorption to polyethylene
TMRE	tetramethyl rhodamine ethyl ester
TRS	Tris borate EDTA buffer
TE	Tris EDTA buffer
TSA	Tris EDTA sodium azide
TPA	tetrafluoroboric acid
Tm	melting temperature
thal	thalassemia
UTR	untranslated region
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WBC(s)	white blood cell(s)

CHAPTER 1

INTRODUCTION

β-Thal is one of the most common single gene disorders worldwide, causing a major health problem especially in underdeveloped countries. It has the highest frequency in areas where malaria was/is endemic, such as the Mediterranean, Southeast Asia, and India, suggesting positive selection by the malaria parasite in sustaining the affected β-globin gene.

β-Thal is a heterogeneous group of disorders, both in terms of molecular defects and in the phenotypic expression. In all cases, however, the molecular defect causes a reduction or absence of β-globin chain synthesis leading to β^+- or β^0-thal, respectively. The β-globin chain deficiency results in a decrease or absence of the adult Hb (Hb A) which is composed of two identical β- and two identical α-globin chains. The levels of the other adult Hb (Hb A_2) and the fetal Hb (Hb F) are variably increased, but this is usually not sufficient to counterbalance the Hb A deficiency.

The clinical course of the β-thal syndromes is variable and ranges from mild (β-thal minor) through intermediate (β-thal intermedia) to extremely severe (β-thal major). In the latter, patients are transfusion-dependent and death rates are high in children and young adults, even with proper transfusion therapy. The severity of the disease is largely determined by the level of α/non-α-globin chain imbalance. The excess α-globin chains precipitate and damage the erythroid precursors in the bone marrow, leading to their premature destruction and an ineffective erythropoiesis. Apart from the degree of β-globin chain deficiency, the globin chain imbalance can also be influenced by the level of α- and γ-globin chain production. Co-inheritance of α-thal (which reduces the α-globin chain excess) and/or genetic factors which increase the γ-globin gene output can ameliorate the clinical course of the disease.

Management strategies in β-thal have focused mainly on two areas: Prevention by means of prenatal diagnosis and development of new therapeutic approaches such as gene therapy and pharmacologic induction of Hb F synthesis. Assessment of different factors that influence the clinical expression of the disease can also provide a more accurate prognosis and indicate the most appropriate treatment for each individual patient.

The main objectives of the work presented in this dissertation are:

To characterize the molecular defects leading to β-thal in the countries of the former Yugoslavia and Bulgaria in order to establish a DNA-based prenatal diagnosis program in this part of the Balkan Peninsula;

to investigate the relative contribution of the different factors that can affect the clinical presentation of the disease;

to evaluate the influence of genetic factors, both linked and unlinked to the β-globin gene complex on Hb F production in β-thal.

HISTORY

The first description of thalassemia, which appeared in 1925, was by the American pediatrician Thomas B. Cooley (1). Although some ancient Greek and early Italian writers also seem to have referred to this condition (2), it is interesting that the disease had been overlooked as a separate clinical entity by Mediterranean physicians who must have frequently encountered it among their patients. Within the following 10 years, many reports of Cooley's anemia appeared in the literature and it became evident that the disease occurred predominantly in the Mediterranean population. To associate the disease with the Mediterranean area, Whipple and Bradford in 1932 gave it the name 'thalassemia', referring to the Greek word 'thalassa' meaning 'the sea' (3). During the 1930s the clinical syndrome of thalassemia had been well described, and in 1938 a genetic determinant underlying the disease was suggested (4). By the end of the 1940s it was already apparent that thalassemia was not a single disorder but a complex syndrome resulting from the interaction of many genetic factors. In the late 1950s detailed Hb analyses were performed and Hb F ($\alpha_2\gamma_2$), Hb A_2 ($\alpha_2\delta_2$), Hb H (β_4), and Hb Bart's (γ_4) were observed in different patients with thalassemia (5-8). In 1959 these findings allowed Ingram and Stretton to define two major classes of thalassemia, namely α and β, and to suggest an inherited defect in the α- or β-globin chain synthesis in these two forms, respectively (9); experimental evidence came with the advancement in biosynthesis studies showing an imbalanced synthesis in both disorders (10). Further heterogeneity among the thalassemias was noted, and forms with decreased or absent α- and β-globin chain synthesis were described. Advancements in recombinant DNA technology during the 1970s made it possible to show a decreased production in globin mRNA (11), and to identify deletions of individual globin genes in certain thalassemic patients (12). Studies at the DNA level allowed the characterization of the genomic organization and structure of individual globin genes (13-19). Further development of molecular biology techniques, and especially the introduction of the PCR procedure in the second half of the 1980s, allowed extensive and almost complete characterization of the molecular defects underlying the thalassemias (20,21). These advancements also allowed the development of successful screening and prenatal diagnostic programs, and formed the basis for future therapeutic modalities such as gene therapy and reactivation of the γ-globin genes.

ETHNIC DISTRIBUTION

The thalassemias probably represent the commonest gene disorder to cause a major public health problem in the world population (22). People of Mediterranean, Asian and African ancestry are primarily affected, but sporadic cases have been reported in many other ethnic groups. It is believed that the geographic distribution of the thalassemias is due to a decreased morbidity of individuals carrying the trait when infected with malarial parasites. The reasons for this protection of the thalassemia heterozygotes are still unknown, but it has been proposed that the red cell membrane in thalassemia heterozygotes is particularly susceptible to damage by oxidation and that infection with the malaria parasite provides sufficient oxidative stress to perturb intracellular metabolism in

a manner that leads to premature death of the parasite (23). This hypothesis also provides a uniform explanation for the decreased malaria susceptibility observed among heterozygotes for α- and β-thal, and for Hb S and G6PD deficiency. More recently, a role for enhanced immune recognition and subsequent clearance of thalassemic red cells infected with the malaria parasite has been suggested (24).

Starting in the mid 1940s, studies have been performed worldwide to estimate the prevalence of thalassemia in various populations (reviewed in Ref. 22). While α-thal appears to be more common in Southeast Asia, India, and the South Pacific (23), β-thal is the most common genetic disorder in Mediterranean countries, with frequencies ranging from 5 to 20% in some of the Mediterranean islands (25). As this dissertation deals mainly with β-thal in the Mediterranean, its incidence in this area will be described in greater detail later. The incidence of heterozygous β-thal in Southeast Asian populations is approximately 5% (23), while in American Blacks it is approximately 0.5% (26). The frequency of heterozygous α-thal in Southeast Asia is also around 5%, but increases to 20% in Thailand, and even to 80% in certain regions of New Guinea and India (23,27). The heterozygosity for α-thal in Southeast Asia includes both α-thal-1, where both α-globin genes are deleted from a single chromosome, and α-thal-2 where only a single α-globin gene is missing. Heterozygosity for an α-thal-2 deletion is found in 25 to 30% of American Blacks; approximately 3% have a homozygosity for this deletion.

A recent compilation of data for 535,000 Mediterranean individuals tested for the incidence of heterozygous β-thal confirmed that β-thal occurs mainly in Cypriots (14.7%), Greeks (8%), Albanians (7.1%), and Italians (3.7%), especially Sardinians (12.6%) and Sicilians (5.9%) (Fig. 1/1). A high incidence of β-thal was also found among Lybians (4.6%) and Tunisians (4.4%). A frequency of 2 to 3% was reported for the populations of Turkey, Lebanon, Israel, Malta, Algeria, Morocco, and Corsica. Lower frequencies were reported in countries of the former Yugoslavia (1.4%) and Spain (0.5%), with the lowest incidence in France (0.1%) (see Ref. 25 for references). The frequency of a β-thal heterozygosity in 1,745 Albanian school children living in Macedonia was only 2% (25). However, a difference in the frequency of β-thal in different regions of Albania has also been observed, with the lowest values in the eastern provinces that border Macedonia (28). The incidence of α-thal in the Mediterranean Basin has not been completely evaluated; some data are available for Spain, Macedonia, Croatia, Greece, Sardinia, Tunisia, and Algeria. The incidence of α-thal in Spain, Macedonia, Croatia, and Greece is low, as judged by the rarity of patients with Hb H disease and by cord blood surveys. Screening of 3,326 Macedonian newborns showed an α-thal incidence of 2.3%, while screening of 1,546 Croatian newborns showed an incidence of 1.4% (25). An incidence of 0.2% was observed among 1,043 newborns of Albanian nationality living in Macedonia. The incidence of α-thal in Spain and Greece is low, namely 0.2 and 0.5%, respectively. However, this condition occurs at a much higher frequency in Sardinia (6.9%), Tunisia (4.8%), and Algeria (9%) (see Ref. 25 for references).

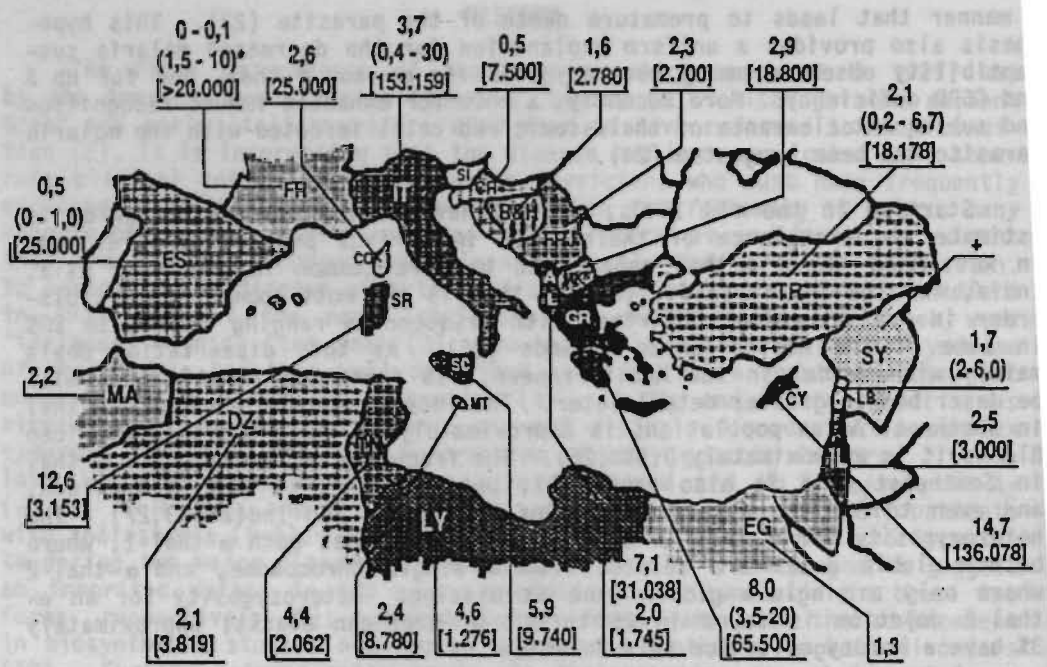


FIG. 1/1. The incidence of β-thal trait in Mediterranean countries determined during population studies. Total number of screened subjects was 535,528; the numbers in parentheses give the ranges and those between brackets the numbers of persons involved in these surveys. The asterisk (*) indicates immigrants.

CLINICAL AND HEMATOLOGICAL FEATURES

The three clinical syndromes associated with differences in the degree of severity have been defined historically as β-thal major, β-thal minor, and β-thal intermedia. While β-thal major and β-thal minor represent the clinical features observed in β-thal homozygotes and heterozygotes, respectively, individuals with β-thal intermedia may belong to both groups. In all cases, however, the basic pathophysiological phenomenon is an imbalance in globin chain synthesis due to a deficient or absent β-globin chain production. The consequence is an excess of α chains which cannot form tetramers and therefore precipitate within the developing RBC precursors in the bone marrow. The precipitated α chains damage the cell membrane and the various organelles, leading to the premature destruction of the erythroblasts in the marrow and an ineffective erythropoiesis. The clinical severity of β-thal is therefore directly proportional to the degree of chain imbalance, and depends mainly on the level of β-globin chain production. However, two additional factors can have a positive influence on the globin chain imbalance, namely the substitution of β chain synthesis by an increased γ chain production, and a decrease in the α chain excess due to a concomitant α-thal condition.

Heterozygous β-Thal (β-thal minor). β-Thal heterozygotes are usually asymptomatic, presenting with only a mild anemia or even normal Hb levels.

Anemia of moderate severity, as seen in β-thal intermedia, has only occasionally been described. The MCV and MCH are usually decreased well below normal, with typical values of 60 to 70 fl (normal range 85-92 fl) and 20 to 25 pg (normal range 27-32 pg), respectively. Further hematologic characteristics include microcytosis, hypochromia, anisocytosis, and poikilocytosis with targeting and basophilic stippling of the red cells in the peripheral blood, and mild erythroid hyperplasia in the bone marrow. Mild to moderate splenomegaly is present in only a few cases. The diagnosis is confirmed by Hb electrophoresis, and by quantitating the levels of Hb A_2 which are about twice the normal values of 2-3%. Hb F levels are slightly elevated in approximately half the cases (1-5%). In vitro globin chain biosynthesis analysis shows an α/β chain synthesis ratio of 1.5 to 2.0 (normal range 0.9-1.1). Less frequently, β-thal heterozygotes can have normal Hb A_2 and Hb F levels, and decreased or even normal MCV and MCH values. These silent carriers are usually distinguished from α-thal heterozygotes by the finding of a clinically significant β-thal syndrome in their offspring and by an impaired α/β-globin chain synthesis ratio. Heterozygotes with normal or slightly decreased Hb A_2 levels and increased Hb F levels (5-20%) carry a δβ-thal trait. Finally, some β-thal heterozygotes are characterized by the presence of a Hb Lepore, which contains normal α chains and hybrid δβ chains; the latter have an amino acid sequence of the δ chain at the N-terminus and that of the β chain at the C-terminus. Apart from 8-12% Hb Lepore, these β-thal heterozygotes have normal Hb A_2 and slightly elevated Hb F levels.

Homozygous β-Thal (β-thal major). The clinical and hematological features of β-thal major become manifest several months after birth, after the completion of the fetal to adult Hb switch and the decrease in Hb F production. Usually within a year, a severe hypochromic, microcytic, hemolytic anemia develops, and a regular transfusion program must be undertaken to maintain adequate Hb levels. In children not receiving transfusion therapy the severe anemia leads to an early death at 3 to 4 years of age for those homozygous for β^0-thal, and at 8 to 12 years of age for those with a β^+-thal homozygosity (29). On the other hand, proper transfusion regimens together with adequate iron chelation therapy have allowed a relatively normal life style and survival well into the third decade (30). Unfortunately, these advancements in the management of thalassemic patients have not yet been reached in the Third World countries where thalassemia is most frequent (29,30). Therefore, most patients still do not receive adequate transfusion and iron chelation therapy, allowing a variety of clinical features to emerge. Early manifestations include progressive enlargement of the liver and spleen, bone changes with a typical facies due to bone marrow expansion, and repeated pathological fractures of the long bones. Gallstones, leg ulcers, and recurrent infections are frequent complications. The latter, along with a neglected anemia, are the most common causes of death in early childhood. Secondary hypersplenism leading to thrombocytopenia, leukopenia, and rapid destruction of transfused red cells can further complicate the clinical picture. Splenectomy is often indicated, which, on the other hand, imposes a high risk of septicemia. Growth retardation becomes most noticeable around 10 years of age, and menarche and secondary sexual characteristics usually do not follow. The course of the disease is further complicated by iron overload due to transfusion or increased iron absorption from the gut in inadequately treated

patients. The iron overload causes significant damage to the heart, liver, pancreas, and other organs. Cardiac failure and arrhythmias due to cardiac siderosis are the most common causes of death in the second and third decades of life. Hematologically, β-thal homozygotes display, in addition to a profound anemia, striking red cell abnormalities, including hypochromia, microcytosis, anisocytosis, poikilocytosis, polychromasia, fragmentation, basophilic stippling, and a frequent occurrence of nucleated RBCs in the peripheral blood. The reticulocyte count is usually not very high because of a massive destruction of erythroid precursor cells in the bone marrow. The bone marrow is hypercellular, with marked erythroid hyperplasia, characterized by poorly hemoglobinized normoblasts.

Untransfused patients with β^0/β^0- and β^0/β^+-thal have average Hb values between 5-6 g/dl, which are slightly higher (7 g/dl) in β^+/β^+-thal homozygotes (29). Hb A_2 is present in variable amounts, while Hb F is always elevated and may account for 10 to 90% of the patient's total Hb. The Hb F is usually heterogeneously distributed among the red cells, due to the increased production and selective survival of F-cells (23).

β-Thal Intermedia. The term 'β-thal intermedia' is used to define the entire spectrum of clinical syndromes between the 'major' and 'minor' forms of β-thal. It refers mainly to patients who maintain functional Hb levels (usually between 6-9 g/dl), an adequate growth and development, and survive into adulthood without blood transfusions (22). The molecular basis of β-thal intermedia is variable, and as yet, not completely resolved, but in general it involves the interaction between different molecular defects and factors that partially correct the globin chain imbalance. An important factor contributing to β-thal intermedia is the simultaneous presence of α-thal (homozygosity for α-thal-2 or heterozygosity for α-thal-1), that, by improving the α/β chain synthesis ratio, can significantly ameliorate the course of the disease. As already mentioned, β-thal intermedia also includes rare cases of unusually severe heterozygous β-thal associated with inclusion body formation and hemolysis, or with features of dyserythropoietic anemia (31). The molecular defects of the β-globin gene expressed as β-thal intermedia will be discussed later in this chapter.

MOLECULAR GENETICS OF THE β-GLOBIN GENE CLUSTER

Human Hbs are tetramers of two identical α-like and two identical β-like chains; their major function is to transport O_2 from the lungs to the tissues. They are produced in a developmentally restricted fashion, with two switches occurring during ontogenesis. The three embryonic Hbs, Hb Gower-I ($\zeta_2\varepsilon_2$), Hb Gower-II ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$), are produced in the yolk sac during the third through eighth week of gestation. From the eighth to the 28th week, the liver becomes the major site of erythropoiesis and the two fetal Hbs ($\alpha_2{}^G\gamma_2$ and $\alpha_2{}^A\gamma_2$) are predominantly produced. The two adult Hbs (Hb A or $\alpha_2\beta_2$ and Hb A_2 or $\alpha_2\delta_2$) are already present in the fetus during the second trimester and their levels slowly increase. At birth, the Hb F level is more than 80%, while 6 months later the Hb switch in a normal baby is almost complete and the level of Hb F falls to less than 2%. During this transition a reversal of the $^G\gamma/^A\gamma$ ratio also occurs in most babies, from the fetal value of ~70% $^G\gamma$ and ~30% $^A\gamma$

to the adult value of ~40% $^{G}\gamma$ and ~60% $^{A}\gamma$. The adult values for Hb A_2 (2.5 ± 0.3%) and for Hb F (<1%) are established within the first year of life (32).

The genes that encode the globin chains are located on two different chromosomes. The α-like genes are clustered on the short arm of chromosome #16, while the β-like genes are located on the short arm of chromosome #11, The arrangement of the genes within each cluster is in the same order as they are expressed during development (Fig. 1/2). The α-globin gene cluster spans a region of about 50 kb and contains three functional genes (ζ, α2, α1), three pseudo genes (ψζ, ψα2, ψα1), and one gene of undetermined function (θ1). The two α genes have the same coding sequence, but α2 is expressed at a higher level (70%). The β-globin gene cluster spans a region of about 90 kb. The genes that are expressed at the same developmental period, i.e. the $^{G}\gamma/^{A}\gamma$ pair and the δ/β pair, are spaced relatively close to one another (5-6 kb), while a considerably longer segment of DNA (15-18 kb) separates the δ/β pair from the $^{G}\gamma/^{A}\gamma$ pair, and the latter from the ε gene. Only one pseudo gene, the ψβ gene, is present in the β-globin gene cluster.

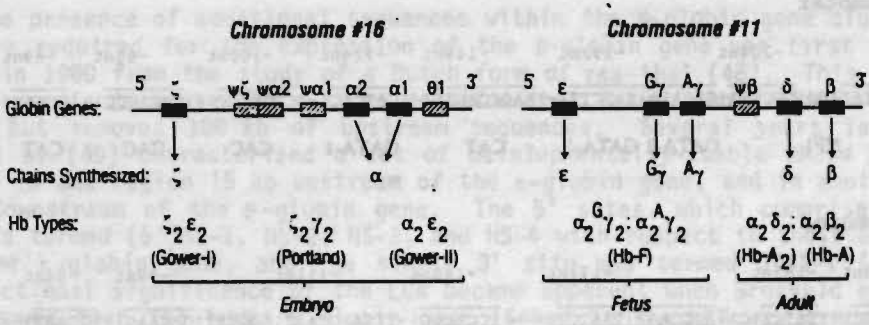


FIG. 1/2. The human α-like and β-like globin genes and their developmental expression.

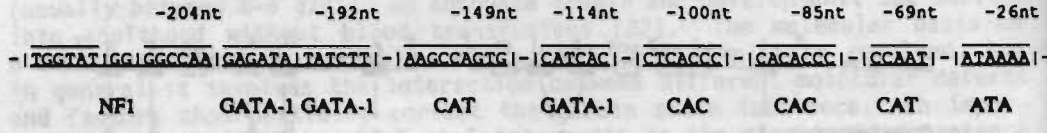
The coding region of each globin gene consists of three exons separated by two introns or IVS. In the β-like globin genes the introns interrupt the sequences between CDs 30 and 31 and between CDs 104 and 105. The intron lengths between different β-like globin genes are rather similar and vary from 122 to 130 nts for IVS-I and 850-904 nts for IVS-II. However, despite a significant homology in the exons, the intron sequences of the β-like genes, especially the IVS-II, have diverged considerably, except in the case of the duplicated $^{G}\gamma$- and $^{A}\gamma$-globin genes (18,19).

The intron sequences are removed from the precursor RNA by the process of RNA splicing. Critical sequences for proper splicing lie at the exon-intron boundaries, and are represented by the two invariant dinucleotides GT and AG at the 5' and 3' ends of the introns, respectively, together with their surrounding consensus sequences. The consensus sequence of the 5' or donor splice site includes the last three nts of the exon and the first six nts of the intron, while the 3' or acceptor site encompasses the last 10 nts of the intron and the first nt of the exon.

The mature globin mRNA contains, in addition to the exon sequences, two UTRs at both ends of the transcripts. These transcripts are modified by the addition of a methylated guanylic acid (Cap structure) and a number of adenylic acid residues constituting the poly A tail at the 5' and 3' ends, respectively. The poly A tail appears to contribute to the stability of the mRNA (33). The hexanucleotide **AATAAA**, located in the 3'UTR approximately 20 nts 3' from the cleavage site, constitutes the signal required for proper processing and polyadenylation of the transcript (34,35).

The first 100 nts flanking the 5' end of the globin genes constitute the minimal promoters, and contain three sets of sequences that are common to all globin genes (18,19,36-40) (Fig. 1/3). The first preserved sequence is **ATAA** (**ATA** box) located approximately 30 nts from the Cap site. Further upstream, approximately 70-80 nts from the Cap site of the β-globin gene, a **CCAAT** sequence (**CAT** box) is present. The δ-globin gene promoter contains three imperfect **CAT**-like sequences (**CCAAC**), whereas two **CAT** boxes are present in the promoters of both γ-globin genes. The third preserved sequence is the **CAC** box [**CA(T)CACCC**] which is duplicated in the β-globin gene promoter and located at around 80 and 100 nts from the Cap site; it is also present further upstream in the ε- and γ-globin genes.

β-promoter



γ-promoter

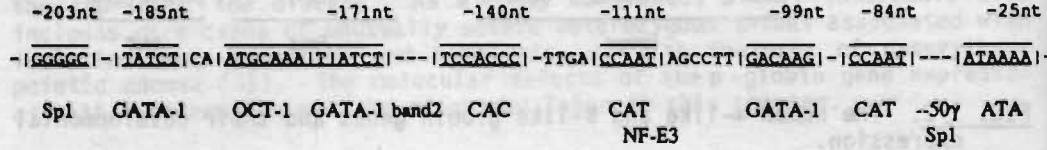


FIG. 1/3. Protein binding regions of the β- and γ-globin genes' promoters (adapted from Refs. 44 and 45). **ATA:** **ATA** binding proteins; **CAT:** **CCATT** binding proteins; **CAC:** **CACCC** binding proteins.

The role of these sequences in gene transcription has been investigated by *in vitro* mutagenesis and expression of the mutant promoters in various cell lines (41,42). Nt substitutions located in these conserved regions result in a significant reduction of transcription, as observed in β-thal patients with mutations in these sequences. Transfection experiments with constructs also containing sequences upstream of the minimal promoter have identified additional elements involved in the regulation of erythroid and developmentally specific expression of the β-globin gene (43,45).

A complex array of erythroid specific and non-specific *trans*-acting factors (reviewed in Ref. 45) bind to the globin gene promoters and other

β-globin gene cluster regulatory elements. Ubiquitous transcription factors such as the TATA-binding protein TBP, the CCAAT binding proteins CP1, CTG/NF1, and CDP, and various other non-erythroid specific factors such as SP1, TEF-2, jun, fos, USF, J-BP, and YY1, have been shown to bind *in vitro* to critical *cis*-acting regulatory sequences; however, their role in regulating the globin genes *in vivo* is at present still unknown.

Several erythroid specific factors have been described which appear to be involved in the regulation of globin gene expression. The best characterized is the GATA-1 protein, which belongs to the family of GATA binding proteins (GATA-1, GATA-2, GATA-3, and GATA-4) that are expressed in different hematopoietic and other cell types (reviewed in Ref. 46). The GATA-1 is expressed only in erythroid, megakaryocytic, and mast cells, and binds to promoter, enhancer, and LCR elements of all known erythroid specific genes, including its own promoter. Other erythroid specific factors include the NF-E1, NF-E2, NF-E3, NF-E4, NF-E5, NF-E6, BGP1, Pal1, and -50 , of which only several (NF-E3, NF-E4, Pal1, BGP1, and -50) seem to be expressed at different levels in cells representing different stages of development, and could therefore be implicated in the control of globin gene switching (47).

The presence of additional sequences within the β-globin gene cluster that are required for the expression of the β-globin gene was first suggested in 1980 from the study of a Dutch form of γδβ-thal (48). This type of thalassemia is caused by a large deletion that leaves the β-globin gene intact but removes 100 kb of upstream sequences. Several years later, Tuan et al (49) characterized a set of developmentally-stable DNase I HS located in one region 15 kb upstream of the ε-globin gene, and in another, 18 kb downstream of the β-globin gene. The 5' sites, which comprise the LCR were termed (5')HS-1, HS-2, HS-3, and HS-4 with respect to their order from the ε-globin gene, and the single 3' site was termed 3'HS-1 (50). The functional significance of the LCR became apparent when Grosveld et al (51) showed that the human β-globin gene linked to the LCR was expressed in transgenic mice at levels comparable to those of the endogenous mouse globin genes. In addition, the level of expression was independent of the integration within the mouse genome, but was dependent on the number of copies integrated. The LCR can thus override the influence of the neighboring chromosomal sequences to create an open chromatin domain capable of supporting high levels of globin gene transcription. Each of the four β-LCR elements has subsequently been mapped to 200-300 bp core regions of DNase I hypersensitivity and has been found to consist of a high density of binding sites for a number of the erythroid and ubiquitous factors described above (52-54). Studies using different subfragments of the LCR, alone or in combination, have shown that sites HS-2 and HS-3 are each responsible for approximately 40-50% of the full enhancing activity of the LCR (55,56). However, all four sites appear to be necessary to obtain the full stimulatory effect, and it has been suggested that the individual β-LCR elements assemble to form a single larger complex capable of interacting with the β-like globin genes (57,58).

Further experiments in transgenic mice have shown competition of the individual β-like globin genes for the LCR, in such a way that the proximal globin genes can suppress the expression of the more distal genes (59,60).

Therefore, to obtain the developmentally regulated switching of the individual globin genes, the interaction of the more proximal ε- and γ-globin genes with the LCR needs to be blocked. Experiments in transgenic mice have shown that this occurs since the γ-globin gene was expressed at much lower levels in adult animals, even when introduced independently from the other globin genes (61). It therefore appears that the developmental regulation depends on the state of the globin gene promoters and their availability to interact with the LCR. In addition, a gene developmental stage specificity has been observed for the HS-3 and HS-4 LCR elements (62).

MOLECULAR BASIS OF β-THALASSEMIA

More than 160 mutations or deletions of the β-globin gene have been described (listed as an addendum to this chapter), approaching an almost complete characterization of the molecular defects causing β-thal in various population groups (63). The identification of the β-globin gene mutations was mainly performed in the last decade, and two major advancements that greatly facilitated this effort were the application of β-globin gene haplotyping and the PCR procedure.

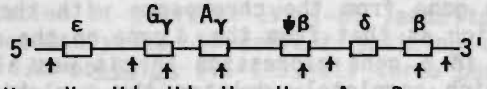
Haplotyping of the β-globin gene cluster refers to the analysis of DNA polymorphisms, namely various RFLP, which have been shown to be associated with different chromosomal backgrounds in various ethnic groups. A pattern of different restriction enzyme polymorphisms spread throughout the β-globin gene cluster determines a particular haplotype (64). Table 1/1 shows the most commonly investigated polymorphisms that define the major haplotypes among Mediterraneans.

Screening for new mutations in the first half of the 1980s took advantage of the observation that, in general, different mutations were associated with different haplotypes. Haplotype analysis, therefore, lowered the chance of repeatedly identifying the same mutation. With the introduction of the PCR methodology in the second half of the 1980s, haplotype analyses became less significant, especially since a large number of identical mutations were already identified as being present on different chromosomal backgrounds. However, some of these mutations differed in their phenotypic expression, especially in Hb F production, which potentiated a new role for haplotype analysis in the identification of sequence variations that can modulate the expression of the γ-globin genes *in cis*.

The β-globin gene mutations described in the following pages are representative examples of the various defects that can affect the function of the β-globin gene. Appendix I contains a complete, updated list of β-thal mutations, including references and other relevant information.

Transcription Mutants. As mentioned earlier, the β-globin gene promoter contains sequences that regulate the efficiency and accuracy of the transcription process. Until recently, 17 different promoter mutations, all causing a β^+-thal, have been reported. They are clustered in the **ATA** box and in the proximal and distal **CAC** boxes of the β-globin gene promoter.

TABLE 1/1. The Common Mediterranean Haplotypes and the Most Frequently Associated Mutations



Haplotypes	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Associated Mutations
I	+	-	-	-	-	-	+	+	IVS-I-110; CD 39; -101; FSC 6; Hb Knossos; IVS-I-116
II	-	-	+	+	-	+	+	+	CD 39; IVS-I-110
III	-	+	+	-	+	+	+	-	IVS-II-1
IV	-	+	+	-	+	+	-	+	FSC 8
V	+	-	-	-	-	-	+	-	FSC 5; IVS-I-5 (G->T); IVS-I-1
VI	-	-	+	+	-	-	-	+	IVS-I-6
VII	+	-	-	-	-	-	-	+	IVS-II-745; IVS-I-6; CD 39
VIII	-	+	+	-	+	-	+	-	-87
IX	-	+	+	-	+	+	+	+	IVS-I-110; FSC 6; CD 39

1 (Hc) = Hinc II 5' to ε; 2 (X) = Xmn I 5' to G_γ; 3 and 4 (Hd) = Hind III at G_γ and A_γ; 5 and 6 (Hc) = Hinc II in ψβ and 3' to it; 7 (A) = Ava II in β; 8 (B) = Bam HI 3' to β.

The seven mutations located at five different positions in the ATA box, between positions -28 and -32 from the Cap site, are found within different ethnic groups. The -29 (A->G) mutation is associated with a very mild clinical phenotype among Blacks, where it accounts for the majority of β-thal mutations (65). This is most likely due to the chromosomal background which carries the C->T substitution at -158 to the G_γ-globin gene. The latter substitution has been shown to be associated with high Hb F production under conditions of erythropoietic stress (66). The Black homozygotes for this mutation have average Hb levels of 10.6 g/dl, with more than 60% Hb F (67). On the other hand, a Chinese homozygote with the same mutation on a different chromosome [lacking the G_γ -158 (C->T) mutation] had a transfusion-dependent thalassemia major (68). Similarly, Japanese homozygotes for the -31 (A->G) mutation have low Hb (6.9 g/dl) and low Hb F (8.8%) levels (69).

Nine relatively mild mutations have been identified in the proximal CACCC box, at positions -86, -87, -88, -90, and -92 relative to the Cap site of the β-globin gene. The -88 (C->T) substitution is the second most common β-thal mutation among Blacks and, as in the case of the -29 (A->G) mutation, is associated with the G_γ -158 (C->T) substitution and high Hb F levels (64.5%) (65,67). It is interesting that unlike other mild β-thal mutations, the proximal CACCC box mutations are also associated with high Hb A_2 levels in the heterozygotes (70). The presence of the δ chain variant Hb B_2, that can be chromatographically separated from Hb A_2, made it possible to evaluate whether the increased δ-globin gene expression is

in cis or in trans to the mutated β-globin gene (71). While the levels of Hb A_2 and Hb B_2 were identical in normal controls and were increased to the same extent in the β° heterozygotes with the CD 47 (+A) mutation, the output of the δ-globin gene from the chromosome with the -88 (C->T) substitution was twice as high as that from the δ gene on the normal chromosome. The same increase in δ gene expression in cis was also observed in the 1393 bp deletion which completely deletes the β-globin gene promoter. These results strongly suggest a competition between sequences within the β- and δ-globin gene promoters for transcription factors or interactions with regulatory elements in cis, such as the LCR.

The mutation in the distal **CACCC** box [-101 (C->T)] was identified in three Turkish and one Bulgarian family (72), and was subsequently found among Italian β-thal subjects (73). The heterozygotes carrying this mutation have the silent β-thal phenotype with normal hematological parameters (MCV and MCH) and normal levels of Hb A_2 and Hb F. Homozygotes for this mutation have not yet been identified, but compound heterozygotes have a β-thal intermedia with relatively mild hematological abnormalities.

It is interesting that even though 17 different β-thal mutations have been identified in the β-globin gene promoter, so far none has been found in the **CCAAT** box.

RNA Splicing Mutants. A large number of β°-thal mutations affect RNA splicing by inactivating the normal splice sites, by activating cryptic splice sites, or by producing novel splice sites. The first β-thal mutation that was identified belongs to the last group and is the most common mutation in the Mediterranean. This **G->A** substitution at IVS-I-110 creates an **AG** dinucleotide within a consensus sequence which is almost identical to the sequence of the acceptor splice site of IVS-I. As a consequence, an alternate acceptor splice site is created 19 nts upstream of the 3' end of the intron, and this site is used for the splicing of 90% of the β-globin mRNA. The improperly spliced β-globin transcripts contain a 19 nt intron remnant which changes the reading frame, and are highly unstable. Even though the IVS-I-110 mutation causes a β^+-thal, the clinical expression is of thalassemia major due to the extremely low β-globin chain output of 10% (74). Three mutations at positions 654, 705, and 745 nts of the second intron create alternative donor splice sites. The latter two mutations greatly reduce the level of normally spliced transcripts, while the IVS-II-654 mutation eliminates normal splicing and is a β° allele. In all three cases, an alternate acceptor splice site at IVS-II-579 is activated, and sequences between this site and the new donor site are retained in the improperly spliced β-globin transcripts.

Twelve mutations have been described that disrupt the invariant **GT** and **AG** dinucleotides in the donor and acceptor splice sites, respectively. The mutations in the **GT** dinucleotide at the 3' end of IVS-I prevent any proper splicing at this site, and lead to the activation of alternative splice sites, of which two are located in the first exon (CDs 18 and 25) and the third at positions 13 and 14 of IVS-I. Similarly, the IVS-II-1 (**G->A**) mutation leads to inactivation of the IVS-II donor splice site and activation of an alternative donor site 47 nts downstream.

Mutations in the consensus sequences of the normal splice sites can lead to their underutilization and again, abnormal use of the alternative splice sites described above. A common Mediterranean β-thal mutation, the T->C substitution at position IVS-I-6, leads to a relatively mild β^+-thal syndrome, presumably due to correct splicing of a substantial number of transcripts. At position IVS-I-5, all three possible nt changes have been reported, associated with different clinical phenotypes. It has been suggested that transversions (G->C or G->T) at this position could have a more profound effect on the tertiary structure of the DNA than the G->A transition, that latter leading to a more moderate reduction of splicing efficiency and a milder clinical phenotype (75).

The two cryptic splice sites in exon 1 can also be activated by mutations that change their consensus sequence, making them more favorable to the splicing mechanism. Three of these mutations activate the alternative splice site around CD 25, while the fourth activates the more upstream site around CD 18. The latter mutation introduces an amino acid substitution which leads to the synthesis of an abnormal Hb variant, Hb Malay. The mutations in CDs 26 and 27 also lead to amino acid replacements and result in the Hb E and Hb Knossos variants, respectively, while the mutation in CD 24 is silent. This mutation is associated with a more severe clinical syndrome and with less normal splicing of precursor β-mRNA molecules than the Hb E and Hb Knossos mutations (23).

Translation Mutants. Most of the mutations that affect translation are nonsense CDs or frameshift mutations that alter the reading frame and ultimately create a new stop CD. The frameshift mutations result from small insertions or deletions of up to 7 nt in the coding region of the β-globin gene. The premature termination of translation is expected to give rise to shorter abnormal peptides in the erythroid cells of the affected individuals. Such peptides have not been identified, probably due to their rapid turnover in red cell precursors. It is interesting that these mutations also affect the level of β-globin mRNA in the erythroid cells. Studies of the CD 39 (C->T) mutation have implicated inefficient nuclear-cytoplasmic transport or nuclear instability as mechanisms that decrease the levels of the β-39 transcripts (76). All frameshift and nonsense mutations result in a β° type of thalassemia, and are usually associated with a severe clinical phenotype.

Several mutations that affect initiation of translation have been reported. Three of them destroy the initiaton CD, while a G->A substitution at position +22 from the Cap site creates a new initiation CD upstream of the normal one.

RNA Cleavage and Poly A Mutants. Four mutations and two small deletions have been identified in the cleavage and poly A signal sequence **AATAAA**. Two of these mutations have been studied by transient expression analysis, and only a small percentage of the transcripts were found to be polyadenylated at the normal site (77,78). Elongated transcripts, cleaved following **AATAAA** signals located 1 to 3 kb 3' to the gene, were present at less than 10% of the normal level, suggesting that the deficient β-globin synthesis in these mutants is due to the instability of the abnormally

elongated transcripts. All of these mutations are associated with a β^+-thal, as they do not completely abolish normal polyadenylation, and they do not affect translation of the abnormal transcripts.

A C->G mutation in nt 6 of the 3'UTR, that probably affects the stability or processing of β-globin RNA, has been associated with silent β-thal. Similarly, heterozygotes for the Cap site mutation [+1 (A->C)] have normal MCV values and borderline normal Hb A_2 levels. The latter mutation could have an effect on transcription or on capping with a secondary effect on translation (79).

Dominant β-Thal Alleles. The dominant forms of β-thal are characterized by a thalassemia intermedia clinical phenotype in heterozygotes, and the presence of inclusion bodies in the erythroid precursors and peripheral RBC. The term 'inclusion body' β-thal that was initially adopted for this syndrome, is now avoided due to the identification of such inclusion bodies in all severe forms of β-thal. The mutations that fall into this category produce highly unstable β chain variants as a result of single base substitutions or CD deletions, truncated β chain variants due to premature termination, or elongated β-globins with an altered carboxy-terminal end. A common determinant of these mutations is the production of β chains that are unable to form viable tetramers with the α chains and are rapidly degraded. The continuous degradation of these non-functional β chain variants overloads the capacity of the proteolytic defense mechanism and compromises the proteolysis of the free α chains, thus leading to their accumulation and precipitation at a greater extent than usually observed in β-thal heterozygotes (reviewed in Refs. 31 and 79). These dominant β-thal mutations [except for the CD 121 (GAA->TAA) mutation) occur at very low frequencies, among widely dispersed ethnic groups, and many have been reported as *de novo* events. These data suggest a lack of positive selection by malaria, as the reduced morbidity to the latter disease would be counterbalanced by the severity of the β-thal trait.

Deletional β°-Thal. Twelve deletions, ranging from 44 bp up to 27 kb, that remove part or the entire β-globin gene, have been described so far. In eight, various portions of the 5' β-globin region are removed. Heterozygotes for each of these deletions, except for a recently discovered 27 kb deletion in Southeast Asians, exhibit significantly higher Hb A_2 levels (5.5-9%) than β-thal heterozygotes with point mutations or deletions in other parts of the gene (80). It has been suggested that the loss of the β-globin gene promoter would allow the δ and γ promoters to react more readily with regulatory elements within the LCR (81). In keeping with this hypothesis is the level of Hb F which is elevated in most cases, but to a variable extent (2.5-14%). Again, the only exception is the Southeast Asian β-thal deletion, which apart from β-globin promoter sequences, also removes the 3'HS-1 site, and is associated with slightly elevated Hb A_2 levels (2.7-5.1%) and markedly elevated Hb F levels, comparable to those observed in HPFH (17.6-26.6%) (82).

HB LEPORE AND ANTI-LEPORE SYNDROMES

The Lepore and anti-Lepore Hbs are abnormal Hbs which contain hybrid β-like chains and are associated with a thalassemia-like syndrome. The first Hb Lepore variant, Hb Lepore-Washington (also known as Hb Lepore-Boston), was described in an Italian family by Gerald and Diamond in 1958 (83). Subsequently, other Hb Lepore variants were described; they are found in various populations but mainly in Mediterranean countries such as the former Yugoslavia, Italy, and Greece (reviewed in Ref. 84).

Hb Lepore heterozygotes are usually asymptomatic, without anemia, and with identical hematologic findings as those seen in β-thal trait. The unique difference that distinguishes Hb Lepore is the presence of ~10% of the abnormal Hb. The amount of Hb F may vary but does not usually exceed 5%, and the level of Hb A_2 is normal or slightly decreased (84,85). The clinical features of homozygotes for Hb Lepore or subjects with Hb Lepore-β-thal are variable, and patients with both mild and severe clinical courses have been described (84). Hb analysis in Hb Lepore homozygotes identifies some 25% Hb Lepore, the rest being Hb F. In compound heterozygotes with β-thal, the Hb Lepore level does not usually exceed 15%; Hb F, Hb A_2, and sometimes Hb A form the remainder of the Hb (84).

The Lepore Hbs are composed of two α chains and two δβ fusion chains which have the amino acid sequence of the δ chain at their N-terminus and that of the β chain at the C-terminus (86). Three Lepore chains, which differ in the point of transition from the δ to the β sequence, and an analogous γβ hybrid chain present in Hb Kenya, have been described (87-89). The Lepore Hbs have arisen through unequal homologous recombination events between parts of the δ gene on one chromosome and parts of the β gene on the complementary chromosome (Fig. 1/4) (87). These events would result in two abnormal chromosomes: A 'Lepore chromosome' which has a single hybrid δβ gene instead of the normal δ- and β-globin genes, and an 'anti-Lepore chromosome' which, apart from the normal δ- and β-globin genes, carries an additional βδ fusion gene in the inter-δβ region (Fig. 1/4). The existence of 'anti-Lepore chromosomes' has been confirmed by the characterization of three anti-Lepore Hbs containing fused βδ chains. The above described mechanism is also applicable in the case of Hb Kenya; the 'anti-Kenya chromosome' has not yet been identified.

Gene mapping and sequence analysis of the δβ gene characteristic for Hb Lepore-Boston have identified the crossover point within a 58 bp region at the junction of exon 2 and IVS-II, where the parental δ and β genes are identical (87,88). The crossover region in the Lepore-Baltimore gene lies within a region of 47 nts where the sequences of the δ and β genes are the same, namely between CDs 69 and 84 of exon 2. The breakpoint in the Lepore-Hollandia gene is located within a 40 nt region at the border of exon 1 and IVS-I, downstream from CD 25 of the δ-globin gene (87).

At least three different types of anti-Lepore Hbs have been observed. The fusions occur between CDs β-12 and δ-22 in Hb Miyada, and between CDs β-22 and δ-50 in Hb P-Nilotic and Hb Lincoln Park (84,89). Hb Coventry, initially described as an anti-Lepore Hb, was recently shown to represent an abnormal β-globin chain variant (90).

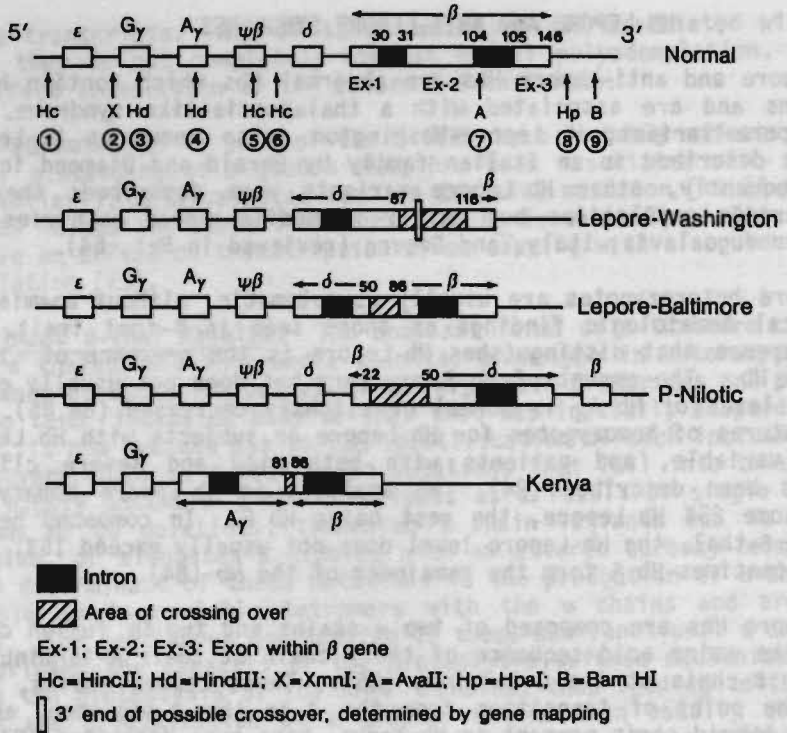


FIG. 1/4. Arrangement of non-α-globin genes on normal chromosome #11 and on chromosomes with the Lepore fusion genes. The β-globin gene and the δβ, βδ , and Yβ fusion genes are drawn out of proportion to allow identification of introns and exons and areas of crossovers. The positions of the polymorphic restriction sites are indicted for the normal chromosome only (85).

The crossover in the Kenya Yβ gene lies within an 18 nt region in exon 2 (CDs 80-87) where the parental $^{A}\gamma$ and β genes are identical (87).

The reasons for the lower levels of Hb Lepore as compared to Hb A are still unknown, and several possibilities such as a relative instability of the δβ-mRNA, or different rates of α/non-α dimer formation, have been proposed (23). Another possibility is a decreased transcription of the Lepore δβ gene due to the presence of a rather inefficient δ-globin gene promoter. However, the expression of the hybrid δβ gene is approximately three times higher than that of the normal δ gene, suggesting that sequences within (such as the β-IVS-II) or flanking the β-globin gene (such as the 3' β-globin gene enhancer) can up-regulate the δ-globin gene promoter. In this respect, it is also interesting to review the data for Hb Kenya, which is characterized by higher levels of the abnormal Hb (up to 20% of the total Hb), and elevated levels of pancellularly distributed fetal Hb (up to 10%). This phenotype can be explained by the deletion of the δ-and β-globin gene promoters, which eliminates competition between γ and δ/β promoter elements for LCR sequences, or by the fact that the deletion places the 3' β-globin gene enhancer in the vicinity of the γ-globin genes. Another possibility is that substitution of the $^{A}\gamma$-IVS-II with the

β-IVS-II can influence the adult silencing of the A_γ-globin gene. An enhancer element has been identified in the β-IVS-II (43), and its elimination may at least in part be responsible for the relatively low level of expression of the anti-Lepore genes, which, despite a functional β-globin promoter, is similar to that of the Lepore δβ genes. Replacement of the β-IVS-II sequence by the δ-IVS-II sequence results in a markedly reduced expression of the β-globin gene in HeLa and MEL cells, while the substitution of the δ-IVS-II with the β-IVS-II is not sufficient to override the low level of expression of the δ-globin gene (91,92). These data suggest that sequences in the β-IVS-II and the 3' β enhancer, together with the β-globin gene promoter, are important for the high level expression of the β-globin gene in normal adult erythroid cells.

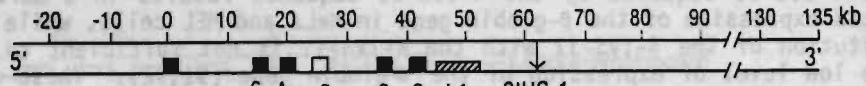
HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN (HPFH)

HPFH refers to a heterogeneous group of disorders that, in the heterozygous state, are associated with increased levels of Hb F and normal RBC indices. In homozygous HPFH the red cells are often somewhat hypochromic and microcytic, reflecting a mild imbalance in the synthesis of α/non-α-globin chains (93). Several classifications of the HPFH disorders are used, based on the molecular defect (deletional or nondeletional), the type of γ-globin chain produced (G_γ, A_γ, or both), and the cellular pattern of Hb F distribution (pancellular HPFH where all the red cells have increased levels of Hb F, and heterocellular HPFH where only a subpopulation of red cells contain Hb F). However, it appears that the last classification is more a reflection of the methodology used, rather than of underlying mechanisms of the HPFH disorders, because certain conditions which give a heterocellular Hb F distribution with an acid elution technique may show a pancellular distribution with a more sensitive immunofluorescent assay (94).

Deletional HPFH. Five different deletions of the β-globin gene cluster have been found in patients with this syndrome, characterized by Hb F levels of 20-30% in the heterozygotes, by a pancellular Hb F distribution, and by the absence of β and δ chain production *in cis* (45). Homozygotes are clinically normal but have an imbalanced α/γ chain synthesis ratio between 1.4 to 2.0, and microcytic, hypochromic red cell indices due to the inability of the γ-globin genes to completely compensate the lack of δ- and β-globin chain production. The increased O_2 affinity of the red cells that contain 100% Hb F is compensated by a mild erythrocytosis with Hb levels of 17 to 19 g/dl (95). Compound heterozygotes of deletional HPFH with Hb S have 30% Hb F and 70% Hb S, while compound heterozygotes with β-thal usually have the hematological features of β-thal trait but with Hb F levels greater than 70%.

The 5' breakpoints of all of these deletions are within the A_γ/β intergenic DNA segment, while the 3' ends are more variable due to the large differences in size between the different HPFH deletions (Fig. 1/5). Several models, not mutually exclusive, have been proposed to explain the increased Hb F levels and the phenotypic differences with the δβ-thalassemias, of which some are characterized by deletions of similar size. These models include lack of competition between the γ-globin genes and

the δ/β-globing gene pair; the removal of the presumed repressor-like sequences between the $^{A}\gamma$- and δ-globin genes; the relocation of enhancer elements from downstream of the 3' breakpoints of these deletions to the vicinity of the γ-globin genes, and extensive changes in chromatin conformation due to the loss of large DNA segments (reviewed in Refs. 45 and 94).



HPFH		Heterozygotes %HbF	Heterozygotes $\%^{G}\gamma$
Black	HPFH-1	24.8±3.1	50.7±4.3
Ghanaian	HPFH-2	24.4±5.0	32.3±4.8
Indian	HPFH-3	22.6±1.0	69.3±3.5
Italian	HPFH-4	17–30	31–39
Italian	HPFH-5	16–20	15

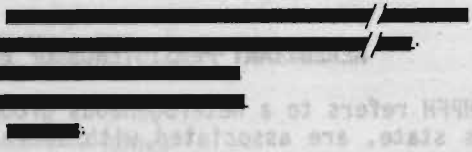


FIG. 1/5. Deletional types of HPFH.

Nondeletional HPFH. The second group of HPFH conditions is characterized by the absence of deletions within the β-globin gene cluster, by a functional β-globin gene on the affected chromosome, and by a mutation (usually a single base substitution) in the promoter of one of the two γ-globin genes. The levels of Hb F in heterozygotes vary and are listed with the particular mutations in Table 1/2. Homozygotes, who have been identified for both the so-called British and Greek types of $^{A}\gamma\beta^{+}$-HPFH, show normal red cell indices and Hb F levels approximately twice those observed in heterozygotes (96,97). Hb analyses of compound heterozygotes for nondeletional HPFH and Hb S have shown that the total output of the γ and β^{A} chains from the HPFH chromosome in such individuals is similar to that of the β^{A} chain in a simple Hb S heterozygote, suggesting a decreased output by the β-globin gene *in cis* to the γ-globin gene mutation (98). This reciprocality in γ and β gene expression could be best explained as a competition between these two genes for *cis*-acting elements such as the LCR (94).

Apart from allowing a further insight into the developmental control of β-globin gene expression, these mutations can also be clinically significant when associated with β-thal. A form of δβ-thal found in Sardinia is characterized by elevated levels of Hb F containing predominantly $^{A}\gamma$-globin chains. The β-globin gene on the affected chromosome carries the common nonsense mutation at CD 39, while the $^{A}\gamma$-globin gene carries the HPFH substitution C->T at position -196 of the $^{A}\gamma$ promoter (99,100).

The presence of specific mutations only in the promoter of the overexpressed γ-globin gene, the absence of these mutations in normal individuals, and the occurrence of identical mutations on different chromosomal backgrounds within different ethnic groups, suggest that these mutations are indeed the cause of the increased level of Hb F. Direct evidence that this is the case was recently obtained by comparing the expression of normal and mutated γ genes in transgenic mice (101).

TABLE 1/2. Nondeletional HPFH Conditions

Type and Racial Group	Mutation in Globin Gene	% Hb F in Heterozygotes
Pancellular $^{G_\gamma}$-HPFH		
Black	G_γ -202 (C->G)	15 -25
Black	G_γ -175 (T->C)	20 -30
Sardinian	G_γ -175 (T->C)	17 -21
Japanese	G_γ -114 (C->T)	11 -14
Pancellular $^{A_\gamma}$-HPFH		
Southern Italian	A_γ -196 (C->T)	12 -16
Chinese	A_γ -196 (C->T)	14 -21
Black	A_γ -175 (T->C)	36 -41
Greek	A_γ -117 (G->A)	10 -20
Sardinian	A_γ -117 (G->A)	12 -16
Black	A_γ -117 (G->A)	11 -16
Black	A_γ -114 to -102 (deleted)	30 -32
Pancellular $^{G_\gamma A_\gamma}$-HPFH		
Chinese	Not known	20 -25
Heterocellular $^{G_\gamma}$-HPFH		
Black (Atlanta)	$G_\gamma G_\gamma$, -158 (C->T)	2.3- 3.8
Heterocellular $^{A_\gamma}$-HPFH		
Black	A_γ -202 (C->T)	1.6- 3.9
British	A_γ -198 (T->C)	3.5-10
Brazilian	A_γ -195 (C->T)	4.5- 7
Heterocellular $^{G_\gamma A_\gamma}$-HPFH		
Swiss	Not known	1 - 4
Black (Seattle)	Normal γ gene promoters	3 - 8
Black (Georgia)	Not known	2.6- 6
$A_{\gamma\delta\beta}$-Thal		
Sardinian	A_γ, -196 (C->T) β, CD 39 (CAG->TAG)	10 -20
$G_\gamma A_{\gamma\delta\beta}$-Thal		
Chinese	Normal γ gene promoters β, -29 (A->G)	22.3

In vitro DNA-protein binding analyses have demonstrated potential binding sites in the γ-globin gene promoters for both ubiquitous and erythroid specific proteins (Fig. 1/3). Most of the nt substitutions associated with nondeletional HPFH conditions are located within or close to the binding sites of these proteins. *In vitro* experiments have demonstrated that these mutations alter the pattern of protein binding which could eventually

result in an increased binding of proteins responsible for activating the γ genes in adult life, or in a decreased binding of repressor molecules, or a combination of these and other mechanisms.

Several mutations have been found in or around a GC-rich area which binds the ubiquitous factor SP1. In the Black $^{G}\gamma\beta^{+}$-HPFH, a single G->C substitution at position -202 is associated with a 30- to 40-fold increase in $^{G}\gamma$-globin chain synthesis (102). DNA binding studies have shown that this mutation increases the binding affinity of the ubiquitous transcription factor SP1 and decreases the binding to the same region of a second undefined factor in a gel retardation assay (103). A mutation at position -198 of the $^{A}\gamma$-globin gene (104) (Table 1/2) also results in an increased SP1 binding in vitro (105), but two other mutations in the $^{A}\gamma$-globin gene promoter (C->T at position -202 and C->T at position -196) seem to decrease SP1 binding (105,106). A uniform explanation for the different effects of the mutations in this region has been proposed recently by Urlich et al (107) who suggested that the sequence between -195 and -218 of the γ-globin promoters has a secondary structure containing a single-stranded region and an intramolecular triplex. The mutations dramatically reduce the stability of this secondary DNA structure, and could therefore affect gene expression by inducing a conformational change that could alter the interaction of (a) critical regulatory molecule(s) with this DNA element.

The T->C mutation at position -175 of both $^{G}\gamma$ and $^{A}\gamma$ genes (108) occurs at a point of partial overlap of the binding domains for the ubiquitous octamer binding protein (OCT-1) and the erythroid specific factor GATA-1. The mutation abolishes the in vitro binding of OCT-1 to its consensus sequence and produces a qualitative change in the GATA-1 binding pattern without a significant quantitative decrease in the binding of this protein (109,110).

In the Greek type of HPFH (Table 1/2), a G->A mutation at -117 is located 2 nts upstream from the distal CCAAT box of the $^{A}\gamma$-globin gene promoter (111). This mutation affects the binding of several different transcription factors, leading to a 4-fold increase in the binding of CP1 (an ubiquitous CCAAT box binding protein), and a 2-fold increase in the binding of CDP, the CCAAT displacement protein (109). On the other hand, the mutation results in an 8-fold reduction of GATA-1 binding to a region downstream of the distal CCAAT box, and also leads to a decrease in binding of another erythroid specific protein (NF-E3) to the distal CCAAT box (112).

In a form of pancellular $^{A}\gamma$-HPFH in Blacks, 13 nts are deleted between positions -114 and -102 of the $^{A}\gamma$ promoter (113). In this condition, the binding of CP1, CDP, and NF-E3 is abolished, while binding of GATA-1 is not significantly affected (112). Binding of CP1 is also abolished in the case of the Japanese $^{G}\gamma$-HPFH associated with the C->T base substitution at position -114 of the distal CCAAT box (114).

Other Inherited Conditions Associated With Increased Hb F in Adult Life. A number of other genetically determined conditions with relatively small increases of Hb F in adult life (<5%) have been described. Some of these conditions have been referred to as the Swiss type of HPFH, which

appears to be a heterogeneous state influenced by multiple genetic factors, both linked and unlinked to the β-globin gene complex on chromosome #11. The Swiss type of HPFH is characterized in heterozygotes by only a slight elevation in Hb F levels of 2-3% (115). Members of several families from the former Yugoslavia had elevated $^{G}\gamma$ and Hb F levels; all had the same haplotype which was apparently associated with the Swiss type of HPFH condition (116). However, this haplotype, which carries the C->T substitution at position -158 of the $^{G}\gamma$-globin gene promoter, was also present in normal individuals with elevated $^{G}\gamma$ and low Hb F levels, suggesting that this Swiss HPFH phenotype results from the interaction of a particular chromosomal background, which also causes high $^{G}\gamma$ values, with other factors not linked to the β-globin gene cluster (116). Using a FACS technique with a monoclonal anti-γ chain antibody, the Hb F production was recently reexamined in normal subjects with and without this C->T mutation (117). A significant correlation of T at -158 ($^{G}\gamma$) with higher levels of F cells was observed, presumably due to slightly higher Hb F/cell in individuals with this mutation.

The -158 (C->T) substitution has also been found to be associated with significantly increased Hb F levels, predominantly of the $^{G}\gamma$ type, in patients with SS, homozygous β-thal, and with Hb S/β-thal (118). Genetic data obtained from the study of β^{S} chromosomes from various world populations have shown a strong association of the C->T at -158 $^{G}\gamma$ with a chromosomal background capable of conferring high Hb F and $^{G}\gamma$ expression upon hematopoietic stress in adult erythroid cells (32,119). A recent finding that this mutation also affects binding of a potential repressor molecule, possibly BP1 (120), suggests a direct effect of the -158 (C->T) substitution in the $^{G}\gamma$ and Hb F expression in SS and β-thal patients.

Another Swiss type of HPFH mutation was recently described in a Czechoslovakian family (121). This A->C mutation at -110 of the $^{G}\gamma$ gene was associated with only a slight increase in Hb F (F_{AD} 2.3%; cation exchange HPLC 0.8%), and with high $^{G}\gamma$ values (95%). The presence of this mutation in two family members who had co-inherited a β° allele on the other chromosome led to a more significant increase in the level of Hb F (cation exchange HPLC 3.1%).

The nature of the HPFH determinants not linked to the β-globin gene cluster is still unknown, but a study of Japanese adults with Swiss HPFH has suggested the existence of an X-linked determinant which affects F cell production (122). More recently, linkage between F cell production and the X chromosome was also observed in SS and AA individuals, and the F cell producing locus was mapped to Xp 22.2 (123). It was suggested that the putative protein product of this locus could either directly affect F cell production or exert its influence through interactions with the β-globin gene cluster (123).

Changes in the arrangement of the γ-globin genes are also frequently associated with a Swiss HPFH phenotype. The Atlanta type of heterocellular HPFH is associated with Hb F levels of 2.5-5% in heterozygotes (in adult AA individuals) and 10-13% (in AS individuals), and $^{G}\gamma$ values of more than 90% (124,125). Instead of the normal $^{G}\gamma/^{A}\gamma$ gene pair, this HPFH chromosome carries two $^{G}\gamma$-globin genes, both of them containing the -158 (C->T) substitution in their promoters (66). Interestingly, individuals with the

same γ-globin gene arrangement but without the $^{G}\gamma$ -158 (C->T) substitution show normal Hb F levels (below 1%) with only slightly elevated $^{G}\gamma$ values (126).

Slightly higher Hb F levels (around 5%, $^{G}\gamma$ around 94%) than those observed in the Atlanta type of HPFH were found in healthy individuals from the former Yugoslavia who had a γ-globin gene triplication. This HPFH chromosome contained two $^{G}\gamma$-globin genes, each with a T at position -158, followed by an $^{A}\gamma$ gene (126). Three other categories of $^{G}\gamma^{G}\gamma^{A}\gamma$ gene triplications have been described and classified according to the Hb F and $^{G}\gamma$ levels in heterozygous adults, and on the presence or absence of the C->T substitution at -158 of the $^{G}\gamma$ promoters (127). The $^{G}\gamma$ genes from both the first and second category lack the -158 (C->T) substitution, but differ in the Hb F (∿0.8 versus ∿3.5%) and the $^{G}\gamma$ (∿35 versus ∿70%) levels. It has been suggested that this difference in γ gene expression is due to a 4 bp deletion at -225 to -222 of the middle γ-globin gene in the chromosome with lower $^{G}\gamma$ and Hb F values. The third category exhibits similar $^{G}\gamma$ and Hb F levels as the second category and contains the -158 (C->T) substitution in the promoter of the 5' $^{G}\gamma$ gene.

A γ-globin gene quadruplication in cis to a β°-thal mutation [CD 8 (-AA)] has been described in a Turkish family (128). This chromosome had a $^{G}\gamma^{G}\gamma^{G}\gamma^{A}\gamma$ arrangement with a C at -158 in the promoters of each of the γ-globin genes. The eight heterozygotes for this chromosome had Hb F levels between 0.5 and 4.2%, with $^{G}\gamma$ values of 87 to 95%. The single homozygote had a thalassemia intermedia phenotype with 99% Hb F, almost completely of the $^{G}\gamma$ type, suggesting a greatly increased output of the $^{G}\gamma$-globin genes from this chromosome under conditions of erythropoietic stress.

A delayed fetal to adult switch has been observed in a Black newborn with five γ-globin genes ($^{G}\gamma^{G}\gamma^{G}\gamma^{G}\gamma^{A}\gamma$) which all lacked the $^{G}\gamma$ -158 (C->T) substitution (129). This baby had Hb F levels of 7.1% at 280 days after birth which declined to 3.3% 60 days later. The $^{G}\gamma$ value during the entire first year was consistently above 80%.

An additional number of γ gene abnormalities and rearrangements have also been identified, such as different γ chain variants, γ-thal, or $^{A}\gamma$ duplications (reviewed in Ref. 130). Further studies of these abnormalities could eventually lead to a better understanding of the mechanisms responsible for the switch in fetal to adult Hb production, and the change in $^{G}\gamma/^{A}\gamma$ ratio that occurs after birth in most infants.

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APPENDIX I

TABLE A/1. Mutations Causing β-Thalassemia

Mutation	Type	Name/Ethnic Group(s)	Reference(s)
A. Transcriptional Mutants			
-101 (C->T)	β^+	Turkish; Bulgarian; Italian	Gonzalez-Redondo et al, 1989c
-92 (C->T)	β^+	Mediterranean	Kazazian, 1990
-90 (C->T)	β^+	Portuguese	Faustino et al, 1992
-88 (C->T)	β^+	American Black; Asian Indian	Orkin et al, 1984a
-88 (C->A)	β^+	Kurdish	Rund et al, 1990
-87 (C->G)	β^+	Mediterranean	Orkin et al, 1982a
-87 (C->T)	β^+	German/Italian	Kulozik et al, 1991
-87 (C->A)	β^+	Yugoslavian; American Black	Dimovski et al, 1991; Coleman et al, 1992
-86 (C->G)	β^+	Lebanese	Kazazian, 1990
-86 (C->A)	β^+	Italian	Meloni et al, 1992
-32 (C->A)	β^+	Taiwanese	Lin et al, 1992
-31 (A->G)	β^+	Japanese	Takihara et al, 1986
-30 (T->A)	β^+	Turkish; Bulgarian	Fei et al, 1988
-30 (T->C)	β^+	Chinese	Cai et al, 1989
-29 (A->G)	β^+	American Black; Chinese	Antonarakis et al, 1984; Huang et al, 1986
-28 (A->C)	β^+	Kurdish	Poncz et al, 1983
-28 (A->G)	β^+	Chinese	Orkin et al, 1983a
+22 (G->A)	β^+	Turkish; Bulgarian Italian	Oner et al, 1991; Cai et al, 1992
+43 to +40 (-AAAC)	β^+	Chinese	Huang et al, 1991
B. RNA Processing Mutants			
1) Splice Junction			
IVS-I-1 (G->A)	β^0	Mediterranean	Orkin et al, 1982a
IVS-I-1 (G->T)	β^0	Asian Indian; Chinese	Kazazian et al, 1984
IVS-II-1 (G->A)	β^0	Mediterranean; Tunisian; American Black	Treisman et al, 1982; Chibani et al, 1988; Wong et al, 1986
IVS-I-2 (T->G)	β^0	Tunisian	Chibani et al, 1988
IVS-I-2 (T->C)	β^0	American Black	Gonzalez-Redondo et al, 1989a
IVS-I-2 (T->A)	β^0	Algerian	Bouhass et al, 1990
IVS-I, 17 nt del (3' end)	β^0	Kuwaiti	Kazazian & Boehm, 1988
IVS-I, 25 nt del (3' end)	β^0	Asian Indian	Orkin et al, 1983b

Mutation	Type	Name/Ethnic Group(s)	Reference(s)
IVS-I-130 (G->C)	β°	Turkish; Japanese	Oner et al, 1990; Yamamoto et al, 1992
IVS-I-130 (G->A)	β°	Egyptian	Deidda et al, 1990
IVS-II-849 (A->G)	β°	American Black	Antonarakis et al, 1984; Atweh et al, 1985
IVS-II-849 (A->C)	β°	American Black	Padanilam & Huisman, 1986
IVS-II-850 (G->C)	β°	Yugoslavian	Jankovic et al, 1992
IVS-II-850 (-G)	β°	Italian	Rosatelli et al, 1992b
IVS-II-850 (G->A)	β°	Irish	Çuruk et al, personal communication (1993)
2) Consensus Sequence and Splicing			
IVS-I-5 (G->C)	β^{+}	Asian Indian; Chinese; Melanesian	Treisman et al, 1983; Kazazian et al, 1984; Cheng et al, 1984
IVS-I-5 (G->T)	β^{+}	Mediterranean; American Black	Atweh et al, 1987; Gonzalez-Redondo et al, 1991
IVS-I-5 (G->A)	β^{+}	Algerian; Mediterranean	Lapoumeroulie et al, 1986
IVS-I-6 (T->C)	β^{+}	Mediterranean	Orkin et al, 1982a
IVS-I, -1 (G->C) (CD 30)	β^{+}	Tunisian; American Black;	Chibani et al, 1988; Gonzalez-Redondo et al, 1989b
IVS-I, -1 (G->A) (CD 30)	?	Bulgarian	Kalaydjieva et al, 1989
IVS-I, -3 (C->T) (CD 29)	?	Lebanese	Chehab et al, 1987
IVS-I-128 (T->G)	β^{+}	Saudi Arabian	Wong et al, 1989
IVS-II-837 (T->G)	?	Asian Indian	Varawalla et al, 1991
IVS-II-843 (T->G)	β^{+}	Algerian	Beldjord et al, 1988
IVS-II-844 (C->G)	β^{+}	Italian	Murru et al, 1991
IVS-II-848 (C->A)	β^{+}	American Black; Egyptian; Iranian	Gonzalez-Redondo et al, 1988; Wong et al, 1989
IVS-II-848 (C->G)	β^{+}	Japanese	Hattori et al, 1992
3) IVS Changes			
IVS-I-110 (G->A)	β^{+}	Mediterranean	Spritz et al, 1981; Westaway & Williamson, 1981
IVS-I-116 (T->G)	β°	Mediterranean	Metherall et al, 1986
IVS-II-4,5 (-AG)	β^{+}	Portuguese	Faustino, personal communication (1992)
IVS-II-654 (C->T)	β^{+}	Chinese	Cheng et al, 1984
IVS-II-705 (T->C)	β^{+}	Mediterranean	Dobkin et al, 1983
IVS-II-745 (C->G)	β^{+}	Mediterranean	Orkin et al, 1982a
4) Cryptic Splice Activation			
CD 19 (A->G) (Asn->Ser)	β^{+}	Malaysian (Hb Malay)	Yang et al, 1989

Mutation	Type	Name/Ethnic Group(s)	Reference(s)
CD 24 (T->A)	β^+	American Black; Japanese	Goldsmith et al, 1983; Hattori et al, 1989
CD 26 (G->A) (Glu->Lys)	β^+	Southeast Asian (Hb E)	Orkin et al, 1982b
CD 27 (G->T) (Ala->Ser)	β^+	Mediterranean (Hb Knossos)	Orkin et al, 1984b
C. Nonsense and Frameshift			
1) Nonsense			
CD 15 (TGG->TAG)	β^0	Asian Indian	Kazazian et al, 1984
CD 15 (TGG->TGA)	β^0	Portuguese	Ribeiro et al, 1992
CD 17 (A->T)	β^0	Chinese	Chang & Kan, 1980
CD 22 (G->T)	β^0	Reunion Islander	Goossens, personal communication (1990)
CD 26 (G->T)	β^0	Thai	Fucharoen et al, 1990c
CD 35 (C->A)	β^0	Thai	Fucharoen et al, 1989
CD 37 (G->A)	β^0	Saudi Arabian	Boehm et al, 1986
CD 39 (C->T)	β^0	Mediterranean	Trecartin et al, 1981
CD 43 (G->T)	β^0	Chinese	Atweh et al, 1988
CD 61 (A->T)	β^0	Black	Gonzalez-Redondo et al, 1988
CD 90 (G->T)	β^0	Japanese	Fucharoen et al, 1990a; Hattori et al, 1992
CD 112 (T->A)	β^0	Slovakian	Divoky et al, 1992
CD 121 (G->T)	β^0	Polish; French-Swiss; Japanese	Kazazian et al, 1986; Fei et al, 1989; Yamamoto et al, 1992
CD 127 (C->T)	β^0	English	Hall et al, 1991
2) Frameshift			
CD 1 (-G)	β^0	Mediterranean	Rosatelli et al, 1992a
CD 5 (-CT)	β^0	Mediterranean	Kollia et al, 1989
CD 6 (-A)	β^0	Mediterranean; American Black	Kazazian et al, 1983; Gonzalez-Redondo et al, 1988
CD 8 (-AA)	β^0	Mediterranean	Orkin & Goff, 1981
CDs 8/9 (+G)	β^0	Asian Indian	Kazazian et al, 1984
CDs 9/10 (+C)	β^0	Turkish	Aulehla-Scholtz et al, 1990
CD 11 (-T)	β^0	Mexican	Economou et al, 1991
CDs 14/15 (+G)	β^0	Chinese	Chan et al, 1988
CD 15 (-T)	β^0	Asian Indian; Thai	Baysal/Fucharoen, personal communications (1992)
CD 16 (-C)	β^0	Asian Indian	Kazazian et al, 1984
CD 22 (-AAGTTGG)	β^0	Turkish	Özçelik et al, 1993
CD 24 (-G,+CAC)	β^0	Egyptian	Deidda et al, 1991
CDs 25/26 (+T)	β^0	Tunisian	Fattoum et al, 1991

Mutation	Type	Name/Ethnic Group(s)	Reference(s)
CDs 27/28 (+C)	β°	Chinese	Lin et al, 1991
CD 35 (-C)	β°	Malaysian	Yang et al, 1989
CDs 36/37 (-T)	β°	Iranian; Kurdish	Rund et al, 1990
CDs 37/38/39 (-GACCCAG)	β°	Turkish	Schnee et al, 1989
CDs 38/39 (-C)	β°	Czech	Indrak et al, 1991
CD 41 (-C)	β°	Thai	Fucharoen et al, 1991
CDs 41/42 (-TTCT)	β°	Chinese	Kimura et al, 1983
CD 44 (-C)	β°	Kurdish	Kinniburgh et al, 1982
CD 47 (+A)	β°	Surinamese	Codrington et al, 1991
CD 51 (-C)	β°	Hungarian	Ringelhann et al, 1993
CD 54 (+G)	β°	Japanese	Fucharoen et al, 1990a
CD 64 (-G)	β°	Swiss	Chehab et al, 1989
CD 71 (+T)	β°	Chinese	Chan et al, 1989
CDs 71/72 (+A)	β°	Chinese	Cheng et al, 1984
CDs 74/75 (-C)	β°	Turkish	Başak et al, 1992
CD 76 (-C)	β°	Italian	Maggio et al, 1988
CDs 82/83 (-G)	β°	Azerbaijani; Czech	Schwartz et al, 1989; Indrak et al, 1992
CD 88 (+T)	β°	Asian Indian	Varawalla et al, 1991
CD 95 (+A)	β°	Thai	Fucharoen, personal communication (1992)
CDs 106/107 (+G)	β°	American Black	Wong et al, 1987
CDs 109/110 (-G)	β°	Lithuanian	Kazazian, 1990
CD 124 (-A)	β°	Russian	Çuruk et al, personal communication (1993)
D. RNA Cleavage and Polyadenylation			
AATAAA->AACAAA	β^{+}	American Black	Orkin et al, 1985
AATTAAA->AATTAAG	β^{+}	Kurdish	Kazazian & Boehm, 1988
AATAAT->AATGAT	β^{+}	Mediterranean	Jankovic et al, 1990
AATTAAA->AATTAGA	β^{+}	Malaysian	Jankovic et al, 1990
AATAAA->A (-ATAAA)	β^{+}	Arabian	Rund et al, 1991
AATAAA->AAAA (-AT)	β^{+}	French	Goossens, personal communication (1991)
E. Cap Site			
+1 (A->C)	β^{+}	Asian Indian	Wong et al, 1987
3'UTR (+1565 to +1577 bp, -GCATCTGGATTCT)	?	Turkish	Başak et al, 1993
3'UTR (Term. CD +6, C->G)	β^{+}	Greek	Jankovic et al, 1991

Mutation	Type	Name/Ethnic Group(s)	Reference(s)
F. Initiation Codon			
ATG->ACG	β'	Yugoslavian	Jankovic et al, 1990
ATG->AGG	β°	Chinese; Japanese; Korean	Kazazian, 1990; Koo et al, 1992
ATG->GTG	β°	Japanese	Hattori et al, 1991
G. Hyperunstable Globins			
CD 60 (GTG->GAG) (Val->Glu)		Italian (Hb Cagliari)	Podda et al, 1991
CD 94 (+TG) Frameshift; extended β chain (Hb Agnana)		Italian	Ristaldi et al, 1989
CD 110 (T->C)		Japanese (Hb Showa-Yakushiji)	Kobayashi et al, 1987
CD 114 (-CT,+G) Frameshift; extended β chain (Hb Geneva)		French-Swiss	Beris et al, 1988
CD 123 (-A) Frameshift; extended β chain (Hb Makabe)		Japanese	Fucharoen et al, 1990b
CDs 123-125 (-ACCCCACC)	β°	Thai	Fucharoen, personal communication (1992)
CD 126 (-T) Frameshift; extended β chain (Hb Vercelli)		Italian	Murru et al, 1991
CD 127 (Gln->Pro)		British (Hb Houston)	Kazazian, 1990
CD 127 (CAG->CGG)		French	Goossens, personal communication (1991)
CDs 127/128 (-AGG)		Japanese (Hb Gunma)	Fucharoen et al, 1990; Hattori et al, 1989
CDs 128/129 (-4,+5) (-GCTG,+CCACA) and CDs 132-135 (-11) (-AAAGTGGTGGC)	β°	Irish	Thein et al, 1990
CDs 134-137 (-10,+4) -(G)TGGCTGGTGT(G) +(G)GCAG(G)	β°	Portuguese	Oner et al, 1991
H. Thalassemic Hemoglobins			
CD 28 (CTG->CGG)	β°	Hb Chesterfield	Thein et al, 1991
CD 30 (G->C)	β°	Hb Monroe	Gonzalez-Redondo et al, 1989b
CDs 32/34 (-GGT)	β°	Hb Korea	Park et al, 1991
CD 69 (GGT->AGT)	β+	Hb City of Hope	Kutlar et al, 1989
CD 114 (T->C)	?	Hb Brescia	Murru, personal communication (1992)
CD 115 (C->A)	β°	Czech [Hb Hradec Kralove (Hb HK)]	Divoky et al, 1993

Mutation	Type	Name/Ethnic Group(s)	Reference(s)
CDs 125/126 (+CCA)	β°	Russian	Çuruk et al, personal communication (1993)
CD 126 (GTG->GGG)	β°	Hb Neapolis	Pagano et al, 1991

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TABLE A/2. Deletions Causing β°-Thalassemia

Deletion	Ethnic Group(s)	Reference(s)
12.6 kb	Dutch[a]	Gilman et al, 1984; Gilman, 1987
~27 kb	Southeast Asian[b]	Dimovski et al, 1993a
>200 kb	Irish	Fortina et al, 1991
619 bp	Asian Indian[c]	Orkin et al, 1979; Flavell et al, 1979; Orkin et al, 1980
1393 bp	Black[d]; English; Anglo-Saxon-Dutch	Padanilam et al, 1984; Anand et al, 1988; Gonzalez-Redondo et al, 1989; Thein et al, 1989
1605 bp (-985 to +621)	Yugoslavian	Dimovski et al, 1993b
4237 bp	Czech[e]	Popovitch et al, 1986
290 bp	Turkish[f]; Jordanian	Diaz-Chico et al, 1987; Gonzalez-Redondo et al, 1989; Aulehla-Scholtz et al, 1989
44 nt	Greek[g]	Gonzalez-Redondo et al, 1989
~3400 bp	Thai[h]	Sanguansermsri et al, 1990
532 bp	American Black[i]	Waye et al, 1991

[a] Removes the entire β gene and its 5' and 3' flanking regions. The δ gene is intact. Extends from 3-4 kb 3' to the δ gene downstream to 6-7 kb 3' to the β gene.

[b] Extends from 1.5-2.2 kb 5' to the β gene to 24 kb 3' to the β gene; the deletion removes the 3'HS-1 regulatory sequence.

[c] Includes one-third of the IVS-II downstream to 209 bp of the 3' flanking DNA.

[d] Removes the promoter and a 5' segment of the β gene (from -484 to the Cap site to IVS-II-415).

[e] Extends from 3.3 kb 5' to the Cap site to the middle of βIVS-II.

[f] Extends from nts -123, -124 or -125 to nts 23, 24 or 25 of the βIVS-I.

[g] From codon 24 downstream to IVS-I-26.

[h] Removes the entire β gene; the 5' breakpoint is between nts -810 and -128, and the 3' breakpoint between Ava II and Xmn I sites downstream to the β gene (1-1.5 kb downstream).

[i] Removes the 5' segment of the β gene.

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TABLE A/3. Mutations Causing δ-Thalassemia

Mutation	Type	Ethnic Group(s)	Reference(s)
A. Transcriptional Mutants			
-77 (T->C)	δ°	Japanese	Nakamura et al, 1987
B. Thalassemic Hemoglobins			
CD 98 (G->T)	δ^{+}	American Black	Codrington et al, 1989
CD 91 (+T)	δ°	Belgian	Losekoot et al, 1989
CD 116 (C->T) (Hb A_2-Troodos)	δ^{+}	Greek Cypriot	Trifillis et al, 1991
CD 141 TT->C) (Hb A_2-Pelenderi)	δ^{+}	Greek Cypriot	Trifillis et al, 1991
C. RNA Processing Mutants			
CD 30 (G->C)	δ°	Southern Italian	Pirastu et al, 1990
IVS-I (T->C)	δ°	Italian	Moi et al, 1988
IVS-II (AG->GG)	δ°	Greek Cypriot	Trifillis et al, 1991
D. Cryptic Splice Site			
CD 27 (G->T) (Hb A_2-Yialousa)	δ^{+}	Greek Cypriot; Sardinian	Trifillis et al, 1991; Pirastu et al, 1990
E. Frameshift			
CD 59 (-A)	δ°	Egyptian	Olds et al, 1991
F. Poly A Tail			
Poly A + 69 bp (G->A)	δ^{+}	?	Pirastu et al, 1990

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CHAPTER 2

MATERIALS AND METHODS

Nakamura, T., Takihara, Y., Ohta, Y., Fujita, S., Takagi, Y., and Fukumaki, Y.: A β-globin gene derived from patients with homozygous β^0-thalassemia functions normally on transient expression in heterologous cells. Blood, 70:809, 1987.

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CHAPTER 2

1. **Materials and Methods.** This chapter describes the hematological, Hb, and DNA analyses that were used for the detection and characterization of the β-thal patients participating in this study. The basic principles of the methodologies used are described, while the particular applications are given in more detail in Chapters 3, 4, 5, and 6.

2. **Patients.** A total of 197 β-thal homozygotes, 214 β-thal heterozygotes, and 10 compound heterozygotes for Hb Lepore and β-thal were studied. These patients originated from various Mediterranean countries (Table 2/1). The blood samples of the patients from the countries of the former Yugoslavia and Bulgaria were collected by Professor Dr. G.D. Efremov, Research Center for New Technologies (RCNT), Macedonian Academy of Sciences and Arts (MASA), Skopje, Republic of Macedonia. These patients were treated at the medical centers in Skopje (Professor Dr. N. Zisovski), Gevgelija (Dr. G.T. Petkov), Veles (Dr. N. Stojanovski), Strumica (Dr. N. Nikolov), all in Macedonia; Zagreb, Croatia (Dr. D. Juricic), Belgrade, Serbia (Professor Dr. E. Stojmirovic), and Stara Zagora, Bulgaria (Professor Dr. G.H. Petkov). The blood samples from Turkey were collected in medical centers in Adana, Ankara, Northern Cyprus, and Istanbul (Professor M. Aksoy, Professor Ç. Altay, and Drs. A. Gurgey and Y. Kilinç), while the samples from Portugal and Sicily were sent by Dr. M.L.S. Ribeiro (Coimbra) and Professor Dr. G. Schiliro (Catania), respectively.

TABLE 2/1. Types of Thalassemia and Country of Origin of Patients Studied

Condition	Former Yugoslavia	Bulgaria	Turkey	Portugal	Sicily	Total
Homozygous β^{Th}	46	64	55	13	8	186
Heterozygous β^{Th}	130	84				214
Hb Lepore/β^{Th}	10					10
TOTAL	186	148	55	13	8	410

The clinical status of the patients was variable in terms of severity and transfusion requirements; these data were provided by the physicians from the local institutions. Hematological and Hb data for all patients residing in the former Yugoslavia and Bulgaria were obtained at the RCNT, MASA, Skopje, Republic of Macedonia. Most of the patients were studied on several occasions, and some were also reevaluated at the laboratory in Augusta, GA, USA to provide standardized data for comparison with patients originating from other Mediterranean countries. The hematological and Hb data of the patients from Turkey, Portugal, and Sicily were obtained at the laboratory in Augusta, GA, USA.

3. **Blood Collection/Shipment.** Blood samples were collected in vacutainers (Becton Dickinson Vacutainer Systems, New Jersey, N.J., USA) with EDTA

as anticoagulant. Samples from patients on transfusion regimens were collected prior to the blood transfusion. All samples were stored at 4°C until the time of shipment and were brought by air by collaborators from participating institutions, or were shipped on ice by fast air mail service to Augusta, GA, USA, where they arrived within 5 days. Informed consent was obtained under the regulations of the local institutions.

4. Hb and Hematological Analyses. The procedures for hematological and Hb analysis are described below in the order routinely used in the laboratories in Augusta, GA, USA, and RCNT, MASA, Skopje, Macedonia.

4/a Hematological Data. Hematological values were obtained with a fully automated cell counter (Sysmex CC-620, Toa Medical Electronics Co., Kobe, Japan). The RBC indices, MCV (in fl) and MCH (in pg) were calculated from the values of the total Hb concentration (g/dl), the PCV (in l/l) and RBC count (x 10^{12}/l). The MCV and MCH values are especially useful parameters in the diagnosis of heterozygous β-thal. MCV values below 80 fl and MCH values below 27 pg are indicative of the presence of a β-thal carrier state. It is noteworthy that the MCV value can be increased by prolonged storage of the RBCs.

4/b Hb Analyses. Plasma was removed by three washes with isotonic saline and the RBCs were hemolyzed in 1.5 volumes of water for 15 minutes at room temperature with occasional stirring. The hemolysates were centrifuged for 20 minutes at 4°C (4,000 rpm) and the supernatant were used for subsequent electrophoretic and chromatographic analyses.

i. IEF. Initial Hb identification was performed by IEF analysis (1) on commercially available agarose gels with buffers supplied by the manufacturer (Isolab, Inc., Akron, OH, USA). Approximately 10 µl of each hemolysate were applied on a gel, followed by 2 hours of electrophoresis at constant power (30 W) using an LKB Multiphor II Horizontal Unit (Pharmacia-LKB, Uppsala, Sweden). The gels were fixed for about 10 minutes in 10% trichloroacetic acid and the bands were visualized with a heme specific dye (O-dianisidine) and H_2O_2. Bands were identified by comparing the distances of migration to control samples containing the known Hb types of A, F, S, C, and Bart's.

ii. Fetal Hb Quantitation. Two procedures were used for quantitation of Hb F. The alkali denaturation method (2-4) was used in earlier studies, and was later replaced by a recently developed HPLC procedure (5,6).

iii. Alkali Denaturation Method. The alkali denaturation method, described by Betke et al (2), is based on the higher resistance of Hb F to denaturation in alkali. The modified procedure used in this study involved the conversion of Hb into its cyanmet derivative before denaturation which prevented false-positives, particularly in patients with increased concentration of the alkali resistant CO-Hb, such as heavy smokers. The method offered a reasonable accuracy for the Hb F concentrations between 5-20%. The disadvantage was that the Hb F levels below 5% were determined too high, and the Hb F levels above 20% were determined too low (6).

iv. Cation Exchange HPLC. The Hb F quantitations were performed on a polyCAT A cation exchange column (4.6 x 200 mm, with a particle size of 5 µm; PolyLC, Columbia, MD, USA) using a Waters HPLC system (Waters Chromatography Division, Milford, MA, USA). The following developers were used: Developer A contains 35 mM Bis-Tris, 3 mM ammonium acetate, 1.5 mM KCN, pH 6.47; Developer B contains 35 mM Bis-Tris, 1.5 mM KCN, 150 mM Na-acetate, 16.85 mM ammonium acetate, pH 7.0. Ten to 15 µl of hemolysate (50-200 µg of Hb) were analyzed. The chromatograms were developed with a gradient of 25 to 85% Developer B in 85 minutes with a flow-rate of 0.8 ml/min. The absorbances of the effluents were continuously recorded at a wavelength of 415 nm. The different Hb types were separated in the following order: F, A, and A_2 (Fig. 2/1).

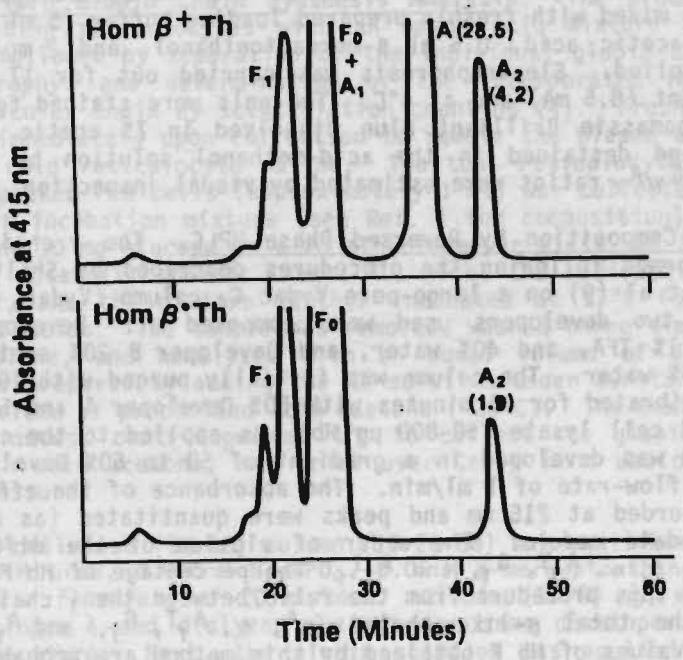

FIG. 2/1. Separation of major and minor Hb components in red cell lysates of one patient with homozygous β^+-thal, and a second with homozygous β°-thal. The numbers between parentheses are pecentages.

v. Analysis of Hb F Composition. Two methods were used for the determination of the γ chain composition of Hb F (G_γ, $A_\gamma I$, and $A_\gamma T$). The visual inspection of the PAGE gel (7) allowed a semi-quantitative analysis of the G_γ/A_γ ratio; it did not discriminate between the $A_\gamma T$ and $A_\gamma I$ variants. A more accurate analysis was obtained by reversed phase HPLC (8,9). Hemolysates that contained less than 20% of Hb F (as estimated by IEF) were enriched by preparative anion exchange DEAE-cellulose chromatography prior to the Hb F composition analysis (10). This was performed on a 30 x 1.5 cm column filled with DEAE-Sephadex equilibrated with 0.2 M glycine, 15 mM KCN buffer, pH 7.5. Hemolysates containing approximately 20-60 mg Hb were applied to the column and the chromatograms were developed with

a 0.2 M glycine, 0.01% KCN, 0.02 M NaCl developer for 15 hours at a flow-rate of 15 ml/hour. The Hb F fractions which were located near the top of the column were collected, poured into a microcolumn (7 x 0.5 cm) and eluted with a stripping buffer (0.01% KCN, 0.2 M NaCl, 0.2 M glycine).

vi. Hb F Composition by PAGE Analysis. The Hb chains were separated on 12% polyacrylamide gels containing 6 M urea and 2% Triton-100 in 5% acetic acid, as described by Alter et al (7). Briefly, 60 minute pre-electrophoresis was performed in 5% acetic acid at a constant voltage (200 V) with the anode at the top. A second pre-electrophoresis was performed with a fresh buffer containing 1 M cysteamine (Aldrich Chemical Co. Inc., Milwaukee, WI, USA) for 45-60 minutes at 150 V. Prior to loading, the cysteamine was removed by flushing the wells with 5% acetic acid. About 5 to 10 µg of Hb mixed with freshly prepared loading buffer (5 ml 8 M urea, 0.5 ml glacial acetic acid, 0.5 ml β-mercaptoethanol, and 2 mg of pyronine Y) were applied. Electrophoresis was carried out for 17 hours at a constant current (8.5 mA) and at 4°C. The gels were stained for 30 minutes in 0.5% Coomassie Brilliant Blue dissolved in 7% acetic acid with 30% methanol, and destained in the acid-methanol solution by overnight diffusion. The G_γ/A_γ ratios were estimated by visual inspection.

vii. Hb F Composition by Reversed Phase HPLC. The γ chain separations were performed following the procedures described by Shelton et al (8) and Kutlar et al (9) on a large-pore Vydac C_4 column (Vydac, Hesperia, CA, USA). The two developers used were composed of: Developer A 60% acetonitrile, 0.1% TFA, and 40% water, and Developer B 20% acetonitrile, 0.1% TFA, and 80% water. The column was initially purged with 100% Developer A and equilibrated for 10 minutes with 50% Developer A and 50% Developer B. The red cell lysate (50-800 µg Hb) was applied to the column and the chromatogram was developed in a gradient of 50 to 60% Developer A in 70 minutes at a flow-rate of 1 ml/min. The absorbance of the effluent was continuously recorded at 215 nm and peaks were quantitated (as area percentages) by a data module. The order of elution of the different Hb chains was: β^A, δ, α, A_γ^T, G_γ, and A_γ^I. The percentage of Hb F can also be determined by this procedure from the ratio between the γ chains (A_γ^T + G_γ + A_γ^I) and the total β-like chains (β^A, δ, A_γ^T, G_γ, and A_γ^I). The slightly higher values of Hb F obtained by this method are probably due to some contamination of G_γ with α chains (9). Fig. 2/2 gives some examples.

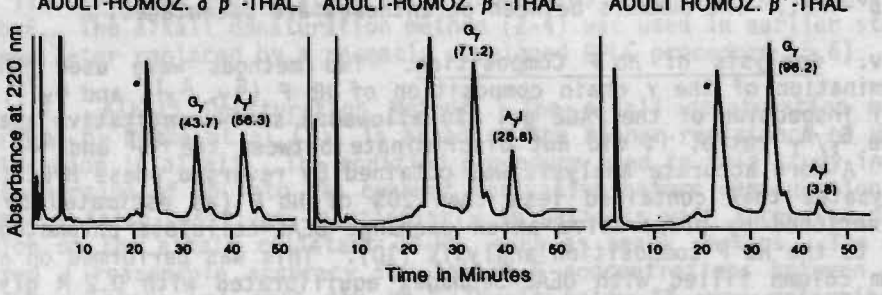

FIG. 2/2. Separation of globin chains by Vydac C_4 reversed phase HPLC. Red cell lysates from three adults with different types of homozygous (δβ)°-or β°-thal were analyzed.

5. **Hb A_2 Quantitation.** The Hb A_2 levels were usually determined with the cation exchange HPLC procedure described above. However, in some instances, Hb A_2 was quantitated by anion exchange chromatography using the Quick-Sep Hb A_2 Test System (Isolab). This procedure allowed the differential elution of Hb A_2 and Hb A under specific concentrations of sodium chloride at constant pH. Five to 10 µl of hemolysate were applied to a column and the Hb A_2 was eluted with Developer A (0.2 M glycine, 0.01% KCN). The remaining Hbs were eluted with Developer B (0.2 M glycine, 0.01% KCN, 0.2 M NaCl). The Hb A_2 concentrations were determined from the ratio of the absorbance values of both fractions at a wavelength of 415 nm.

6. **In Vitro Globin Chain Synthesis Analysis.** The procedure consisted of incubating reticulocytes with an amino acid mixture containing [^{3}H] leucine, followed by separation of the individual globin chains by column chromatography, and determination of the incorporated radioactivity in each particular chain by scintillation counting (4). Fresh blood was centrifuged immediately upon collection to remove the plasma and washed three times in cold reticulocyte saline. The top, reticulocyte rich, fraction from the packed red cells (approximately 1 ml) was collected and incubated in 5 ml of incubation mixture (see Ref. 4 for composition), 0.1 ml transferrin, and 10 mg glucose in a 25 ml Erlenmeyer flask, at 37°C for 10 minutes in a metabolic shaker. Next, 0.1 ml of the radioactive [^{3}H] leucine was added, and the mixture further incubated at 37°C for 2 hours with gentle agitation. The sample was removed, washed three times with reticulocyte saline, and hemolyzed with an equal volume of distilled water. Globin was prepared by adding the Hb solution under constant stirring to a 20-fold volume of prechilled acid acetone (-20°C). The mixture was stirred for 30 minutes, centrifuged at 4°C to collect the globin, washed three times with cold acetone, and dried over $CaCl_2$ in a desiccator at atmospheric pressure.

A CM-cellulose (CM-52) column (18-20 cm) was equilibrated with Developer A (8 M urea, 0.005 M Na_2HPO_4, 0.05 M β-mercaptoethanol, pH 6.5) for 1 hour at a flow-rate of 20 ml/hour. Globin (50-60 mg) was dissolved in 2 ml Developer A and dialyzed against the same developer for 2 hours at room temperature. Next, the globin solution was applied to the column, allowed to drain under gravity, and elution with Developer A was performed for 3 hours. The chromatography was continued with a linear Na^+ gradient obtained by mixing 300 ml of Developer A with 300 ml Developer B (8 M urea, 0.04 M Na_2HPO_4, 0.05 M β-mercaptoethanol, pH 6.5), both in open cylinders. The procedure was carried out at room temperature, at a constant flow-rate of 20 ml/hour. Five ml fractions were collected with an automated fraction collector and analyzed at 280 nm. The radioactivity of each isolated fraction was determined in a liquid scintillation counter.

7. **DNA Analyses**

i. Isolation of Genomic DNA From WBCs. High MW genomic DNA was isolated from WBCs by the method of Poncz et al (11). About 10 ml of blood were washed three times with 30-40 ml of 1 x reticulocyte saline (140 mM

NaCl, 4 mM KCl, 6.8 mM $MgCl_2$) by pelleting the cells with centrifugation at 2,500 g for 10 minutes at 4°C. The supernatant was removed after each centrifugation by aspiration. Next, the RBCs were hemolyzed with 30 ml freshly prepared lysing solution [131 mM NH_4Cl, 0.9 mM $(NH_4)_2CO_3$, pH 6.5] for 20 minutes at room temperature with gentle mixing. The WBCs were collected by centrifugation at 2,500 g for 10 minutes at 4°C and the supernatant (hemolysate) was carefully removed and stored at -20°C for subsequent Hb analysis. The lysis was repeated once more to remove the remaining RBCs, and the pellet of WBCs was resuspended in 10 ml of STE buffer (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4, 1 M EDTA). The WBCs were dispersed thoroughly by vigorous shaking and 0.5 ml of a 10% SDS solution and 0.1 ml of proteinase K (10 mg/ml in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) were added. The sample was incubated overnight at 37°C without shaking; the mixture became viscous upon incubation. Proteins were removed by several phenol/chloroform/isoamyl extractions. An equal volume of redistilled phenol saturated with 200 mM Tris-HCl, pH 8.0, and containing 0.1% hydroxyquinoline as an anti-oxidant, was added; the mixture was shaken gently for 10 minutes at room temperature and cooled on ice for 10 minutes. After 10 minutes of centrifugation at 2,500 g and at 4°C, the aqueous layer was transferred with a large bore pipet to a second tube and the phenol extraction was repeated. An equal volume of chloroform/isoamyl (49:1) was added, and the mixture was again shaken gently at room temperature for 10 minutes, cooled on ice for 10 minutes, and centrifuged for 10 minutes at 4°C. Following centrifugation, the aqueous phase containing the DNA was transferred to a clean tube. The DNA was precipitated with 3-4 volumes of ice-cold absolute ethanol by gentle mixing. The precipitate was transferred to a 1.5 ml Eppendorf tube using a sterile Pasteur pipet, and washed three times with 70% ethanol to remove any residual phenol or salt. After drying under vacuum, the DNA pellet was dissolved overnight at 37°C in 1 ml of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The DNA concentration was determined by measuring the OD at a wavelength of 260 nm. An OD_{260} of 1.0 corresponds to approximately 50 µg/ml DNA. The DNA was stored at 4°C until further use.

ii. Gene Mapping. Gene mapping analyses were performed to determine the β-globin gene haplotypes, the number of α-globin genes, and the type of Hb Lepore, using procedures described in Refs. 12, 13, and 14, with slight modifications as described below.

iii. Restriction Enzyme Digestion, Gel Electrophoresis, and Southern Transfer. Five µg of genomic DNA were digested with the appropriate restriction enzyme for 6 hours under conditions recommended by the manufacturer (temperature and buffer). The resulting DNA fragments were separated on a 0.8% agarose gel prepared with 1 x TEA buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.3). The same buffer was used for gel electrophoresis. Electrophoresis was performed overnight, at room temperature, in a horizontal electrophoresis chamber (Bio-Rad DNA Sub Cell; Bio-Rad, Richmond, CA, USA) at a constant voltage of 25 to 30 volts. Hind III digested λ-DNA was always included as a size marker. Following electrophoresis, the DNA was stained for 5-10 minutes with ethidium bromide (0.5 mg/ml) and the gel was photographed under long wavelength UV light. The DNA was then depurinated, to facilitate subsequent transfer, by soaking the gel for

30 minutes in 0.1 N HCl. After one wash with distilled water, the gel was soaked for 60 minutes in denaturation solution (0.5 M NaOH, 1.5 M NaCl), and next washed for at least 1 hour in a neutralizing solution (3 M NaCl, 1.5 M Tris-HCl, pH 7.0). The depurination, denaturation, and neutralization steps were performed at room temperature with gentle agitation on a rocking platform. The DNA was then transferred to either a nitrocellulose (Trans-Blot Transfer Membrane; Bio-Rad) or nylon membrane (Hybond C Membrane; Amersham International, Amersham, Bucks, England) using 20 x SSC as a transfer solution for the Southern blotting. The transfer was performed for 20-24 hours. The DNA was fixed by baking the membrane for 2-3 hours in a vacuum oven at 70-80°C. Membranes were stored at 4°C until use.

iv. Prehybridization. The membrane was wetted with a few ml of 6 x SSC plus Denhardt's solution (0.5% SDS, 0.02% BSA 0.02% Ficoll 400, 0.02% PVP, 0.09 M Na-citrate, pH 7.0, and 0.9 M NaCl), and prehybridized overnight at 65°C in a sealed plastic bag with 15-20 ml of 1 x Flavel Modified Solution (3 X SSC, 0.1% SDS, 0.2% BSA, 0.2% PVP, 0.02 M phosphate buffer, pH 6.0) containing denatured, sonicated salmon sperm DNA at 50-100 µg/ml.

v. Radioactive Labeling of Probes. Probes were labeled to high specific activity using the random-primer oligolabeling procedure (15) as recommended by the manufacturer (Oligolabeling Kit, Pharmacia-LKB). An average of 50-100 ng of probe was used per labeling reaction. The probe was denatured by boiling for 10 minutes in a water-bath and immediately placed on ice to prevent reannealing. After a brief centrifugation to spin down the condensation inside the tube, a reaction mixture of a buffered aqueous solution of dATP, dCTP, dGTP, dTTP, and random hexanucleotides was added together with 50 µCi of α^{32}P-dCTP (ICN Biochemicals, Inc., Irvine, CA, USA) and 10 units of Klenow fragment, up to a total volume of 50 µl. The reaction was incubated for 3 hours at 37°C, and the labeled probe was purified from unincorporated nts by gel filtration through a G-50 Sephadex column with TE elution buffer. The total radioactivity was calculated from the counts obtained for 5 µl of the effluent using a liquid scintillation counter. The labeled probes were stored at -20°C.

vi. Hybridization. An average of 10×10^6 cpm of an α^{32}P-labeled probe was used per 10 ml of hybridization solution. The probes were denatured for 10 minutes in a boiling water-bath and immediately diluted into the prehybridization solution. Hybridizations were performed overnight (at least 16 hours), at 65°C in an incubator with a rocking platform.

vii. Washing and Autoradiography. The membranes were washed at 65°C in 3 x SSC and 0.5% SDS with several (at least three) half-hourly changes of washing solution. The washing was performed until only a few counts above background could be detected with a Geiger counter. The membranes were air-dried, wrapped in plastic wrap and exposed for 5-6 days to a Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y., USA) with DuPont Quanta III intensifying screens (DuPont, Boston, MA, USA) at -70°C. Films were developed either manually or using a Kodak X-ray film processor.

The sizes of particular DNA fragments were determined by plotting the migration distances of the corresponding bands against the standard curve derived from the migration of the λ-DNA-Hind III marker.

viii. Restriction Endonucleases and DNA Probes. Nine polymorphic restriction sites in the β-globin gene cluster were analyzed to determine the haplotypes of the β-thal patients in this study (16). These polymorphic sites were: a) Hinc II 5' to ε, detected with a 1.2 kb Bam HI/Eco RI probe spanning the entire ε-globin gene, b) Xmn I 5' to $^{G}\gamma$, and Hind III at $^{G}\gamma$ and $^{A}\gamma$; all three sites were analyzed with the γ-IVS-II probe, a 457 bp Bam HI/Pvu II fragment complementary to the $^{G}\gamma$- and $^{A}\gamma$-IVS-II regions, c) Hinc II at ψβ and 3' to it, both sites were detected with the ψβ probe, a 1.6 kb Eco RI/Xba I fragment spanning the entire ψβ-globin gene, and d) Ava II within β, and Hpa I and Bam HI 3' to the β-globin gene, all three sites analyzed with the β-IVS-II probe, a 1.2 kb Bam HI/Eco RI fragment of the β-IVS-II region.

The 7 kb deletion involving the 3' end of the δ-globin gene, the intergenic region, and the 5' end of the β-globin gene, which leads to the fusion of the δ- and β-globin genes into a hybrid Lepore-Boston-Washington δβ gene, was detected by restriction enzyme digestion with Xba I and Bgl II, and hybridization with the β-IVS-II probe (17).

The number of α-globin genes was determined through gene mapping analysis with the restriction enzymes Eco RI, Bgl II, and Bam HI, and hybridization with the α (a 1.5 kb Bam HI fragment containing the entire α-globin gene), ζ (a 1.9 kb Hinf I fragment containing the entire ζ-globin gene), and θ (a 0.8 kb Bam HI fragment of the θ-globin gene) probes (18,19).

The DNA probes discussed above were cloned into plasmid vectors (pUC 19 or pBR 322) and were isolated when necessary by mini-preparation of plasmid DNA (14) and digestion with the appropriate restriction enzymes. The restriction fragments corresponding to the probes of interest were separated on 1% agarose gels, sliced out from the gels, and the DNA was eluted either with a Gene Clean Kit (Bio 101; Bio-Rad) or by electroelution with a dialysis bag (14).

8. PCR Methodology. The discovery of the PCR procedure in 1985 (20) has had a significant impact on DNA research in general, and especially on studies of molecular defects and other sequence variations. Over the years, there have been considerable improvements both in the PCR reaction itself, and the techniques with which it is associated. Such improvements include substitution of the Klenow fragment with the thermostable Taq polymerase (21), non-radioactive procedures for the detection of point mutations (22-24), and the identification of sequence variations by direct sequencing of the PCR amplified DNA (25). As the work described in this study extends over a period of several years, it has followed and included the methodological improvements, and has also contributed to the development of PCR based procedures for the detection of point mutations (26-28). Therefore, while this section will cover all the procedures used, the present ones will be described in greater detail.

i. Oligonucleotide Synthesis. All oligonucleotides used in this study were synthesized by a solid-phase oligonucleotide synthesis method (29) either on an Applied BioSystems 380B DNA Synthesizer (Applied BioSystems, Inc., Foster City, CA, USA) or on a Beckman Plus 1 DNA Synthesizer

(Beckman, Palo Alto, CA, USA). Following synthesis, the oligonucleotides were removed from the solid support by an ammonia treatment (55°C) overnight, dried under vacuum, and purified by G-50 Sephadex molecular sieve chromatography using TE elution buffer. These oligonucleotides were used as PCR primers, oligonucleotide probes for ASO hybridization, and as sequencing primers. The sequences of these oligonucleotides are given in Chapters 3, 4, 5, and 6, together with appropriate references.

ii. PCR Using Klenow Fragment. Amplifications of 1 µg of genomic DNA were performed in 1.5 ml Eppendorf tubes containing the following in a total volume of 100 µL; 50 mM NaCl, 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 pM of each primer, 100-125 pM of each dNTP, and 10% DMSO. A hole was made in the cap of each tube with a small needle and the samples were denatured for 10 minutes at 95°C. After centrifugation for 10 seconds the samples were kept at 37°C for 2.25 minutes to allow the primers to anneal to the DNA, and 1 unit of Klenow fragment was added. The samples were incubated at 37°C for 2-3.5 minutes. This cycle of denaturation, annealing, and synthesis was repeated once more under the same conditions, and then 25 times at a denaturation time of 1 minutes (1 unit of Klenow fragment was added at each cycle). One-fifth of each sample was then used for ASO hybridization as described below.

iii. PCR Using Taq Polymerase. In general, amplifications were performed on 1 µg of genomic DNA using Taq polymerase from various manufacturers. The reaction mixtures contained 200 µM of each dNTP, 50-100 pM of each oligonucleotide primer, 2.5 units of Taq polymerase, and reaction buffer supplied by the manufacturer, in a total volume of 100 µL. The samples were overlayed with 60 µL of mineral oil to prevent evaporation, and were subjected to 30-35 repeated cycles of denaturation, annealing, and extension. The denaturation step in the first cycle was at 95°C for 4 minutes, at the same temperature but lasting for only 1 minute in subsequent cycles. The annealing was always for 1 minute, while the temperatures used varied from 58 to 65°C, and were usually several degrees above the T_m of the primers (see below). The extension reactions were performed at 72°C for 1-5 minutes, depending on the lengths of the expected PCR fragments. The extension step in the last cycle was always prolonged for at least 7 minutes, to allow complete extension of all DNA strands synthesized in the previous steps. The efficiency and specificity of the PCR was estimated by analyzing a 10 µL aliquot from each reaction on an ethidium bromide stained (10 µg/ml) 2% agarose gel under UV light. The samples were stored at 4°C until further use.

iv. ASO Hybridization With ^{32}P-Labeled Oligonucleotides. Screening for previously characterized point mutations was by ASO hybridization (30). Two 19 mer oligonucleotides were used; one was complementary to the sequence carrying the mutation of interest in such a way that the mutation was positioned in the middle (mutant oligonucleotide), while the second (normal oligonucleotide) was complementary to the corresponding sequence from the normal allele.

v. ^{32}P 5' End Labeling of Oligonucleotides. Both oligonucleotides were labeled at the 5' end to a high specific activity with $\gamma^{32}P$-dATP (7,000 Ci/mM, 160 mCi/ml; ICN) by T_4 polynucleotide kinase [United States

Biochemical (USB), Cleveland, OH, USA] (14). After 1-2 hours of incubation at 37°C, the unincorporated nt was removed from the probes using either G-50 Sephadex molecular sieve chromatography with the TE elution buffer or by commercially available Nensorb (NEN) columns (NEN Products, DuPont) under the conditions recommended by the manufacturer. The activity of the probes was determined as cpm in a scintillation counter (Beckman).

vi. Dot-Blot Hybridization. Two 20 µL aliquots of each PCR reaction were denatured in the presence of 0.4 M NaOH, 25 mM EDTA. Both samples were applied to a zeta probe nylon membrane using a Bio-Dot spotting apparatus (both from Bio-Rad), so that one of the spots could later be used for hybridization to the normal and the other to the mutant probe. The membrane was cut in the middle, and the DNA was fixed to the membranes by baking in a vacuum oven at 80°C for 2 hours. The duplicate membranes were prehybridized in separate plastic bags in a solution of 5 x SSPE, 5 x Denhardt's, 0.5% SDS, for 30 minutes at the hybridization temperature (see below). Following prehybridization, one probe (50-100 x 10^6 cpm) was added to each membrane, and hybridization was performed in a circulating waterbath for 1-2 hours. Next, the membranes were washed twice for 10 minutes at room temperature with 2 x SSPE, 0.1% SDS, followed by a stringent 10-minute wash with 5 x SSPE, 0.5% SDS, usually at the same temperature as for the hybridization (see below). After washing, the membranes were air-dried, wrapped in plastic film, and autoradiographed for 2-12 hours at -70°C.

The hybridization and subsequent washing temperatures were crucial for the specific binding of the oligonucleotides to perfectly matched PCR amplified sequence. These temperatures were determined empirically (using positive and negative controls) for each pair of oligonucleotides, but were generally near the T_m of the oligonucleotides (T_m -2 to T_m +4°C). The T_m depend mainly on the nt content of the oligonucleotides and were calculated by the following formula: $T_m = 4 \times (G+C) + 2 \times (A+T)$. The hybridization and washing conditions for the detection of the most common β-thal mutations in the Mediterranean Basin have been reported in a separate publication (25).

vii. ASO Hybridization With DIG-ddUTP Labeled Oligonucleotides. There has been a considerable effort in the past few years to develop simple, safe, and reliable nonradioactive procedures for the detection of mutations that could also be easily implemented in the setting of a clinical laboratory. We therefore modified the standard ASO hybridization procedure by replacing the 5' ^{32}P-end labeled oligonucleotides with oligonucleotides labeled at the 3' end with DIG (28). The 3' end labeling reaction was performed with 100 pM oligonucleotide, 1 nM DIG-ddUTP (Boehringer, Mannheim, Germany), 5 units Terminal transferase [Bethesda Research Laboratories (BRL), Inc., Gaithersburg, MD, USA], and a reaction buffer supplied by the manufacturer in a 20 µL final volume. After incubation at 37°C for 15 minutes, the reaction was stopped with 2 µL 0.2 M EDTA, pH 8.0. The labeled oligonucleotide was precipitated with 2.5 µL 4 M LiCl and 75 µL prechilled absolute ethanol, incubated at -70°C for 30 minutes and centrifuged at 12,000 g, 4°C for 15 minutes. The pellet was washed with 70% cold ethanol, dried under vacuum and dissolved in sterile redistilled

water. The prehybridization, hybridization, and washing of the nylon membranes was performed as described above for the radioactive ASO procedure. The hybridized oligonucleotides were detected with an anti-DIG antibody (DIG Nucleic Acids Detection Kit; Boehringer). Rehybridization of the membranes was done after color removal with N' N'-DMF at 65°C in a glass tray (approximately 4 x 30 minutes). Next, the membranes were washed twice at room temperature in 2 x SSC, 0.1% SDS, and incubated at 65°C for 2 hours with 0.5 mg/ml proteinase K in 2 x SSC, 0.1% SDS, followed by three 5-minute washes in 2 x SSC, 0.1% SDS. The probes were finally removed with 50% formamide, 10 mM sodium phosphate, pH 6.5 at 65°C for 1 hour.

viii. Direct Sequencing of PCR Amplified DNA. Two procedures were followed to obtain the PCR fragments that were used in the dideoxy chain termination sequencing reactions (31). The first procedure included a standard PCR reaction, as described above, to generate a double-stranded PCR product that was denatured with NaOH and purified from the remaining PCR primers with a Miniprep Spun Column (Pharmacia-LKB). The second procedure involved an asymmetric PCR reaction to generate a single-stranded PCR product for sequencing. These two procedures also differed in the annealing step of the sequencing reaction, while the labeling and termination steps were identical.

ix. Miniprep Spun Column for the Purification of the PCR Product. The PCR sample was transferred to a clean Eppendorf tube and an equal volume of a phenol-chloroform solution was added to remove the remaining mineral oil and protein. The sample was vortexed, centrifuged for 10 minutes at top speed, and the supernatant was transferred to a clean tube. The sample was then precipitated in 0.3 M sodium acetate, pH 5.2, and 2.5 volumes of absolute ethanol for 30 minutes at -20°C. After a 15 minute centrifugation at 4°C and at top speed, the recovered pellet was dried under vacuum in a Speed-Vac (Savant, Farmingdale, N.Y., USA) and dissolved in 20 µL water. The amplified DNA was denatured with 5 µL of 2 M NaOH for at least 10 minutes at room temperature. Meanwhile, a Miniprep Spun column was prepared as described in the manufacturer's instructions. Briefly, the Miniprep Spun column was equilibrated with 5 ml of TE buffer, placed in a 15 ml Corex tube, and centrifuged at 400 g for 2 minutes in a centrifuge with a swinging bucket rotor. Twenty-five µL of TE buffer were applied to the center of the flat upper surface of the compacted bed of Sephacryl S-400. The centrifugation was repeated and the 25 µL denatured DNA sample was applied to the upper surface of the Sephacryl. A capless Eppendorf tube was placed in the bottom of the Corex tube to recover the eluted DNA. The centrifugation was repeated and the eluted 25 µL sample was transferred to a clean tube. Two more elutions were performed with 25 µL of TE buffer in the same manner. Ten µL aliquots of each of the three effluents were analyzed on an ethidium bromide stained 2% agarose gel to identify the fraction with the highest DNA yield. Seven µL of this fraction were used per sequencing reaction with the Sequenase Version 2.0 DNA Sequencing Kit (USB).

x. Annealing Reaction. One µL (50 pM) of sequencing primer and 2 µL of 5x reaction buffer were added to the denatured DNA sample up to a total volume of 10 µL. The reaction mixture was vortexed briefly, centrifuged, and incubated at 37°C for 15 minutes.

xi. Labeling Reaction. The labeling mix provided in the sequencing kit was diluted 10 times with sterile water. The Sequenase enzyme (13 U/μL) was then diluted (1:8) in Enzyme Dilution Buffer (USB) up to a total volume of 2 μL. Next, 0.5 μL of α^{35}S-dATP (800 Ci/mM, 10.0 mCi/ml; DuPont) were added to the diluted enzyme. All the dilutions were performed on ice. The labeling reaction was started with the addition of 1 μL 0.1 M DTT, 1 μL diluted Labeling Mix, and 2.3 μL of diluted enzyme/α^{35}S-dATP mixture to the tube containing the annealed template/primer. The sample was incubated at room temperature for 2 minutes.

xii. Termination Reaction. Two and a half μL of each Termination Mixture (ddATP, ddCTP, ddGTP, and ddTTP) were placed in clean 1.5 ml Eppendorf tubes labeled A, C, G, and T, respectively. The tubes were prewarmed for at least 1 minute at 37°C. When the labeling reaction was complete, 3.5 μL of the reaction mixture was added to each of the labeled tubes and incubated at 37°C for 5 minutes. The reactions were stopped with 4 μL of Stop Solution and the tubes were kept on ice until loaded onto the gel.

xiii. Denaturing PAGE. The sequencing reactions were separated on a 0.04 mm thick 7 M urea/8% polyacrylamide/TBE gel using a vertical electrophoresis apparatus for DNA sequencing (Model S2; BRL-GIBCO) and a high voltage constant power supply (2297-Microdrives; LBK-Broma, Uppsala, Sweden). The gel was allowed to polymerize for at least 1 hour, and next was pre-electrophoresed at 70 W constant power (1400-1600 V, 50-60 mA) for at least 30 minutes. The DNA samples were denatured for 3 minutes at 80°C, placed on ice for 3-5 minutes, and loaded onto the gel (2-3 μL per well). The gel was run at constant power (75 W) for 1.5 to 5 hours, depending upon the proximity of the desired sequence to the sequencing primer. Following electrophoresis, the gel was fixed in a 10% acetic acid/12% methanol solution for 30 minutes, transferred to a Whatman 3MM paper (Whatman, Maidstone, Kent, England), covered with plastic film, and dried under vacuum on a gel dryer (Slab Dryer Model 483; Bio-Rad) at 80°C for 1 hour. The gel was then unwrapped and autoradiographed by overnight exposure to a Kodak X-ray film.

xiv. Sequencing of Single-Stranded PCR Fragment. To generate single-stranded PCR products, the standard PCR reaction was adjusted by decreasing the concentration of one of the primers to 1 pM/100 μL, resulting in an imbalanced primer ratio of 1:100. The PCR primers that remained after the PCR reaction were eliminated by differential precipitation of the amplified PCR product with 1 volume of 2.5 M ammonium acetate and 2 volumes of absolute ethanol at room temperature for 15 minutes. The pellet was recovered by centrifugation for 15 minutes at 4°C, washed three times with 70% ethanol, dried under vacuum, and dissolved in 10 μL water. The recovery was estimated by analyzing 2 μL of the PCR product on a 2% agarose gel. The amount of amplified DNA corresponding to 0.3 μg M-13 single-strand DNA was used for the sequencing reaction. The sequencing reaction was performed as described above except that the annealing was at 65°C for 3 minutes followed by a 30 minute incubation on ice.

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CHAPTER 3

MOLECULAR CHARACTERIZATION OF β-THALASSEMIA MUTATIONS

CHAPTER 3a

β-THALASSEMIA IN YUGOSLAVIA

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ABSTRACT

This study concerned the evaluation of β-thal alleles in nearly 50 patients with β-thal major and in 130 β-thal heterozygotes using gene amplification and dot-blot hybridization with synthetic probes. Fourteen different mutations were observed (Table 3a/1); of these, three (IVS-I-110; IVS-I-6; IVS-I-1) account for some 75% of all β-thal alleles. Newly discovered variants, i.e. T->C in the initiation CD and AATAAA->AATGAA in the poly A site were observed in a few patients. The poly A mutation with classical β-thal alleles result in thalassemia intermedia. Hb Lepore is a rather common abnormality and combinations of this variant with β-thal often result in severe disease; a search for β-thal mutations among patients affected with this disease should include an analysis to detect this Hb abnormality.

INTRODUCTION

Several reports have been published describing the occurrence of β-thal and the related δβ-thal in the Yugoslavian population (1-6). These abnormalities were observed as simple heterozygotes, and in patients with thalassemia major or with a compound heterozygosity for β-thal and an abnormal Hb. We have recently completed a program to identify the various mutations and frameshifts in the β-globin gene which result in the different types of thal found in Yugoslavia. The results of this study involving nearly 50 subjects with thalassemia major and 130 β-thal heterozygotes are summarized here.

MATERIALS AND METHODS

Patients. Blood samples were collected from 46 subjects with thalassemia major, from 68 of their heterozygous relatives, and from 62 randomly selected, unrelated, β-thal heterozygotes. Most patients (i.e. 80%) came from Macedonia (one of the six republics of Yugoslavia which is located in the southern part of the country), where they attended clinics in the cities of Skopje, Strumica, Gevgelija, Titov Veles, and others. Patients from other areas were followed in clinics in Zagreb (Croatia) and Belgrade (Serbia). Blood samples were collected with EDTA as anticoagulant and shipped to the general laboratory in Skopje, Macedonia.

Hematological data were collected with an automated cell counter in the laboratory in Zagreb. Hb analyses were done in Skopje by the same procedures as listed in the preceding paper (7) with the following exceptions: Hb A_2 was quantitated by microcolumn chromatography using Tris-HCl developers (8), while the $^{G}\gamma$ and $^{A}\gamma$ levels in isolated Hb F were determined by PAGE (9,10).

DNA methodology, including gene mapping and dot-blot analysis of amplified DNA, was the same as described in the preceding paper (7). The analyses of the samples from the β-thal major patients were done in the laboratory in Skopje. The β-thal mutations in the majority of the β-thal heterozygotes were determined in the laboratories in Augusta, GA, USA.

RESULTS AND DISCUSSION

The Frequencies. Fourteen different mutations have been observed (Table 3a/1). Eight of these were present in the patients with thalassemia major and in β-thal heterozygotes, while the remaining six were observed only among the β-thal heterozygotes who participated in the random survey (see Ref. 11 for references describing individual mutations and frameshifts). About 10% of the β-thal genes present in the patients remained unknown mainly because the survey of these DNA samples was limited to an evaluation of the presence of the 12 most common β-thal mutations. Moreover, deletional types of β-thal are not included in the data listed in Table 3a/1. Nearly 40% of the chromosomes carried the IVS-I-110 mutation (45.4% in the survey of the heterozygotes) and 29% the IVS-I-6 mutation.

This high percentage likely does not reflect the true incidence because the mild clinical course of the β-thal conditions due to a homozygosity of this mutation, or to a combination of the IVS-I-6 and other mutations, affects the frequency values calculated for this relatively small group of patients. The frequency value of 19.2% found for the IVS-I-6 mutation among β-thal heterozygotes may be too low because the hematological and Hb A_2 data may be closer to normal which interferes with its detection in a field survey. The third most frequently occurring variant is the G->A mutation at IVS-I-1 (9-11%). Thus, three mutations, IVS-I-110 (G->A), IVS-I-6 (T->C), and IVS-I-1 (G->A), account for more than 75% of the β-thal genes observed in Yugoslavia.

TABLE 3a/1. The Frequencies of 14 Different β-Thal Alleles in the Yugoslavian Population

Mutation	Thalassemia Major Patients # Chroms.	%	Heterozygotes # Chroms.	%
-87 (C->G)			2	1.54
Initiation CD (T->C)			1	0.77
CD 5 (-CT)			2	1.54
CD 6 (-A)	1	1.16	2	1.54
CD 8 (-AA)			1	0.77
CD 29 (C->T)	1	1.16	1	0.77
CD 39 (C->T)	3	3.49	5	3.85
IVS-I-1 (G->A)	8	9.31	15	11.54
IVS-I-6 (T->C)	25	29.07	25	19.23
IVS-I-110 (G->A)	34	39.53	59	45.37
IVS-II-1 (G->A)			2	1.54
IVS-II-745 (C->G)	1	1.16	2	1.54
IVS-II-848 (C->A)			2	1.54
Poly A (AATAAA->AATGAA)	4	4.65	5	3.95
Unknown	9	10.47	6	4.61
Total	86	100.00	130	100.00

The CD 39 (C->T) mutation appears to be relatively rare in Yugoslavia (3.5-4.0%) but the newly discovered poly A mutation (AATAAA->AATGAA; Ref. 12) was present in four unrelated families, suggesting that this mild β^+-type of thalassemia may be present with a frequency of some 4%. Another newly discovered mutation, i.e. the T->C mutation in the initiation CD (13), was found in only one β-thal heterozygote.

No significant differences were observed between the types of β-thal mutations among Macedonians and Yugoslavians living in the northern part of Yugoslavia; however, the numbers are too small for a definitive comparison.

β-Thal Homozygotes. Ten of the β-thal major patients (i.e. 23% of the total) were homozygous for the IVS-I-110 (G->A) mutation and one was homozygous for the IVS-I-1 (G->A) mutation. All 11 subjects were severely affected and were transfusion-dependent. Of the 16 chromosomes with the IVS-I-110 mutation tested, 15 had haplotype I, and one had haplotype IX (see Ref. 14 for details). Five subjects were homozygous for the IVS-I-6 (T->C) mutation, and some of the data observed for these patients are listed in Table 3a/2. All were mildly affected by their disease but were not transfusion-dependent. Patients #1, #2, and #3 were homozygous for haplotype VI and their Hb F levels averaged about 8%. In contrast, patients #4 and #5 (who are brother and sister) were homozygous for haplotype VII and had much higher Hb F levels of 15% and 35%; such differences have also been observed for patients in other countries (7).

TABLE 3a/2. Hematological and Hb Composition Data for Five Yugoslavian Subjects With a Homozygosity for the IVS-I-6 (T->C) Mutation

Subject	Sex-Age	Hb g/dl	MCV fl	MCH pg	Hb A_2 %[a]	Hb F %[b]	G_γ %[c]	A_γ^T %[d]	Haplotype[e]
1	F-34	11.3	75	22	6.3	3.9	57	43	VI/VI
2	M-23	10.2	61	21	3.0	12.0	52	48	VI/VI
3	M-32	10.9	65	24	5.9	7.7	61	39	VI/VI
4	F-14	9.1	76	22	6.2	15.0	42	0	VII/VII
5	M-12	9.6	70	20	4.7	35.0	45	0	VII/VII

[a] By microcolumn chromatography using Tris-HCl developers (8).

[b] By alkali denaturation (15).

[c] By PAGE (9,10).

[d] By reversed phase HPLC (16) showing the absence of A_γ (haplotype VI/VI) or of A_γ^T (haplotype VII/VII).

[e] Haplotype VI or [- - + + - - + + +] and haplotype VII or [+ - - - - - - + +]. Restriction sites: Hinc II 5' to ε; Xmn I 5' to G_γ; Hind III at G_γ and A_γ; Hinc II at ψβ and 3' to it; Ava II at β, and Hpa I and Bam HI 3' to β.

Compound Heterozygotes. Twenty-eight of the 44 patients were compound heterozygotes; most were severely affected by their disease and were transfusion-dependent. Exceptions were two patients with the IVS-I-110/IVS-I-6 combination, both having haplotype I for the IVS-I-110 chromosome and haplotype VII for the IVS-I-6 chromosome, one patient with the IVS-I-1/CD 6 (-A) combination and four patients with a combination which involved the AATAAA->AATGAA mutation at the poly A site (Table 3a/3). Patient #3 (listed in this table) was mildly affected and had not been transfused; her clinical condition was diagnosed as thalassemia intermedia. The same was the case for patient #4, although he had a more severe disease.

TABLE 3a/3. Hematological and Hb Composition Data for Four Patients With a Compound Heterozygosity Involving Different β-Thal Mutations and the A->G Mutation at the Poly A Site

Subject	Sex-Age	Combination	Hb g/dl	MCV fl	MCH pg	Hb A_2 %[a]	Hb F %[b]	G_γ %[c]
1	F- 4	Poly A/IVS-I-110	5.9	74	26	2.8	13.3	54
2	M-17	Poly A/IVS-I-1	7.9	84	28	2.3	19.3	47
3	F- 7	Poly A/IVS-I-6	10.2	60	20	5.9	10.2	50
4	M-23	Poly A/CD 29	9.0	65	17	5.6	5.0	47

a,b,c See footnotes to Table 3a/2.

Combinations of an Abnormal Hb and β-Thal. Three patients fell into this group; they are excluded from Table 3a/1. Two had Hb Lepore-β-thal (IVS-I-110 and CD 39) and one had Hb O-Arab-β°-thal (the frameshift at CD 6). Hb Lepore is a frequently occurring variant in Yugoslavia (17,18); data from a previous study involving patients with Hb Lepore and β-thal have indicated that the considerable variation from severe disease to a moderate compensated anemia is relate to the type of β-thal that is present (19).

The β-Thal Heterozygote. The frequencies of the 14 different mutations observed in the 130 β-thal heterozygotes are listed in Table 3a/1. Newly discovered variants are the T->C mutation at the initiation CD (13) and the A->G mutation at the poly A site (12). Table 3a/4 lists average data and standard deviations for different parameters for five groups of subjects with different β-thal alleles.

TABLE 3a/4. Hematological and Hb Composition Data for Yugoslavian Adults With a Heterozygosity for β-Thal Caused by Different Mutations or Frameshifts (average values and standard deviations only)

Mutation	No. of Cases	Hb g/dl	MCV fl	MCH pg	Hb A_2 %[a]	Hb F %[b]	G_γ %[c]
IVS-I-110 (G->A)	62	12.05 (± 2.45)	69.8 (± 6.5)	22.2 (± 2.3)	4.9 (± 0.6)	1.3 (± 1.3)	Variable
IVS-I-6 (T->C)	26	13.45 (± 1.6)	72.7 (± 3.4)	23.2 (± 1.2)	4.5 (± 0.55)	1.0 (± 0.35)	Variable
IVS-I-1 (G->A)	16	12.05 (± 1.55)	70.7 (± 6.5)	23.0 (± 2.4)	4.8 (± 0.95)	2.65 (± 2.3)	37.0 (± 11.0)
Poly A (A->G)	5	13.45 (± 0.9)	78.3 (± 1.3)	24.3 (± 0.4)	3.95 (± 0.6)	0.55 (± 0.3)	n.d.
IVS-I-1 (G->A)	3	11.2 (± 1.8)	62.7 (± 1.2)	20.0 (± 0.8)	5.55 (± 0.25)	1.15 (± 0.6)	76.0 (± 1.0)

a,b,c See footnotes to Table 3a/2.

The data are somewhat different from those listed for the Turkish β-thal heterozygotes (7), presumably because of the different methodology that was used, especially in the quantitation of Hb A_2. Of interest are the data for the five subjects with the A->G mutation in the poly A site. These subjects have the highest MCV and MCH values and the lowest level of Hb A_2 and might easily be overlooked in a simple field survey.

CONCLUSIONS

β-Thal in Yugoslavia is a heterogeneous condition since 14 different mutations have been detected. However, three mutations, i.e. G->A at IVS-I-110, T->C at IVS-I-6, and G->A at IVS-I-1 occur most frequently and were present in 75 to 80% of all chromosomes tested. This information is of considerable importance for centers interested in prenatal diagnostic procedures, particularly when simplified methodology to detect these mutations becomes available (20,21). However, such centers should also be equipped to detect certain Hb variants because combinations of some of these (for instance, Hb Lepore) with certain types of β-thal (mainly the IVS-I-110, IVS-I-1, and CD 39 mutations) cause a severe thalassemic condition.

Thalassemia intermedia, i.e. a relatively mild clinical condition without the requirement for regular blood transfusions, has been observed in patients who are homozygous for the IVS-I-6 (T->C) mutation and in a few patients with combinations involving the newly discovered A->G mutation in the poly A site. The high frequencies of the IVS-I-110 (G->A) and the IVS-I-1 (G->A) mutations, and the relatively high incidence of Hb Lepore leads to the conclusion that at least 80% of all Yugoslavian patients with a homozygosity or a compound heterozygosity for one (or two) of the discovered β-thal alleles, is affected by severe disease and a complete blood transfusion dependency.

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CHAPTER 3b

β-THALASSEMIA IN BULGARIA

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ABSTRACT

Analyses of DNA from 64 patients with thalassemia major using the hybridization technique of amplified DNA with radio-labeled synthetic oligonucleotide probes identified 13 different β-thal mutations. The CD 39 (C->T) and IVS-I-110 (G->A) mutations occurred most frequently but seven additional mutations were observed which were present at frequencies of 3.9 to 10.2%. This broad spectrum of β-thal alleles complicates the analyses for institutions involved in prenatal diagnosis. Promoter mutations were rare and the frequencies of two other mild mutations [IVS-I-6 (T->C) and the poly A mutation] were relatively low, indicating that β-thal is a severe disease among Bulgarians. The high frequencies of 4.7-5.5% for the four frameshifts at CDs 5, 6, 8, and 8/9 may be specific for this population.

INTRODUCTION

Since 1937, when the first patients with Cooley's anemia in Bulgaria were described (1), numerous additional cases have been reported from all parts of the country (2-9). Over the past 20 years population studies have been carried in most parts of the country (10-18); different approaches for the detection of β-thal carriers were used and a frequency of 0.05 to 19.9% was found (11,13). It is estimated that the average frequency of β-thal in Bulgaria is about 2% (9). At present, more than 250 patients with Cooley's anemia are registered in different hospitals. Here we describe the identification of numerous β-thal alleles among more than 60 patients with β-thal major and over 80 of their relatives. The data indicate a considerable heterogeneity which may complicate the general activities of the prenatal diagnostic programs.

MATERIALS AND METHODS

Patients. The total number of patients was 64, and 82 heterozygous relatives were also evaluated. Most patients attended a pediatric clinic at the university at Stara Zagora but others were seen in local clinics in various provinces (Fig. 3b/1). Nearly all patients were children or young adults. Blood samples were collected in vacutainers with EDTA as anticoagulant and studied at the local institution, at the laboratory in Skopje, Yugoslavia, and were shipped by fast air mail service to Augusta, GA, USA for detailed analysis. Informed consent was obtained.

FIG. 3b/1. The provinces of Bulgaria where the patients and their parents were located. A major β-thal center is located in Stara Zagora.

Methods. Hematological and Hb composition data were collected as described in a previous study (19). The same is the case for some gene mapping analysis and for the identification of the various mutations and frameshifts.

RESULTS AND DISCUSSION

The Frequencies. Table 3b/1 lists the 13 different β-thal alleles that were discovered. The other abnormalities tested for were: The 44 bp deletion, -88 (C->T), -30 (T->A), -29 (A->G), -28 (A->C), IVS-I-5 (G->T; G->C; G->A), IVS-II-848 (C->A), CD 30 (G->A or G->C), and the 7 bp deletion [for references see the β-thalassemia repository (20)]. Two mutations, i.e. C->T at CD 39 and G->A at IVS-I-110, occurred at about equal percentages and accounted for 46% of the 128 β-thal chromosomes studied. Next are the T->C mutation at IVS-I-6 and the C->G mutation at IVS-II-745, each occurring in about 10% of all chromosomes tested. Most interesting was the relatively high frequencies of frameshifts at CD 5 (21), CD 6, CD 8, and CDs 8/9; each of these alleles occurred with a frequency of about 5%. Promoter mutations were relatively rare; only one chromosome with C->T at -101 and five chromosomes with C->G at -87 were observed. Thus, the occurrence of alleles with relatively mild clinical consequences (i.e. C->T at -101; C->G at -87; T->C at IVS-I-6; A->G at the poly A site) was a low 15 to 16%. Only four chromosomes had β-thal mutations which remained unidentified.

TABLE 3b/1. The Frequencies of 13 Different β-Thal Alleles in the Bulgarian Population

Mutation	Thalassemia Major Patients # Chroms.	%
-101 (C->T)	1	0.78
-87 (C->G)	5	3.91
CD 5 (-CT)	6	4.69
CD 6 (-A)	6	4.69
CD 8 (-AA)	7	5.46
CDs 8/9 (+G)	7	5.46
CD 39 (C->T)	28	21.88
IVS-I-1 (G->A)	4	3.14
IVS-I-6 (T->C)	13	10.15
IVS-I-110 (G->A)	31	24.21
IVS-II-1 (G->A)	2	1.56
IVS-II-745 (C->G)	13	10.15
Poly A (AATAAA->AATGAA)	1	0.78
Unknown	4	3.14
Total	128	100.00

β-Thal Homozygotes. Only 15 of the 64 patients were found to be homozygous; this reflects the large number of alleles which occurred at relatively high frequencies. Three were homozygous for the IVS-I-110 mutation, three for the CD 39 mutation, and two for the IVS-II-745 mutation; all eight patients were transfused at regular intervals. One 6-year-old girl was homozygous for the IVS-I-6 (T->C) mutation and one 33-year-old male

was homozygous for the C->G mutation at position -87 of the β-globin gene promoter (Hb 8.3 g/dl; MCV 82 fl; MCH 24 pg; Hb F 12.2%). The latter patient had been diagnosed as having a "thalassemia minor" and was the parent of a child with thalassemia major and a compound heterozygosity [CD 5 (-CT) and C->G at -87]. Additional patients included three children with a CD 8 (-AA) homozygosity. All three had high levels of Hb F and high $^{G}\gamma$ values, as similarly observed among Turkish patients with the same frameshift; one 16-year-old teenager was transfused. There were also two with a homozygosity for the frameshift at CDs 8/9 (+G). Both were young children with a marked anemia who were regularly transfused; one 2-year-old child, studied prior to transfusion, had a Hb level of 4.9 g/dl; MCV 78 fl; MCH 27.2 pg; Hb A_2 2.3%; Hb F 24.3%; $^{G}\gamma$ 57.6%.

Compound Heterozygotes. Eleven subjects had the IVS-I-110/CD 39 combination; only five had the IVS-I-110 mutation on one chromosome and the IVS-I-6 mutation on the other. Various other combinations were observed, many causing severe disease and a blood transfusion dependency. Only a few patients had a somewhat less severe disease, notably those with the IVS-I-110/poly A combination (one patient), the CD 39/-101 combination (one patient), and the CD 39/-87 combination (one patient). Two children were compound heterozygotes for the frameshift at CD 6 (-A) and the C->G mutation at position -87 of the β-globin gene promoter. Both were not transfused and had nearly identical hematological data: Hb 7.4/7.4 g/dl; MCV 84/87 fl; MCH 28.1/29.6 pg; Hb A_2 2.9/3.3%, and high Hb F values of 87.8/85.9%, respectively, indicating that some 10% of their Hb was Hb A.

Combinations of an Abnormal Hb and β-Thal. Four patients with Hb O-Arab/β-thal and three with Hb Lepore/β-thal were studied. The β-thal mutations were: IVS-I-110 (three), CD 39 (two), IVS-I-6 (one), and IVS-II-745 (one).

The β-Thal Heterozygotes. Only relatives of patients with β-thalassemia major participated in this study; their total number was 82. The distribution pattern was nearly the same as observed for their children. The relatively large number of alleles occurring at high frequencies allowed an evaluation of nine groups of adult β-thal heterozygotes, each with a specific mutation. These data are listed in Table 3b/2. The results are comparable for each of eight groups indicating the mild anemia (Hb 10.8-11.7 g/dl) with microcytosis and hypochromia (65-72 fl; MCH 19-22 pg), elevated Hb A_2 (4.5-5.5%), and slight elevations of Hb F [1.04.4%; this rather high value may be characteristic for the CD 5 (-CT) frameshift]. Three patients with the IVS-I-6 (->C) mutation had a higher Hb level, less microcytosis, and lower Hb A_2 and Hb F values than most other groups.

CONCLUSIONS

The incidence of β-thal among Bulgarians is relatively small and has been estimated at 2% (9). The distribution pattern has not been clearly defined, but the highest incidence appears to occur among Bulgarians in the south where this country borders with Greece and European Turkey, and where the patients and their parents participated in this study were located.

The frequency data listed in Table 3b/1 indicate that about half of all β-thal chromosomes have either the IVS-I-110 (G->A) or the CD 39 (C->T) mutations. Six additional mutations occur at a frequency of 5 to 10%. This rather broad spectrum is not advantageous for any institution involved in prenatal diagnostic programs because testing for multiple alleles is required.

TABLE 3b/2. Hematological and Hb Composition Data for Bulgarian Adults With a Heterozygosity for β-Thal Caused by Different Mutations or Frameshifts (average values and standard deviations only)

Mutation	# of Cases	Hb g/dl	MCV fl	MCH pg	Hb A_2 %	Hb F %	G_γ %[a]
CD 39 (C->T)	23	10.6 (± 1.8)	69.45 (± 7.6)	20.25 (± 1.8)	5.1 (± 0.75)	2.05 (± 1.45)	40.6 (± 13.1)
IVS-I-110 (G->A)	19	11.65 (± 2.0)	69.5 (± 6.6)	21.1 (± 1.5)	5.1 (± 0.55)	1.65 (± 2.5)	Variable
IVS-I-1 (G->A)	9	11.45 (± 1.05)	71.65 (± 6.4)	20.2 (± 1.7)	5.25 (± 0.6)	1.35 (± 1.1)	44.8 (± 14.1)
IVS-I-6 (T->C)	3	12.9 (± 1.2)	81.7 (± 5.3)	22.8 (± 1.65)	3.8 (± 0.4)	0.2 (± 0.1)	n.d.
IVS-II-745 (C->G)	5	11.2 (± 2.1)	72.2 (± 4.7)	20.3 (± 1.15)	5.25 (± 0.15)	1.0 (± 0.9)	Variable
CD 5 (-CT)	6	11.0 (± 1.1)	71.8 (± 6.6)	20.6 (± 1.8)	4.95 (± 0.7)	4.4 (± 3.2)	Variable
CD 6 (-A)	4	11.1 (± 0.8)	67.8 (± 4.7)	22.3 (± 1.5)	4.5 (± 0.9)	2.1 (± 1.2)	29.7 (± 4.5)
CD 8 (-AA)	5	11.7 (± 1.1)	64.8 (± 1.7)	20.25 (± 0.55)	5.4 (± 0.65)	1.4 (± 0.8)	66.3 (± 6.3)
CDs 8/9 (+G)	5	10.8 (± 2.7)	67.6 (± 7.9)	19.0 (± 1.7)	5.5 (± 0.25)	1.1 (± 0.8)	30.8 (± 12.9)

[a] Variable: To indicate that values fell into two groups, i.e. low G_γ (20-45%) and high G_γ (45-70%) suggesting differences in haplotypes of the β-thal chromosome.

β-Thal is a severe disease among Bulgarians because the milder allele, such as the two promoter mutations (-101; -87), the IVS-I-6 mutation, and the mutation in the poly A site, which are known to be associated with thalassemia intermedia, were observed for only 16% of the β-thalassemic chromosomes. This is quite different from what has been observed in neighboring countries, mainly because of the low frequency of the IVS-I-6 (T->C) mutation among Bulgarians. The relatively high frequencies of frameshifts at CDs 5, 6, 8, and 8/9 (together accounting for 20% of all β-thal alleles) were a surprise and may be specific for the population (of some province) of Bulgaria.

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CHAPTER 4

CORRELATION OF CLINICAL SEVERITY WITH DIFFERENT β-GLOBIN GENE DEFECTS

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CHAPTER 4a

VARIATION IN CLINICAL SEVERITY AMONG PATIENTS WITH HB LEPORE-BOSTON-β-THALASSEMIA IS RELATED TO THE TYPE OF β-THALASSEMIA

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SUMMARY

Clinical and hematological observations, made for 10 Yugoslavian patients with the Hb Lepore-β-thal condition, suggested a considerable variation from severe disease and complete blood transfusion dependency to a moderate, compensated, anemia without major complications and without a need for regular blood transfusions. As the type of Hb Lepore was the same in all patients (Lepore-Washington-Boston) and an α-globin gene deficiency was absent, it was concluded that the type of β-thal determined the severity of the disease. Six patients with severe disease had one of the following three β-thal determinants: IVS-I-110 (G->A), exon 2 CD 39 (C->T), and IVS-I-1 (G->A), while the three patients with mild disease had the Portuguese type of thalassemia which is caused by the T->C substitution at IVS-I-6. In one patient with severe disease the β-thal determinant remained unknown. Our observations are consistent with those made for thalassemia patients with a homozygosity for these determinants.

INTRODUCTION

Hb Lepore-Washington-Boston (Lepore-W-B) is a variant with a δβ hybrid chain which results from a crossover between CDs 87 and 116 of the δ- and β-globin genes (Gerald and Diamond, 1958; Baglioni, 1965). Heterozygotes have a mild anemia resembling a thalassemia trait, while homozygotes are severely affected with a severe β-thalassemic type of disease. Hb Lepore-W-B is found in various Mediterranean populations; its highest incidence is in Italy, Yugoslavia, and Greece (reviewed in Efremov, 1978).

Hb Lepore-W-B has been observed in association with β chain variants (i.e. S and C) and with β- and δβ-thal (references in Efremov, 1978). The clinical features of most Lepore-β-thal patients resemble those of patients with homozygous β-thal (Duma et al, 1968; Weatherall and Clegg, 1981) but there are reports of cases with a milder clinical course (Quattrin et al, 1967; Efremov, 1978). In the study to be described here, we have compared clinical and hematological data for 10 patients with the Lepore-β-thal condition, and have determined the haplotypes and mutations that are associated with the β-thal. The data offer a satisfactory explanation for the milder form of the disease seen in some of the patients.

MATERIALS AND METHODS

Blood Samples. These were collected in vacutainers with EDTA as anticoagulant at various locations in Yugoslavia and transported to the Central Laboratory in Skopje, Yugoslavia. Most of this material, together with blood samples from parents and/or siblings, were mailed by air at 40°C to Augusta, GA, USA, where it arrived within 5 days. Informed consent was obtained.

Hematological Analyses. Hematological parameters were determined on freshly collected samples at local institutions using automated cell counters. Quantitation of the various Hb components was by DEAE-cellulose chromatography (Schroeder and Huisman, 1980), while the percentages of the $A_\gamma T$, G_γ, and A_γ chains were determined by reversed phase HPLC (Shelton et al, 1984; Kutlar et al, 1986). *In vitro* chain synthesis analyses followed procedures detailed before (Huisman and Jonxis, 1977); these analyses were made on different blood specimens, collected a few years prior to the present study.

DNA Studies. DNA was isolated from white blood cells following the method of Poncz et al (1982). The determination of the presence or absence of several restriction sites (listed in the footnote of Table 4a/3) followed procedures routinely in use in our laboratories (Harano et al, 1985; Lanclos et al, 1987; Efremov et al, 1987); the numbering system was that which was introduced by Orkin et al (1982). The number of α-globin genes was determined through gene mapping with the restriction enzymes Eco RI, Bgl II, and Bam HI as described before (Felice et al, 1984; 1986).

The types of β-thal were determined by a modification of the procedure of Saiki et al (1986) using gene amplification with specific primers and

with synthetic oligonucleotide probes for the identification of the mutation. Details about this method have been described elsewhere (Diaz-Chico et al, 1988).

RESULTS

The Patients. These persons were located in various areas of Yugoslavia, but five regularly attend clinics in Skopje and Titov Veles, SR Macedonia. Some clinical data are listed in Table 4a/1. Their ages in 1986 ranged from 2 to 41 years; six patients were diagnosed before they were 1 year of age, while the other four were 3-19 years old before the diagnosis of Lepore-β-thal was made. Patient P-361B is the brother of P-361. The clinical severity varied considerably as indicated. Patient P-303 is married and has two sons, both with a β-thal heterozygosity. This patient and patients P-4 and R-27 are doing clinically well having a moderate, compensated, anemia with mild hemolysis and most modest (if any) blood transfusion requirements.

TABLE 4a/1. Some Clinical and X-ray Data for 10 Patients With Lepore-W-B-β-Thal

Patient	Sex-Age	Age of Dx	Enlarged (cm) Spleen	Liver	Splenectomy Age (yr)	Facies and Bone Changes[a]	Transfusion[b]
P1	F-24	6 mos	7	4	7	++	Often
P-20	F-22	1 yr	8	5	8	+++	Very often
I-C	F- 8	8 mos	-	-	7	++	Very often
R-13	F-10	6 mos	7	5	No	+++	Very often
P-361	F-10	6 mos	10	6	No	++	Very often
P-361B	M- 2	6 mos	4	4	No	+	Very often
R-927	M- 7	3 yrs	6	2	No	+	Often
P-4	M-25	3 yrs	7	4	9	+	Rarely
P-303	M-41	19 yrs	10	12	20	+	Rarely
R-27	F-17	8 yrs	4	0	No	-	None

[a] Moderate (+); severe (++); very severe (+++).

[b] Rarely = once in 2 or 3 years; often = once in 2-3 months; very often = once a month.

Hematological and Hb Analyses (Table 4a/2). Hematological data were comparable for all patients but were influenced by the (often frequent) blood transfusions. Blood transfusions also affected the quantitative Hb data; only a few patients (P-1, R-927, R-27) had Hb F levels of 60-80% as expected with Hb Lepore levels of 8-11%. As patient R-27 had not received any blood, it can be assumed that his β^+-thal chromosome was responsible for the production of some 25% Hb A. No such calculations were possible for the other patients.

TABLE 4a/2. Hematological and Hb Data for 10 Patients With the Lepore-W-B-β-Thal[a]

Patient	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	A_2[b] %	Lepore[b] %	F[b] %	α/non-α	β/α
P-1	F-24	7.7	0.300	3.30	2.3	6.2	82.7	3.5	0.16
P-20	F-22	7.9	0.290	2.99	3.1	3.7	29.0	3.3	0.19
I-C	F- 8	9.4	0.300	3.60	1.9	<1.0	3.5	n.d.	n.d.
R-13	F-10	7.4	0.240	2.80	2.7	0.5	4.6	2.8	0
P-361	F-10	8.0	0.264	2.82	2.6	6.7	22.0	3.3	0
P-361B	M- 2	7.8	0.260	2.90	2.1	6.4	14.0	3.1	0
R-927	M- 7	7.5	0.230	3.25	1.9	7.5	80.1	2.0	0
P-4	M-25	9.2	0.325	3.71	2.9	8.7	49.5	1.7	0.21
P-303	M-41	8.6	0.290	3.40	2.5	10.1	63.0	2.2	0.18
R-27	F-17	8.6	0.255	3.35	2.0	6.7	62.5	1.6	0.24

[a] Hematology data were collected prior to the next blood transfusion.

[b] Hbs A_2, Lepore, and F were determined by DEAE-cellulose chromatography in blood specimens collected prior to (the next) blood transfusion.

n.d. = not determined.

The $^{G}\gamma$ values in the Hb F varied from 41.8 to 60.6%, while three patients were positive for the $^{A}\gamma^{T}$ chain (Huisman et al, 1985, and references quoted). In vitro chain synthesis analyses showed a severe deficiency for four of the first seven patients (P-1, P-361, P-361B, and R-927) with an average α/non-α ratio of 3.3 (range 3.0-3.5); these values were nearly 2 for the remaining three patients. In vitro chain synthesis was demonstrable for four patients (P-1, P-4, P-303, and R-27). Structural analyses of the isolated non-α chain of Hb Lepore were made during the past several years for six of the 10 patients (P-1, P-20, P-361, P-4, P-303, and R-27). In all instances the δβ hybrid chain was type Lepore-W-B with a crossover between residues 87 and 116 (data not shown; for structural information see Efremov, 1978).

DNA Analyses. Haplotype analyses involved the nine restriction sites listed in Table 4a/3; family studies facilitated these analyses. As expected from previous studies (Lanclos et al, 1987) the haplotype of the Lepore-W-B chromosomes was the same in all nine families. Six different haplotypes were observed for the β-thal chromosomes (Table 4a/3); haplotypes I and II are the most common among Mediterranean populations (Orkin et al, 1982).

Identification of the molecular abnormality through hybridization of amplified DNA with synthetic probes was possible for nine patients. Table 4a/3 lists the final data for the different types of β-thal; patients P-1, P-20, and I-C had the β-thal with the G->A substitution at IVS-I-110 (haplotype I); patient R-13 that of a C->T replacement in CD 39 of the second exon (haplotype II). The two patients of Family 361 had the G->A

substitution at position 1 of the first intron (haplotype V); The β-thal determinant in patient R-927 with haplotype IV remained unknown; as many as 11 known substitutions [in CDs 6, 8, 39, and 121; at positions 1, 5, 6, and 110 of IVS-I; at positions 1, 705, and 745 of IVS-II (see Lanclos and Kutlar, 1986, for references)] were excluded. The three patients P-4, P-303, and R-27 with the milder form of the disease, each had the T->C substitution at IVS-I-6 (haplotypes VII and VI).

TABLE 4a/3. Determination of the Molecular Lesion for 10 Patients With the Lepore-W-B-β-Thal [all 10 patients had four α-globin genes (αα/αα)]

Patient	Sex-Age	Haplotype[a]	$A_\gamma T$	Type of Anomaly	β-Thal
P-1	F-24	I [+ - - - - - + + +]	-	IVS-I-110 (G->A)	β^+
P-20	F-22	I Same	-	Same	β^+
I-C	F- 8	I Same	-	Same	β^+
R-13	F-10	II [- - + + - + + + +]	+	Exon 2; CD 39 (C->T)	β°
P-361	F-10	V [+ - - - - - + + -]	-	IVS-I-1 (G->A)	β°
P-361B	M- 2	V Same	-	Same	β°
R-927	M- 7	IV [- - - - + + - + +]	-	Unknown	β°
P-4	M-25	VII [+ - - - - - - + +]	-	IVS-I-6 (T->C)	β°<-+
P-303	M-41	VI [- - + + - - - + +]	+	Same	β^+
R-27	F-17	VI Same	+	Same	β^+

[a] Based on family studies. The numbering system is according to Orkin et al (1982). Restriction sites: Hinc II (5' to ε); Xmn I (5' to G_γ); Hind III (G_γ and A_γ); Hinc II (at ψβ and 3' to it); Ava II (at β); Hpa I and Bam HI (3' to β). Haplotype of the Lepore chromosome [+ - - - - - + + -].

DISCUSSION

Variation in clinical severity for patients with the Lepore-β-thal has been noted and is adequately documented (Duma et al, 1968; Quattrin et al, 1967; Efremov, 1978). Three possible explanations can be considered; different types of Lepore δβ chains are involved (Efremov, 1978; Lanclos et al, 1987), an α-thal is present which would alleviate the excessive overproduction of α chains in these conditions (Weatherall et al, 1981; Wainscoat et al, 1983), and different types of β-thal are associated with the Hb Lepore abnormality. The data listed here excluded the first two possibilities.

Four different types of β-thal were observed in the seven patients with the severe form of Hb Lepore-β-thal. Three patients had the G->A base substitution at position 110 of the first intron (Spritz et al, 1981; Westaway and Williamson, 1981); this β^+-thal, in which some 90% of mRNA is abnormal and rapidly turned over (Busslinger et al, 1981), occurs mainly

among Greeks, Cypriots, and Turks, and is primarily associated with haplotype I (Orkin et al, 1982). The C->T mutation at CD 39 of exon 2 (patient R-13) is the cause of the most common β^0-thal among Sardinians (Trecartin et al, 1981; Thein et al, 1985); it is present primarily on chromosomes with haplotypes I and II. The single G->A mutation at position 1 of the first intron (patients P-361 and P-361B, Table 4a/3) eliminates the 5' splicing site resulting in a complete absence of β chain production (Kazazian et al, 1984a). The abnormality in the seventh patient (R-927) needs to be analyzed further. Possible clinical differences between the patients with the Lepore-W-B in combination with any of these types of β-thal are minimal if any.

The β-thal present in the last three patients (P-4, P-303, and R-27, Table 4a/3) is also known as the Portuguese type and has been observed primarily among Portuguese and Yugoslavians, although it has also been seen in other Mediterranean populations (Tamagnini et al, 1983; Orkin et al, 1982; Kazazian et al, 1984b; Gilman et al, 1984; Treisman et al, 1983). It leads to a relatively mild β-thalassemic syndrome. The T->C substitution is known to be associated with haplotypes VI, VII, and X; the first two were present in our patients. Thus, this type of β^+-thal in association with the Hb Lepore-W-B abnormality will result in a relatively mild condition, which was indeed observed in the three patients.

In summary, the heterogeneity among the types of β-thal present in a small group of patients with Lepore-β-thal has offered a satisfactory explanation for the mild condition observed in three patients; the presence of any one of the types (β^+ or β^0), known to be associated with severe disease, can be considered the reason for severe thalassemia in the remaining seven patients.

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CHAPTER 4b

β-THALASSEMIA DUE TO A T->A MUTATION WITHIN THE ATA BOX

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ABSTRACT

Sequence analyses of amplified DNA from a Yugoslavian patient with Hb Lepore-β-thal and from his father with a simple β-thal trait have revealed a T->A mutation within the ATA box at a position 30 bp upstream from the Cap site. The nt substitution was confirmed through dot-blot analysis of amplified DNA with specific ^{32}P-labeled synthetic oligonucleotide probes. The patient had a clinically severe condition; his Hb Lepore-β-thal was of the β^+ type, as about 8-10% of the non-α chain was normal β^A. The same T->A mutation at nt -30 was present on both chromosomes of a young Turkish patient who suffered from a thalassemia intermedia with a low level of Hb F (13.1%) and a relatively high β^A chain synthesis. These data are similar to those obtained for other types of β^+-thal caused by comparable substitutions at positions 31, 29, and 28 bp upstream from the Cap site of the β-globin gene.

INTRODUCTION

Mutations in the human β-globin gene leading to a deficiency in the synthesis of the β chain of human Hb A, characteristic of β-thal, are numerous and over 40 different types have been discovered [summarized in (1)]. Four mutations were found in the proximal segment at -31 to -28 bp (the **ATA** box) relative to the Cap site of the β-globin gene. These mutations [**A->G** at nt -31 (2), **A->G** at nt -29 (3), and **A->G** and **A->C** at nt -28 (4,5)] decrease the efficiency of transcription leading to mild to severe β^+ types of β-thal. In this communication we describe an additional transcription mutation found in a Yugoslavian and two Turkish families, which involves a **T->A** substitution at nt -30 and results in a relatively mild type of β^+-thal in the homozygote.

METHODS

The first patient (R-927) was an 8-year-old Yugoslavian boy with a clinically severe Hb Lepore-β-thal who was studied earlier when the β-thal chromosomes of 10 patients with this condition were analyzed (6). Hematological and Hb composition data for the propositus and his parents are given in Table 4b/1. The patient and his father were chosen for further investigation of their DNA because an extensive search for the mutation causing their β-thal was negative. Haplotype analysis showed that their β-thal chromosome was type IV (6). The second patient was a Turkish teenager who suffered from an intermedia type of β^+-thal with a low level (13%) of Hb F and Hb levels between 9 and 10 g/dl. His hematological data and that for his mother are also included in Table 4b/1. A third, Turkish, patient had the same rare mutation on one chromosome and the common IVS-I-110 (**G->A**) mutation on the other.

DNA was isolated from white cells of peripheral blood using the method of Poncz et al (7). An ~700 bp segment of DNA, which includes exon 1, exon 2, intron 1, a large segment 5' to exon 1, and a smaller segment 3' to exon 2 was amplified with Taq polymerase using methodology developed by Saiki and collaborators (8,9) and described in detail before (10). Sequence analyses of amplified DNA was with the dideoxy procedure of Sanger et al (11) using three 19-20 nt long primers, as indicated elsewhere (10). Details about this procedure can be found in Refs. 9,12-14). The presence of the newly discovered mutation was confirmed by dot-blot hybridization of amplified DNA using appropriate probes (10,15,16).

In vitro chain synthesis analyses involved the incorporation of ^{14}C-leucine into the various globin chains synthesized by reticulocytes from blood of the Yugoslavian patient; the methodology has been detailed before (17,18).

RESULTS AND DISCUSSION

It is often difficult to determine if normal β^A chain synthesis exists *in cis* to the β-thal determinant in patients with a combination of a β-thal mutation and an abnormal Hb, mainly because the severity of the condition

may require frequent blood transfusions. The propositus in this study has been reported to have β^{o}-thal (6). However, more recent *in vitro* chain synthesis analyses using a blood sample which was collected several months after the last transfusion showed that about 10% of the non-α (i.e β^{+} $\delta\beta$-Lepore + γ) chain production is contributed by normal β^{A} chains. This number agrees reasonably well with the 7.8% Hb A that was present in that blood sample (Table 4b/1). Thus, the propositus has a Hb Lepore-β^{+}-thal condition of a clinical severity comparable to that seen in other patients with this condition but with other β-thal determinants (6).

TABLE 4b/1. Hematological and Hb Composition Data[a]

	R-927[b]	Father	Mother	O.K.[c]	Mother
Origin Sex-Age Diagnosis	Yugoslavia M- 8 Lepore-β^{Th}	Yugoslavia M-37 A-β^{Th}	Yugoslavia F-30 A-Lepore	Turkey M- Homoz. β^{Th}	Turkey F- A-β^{Th}
Hb (g/dl)	9.2	14.2	12.4	10.0	12.9
RBC (10^{12}/l)	3.9	6.0	6.1	4.0	5.9
MCV (fl)	75.0	71.0	64.0	82.0	75.0
MCH (pg)	24.0	23.7	20.2	24.4	21.6
Hb F (%)	80.0	2.0	1.5	13.1	0.8
Hb A (%)	7.8	92.5	86.9	82.7	93.9
Hb A_2 (%)	2.4	5.5	2.5	4.2	5.3
Hb Lepore (%)	9.8	0	9.1	0	0
G_γ in Hb F (%)	61.4	74.2	19.2	33.0	13.5

[a] Hbs F, A, A_2 and Lepore quantitated by cation exchange HPLC (19); G_γ quantitated in isolated Hb F by reversed phase HPLC (20,21).

[b] Sample was collected 8 months after the last blood transfusion. The β-thal mutation occurred on a chromosome with haplotype IV (6,22) or [- + + - + + - + +]; restriction sites: Hinc II at ε ; Xmn I 5' to G_γ; Hind III at G_γ and A_γ; Hinc II at $\psi\beta$ and 3' to it; Ava II at β; Hpa I and Bam HI 3' to β.

[c] Patient was not transfused. The mutation occurred on both chromosomes, each with haplotype VII or [+ - - - - - - + +].

Amplified DNA samples from both the patient and his father were sequenced. One can assume that only DNA from the β-thal chromosome of the propositus was amplified because the second chromosome is characterized by a large deletion of 7 kb involving the 3' end of the δ-globin gene, the intergenic region, and the 5' end of the β-globin gene (23). Sequence analyses identified one single base substitution at nt position -30 to the Cap site; in normal DNA this nt is T but in the DNA of the propositus it was replaced by A. Similar analyses of DNA from the father showed that the same position was occupied by a T and an A, indicating the presence of two species of DNA. Dot-blot analyses with synthetic probes specific for the T->A mutation at nt -30 to the Cap site confirmed the substitution (data not shown). DNA samples from several Mediterranean patients with β-thal were tested with the same procedure. This survey resulted in the detection of one heterozygous and one homozygous patient.

The **ATAAA** (nts -31 through -26) element of the promoter region 5' to the β-globin gene is a determinant in the efficiency of transcription, and mutations within this **ATA** box will have a quantitative effect on the expression of the β-globin gene. Four substitutions have been described thus far, all leading to a mild to severe type of β-thal. Nt **A** at -31 was replaced by **G** in a Japanese β-thal patient (2); an **A->G** substitution at nt -29 is common in Black β-thal patients (3,10); **A->G** and **A->C** substitutions at nt -28 have been found in Chinese β-thal patients (4), and in a Kurdish Jew (5), respectively. All subjects have a β^+-thal with a rather variable β chain synthesis *in cis* to the β-thal determinant and a variable clinical condition. The newly discovered **T->A** substitution at nt -30 is no exception; the β-thal condition in combination with Hb Lepore appears to be rather severe with a modest β^A chain synthesis *in cis*. The **T->A** at nt -30 occurred on a chromosome with haplotype IV, which also has a **C->T** substitution at position -158 to the Cap site of the $^G\gamma$-globin gene. The presence of such a chromosome may be advantageous because an increase in γ chain production (mainly $^G\gamma$) has been noted in patients with SS (24,25) and β-thal (10,16) who have one or two chromosomes with this polymorphism. The Hb F production in our propositus was also considerable and nearly two-thirds of the γ chains were of the $^G\gamma$ type (Table 4b/1).

The low Hb F level in the untransfused patient O.K. with a homozygosity for this mutation is surprising, while the β chain production is considerable and much higher than expected from the data obtained for R-927 with Hb Lepore-β^+-thal. The difference between these two patients is in the haplotypes characterizing their β^+-thal chromosome; perhaps the β-thal chromosome with haplotype VII (patient O.K.) is capable of directing a higher *in vitro* β^A chain synthesis than is the β-thal chromosome with haplotype IV (patient R-927).

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CHAPTER 4c

MILD AND SEVERE β-THALASSEMIA AMONG HOMOZYGOTES FROM TURKEY: AMPLIFICATION OF THE TYPES BY HYBRIDIZATION OF AMPLIFIED DNA WITH SYNTHETIC PROBES

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ABSTRACT

Through the procedure of gene amplification combined with hybridization to synthetic 19 bp oligonucleotide probes, it has been possible to identify nine different mutations in the DNA of 47 subjects from Turkey and Northern Cyprus with a β-thal homozygosity. The IVS-I-110 (G->A) and the IVS-I-6 (T->C) substitutions and the frameshift at CD 8 were most frequently observed. Direct correlations were made between these data and clinical observations; mild disease was associated with homozygosity for IVS-I-6 (T->C), for frameshift at CD 8, for the C->G substitution at nt -87, and for IVS-I-5 (G->T), and for a double heterozygosity for some of these conditions. Moderate disease, observed in some of the patients, could be explained by combinations of specific mutations. All mutations were associated with specific haplotypes, while in some, the observed β^{o}-thal was of the mild type due to a considerable production of Hb F.

INTRODUCTION

The clinical condition of the β-thal homozygotes, attending major hematology clinics in Istanbul, Ankara, Adana, and in Northern (Turkish) Cyprus, varies from mild to severe, which has been documented in different reports (1-5). The present study was designed to identify the different types of β-thal in a large number of patients through hybridization of amplified DNA with various synthetic probes. These results will be compared with clinical and hematological data, and with the haplotypes that were observed.

MATERIALS AND METHODS

Blood samples were collected in vacutainers with EDTA as anticoagulant and studied at the home institutions, and at the laboratories in Augusta, GA, USA. Most samples were brought by air by some of the authors, which greatly shortened the transportation time. The patient distribution was: Adana, 14; Ankara, 14; Cyprus, 14; Istanbul, five. The selection of the patients attending the various clinics was at random; they originated from Northern Cyprus; Thrace or Northwestern Turkey (attending the Istanbul clinic); the Mediterranean coast, Central and Western Turkey (attending the Ankara clinic); Southeastern Turkey (Adana and surrounding villages); the Black Sea coast in Northern Turkey (one patient), and Eastern Turkey near the Syrian border (one patient). None of the patients were related. Informed consent was obtained.

Hematological data were collected with automated cell counters. The level of fetal Hb was determined by alkali denaturation (6) or by cation exchange HPLC (7) when Hb A was absent. The γ chain composition of (isolated) Hb F was determined by reversed phase HPLC (8,9).

DNA was isolated from white cells using the method described by Poncz et al (10). Haplotyping followed procedures routinely in use in our laboratories (11,12). Restriction sites included Hinc II 5' to ε, Xmn I 5' to $^{G}\gamma$, Hind III at $^{G}\gamma$ and $^{A}\gamma$, Hinc II at ψβ and 3' to it, Ava II within β, and Hpa I and Bam HI 3' to β. Nomenclature used was the one introduced by Orkin et al (13). Family studies facilitated the determination of the haplotypes of the various β-thal chromosomes.

The method of DNA amplification was a modification of the procedure of Saiki et al (14) and has been described in detail elsewhere (15). Each amplification required two deoxynucleotide primers, 19 or 20 bases long, chosen so they flanked the suspected mutation point, and had sufficient sequence differences with the δ-globin gene to prevent amplification of the comparable segment of this gene. Hybridization was with 9 bp oligonucleotide probes, synthesized so that the mutation points were in the center. The amplified DNA samples were applied to a nylon membrane using a Bio-Dot spotting apparatus (both from Bio-Rad, Rockville Center, NY, USA). Application was in duplicate; one of the spots was used for hybridization to the normal probe, and the other to the mutant probe. All probes were labeled at the 5' end with ^{32}P-γ-ATP as previously reported (16). DNA primers and probes were synthesized using an Applied BioSystems 380 B DNA synthesizer (Applied BioSystems Inc., Foster City, CA, USA).

RESULTS

Identification of β-Thal Mutations. Hybridization of amplified DNA with synthetic probes identified the following mutations among the 94 chromosomes of the 47 patients who were studied: The G->A substitution at IVS-I-110 (19,20) 40 chromosomes; the T->C substitution at IVS-I-6 (21,22), 22 chromosomes; the frameshift at CD 8 (17,18), 13 chromosomes; the G->A substitution at IVS-II-1 (23), eight chromosomes; the G->A substitution at IVS-I-1 (24), four chromosomes; the C->T substitution at CD 49 (29), two chromosomes; the G->T substitution at IVS-I-6 (26), two chromosomes; the C->G substitution at nt -87, 5' to β (27), two chromosomes, and the C->G substitution at IVS-II-745 (27), one chromosome.

Correlation of Specific Mutations With Haplotypes and Clinical Observations. The data are summarized in Table 4c/1. Presentation of the results is facilitated by listing average values only; some of the ranges will be listed in the text.

The mutation, most frequently observed, was the G->A substitution at IVS-I-110; 15 patients were homozygous for this condition (10 were from Cyprus, four from Adana, and one from Istanbul) and all were severely affected. Hb F values varied from 1.3 to 36.6% and were greatly influence by the blood transfusion schedule. As many as 27 chromosomes had haplotype I, one patient was homozygous for haplotype II [which also carries the $A_\gamma T$ substitution resulting in the $A_\gamma T$ variant (28)], and one patient was heterozygous for haplotype IX [which has an Xmn I site 5' to G_γ being associated with high G_γ values (29)].

Seven of the eight patients with the Portuguese type of β-thal (the T->C substitution at IVS-I-6) were homozygous for homozygotes for haplotype VI (and for the $A_\gamma T$ chain), and one for haplotype VII. All eight (three from Adana and five from Ankara) were mildly affected with marked splenomegaly (two were splenectomized) and low Hb F levels (range 3 to 12.2%).

Among the various patients with relatively mild disease were four with a homozygosity for the frameshift at CD 8 (one from Adana, three from Ankara), one patient from Istanbul with a homozygosity for the C->G substitution at nt -87, one Ankara patient with a homozygosity for the G->T for the G->T substitution at IVS-I-5, and two patients (from Ankara) with a homozygosity for the G->A substitution at IVS-II-1. Most of these subjects produced high levels of Hb F (96% or higher; one patient with the frameshift at CD 8 was transfused prior to collection) or had a modest decrease in β chain production (the 52-year-old patient with the C->G substitution at nt -87), while characteristic haplotypes were associated with each of the conditions.

The lower part of Table 4c/1 lists comparable data for 16 subjects who were double heterozygotes for the stated mutations. Moderate to severe disease was present in all patients with the G->A substitution at IVS-I-110 on one chromosome. When the second chromosome carried the G->T mutation at CD 39 or the G->A substitution at IVS-I-1, the transfusion requirements

TABLE 4c/1. Summary for 47 β-Thalassemia Homozygotes From Turkey and Northern Cyprus

β-Thal Type	Haplotype	n	Average Age	Clin. Cond.; Transfusion[a]	Hb F %	$A_\gamma T$ %	G_γ %	A_γ %	Xmn I
IVS-I-110/IVS-I-110 (β^+)[b]	I/IX	1	10	Severe; +++	17.3	0	68.4	31.6	-/+
	I/I	13	11	Severe; +++	9.9	0	49.5	50.5	-/+
	II/II	1	8	Severe; +++	8.6	38.6	61.4	0	-/-
IVS-I-6/IVS-I-6 (β^+)[b]	VI/VI	7	13	Mild; -	7.5	47.2	52.8	0	-/-
	VII/VII	1	10	Mild; -	7.9	0	51.6	48.4	-/-
CD 8/CD 8 (β°)	IV/IV	4	12	Mild; ±[c]	82.6	0	72.5	27.5	+/+
-87/-87 (β^+)	VII/VII	1	82	Mild; -	31.2	0	77.3	22.7	+/+
IVS-I-5/IVS-I-5 (β^+)	IX/IX	1	17	Mild; -	96.0	0	72.1	27.9	+/+
IVS-II-1/IVS-II-1 (β°)	III/III	2	11	Moderate; +	98.3	0	71.5	28.5	+/+
IVS-I-110/CD 39[b]	I/II[b]	1	16	Severe; +++	8.8	35.5	52.0	14.5	-/-
IVS-I-110/IVS-I-1	I/V	2	5	Severe; +++	2.0	0	55.5	44.5	-/-
IVS-I-110/CD 8[b]	I/IV	3	9	Moderate; ++	8.3	0	59.8	40.2	-/+
	II/VII	1	3	Moderate; ++	1.4	21.7	52.8	25.5	-/-
IVS-I-110/IVS-I-6	I/VI	3	24	Moderate; ++	17.2	17.5	52.2	30.3	-/-
IVS-I-6/CD 8	VI/VI	1	8	Mild; -	7.2	18.0	63.9	18.1	+/-
IVS-I-6/IVS-II-1	VI/?	1	7	Mild; -	50.0	11.5	65.1	23.4	?/?
IVS-II-1/CD 39	III/II	1	12	Moderate; +	98.0	17.3	65.1	17.6	-/+
IVS-I-1/IVS-I-6	V/VI	1	20	Moderate; +	54.0	13.9	58.4	27.6	-/-
IVS-II-1/IVS-II-745	V/VII	1	10	Moderate; +	11.0	0	67.7	32.3	-/-
IVS-I-1/IVS-II-1	V/V	1	20	Moderate; +	49.8	0	58.7	41.7	-/-

[a] Evaluation of clinical condition is based on clinical and hematological data, on the age at which the diagnosis was made (all patients listed as severe were diagnosed before the age of 2 years), and on the transfusion frequency required to maintain a functional Hb level; +++ indicates a transfusion every 3-5 weeks; ++ same but three to four times a year, and + an occasional transfusion, mainly following a severe infection, etc.

[b] The molecular defects and the haplotypes are listed in the same order; haplotyping was facilitated by extensive family data (not shown). α-Thal-1 was absent; an α-thal-2 heterozygosity was found in one patient with IVS-I-110/IVS-I-110, one patient with IVS-I-6/IVS-I-6, and one patient with IVS-I-110/CD 8.

[c] Only one patient was transfused before collection because of an infection.

were considerably higher than seen for patients with the second chromosome having the frameshift at CD 8 or the T->C substitution at IVS-I-6. Comparable observations were made for the six patients with the rarer combinations; milder disease was present when one chromosome carried the Portuguese β-thal mutation.

DISCUSSION

The relative simplicity of the DNA amplification method, combined with hybridization to radio-labeled synthetic oligonucleotide probes, has made it possible to identify specific mutations in the DNA of a large number of β-thal homozygotes. Nine different substitutions were identified, with the IVS-I-110 (G->A), the IVS-I-6 (T->C), and the frameshift at CD 8 mutations being the most frequent ones. Several of the patients were homozygous for one specific abnormality, which finds its basis in the fact that parents of some of these subjects were related.

Some of the observations reported here are not surprising, such as the severity and high incidence of the IVS-I-110 (G->A) substitution among the patients from Cyprus and Southern Turkey, and the mild condition with low Hb F levels in subjects with the IVS-I-6 (T->C) mutation. These patients formed the largest group among the 16 subjects clinically characterized as being mildly affected; eight were homozygous, while two had this mutation in combination with another, relatively mild, mutation (Table 4c/1). Rather surprisingly, three mutations were found among the mild homozygous patients who also produced large quantities of Hb F; four such patients had the frameshift at CD 8, one the G->T substitution at IVS-I-5, and two the IVS-II-1 (G->A) substitution. All except the last two patients could be managed without blood transfusions, except for surgical conditions or during periods of severe infections. Indeed, identification of specific mutations is of considerable importance for the management of the patients.

The reasons for the high Hb F production in some of the patients remain unclear, but it may be directly related to the chromosomal haplotype (Table 4c/1). Moreover, the presence of an Xmn I site, due to the C->T substitution at position -158 5' to $^{G}\gamma$ (29), invariably results in $^{G}\gamma$ values in excess of 70%, confirming earlier observations (3). Careful examination of mutations within the promoter regions 5' to $^{G}\gamma$ and to $^{A}\gamma$, and within the enhancer regions 3' to $^{A}\gamma$ of the DNA of some of these patients might provide further insight into the observed variations in Hb F levels.

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CHAPTER 5

POSSIBLE FACTORS INFLUENCING THE HEMOGLOBIN AND FETAL HEMOGLOBIN LEVELS IN PATIENTS WITH β-THALASSEMIA DUE TO A HOMOZYGOSITY FOR THE IVS-I-6 (T->C) MUTATION

CHAPTER 5

POSSIBLE FACTORS INFLUENCING THE HEMOGLOBIN AND FETAL HEMOGLOBIN LEVELS IN PATIENTS WITH β-THALASSEMIA DUE TO A HOMOZYGOSITY FOR THE IVS-I-6 (T->C) MUTATION

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ABSTRACT

We have collected hematological, Hb, and DNA sequence data for 29 patients with a homozygosity for the IVS-I-6 (T->C) mutation with the intention of identifying factors contributing to the observed variability in the severity of the disease. None of the patients had received blood transfusion therapy for at least six months prior to the study. Hb levels varied from 5.0 to 9.9 g/dl. Patients with high Hb F (more than 1.5 g/dl or >20%) had high total Hb levels (7.5 to 9.7 g/dl) but some with low Hb F also had high total Hb levels; two had a concomitant α-thal-2 heterozygosity. An inverse correlation between the Hb F and Hb A_2 levels was observed. The majority of the patients was homozygous for haplotype VI (49/58 chromosomes) but haplotypes IV (2/58) and VII (7/58) were also present. The only haplotype IV homozygote had high Hb F levels with high G_γ

values and the C->T mutation at position -158 in the $^{G}\gamma$ promoter, while both high and low Hb F levels were observed among patients with haplotypes VI and VII. Analysis of sequence variations in regulatory regions included the 5' HS 4, 3, and 2 of the LCR, the $^{G}\gamma$ and $^{A}\gamma$ 5' flanking regions, the IVS-II, and the 5' β-globin gene region in two patients with high Hb F (one homozygote each for haplotypes VI and IV), and in two patients with low Hb F levels (one homozygote each for haplotypes VI and VII). Haplotype specific differences were observed in the LCR 5' HS-2 and in the $^{G}\gamma$ and $^{A}\gamma$ flanking and IVS-II regions; however, no differences were present between the low and high Hb F-producing haplotype VI chromosomes, suggesting a major role for factors which are not linked to the β-globin gene cluster in mediating γ-globin gene expression in patients with this type of β-thal.

INTRODUCTION

The clinical severity of β-thal mainly depends on the degree of β chain deficiency, while a co-inheritance of an α-thal determinant or of factors that increase γ-globin gene expression can moderate the α/non-α-globin chain imbalance and ameliorate the course of the disease (Weatherall and Clegg, 1981). Increased Hb F levels have been associated with certain β-globin gene promoter mutations such as those in the proximal **CACCC** box (Gonzalez-Redondo et al, 1988; Camaschella et al, 1990), with the co-inheritance of an HPFH determinant in cis to the mutated β gene as in the Sardinian δβ-thal (Ottolenghi et al, 1987), and most frequently with a chromosome that carries the -158 (C->T) substitution in the $^{G}\gamma$ promoter (Gilman and Huisman, 1985; Thein et al, 1987; Diaz-Chico et al, 1988). Sequence variations located in the LCR 5' HS-2 (Oner et al, 1992), the $^{A}\gamma$ IVS-II (Ragusa et al, 1992a), and the 5' flanking region of the β-globin gene (Ragusa et al, 1992b) have also been implicated in the regulation of Hb F expression, mainly in patients with SS and β-thal. An X-linked determinant has also been associated with elevated Hb F levels and F-cell production in normal adults and SS patients (Miyoshi et al, 1988; Dover et al, 1992).

The influence of these factors on Hb F production and on the total Hb levels in β-thal patients is difficult to assess due to the large number of mutations that affect β chain synthesis, while blood transfusions received by most of these patients also complicate the studies. We have analyzed a group of 29 patients homozygous for the IVS-I-6 (T->C) mutation who had not received blood transfusions for at least six months prior to the study. This mutation is one of the more common β-thal defects in the Mediterranean population, with high frequencies in Sicily, the former Yugoslavia, Turkey, and Portugal (Huisman, 1990a). Homozygotes are often mildly affected due to the considerable β-globin chain synthesis by the mutated chromosome and usually have low Hb F and high Hb A_2 levels (Huisman, 1990b). The mutations present in the subjects of this study were previously characterized by PCR and by dot-blot hybridization during the molecular characterization of β-thal alleles in Portugal, Sicily, Turkey, and the former Yugoslavia (Tamagnini et al, 1993; Schiliro et al, in preparation; Oner et al, 1990; Dimovski et al, 1990). Fresh blood samples were collected from all patients and re-analyzed to investigate the factors that could account for variations in total Hb, Hb F, and Hb A_2 levels.

PATIENTS AND METHODS

Patients. Blood samples from 29 homozygotes (13 from Portugal, seven from Sicily, seven from Turkey, and two from Macedonia) and 22 family members were collected in EDTA and transported to Augusta, GA, on ice by fast air service. The ages of the patients ranged from 2 to 58 years; about half had been splenectomized. All patients were clinically classified as thalassemia intermedia with Hb levels at steady state ranging from 5.0 to 9.7 g/dl. More than two-thirds of the patients had never been transfused, and none had received blood transfusions during the last six months prior to blood sampling. Variability in the severity of the disease has been reported by several of the participating physicians; we have relied mainly on the hematological observations, especially the total Hb levels, in comparing the severity of the disease in the 29 homozygotes, since these data were obtained in the same laboratory and were confirmed in repeated analyses. Informed consent was obtained in all cases.

Hematological and Hb Analysis. Complete blood counts and red cell indices were obtained with an automated cell counter. The Hb F and Hb A_2 levels were quantified by cation exchange HPLC (Bisse and Wieland, 1988; Kutlar et al, 1990). The relative quantities of the three γ chains in isolated Hb F were determined by reversed phase HPLC (Shelton et al, 1984; Kutlar et al, 1986).

DNA Analysis. DNA was isolated from white blood cells with the method of Poncz et al (1982). Haplotypes of the β-globin gene cluster were determined with methodology described previously (Aksoy et al, 1985); the restriction sites were Hinc II 5' to ε, Xmn I 5' to $^{G}\gamma$, Hind III at $^{G}\gamma$ and $^{A}\gamma$, Hinc II at $\psi\beta$ and 3' to it; Ava II at β, and Bam HI 3' to β.

The number of α-globin genes was determined by digestion with Bam HI, Hind III, Hpa I, and Bgl II, and hybridization with α, ζ, and θ1 probes (Felice et al, 1984; Gu et al, 1987). The possible presence of α2-globin gene mutations commonly found in the Mediterranean populations (initiation codon mutation **ATG->ACG**; 5 bp deletion in the IVS-I donor splice site; **AATAAA->AATAAG** and **AATAAA->AATGAA** in the poly A signal, and the termination codon **TAA->AAA** mutation) (Higgs et al, 1989) was evaluated by hybridization of PCR amplified DNA with mutation specific oligonucleotide probes as previously described (Yuregir et al, 1992).

Homozygosity for the IVS-I-6 (T->C) mutation was confirmed for all patients by direct sequencing of PCR amplified DNA. Sequence variations in regulatory regions of the β-globin gene cluster were analyzed by amplification and direct sequencing in two patients with high Hb F levels and in two patients with low Hb F levels. The sequenced regions included the 739 bp fragment between the Hind III and Bgl II sites of LCR 5' HS-2; the 300 bp fragment between the Hph I and Fnu 4HI sites of LCR 5' HS-3; the 280 bp fragment between the Sst I and Ava I sites of LCR 5' HS-4; the $\sim$1.3 kb 5' flanking regions of the $^{G}\gamma$- and $^{A}\gamma$-globin genes; the $\sim$400 nts in the 3' halves of the IVS-II ($^{G}\gamma$ and $^{A}\gamma$), and the 5' flanking region of the β-globin gene between positions -550 to -200 relative to the Cap site. Procedural details have been described previously (Lanclos et al, 1991; Oner et al, 1992; Adekile et al, 1993; Dimovski et al, 1993).

RESULTS

Hematological data for the 29 β-thal homozygotes are listed in Table 5/1. Considerable variation in total Hb levels (5.0-9.9 g/dl) was observed which did not correlate with gender, age, splenectomy, or Hb F levels. All patients with high Hb F (>1.5 g/dl) also had high total Hb levels (>8.0 g/dl). Furthermore, an inverse correlation was observed between the Hb F and Hb A_2 levels ($r = -0.87$, $p < 0.00001$) (Fig. 5/1). Repeated analyses were made over the past three years for many Portuguese, Turkish, and Macedonian patients; the data showed that the Hb A_2 and Hb F levels did not change significantly for each individual subject. All 22 heterozygous family members had Hb F levels lower than 1%.

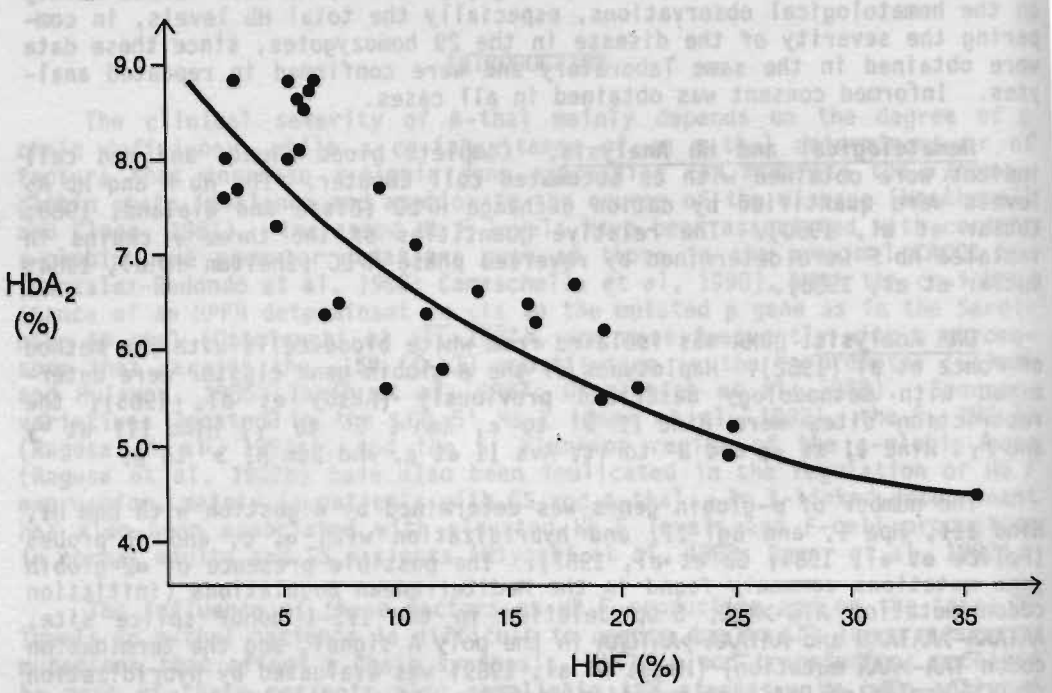

FIG. 5/1. The relationship between the levels of Hb F and Hb A_2.

Twenty-four patients were homozygous for the $A_\gamma T$ variant [A_γ75(E19) Ile->Thr] with an average level of 47.0 ± 11.3%. Four homozygotes for the $A_\gamma I$ allele were observed; the G_γ to A_γ ratio in three averaged 47 to 53, while the fourth patient had a G_γ to A_γ ratio of 74:26. The one patient with all three γ chains (#380) had almost equal amounts of the two types of A_γ chain with a G_γ level of 49.7%. These data suggested that the IVS-I-6 (T->C) mutation occurs on at least three different chromosomes: One characterized by the presence of the $A_\gamma T$ variant, the second with $A_\gamma I$ and low G_γ levels, and the third with $A_\gamma I$ and high G_γ levels. This was confirmed by DNA analysis identifying the mutation on Mediterranean haplotypes VI ([- - + + - - - +], $A_\gamma T$ positive, 49 chromosomes), VII ([+ - - - - - - +], $A_\gamma T$ negative; low G_γ, 7 chromosomes), and IV ([- + + -+ + - +], $A_\gamma T$ negative; high G_γ, 2 chromosomes).

TABLE 5/1. Hematological and Hb Composition Data

Patient	Co.[a]	Sex-Age	RBC 10^{12}/l	Hb g/dl	PCV l/l	MCV fl	MCH pg	MCHC g/dl	A_2 %[b]	F %[b]	$A_\gamma T$ %[c]	G_γ %[c]	$A_\gamma I$ %[c]	Haplo-types	#α Genes
L.R.	P	M-14	3.94	7.6	.255	64.7	19.3	29.8	4.5	35.4	43.0	57.0	0	VI/VI	4
M.S.	P	F-24	4.26	8.2	.275	64.6	19.2	29.8	4.9	24.6	48.8	51.2	0	VI/VI	4
F.R.I[d]	P	M-28	4.87	8.6	.284	58.3	17.7	30.3	6.4	11.4	47.6	52.4	0	VI/VI	4
F.R.II[d]	P	M-23	5.22	9.7	.315	60.3	18.6	30.8	5.6	20.8	46.2	53.8	0	VI/VI	4
C.L.	P	F-19	3.07	6.7	.262	85.3	21.8	25.6	6.3	16.2	57.9	42.1	0	VI/VI	4
V.R.	P	F-38	3.29	6.8	.282	85.7	20.7	24.1	7.1	11.0	57.1	42.9	0	VI/VI	4
H.B.	P	M-10	3.58	7.0	.252	70.4	19.6	27.8	6.6	13.6	46.3	53.7	0	VI/VI	4
H.S.II	P	M-43	4.83	8.6	.331	68.5	17.8	26.0	7.7	9.2	57.8	42.2	0	VI/VI	3
O.R.	P	M-42	4.80	8.0	.268	55.8	16.7	29.9	6.5	7.4	46.7	53.3	0	VI/VI	4
M.H.	P	F-32	5.41	8.8	.293	54.6	16.3	30.0	7.3	4.8	47.8	52.2	0	VI/VI	4
M.A.	P	F-58	4.08	6.2	.290	71.1	15.2	21.4	8.0	2.4	53.3	46.7	0	VI/VI	4
S.B.	P	M-26	4.71	7.8	.300	63.7	16.6	26.0	8.8	2.9	0	48.6	51.4	VII/VII	4
P.S.	P	F-36	4.69	8.5	.303	64.6	18.1	28.1	5.2	21.5	0	74.0	26.0	IV/IV	4
464	I	F-11	4.73	8.0	.287	60.7	16.9	27.9	5.2	24.7	48.8	51.2	0	VI/VI	4
375	I	F- 2	4.95	7.6	.274	55.4	15.4	27.7	5.8	12.1	35.6	64.4	0	VI/VI	4
386	I	M-19	4.15	7.3	.281	67.7	17.6	26.0	6.7	18.1	46.2	53.8	0	VI/VI	4
242	I	F- 8	3.70	6.6	.253	68.4	17.8	26.1	5.6	9.6	43.5	56.5	0	VI/VI	4
385	I	M- 4	3.62	6.6	.249	67.5	17.9	26.5	6.4	6.8	47.9	52.1	0	VI/VI	4
376	I	F-12	5.50	7.8	.279	50.7	14.2	28.0	7.7	2.0	50.5	49.5	0	VI/VI	4
380	I	F-13	4.01	7.7	.292	72.8	19.3	26.4	6.5	15.9	23.3	49.7	27.0	VI/VII	4
74	T	F- 9	5.19	8.2	.303	58.4	15.8	27.1	8.0	5.2	-	-	-	VI/VI	4
686[d]	T	M-21	2.77	5.0	.217	78.3	18.1	23.0	8.8	6.4	47.1	52.9	0	VI/VI	4
687[d]	T	F-15	2.94	5.5	.235	79.9	18.7	23.4	8.1	5.8	48.4	51.6	0	VI/VI	4
688	T	M-42	4.83	9.6	.309	64.0	19.9	31.1	7.6	2.5	35.1	64.9	0	VI/VI	3
689	T	M-46	3.16	6.9	.254	80.4	21.8	27.2	8.8	5.3	40.8	59.2	0	VI/VI	4
690	T	M-18	4.33	7.1	.245	56.6	16.4	29.0	8.6	5.7	44.0	56.0	0	VI/VI	4
692	T	F- 9	5.22	8.5	.293	56.1	16.3	29.0	8.7	6.2	40.3	59.7	0	VI/VI	4
P-01[d]	M	F-37	4.23	7.3	.315	74.5	17.3	25.2	8.5	6.0	0	46.2	53.8	VII/VII	4
P-01B[d]	M	M-31	5.45	9.9	.388	71.2	18.2	25.5	6.2	19.2	0	45.8	54.6	VII/VII	4

[a] P = Portugal; I = Italy; T = Turkey; M = Macedonia. [b] By cation exchange HPLC (Bisse and Wieland, 1988; Kutlar et al, 1990). [c] By reversed phase HPLC (Shelton et al, 1984; Kutlar et al, 1986). [d] Relatives.

Two patients had an α-thal determinant: A 3.7 kb deletion was present in patient #688, while a 5.3 kb deletion was identified in patient H.S.II (Higgs, 1993). Both had high total Hb levels (9.6 and 8.6 g/dl, respectively) with low Hb F values (2.5 and 9.2%, respectively). None of the patients carried an α-globin gene triplication, nor were any of the five common Mediterranean nondeletional α-thal determinants present.

Data from the sequence analysis of the regulatory regions within the β-globin gene cluster are summarized in Fig. 5/2. These analyses were performed for four Portuguese patients; two with high Hb F levels (P.S., 21.5% and L.R., 35.4%) were homozygotes for haplotypes IV and VI, respectively, and two patients with low Hb F levels were homozygous for haplotype VI (M.A., 2.4%) and haplotype VII (S.B., 2.9%). Sequencing of the LCR 5' HS revealed the absence of any sequence variations except for the presence of haplotype specific **AT** repeats in HS-2. Differences were observed at three positions in the 5' $^{G}\gamma$ flanking region, namely -1067, -807, and -158; haplotype VII differed at all three positions, while haplotypes VI and IV differed only by the **C->T** substitution at position -158. The sequences of the $^{G}\gamma$ IVS-II regions for haplotypes IV and VI were also identical, while haplotype VII had a different **TG** motif from position +1061 and a **T->G** substitution at position +1295. This substitution abolishes a Hind III restriction site and creates a duplicated **CACCC** motif (**GGGTGT**) (Fig. 5/2). Other differences were present in the 5' $^{A}\gamma$ flanking region; haplotype VI was identical to haplotype IV at position -1072 and to haplotypes VII at position -588, but differed from these two haplotypes by a 4 bp deletion between positions -225 and -222. This deletion was previously described as a promoter mutation characteristic for $^{A}\gamma^{T}$ alleles (Gilman et al, 1988). All three haplotypes had characteristic **TG** motifs in the $^{A}\gamma$ IVS-II sequence from position +1061, and also differed at positions +1143, +1268, and +1275 (Fig. 5/2). The last two sequence variations create a duplicated **CACCC** motif in haplotype IV, while only a single **CACCC** motif is present around +1268 in haplotype VI and around +1275 in haplotype VII. All three haplotypes contained the same $(AT)_7(T)_7$ motif at -540 in the β-globin gene flanking region.

The sequence data obtained for four chromosomes from the two patients with haplotype VI demonstrated complete sequence identity. The chromosome with haplotype IV was identical to the previously characterized β^{S} chromosome with haplotype #3 (Senegal), except for the **AT** repeats in the 5' β-globin gene flanking region (Lanclos et al, 1991; Oner et al, 1992; Elion et al, 1992), while the chromosomes with haplotypes VI and VII differed from all previously described chromosomes.

DISCUSSION

Approximately half the patients described here (15/29) had steady state Hb levels between 6.5 and 8.0 g/dl. Higher Hb levels (>8.0 g/dl) seen in six patients (L.R., M.S., F.R.II, P.S., #464, and P-01B) could be accounted for by their high Hb F values (>20.0%), because elevated γ chain production decreases the imbalance in α/non-α ratio, and thus prolongs red cell survival. A second group of six patients (H.S.II, O.R.,

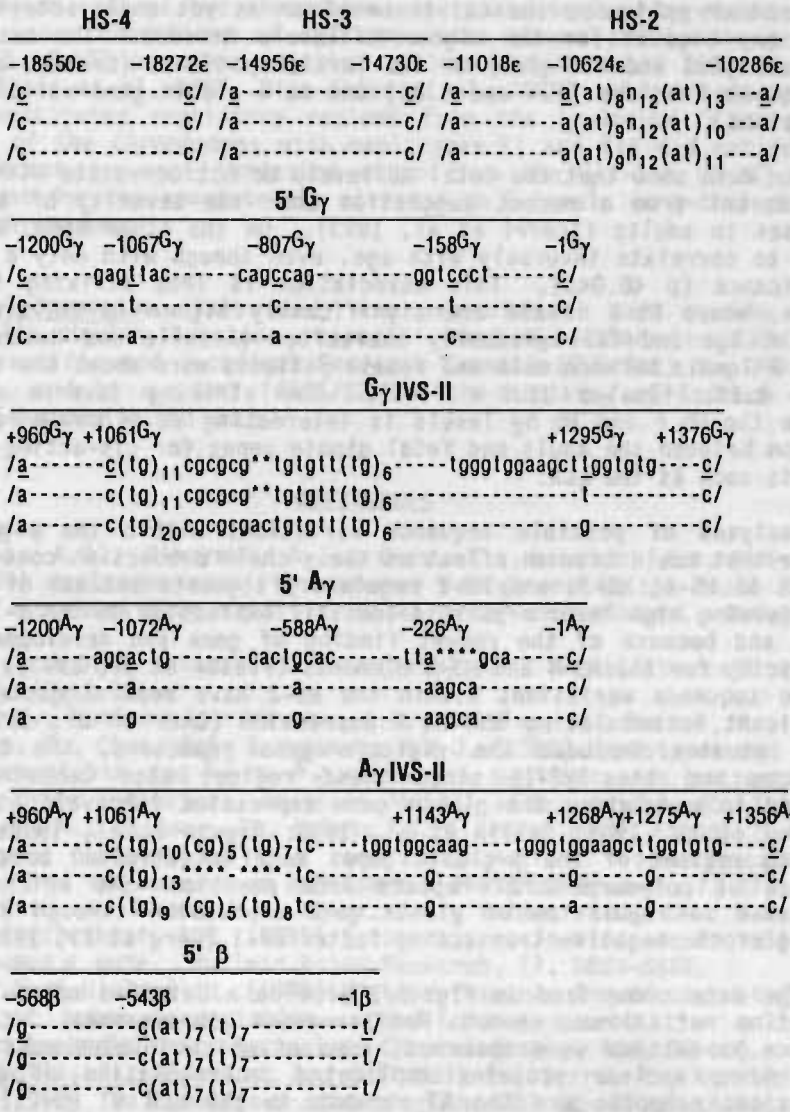

FIG. 5/2. Sequence comparison of β-globin gene cluster regulatory regions in patients homozygous for haplotypes VI (L.R. and M.A.), IV (P.S.), and VII (S.B.). No nt differences were observed between the two patients with haplotype VI and high (L.R.) or low (M.A.) Hb F levels. The numbers above the sequences refer to the positions of the underlined nts, and are relative to the Cap sites of the particular genes as located on chromosome A (Slightom et al, 1989). Asterisks (*) indicate the absence of nts at these positions. n_{12} = acacatatacgt.

M.H., #74, #688, and #692) had similar Hb levels (>8.0 g/dl) but low Hb F values (<10.0%). In two (#688 and H.S.II) a concomitant α-thal-2 deletion (-3.7 and -5.3 kb, respectively) was identified. Even though the most common α-thal determinants in the Mediterranean population were excluded

in all other patients, the existence of an as yet uncharacterized α-thal allele may account for the higher Hb levels in some. The extremely low Hb levels (5.0 and 5.5 g/dl) in two Turkish patients (brother and sister) with low Hb F values (6.4 and 5.8%) and no α-globin gene variation remain unexplained.

Our data show that the total Hb levels do not correlate with age which is different from a recent suggestion that the severity of the disease decreases in adults (Scerri et al, 1993). On the other hand, Hb F levels appear to correlate inversely with age, even though with only a borderline significance ($p < 0.045$). This association is less striking than in SS disease, where Hb F levels are significantly higher in children below 5 years of age and fall gradually thereafter (Adekile and Huisman, 1993). The Hb F levels between male and female patients were about the same (males 11.8 ± 8.7%; females 11.3 ± 7.4%). The striking inverse correlation between the Hb F and Hb A_2 levels is interesting as it could reflect competition between the adult and fetal globin genes for cis-acting regulatory elements such as the LCR.

Analyses of possible sequence variations within the β-globin gene cluster that could have an effect on the γ chain production concerned first the LCR 5' HS-4, HS-3, and HS-2 regulatory segments because of their role in conferring high level erythroid-specific expression on the β-like globin genes, and because of the recent finding of gene and developmental stage specificity for the HS-4 and HS-3 elements (Fraser et al, 1993). Moreover, certain sequence variations within the HS-2 have been suggested as being significant in modulating the Hb F expression (Oner et al, 1992). Additional studies included the γ-globin gene promoters, the 5' flanking sequences and the IVS-II since these regions also contain sequences involved in modulating the globin gene expression (Grosveld et al, 1993; Antoniou et al, 1988; Collis et al, 1990; Ragusa et al, 1992a). The 5' flanking regions of the β-globin genes were investigated because of the presence of polymorphic **AT** repeats from position -540 which have been implicated in regulation of globin gene expression through differential binding of the negative transacting factor BP-1 (Berg et al, 1989).

The data summarized in Fig. 5/2 provide a detailed molecular characterization of three common Mediterranean chromosomes. Considerable sequence variations were observed, some of which involve possible motifs for binding nuclear proteins implicated in regulation of globin gene expression; examples are the **AT** repeats in the LCR 5' HS-2, and the **TG** repeats and **CACCC** motifs in the $^{G}\gamma$ and $^{A}\gamma$ IVS-II. However, these sequence variations are all confined to specific haplotypes and cannot account for variations in the Hb F levels among the patients with the same haplotypes. Our findings, therefore, suggest a major role for unknown genetic loci, separated from the β-globin gene cluster, in controlling γ chain production in β-thal patients. This is further supported by the differences in total Hb and Hb F levels between the siblings in two families, namely P-01 and P-01B, and F.R.I and F.R.II.

Sequence analysis of the DNA from the homozygote for haplotype IV (P.S.) showed that this chromosome is identical to the β^S chromosome with haplotype #3 (Senegal), except for the **AT** repeats in the 5' β-globin gene

flanking region. These two chromosomes are characterized by elevated Hb F expression under conditions of erythropoietic stress and by the presence of the -158 (C->T) promoter mutation (Gilman and Huisman, 1985; Labie et al, 1985; Thein et al, 1987; Diaz-Chico et al, 1988). Sequence comparison of the investigated regulatory regions from the haplotype IV chromosome with those of the chromosomes with haplotypes VI and VII did not identify any significant sequence variation other than the G_γ -158 (C->T), which could account for the consistently elevated Hb F levels in β-thal patients with this chromosome.

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CHAPTER 6

THE -158 (C->T) PROMOTER MUTATION IS RESPONSIBLE FOR THE INCREASED TRANSCRIPTION OF THE 3' Gγ GENE IN THE ATLANTA TYPE OF HPFH

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THE -158 (C->T) PROMOTER MUTATION IS RESPONSIBLE FOR THE INCREASED TRANSCRIPTION OF THE 3' γ GENE IN THE ATLANTA TYPE OF HPFH

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ABSTRACT

The Atlanta type of HPFH is characterized by a mild elevation of Hb F (2-5% in heterozygotes), almost exclusively of the $^{G}\gamma$ type (<90%). Gene mapping analysis has identified this condition as a -$^{G}\gamma$-$^{G}\gamma$- arrangement with the -158 (C->T) substitution in the promoters of both $^{G}\gamma$ genes. We have reevaluated this condition in members of two families. Sequence analysis identified only two changes in the 3' γ gene as compared to the $^{A}\gamma$ gene from a chromosome with haplotype #3 (or Senegal), namely the -158 (C->T) promoter mutation and the C->G change in CD 136, which accounts for the -$^{G}\gamma$-$^{G}\gamma$- phenotype. The absence of any other nt substitution provides genetic evidence that the -158 (C->T) change is primarily responsible for the elevated Hb F levels associated with this condition. A quantitative RT/PCR procedure, presented in detail in this report, was developed to determine the effect of this mutation on the transcription of individual γ genes in four individuals with the Atlanta type of HPFH. Both γ-globin genes, i.e. the (5') $^{G}\gamma$ and the (3') $^{G}\gamma$-Atlanta genes of the Atlanta type of HPFH chromosome, expressed elevated amounts of transcripts, which were present in nearly equal amounts. This demonstrates that the -158 (C->T) mutation exerts its effect on the transcriptional rate of the gene with which it is associated.

INTRODUCTION

The -G_γ-G_γ- gene arrangement is a common anomaly observed with a frequency of 4.6% among Blacks (1). Adults with this condition have low Hb F (<1.3%) and high G_γ (71-100%) values (2). The same γ-globin gene arrangement has been observed in two families with the Atlanta type of HPFH (3,4). Heterozygotes for this condition have elevated Hb F levels of 2-5% which are higher in young children and in compound heterozygotes with Hb S (2-5). The G_γ values in all affected individuals exceed 90% (2). Gene mapping analysis has shown that both G_γ genes on this HPFH chromosome contain the -158 (C->T) change in their promoters (5).

The -158 (C->T, G_γ) mutation has been found on normal, β-thal, and β^S chromosomes. Chromosomes with T at -158 (G_γ) are capable of conferring an increase in Hb F expression under conditions of erythropoietic stress as is seen in patients with β-thal major or SS (5-8). Both increased and normal Hb F levels with high G_γ values have been observed in β-thal heterozygotes with a T at -158 (G_γ) on the thalassemic chromosome (2). Increased Hb F levels (1-3%) with high G values have also been observed in normal adults with the Swiss type of HPFH (9). Many have at least one chromosome with T at -158 (G_γ); however, others with the same C->T substitution on one or even two chromosomes, have only high G_γ values (60-98%) with normal Hb F levels (1%) (9). More recently, flow-cytometric analysis with an anti Hb F monoclonal antibody demonstrated a small increase in F-cell percentages in individuals homozygous or heterozygous for the C->T change at -158 (G_γ) (10).

To investigate the possible role of the -158 (C->T) substitution on γ-globin gene expression in the Atlanta type of HPFH, we studied the sequences of the 5' γ (G_γ) and 3' γ (G_γ-Atlanta) globin genes in four members of two families with this condition. A competitive RT/PCR method was developed to evaluate the relative amounts of the transcripts from the individual γ genes in this condition.

MATERIALS AND METHODS

Hematological and Hb Composition Data. Blood samples from three members of a previously reported family with the Atlanta type of HPFH (4) and from two new cases (a 19-year-old Black female and her 2-year-old son) were collected in vacutainers with EDTA as anticoagulant and transported in ice to the laboratory. Informed consent was obtained. Three normal adults served as controls. Complete blood counts and red cell indices were obtained with an automated cell counter. The Hb F and Hb A_2 were quantified by cation exchange HPLC (2,11). The relative quantities of the G_γ and A_γ chains in isolated Hb F were determined by reversed phase HPLC (12,13). Serum ferritin levels were analyzed with the Quantimune Ferritin IRMA (Bio-Rad Laboratories, Richmond, CA, USA).

DNA Analysis. DNA was isolated from the white blood cells with the method of Poncz et al (14). Gene mapping analysis was with Bgl II, Xmn I, and Pst I, and the γ-IVS-II probe as previously described (5). The 5'

flanking regions of the $^{G}\gamma$- and $^{A}\gamma$-globin genes were amplified independently with 5' oligonucleotide primers specific for $^{G}\gamma$ (5'-ACGTCATAATCTACCAAGGTCATG-3', positions -1227 to -1204) or $^{A}\gamma$ (5'-AGCTTAGGGGATAAACTAATTTG-3', positions -1279 to -1257) (all numbers are relative to the Cap site of the nearest γ gene). A common 3' amplification primer was used (5'-GGCGTCTGGACTAGGAGCTTATTG-3', positions +53 to +30). The IVS-II regions were amplified with a common 5' primer (5'-TGGACCCAGAGGTTCTTTGA-3', positions +287 to +306), and 3' primers specific for $^{G}\gamma$ (5'-TGTGCTGCAATCCAGGGAAGGGGGT-3', positions +1948 to +1924) or $^{A}\gamma$ (5'-CTGCAATCAATCCAGCCCCCAGGTC-3', positions +1908 to +1885). The specificity of the PCR procedures in amplifying only $^{G}\gamma$ or $^{A}\gamma$ sequences has been established in a previous study, which also gives details of the PCR and sequencing reactions (15). The presence of the most common α-thal-2 determinant among Blacks (the $-\alpha^{3.7}$ kb deletion) was investigated with a PCR-based procedure (16).

RNA Analysis. Total cellular RNA was isolated with the procedure of Chomczynski and Sacchi (17). Briefly, the cells in 10 ml of freshly collected blood were washed twice with PBS, centrifuged at 2,500 rpm for 10 minutes at 4°C, and the reticulocyte-enriched top fraction (1 ml) was transferred into four 1.5 ml Eppendorf tubes. Five hundred µl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M -mercaptoethanol) were added to each tube, followed by 50 µl of 2 M sodium acetate, pH 4, 0.5 ml of acid phenol (water saturated), and 100 µl of a chloroform-isoamylalcohol mixture (49:1). After centrifugation at 10,000 g for 20 minutes at 4°C, the RNA present in the aqueous phase was precipitated with 0.5 ml of isopropanol at -20°C for at least 1 hour, collected by centrifugation, and dissolved in 15 µl water.

Three µl (1-2 µg of RNA) were RT with an oligo-dT primer, using the Gene Amp RNA/PCR kit (Perkin Elmer Cetus, Norwalk, CT, USA) and the procedure recommended by the manufacturer. The entire RT sample was next subjected to PCR amplification with two oligonucleotide primers (50 pM each) complementary to two regions in exon 2 and in the 3' UTR where the $^{G}\gamma$, $^{A}\gamma$, and $^{G}\gamma$-Atlanta globin genes have identical sequences. Their sequences and positions were: GF 5'-CTGACTTCCTTGGGAGAT-3', positions +381 to +397, and GR 5'-GCTTGCAGAATAAAGCCTA-3' located from nt +55 to nt +33, 3' to the termination CD. The PCR was done as recommended by the manufacturer of the RNA/PCR kit, except that the Taq polymerase was added during the first cycle of denaturation (95°C, 5 minutes). The PCR required 30 cycles of one minute of denaturation at 95°C, one minute of annealing at 54°C, and one minute of extension at 72°C, in a Perkin Elmer Cetus thermal cycler. Fifteen µl of the PCR product were next radioactively labeled with the ^{32}P-labeled GF oligonucleotide primer through only one cycle of extension, which prevented the formation of ^{32}P-labeled $-^{G}\gamma-^{A}\gamma-$, $-^{G}\gamma-^{G}\gamma$-Atlanta, and $-^{A}\gamma-^{G}\gamma$-Atlanta heteroduplexes. The reaction mixture contained, besides the PCR product, 40 pM of the ^{32}P-labeled GF oligonucleotide, 5 µl of reaction buffer (200 mM/L MOPS, pH 7.8, 500 mM/L NaCl, 30 mM/L $MgCl_2$), 2.5 µl of 6 mM dNTP, and 2.5 U of Taq polymerase (Perkin Elmer Cetus) in a final volume of 50 µl. The thermal cycler profile was: Denaturation for 5 minutes at 95°C, annealing for 2 minutes at 54°C, and extension for 12 minutes at 72°C. The GF oligonucleotide primer (800 pM) was previously labeled to high specific activity with 2 µl of ^{32}P-ATP (7,000 Ci/mM; ICN

Biochemicals, Irvine, CA, USA) and 30 U Polynucleotide kinase (USB, Cleveland, OH, USA) (18), and purified by elution through a Nick column (Pharmacia, Uppsala, Sweden). A double restriction enzyme digestion was done on the radioactively labeled PCR product with Pst I (New England Biolabs), which recognizes only the $^{A}\gamma$ sequence at CD 136 (207 bp from the ^{32}P-labeled oligonucleotide), and Hinf I (New England Biolabs), which cuts the $^{A}\gamma$ and the $^{G}\gamma$-Atlanta PCR products at nt position +1502 from the Cap site (254 bp from ^{32}P GF, Fig. 6/1). Four µl of each digestion were analyzed on a non-denaturing 6% polyacrylamide gel and autoradiographed for 4 to 12 hours. The sizes of the fragments were confirmed by analyzing the remainder of the digestion on an ethidium bromide stained 2% agarose gel with a 1 kb ladder (Gibco-BRL, Gaithersburg, MD, USA) as a marker. Densitometric scanning of the autoradiograms was done on a Shimadzu CS4000 densitometer (Columbia, MD, USA).

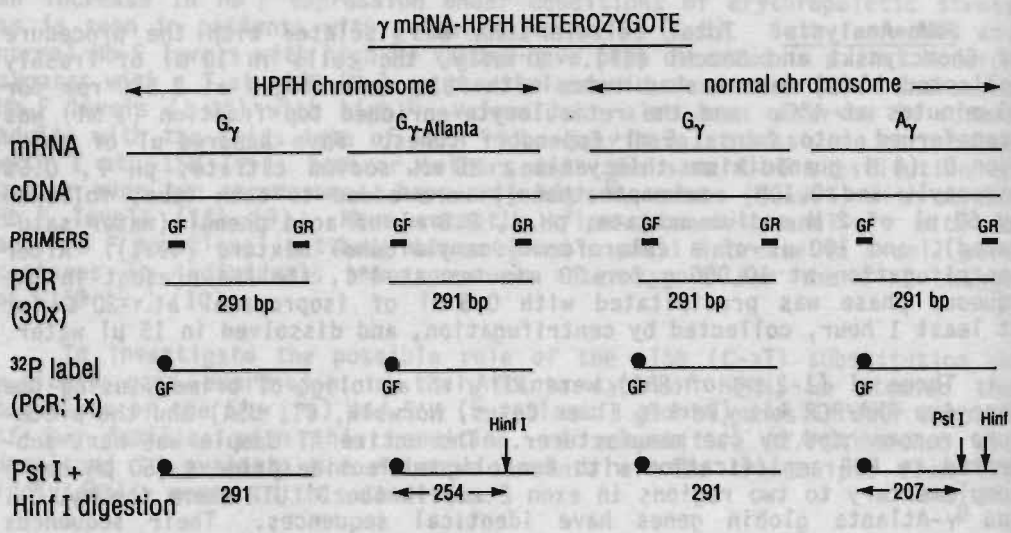

FIG. 6/1. Schematic representation of the RT/PCR analysis for the quantitation of the relative amounts of globin gene transcript from the $^{G}\gamma$, $^{G}\gamma$-Atlanta, and $^{A}\gamma$ genes.

RESULTS

Family W. Two members of this Black family were investigated because of the high Hb F levels (26.2%) in the 2-year-old Hb S heterozygote (Table 6/1). Other hematological and Hb parameters were normal except for a mild microcytosis and hypochromia, possibly of nutritional origin. The mother had only a slightly elevated Hb F level (1.6%), almost exclusively of the $^{G}\gamma$ type (98%). The father was not available for study.

Family B. This family was studied in 1984 when six members were characterized as heterozygotes for the Atlanta type of HPFH (4). A 32-year-old female (S.B.) and her two daughters (N.B. and M.B.), both with an additional Hb S heterozygosity, were reevaluated (Table 6/1). All three had

TABLE 6/1. Hematological/Hb Composition Data for Members of Two Families With the Atlanta Type of HPFH

Patient; Relationship	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	S %	F %	G_γ[a] %	Ferritin[b] ng/dl	#α Genes
Family W												
D.W.; proband	F-19	12.7	.401	4.52	88.7	28.1	2.3	0	1.6[c]	98.0	4	3
C.W.; son	M- 2	13.5	.387	5.05	76.6	26.7	2.3	39.2	26.2[c]	92.6	n.d.	4
Family B												
S.B.; proband	F-32	11.5	.357	4.20	85.0	27.4	2.1	0	4.2[c]	100.0	4	3
1984 data	F-23	10.5	.325	3.59	91.0	28.7	2.6	0	5.5[d]	100.0		
N.B.; daughter	F-15	7.8	.274	4.66	58.8	16.7	2.1	34.4	5.3[c]	100.0	7	4
1984 data	F- 6	13.0	.350	4.64	75.0	28.0	2.9	40.6	9.8[d]	92.1		
M.B.; daughter	F- 9	10.6	.317	4.21	75.3	25.2	2.9	33.8	6.2[c]	100.0	25	3
1984 data	F- 3/12	9.8	.260	3.40	76.0	28.8	1.8	21.2	40.0[d]	86.1		

[a] By reversed phase HPLC (12,13).

[b] Normal range = 10-260 ng/ml.

[c] By cation exchange HPLC (2,11).

[d] By alkali denaturation (19).

in Table 6/2. The $^{G}\gamma$-Atlanta transcripts were present only in the four individuals with the Atlanta type of HPFH (Fig. 6/3). The levels of these transcripts were equal or slightly higher than those of the $^{G}\gamma$ transcripts, while the $^{A}\gamma$ transcripts were absent (in S.B.) or below 4% (Table 6/2). Higher levels of $^{G}\gamma$ than $^{A}\gamma$ transcripts were present in the three controls (Table 6/2). The values were comparable to those obtained for the $^{G}\gamma$ and $^{A}\gamma$ chains in isolated Hb F by reversed phase HPLC.

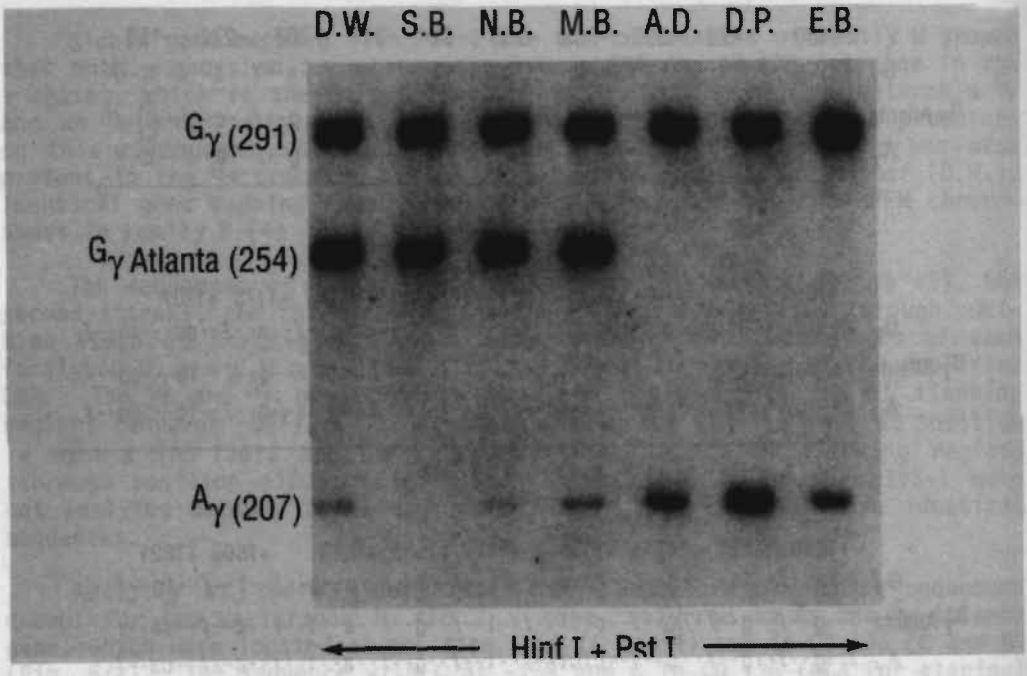

FIG. 6/3. Autoradiography of digested ^{32}P-labeled RT/PCR products for the analysis of $^{G}\gamma$, $^{G}\gamma$-Atlanta, and $^{A}\gamma$ mRNA in heterozygotes for the Atlanta type of HPFH and in three controls. The values obtained after densitometric scanning of the autoradiograms are listed in Table 6/2.

DISCUSSION

Gene mapping studies of the Atlanta type of HPFH have identified two $^{G}\gamma$ genes on the HPFH chromosome, both having a T at position -158 (4,5). The sequence data presented here show that the $^{G}\gamma$-Atlanta gene of the HPFH chromosome, located in 3' position, is similar to an $^{A}\gamma$ gene with only two nt substitutions: The C->T at -158 and the GCA->GGA at CD 136. Analysis of the LCR 5'HS-2, the 5' flanking and the IVS-II regions of the 5' ($^{G}\gamma$) and 3' ($^{A}\gamma$) γ genes, and the 5' flanking region of the β-globin gene, identified sequence variations which are characteristic for a chromosome with haplotype #3 (15) (some of these data are not shown here). Therefore, the Atlanta type of HPFH chromosome may have arisen either by two independent mutations in the $^{A}\gamma$ gene of a chromosome with haplotype #3, or more likely by a "patchy" conversion between an $^{A}\gamma$ gene and a $^{G}\gamma$ gene containing a T at -158 (20-22). Chromosomes with haplotype #3 have been found in

various world populations and have consistently been associated with high $G\gamma$ and low $A\gamma$ values, presumably due to the presence of T at -158 in the $G\gamma$ promoter (5,8,15).

TABLE 6/2. Relative Amounts of $G\gamma$, $G\gamma$-Atlanta, and $A\gamma$ Transcripts in Four Individuals With the Atlanta Type of HPFH Condition and in Three Normal Controls[a]

	Pst I/Hinf I Digests			
	$G\gamma$	$G\gamma$-Atlanta	$A\gamma$	Genotype
D.W.	44.1	52.6	3.3	$+G\gamma+G\gamma/+G\gamma-A\gamma$
S.B.	53.2	46.8	0	$+G\gamma+G\gamma/-G\gamma-G\gamma$
M.B.	47.9	51.1	1.0	$+G\gamma+G\gamma/-G\gamma-A\gamma$
N.B.	43.9	52.1	4.0	$+G\gamma+G\gamma/-G\gamma-A\gamma$
A.D.	82.5	0	17.5	$+G\gamma-A\gamma/+G\gamma-A\gamma$
D.P.	62.8	0	37.2	$+G\gamma-A\gamma/-G\gamma-A\gamma$
E.B.	87.0	0	13.0	$+G\gamma-A\gamma/+G\gamma-A\gamma$

[a] Two adult males (A.D. and E.B.) and one female (D.P.) served as controls. All three had normal hematological values; serum ferritin levels were low for A.D. (25 ng/ml) and D.P. (22 ng/ml), and normal for E.B. (105 ng/ml). The + or - in front of $G\gamma$ refers to the presence or absence of the -158 (C->T) mutation.

The unexpected observation of a T at -158 in the $A\gamma$ promoter of the Atlanta type of HPFH chromosome provided a unique opportunity to study the effect of this mutation on the level of transcription of an $A\gamma$ gene. Comparison of the levels of $G\gamma$, $A\gamma$, and 3' γ (= $G\gamma$-Atlanta) transcripts showed similar values for the $G\gamma$ and $G\gamma$-Atlanta transcripts in three individuals with the Atlanta type of HPFH ($G\gamma$ 43.9-48.0%, $G\gamma$-Atlanta 51.0-52.6%). This ratio is similar to the $G\gamma/A\gamma$-globin chain ratio observed in normal adults (40:60), taking into account that the $G\gamma$ mRNA value includes the output of the $G\gamma$-globin gene of the second chromosome. The values for the $A\gamma$ transcript in these three individuals were low (1-4%) indicating a low output from the non-HPFH chromosome. No $A\gamma$ transcripts were present in S.B., who had one chromosome with the Atlanta type of HPFH ($+G\gamma+G\gamma$) and a second with the common $-G\gamma-G\gamma-$ arrangement.

Using a similar approach we have also attempted to evaluate the relative expression of the γ- versus β-globin gene mRNA transcripts in the individuals with the Atlanta type of HPFH. Oligonucleotide primers complementary to two regions in exons 1 and 2, which have identical sequences in the γ- and β-globin genes, have been used in the RT/PCR analysis, followed by differential digestion with restriction enzymes that recognize either γ- or β-globin gene transcripts. The levels of γ mRNA transcripts obtained

by this approach were significantly higher than expected from the levels of Hb F observed in the individuals with the Atlanta type of HPFH, as well as in several normal controls, suggesting the preferential amplification of the γ at the expense of the β mRNA transcripts. The analysis of serial dilution of cloned γ and β RT/PCR fragments confirmed this observation, and we are currently investigating this phenomenon; details of these analyses will be published separately.

The quantitative RT/PCR procedure described here has been shown to be useful for the determination of the relative amounts of individual γ mRNA transcripts, even in individuals with low Hb F levels and reticulocyte counts of less than 1%. The $^{G}\gamma$, $^{A}\gamma$, and $^{G}\gamma$-Atlanta transcripts are co-amplified in a single competitive PCR, followed by endonuclease digestion with restriction enzymes that recognize sequences specific for the individual transcripts. This approach has been used for the quantification of GM-CSF mRNA by comparison with serial dilutions of external competitor DNA containing a unique restriction enzyme site (23). We have modified this approach by competing between different mRNA molecules present within the same cell. No preferential amplification can occur because the template sizes and the target sequences for the oligonucleotide primers are identical in the different transcripts. Moreover, high sequence homology between the sequences of individual transcripts exclude the possibility of preferential amplification of particular templates. A further development is the absence of -$^{G}\gamma$-$^{A}\gamma$-, -$^{G}\gamma$-$^{G}\gamma$-Atlanta, and -$^{A}\gamma$-$^{G}\gamma$-Atlanta heteroduplexes which will remain uncleaved by the restriction enzymes and will not affect the ratios of the different transcripts (23, and unpublished observations). The high sequence homology between the coding regions of the different globin genes allows further use of this procedure in studies pertaining to their expression. However, a possible preferential amplification of transcripts that have a high degree of sequence differences as, for instance, observed for the γ and β mRNA transcripts, should be considered in evaluating the results of these experiments.

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CHAPTER 7

DISCUSSION

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DISCUSSION

Characterization of the molecular defects leading to β-thal in a given population is an important prerequisite for the implementation of a successful prenatal diagnosis program (1). Identification of the β-thal mutations and the factors that can influence the clinical phenotype can also provide useful information needed for a more accurate prognosis, a more adequate patient management, and appropriate usage of new therapeutic modalities such as bone marrow transplantation, pharmacological manipulation of Hb F expression or gene therapy.

Molecular characterization of β-thal alleles was therefore undertaken to identify the mutations leading to β-thal in the countries of the former Yugoslavia and Bulgaria. The frequencies of the various mutations were established which facilitated the identification of the β-thal defect in other β-thal patients. The development of a new nonradioactive PCR-based procedure allowed rapid and safe detection of the common β-thal mutations (2).

The study was extended to β-thal homozygotes from Turkey and compound heterozygotes for β-thal and Hb Lepore from the former Yugoslavia, which allowed correlation of the different β-thal mutations with the clinical severity of the disease. Additional factors that can ameliorate the clinical phenotype were investigated by studying 29 Mediterranean β-thal intermedia patients, all homozygous for the same mutation. The factors investigated included sex, age, the presence of α-thal, and the Hb F levels.

The study of the factors that influence the levels of Hb F was undertaken through sequence analysis of regulatory regions within the β-globin gene cluster in four β-thal patients. A number of sequence variations were observed which appeared to be unrelated to Hb F expression but were characteristic for certain chromosome backgrounds, previously defined by haplotype analysis. The only exception was the -158 (C->T) substitution in the promoter of the $^{G}\gamma$ gene which was consistently associated with increased Hb F levels, mainly of the $^{G}\gamma$ type.

The role of the -158 (C->T) mutation was further studied through the molecular characterization of the Atlanta type of HPFH. This condition was reevaluated because of its association with a chromosome that contained the -158 (C->T) mutation in the promoters of both γ genes, previously characterized as $^{G}\gamma$ genes. A new quantitative RT/PCR procedure was developed which allowed comparison of the levels of different γ transcripts in this condition, and demonstrated a direct effect of the -158 (C->T) mutation on the transcriptional activity of the gene with which it is associated.

Molecular Characterization of β-Thal Mutations in the Former Yugoslavia and Bulgaria. The incidence of β-thal trait in the former Yugoslavia has been estimated at about 1% (3) and is slightly higher in neighboring Bulgaria (around 2%) (4). A broad spectrum of β-thal mutations

were identified in these two countries by testing 50 β-thal homozygotes and 62 unrelated heterozygotes from the former Yugoslavia and 64 homozygotes from Bulgaria for the presence of 28 different β-thal alleles. The 14 different β-thal mutations identified in Yugoslavia are listed in Table 3a/1. Most frequent were the IVS-I-110 (G->G) (39.5%), the IVS-I-6 (T->C) (29%), and IVS-I-1 (G->A) (9.3%) mutations, accounting for more than 75% of all β-thal alleles. The frequencies of the 13 mutations identified in Bulgaria were more evenly distributed (Table 3b/1). Again, the most common mutation was the IVS-I-110 (G->A) mutation, but at a much lower frequency (24%). A similar frequency (22%) was observed for the CD 39 (C->T) mutation, which on the other hand, was infrequent in the former Yugoslavia (only 3.5%). Relatively high frequencies were observed for an additional seven mutations (3.1-10.2%) which would present a disadvantage for a prenatal diagnosis program where rapid identification of the mutations in the parents is required.

Comparison of the allele frequencies with those reported for other Mediterranean countries (Table 7/1) showed similarities regarding the relative percentages of the most common Mediterranean mutations, except for the CD 39 (C->T) substitution, which was infrequent in Macedonia (3.9%), but accounted for approximately 20% of the β-thal alleles in the surrounding countries. On the other hand, some of the less common Mediterranean β-thal alleles were frequently observed in this survey. The CD 5 (-CT), CD 6 (-A), and CDs 8/9 (+G) frameshifts were relatively frequent in Bulgaria, and together accounted for approximately 15% of the β-thal alleles. The poly A mutation (AATAAA->AATGAA) was identified in four unrelated families in Macedonia and had a similar frequency (4%). These mutations appear to be specific for these populations, possibly due to a more recent origin.

The number of chromosomes that remained uncharacterized during this survey was low and accounted for only 4.6 and 3.1% of all β-thal alleles in the former Yugoslavia and Bulgaria, respectively. Some of these alleles have more recently been characterized as novel mutations or deletions and have been reported in subsequent studies (6-8).

Correlation of β-Thal Mutations With Clinical Phenotype. The introduction of PCR methodology (9) and its implementation in the study of β-thal (10) made it possible to characterize the underlying defect in large series of β-thal patients with the aim to correlate particular molecular defects with the clinical expression of the disease. To that extent, characterization of the β-globin gene mutations was undertaken in 10 Macedonian Hb Lepore/β-thal compound heterozygotes, six with a severe, and four with a mild condition. Since the δβ hybrid gene in all cases was of the Lepore-Boston-Washington type, it was expected that different β-thal mutations will account for the difference in the clinical phenotype among these patients. Indeed, two of the three mutations identified in the six patients with severe disease were of the β° type [CD 39 (C->T) and IVS-I-1 (G->A)], while the third mutation was the common Mediterranean IVS-I-110 (G->A) substitution which prevents normal splicing of more than 90% of the β-mRNA. Three of the four patients with mild disease had the IVS-I-6 (C->T) mutation, while the β-thal defect in the fourth patient was subsequently characterized through sequencing analysis as a novel promoter mutation at position -30 (T->A).

TABLE 7/1. Frequency Distribution of 11 Common β-Thal Alleles in 17 Mediterranean Countries[a] (data in %)

Country	No. of Chroms.	-28 A->C	CD 6 -A	CD 8 -AA	CD 15 G->A	CD 39 C->T	CD 44 -C	I-1 G->A	I-6 T->C	I-110 G->A	II-1 G->A	II-745 C->G	Others
Albania	58	0[b]	0	0	0	22.4	3.4	5.2	17.2	**43.2**	1.7	0	6.9
Algeria	129	0	**17.9**	0	0	26.0	0	14.5	3.5	25.4	0	0	12.7
Azerbaijan	99	0	0	**21.2**	0	2.0	3.1	2.0	7.1	20.2	**21.2**	3.1	20.1
Bulgaria	483	0	6.1	5.5	0	21.9	0	5.3	9.2	27.4	1.7	6.6	16.3
Cyprus	937	0	0.1	0.2	0	1.9	0	6.3	6.7	**77.0**	0	5.8	1.4
Czech/Slovakia	93	0.4	0	0	0	2.1	0	**45.2**	0	5.9	14.1	4.3	28.4
Egypt	50	0	4.2	**21.2**	0	2.1	3.1	10.4	18.8	27.1	0	8.3	4.8
Greece	348	0	2.9	0.6	0	17.0	0	13.2	7.2	**42.6**	2.0	6.9	7.6
Hungary	17	0	0	0	0	29.4	0	**29.4**	5.9	0	11.8	5.9	17.6
Israel	46	**15.0**	0	0	0	12.0	**19.5**	4.0	2.5	18.0	13.5	0.8	14.7
Italy	914	0	1.2	0	0	**40.1**	0.7	10.2	9.9	23.0	3.9	5.0	6.0
(Sardinia)	494	0	2.2	0	0	**95.4**	0	0	0	0.4	0	0.4	1.6
(Sicily)	399	0	2.2	0	0	**37.6**	0	5.5	**23.9**	24.1	0	3.5	3.2
Lebanon	50	0	0	6.0	0	4.0	0	0	6.0	**62.0**	4.0	4.0	14.0
Portugal	334	0	0	0	**15.2**	**40.1**	0	18.7	**20.3**	15.2	0	0	5.7
Spain	58	0	5.0	1.7	0	**64.0**	0	3.5	15.5	8.5	0	0	1.8
Tunisia	48	0	2.3	2.3	0	**40.8**	9.1	0	0	20.5	0	0	25.0
Turkey	859	0.4	0.4	5.8	0	3.2	0	3.7	17.2	**41.2**	8.0	1.9	18.2
Former Yugoslavia	469	0	1.6	0.8	0	3.9	0	11.5	19.2	**45.4**	3.9	1.6	12.1

[a] Adapted from Ref. 5.

[b] Zero = not observed or not reported.

Data from studies on β-thal homozygotes from Turkey (47 subjects) and from those mentioned in the previous section, allowed classification of other, less frequently occurring mutations, according to their phenotypic expression (Table 7/2). The majority of β-thal mutations were associated with a severe anemia and regular blood transfusion requirements. Both β^0 and β^+ alleles fell into this category, including some of the most common defects, such as the IVS-I-110 (**G->A**), the IVS-I-1 (**G->A**), and the CD 39 (**C->T**) mutations. Their frequent occurrence in the homozygous state or together with another severe β-thal allele leads to the conclusion that most β-thal patients in these populations are affected by a severe disease.

TABLE 7/2. The Clinical Phenotypes of the Identified β-Thal Alleles With the Haplotypes of the Chromosomes on Which They Occured

Clinical Phenotype		β-Thal Allele	Haplotype
1. Silent β-thal	β^+	-101 (C->T)	I
2. Mild β-thal	β^+	-87 (C->G)	VIII
		-30 (T->A)	IV; VII
		Poly A (AATAAA->AATGAA)	II
		IVS-I-6 (T->C)	IV; VI; VII
3. Moderate β-thal (↑ Hb F production)	β^0	CD 8 (-AA)	IV
		IVS-II-1 (G->A)	III
	β^+	IVS-I-5 (G->T)	IX
		Hb Lepore	V
4. Severe β-thal	β^+	IVS-I-110 (G->A)	I; II
		IVS-II-745 (C->G)	VII
		IVS-I-5 (G->T)	V
	β^0	CD 5 (-CT)	V
		CD 6 (-A)	I
		CDs 8/9 (+G)	I
		CD 39 (C->T)	I; II
		IVS-I-1 (G->A)	V

On the other end of the spectrum were the silent and mild β-thal alleles which included promoter mutations and mutations that affect proper processing of mRNA. The **C->T** mutation at position -101 occurs in the distal **CACCC** box of the β-globin gene promoter, and appears to be a true "silent" β-thal mutation with normal hematological parameters, and normal Hb A_2 and Hb F levels in heterozygotes (11). This mutation was initially identified in Turkish and Bulgarian patients but appears to be more frequent in Italy (12). Compound heterozygotes for this mutation and a severe β-thal allele have a thalassemia intermedia phenotype.

Other promoter mutations, such as the -87 (**C->G**) mutation which occurs in the proximal **CACCC** box, or the -30 (**T->A**) mutation in the **ATAAAA** box, are associated with only a moderate anemia and elevated Hb F levels when present in the homozygous state. However, at least in the case of the

ATAAAA box mutation, the increased Hb F level might be a consequence of a particular chromosomal background that contains the -158 (C->T) mutation in the promoter of the G_γ gene. In combination with severe β-thal alleles these promoter mutations will produce either β-thal intermedia or β-thal major. The same is true for the IVS-I-6 (T->C) mutation which is the most frequently occurring mild β-thal allele in the Mediterranean populations. Homozygotes for this mutation are mildly affected and do not usually require blood transfusions.

Two β-thal mutations [CD 8 (-AA) and IVS-II-1 (G->A)] were also associated with only a moderate anemia, despite the absence of any β chain production. These mutations occurred on particular chromosomal backgrounds, defined by haplotypes III and IV, which appeared to be responsible for the markedly increased Hb F production seen in these patients. Another interesting example of the effect of chromosomal background on the clinical phenotype was the IVS-I-5 (G->T) mutation. This mutation was present in the homozygous form, both chromosomes having haplotype IX, in a Turkish patient with a mild disease, no transfusion requirements, and 95% Hb F. Mutations at the IVS-I-5 position have been found in various world populations and associated with different haplotypes produce diverse clinical phenotypes (1). The three mutations discussed above occurred on three Mediterranean haplotypes (III, IV, and IX) which share the same pattern of the polymorphic restriction enzyme sites in the 5' part of the β-globin gene cluster. A significant characteristic of these haplotypes is the presence of an Xmn I site created by the -158 (C->T) substitution in the G_γ promoter.

In summary, these data provide a satisfactory explanation for the variability in the severity of the disease in most of the studied β-thal patients. Many of the β° mutations, and some of the β^+ mutations, are associated with a severe disease, while the promoter mutations, and some of the mutations affecting mRNA processing, are found in patients with a milder condition. Interactions between these two types of mutations result in various clinical phenotypes, but in most instances a β-thal intermedia is observed. However, a role of additional factors in ameliorating the course of the disease is obvious, and is most striking among the patients homozygous for β°-thal mutations with high Hb F levels.

Factors Affecting the Total Hb and Hb F Levels in β-Thal Patients. The influence of additional factors on the clinical phenotype of β-thal is difficult to assess because most β-thal patients require regular blood transfusions, and are therefore uninformative regarding their steady state Hb levels. The heterogeneity of the molecular defects leading to β-thal further complicates such studies since particular mutations affect the β-globin gene output to a different extent.

The study of the 29 homozygotes for the IVS-I-6 (T->C) mutation overcame these problems. All patients had the same molecular defect and none of them had received a blood transfusion for at least six months prior to the study. In most patients, the Hb levels were between 6.5 and 8.0 g/dl, but higher and lower values were also observed. The Hb levels were not affected by the age or sex of the patients, but correlated well with the Hb F levels, except in six patients with low Hb F and high total Hb. A concomitant α-thal-2 determinant was identified in two of these patients.

The co-inheritance of α-thal or increased γ chain production can reduce the overall α/non-α chain imbalance and decrease the peripheral hemolysis of the RBCs (13). Both α-thal and increased γ chain production might have the same effect on the ineffective erythropoiesis in the bone marrow of β-thal patients, recently shown to be due to accelerated programmed cell death (apoptosis) of erythroid progenitors containing α-globin chain deposits (14). The absence of increased Hb F levels and of a common α-thal determinant in some of the patients with high total Hb could imply the presence of as yet uncharacterized nondeletional α-thal alleles, or the existence of other factors that can increase the Hb A levels in homozygotes for the IVS-I-6 (T->C) mutation.

Further investigation focused on the factors that could affect the Hb F levels. Recent studies have suggested the existence of an X-linked determinant which regulates Hb F and F-cell production (15,16). However, no association between sex and Hb F levels was observed in the IVS-I-6 homozygotes. On the other hand, the Hb F levels correlated inversely with age, even though with only a borderline significance. A more significant effect of age on Hb F levels has been observed in SS patients (17).

Sequence Variations in Regulatory Regions of the β-Globin Gene Cluster. To investigate the influence of different chromosomal backgrounds on Hb F expression, haplotype analysis was performed on all IVS-I-6 homozygotes. Three haplotypes were identified (IV, VI, and VII). Haplotype IV was found on both chromosomes in one patient with high Hb F levels, while the other two haplotypes were associated with both high and low Hb F production. Since the haplotypes are defined only by the presence or absence of certain polymorphic restriction enzyme sites on a particular chromosome, they do not rule out the presence of variations in regulatory cis-acting sequences between chromosomes with identical haplotypes but different Hb F expression. To further explore such a possibility, sequence analysis of regulatory regions within the β-globin gene cluster was undertaken in four patients, two with high Hb F levels (homozygotes for haplotypes IV or VI), and two with low Hb F levels (homozygotes for haplotypes VI or VII). Sequence analysis of the LCR showed identical 5' HS-4, HS-3, and HS-2 elements in all four patients, except for the presence of haplotype-specific **AT** repeats in HS-2 (Chapter 5, Fig. 5/2). Sequence variations between the three different haplotypes were common in the 5' flanking and IVS-II regions of both γ-globin genes, while the **AT** repeat motif in the 5' flanking region of the β-globin gene was identical in all three.

Some of these sequence variations occurred in possible motifs for binding of regulatory proteins. The **AT** repeats in the LCR 5' HS-2 and the **TG** repeats in the $^{G}\gamma$- and $^{A}\gamma$-IVS-II regions have been implicated through genetic studies in modulating γ-globin gene expression (18,19). Moreover, binding of a nuclear protein factor has been reported for the **TG** motif in the $^{A}\gamma$-IVS-II (19). The two polymorphic motifs that have been associated with higher Hb F levels [$(AT)_9n_{12}(AT)_{10}$ in LCR 5' HS-2 and $(TG)_{13}$ in $^{A}\gamma$-IVS-II)] were found only on the chromosome with haplotype IV. A possible effect of the negative regulatory factor BP1 (20) on γ-globin gene expression appeared unlikely, since no variations were present in the binding site for this protein in the 5' flanking region of the β-globin gene [$(AT)_7(T)_7$ was found in all patients]. An interesting observation was the pres-

ence of duplicated CACCC sequences in the $^{G}\gamma$-IVS-II on haplotype VII and in the $^{A}\gamma$-IVS-II on haplotype IV. Whether these sequence variations have any effect on γ-globin gene expression, as shown for the same sequences in the γ-globin gene promoters (21) remains to be determined through nuclear protein binding and transfection experiments.

The sequence variations described above were all confined to the specific haplotypes and no nt differences were observed between the two homozygotes for haplotype VI with various Hb F levels. The existence of other unidentified cis-acting sequence variations is unlikely because differences in Hb F levels were also observed between the siblings in two families. These findings, therefore, suggest a major role for unknown genetic loci, separated from the β-globin gene cluster, in controlling Hb F production in β-thal.

The only exception appears to be the chromosome with haplotype IV, which was consistently found to be associated with increased Hb F levels. The chromosome with this haplotypes shares identical RFLPs in the 5' part of the β-globin gene cluster with two other Mediterranean β-thal chromosomes associated with elevated Hb F production (haplotypes III and IX). Furthermore, sequence analysis of this chromosome found it to be identical to the β^{S} chromosome with haplotype #3 (Senegal), which has also been associated with increased Hb F production under conditions of erythropoietic stress (22-24). These two chromosomes differ only in the 3' subhaplotype, suggesting that cis-acting factors are likely to exist in the 5' part of the β-globin gene cluster.

The -158 (C->T Promoter Mutation. The most significant feature of the Mediterranean β-thal chromosomes with haplotypes III, IV, and IX, and the β^{S} chromosome with haplotype #3 appears to be the -158 (C->T) substitution in the promoter of the $^{G}\gamma$ gene (25). Normal individuals with this mutation (either in the homozygous or heterozygous state) have normal Hb F levels with elevated $^{G}\gamma$ values (60-98%); however, in a few families slightly increased Hb F levels (1-3%) have been observed. It has been suggested that this substitution is a promoter mutation which increases the expression of the $^{G}\gamma$ gene, but additional factors can also accentuate the effect of this mutation, and result in increased Hb F levels (26). One factor appears to be the chronic erythropoietic stress present in β-thal or SS disease.

To further investigate the effect of this mutation on γ-globin gene expression, a rare HPFH condition known as the Atlanta type of HPFH was reevaluated. Individuals with this condition have variable but usually moderately increased Hb F levels (2-12%), mainly of the $^{G}\gamma$ type (>90%). Previous gene mapping studies had identified a $^{G}\gamma$-$^{G}\gamma$ arrangement on this HPFH chromosome with the -158 (C->T) substitution in the promoters of both γ gene (25,27).

Sequence analysis of the γ-globin genes from the HPFH chromosome was undertaken to investigate the possible presence of additional sequence variations that could account for the elevated Hb F levels in this condition. The 5' $^{G}\gamma$ gene was found to be completely identical to the $^{G}\gamma$ gene

found on the previously discussed chromosome with haplotype #3 (Senegal), while the 3' γ gene contained only two nt substitutions with respect to the $^{A}\gamma$ gene from the same chromosome. These substitutions were the ones detected by the restriction enzymes in the gene mapping analysis, i.e. the -158 (C->T) and the CD 136 (GCA->GGA) mutations. The CD 136 mutation introduced the Ala->Gly amino acid substitution which converted this gene into a $^{G}\gamma$ gene. The absence of sequence variations, other than the -158 (C->T) mutation, in the investigated regulatory regions of the HPFH chromosome (the LCR 5' HS-2, the 5' flanking and IVS-II regions of both γ genes, and the 5' flanking region of the β-globin gene) provided genetic evidence that this promoter mutation is responsible for the increased Hb F levels in the Atlanta type of HPFH.

The individual contribution of the two γ-globin genes from the HPFH chromosome on the Hb F levels was assessed by a new, competitive RT/PCR procedure. This procedure allowed quantitation of the relative amounts of transcripts expressed by the $^{G}\gamma$, $^{A}\gamma$, and HPFH 3' γ genes. Similar values for the $^{G}\gamma$ and 3' γ transcripts were obtained in the four investigated heterozygotes for the Atlanta type of HPFH. This was expected since both γ genes on the HPFH chromosome contained the -158 (C->T) mutation. The possibility of discriminating between the $^{A}\gamma$ and 3' γ transcripts also allowed comparison of the outputs of the HPFH 3' γ gene with different $^{A}\gamma$ genes. In this respect especially interesting was the comparison with the $^{A}\gamma$ gene from the chromosome with haplotype #3 (Senegal) which differed from the HPFH 3' γ gene only in the -158 and CD 136 positions. The data showed a 15-fold higher level of transcripts from the HPFH 3' γ gene, demonstrating that the -158 (C->T) mutation can also increase the expression of an $^{A}\gamma$ gene.

To investigate whether the observed differences in Hb F levels among the Atlanta type of HPFH heterozygotes were due to differences in γ gene expression, the relative amounts of γ- and β-mRNA were determined with the same RT/PCR method. The levels of γ transcripts varied from 9-38%, and were highest in the individual with a moderate iron deficiency anemia (Chapter 6, Table 6/2). These data further support the existence of additional genetic factors, separated from the β-globin gene cluster, which can affect the expression of the γ-globin genes. It is tempting to speculate that such factors could affect the silencing of the γ-globin genes; when they are present the effect of the -158 (C->T) mutation would be more pronounced, resulting in higher Hb F levels. Incomplete silencing of the γ-globin genes could also occur in conditions with erythropoietic stress, such as β-thal, SS disease, or iron deficiency anemia which would accentuate the effect of the -158 (C->T) mutation on the expression of the associated γ-globin gene.

Quantitative RT/PCR Analysis of Globin Gene Transcripts. The newly developed competitive RT/PCR assay allowed the accurate quantitation of the relative amounts of different globin gene transcripts even in individuals with Hb F levels and reticulocyte counts of less than 1%. The γ and β, or the $^{G}\gamma$, $^{A}\gamma$, and 3' γ transcripts were co-amplified in a single competitive PCR, followed by endonuclease digestion with restriction enzymes that recognize sequences specific for the individual transcripts. Since

the template sizes and the target sequences for the PCR primers were identical between the different transcripts, no preferential amplification was expected. However, the obtained values for the γ transcripts were higher than the corresponding Hb F levels which prompted further experiments with cloned γ- and β-cDNA fragments. These experiments confirmed a preferential amplification of the γ transcripts, which was more pronounced when the PCR was performed at lower denaturation temperatures. The inability of completely overcoming this bias in amplification with extended denaturation times suggested that other factors, such as sequence specificity of the Taq polymerase, also contribute to the preferential γ amplification. Therefore, all subsequent experiments were performed with controls containing different relative amounts of known quantities of cloned γ and β fragments. The values obtained for the controls were plotted against the actual values, and the γ/β ratios of the analyzed samples were intrapolated from the curve.

The quantitative RT/PCR analysis of globin mRNAs has further applicability in the study of the β-globin gene non-linked factors that affect the Hb F levels in β-thal, and should clarify whether the latter act through transcriptional or post-transcriptional mechanisms. Furthermore, the high sequence homology between the coding regions of the different globin genes should allow implementation of this technique in other studies pertaining to globin gene expression. Its sensitivity may also prove useful in monitoring the effects of pharmacological manipulation of γ-globin gene expression in ongoing clinical trials for the treatment of β-thal and sickle cell anemia.

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CHAPTER 8

SUMMARY

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The main objectives of the studies presented in this thesis are: 1) The characterization of the molecular defects leading to β-thal in the countries of the former Yugoslavia and Bulgaria; 2) the correlation of these defects with different clinical phenotypes, and 3) a study of possible ameliorating factors, with special emphasis on those that can influence the Hb F levels in patients with β-thal.

A review of the literature is presented in **Chapter 1**, which includes a short historical perspective of some of the more important accomplishments in the study of the thalassemias. The ethnic distribution of the thalassemias is presented, with special emphasis on the frequency of β-thal in various Mediterranean countries. This chapter also provides a short description of the basic pathophysiological phenomena in β-thal, together with the more important clinical and hematological features of the disease. The review of the common molecular defects leading to β-thal is preceded by a description of the structure and function of the globin genes, their organization, and developmental expression. The possible role of different *cis*-acting elements in regulating the high level of expression of the β-globin gene in adult erythroid cells is discussed together with a brief description of the clinical features and molecular defects leading to the Lepore Hbs. Regulation of the γ-globin gene expression by genetic factors, both linked and non-linked to the β-globin gene cluster, is presented in the section dealing with the different HPFH syndromes. Special emphasis is given to the different γ-globin gene arrangements, and to the most frequent sequence variation associated with particular β-thal chromosomes, the -158 (C->T) mutation. An updated list of known β-thalassemic mutations is provided as an appendix to this chapter.

Relevant patient data and a description of some of the methodology used are presented in **Chapter 2**.

Chapter 3 provides detailed data about the occurrence of various β-thal alleles in the populations of the former Yugoslavia and Bulgaria. The observed frequencies of the different mutations are compared to those of the neighboring countries, and the implications of this study in initiating a prenatal diagnosis program are discussed.

Characterization of the molecular defects in 50 β-thal homozygotes and 62 unrelated β-thal heterozygotes from the former Yugoslavia is presented in **Chapter 3a**. Fourteen different mutations were observed; of these, three [IVS-I-110 (G->A); IVS-I-6 (T->C); IVS-I-1 (G->A)] accounted for approximately 75% of all β-thal alleles. A newly discovered mutation (**AATAAA->AATGAA** in the poly A site) appeared to be rather common as it was observed in four unrelated families with a frequency of 4.7%. Hb Lepore was found to be a frequent and clinically significant abnormality since its association with β-thal often resulted in a severe disease. The high frequencies of the IVS-I-110 (G->A) and IVS-I-1 (G->A) mutations led to the conclusion that most β-thal patients originating from the countries of the former Yugoslavia will be severely affected by their diseases.

Chapter 3b presents data from the DNA analysis in 64 β-thal homozygotes from Bulgaria using ASO hybridization of PCR amplified DNA. Thirteen different β-thal alleles were identified; most frequent were the IVS-I-110 (G->A) (24.2%) and CD 39 (T->C) (21.9%) mutations. However, seven additional mutations were observed which were present at frequencies of 3.9 to 10.2%. The even distribution of nine different mutations is likely to complicate the analyses for institutions involved in prenatal diagnosis. High frequencies of 4.7 to 5.5% were observed for four rare mutations. These frameshift mutations (at CDs 5, 6, 8, and 8/9), as well as the poly A mutation identified in Macedonia, appear to be specific for these populations.

The correlation of particular clinical phenotypes with the different β-thal mutations is described in Chapter 4. Chapter 4a presents the study of 10 patients with the Hb Lepore/β-thal condition, originating from the countries of the former Yugoslavia, who showed significant variations in the severity of the disease. As the type of Hb Lepore was the same in all patients (Lepore-Boston-Washington) and an α-globin gene deficiency was absent, it was concluded that the type of β-thal determined the severity of the disease. The latter was confirmed through characterization of the β-thal alleles; six patients with severe disease had either a β° or a severe β^{+} mutation [CD 39 (C->T); IVS-I-1 (G->A); IVS-I-110 (G->A)], three patients with mild disease had the IVS-I-6 (T->C) mutation, while the β-thal determinant in one patient remained unknown. Further characterization of the β-thal allele in this patient is presented in Chapter 4b. Sequence analysis of PCR amplified DNA revealed a T->A mutation within the ATA box at position -30 nt from the Cap site. The same mutation was present on both chromosomes in a young Turkish patient with thalassemia intermedia. While the Hb F levels in the Turkish patient were low (13.1%), they were extremely high in the Hb Lepore/β-thal compound heterozygote (80%). The mutation was found on two different haplotypes (IV in the patient with high Hb F, and VII in the patient with low Hb F), suggesting a role for the chromosomal background in determining the level of Hb F expression.

The molecular characterization of β-thal alleles in 47 homozygotes from Turkey, presented in Chapter 4c, provided sufficient data to correlate some less frequent mutations with particular phenotypes. Mild disease was observed in patients homozygous for the IVS-I-6 (T->C), CD 8 (-AA), -87 (C->G), and IVS-I-5 (G->T) mutations, or compound heterozygotes for combinations of these mutations. Moderate disease was present in patients with one of these mutations and a severe mutation such as the IVS-I-110 (G->A), IVS-I-1 (G->A), and CD 39 (C->T). Two β° mutations (IVS-II-1 and CD 8) were consistently associated with a mild phenotype and high total Hb and Hb F levels. These mutations were found on two different haplotypes (III and IV), both containing the C->T substitution at position -158 ($^{G}\gamma$).

The effect of different chromosomal backgrounds was analyzed in detail in 29 homozygotes for the IVS-I-6 (T->C) mutation, none of whom had received a blood transfusion for at least six months prior to the study. The obtained data, described in Chapter 5, showed higher Hb levels in patients with high Hb F of a concomitant α-thal-2 heterozygosity. The IVS-I-6 (T->C) mutation was found in three different haplotypes; most of

the patients were homozygous for haplotype VI, but haplotypes IV and VII were also present. The only haplotype IV homozygote had high Hb F levels, while both high and low Hb F levels were observed among patients with haplotypes VI and VII. Sequence analysis of regulatory cis-acting elements was undertaken in two patients with high Hb F (one homozygote each for haplotypes VI and IV) and in two patients with low Hb F (one homozygote each for haplotype VI and VII). Haplotype specific differences were observed in the LCR 5' HS-2 and in the $^{G}\gamma$ and $^{A}\gamma$ 5' flanking and IVS-II regions, while all chromosomes were identical in the 5' LCR HS-4, HS-3, and the 5' flanking region of the β-globin gene. The -158 (C->T) substitution appeared most likely to be responsible for the elevated Hb F levels observed in association with haplotype IV. The absence of nt differences between the low and high Hb F-producing haplotype VI chromosomes suggested a major role for unknown genetic factors, separated from the β-globin gene cluster, in controlling the Hb F production in these β-thal patients.

The effect of the -158 (C->T) substitution was further evaluated through the study of the Atlanta type of HPFH. Chapter 6 provides details on the molecular analysis of this condition, previously characterized as a $^{G}\gamma^{G}\gamma$ arrangement with the -158 (C->T) substitution in the promoters of both $^{G}\gamma$ genes. Sequence analysis identified the -158 (C->T) mutation and the CD 136 (C->G) substitution as the only differences between the 3'γ gene from this arrangement, and the $^{A}\gamma$ gene from a chromosome with haplotype #3 (Senegal). The absence of any other nt substitutions provided genetic evidence that the -158 (C->T) mutation is primarily responsible for the elevated Hb F levels associated with this condition. A quantitative RT/PCR procedure was developed to determine the effect of this mutation on γ-globin gene transcription in four individuals with the Atlanta type of HPFH. High levels of γ transcripts were observed in all individuals (9-38%), and were highest in one female with a moderate anemia. The $^{G}\gamma$ and the 3'γ genes of the Atlanta type of HPFH chromosome expressed similar amounts of transcripts, which were over 10 times higher than those of the $^{A}\gamma$ gene from the normal chromosomes. These data demonstrated that the -158 (C->T) mutation directly affects the transcriptional rate of the gene to which it is associated.

The data presented in this dissertation provide a satisfactory explanation for the observed phenotypic variations in most of the studied β-thal patients. Apart from the presence of a mild β-thal allele, increased Hb F expression is the most important factor which can contribute to a mild clinical phenotype. Genetic factors, both linked and non-linked to the β-globin gene cluster, can affect the levels of Hb F in β-thal patients. Clinically, the most significant cis-acting factor is the -158 (C->T) mutation which exerts a positive regulatory effect on the associated γ-globin gene. The trans-acting factors influencing the levels of Hb F in β-thal require further characterization. The competitive RT/PCR assay may prove useful in investigating these factors and in monitoring the effects of pharmacological manipulation of γ-globin gene expression.

SAMENVATTING

Samenvatting

Het onderzoek beschreven in dit proefschrift heeft de volgende doelstellingen: (1) Het karakteriseren van de moleculaire defecten welke aanleiding geven tot β-thalassemie in het voormalig Joegoslavië en in Bulgarije; (2) de correlatie van deze defecten met de verschillende klinische phenotypen; en (3) een onderzoek naar mogelijk factoren met een positief effect op de ziekte, met speciale aandacht voor factoren die de HbF niveaus beïnvloeden bij patiënten met β-thalassemie.

Hoofdstuk 1 is een overzicht van de literatuur. Het begint met een kort historisch overzicht van de belangrijkste resultaten van de onderzoekingen aan thalassemie. De etnische distributie van thalassemieën wordt beschreven, met speciale aandacht voor de frequentie van β-thalassemie in de verschillende landen rond de Middellandse Zee. Het hoofdstuk geeft ook een beschrijving van de basale pathofysiologische karakteristieken van β-thalassemie, samen met de belangrijke klinische en haematologische bevindingen bij deze ziekte. Het overzicht van de genetische defecten die aanleiding geven tot β-thalassemie wordt voorafgegaan door een beschrijving van de structuur, functie, organisatie en ontwikkelingsafhankelijke expressie van de globine genen. De mogelijke rol van de verschillende cis-werkende elementen bij het reguleren van de hoge expressie niveaus van de β-globine genen in volwassen erythroide cellen wordt aan de orde gesteld, samen met een korte beschrijving van de klinische karakteristieken en moleculaire defecten die leiden tot de Lepore haemoglobinen. Regulatie van de γ-globine gen expressie door genetische factoren, die al of niet gekoppeld zijn aan het β-globine gen cluster, wordt bediscussieerd in het gedeelte dat gaat over de verschillende HPFH syndromen. Speciale aandacht wordt geschonken aan de verschillende γ-globine gen rangschikkingen, en aan de meest voorkomende sequentie variatie geassocieerd met bepaalde β-thalassemie chromosomen, de -158 (C→T) mutatie. Een bijgewerkte lijst van bekende β-thalassemie mutaties wordt als appendix toegevoegd aan dit hoofdstuk.

Relevante patiënt gegevens en een beschrijving van verschillende gebruikte technieken worden beschreven in hoofdstuk 2.

Hoofdstuk 3 geeft gedetailleerde informatie over het voorkomen van verschillende β-thalassemie allelen in de bevolkingsgroepen van het voormalig Joegoslavië en Bulgarije. De waargenomen frequenties van de verschillende mutaties worden vergeleken met die van de buurlanden. De implicaties van deze studie, met name voor de prenatale diagnostiek, worden bediscussieerd.

Hoofdstuk 3a beschrijft de karakterisering van de moleculaire defecten in 50 patiënten die homozygoot zijn voor β-thalassemie, en 62 ongerelateerde heterozygote β-thalassemie patiënten uit het voormalig Joegoslavië. Veertien verschillende mutaties werden gevonden; drie hiervan [IVS-I-110 (G→A); IVS-1-6 (T→C); IVS-I-1 (G→A)] namen ongeveer 75% van alle β-thalassemie allelen voor hun rekening. Een nieuw ontdekte mutatie (AATAAA→AATGAA in het poly-A gebied) leek een vrij algemeen voorkomende mutatie, daar deze werd aangetroffen in vier ongerelateerde families met een frequentie van 4,7%. Van Hb Lepore werd vastgesteld dat het een frequente en klinisch significante aandoening is, omdat de associatie met β-thalassemie vaak resulteerde in ernstige ziekte. De hoge frequenties van de IVS-I-110 (G→A) en de IVS-I-1 (G→A) mutaties gaven aanleiding tot de conclusie dat de meeste β-thalassemie patiënten uit het vroeger Joegoslavië ernstig aangedaan zullen zijn door hun ziekte.

In hoofdstuk 3b worden de gegevens gepresenteerd met betrekking tot de DNA analyse van 64 β-thalassemie homozygoten uit Bulgarije, waarbij gebruik werd gemaakt van ASO hybridisatie m.b.v. PCR geamplificeerd DNA. Dertien verschillende β-thalassemie allelen werden geïdentificeerd; het meest frequent de IVS-I-110 (G→A) (24,2%) en de CD 39 (T→C) (21,9%) mutaties. Er werden echter ook zeven additionele mutaties gevonden, welke aanwezig waren met een frequentie van 3,9 tot 10,2%. De gelijkelijke verdeling van negen verschillende mutaties compliceert de prenatale diagnostiek. Hoge frequenties, van 4,7 tot 5,5%, werden gevonden voor vier bijzondere mutaties. Deze frame-shift mutaties (op CDs 5, 6, 8 en 8/9), en de poly-A mutatie geïdentificeerd in Macedonië, leken specifiek voor deze populatie.

De correlatie van specifieke klinische phenotypen met de verschillende β-thalassemie mutaties wordt beschreven in hoofdstuk 4. Hoofdstuk 4a beschrijft een studie van 10 patiënten met de Hb Lepore/β-thalassemie constitutie, die afkomstig waren uit landen van het voormalig Joegoslavië, welke een significante variatie in de ernst van hun ziekte lieten zien. Omdat het type van Hb-Lepore in alle patiënten hetzelfde was (Lepore-Boston-Washington) en een α-globine gen deficiëntie afwezig was, werd geconcludeerd dat het type β-thalassemie de ernst van de ziekte bepaalt. Dit werd mede bevestigd door karakterisering van de β-thalassemie allelen; zes patiënten met ernstige ziekte hadden of een β^0 of een ernstige β^+ mutatie [cd 39 (C→T); IVS-I-1 (G→A); IVS-I-110 (G→A)], drie patiënten met een milde ziekte hadden de IVS-I-6 (T→C) mutatie, terwijl de β-thalassemie determinant in één patiënt onopgehelderd bleef. Verdere karakterisering van het β-thalassemie allel in deze patiënt wordt gepresenteerd in hoofdstuk 4b. Sequentie analyse met PCR geamplificeerd DNA resulteerde in een T→A mutatie in de ATA-box op positie -30 nt van de Cap site. Dezelfde mutatie was aanwezig in beide chromosomen van een jonge Turkse patiënt met een intermediaire thalassemie. Terwijl het HbF niveau in de Turkse patiënt zeer laag was (13,1%), was dit niveau in de Hb Lepore/β-thalassemie compound heterozygoot zeer hoog (81%). De mutatie werd gevonden bij twee verschillende haplotypen (IV in de patiënt met een hoge HbF en VII in de patiënt met een lage HbF), hetgeen suggestief is voor een rol van de chromosomale achtergrond in het bepalen van het HbF expressie niveau.
De moleculaire karakterisering van β-thalassemie allelen in 47 homozygoten uit Turkije, beschreven in hoofdstuk 4c, verschafte voldoende materiaal om enkele minder frequent voorkomende mutaties te correleren met bepaalde phenotypen. Milde ziekte werd gevonden in patiënten die homozygoot waren voor de IVS-I-6 (T→C), CD 8 (-AA), -87 (C→G), en IVS-I-5 (G→T) mutaties, of compound heterozygoot waren met combinaties van deze mutaties. Middelmatig ernstige ziekte werd aangetroffen in patiënten met één van de voornoemde mutaties in kombinatie met een ernstige mutatie, zoals de IVS-I-110 (G→A), IVS-I-1 (G→A) of CD 39 (C→T) mutatie. Twee β^0 mutaties (IVS-II-! en CD 8) waren consistent geassocieerd met een mild fenotype en een hoog totaal Hb en HbF niveau. Deze mutaties werden gevonden bij twee verschillende haplotypen (III en IV), welke beide de C→T substitutie op positie -158 (Gγ) lieten zien.

Het effect van de verschillende chromosomale achtergronden werd gedetailleerd geanalyseerd in 29 homozygoten voor de IVS-I-6 (T→C) mutatie, waarvan niemand een bloedtransfusie had ontvangen gedurende de laatste 6 maanden voorafgaand aan het onderzoek. De verkregen data, beschreven in hoofdstuk 5, lieten hogere Hb niveaus zien in patiënten met hoge HbF en een additionele α-thalassemie-2 heterozygotie. De IVS-I-6 (T→C) mutatie werd gevonden in drie verschillende haplotypen; de meeste van de

patiënten waren heterozygoot voor haplotype VI, maar de haplotypen IV en VII waren ook vertegenwoordigd. De enige patiënt met haplotype IV homozygotie vertoonde hoge HbF niveaus, terwijl zowel hoge als lage HbF niveaus werden gezien bij patiënten met de haplotypes VI en VII. Sequentie analyse van de regulatoire, cis-werkende elementen vond plaats bij twee patiënten met hoge HbF (één homozygoot voor haplotype VI, de ander voor haplotype IV) en in twee patiënten met een lage HbF (één homozygoot voor haplotype VI, de ander voor haplotype VII). Haplotype specifieke verschillen werden gevonden in de LCR 5' HS-2, in de Gγ en Aγ 5' flankerende en in de IVS-II regionen, terwijl alle chromosomen identiek waren m.b.t. de 5'LCR HS-4, HS-3 en de 5' flankerende regionen van het β-globine gen. De -158 (C→T) substitutie, in combinatie met haplotype IV, leek verantwoordelijk voor verhoogde HbF niveaus te zijn. De afwezigheid van nucleotide verschillen tussen de lage en hoge HbF producerende haplotype VI chromosomen, suggereerde een rol van onbekende genetische factoren, die gescheiden zijn van het β-globine gen cluster, en die de HbF produktie in deze β-thalassemie patienten controleren.

Het effect van de -158 (C→T) substitutie werd verder geëvalueerd door de bestudering van de Atlanta type HPFH. Hoofdstuk 6 geeft de details van de moleculaire analyse van deze aandoening, die eerder werd gekarakteriseerd als een GγGγ met de -158 (C→T) mutatie en de CD 136 (C→G) substitutie als enig verschil tussen het 3'γ gen van deze toestand, en het Aγ gen van een chromosoom van haplotype #3 (Senegal). De afwezigheid van alle andere nucleotide substituties vormde een genetisch bewijs voor het feit dat de -158 (C→T) mutatie primair verantwoordelijk is voor de verhoogde HbF niveaus die gevonden worden bij deze aandoening. Een quantitatieve RT/PCR procedure werd ontwikkeld om het effect van deze mutatie op γ-globine gen transcriptie te bepalen bij vier individuen met het Atlanta type HPFH. Hoge niveaus van γ-transcripten werden gevonden bij alle individuen (9-38%), het hoogste niveau bij een vrouw met een middelmatige anemie. De Gγ en de 3'γ genen van het Atlanta type HPFH chromosoom brachten vergelijkbare hoeveelheden transcript tot expressie, welke 10 maal hoger waren dan die van het Aγ gen van de normale chromosomen. Deze gegevens laten zien dat de -158 (C→T) mutatie een direct effect heeft op de transcriptie snelheid van het gen waarmee het geassocieerd is.

De gegevens die in dit proefschrift worden gepresenteerd geven een bevredigende uitleg voor de waargenomen phenotypische variatie in de meeste van de bestudeerde β-thalassemie patiënten. Naast de aanwezigheid van een mild β-thalassemie allel, is de aanwezigheid van verhoogde niveaus van het HbF de meest belangrijke factor die aan een mild klinisch fenotype bijdraagt. Genetische factoren, zowel gekoppeld als niet-gekoppeld aan het β-globine genen cluster, kunnen de niveaus van het HbF in β-thalassemie patiënten beïnvloeden. Klinisch is de cis-werkende factor, de -158 (C→T) mutatie, het belangrijkst, welke een positief regulerend effect heeft op het geassocieerde γ-globine gen. De trans-werkende factoren, die de niveaus van HbF in β-thalassemie beïnvloeden, verdienen nog verder onderzoek. De competitieve RT/PCR assay kan een bruikbaar instrument zijn bij het bestuderen van deze factoren en voor het onderzoeken van de effecten van farmacologische manipulatie van de γ-globine gen expressie.

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