β-THALASSEMIA IN TURKEY: DISTRIBUTION, DIVERSITY, EVOLUTION AND PHENOTYPE-GENOTYPE CORRELATIONS

by

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TO MY PARENTS,

MY BELOVED NISRINE,

AND TO THE MEMORY OF MY

GRANDPARENTS

& MY AUNT

IN'AA'M

FOREWORD

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ABSTRACT

The present study illustrates the results of five years of research on different aspects of β-thalassemia in Turkey. Methods to detect the C-T change at position -158 upstream of the Gγ-globin gene and the $(AT)_xT_y$ motif 5' to the β-globin gene were established and implemented. Analysis of these polymorphisms explained the reason behind the increased levels of fetal hemoglobin in nine out of 31 β-thalassemia patients analyzed and demonstrated a dominant effect exerted by the *XmnI* Gγ-globin polymorphism.

Molecular screening of β-globin genes in 19 β-thalassemia individuals by genomic DNA sequencing uncovered the presence of 14 mutations; three of these are seen for the first time in Turkey. Another achievement made during this study is the compilation of β-globin gene data collected since 1988 in a single repository. This allowed an easy mean to investigate the distribution of β-globin gene mutations in various regions and towns of Turkey. This also demonstrated that the distribution of β-thalassemia mutant alleles differed within each geographical area with a decreased gradient of mutation numbers from the East to the West of Anatolia.

Analysis of nine polymorphic nucleotides and the $(AT)_xT_y$ motif 5' to the β-globin gene in 204 non-related β-globin genes from Turkey exhibited 12 sequence haplotypes. Samples from the Black Sea region demonstrated a remarkable level of genetic heterogeneity in contrast to the homogeneity in Central Anatolian samples. Of the 22 β-globin mutations analyzed, 18 were related with single sequence haplotypes and each of the other four were associated with a minimum of two sequence haplotypes. Our results demonstrate that the heterozygote advantage against malaria in Anatolia may have occurred at 6500-2000 BC by the oldest β-thalassemia allele (i.e., IVS-I-110 G-A). From that date on, most of the common β-thalassemia mutations in Turkey were established and by the 13^{th} century AD most of them were brought to frequencies close to what is observed at present.

ÖZET

Bu tez, Türkiye'de β-talaseminin değişik yönlerini kapsayan beş senelik bir araştırmanın sonuçlarını içermektedir. Çalışma çerçevesinde Gγ-geninin -158. pozisyonundaki C-T değişimini ve β-globin geninin 5' ucundaki $(AT)_xT_y$ dizisini tanımlamak için gerekli yöntemler kurulmuş ve uygulanmıştır. Bu polimorfizmlerin incelenmesi 31 hastadan dokuzunda HbF değerinin normaldan yüksek olmasını açıklayabilmiş ve *XmnI* Gγ-globin polimorfizminin dominant bir mekanizma ile etki ettiğini göstermiştir.

Ondokuz β-talasemili bireye uygulanan DNA dizi analizi ile 14 değişik mutasyon tanımlanmıştır; bu mutasyonlardan üçü ilk defa Türkiy'de tarif edilmiştir. Bu tezin önemli bir katkısı, 1988'den beri toplanan β-globin geni ile ilgili tüm sonuçların ilk defa dijital ortamda bir araya getirilmiş olmasıdır. Bu şekilde Türkiye'de görülen mutasyonların yörelere göre sınıflandırılması mümkün olmuştur. Mutasyon dağılımının coğrafi bölgeler arasında farklılıklar gösterdiği ve Anadolu'nun doğusundan batısına doğru mutasyon çeşitliliğinin azaldığı bu çalışma sonunda ortaya çıkmıştır.

Birbiri ile akrabalık bağı olmayan 204 bireyin β-globin genleri dokuz polimorfik nükleotid ve $(AT)_xT_y$ dizisi açısından incelendiğinde, toplam 12 değişik haplotip tanımlanmıştır. Karadeniz bölgesine ait örnekler Orta Anadolu'dan gelen örneklere göre oldukça belirgin bir genetik heterojenite göstermişlerdir. İncelenen 22 β-globin mutasyonundan 18'i tek bir haplotipte görülürken, diğer dördü en az iki haplotiple birliktelik göstermiştir. Sonuçlarımız Anadolu'daki muhtemelen en eski β-talasemi mutasyonu olan IVS-I-110'un yaklaşık M.Ö. 6500-2000 senelerinde sıtma seleksiyonu ile çoğaldığı yönündedir. Türkiye'de sıklıkla görülen diğer β-talasemi mutasyonlarının bu tarihten sonra oluştuğu ve 13. y.y. civarında yaklaşık bugünkü oranlarına ulaştıkları düşünülmektedir.

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ABBREVIATIONS

aDNA	Ancient DNA
AM	Aegean-Mediterranean region
ASO	Allele specific oligonucleotide
BLK	Immigrant Turks from the Balkan
bp	Base pair
BPB	Bromophenol blue
BSR	Black Sea region
CA	Central Anatolia
Cd	Codon
DGGE	Denaturing gradient gel electrophoresis
dNTP	2'-Deoxynucleoside 5'-triphosphate
EA	East Anatolia
EDTA	Ethylenedinitrilo tetraacetate
EtBr	Ethidium bromide
FSC	Frameshift codon
Hb	Hemoglobin
Hct	Hematocrit
HT	Haplotype
IVS	Intervening sequence or intron
Kb	Kilobase
LCR	Locus control region
M	Molar
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MR	Marmara region
mtDNA	Mitochondrial DNA
OD	Optical density

P	Probability
PCR	Polymerase chain reaction
pmole	Picomole
RBC	Red blood cell or erythrocyte
rpm	Revolution per minute
SCD	Sickle cell disease
SDS	Sodium dodecyl sulphate
SEA	Southeastern Anatolia
Tris	Tris (hydroxymethyl)-aminomethan
WBC	White blood cell or leukocyte
wt	Wild-type
χ^2	Chi-square test

I. INTRODUCTION

β-Thalassemia constitutes one of the most serious health problems worldwide, accounting for hundreds of thousands of child deaths per year primarily in regions of the world endemic for malaria (WHO, 1983). β-Thalassemia is an autosomal recessive disorder characterized by microcytosis and hemolytic anemia. It results from a variety of molecular defects that reduce (β^+-thalassemia) or abolish (β^0-thalassemia) the normal synthesis of the β-globin chains of hemoglobin (Weatherall and Clegg, 1981).

A. Clinical Features of β-Thalassemia

Homozygotes and compound heterozygotes for β-thalassemia have a wide spectrum of clinical phenotypes, ranging from undetectable to a life-threatening transfusion-dependent disease (Table I.1). Generally, most homozygous patients present a severe, transfusion-dependent anemia within the first two years of life and later suffer from the long-term consequences of iron overload (thalassemia major). However, many other patients may have minor clinical manifestations that may not require transfusion (thalassemia intermedia).

Heterozygous (carrier) β-thalassemia subjects are usually asymptomatic at clinical level. Their hematology is characterized by a slight to moderate anemia with marked hypochromia, microcytosis, a slightly raised level of the minor adult hemoglobin, HbA_2, and an unbalanced α/β globin chain synthesis ratio (thalassemia minor; Lin et al., 1994).

TABLE I.1. Clinical symptoms resulting from molecular defects of the β-globin gene (modified from OMIM, 1999).

Hematology:	Anemia, microcytosis, hypochromia, mild hemolytic anemia, hemolytic microcytic anemia in compound heterozygosity, macrocytic hemolytic disease, erythrocytosis, congenital Heinz body anemia, acute splenic sequestration, splenomegaly, dactylitis, ischemia, avascular necrosis, leg ulcers, cholelithiasis, priapism, osteonecrosis, osteomyelitis, and drug-induced hemolysis.
Skin:	Jaundice, and cyanosis (e.g., Hb M Saskatoon).
Gastro-Intestinal:	Cholelithiasis, splenomegaly, and splenic syndrome.
Genito-Urinary:	Hematuria.
Miscellaneous:	Intact β-globin structural gene in β^0-thalassemia, deleted β-globin structural gene in both hereditary persistence of fetal hemoglobin and $\delta^0\beta^0$-thalassemia, resistance to *Falciparum* malaria, and β-thalassemic mechanisms: (a) gene deletion, (b) chain termination (nonsense), (c) point mutation in an intervening sequence, (d) point mutation at an intervening sequence splice junction, (e) frame-shift deletion, (f) fusion genes, and (g) single amino acid mutation leading to very unstable globin.
Laboratory Findings:	Abnormal red cell morphology, bone marrow erythroid hyperplasia, increased numbers of multinucleate red cell precursors, inclusion bodies in normoblasts, altered hemoglobin A_2 levels, altered hemoglobin F levels, unstable hemoglobin (e.g., Hb Köln), diminished oxygen affinity (e.g., Hb Chico), increased oxygen affinity (e.g., Hb Heathrow), increased N-terminal glycation (e.g., Hb Himeji), discrepant HbA_{1c} measurement (e.g., Hb Marseille), unusually low HbA_{1c} level (e.g., Hb Kodaira), red cell inclusion bodies (e.g., Hb Matera), non-hemoglobin S (HbS) red cell sickling [e.g., HbC (Georgetown)], and electrophoretic migration as HbS (e.g., Hb Muskegon).

B. Treatment and Prevention of β-Thalassemia

Initially, patients with β-thalassemia major were blood transfused only when their anemia became symptomatic, but since the late 1960s, regular transfusion regimes have been designed to suppress the patients' bone marrow by maintaining a relatively normal baseline level of hemoglobin. Such treatment prevents complications because of expansion of the bone marrow, but leads to progressive iron overload, which, within few years, results

in endocrinopathies and intractable cardiac failure. Current regimen, therefore, also includes the use of long-term iron chelation with desferrioxamine (commercially known as Desferal) which, unfortunately, is expensive and has to be administered parenterally through an infusion pump (Fosburg and Nathan, 1990). At present, considerable effort is directed towards developing a safe and efficient oral iron chelator; the most promising of these (referred to as L_1) is currently under clinical trial (Porter et al., 1990).

Bone marrow transplantation is the only treatment that can cure the severe forms of thalassemia (Lucarelli and Weatherall, 1991), but is dependent on the availability of marrow from an HLA-matched normal sibling. Paradoxically, the best candidates for transplantation are young patients who have not yet developed complications of thalassemia or its treatment.

Since β-thalassemia is predominantly a disease that occurs in individuals from relatively poor areas of the world, treatment of any kind is available to only a limited number of patients. In such cases, it seems reasonable to develop preventive strategies to avoid this serious genetic disorder. When the range of molecular defects within an affected population was known, it became possible to combine this approach with first trimester prenatal diagnosis. Such programs have greatly reduced the incidence of β-thalassemia in various populations (Alter, 1990).

For many years, investigators have concentrated on β-thalassemia as the most likely initial candidate for gene therapy. However, it was clear by the year 1984 that diseases of hemoglobin synthesis were going to be particularly difficult to correct by gene therapy because of the complex regulation involved (Anderson, 1992). A possible genuine alternative would be preimplantation diagnosis by the polymerase chain reaction (PCR). This method involves the isolation of 1-2 blastomeres from embryos or, alternatively, aspiration of a polar body from oocytes. If a genetic defect causing β-thalassemia is excluded from the biopsied cells, the remaining blastomeres are transferred into the

mother's womb for normal fetal development (Monk et al., 1993). Micromanipulation of the gamete and embryo biopsy combined with the sensitive PCR technology are expected to provide a comprehensive genetic screening of embryos fertilized and developed in vitro with the resultant reduction or elimination of β-thalassemia (Baysal et al., 1995).

C. Molecular Anatomy and Physiology of the β-Globin Gene Complex

The β-globin gene is a small gene of 1.6 kb that occurs as a single copy in the haploid genome. It is arranged together with the other β-like globin genes (ε, Gγ and Aγ, and δ) in an ~60 kb long gene complex on the short arm of chromosome 11 (11p15; Lin et al., 1985). Each gene is a separate transcriptional unit consisting of upstream regulatory sequences and the coding sequence (always split by two introns), followed by transcriptional termination signals that are probably important for RNA processing, mRNA stability, and efficient protein synthesis. Besides these local regulatory elements there is a region 6-18 kbp upstream from the ε-globin gene that controls the expression of the entire β-globin gene complex during development and has been termed β-globin locus control region (β-LCR). The β-LCR is characterized by a set of developmentally stable, DNaseI hypersensitive sites, HS1, 2, 3, and 4 (Figure I.1). Without the β-LCR, the human β-like globin genes are expressed at low levels. The β-LCR achieves activation of transcription in some dominant fashion by opening the chromatin structure and possibly creating very stable interactions between the β-LCR and the promoters of the β-globin gene family (Orkin, 1990; Figure I.2).

The first step of β-globin gene expression requires the binding of various transcription factors to the promoter and to the LCR. The transcript is immediately modified at its 5' and 3' ends by the addition of a methylated guanosine nucleotide (Cap) and a string of adenosine residues (Poly A tail), respectively. The introns are subsequently removed from the pre-RNA by exon splicing generating the mature mRNA. The splicing

mechanism depends on signal sequences at the 5' (GT, donor) and at the 3' (AG, acceptor) exon/intron junctions and within the introns that have been well conserved during evolution. The splicing process is a three-step mechanism with the cleavage of the 5' end of the intron from the preceding exon. The free 5' end is subsequently joined to an adenine at about 25 bp upstream from the 3' end of the intron. Finally, the 3' end is cleaved from the next exon and the two exons are joined. The intron is released as a lariat and degraded within the nucleus. The mature RNA is then transported into the cytoplasm where it is translated into β-globin protein.

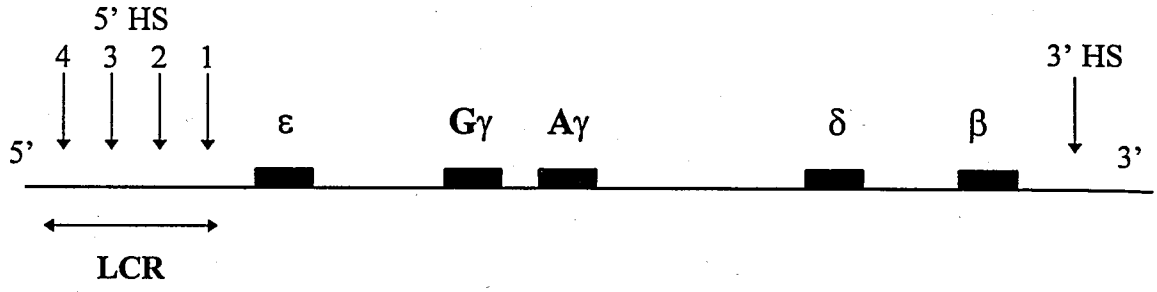


FIGURE I.1. The β-globin gene cluster. The position of the major upstream and downstream regulatory regions as defined by DNase I hypersensitive sites (HS) is indicated by arrows; the 5' HS forms the locus control region (LCR).

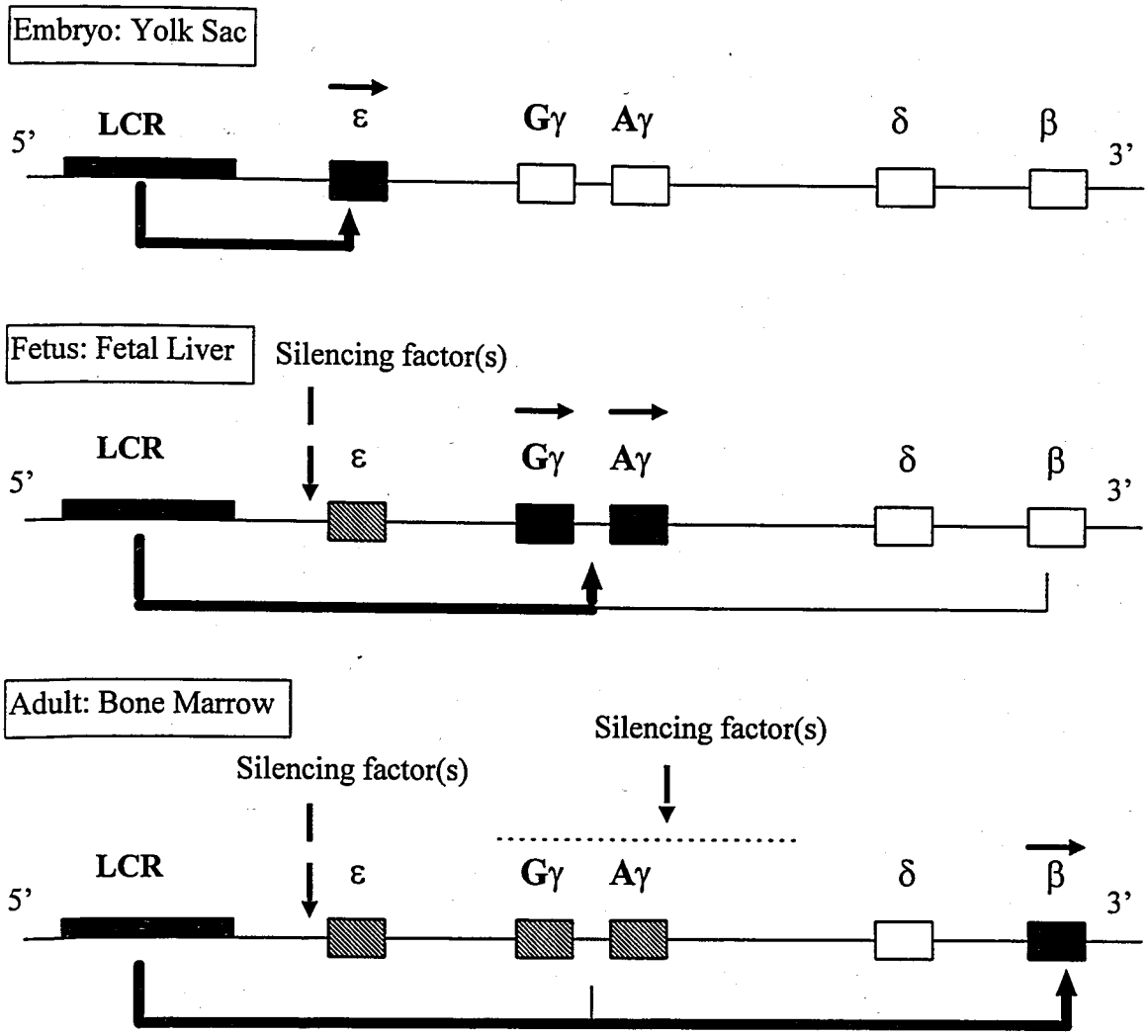


FIGURE I.2. Hemoglobin switching in the human β-gene cluster. In this model the positive effects of the LCR, the competitive relationship between the γ- and β-genes, and the actions of putative silencing factors are depicted for stages of human ontogeny. The strong, positive influence of the LCR is depicted by thick lines with a solid arrow. Unfavorable interactions with the LCR are shown by thin lines without an arrow. Active transcription of a gene is shown by a horizontal arrow. Potential targets for "silencing" near the γ-genes are unknown and are therefore depicted by a dashed line (Orkin, 1990).

D. Pathogenesis of β-Thalassemia

At present, more than 180 mutations produce β-thalassemia (Huisman et al., 1997). They affect not only the actual amino acid coding regions of the β-globin exons, but also sites surrounding the gene and even within the non-coding introns. Most of these mutations cause defects in transcription, RNA splicing, RNA modification and translation because of frameshifts and nonsense codons or produce highly unstable β-globin products. Other alleles range from extensive deletions that remove the 5' end of the β-globin gene cluster, but leave the β-gene intact (Figure I.3), to point mutations that affect sequences critical to the β-globin gene function. The vast majority of β-thalassemia syndromes, however, are caused by point mutations within the β-globin gene itself or in its immediate flanking sequences (Huisman et al., 1997).

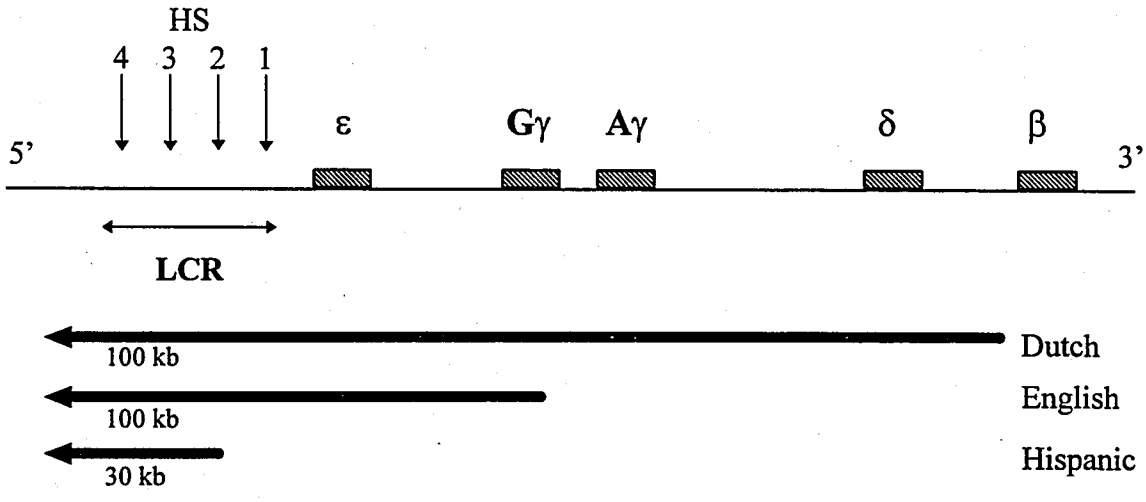


FIGURE I.3. Three of the deletions in the β-globin gene cluster that leave the β-globin gene intact but cause a β-thalassemia phenotype. These deletions remove in common three of the 5' most HS (HS 2, HS 3, and HS 4) leading to inactivation of the β-globin gene in *cis* (Thein, 1993).

β-Thalassemia mutations differ greatly in their phenotypic effects which range from the extremely mild mutations, which are both clinically and phenotypically silent in the heterozygous state (Rosatelli et al., 1994), to those which are rare and produce a phenotype of thalassemia intermedia despite of the inheritance of a single copy of abnormal gene (dominant β-thalassemia; Thein, 1992). Between these two extremes lay the majority of β-thalassemia mutations whose carriers are asymptomatic, whereas homozygotes and compound heterozygotes suffer from a transfusion-dependent anemia (Figure I.4; reviewed by Weatherall et al., 1989).

1. Silent β-Thalassemia

The silent β-thalassemia does not produce any evident hematological phenotype; the only abnormality is an unbalanced α/β-chain synthesis. Perhaps the truly silent β-thalassemias are because of the C-T mutation at position -101 (Gonzalez-Redondo et al., 1989a) and the A-C mutation at Cap +1 position (Wong et al., 1986).

2. Dominant β-Thalassemia

This unusual form of β-thalassemia was probably first identified in an Irish family in 1973 (Weatherall et al., 1973) and subsequently in a Swiss-French family (Stamatoyannopoulos et al., 1974). Later on, several similarly affected kindreds have been reported in dispersed geographical non-malarial regions where the gene frequency of β-thalassemia is very low (e.g., Northern Europe, Japan, and Korea). At least 16 mutations underlying these dominantly inherited forms of β-thalassemia have been identified so far (Figure I.5). Most of these mutations are *de novo* events which fall into three main groups:

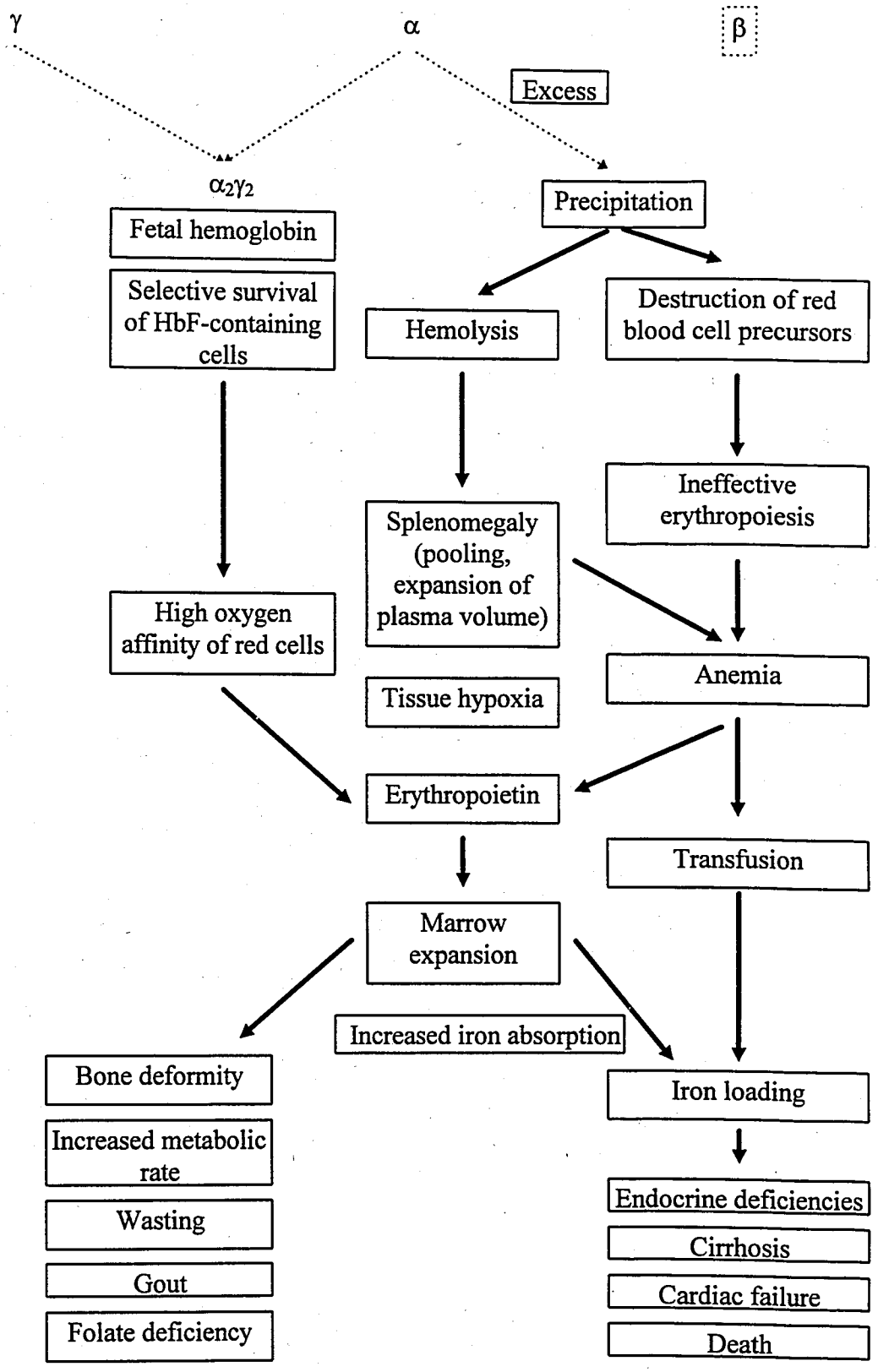


FIGURE I.4. Schematic representation summarizing the pathophysiology of β-thalassemia (adapted from Weatherall, 1997).

(a) Those resulting in a highly unstable β-variant as a result of a single base substitution or deletion of intact codons, (b) those with a truncated β-chain because of premature termination as a result of a base substitution, (c) and those with an elongated β-globin with an altered carboxy-terminal end as a result of a frameshift mutation (reviewed by Thein, 1992).

The dominantly inherited β-thalassemias are generally characterized by the synthesis of a highly unstable β-variant (Hall and Thein, 1994). These resemble the intermediate forms of β-thalassemia because of ineffective erythropoiesis. They also bear resemblance to the congenital hemolytic anemias with the variable amount of peripheral hemolysis.

Clinically, since spontaneous mutations are common in dominant β-thalassemia, it is important that the disorder should be suspected in any patient with a thalassemia intermedia phenotype even if both parents are hematologically normal and the patient is from an ethnic background where β-thalassemia is rare (Thein, 1992).

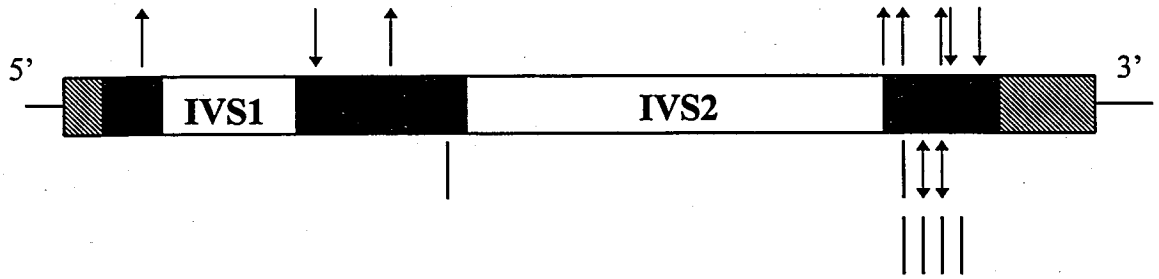


FIGURE I.5. Mutations causing dominantly inherited β-thalassemia. Upper headed arrows: single base substitutions, down-headed arrows: deletion of intact codons, double-headed arrows: premature termination, simple vertical lines: frameshift mutations (reviewed in Thein, 1993).

3. β-Thalassemia With Unusually High HbA_2

Recently, a subgroup of β-thalassemia has been recognized. This subgroup is characterized by unusually high levels of HbA_2 (>6.5 per cent) and variable increases in the fetal hemoglobin F (HbF) levels in the heterozygous state. In the majority of cases, such a phenotype appears to be caused by point mutations and deletions in the β-promoter that includes the TATA box and the proximal and distal transcriptional elements at positions -90 and -105 (Gilman and Huisman, 1985). This would remove competition for the upstream regulatory elements allowing an increased interaction of the LCR with the δ- and γ-genes *in cis*, thereby enhancing their expression (Figure I.6, Hanscombe et al., 1991).

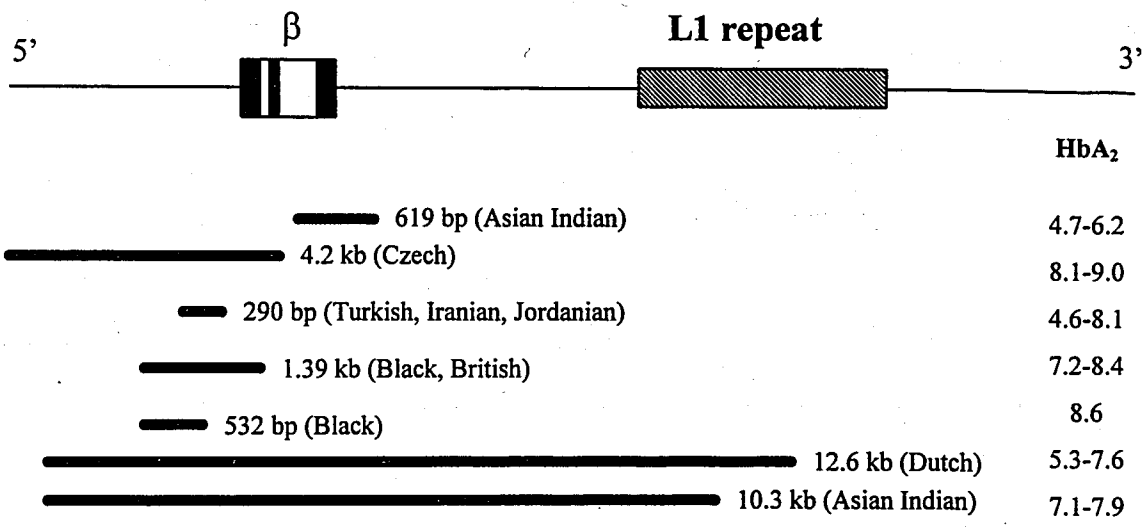


FIGURE I.6. Some of the deletions in the β-globin gene cluster that remove only the β-globin gene either partially or completely. Black boxes indicate extent of deletions. The hatched box indicates a 6.4 kbp L1 repeat sequence that is located downstream of the β-globin gene. The corresponding levels of HbA_2 (per cent) seen in heterozygotes for the different deletions are shown on the right (Thein, 1993).

4. β-Thalassemia Caused by Unknown Mutations

It has been estimated that about one per cent of the β-thalassemia genes in the world remain uncharacterized (Kazazian and Boehm, 1988). In such cases, it has been postulated that mutations may either be found in the upstream β-LCR region or may be completely unlinked to the β-globin gene cluster (Murru et al., 1992; Thein et al., 1993).

E. Factors Modulating the Phenotypes of β-Thalassemia and Sickle Cell Disease

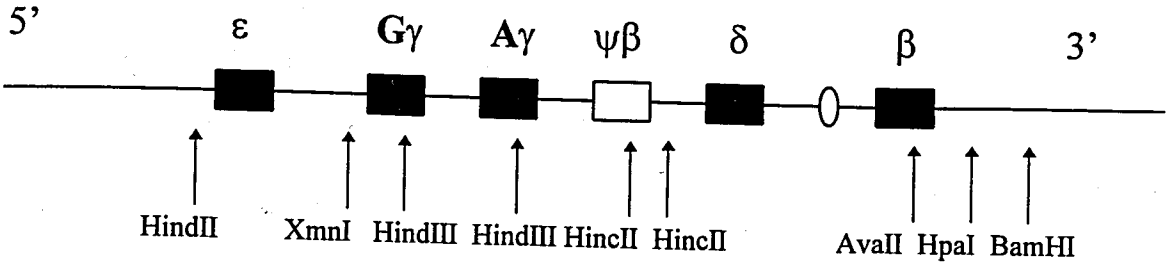
The human β-globin gene cluster contains a tandem sequence of genes that are expressed in erythroid tissues in a developmentally specific manner (Karlsson and Nienhuis, 1985). The γ-globin genes are coordinately switched off before birth, and by six months of age, the level of HbF is only approximately one per cent of the total hemoglobin.

As previously mentioned, β-thalassemia homozygotes usually show a severe transfusion-dependent anemia starting from the first year of life (thalassemia major). However, a minority of patients (10-20 per cent) present a more benign clinical phenotype with survival into adulthood without the need for regular blood transfusions (thalassemia intermedia). One of the characteristics of these patients is the relative increase in the synthesis of fetal hemoglobin (HbF) that partially corrects the unbalanced α/non-α ratio.

Several genetic and non-genetic factors have been proposed to influence levels of HbF in adults carrying mutations in the β-globin gene. These variables include age, sex, α-globin gene number, regulatory sequences linked to the β-globin gene region, and genetic factors not linked to the β-globin gene region (reviewed by Steinberg, 1996).

A variety of mutations involving deletions in the region spanning from the Aγ-globin gene to the 3' end of the β-globin gene or base substitutions in the promoter region of the γ-globin gene have been associated with hereditary persistence of HbF. Of these substitutions, the C-T variation at position -158 upstream from the Cap site of the Gγ-globin gene (causing polymorphism for an *XmnI* cleavage site) is consistently found in association with specific phenotypic features in patients under hematopoietic stress. These features include: the persistence of the fetal Gγ/Aγ ratio at the adult stage and most often an increased HbF level in sickle cell disease (SCD), β-thalassemia, and HbE/β-thalassemia patients (Gilman and Huisman, 1985).

On the other hand, several investigators have suggested that β-globin haplotypes as defined by restriction fragment length polymorphisms (RFLPs) may have direct effects on HbF levels in patients with β-thalassemia or SCD. In SCD patients five main haplotypes are observed. These have been named Benin, Bantu (also called Central African Republic), Cameroon, Senegal, and the Saudi-Indian types to refer to the places where they most commonly occur, and have also been numbered as XIX, XX, XVII, III, and XXXI, respectively (Pagnier et al., 1984). Patients with haplotypes Benin, Bantu, and Cameroon generally have low levels of HbF (one to ten per cent), while haplotypes Senegal and Saudi-Indian are associated with higher levels of HbF (15-30 per cent) accompanied by a high Gγ- to Aγ-globin synthesis ratio (Figure I.7; Steinberg, 1996). The Saudi-Indian haplotype is associated with an $(AT)_9T_5$ motif present at 0.5 kbp upstream from the β-globin gene (Zeng et al., 1994). The $(AT)_xT_y$ polymorphic motif lies with a negative regulatory region that acts as a binding site for a protein (BP1) present in nuclear extracts from human K562 cells (Berg et al., 1989). BP1 binding to various configurations of the $(AT)_xT_y$ motif was shown to vary *in vitro*. Binding is weakest to the configuration linked to the Bantu haplotype and strongest to the configuration of the Saudi-Indian haplotype (Berg et al., 1989).



										HbF
Benin	-	-	-	-	-	+	+	-	+	8.0±5.2
Bantu	-	-	+	-	-	-	+	+	+	10.4±4.9
Cameroon	-	-	+	+	-	+	+	+	+	4.0
Senegal	-	+	+	-	+	+	+	+	+	12.3±5.3
Saud/Ind	+	+	+	-	+	+	+	+	-	20.6±7.5

FIGURE I.7. The organization of the β-globin gene cluster and the position of polymorphic restriction enzyme sites frequently used in the construction of the RFLP β-globin haplotype (map not to scale). The haplotypes associated with sickle cell genes from various regions are shown. Sites that are cut by the enzymes are designated by a (+) sign while those which remain uncut are indicated as (-). The *XmnI* site shown is that at position -158 upstream from the Cap site of the Gγ-globin gene. On the right is shown the mean (± standard deviation) HbF levels (in per cent) in SCD cases homozygous for the respective haplotypes. The empty circle indicates the relative position of the $(AT)_xT_y$ motif located at position -530 upstream from the β-globin gene (Adapted from Lanclos et al., 1991; Flint et al., 1993).

F. Interactions of β-Thalassemia With Other Genetic Defects

Imbalance in the relative amounts of α- and β-globin chain is an important factor in the pathophysiology of β-thalassemia. In this disorder, red blood cell destruction is caused by membrane damage because of precipitation of denatured excess α-globin chains (Shinar and Rachmilewitz, 1990).. The deleterious effects of excess α-globin chains are also

apparent in heterozygotes for β-thalassemia, as reflected in the variable depression of their mean corpuscular volume (MCV) values (Rund et al., 1992).

Because of their relatively common presence in the world, both α- and β-thalassemia alleles may be co-inherited in the same individuals. Thus, heterozygosity for α-thalassemia in conjunction with β-thalassemia ameliorates the clinical condition of the patient (Weatherall and Clegg, 1981). Conversely, excess α-globin genes have been implicated in increasing the severity of β-thalassemia (Sampietro et al., 1983; Thein et al., 1984).

The marked chain imbalance in homozygous β-thalassemia can also be reduced by increasing the production of γ-globin chains since the γ-chain combines with the excess α-chain to form HbF (Figure I.4). The degree of such a hereditary persistence of fetal globin synthesis and the resulting clinical effect depends on its molecular basis. It is quite clear that two main genetic factors are involved in modifying γ-chain production: (a) DNA deletions removing the β-globin gene and more or less of its flanking sequences, thus resulting in a picture characterized by a virtually normal hematology whereas others result in a thalassemia phenotype of variable severity; (b) Point mutations of the γ-globin gene promoters (as described above) increase their transcriptional efficiency (reviewed by Wood, 1993).

G. The Evolution of β-Globin Mutations in the World

SCD and β-thalassemia are the most common hemoglobinopathies with a distribution corresponding to areas of the world where malaria is currently, or until recently, endemic (WHO, 1983). The high prevalence of these two disorders in malaria-infested regions led Haldane (1949) to propose that heterozygous carriers for these hemoglobinopathies are less susceptible to severe malarial infection since cells containing

abnormal hemoglobins are not very conductive to the malarial parasite's proper development. Angel (1966) suggested that these diseases are ancient and they followed the establishment of permanent human settlements near standing water in the Neolithic period. Thus, the age of SCD and β-thalassemia as a human problem can then be related to the evolution of malaria.

1. Brief History of Malaria

The malarial parasite must have been with modern humans for a long time, perhaps for the entire existence of the genus *Homo* (Cavalli-Sforza et al., 1996), and it may have originated in tropical areas of the Old World, but the Pleistocene glaciations (130,000-10,000 years ago) delayed its spread in the Northern Hemisphere. When temperatures approximately equal to those of today were reached, 10,000 years ago, the disease spread to the North (i.e., Europe). Malaria may have spread earlier in the Levant and parts of Asia, because of a less marked drop of temperature during the last glaciation (10,000 years ago; de Zulueta, 1994). Before the advent of agriculture, roughly 5,000 years ago, it is unlikely that humans were exposed to large malarial outbreaks and that this stage was only reached whenever Neolithic (early) farmers settled in mosquito-infested soft and marshy soil, near standing water (Weiss and Mann, 1975). It is believed that the recrudescence of malaria in Anatolia could have occurred at the Neolithic period (5,000 years ago; de Zulueta, 1994). However, from Hellenistic to Romantic times it again increased (Hellenistic 10%, Roman 24%, Medieval 12%, Turkish 45%, Romantic 37%; Angel, 1966) with a high impact during the beginning of the Turkish conquest 700 years ago (Angel 1966; Grmek, 1994).

Besides malaria, the evolution of SCD and β-thalassemia has been studied from two other perspectives: macroscopic examination of ancient archeological skeletal remains on the basis of bone pathology and analysis of distribution of mutations in various present day living populations.

2. Analysis of Archeological Remains Suggestive of Hereditary Anemias

Several documented skeletal remains with bone pathology suggestive of either SCD or β-thalassemia were discovered in various prehistoric sites of the world such as in Greece, Albania, Australia (Webb, 1990), the Middle East (Hershkovitz et al., 1991), and Southeast Asia (Tayles, 1996). However, with the knowledge that significant amount of genetic information can be recovered from ancient skeletal remains, analysis of genetic markers in 'ancient DNA' has become an important research tool in archeology and contributed to the development of hypotheses about past populations (reviewed by Brown and Brown, 1994). In 1995, Filon et al. presented the first direct proof of the occurrence of an inherited anemia in the archeological remains of a child with severe bone pathology consistent with β-thalassemia. The remains came from a grave thought to date to the Ottoman period, sometime between the 16^{th} and the 19^{th} centuries. DNA analysis has shown that the child was homozygous for the frameshift mutation at codon 8 (-AA) as well as the polymorphism (C-T) in the second codon of the β-globin gene (Filon et al., 1995a). Thus, we are beginning to realize the promise of ancient DNA analysis to experimentally answer heretofore unapproachable questions regarding human prehistory and genetic change.

3. β-Globin Mutations and the History of Populations

The comparison of regional patterns of recessive disease mutations is a new source of information for studies of population genetics since they can serve as molecular tags in the genetic heritage of human populations. The geographic distribution of a particular allele may give information on the place of origin of the genetic change (mutation) that generated it. Therefore, such analysis allows the identification of specific genetic links between different populations and of ancient peoples who did or did not contribute significantly to the gene pool of a modern population.

Extensive and reliable data about the distribution and exact incidence of the 180 β-globin gene mutations in various world populations were made available during the last 15 years (Huisman et al., 1997). Interestingly, in countries relatively free from malaria and where β-thalassemia is uncommon (e.g., Northern Europe and Japan), a diversity of β-thalassemia mutations have been found. On the contrary, in regions with long histories of exposure to malaria, probably only 13 alleles account for >80 per cent of the β-thalassemia genes. From an evolutionary point of view, these mutations are population-specific, different sets of which are found in Mediterraneans, Asian Indians, Blacks, and Chinese; together with a varying number of rare ones (Huisman et al., 1997). It is therefore possible to identify certain 'marker' mutations, track their distribution in various world populations and to infer from this the genetic relationships between different peoples. Several studies of this kind have been published (reviewed by Livingstone, 1989; Flint et al., 1993; Adekile, 1997; Huisman et al., 1997); their conclusions were inevitably hypothetical, but nevertheless provided important information on population genetics.

In brief, it is believed that β-thalassemia mutations may have become established at similar times throughout the world (Livingstone, 1989; Flint et al., 1993). There is evidence that modern humans (*Homo sapiens sapiens*) originated in Africa, and that a small number of them migrated out of Africa into Europe and Asia (Cavalli-Sforza et al., 1996). Leaning on this fact, it is possible to conclude that the regional specificity of β-thalassemia mutations provides further evidence of their relatively recent origin. Had β-thalassemia been present at significant frequencies in the founder population leaving Africa, then it is likely that the present inhabitants of Asia and Europe would share the same mutations (Flint et al., 1993), just as they share other genetic markers (for instance sickle cell disease or β-globin haplotypes). The contiguous distribution of β-thalassemia as well as other β-globin gene mutations in localized areas of the world led Livingstone (1989) to conclude that these mutations may postdate the racial divergence of modern humans (100,000-30,000 years ago) and have emerged before malaria became an important selective factor. Afterwards, modern humans dispersed throughout different areas of the

(Old) world and when malaria spread to most populations with the advent of agriculture, it first began to select for and, thus, increase the incidence of any 'malaria-resistant' mutation that happened to be present (Livingstone, 1989). In fact, there is a certain type of correlation between the early migrations of Phoenicians, Carthaginians, and Greek colonists and the distribution of some major molecular mutants. This correspondence led Cavalli-Sforza and his colleagues (1996) to assume that the date of origin of most of the β-thalassemia mutants, or at least their arrival in the colonies established by these people, was 3,000-2,500 years ago. The most frequent and established mutations almost certainly originated earlier than 3,000 years ago in the places of origin of the colonists (Cavalli-Sforza et al., 1996).

4. β-Globin Haplotypes

By far the most common readily detectable type of variation between individuals in the globin loci is produced by neutral DNA sequence differences named 'polymorphisms'. Such polymorphisms are estimated to occur at every hundred bases or so throughout the genome (Jeffreys, 1979), representing a huge reservoir of genetic variation. Many polymorphisms have been found in the β-globin gene cluster (reviewed by Labie and Elion, 1996). Given the considerable number of such polymorphisms, it may be calculated that, potentially, a very large number of combinations of these sites along a chromosome, or 'haplotypes', might exist. Since their first description in 1982, many haplotype polymorphisms detectable with restriction endonucleases (RFLPs) have been observed in the ~60 kbp DNA fragment spanning the β-globin gene cluster (Antonarakis et al., 1982; Antonarakis et al., 1985). These were subsequently used for the discrimination between diverse epistatic events linked to the β-gene that may modulate the phenotypic expression of a structural mutation and for the determination of the date of origin and track of gene flow of a particular β-globin gene mutation.

By calculating linkage disequilibrium in population data, Chakravarti et al. (1984) estimated that 75 per cent of the recombination events in the β-gene cluster occur within a 9.1 kbp DNA sequence present between the δ- and β-globin genes and that recombination rate in this segment is about 30 times the average expected rate for a DNA stretch of this length. Subsequently, several working groups observed an apparent crossover within this region (Gerhard et al., 1984; Camaschella et al., 1988; Hall et al., 1993). This relatively increased rate of recombination is believed to be the reason behind the subdivision of the full RFLP haplotypes into 5' and 3' sub-haplotypes. Five sites, scattered over a 34 kbp DNA portion, make up a 5' sub-haplotype, and two sites separated by a 19 kbp distance (containing the β-globin gene) make a 3' sub-haplotype. With the advent of easy DNA sequencing methods, a new haplotyping system attracted increased attention. The system, named as sequence haplotyping, incorporates the repetitive sequencing of a highly mutable sequence, spanning the region between positions -1069 and -491 relative to the cap site of the b-globin gene, in large series of subjects. This new methodology allowed the detection of all nucleotide changes within the sequence of interest and opened access to new polymorphic markers not recognized by traditional restriction enzyme analysis (Trabuchet et al., 1991a; Perrin et al., 1998), as explained in the following sections of this thesis.

Despite the molecular differences among the two models of haplotypes, most of them are found in many studied populations, but some are confined to single population groups. Interestingly, it was also observed that various alleles associated with β-globin disorders tend to be related with particular haplotypes (Hill and Wainscoat, 1986). Thus, the combined analysis of the frequency differences of common β-globin mutations and haplotypes between populations, is what adds a new dimension to the discipline of genetic anthropology (Wainscoat et al., 1986; Wainscoat, 1987; Trabuchet et al., 1991a; Trabuchet et al., 1991b; Flint et al., 1993; Fullerton et al., 1994; Harding et al., 1997a; Harding et al., 1997b). This approach has provided a much clearer picture about the population distribution of the β-thalassemias as well as the evolution of human β-globin genes.

Although the 'one haplotype - one mutation' rule is generally true, exceptions of both types occur since the β-globin gene cluster is far from being static. Occasionally, more than one mutation may be found on a single haplotype, an indication that haplotypes are usually older than the mutations that they carry. Unexpectedly, some of the β-globin gene mutations may be found associated with different haplotypes in various populations. At first sight these results suggest that the mutation may have multiple origins. On the other hand, these data must be interpreted with caution because of the possibility that a number of recombination events could have caused at least some of the variability in mutation/haplotype associations (Flint et al., 1993).

The example of mutation/haplotype association of β-globin alleles shows the complexities that must be entertained when we try to deduce reasons for the presence of a genetic disease from the haplotype and gene frequency data derived from large numbers of various populations. Although there are yet no studies in any part of the world that tried to combine the β-thalassemia data with the archeological and historical record to confirm the origin of descent of mutations, a more clear picture about the scenario of origin and spread of the β^S-globin gene has been accomplished recently (Adekile, 1992).

5. The β^S-Globin Gene as a Model

The β^S-globin mutation has been one of the most intensively studied mutations of all human DNA alterations. The study of the molecular history of the HbS gene started with the pioneering work of Kan and Dozy (1978), who described the polymorphism of the *HpaI* site located 3' to the β-globin gene. A more extensive investigation of 11 polymorphic sites of the β-globin cluster established that the β^S-gene is associated with five major haplotypes each virtually exclusively present in five separate geographic areas of the world (Antonarakis et al., 1982; Pagnier et al., 1984; Kulozik et al., 1986; Lapoumeroulie et al., 1992), as described in section I.E. Further evidence of the separate origin of these five major haplotypes came with studies of the area upstream from the β-

globin gene that demonstrated that the combination of polymorphisms in that region was unique for each haplotype (Chebloune et al., 1988; Trabuchet et al., 1991a). Considering the combined analysis of these findings and anthropological correlates, it was proposed that the β^S-globin mutation has originated in West Africa, most probably on haplotype Benin, some 50,000 years ago (Adekile, 1992). Considerable dispersal of the gene followed the massive migration that occurred after the desertification of large areas of North Africa (2,000-500 BC). It is at this time, that the β^S-mutation introduced by immigrant individuals may have spread by recombination and gene conversion mechanism, that occur more frequently than mutations, on to new, locally common, haplotypes (i.e., Bantu, Cameroon, and Senegal). On the other hand, the haplotype on which β^S occurs in India and the Eastern coasts of Arabia is rare among normal β-globin chromosomes in that region, unknown in Africa, and extremely rare elsewhere in the world (Wainscoat et al., 1986). Thus, it is proposed that the mutation on the Saudi-Indian haplotype independently originated, probably recently, in the Indus Valley and was dispersed along trade routes established by Arabs (Flint et al., 1993; Adekile, 1997).

H. A Brief History of Research on the β-Globin Gene in Turkey

1. Sickle Cell Disease

SCD was the first abnormal hemoglobin to be described in Turkey. It was first characterized in 1946 in a Greek individual living on a small island (Egeli and Ergün, 1946). Later, it was shown that the sickle cell gene existed with a variable frequency in different regions of the country. In 1955, Prof. Aksoy started population surveys and research studies on thalassemia and abnormal hemoglobins in Turkey. He noted that the Eti-Turks, an Arab-speaking population living in the south of Turkey, possess the highest incidence of SCD (16.8 per cent) in the white race (Aksoy, 1961a). Among this population, however, there was a wide range of sickling, fluctuating between 5.4 (Antakya)

and 27.3 (Mersin) per cent (Aksoy, 1961b). Prof. Aksoy also pointed out that the Eti-Turks and the Allewits of Syria and Lebanon may have the same racial background. In 1980 and 1985, Aksoy and colleagues reported an incidence value of 2.3 and 2.9 per cent for SCD trait among Turks from Antalya (Manavgat, Serik and Boztepe) and Western Thrace, respectively. In 1987, Yüreğir and colleagues reported the results of four projects performed during the years 1975-1984. According to their study, the frequency of SCD gene hospital population of Tarsus (mostly inhabited by Eti-Turks), Adana, and Antakya was found to be 22.8, 2.2, and 2.8 per cent, respectively, with an average rate of around 3.9 per cent (Kocak et al., 1995). Interestingly, it was not found at all in the Kurdish speaking people of that region (Kocak et al., 1995). In brief, the frequency of SCD in Turkey is about 0.5 per cent (reviewed by Arcasoy, 1992) and in homozygous patients it expresses itself as severe as that seen among the Black population of Africa and the Americas. Haplotyping, involving nine restriction sites, identified RFLP haplotype Benin as the major type among the Eti-Turks (Aluoch et al., 1986). The historical events that may explain this observation are as follows:

With the desertification of large areas of land in Africa (4,000-2,500 BC), the β^S gene, with the Benin haplotype, migrated in many directions one of which was the Western Coast of Arabia, later inhabited by the Semites (Adekile, 1997). Following the desiccation of Arabia, there were waves of migration out of the Arabian Peninsula, and it is most probable that the Semites may have transferred the β^S (Benin) genes to their descendants some of whom are the Canaanites and Arameans who may have contributed to a great extent to the genetic constitution of the populations that inhabited the Eastern Coasts of the Mediterranean (Allewits in Syria and Lebanon) and Southern Anatolia (Eti-Turks in Turkey) some 3,000 years ago. In the year 904 AD, the Saraceans conquered Salonika and transported around 20,000 hostages to South Turkey, where they remained in bondage and in tight contact with the Eti-Turks. Twenty years later, they were released to return to the fertile, but isolated, villages of Salonika and Western Thrace and islands of the Aegean Sea, evidently carrying along a fair number of relatives with β^S genes (Boussiou et al., 1991). In addition, the recent importation of great numbers of North African slaves by the Franks, Venetians, and Ottomans between the 10^{th} and the 18^{th} centuries may have further

created foci of β^S (Benin) carriers, who later merged with the local population and expanded through endogamy and malaria (Aksoy, 1961b; Boussiou et al., 1991; Adekile, 1992).

2. β-Thalassemia

The first two patients with β-thalassemia major in Turkey, were reported in 1941 (reviewed in Aksoy, 1991). However, the importance of β-thalassemia as a health problem was brought to the attention of physicians only in the late 1950s (Aksoy, 1959). Common problems encountered in Turkish thalassemia major patients included below-average height, growth retardation (mainly in patients of 10 years of age or more), delay in bone age, and delayed puberty (Yeşilipek et al., 1993). These results showed that growth and endocrine disturbances have significant negative effects in the quality of life of thalassemia patients. The first report about the prevalence of β-thalassemia carriers in Turkey was in 1971 as two per cent (Çavdar et al., 1971). Dinçol and colleagues were the first investigators to indicate regional differences in prevalence rate of β-thalassemia trait in Turkey and to demonstrate the presence of both β^+- and β^0-thalassemia genes in the country (Dinçol et al., 1979). Studies conducted in the years 1985 and 1986 confirmed this observation and noted a frequency variability ranging between 3.4 (East Anatolia) and 11 (Western Thrace and Antalya) per cent (Aksoy et al., 1980; Aksoy et al., 1985; Kurkçuoğlu et al., 1986; Bircan et al., 1993; Kocak et al., 1995). Interestingly, the highest occurrence (7.1 per cent) of β-thalassemia in Southern Anatolia was mostly observed in the Kurdish speaking people living in that region (Kocak et al., 1995). Because of this relatively high incidence of β-thalassemia in the Turkish population, the presence of patients co-inheriting β-thalassemia along with another congenital disorder is not surprising. So far, there has been several reports about the co-inheritance of β-thalassemia along with Immerslund Grasbeck syndrome (Saylı et al., 1994), Fanconi anemia (Altay et al., 1996a), and familial Mediterranean fever (Canatan et al., 1999). The co-inheritance of β-thalassemia and SCD,

that is usually expressed as a severe type of disease in Turkish patients (Altay et al., 1997), cannot be neglected either (reviewed in Altay and Başak, 1995).

It was only in the year 1987 that the first study discussing the molecular basis of β-thalassemia in the Turkish population was published (Akar et al., 1987). Progress in the methodology to analyze mutations of the β-globin gene has made it possible to understand some of the mechanisms that are responsible for the occurrence of β-thalassemia in Turkey. Since then, several country-scale studies have been conducted in order to elucidate the molecular basis of β-thalassemia (Diaz-Chico et al., 1988; Gürgey et al., 1989; Aulehla-Scholz et al., 1990; Öner et al., 1990; Başak et al., 1992a; Atalay, et al., 1993; Altay and Başak, 1995; Nişli et al., 1997; Tadmouri et al., 1998a). These studies showed that β-thalassemia in Turkey is quite heterogeneous at molecular level and that more than 30 mutations are behind the great variability in clinical expression of this disorder. In addition to these studies, many reports of single cases of Turkish β-thalassemia patients, either living in Turkey or abroad, contributed to the wealth of information about the presence of many rare and several novel β-globin mutations responsible for the disease in the Turkish population (Diaz-Chico et al., 1987; Gonzalez-Redondo et al., 1989a; Gonzalez-Redondo et al., 1989b; Schnee et al., 1989; Öner et al., 1991a; Başak et al., 1992b; Özçelik et al., 1993; Başak et al., 1993; Jankovic et al., 1994; Tadmouri et al., 1997; Tüzmen et al., 1997; Tadmouri et al., 1998b; Tadmouri et al., 1999a; Tadmouri et al., 1999b). Furthermore, several other papers described non-common forms of molecular alterations leading to β-thalassemia such as a deletion/inversion rearrangement of the β-globin gene cluster in a Turkish family with $\delta\beta^0$-thalassemia intermedia (Kulozik, et al., 1992; Öner et al., 1996; Öner et al., 1997) and a new type of β-thalassemia major with homozygosity for two non-consecutive 7.6 Kbp deletions of the ψβ and β-genes (Öner et al., 1995; Table I.2). All these alleles can surely be considered as a testimony of past colonies in Anatolia.

TABLE I.2. β-Thalassemia Alleles Described in Turkish People.

	Mutation	Reference
1.	-101 (C-T)	Gonzalez-Redondo et al., 1989b
2.	-87 (C-G)	Diaz-Chico et al., 1988
3.	-30 (T-A)	Öner et al., 1990
4.	-28 (A-C)	Öner et al., 1990
5.	5'-UTR +22 (G-A)	Öner et al., 1991a
6.	FSC-5 (-CT)	Öner et al., 1990
7.	Cd 6 (A-T) [HbS]	Egeli and Ergün, 1946
8.	FSC-6 (-A)	Öner et al., 1990
9.	FSC-8 (-AA)	Diaz-Chico et al., 1988
10.	FSC-8/9 (+G)	Öner et al., 1990
11.	Cd 15 (G-A)	Aulehla-Scholz et al., 1990
12.	FSC 22/23/24 (-AAGTTGG)	Özçelik et al., 1993
13.	Cd 26 (G-A) [HbE]	Altay and Başak, 1995
14.	Cd 27 (G-T) [Hb Knossos]	Gürgey et al., 1989
15.	Cd 30 (G-C)	Jankovic et al., 1994
16.	IVS-I-1 (G-A)	Diaz-Chico et al., 1988
17.	IVS-I-1 (G-C)	Altay and Başak, 1995
18.	IVS-I-1 (G-T)	Altay and Başak, 1995
19.	IVS-I-5 (G-A)	Öner et al., 1990
20.	IVS-I-5 (G-C)	Diaz-Chico et al., 1988
21.	IVS-I-5 (G-T)	Öner et al., 1990
22.	IVS-I-6 (T-C)	Diaz-Chico et al., 1988
23.	IVS-I-110 (G-A)	Diaz-Chico et al., 1988
24.	IVS-I-116 (T-G)	Başak et al., 1992a
25.	IVS-I-130 (G-A)	Tadmouri et al., 1999b
26.	IVS-I-130 (G-C)	Öner et al., 1990
27.	FSC-36/37 (-T)	Jankovic et al., 1994
28.	Cd 37 (G-A)	Altay and Başak, 1995
29.	FSC 37-39 (-7 bp)	Schnee et al., 1989
30.	Cd 39 (C-T)	Diaz-Chico et al., 1988
31.	FSC-44 (-C)	Altay and Başak, 1995
32.	FSC-74/75 (-C)	Başak et al., 1992b
33.	IVS-II-1 (G-A)	Diaz-Chico et al., 1988
34.	IVS-II-654 (C-T)	Tadmouri et al., 1999a
35.	IVS-II-745 (C-G)	Diaz-Chico et al., 1988
36.	IVS-II-848 (C-A)	Altay and Başak, 1995
37.	3'-UTR +1,565 to +1,577 (-13 bp)	Başak et al., 1993
38.	Poly A (AATAAA-AATAAG)	Altay and Başak, 1995
39.	Poly A (AATAAA-AATGAA)	Tadmouri et al., 1998a
40.	290 bp deletion	Diaz-Chico et al., 1987
41.	7.6 kb deletion	Öner et al., 1995
42.	30 kb deletion	Öner et al., 1996

Advances in the molecular understanding of β-thalassemia in Turkey did greatly improve preventive medical services such as genetic counseling and prenatal diagnosis and provided information that helped in understanding the clinical and hematological variations of this disorder. For this, specialized medical faculties at various institutes were established to conduct treatment and investigation for β-thalassemia patients. Despite the difficulties imposed by the presence of various kinds of mutations leading to β-thalassemia in Turkey, prenatal diagnosis is feasible when early methods of fetal sampling are combined with the advent of PCR-based techniques such as allele specific oligonucleotide hybridization, the amplification refractory mutation system (ARMS), restriction endonuclease digestion analysis, DNA sequencing (Tüzmen et al., 1996), and, more recently, the reverse dot-blot hybridization technique (Bilenoğlu, 1996). At present, prenatal diagnosis of β-thalassemia and SCD are performed in several centers in Turkey, some of which are Hacettepe University (Ankara; Gürgey et al., 1996), Boğaziçi University (Istanbul), and Çukorova University (Adana; reviewed by Altay and Başak, 1995).

Besides the elevated rate of consanguineous marriages (21 per cent) within certain communities having a high incidence of thalassemia (Başaran et al., 1988), Turkey is one of the countries showing the highest rates of population increase in the world (36:1000, Census 1994). Both figures appear to contribute drastically to the frequency of affected births. The expected number of infants born annually with β-thalassemia and SCD in Turkey have been calculated to be around 150 and 40, respectively. Hence, approximately 800 pregnant women should seek prenatal diagnosis each year. Unfortunately, the total number of prenatal diagnoses performed in all operating centers barely exceeds $1/8^{th}$ of the expected value each year (reviewed by Altay and Başak, 1995). This indicates the need for implementing a comprehensive genetic preventive program for the eradication of β-thalassemia and SCD in Turkey like those going on in many Mediterranean countries. This could be performed either by screening of reproductive couples when they register for marriage (Altay et al., 1996b) or by educating the population at risk and their physicians (Tüzmen et al., 1996). Intensive involvement of the population, e.g., community education and informed genetic counseling, is an important prerequisite. The materialization and realization of such a program rely greatly on the resources for education of the population

and the nation-wide transfer of know-how and technical facilities, present in research centers, to routine clinical laboratories, that may be playing an important role in the near future (Tüzmen et al., 1996).

It is important to note that the large migration flows of Turkish citizens to European countries, mainly to Germany, during the last several decades contributed to a large extent to the presence of β-thalassemia and other hereditary anemias in such non-malarial countries. Socio-medical surveys conducted in Turkish societies living in those countries demonstrated that the knowledge and perception of thalassemias were extremely limited, making large-scale screening programs a difficult task especially when taking into account the cultural and educational conditions of the Turkish minority (Holzgreve et al., 1990).

II. PURPOSE

During the past 11 years, research on β-thalassemia in Turkey followed a new direction by the application of several types of molecular-based analyses to investigate this serious genetic disorder. Starting from this point, some of the goals set for the present study were to:

A) Delineate the molecular reasons in β-thalassemia individuals with 34 uncharacterized chromosomes, thus offer the chance of prenatal diagnosis to more families at risk of β-thalassemia in Turkey, and develop simple methods for the detection of rare mutants.

B) Investigate some inter- or intra-genetic factors contributing to the variability in the clinical phenotypes in β-thalassemia patients and establish easy methods for their detection.

The data obtained will be combined with our previous knowledge about the spectrum of β-thalassemia mutations in Turkey to:

A) Permit the elucidation of the dynamics of the probable events which may have led to the presence of those previously non-described mutations in Turkey.

B) Analyze the geographic distribution of these alleles in Turkey at regional and sub-regional levels.

In addition, sequence haplotype analysis of Turkish β-globin genes will be used to:

A) Provide valuable information about the timing of racial diversions, population movements, and the molecular events which have helped to form and maintain β-thalassemia at high gene frequencies in Turkey and the Mediterranean.

B) Test for the feasibility of applying polymorphic nuclear DNA marker analysis in delineating important historical events in modern humans.

Results recorded in the framework of this thesis are not only expected to be useful for researchers in the field of β-thalassemia, but they will also serve as a model for scientists working in the field of population genetics to understand human evolution and history in Turkey, the Middle East, and Europe.

III. MATERIALS

A. Human Blood and DNA Samples

A total of 795 randomly selected Turkish β-thalassemia and HbS chromosomes from patients and carriers whose blood samples were sent to our laboratory from hospitals in Istanbul, Ankara, Izmir, Adana, and Antalya participated in this study. These chromosomes formed the basis of an extensive analysis to depict the frequency distribution of common β-globin gene mutations in various geographical regions of Turkey. The majority of individuals selected had detailed family histories and were classified to 102 towns in Turkey; the rest consisted of immigrant Turks from the Balkan countries, Cyprus, and the Near East (Figure III.1). Alternatively, Turkish individuals investigated in the framework of this thesis were also classified according to the geographical regions of Turkey which were: the Marmara region (MR), Aegean-Mediterranean region (AM), Central Anatolia (CA), Black Sea region (BSR), Eastern Anatolia (EA), and Southeastern Anatolia (SEA; Figure III.2). Immigrant Turks from Bulgaria, Greece, Cyprus, and Yugoslavia were all grouped as of Balkan origin (BLK). Of the 795 chromosomes investigated, 34 were subjected to DNA sequencing to characterize the molecular reasons behind the β-thalassemia phenotype encountered in 19 individuals (14 homozygous and 6 heterozygous).

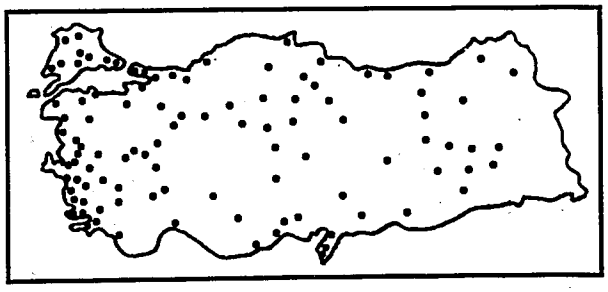


FIGURE III.1. Map of Turkey. Dots indicate the geographical origin of Turkish chromosomes analyzed in the framework of this thesis.

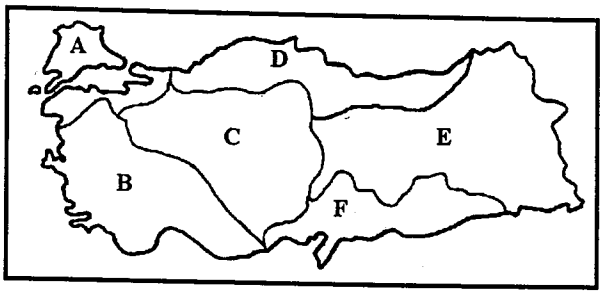


FIGURE III.2. Geographical regions of Turkey analyzed in the framework of this thesis: (A) Marmara region, (B) Aegean and Mediterranean region, (C) Central Anatolia, (D) Black Sea region, (E) East Anatolia, (F) Southeastern Anatolia.

The association of the *XmnI* polymorphism at position -158 of the Gγ-globin gene with the clinical course was investigated in 62 chromosomes from 31 in β-thalassemia homozygotes (AM: 4; BSR: 8, CA: 22, EA: 14, SEA: 14) with high levels of HbF. The evaluation of the $(AT)_xT_y$ motif, present at -530 bp upstream from the β-globin gene and its relation to the (a) variable HbF levels and (b) geographical origin in 143 β-thalassemia and normal individuals involved the analysis of 259 chromosomes (AM: 35, BSR: 24, CA: 43, EA: 23, MR: 21, SEA: 29, BLK: 23, Lebanon: 3, Syria: 2, Pakistan: 2, and 54 chromosomes with undefined origin). All individuals were non-related and their selection depended on their well-defined phenotypes, transfusion-dependency, and/or geographical origins.

A total of 191 non-related β-thalassemia chromosomes from Turkey was typed for sequence haplotype analysis in the framework of this thesis. Most of the β-thalassemia chromosomes were previously included in a study of the regional distribution of β-thalassemia alleles in Turkey (Tadmouri et al., 1998a). Chromosomes analyzed belonged to different geographical origins: MR (15), BSR (19), AM (30), CA (33), SEA (25), EA (18), and BLK (21). An additional group included 30 chromosomes with undefined

origins. Of the 191 β-thalassemia chromosomes investigated, 181 were found to carry the following 22 mutations: IVS-I-110 (G-A), IVS-I-6 (T-C), -30 (T-A), Cd39 (C-T), -87 (C-G), FSC-8/9 (+G), IVS-I-5 (G-C), IVS-I-1 (G-A), FSC-8 (-AA), IVS-II-1 (G-A), IVS-II-745 (C-G), Cd27 (G-T; Hb Knossos), FSC-5 (-CT), IVS-I-116 (T-G), FSC-74/75 (-C), Cd15 (G-A), HbE Saskatoon, HbS, HbD Los Angeles, IVS-II-654 (C-T), IVS-I-130 (G-A) and IVS-II-848 (C-A). In the remaining 10 chromosomes the molecular defect could not be characterized by the methods applied. Additionally, 18 non-related wild type β-globin chromosomes from Turkey, Lebanon, and Syria were also included for comparative analysis.

B. Equipment

Facilities of the Department of Molecular Biology & Genetics at the Boğaziçi University (Istanbul, Turkey) and of the Biodiversity Molecular Analysis Center at the University Claude-Bernard Lyon I (Lyon, France) were used in the framework of this thesis (Table III.1).

C. Buffers and Solutions

All chemicals and solutions used in this study (Table III.2) were purchased from Merck (Germany), Fluka Chemica (Germany), and Sigma (USA), unless stated otherwise in the text.

TABLE III.1. Facilities used in the framework of this thesis.

Autoclaves:	Eyela Autoclave, MAC-601 (Eyela, Japan)
Balances:	Electronic Balance Model VA124-1AAZM13AAE (Gec Avery, UK) Electronic Balance Model CC081-10ABAAGA (Gec Avery, UK)
Cameras:	DS34 (Polaroid, USA) BioDoc Video Documentation System (Biometra, Germany)
Centrifuges:	SORVALL RC-5B Refrigerated Superspeed Centrifuge (DuPont, USA) Centrifuge 5415C, (Eppendorf, Germany) Microfuge®R (Beckman, Germany)*
Refrigerators:	4°C Medicool (Sanyo, Japan)
Deep-freezers:	-20°C (Bosch, Germany) -70°C (Sanyo, Japan) -70°C (GFL, Germany)
Electric pipette:	Acuboy (Tecnomara, Switzerland)*
Electrophoresis systems:	Horizon 58, Model 200 (BRL, USA) Sequencing Gel Electrophoresis System, Model S2 (BRL, USA) BaseAce® Jr. Vertical Sequencing Apparatus (Stratagene, USA) Bio Co. LTD (Europe)* 370A, DNA Sequencer (Applied Biosystems, USA)*
Gel drier:	Mididry Gel Drier (Biometra, Germany)
Heat blocks:	Thermostat Heater 5320 (Eppendorf, Germany) Bioblock Scientific 92617 (Bioblock Scientific, USA)*
Incubators:	Shaking Incubator (Hybaid, UK) Oven, EN400 (Nuve, Turkey) Oven 300 Plus Series (Gallenkamp, Germany) Orbital Incubator (Gallenkamp, Germany)
Laminar flow:	C.B.S. Scientific Co. (CBS, USA)*
Magnetic stirrers:	Chiltern Hotplate Magnetic Stirrer, HS31 (UK)
Ovens:	Microwave Oven (Vestel, Turkey) 65dC EN400 (Nuve, Turkey) 56dC (LEEC, UK)
Power supplies:	Model 100 (BRL, USA) ECPS 3000/150 Constant Power Supply (Pharmacia, Sweden)
Shaker:	VIB (InterMed, Denmark)
Speed vacuum concentrator:	Savant, Speed Vac SC 100 (Savant Instruments Inc., USA)*
Thermal cyclers:	UNO-Thermoblock (Biometra, Germany) Techne (Progene, UK) Gene Amp PCR System 2400 (Perkin-Elmer, USA)*
UV-Transilluminator:	Chromato-Vue Transilluminator, Model 1TM-20UVP (USA)
Vortex:	Rotalab, Vortex Machine (OSI, France)*
Water baths:	Thermomix BU (Braun, Germany) Thermomix 1441 (Braun, Germany) Kottermann (Labortechnik, Germany)
Water purification:	UHQ, Ultra-pure water (Elga Inc., UK)* Prima Reverse Osmosis (Elga Inc., UK)*

*Facilities of the Biodiversity Molecular Analysis Center at the University Claude-Bernard Lyon I (Lyon, France).

TABLE III.2. Buffers and solutions used in the framework of this thesis.

DNA extraction from blood	**Cell lysis buffer:**	155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM Na_2EDTA (pH 7.4)
	Nucleus lysis buffer:	10 mM Tris (pH 8.0), 400 mM NaCl, 2 mM Na_2EDTA
	Proteinase K:	20 mg/ml in dH_2O
	SDS:	10% stock solution
	NaCl:	5 M saturated stock solution
	Alcohol:	Absolute ethanol
	TE buffer:	20 mM Tris (pH 8.0), 0.1 mM Na_2EDTA (pH 8.0)
Gel electrophoresis	**10X Loading buffer:**	2.5 mg/ml BPB, 1% SDS in 2 ml glycerol
	Ethidium bromide:	10 mg/ml
	10X TBE buffer:	1 M Tris-Base, 900 mM Boric Acid, 20 mM Na_2EDTA (pH 8.3)
	Agarose gels:	0.8-2.0% agarose (w/v) in 0.5X TBE buffer containing (0.5 μg/ml) ethidium bromide
	40% Acrylamide:	19:1 acrylamide-bisacrylamide in H_2O
	8% Instagel:	8% Acrylamide-bisacrylamide (19:1), 8 M Urea, 1X TBE buffer (pH 8.3)
	10% APS:	10% Ammoniumpersulfate in H_2O
Polymerase chain reaction	**1X PCR buffer:**	10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100
	$MgCl_2$:	1.5 mM $MgCl_2$
	dNTPs:	25 mM of each dNTP

D. Fine Chemicals

1. Enzymes

The *Taq* DNA polymerase was from Advanced Biotechnologies (USA), Perkin-Elmer Cetus (USA), Sigma (Germany) or MBI Fermentas (Lithuania). The restriction enzymes with their appropriate buffers were from New England Biolabs (USA), Boehringer Mannheim (Germany), Promega (USA), or MBI Fermentas (Lithuania).

2. Primers

Primers used in the framework of this thesis are listed in Table II.3 and they were synthesized at Biometra (Germany) or Genset (France). Some of the primers used for the analysis of the polymorphic sequence 5' to the β-globin gene were kindly provided by Dr. Pascale Perrin (University of Claude Bernard, Lyon I, the Biodiversity Molecular Analysis Center, Lyon, France).

3. Other Fine Chemicals

dNTPs used for PCR were either from Promega (USA) or Sigma (Germany). PCR products were purified by using QIAquick PCR Purification Kit (Qiagen, Germany) that contains commercial PCR purification and washing buffers PB and PE (55 mls to which 220 ml of pure ethanol are added).

TABLE III.3. Primers used in the framework of this thesis (stated 5' to 3'). Numbers in parentheses denote the position of the corresponding primer.

β-Globin amplification	**Forward:**	**CD1**	TGC CTC TTT GCA CCA TTC TAA
		CD7	TCC TAA GCC AGT GCC AGA AG
		F 108	GCC AAG GAC AGG TAC GGC TGT CAT C
		F 16	GCA GGT TGG TAT CAA GGT T
		F 229	ATA CAA TGT ATC ATG CCT CTT TGC ACC
		F 378	ACT TAG ACC TCA CCC TGT GGA GC
		F 58	AAT CCA GCT ACC ATT CTG C
		F 70	GGG TTA AGG CAA TAG CAA T
		GH 20	GAA GAG CCA AGG ACA GGT AC
		KM 29	GGT TGG CCA ATC TAC TCC CAG G
		PCO3	ACA CAA CTG TGT TCA CTA GC
		R 35	CAA GAC AGG TTT AAG GAG ACC
		R 37	CCA ATC TAC TCC CAG GAG CA
		R 92	TGC ATA TAA ATT GTA ACT GAT
		R 95	TAA GCC AGT GCC AGA AGA GCC
		S 16	GCA GGTBTGG TAT CAA GGT T
		SR 96	CTG GCC CAT CAC TTT GGC AA
		TCG1	CAA TGT ATC ATG CCT CTT TGC ACC
	Reverse:	**CD2**	TGA CCT CCC ACA TTC CCT TTT
		CD6	ATC ATT CGT CTG TTT CCC ATT CTA AAC
		R 230	GTA TTT TCC CAA GGT TTG AAC TAG CTC T
		R 47	CAC TCA GTG TGG CAA AGG TG
		R 61	CAC TGA CCT CCC ACA TTC CC
		R 93	GAA ATT GGA CAG CAA GAA AGC G
		R 94	TCC TAT GAC ATG AAC TTA ACC
		R109	CCC TTC CTA TGA CAT GAA CTT AAC CAT
		RS 43	GCT CAC TCA GTG TGG CAA AG
		SR 14	GTG CCC TTG AGG TTG TCC AGG T
		SR 16	GAC CAC CAG CAG CCT AAG GGT G
		TCG2	GGA GTC AAG GCT GAG AGA TGC AGG A
***XmnI* Gγ-polymorphism**	**Forward:**	**G5**	GCT ACA GAC AAG AAG GTG
	Reverse:	**G4**	TTT TAT TCT TCA TCC CTA GC
Sequence haplotype analysis	**Forward:**	**A**	CCA GGC AGA AAC AGT TAG AT (-844,-825)
		D	CCT CAC CTG AGG AGT TAA TT (-1140,-1121)
		P	CGC TGA CCT CAT AAA TGC T (-1228,-1210)
	Reverse:	**B**	CTA CCA TAA TTC AGC TTT GGG AT (-390,-373)
		H	CTC TTG TTT CCC AAA ACC TA (-702,-683)
		Q	GGA TCT CTT CCT GCG TCT C (-419,-401)
ARMS for mutation detection	**Control:**	**F Control A**	CAA TGT ATC ATG CCT CTT TGC ACC
		R Control B	GAG TCA AGG CTG AGA GAT GCA GGA
	Common:	**F Common C**	ACC TCA CCC TGT GGA GCC AC
	Mutation specific:	**IVS-I-110 mt**	ACC AGC AGC CTA AGG GTG GGA AAA TAG ACT
		IVS-I-110 wt	ACC AGC AGC CTA AGG GTG GGA AAA TAG ACC
		IVS-I-6 mt	TCT CCT TAA ACC TGT CTT GTA ACC TTC ATG
		IVS-I-6 wt	TCT CCT TAA ACC TGT CTT GTA ACC TTC ATA
		FSC-8 mt	CCT TGC CCC ACA GGG CAG TAA CGG CAG ACC
		FSC-8 wt	CCT TGC CCC ACA GGG CAG TAA CGG CAG ACT
		IVS-I-1 mt	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAT
		IVS-I-1 wt	TTA AAC CTG TCT TGT AAC CTT GAT ACG AAC
		IVS-II-745 mt	TCA TAT TGC TAA TAG CAG CTA CAA TCG AGG
		IVS-II-745 wt	TCA TAT TGC TA TAG CAG CTA CAA TCG AGC
		IVS-II-1 mt	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAT
		IVS-II-1 wt	AAG AAA ACA TCA AGG GTC CCA TAG ACT GAC

DNA molecular weight standards included (a) ΦX 174 DNA/*BsuRI* (*HinfI*) marker with fragments of 726, 553, 500, 427, 417, 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 24 bp, (b) λDNA/*HindIII* marker with fragments of 23.1, 9.4, 6.7, 4.4, 2.3, 2, 0.6, 0.13 Kbp, (c) 100 bp Ladder marker with fragments of 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, and 80 bp (MBI Fermentas, Lithuania), (d) GT3 with fragments of 1801, 862, 707, 644, 533, 453, and 390 bp, (e) GT4 with fragments of 1801, 861, 762, 676, 576, 453, 282, and 281 bp, (f) GT5 with fragments of 1801, 861, 688, 453, 390, and 269 bp, and (g) GT6 with fragments of 861, 707, 634, and 394 bp (manually prepared in the laboratory).

The radionucleotide α-[^{35}S]-dATP (>1000 Ci/mmole) was either from Amersham (USA) or Izotop (Hungary). X-Ray film developing solutions were from Kodak (USA). Two different sequencing kits were used:

(A) Sequenase Version 2.0 (USB, USA): This kit includes 5X Sequenase buffer (200 mM Tris pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), Mn Buffer (0.15 M Sodium Isocitrate, 0.1 M $MnCl_2$), DDT (0.1 M Dithiothreitol), 5X Labeling Mix "dGTP" (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP), A Termination Mix (80 μM of each dNTP, 8 μM ddATP, 50 mM NaCl), C Termination Mix (80 μM of each dNTP, 8 μM ddCTP, 50 mM NaCl), G Termination Mix (80 μM of each dNTP, 8 μM ddGTP, 50 mM NaCl), T Termination Mix (80 μM of each dNTP, 8 μM ddTTP, 50 mM NaCl), Enzyme Dilution Buffer (10 mM Tris pH 7.5, 5 mM DTT, 0.5 mg/ml BSA), Stop Solution (95% formamide, 20 mM EDTA, 0.05% BPB, 0.05% XC), and Sequenase Version 2.0 T7 DNA Polymerase (13 units/μl in 20 mM KPO_4 pH 7.4, 1 mM DTT, 0.1 mM EDTA, and 50% Glycerol).

(B) PRISM™ Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., USA): This kit includes a Terminator Ready Reaction Mix with AmpliTaq® FS (Perkin-Elmer, USA).

E. Other Materials

Instant films (type 667) were from Polaroid (USA) or (heat-sensitive papers) from Sanyo (Japan). Centricon®-30 concentrators were purchased from Amicon Inc. (USA). Bottle top filters were from Nalgene (USA). Terasaki plates were from Greiner (Germany). 3MM Whatman papers were from Whatman Inc. (USA). X-ray films X-OMAT AR5, X-OMAT RP and Agfa Curix RP1 were from Kodak (USA) and Agfa (Japan), respectively.

IV. METHODS

A. DNA Extraction from Whole Blood Using the NaCl Method

Peripheral blood samples are collected from individuals into vacutainer tubes containing EDTA as anticoagulant and stored at -20°C or -70°C until DNA extraction. Before NaCl extraction of leukocyte DNA, blood samples are allowed to thaw before their transfer to 50 ml polypropylene centrifuge tubes. Ice-cold cell lysis buffer (30 ml/10 ml of blood) is added to the blood sample and mixed thoroughly. The samples are then left on ice for 15 minutes during which the positively charged ions of the RBC lysis buffer avoid plumping of the cells and form an osmotic pressure inside the RBCs, leading to the lysis of their membranes. The EDTA present in the buffer acts in the chelation of free magnesium ions, and any other divalent cations if present, to disrupt the activity of any nucleases that might be present in the solution (Blackburn and Gait, 1990).

Leukocyte nuclei are collected after centrifugation of the lysed mix at 5000 rpm at 4°C for 10 minutes using an SS-34 rotor. The supernatants containing the RBC debris are discarded and the nuclei are re-suspended in 10 ml RBC lysis buffer by vortexing. At this step the nuclei may be stored at -70°C until DNA isolation. Centrifugation of the re-suspended mix is repeated at 5000 rpm at 4°C for 10 minutes. After discarding the supernatant, the nuclear pellet is re-suspended in three milliliters of nuclei lysis buffer and vortexed until all the clumps are broken. Proteinase K (final concentration 150 μg/ml) and SDS (final concentration 0.14 per cent) are added and lysates are gently mixed by rolling the tubes several times. The mixture is then incubated either at 56°C for three hours or at 37°C overnight. When the incubation is completed, five ml H_2O and five ml of saturated (5 M) NaCl are added to each mixture. The tubes are then shaken vigorously to ensure protein precipitation and centrifuged at 10,000 rpm at room temperature for 20 minutes. The supernatant containing the DNA is precipitated with two volumes of absolute ethanol. The tubes are gently inverted for several times until DNA threads are visible. The DNA

lump is then quickly transferred to an Eppendorf tube and should not be allowed to stay in the alcohol solution for long time to avoid dehydration of DNA. The DNA lump is then dissolved overnight at room temperature in 200-500 µl of a low ionic strength buffer such as TE buffer. Before using TE buffer, it is very important to ensure its neutral pH since low pH will depurinate DNA and high pH will cause degradation of DNA especially during restriction endonuclease digestion or other manipulations (Bothwell et al., 1990).

B. Qualitative and Quantitative Analysis of the Extracted DNA

The first evaluation of the isolated DNA is done by agarose gel electrophoresis. This technique is a standard method that separates and identifies DNA fragments according to their molecular weights. Agarose gels are prepared as 0.6-1.8 per cent in 0.5X TBE buffer by boiling the solution in a microwave oven. Various agarose concentrations provide different resolution capacities, higher concentrations being more specific in separating shorter fragments. To avoid degradation of the DNA-coloring dye, ethidium bromide is added to a final concentration of 0.5 mg/ml after the agarose solution cools down to less than 50°C. Warm agarose is then cast and a comb is positioned into the solution. After the solution jellies, the plate is placed into an electrophoresis tank containing 0.5X TBE buffer and the comb is gently removed from the gel to form the slots for loading the DNA samples. DNA samples are mixed with the BPB loading buffer to a final concentration of 1X and loaded into the slots using a micropipette. When an electric power is applied, DNA molecules run towards the positively charged electrode, because of the presence of negatively charged phosphate groups at the DNA backbone. Generally, agarose gels are run at voltages between 100-150 for 10-45 minutes, depending on the fragments to be separated. The effective range of separation of agarose gels decreases as the voltage increases. To avoid gel deterioration and to obtain a maximum resolution of DNA fragments, a current of no more than 5V/cm should be applied (Dickerson and Geis, 1983). When the electrophoresis migration is complete, the DNA bands can be visualized under UV light and documented. The amount and quality of an aliquot of DNA are then roughly

estimated by comparing its intensity with DNA standards of known characteristics. To determine the exact concentration of the isolated DNA, spectrophotometry is applied. Knowing that 50 μg of double-stranded DNA has an absorbance of 1.0 at 260 nm (OD_{260}), thus 50 μg/ml x OD_{260} x dilution factor = Concentration in μg/ml. The ratio between the spectrophotometric measurements at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the DNA sample. Pure preparations have a value of 1.8. Values greater than 1.8 indicate RNA contamination, whereas values less than 1.8 are the indication of contamination with protein.

C. Polymerase Chain Reaction

The idea of the PCR is to attach short DNA primers flanking the region of interest, one on each and opposite end of the two pairs of homologous DNA strands. A repetitive cycle of denaturation, primer annealing, and polymerization of the annealed primers by the *Taq* polymerase enzyme (a heat-stable DNA polymerase) results in the exponential accumulation of a specific fragment, almost doubling at every cycle. Thus, 30 cycles of PCR yield about a million-fold amplification of the target DNA in question (Saiki et al., 1988). In this study, three PCR approaches were used.

1. Conventional Double-Stranded PCR (dsPCR)

In each dsPCR reaction, 0.5-1.0 μg of genomic DNA is amplified in a 50 μl reaction mixture containing 15-50 pmoles of each primer, 1X PCR buffer, 1.5-2.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 1-2 units of *Taq* DNA polymerase. Dependent on the thermal cycler used, the tubes containing the PCR mix are covered with a few drops (50 μl) of mineral oil to prevent evaporation. The samples are then placed into the thermal cycler that runs according to the program required (Table IV.1).

To check the quality and quantity of the amplification, five µl of an aliquot of the PCR product are mixed with loading BPB buffer to a final concentration of 1X and electrophoresed on a 1-1.5 per cent agarose gel. The gel is then visualized on a UV-transilluminator and documented. In certain circumstances, purification of the resulting dsPCR product may be necessary. This is done by either of four methods:

Elution from agarose gels: In this technique, the complete volume of a PCR product is loaded on an agarose gel as described before. When the PCR product reaches the center of the gel, the band is sliced out by dissection using a sterile scalpel under a low-power UV light. The sliced part is then placed in an Eppendorf tube in 0.25 ml of ultra-pure H_2O. The tube is then subjected to repetitive cycles of freezing (-70°C, 15 minutes) and thawing (37°C, 15 minutes) until most of the pure PCR products ooze out of the gel into the H_2O.

Filtration through Centricon© filters: In this method, all the PCR product is placed in a Centricon© filter along with two ml of ultra-pure water and then centrifuged at 5,000 rpm for 20 minutes. The step is repeated for a second time for 45 minutes. The filter is inverted in a clean Eppendorf tube and spun briefly to collect an approximate volume of 40 µl of purified PCR product.

TABLE IV.1. dsPCR profiles used in the framework of this thesis.

β-Globin gene mutation detection:	95°C, 4'; (95°C, 30"; 55°C, 30"; 72°C, 2') 30 cycles; 72°C, 5'
Sequence haplotype analysis:	94°C, 1'; (94°C, 30"; 50°C, 30"; 72°C, 1') 35 cycles

Precipitation by ammonium acetate and ethanol: To the resulting PCR product, two µl of ammonium acetate (3M, pH 4.6) are added along with 50 µl of 95 per cent ice-cold ethanol to wash out the extra dNTPs unused in the reaction. The resulting mix is vortexed, stored at -20°C for 10 minutes, and centrifuged at 14,000 rpm for 30 minutes. The supernatant is discarded and 250 µl of 70 per cent ice-cold ethanol is added to the pellet to rinse any salts present. The solution is then centrifuged for a second time at 14,000 rpm for 30 minutes. The supernatant is discarded and the pellet is dried either by letting the tube open at 37°C for overnight or by lyophilization for 3-5 minutes using a speed vacuum concentrator.

PCR purification by QIAquick filters: For 50 µl of a PCR product, 250 µl of PB buffer are added and the total mix is then placed on top of the filter that is placed in an Eppendorf tube. The mix is centrifuged at maximum speed for one minute, the filter is washed with 375 µl of PE buffer, and then centrifuged for a second time at maximum speed for one minute. The Eppendorf tube placed below the filter is discarded and replaced by a clean one. Twenty µl of pre-warmed (37°C) ultra-pure water (pH 7.0) is placed on the filter and centrifugation at maximum speed is allowed for one minute. This step is repeated for a second time to ensure the collection of a total volume of ~40 µl of purified PCR product.

2. Single-Stranded PCR (ssPCR)

In the framework of this thesis, ssPCR was applied as an alternative approach to prepare DNA templates for sequencing, using the Sequenase Version 2.0 Kit (Amersham, USA). A volume of 5-20 µl of dsPCR products is used as a template to make a single ssPCR reaction. Each sample is taken into a 100 ml of PCR mixture containing unequal amounts of primers (100 pmole:1 pmole) and two units of *Taq* polymerase. The PCR conditions for generating ssDNA products are: 94°C, 4', (95°C, 30"; 57°C, 30"; 72°C, 2') 35-40 cycles; 72°C, 4'. During the initial 15-25 cycles, most of the product generated is

double stranded and accumulates exponentially. As the low concentration primer becomes depleted, further cycles generate an excess of one of the two strands, depending on the limiting primer (Gyllensten and Erlich, 1988). This single stranded DNA accumulates linearly and is complementary to the limiting primer. Before performing the sequencing reaction, ssPCR products are purified either by Centricon filtration or precipitation using ammonium acetate and ethanol as described previously.

3. The Amplification Refractory Mutation System

Mutations in the β-globin gene may be diagnosed directly by the presence or absence of amplification, using allele-specific primers. This method (ARMS) includes the use of two allele-specific primers (Table III.3) which have their 3' terminal nucleotides complementary to either the mutation (mutant ARMS primer) or the wild-type sequence at that point (wild-type ARMS primer); these primers amplify DNA only when their 3' terminal nucleotides have a perfect match with the target DNA. The method is based on the observation that, in many cases, oligonucleotides with a 3' mismatched residue will, under appropriate conditions, not function as primers in the PCR (Newton et al., 1989). The ARMS technique was implemented in the framework of this thesis for three purposes, the conditions for the PCR are shown in Table IV.2.

TABLE IV.2. ARMS-PCR profiles used in the framework of this thesis.

β-Globin mutation detection:	93°C, 6'; (93°C, 1'; 60°C, 3') 30 cycles; 60°C, 7'
***XmnI* Gγ-polymorphism:**	94°C,1'; (94°C, 30"; 52°C, 30"; 72°C, 1') 35 cycles; 72°C, 5'
Haplotype analysis:	94°C, 1'; (94°C, 1'; 55°C, 1'; 72°C, 2') 40 cycles; 72°C, 7'

Confirm the results of some β-globin mutations deduced from DNA sequencing: In this system a second pair of primers is always included in the reaction mixture to simultaneously amplify an unrelated DNA sequence as a control of the ARMS analysis. The reaction is performed in a mixture of 50 µl PCR mix containing approximately 0.5-1 µg of genomic DNA, and 50 pmoles of each of the four primers.

Differential amplification of Gγ-globin promoter sequences: Despite of the strong homology between promoter sequences of Gγ- and Aγ-globin genes, the use of a specific primer, only hybridizing to the Gγ-globin promoter sequence, enables the selective amplification of the Gγ-globin gene sequences and avoids the possibility of contamination with Aγ-globin gene sequences. In this reaction control primers are not used since (a) the amplified target is always expected to give a positive signal and (b) the presence of foreign sequences other than those of the promoter region of the Gγ-globin genes will interfere with the following restriction endonuclease analysis by the *XmnI* enzyme.

Differential amplification of polymorphisms and mutations of the β-globin gene: To assign the haplotypes to heterozygous patients carrying either the IVS-I-110 (G-A) or Cd39 (C-T) alleles along with a different mutation, the ARMS technique is applied using specific primers. In the case of the IVS-I-110 (G-A) mutation, forward primer D and reverse primers IVS-I-110 mt and IVS-I-110 wt are used to selectively amplify the IVS-I-110 (G-A) and other chromosomes, respectively. In the case of the Cd39 (C-T) mutation, forward primer D and reverse primers Cd39 mt and Cd39 wt are used to selectively amplify the Cd39 (C-T) and other chromosomes, respectively. This system allowed a further confirmation of the presence of the IVS-I-110 and Cd39 mutations in the samples analyzed and opened access to the analysis of haplotypes of rare mutations not present in homozygous forms. In this amplification, the PCR mixture (50 µl) contains 10 pmol of each primer and three nmol of each dNTP in *Taq* buffer with two units of DNA polymerase (Perrin et al., 1998). The resulting PCR products are purified before sequencing using QIAquick filters as previously described.

During the experiments conducted in the framework of this study in the Biodiversity Molecular Analysis Center at the University of Claude Bernard Lyon I (Lyon, France), a full use of the present PCR laboratory was practiced. This laboratory includes two separate chambers, each containing a positive pressure producing aeration system, a refrigerator-freezer, a laminar flow, a micropipette set, sterilized filtered tips, and a room UV-sterilizing system. DNA handling is only allowed in one chamber, the PCR mix preparation is practiced in the second chamber. To avoid DNA contamination of PCR reactions, PCR mixes are transported to the DNA chamber, but not in the reverse direction. When PCR preparation is complete, the rooms are emptied from waste and exposed to UV-light sterilization for 1-2 hours to be ready for another user. No equipment and laboratory instruments are allowed to be exchanged between the PCR laboratory and other laboratories of the institute unless they are extensively sterilized by conventional methods.

E. Restriction Endonuclease Analysis

This non-radioactive method is used to cleave an amplified DNA fragment with the appropriate endonuclease. Dependent on the case, the digestion pattern indicates the presence or absence of the mutant allele after agarose gel electrophoresis. The method is used in the framework of this thesis to (a) confirm β-globin mutations diagnosed by sequencing and (b) analyze the C-T variation at position -158 upstream from the Cap site of the Gγ-globin gene by digestion with the *XmnI* restriction endonuclease that recognizes the sequence 166-157 bp 5' of the Gγ cap site only if T is at -158 (GAAACGGTTC); the site is abolished if C is at that position.

In any of these cases, a 10-17 μl aliquot of the amplified DNA is digested with 2-5 units of the appropriate restriction endonuclease enzyme and its reaction buffer in a 20 μl reaction volume. The mixture is incubated at 37°C or 56°C for 3-4 hours or, sometimes for overnight, dependent on the characteristics of the enzyme used. The digested DNA is

applied on a 1.2-1.8 per cent agarose gel depending on the length of the expected fragments. The electrophoresis is carried out at 100V for 30-45 minutes, the band pattern is visualized on a UV-transilluminator and then documented.

F. DNA Sequencing for Detection of Polymorphisms and Rare Mutations in the β-Globin Gene

1. Denaturing Polyacrylamide (Sequencing) Gel Preparation

The sequencing reactions performed in the framework of this study are analyzed on eight per cent denaturing polyacrylamide (Insta-) gels containing 7 M urea for denaturation of the DNA fragments. Ultra-thin gels (0.4 mm) are used to increase the resolution of the sequence bands. A shark-tooth comb is used to eliminate space between adjacent sample lanes and increase the number of samples electrophoresed on a single gel. In conventional radioactive sequencing 48-96 slots are used. Because of technical restrictions, only 24 slots are used in non-radioactive automated sequencing electrophoresis. Migration of the two tracking dyes, BPB and XC, is used in conventional radioactive sequencing to indicate the location of the DNA fragments.

Glass plates, cleaned with ultra-pure water, are assembled by clamps, placed on two sides, and then layered almost horizontally. Before pouring the Instagel, the solution is filtered by a Nalgene bottle top filter to avoid the presence of any particle that may disrupt the proper migration of DNA bands during electrophoresis. After filtration, the solution is optionally degased by vacuum treatment to avoid the presence of any air bubbles. Appropriate amounts of TEMED and APS are mixed gently with the Instagel solution, which will then start to polymerize in 10-20 minutes, depending on present room temperature. With the aid of a syringe or a squeeze-able bottle, 55 ml of Instagel are gently

poured between the plates. The flat edge of the shark tooth comb is placed into the gel in a horizontal position. Three hours are allowed for the gel polymerization to occur (an overnight stay is also possible). The comb is carefully removed and the well is thoroughly rinsed with 1X TBE to avoid crystals of urea to clog the slots. After the gel is connected to the apparatus, its top is again rinsed with 1X TBE buffer to remove any non-polymerized acrylamide together with excess urea oozing out of the gel. The buffer chambers are filled with fresh 1X TBE buffer and the comb is carefully inserted so that the teeth make a slight contact with the gel surface. Before loading the sequencing samples, the gel is pre-heated by applying 1600 Volts for 30 minutes until a temperature of 50-55°C is reached to help further denaturation of the DNA sequences to be run. Finally, the wells are flushed with 1X TBE buffer several times to remove the urea diffusing out of the gel before loading the sequencing products.

Three different DNA sequencing protocols were implemented in the framework of this thesis; all protocols involve the same principle with slight modifications. Basically, the chain-termination method includes the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded or double-stranded template (Sanger et al., 1975). Synthesis is only initiated at the location where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a nucleotide analog that will not support continued DNA elongation (hence the name chain-termination). The chain-terminating nucleotide analogs are the 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs). These lack the 3'-OH group necessary for DNA chain elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalyzed polymerization will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated (Sanger et al., 1975). In radioactive sequencing, four separate reactions are performed for each sample, each with a different ddNTP giving complete sequence information. A radioactively labeled nucleotide also is included in the synthesis, so the labeled chains of various lengths can be visualized by autoradiography after separation by high-resolution electrophoresis. The Sanger technique, which is conceptually elegant and efficacious, is in practice time-consuming and labor-intensive, partly because a single radioisotopic reporter is used for detection. Using one reporter to

analyze each of the four bases requires four separate reactions and four gel lanes. The resulting autoradiographic patterns, obtained after a delay for exposure and development, are complex and require skilled interpretation and data transcription.

These deficiencies are corrected by switching from a radioisotopic to a fluorescent reporter. In this system, four chemically related, yet distinguishable, fluorescence-tagged dideoxynuxleotides are used to label DNA by a modified Sanger protocol with a suitable chain-extending DNA polymerase (Prober et al., 1987). The fluorescent sequencing fragments are resolved temporally rather than spatially in a single lane by conventional polyacrylamide gel electrophoresis. Analysis of the fluorescent emission of each fragment permits the identification of the terminating nucleotide and assigns the sequence directly in real time (Prober et al., 1987).

2. Radioactive DNA Sequencing for β-Globin Gene Mutation Detection

In this method, the sequencing reactions are carried out by using the Sequenase Version 2.0 Sequencing Kit. The kit includes a specific DNA sequencing polymerase that has high process rate, no 3' to 5' exonuclease activity, and efficient use of nucleotide analogs important for sequencing. The sequencing reaction is applied on ssPCR products in a three-step procedure.

(A) Annealing: To seven μl of template DNA, two μl of 5X reaction buffer and 1 μl (1-100 pmol) primer are added. The resulting 10 μl mix is then heated at 70°C for three minutes and quickly cooled down on ice.

(B) Labeling: In a separate tube one µl of 10 mM DTT, 0.5-1 µl α-[^{35}S]-dATP (5-10 µCi), two µl 2-10 folds diluted labeling mix, and two µl Sequenase enzyme (two units) are mixed together and allowed to react for five minutes at room temperature.

(C) Termination: The labeling mix is divided among four tubes each of which contain 2.5 µl of each termination mixture (pre-warmed at 37°C). The duration of the labeling step is five minutes at 37°C. The reaction is stopped upon addition of four µl stop solution.

Subsequently, the samples are either stored at -20°C or loaded on a pre-heated sequencing gel after denaturation at 95°C for five minutes. Three ml of each termination tube is loaded in each lane in the order A, C, G, and T. Generally, sequencing gels are run at an electric powers of 70-80 Watts, 35-45 mA, and 1600-2000 V for 2-6 hours, dependent on the location of the sequence to be resolved and examined.

After electrophoresis, the gel is transferred onto a 3MM Whatman© paper, covered with a nylon sheet, and dried in a gel-drier at 100°C for one hour. The blotted gel is then layered in an X-ray film cassette and exposed to an X-ray film at room temperature for few hours to a few days, dependent on the freshness of the radionucleotides used. The X-ray film is developed manually for 1.5 minutes until the gel pattern appears. The film is then water-rinsed for 20 seconds, fixed for 1-5 minutes, and extensively washed under tap water. The blotted-gel may be stored for a couple of weeks in a dry and dark place for re-exposure, if necessary.

3. Radioactive DNA Sequencing for β-Globin Gene Haplotype Analysis

The sequencing reaction followed in this method is, basically, the same as the procedure described above with slight modifications. Only the differences from the above procedure are mentioned herein. The present technique uses dsPCR products as templates for the sequencing reaction in a three-step procedure.

(A) Annealing: In an Eppendorf tube, 8.5 µl of template DNA and 1.5 µl (20 pmol) primer are put together. The resulting 10 µl mix is then heated at 95°C for three minutes and quickly cooled down on ice.

(B) Labeling: In a separate tube, one µl of 10 mM DTT, two µl of 5X Sequenase reaction buffer, two µl of 20 per cent 1:4 diluted labeling mix, 0.5-1 µl α-[^{35}S]-dATP (5-10 µCi), 0.5 µl Mn buffer, and 0.5 µl Sequenase enzyme are mixed to each annealing tube and allowed to react for five minutes at room temperature.

(C) Termination: The labeling mix is mixed with the 10 µl denatured DNA mix, and 3.5 ml are divided among four slots in a Terasaki plate each of which contain 2.5 µl of each termination mixture (pre-warmed at 37°C). The reaction is then conducted for five minutes at 37°C and stopped upon addition of four µl stop solution in each slot.

Before loading the samples onto the sequencing gel, the Terasaki plate is heated to 95°C for two minutes; the subsequent steps are as described above. In conclusion, this method (a) reduces the time loss by omitting the production of single-stranded PCR-purified products and the subsequent manipulations performed, (b) eliminates the inconvenient handling of four tubes for each sequencing reaction by combining all reactions in one Terasaki plate, and (c) allows the completion of a dozen sequencing

reactions at a time in comparison to a maximum of 5-6 reactions with the classical method mentioned in section IV.F.2.

4. Non-Radioactive DNA Sequencing for β-Globin Gene Haplotype Analysis

The automated non-radioactive DNA sequencing procedure applied in the framework of this thesis is performed by using dsPCR templates purified by the QIAquick PCR Purification Kit as previously described. To four µl of purified dsPCR, four µl of the Ready Reaction Mix with AmpliTaq FS included in the Applied Biosystems Dye DeoxyTerminator Cycle Sequencing Kit and two µl of 10 pmole/µl primer are added. This and subsequent steps performed in this procedure are all done in light-free environments to avoid degradation of the sensitive fluorescent chemicals included in the mix. The resulting 10 µl mix is then subjected to PCR amplification for (96°C, 30"; 50°C, 30"; 60°C, 4') 25 cycles. When the PCR reaction is complete, the amplified sequencing products are purified and precipitated by using ammonium acetate and ethanol as described in section IV.C.1. The resulting pellet is either processed and prepared for gel electrophoresis or, alternatively, it can be stored in a dark place at -20°C for some days.

Before electrophoresis, the pellet is re-suspended by vortexing in a five µl solution containing formamide and EDTA at a ratio of 4:1. The sequencing products are then denatured by incubation for two minutes at 95°C and quickly cooled down on ice. The products are deposited on the gel and then subjected to electrophoresis at 1200V for 14 hours. The gel is automatically read by using a color sensitive laser beam and analyzed through a computer that is monitoring the whole process. The obtained sequences are compared and nine polymorphic nucleotides and a hypervariable motif of composite sequence $(AT)_xT_y$ present at -490 to -1069 bp 5'to the β-globin gene are analyzed. Each combination of these specific polymorphic sites defines a sequence haplotype.

G. Statistical Analysis

χ^2 test of independence was applied in all the statistical analysis related to sequence haplotypes in the population studied. The equation: $\chi^2=(ad-bc)^2n/((a+b)(b+d)(b+d)(d+c))$ is applied, where a and b are the number of observed chromosomes for a given haplotype in wild-type and β-thalassemia chromosomes, respectively. The remaining number of wild-type and β-thalassemia chromosomes is designated by the letters c and d, respectively; n is the total number of chromosomes studied (including both wild-type and β-thalassemia). The χ^2 test of independence is only applied for a total size that is greater than 20 chromosomes and when a, b, c, and d are greater than five. A χ^2 value less than 3.78, that corresponds to a probability (P) greater than five per cent, indicates the significance of a result (Wayne, 1995). Probabilities were calculated using the online version of the software "Enigma's Chi-Square Probality Calculator" (http://www.enigma.ene.unb.br/pub/crypto/chi2.htm)

As for β-globin alleles statistical analysis, the application of χ^2 test suffers from great deviations mainly because of the effect of malaria mortality in modulating the distribution of individuals carrying β-thalassemia or SCD genes in a given population (Cavalli-Sforza et al., 1996). In addition, the definition of Mendelian populations based on panmixia (that is general random mating within a population), and hence the χ^2 test, are, in practice of limited use for our purposes. If applied, the χ^2 test will continuously demonstrate non-perceptible results with variable sample sizes, and it will be difficult to set a size threshold. The main reason for this observed phenomenon is the tendency to marry at a short distance between places of residence or of birth, because of consanguinity or other factors, that generates a geographic heterogeneity unless the area investigated is very small. In the latter case, the sample size is limited and fails to obey the χ^2 test rules. Thus, validity of the χ^2 test was not crucial for this purpose and gene frequency data comparisons were applied instead (extensively reviewed by Cavalli-Sforza et al., 1996).

V. RESULTS

A. Rare Mutations Identified by Genomic Sequencing

A total of 19 Turkish individuals whose blood samples were sent to our laboratory from various medical centers in Turkey were screened for possible rare forms of β-globin gene mutations by genomic DNA sequencing. To nominate a DNA sample for sequencing, three factors were considered: (a) the sample should prove negative for the most common 15-20 Mediterranean β-thalassemia mutations, (b) the hematology of the patient should present an abnormal picture (e.g., abnormally increased levels of HbF or HbA_2, presence of an abnormal hemoglobin, or others), and/or (c) the patient might be related to a certain geographical region or an ethnic group that is characterized by heterogeneity of genes (e.g., those originating from the Black Sea region of Turkey or immigrants from Balkan countries or others).

DNA sequencing of these genes, with primers covering the whole β-globin gene and its flanking 5' and 3' regions (Table III.3), allowed the detection of 14 mutations in 30 (88.1%) out of 34 chromosomes sequenced in the framework of this thesis (Table V.1, Figure V.1). The presence of some mutations was confirmed by restriction endonuclease digestion (Figure V.2). The two most common mutations in the chromosome group analyzed are the IVS-I-110 (29.4%) and IVS-II-1 (11.9%) lesions. The IVS-II-745 mutation, thought to be rare in the Turkish population (Başak et al., 1992a), is present in 5.9% of the chromosomes sequenced. Of the remaining chromosomes, 12 carry 10 rare β-thalassemia mutations, two of which (namely, IVS-II-654, and IVS-I-130) were not previously described in the Turkish people (Tadmouri et al., 1998a; Tadmouri et al., 1999a; Tadmouri et al., 1999b). Because of their clinical and geographical importance, five of these 10 rare mutations are presented in detail.

TABLE V.1. Frequencies of mutations encountered among 34 Turkish β-globin genes subjected to genomic DNA sequencing.

Mutation	Number of chromosomes	Per cent
IVS-I-110 (G-A)	10	29.4
IVS-II-1 (G-A)	4	11.9
IVS-II-745 (C-G)	2	5.9
IVS-I-5 (G-C)	2	5.9
IVS-I-116 (T-G)	2	5.9
Cd 6 (A-T) [HbS]	2	5.9
IVS-I-6 (T-C)	1	2.9
FSC-8 (-AA)	1	2.9
-30 (T-A)	1	2.9
290 bp deletion	1	2.9
FSC-36/37 (-T)	1	2.9
Cd22 (G-A) [HbE Saskatoon]	1	2.9
IVS-II-654 (C-T)	1	2.9
IVS-I-130 (G-A)	1	2.9
Unknown	4	11.9
Total	34	100

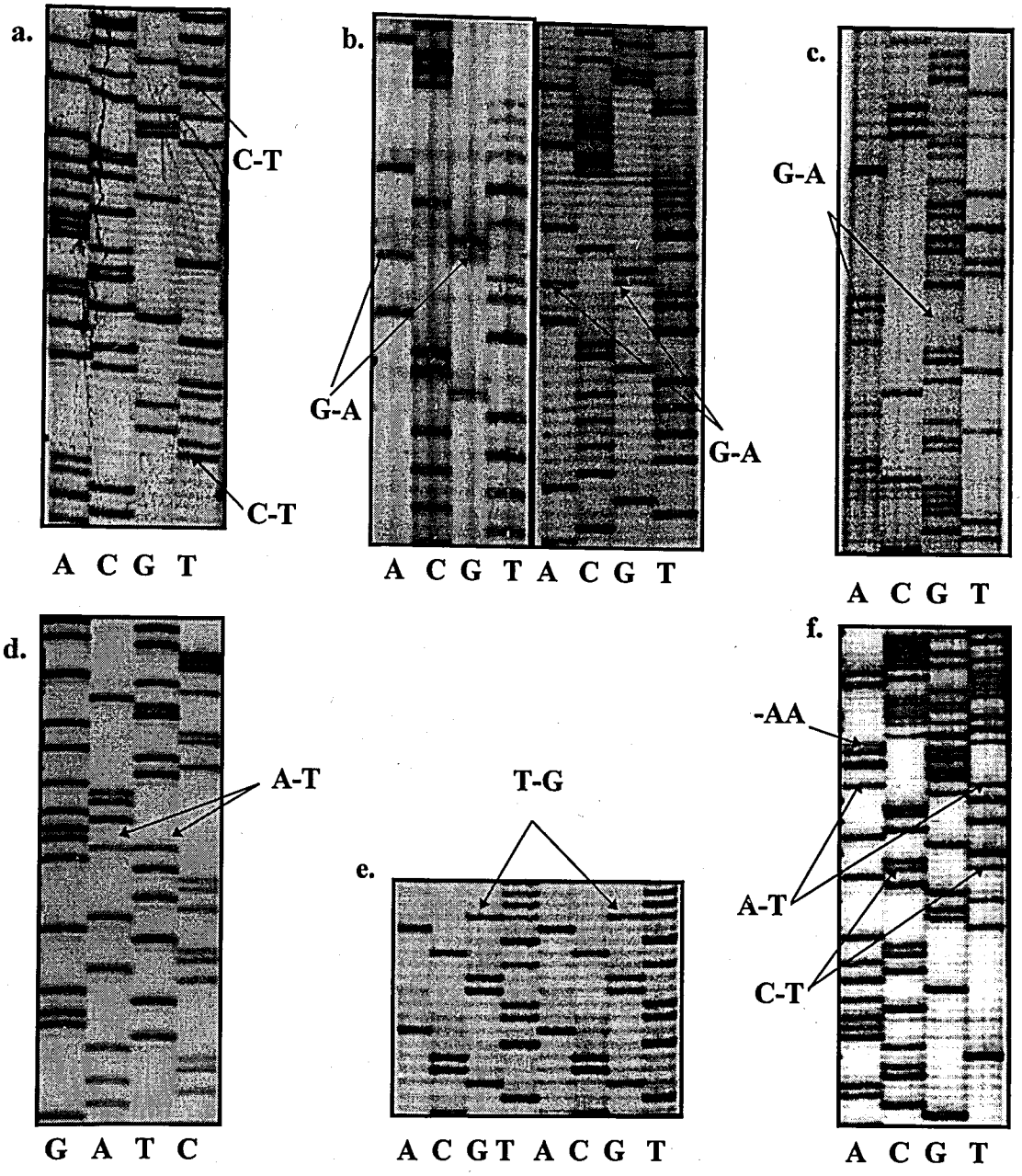


FIGURE V.1. Genomic DNA sequencing results. (a) Homozygosity for the C-T polymorphisms at positions +20 of the β-globin gene cap site (lower arrow) and at Cd2 (upper arrow) in a homozygous patient for the IVS-II-745 (C-G) β-thalassemia mutation. (b) Two examples of heterozygous IVS-I-110 (G-A) carriers. (c) G-A heterozygosity at Cd22 of the β-globin gene leading to the formation of HbE Saskatoon (Glu-22-Lys) in a heterozygous carrier. (d) Heterozygosity for the A-T substitution at codon 6 leading to HbS (Glu-6-Val) formation. (e) Two heterozygous carriers for the rare mutation IVS-I-116 (T-G). (f) A compound heterozygous patient carrying the C-T polymorphism at Cd2 (lower arrow), the A-T substitution at codon 6 leading to HbS (middle arrow), and the -AA microdeletion leading to a frameshift starting from Cd8 (upper arrow).

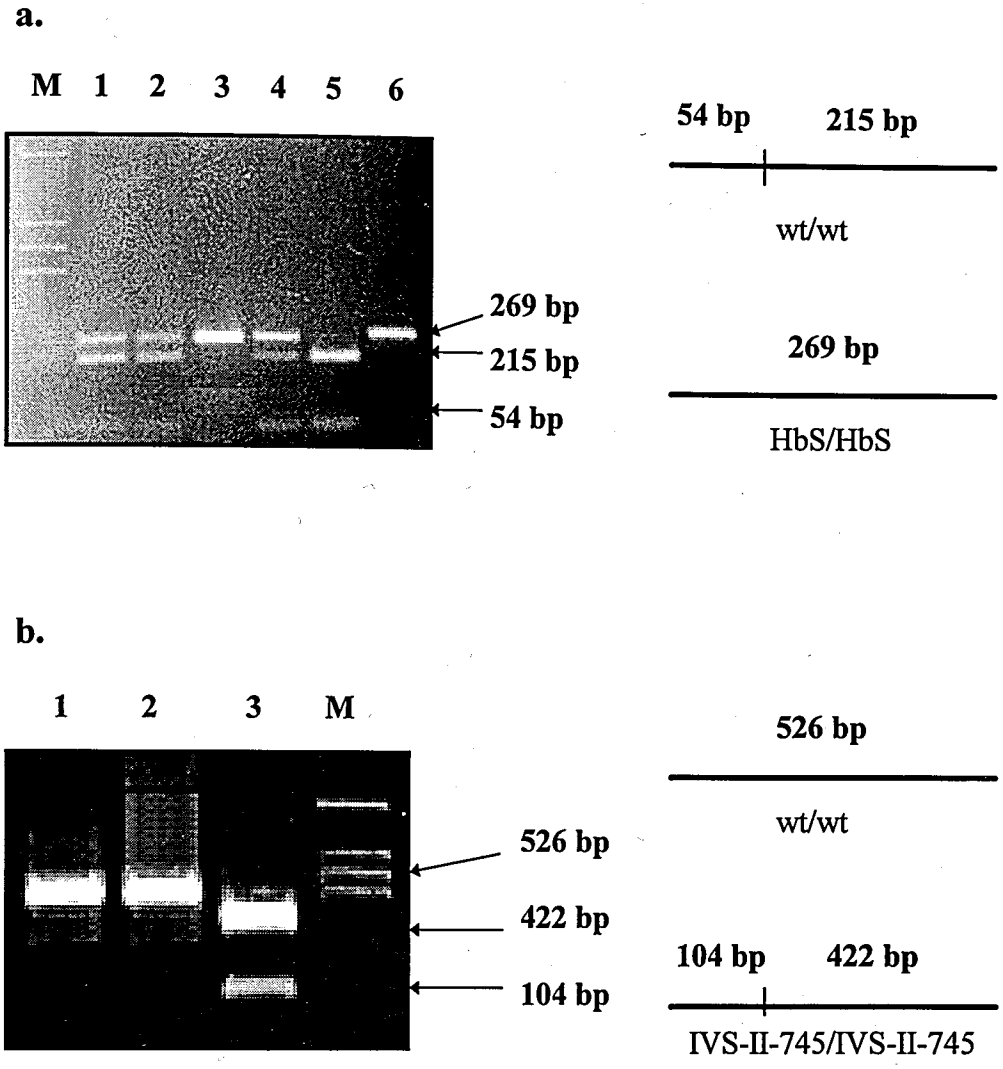


FIGURE V.2. Agarose gels (1.5%) illustrating restriction endonuclease digestions to confirm the DNA sequencing results. (a) DdeI digestion and band pattern specific for the HbS mutation. Lanes 1, 2, and 4 are HbS heterozygous carriers, lane 3 is a HbS homozygous control, lane 5 is a healthy control, and lane 6 is an undigested PCR product amplified using primers PCO3 and SR16. (b) RsaI digestion and band pattern specific for the IVS-II-745 (C-G) mutation. Lane 1 is an undigested PCR product of primers F Control A and CD2, lane 2 is a healthy control, lane 3 is a β-thalassemia patient homozygous for the IVS-II-745 (C-G) mutation. In both gels, M is the DNA marker GT6.

1. The Rare FSC-36/37 (-T) Mutation in a Turkish β-Thalassemia Patient

The blood sample of a five-year-old Turkish girl from Istanbul was referred to our laboratory. At the age of 18 months she had been referred to the Medical School of Adana because of severe anemia (Table V.2). DNA samples of the patient and her parents were screened for the presence of β-thalassemia mutations by dot-blot hybridization of the β-globin gene with allele-specific oligonucleotide (ASO) probes and by the denaturing gradient gel electrophoresis (DGGE) system (Tüzmen, 1995). ASO hybridization with 18 probes, specific for Mediterranean countries, revealed the presence of the IVS-I-110 (G-A) mutation on one chromosome of the child and of her heterozygous father. The mutation of the heterozygous mother, thus the other allele of the child, could not be identified by the two approaches mentioned above. For this, further analysis was done by direct sequencing of the β-globin genes using the dideoxy chain termination method (see section IV.F.2). This uncovered the presence of the rare FSC-36/37 (-T) mutation in the chromosomes of the patient and her mother (Tadmouri et al., 1997; Figure V.3).

TABLE V.2. Laboratory data of the proband with the β-thalassemia FSC-36/37 (-T) mutation and her parents.

Laboratory data	Father	Mother	Propositus
Age	34	26	5
Origin	Osmaniye	Osmaniye	Gebze
RBC (10^{12}/L)	-	3.54*	-
Hb (g/dl)	13.9	7.3*	7.9
Hematocrit (per cent)	46	22.9	24
MCV (Fl)	69.1	64.6	79.3
MCH (pg)	20.9	20.6	25.7
MCHC (g/dl)	30.2	31.9	32.4
Genotype	IVS-I-110/wt	FSC-36/37/wt	IVS-I-110/FSC-36/37

* The low RBC count and hemoglobin values may be caused by iron deficiency in addition to the β-thalassemia trait condition.

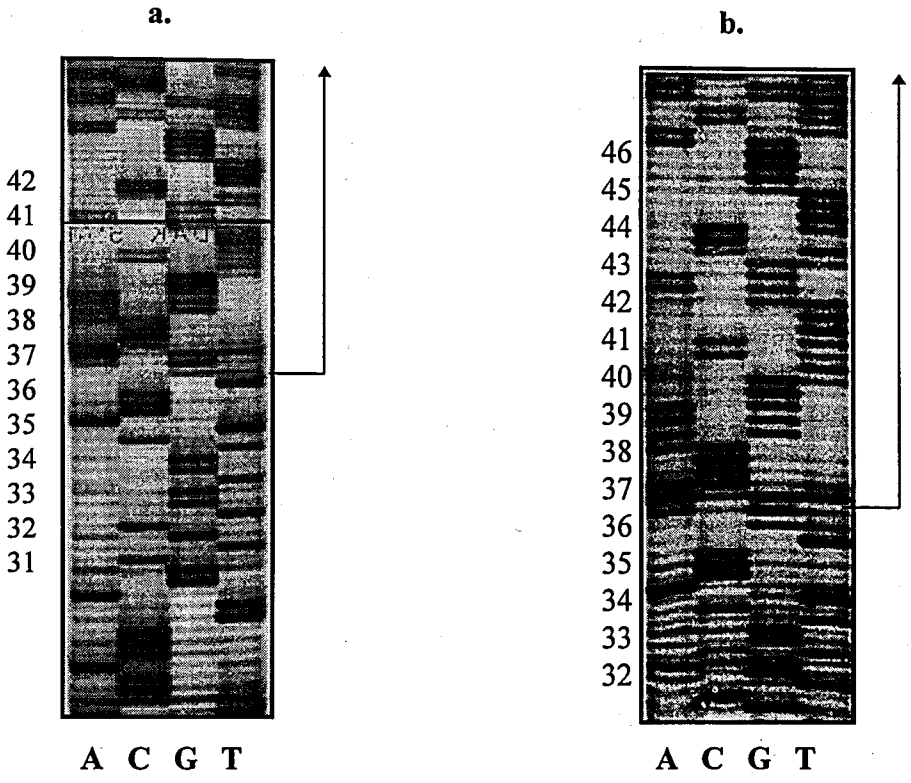


FIGURE V.3. β-Globin gene sequences showing the FSC-36/37 (-T) microdeletion. Numbers indicate the relative positions of β-globin codons. Broken arrows indicate the start and direction of the resulting frameshift. (a) The proband: IVS-I-110 (G-A)/FSC-36/37 (-T). (b) The mother: heterozygous carrier for the FSC-36/37 (-T).

2. Identification of the IVS-II-654 (C-T) β-Thalassemia Mutation in an Immigrant Turkish Family

The proband was a 26-year-old pregnant woman who presented with her spouse for prenatal diagnosis. The couple had been diagnosed as β-thalassemia carriers in their hometown Xanthe, Greece, from where they had migrated to Turkey three years ago. The hematological data of the proband and her family are given in Table V.3. All family members except her mother show typical features of heterozygous β-thalassemia with decreased MCV, MCH, and MCHC levels and increased HbA_2 levels.

The initial investigation of the β-globin genes of the couple by reverse dot-blot analysis (Bilenoğlu, 1996) revealed the presence of the IVS-I-110 (G-A) mutation in the spouse. The DNA of the proband was negative for all the common β-thalassemia mutations tested by the system, i.e., -87 (C-G), IVS-I-1 (G-A), IVS-I-6 (T-C), IVS-I-110 (G-A), Cd39 (C-T), IVS-II-1 (G-A), and IVS-II-745 (C-G). Consequently, the proband's DNA was subjected to both manual and automatic fluorescent sequencing (Tadmouri et al., 1999a). This technique revealed that she had heterozygously inherited the IVS-II-654 (C-T) mutation. The presence of this mutation was confirmed in the father, grandmother, and brother of the proband (Table V.3).

Analysis of the -158 (C-T) polymorphism in the Gγ-globin gene promoter showed no change from the reference sequence of Poncz et al. (1983). Sequence analysis of the polymorphic nucleotides -1069, -989, -780, -710, -703, -551, -543, -521, and -491 and the hypervariable microsatellite of composite sequences $(AT)_xT_y$ located near the 5' end of the IVS-II-654 β-globin genes of the proband and members of her family showed the occurrence of ACATCCCCA and $(AT)_9T_5$ at the positions respectively (Tadmouri et al., 1999a).

TABLE V.3. Laboratory data of the proband heterozygous for the β-thalassemia IVS-II-654 (C-T) mutation and of her family.

Laboratory data	Grandmother	Father	Proband	Brother	Mother
Age	68	48	26	25	50
WBC (10^3/ml)	5.91	5.63	5.69	6.66	5.72
RBC (10^6/ml)	5.34	5.72	4.47	6.42	4.10
Hb (g/dl)	10.9	11.9	9.72	12.5	11.9
Hct (per cent)	34.6	38.1	29.7	39.6	34.8
MCV (Fl)	64.8	66.6	66.3	61.6	84.7
MCH (pg)	20.4	20.8	21.7	19.5	29.1
MCHC (g/dl)	31.5	31.3	32.7	31.6	34.3
Platelet (10^3/ml)	201	193	318	258	235
HbA_2 (per cent)	5.50	5.20	5.40	5.40	2.90
HbF (per cent)	-	1.1	2.2	2.0	-
Genotype	IVS-II-654/wt	IVS-II-654/wt	IVS-II-654/wt	IVS-II-654/wt	wt/wt

3. The Rare IVS-I-130 (G-A) Mutation in a Turkish β-Thalassemia Major Patient

The patient is a 4-year old Turkish girl from Istanbul who was referred to the Pediatric Hematology Division of Istanbul University Medical School at the age of two years because of paleness, poor appetite and no gain of weight (Tadmouri et al., 1999b). The hematological evaluation of the patient showed classical signs of β-thalassemia major. After the definite diagnosis, she received two blood transfusions, three months apart. Since then, her clinical and laboratory data made regular blood transfusions necessary, e.g., every 30-35 days (Table V.4).

Using the reverse dot-blot system (Bilenoğlu, 1996), one allele of the patient was found to be IVS-I-6 (T-C). This mutation was confirmed to be also present in the DNA of her father as well as in her twin brother. Further analysis included *DdeI* digestion of a PCR fragment, spanning between positions +26 and Cd49 (Table III.3) to exclude the possibility of the common FSC-5 (-CT) and FSC-6 (-A) mutations. This analysis showed an unexpected pattern because of an abolished DdeI site that was calculated to be at the acceptor splice site of IVS-I. Subsequently, PCR amplified β-globin genes of the patient and members of her family were directly sequenced by the dideoxy chain termination method in combination with ^{35}S-dATP (see section IV.F.3). This technique uncovered the presence of the rare IVS-I-130 (G-A) mutation in the patient, her mother, and her grandmother (Table V.4; Figure V.4).

The patient's and her family's DNA samples were subjected to sequence haplotype analysis as described in sections IV.C.1 and IV.F.3. Sequence analysis of the polymorphic nucleotides -1069, -989, -780, -710, -703, -551, -543, -521, and -491, and the hypervariable microsatellite of composite sequences $(AT)_xT_y$ upstream of the β-globin gene of the patient and her family showed the occurrence of a distinct sequence haplotype for each of the two mutations IVS-I-6 (T-C) and IVS-I-130 (G-A). This will be discussed in detail in section V.D.

To trace back the origin of the IVS-I-130 (G-A) mutation in the family of our patient, we studied DNA samples belonging to members of her mother's family and found that the mutation was inherited from her grandmother. An investigation of the grandmother's parents' past revealed that the family immigrated to Turkey from Albania, most probably, early in the 20th century (Tadmouri et al., 1999b).

TABLE V.4. Laboratory data of the patient carrying the β-thalassemia IVS-I-130 (G-A) mutation and of her family.

Laboratory data	Grandmother	Mother	Brother	Patient	Father
Hb (g/dl)	-	9.2	-	7.4	14
Hct (per cent)	-	26.4	-	21.1	41.2
RBC (10^{12}/L)	-	4.78	-	3.52	6.06
MCV (Fl)	-	58	-	60	68
MCH (pg)	-	19.2	-	21	23.1
MCHC (g/dl)	-	33.2	-	35.1	34.0
HbA_2 (per cent)	6	6.6	7.4	1.8	4.4
HbF (per cent)	1	2.6	1	59	1.05
Genotype	IVS-I-130/wt	IVS-I-130/wt	IVS-I-6/wt	IVS-I-6/ IVS-I-130	IVS-I-6/wt

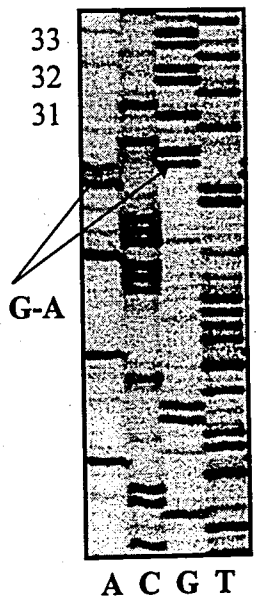


FIGURE V.4. β-Globin gene DNA sequencing result showing the IVS-I-130 (G-A) mutation. Numbers indicate the relative positions of β-globin codons.

4. β-Thalassemia Trait Associated With Fanconi Aplastic Anemia

The blood sample of a 10-year-old girl was referred to our laboratory with a severe type of anemia. She was the third child of a consanguineous marriage; one of her siblings had died of anemia and bleeding at the age of eight. Her history revealed that she was diagnosed as having patent ductus arteriosis, which was corrected at the age of 15 months. Physical examination at the age of 10 years revealed growth retardation and mild microcephaly. Her height was 127 cm, weight 23 kg (both measurements were less than the 3rd percentile of her age), and head circumference 50 cm. She had two café-au-lait spots, and her right thumb was dislocated (Altay et al., 1996a). The results of laboratory examination indicated the presence of a possible β-thalassemia trait in the patient and in one of her parents (Table V.5). Karyotype analysis revealed 46 chromosomes with an XX pattern; an increased rate of spontaneous and induced chromosomal breakage by diepoxy butane was observed (Altay et al., 1996a). The diagnosis of Fanconi Anemia was made, and, accordingly, a treatment program was designed for the patient to which she responded well at least for one year (Table V.5; Altay et al., 1996a). DNA sequencing of her β-globin genes revealed heterozygosity for the IVS-I-5 (G-C) mutation (Figure V.5), which she had inherited paternally. During the 4-year-follow-up period, the patient remained well as it can be seen from her hematological values (Table V.5).

TABLE V.5. Laboratory data of the patient with Fanconi's anemia and β-thalassemia trait and of her parents.

Laboratory data	Father	Mother	Propositus		
Age	40	38	10	11	14
Hb (g/dl)	12.0	12.0	4.2	12.4	11.0
WBC (mm^3)	7,800	6,800	3,000	5,300	6,000
MCV (Fl)	56-61	87	113	68	68
Platelet (10^9/L)	sufficient	sufficient	20	220	sufficient
HbA_2 (per cent)	4.0-4.7	2.6	-	3.6	3.7
HbF (per cent)	0.5-0.6	0.6	16	7	8
Genotype	IVS-I-5/wt	wt/wt	IVS-I-5/wt		

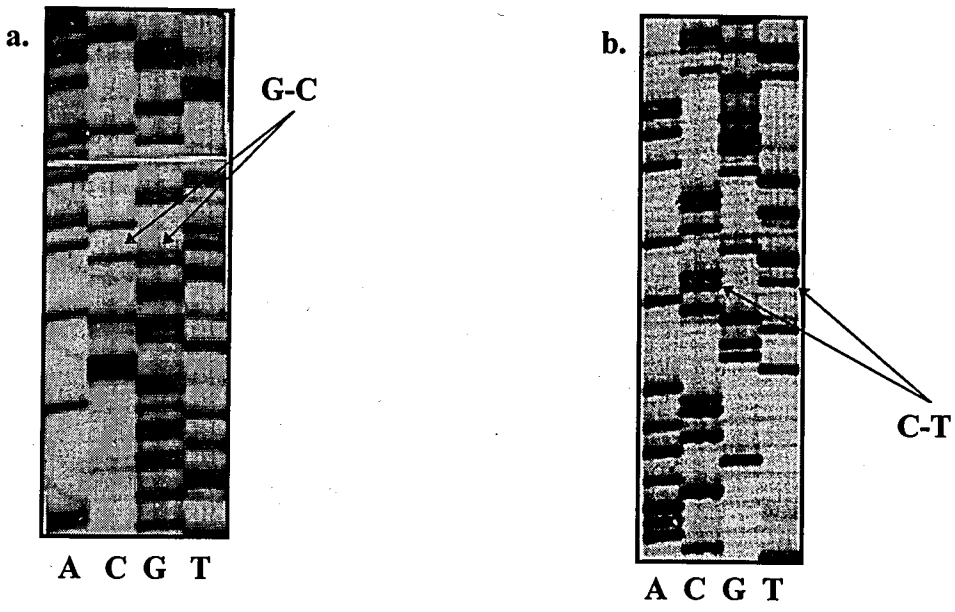


FIGURE V.5. DNA sequences of the β-globin gene of the Fanconi anemia/β-thalassemia compound patient. (a) Heterozygosity for the G-C mutation at IVS-I-5. (b) Heterozygosity for the C-T polymorphism at Cd2 of the β-globin gene.

5. HbS/β^{del}-Thalassemia Associated With High Levels of HbA_2 and HbF in a Turkish Family

A family from Batman, composed of a father, mother, and three sibs, was referred to our laboratory from the Hematology Clinic of Cerrahpaşa Medical School, Istanbul, because of a possible hemoglobinopathy. HbS, HbF, and HbA_2 values had been determined by hemoglobin electrophoresis. Results had been confirmed for HbF and HbA_2 by alkaline denaturation and column chromatography, respectively (Tadmouri et al., 1998b). The father had an unusually elevated HbA_2 (9.0%) and normal HbF levels. The mother showed a typical HbS heterozygous hematology (Table V.6). One of the two affected siblings was a 7-year-old boy at the time of diagnosis (1994). From his fourth year of life, he had pains in his hand and foot joints, and occasionally the color of his urine turned dark. Physical examination of the patient showed paleness, 3.5 cm hepato- and 8 cm splenomegaly. Up to this age, he was not transfusion-dependent. These findings seemed to be in accordance with a β^S/β^0-thalassemia condition. The child is currently transfused, not because of anemia, but because of recurrent pains in his extremities, every two months. When he was 11 years old (1998), his liver and spleen were 2.5 and 10 cm, respectively. The same diagnosis was made in the patient's sister. At the time she was 1-year-old, she did not show hepatosplenomegaly and did not need blood transfusion. At 1998, she, at the age of six, had a very stable and symptomless condition. Her liver was 2 cm, and she had no splenomegaly. These findings mistakenly predicted the presence of a β^S/β^+-thalassemia genotype but were corrected later by the results of the molecular analysis (Tadmouri et al., 1998b).

Genotypes of all the family members were determined by PCR and genomic sequencing. The presence of a 290 bp deletion was shown by PCR amplification of the β-globin gene using primers flanking the deletion (i.e., CD7 and R109, see Table II.3). Sequencing of the abnormal PCR product allowed the definition of the breakpoints of the deletion. The 5'-breakpoint of the 290 bp deletion is 123-125 bp upstream from the β-

globin gene cap site and the 3' end is at nucleotide 23-25 of IVS-I of the β-globin gene (Figure V.6).

Putative *cis*-acting determinants modulating HbF levels within the β-globin cluster were analyzed. The -158 C-T polymorphism in the Gγ-globin gene promoter was assessed by *XmnI* restriction of a 351-bp amplified sequence from that region (see Table II.3 and section IV.C.3). The AT-rich region at 530 bp upstream from the β-globin gene was analyzed by amplifying and directly sequencing a 790 bp DNA fragment as described in sections IV.C.1 and IV.F.3.

TABLE V.6. Laboratory data of the family with HbS/β^{del}-thalassemia.

Laboratory data	**Father**	**Mother**	**Son**	**Son**	**Daughter**
Age	32	28	10	9	5
Hb (g/dl)	13.2	13.2	9.5	12.9	10.7
Hct (per cent)	43.4	40.2	27.5	39	32.5
RBC (1012/L)	6.54	4.81	4.05	4.63	4.76
MCV (Fl)	66.4	83.7	71	84.2	69.1
MCH (pg)	20.2	27.5	31	27.8	31.9
MCHC (g/dl)	30.4	32.9	22	33	22
Reticulocyte (per cent)	2.6	1.6	31	1	1.2-5.8
HbA_2 (per cent)	9.0	3.3	6.7	3.5	6.0
HbF (per cent)	0.8	0.5	18.1	0.6	33.6
HbS (per cent)	0.0	43.2	50.7*	0.0	60.4
XmnI	-/nd	-/nd	-/-	nd/nd	-/-
$(AT)_xT_y$	$(AT)_7T_7$/ nd	$(AT)_7T_7$/ nd	$(AT)_7T_7$/ $(AT)_7T_7$	nd/ nd	$(AT)_7T_7$/ $(AT)_7T_7$
Genotype	β^{del}/wt	HbS/wt	HbS/β^{del}	wt/wt	HbS/β^{del}

*The total quantity of hemoglobin types in the proband is 75.5%, the remaining 24.5% is transfused HbA. The results of *XmnI* and $(AT)_xT_y$ for the parents are deduced from the results of their children.
nd: not determined

Analysis of the -158 C-T polymorphism in the Gγ-globin gene promoter showed no change from the normal sequence. The same result was obtained for the $(AT)_xT_y$ microsatellite, which showed an $(AT)_7T_7$ repeat (Table V.6), as in the reference sequence of Poncz et al. (1983).

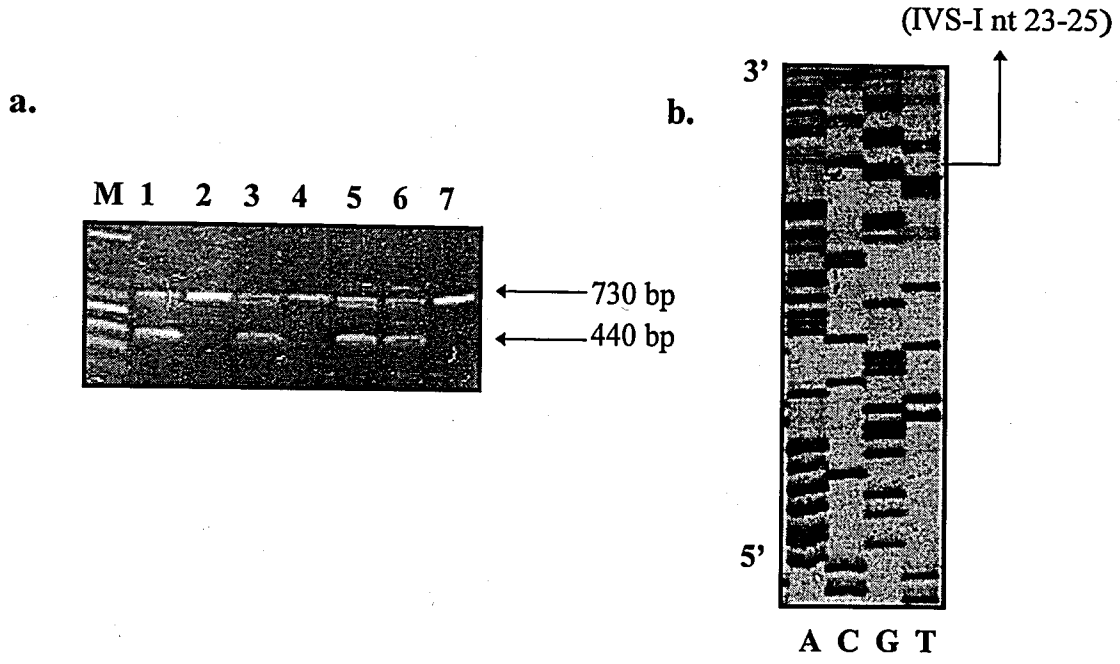


FIGURE V.6. PCR and genomic DNA sequencing results showing the 290 bp deletion. (a) PCR amplification using primers CD7 and R 109. Lanes 1, 3, 5, and 6 are heterozygous for the deletion and present two amplification products of 290 bp difference. M is DNA marker GT4. (b) The 3' breakpoint of the 290 bp deletion in the β-globin gene as demonstrated by sequencing. The bended arrow indicates the beginning of the 3' breakpoint of the deletion at nucleotide 23-25 of IVS-I of the β-globin gene.

B. Regional Distribution of β-Globin Gene Mutations in Turkey

Detailed family histories of patients referred to our laboratory since 1988 were collected, reviewed, and stored in electronic medium. A total of 32 β-globin gene mutations were encountered (Table V.7), 13 of which were described after 1992 (Başak et al., 1992b; Başak et al., 1993; Özçelik et al., 1993; Tüzmen et al., 1997; Tadmouri et al., 1998a; Tadmouri et al., 1998b; Tadmouri et al., 1999a; Tadmouri et al., 1999b). The newly compiled data confirms, as expected, that the IVS-I-110 (G-A) mutation is the most common β-thalassemia defect in Turkey (Table V.7). This is followed in decreasing order by IVS-I-6 (T-C), FSC-8 (-AA), IVS-I-1 (G-A), IVS-II-745 (C-G), IVS-II-1 (G-A), Cd39 (C-T), -30 (T-A), FSC-5 (-CT) lesions, all of which have frequencies above two per cent. β-Globin gene mutations, identified in the framework of this study, comprise 90.9% of the diseased β-globin genes identified in Turkey (Tadmouri et al., 1998a). The mutations in 72 out of 795 chromosomes (9.1%) could not be identified so far (Table V.7).

Table V.8 lists the relative frequencies of the 32 mutations, classified to six regions of Turkey. Turks from Balkan countries, forming the vast majority of samples, a few samples from Cyprus, and one sample from Near East are considered as a seventh distinct group (BLK). The remaining chromosomes, whose origins could not be traced, are displayed in a separate column (Table V.8). At a regional level, frequency differences among β-globin gene mutations are present in the Turkish population groups analyzed. A major example is the common IVS-I-110 (G-A) mutation, the frequencies of which vary from 26.4% to 52.3% among samples from SEA and CA, respectively. Some mutations tend to be more represented in specific regions. Chromosomes from the BLK group demonstrate the highest frequencies of the Cd39 (C-T) mutation, frequently encountered in patients of Western Mediterranean ancestry (Huisman et al., 1997). In addition, several rare mutations are observed in this group, the most important of which are: Cd27 (G-T), IVS-I-130 (G-A), and IVS-II-654 (C-T). The mild promoter mutations -30 (T-A) and -87 (C-G) occur in their highest frequencies in the BSR group, in which the rare Cd15 (G-A) mutation is also encountered (Tüzmen, 1995). The EA and SEA groups are characterized

by the presence of many Asian [e.g., FSC-8/9 (+T), FSC-44 (-C), FSC-36/37 (-T), and 290 bp deletion], rare [e.g., Cd121 (G-C)], and novel mutations [e.g., FSC-22-24 (-7 bp), and FSC-74/75 (-C)]. As expected, the highest frequencies of the HbS gene occurs in the SEA group (Table V.8).

Another important achievement made possible by the collected data is the possibility to look at the frequency distributions of β-globin gene mutations in Turkey at the level of individual towns (Table V.9). It is easily noticed that the mutation IVS-I-110 (G-A) occurs mainly in the southern and western cities of Turkey such as Antalya, Muğla, Tekirdağ, Çanakkale, Tunceli, Gaziantep, Edirne, Diyarbakır, and also from Batman, Zonguldak, Kayseri, and Niğde where frequencies between 50% and 100% are observed. The absence of this mutation in the eastern and northern cities of Kars, Iskenderun, and Merzifon may be because of limited sampling. The IVS-I-6 (T-C), FSC-8 (-AA), IVS-I-1 (G-A), IVS-II-1 (G-A), Cd39 (C-T), and IVS-II-745 (C-G) mutations occur almost everywhere in the country with a limited presence in coastal towns of the Black Sea region (Table V.9). The -30 (T-A) mutation, however, has a more restricted distribution since it mainly occurs in towns of northern, eastern, and southeastern Anatolia such as, Tokat, Isparta, Siirt, Diyarbakır, Batman, Kars, Iskenderun, Merzifon, Mardin, and Ordu where frequencies between 1.1-66.7% are observed. The FSC-5 (-CT) is mostly found in southern and western towns of Turkey such as, Tekirdağ, Çanakkale, Isparta, Salihli, Gaziantep, and Antakya where frequencies between 14.3-23.1% are seen. The FSC-8/9 (+G) has a much more restricted distribution since it is only detected in towns of inner central and eastern Turkey such as Ankara, Sivas, Erzincan, and Tunceli with frequencies ranging between 8-40% (Table V.9).

TABLE V.7. Comparison of β-globin gene mutation data in the Turkish population as described in our laboratory in 1992 and 1998.

	Mutation	Type	1992 *Başak et al., 1992a*		1998 *Tadmouri et al., 1998a*	
			n*	per cent	n*	per cent
1.	**IVS-I-110 (G-A)**	β^+	181	42.2	312	39.3
2.	**IVS-I-6 (T-C)**	β^+	43	10.0	80	10.1
3.	**FSC-8 (-AA)**	β^0	21	4.9	43	5.5
4.	**IVS-I-1 (G-A)**	β^0	19	4.5	40	5.0
5.	**IVS-II-745 (C-G)**	β^+	10	2.4	40	5.0
6.	**IVS-II-1 (G-A)**	β^0	15	3.5	37	4.7
7.	**Cd 39 (C-T)**	β^0	16	3.7	30	3.8
8.	**-30 (T-A)**	β^+	17	4.0	25	3.1
9.	**FSC-5 (-CT)**	β^0	16	3.7	17	2.2
10.	**FSC-8/9 (+G)**	β^0	13	3.0	10	1.3
11.	**FSC-44 (-C)**	β^0	-	-	10	1.3
12.	**IVS-I-5 (G-C)**	β^+	5	1.2	9	1.1
13.	**-87 (C-G)**	β^+	6	1.4	6	0.8
14.	**Poly A (ATA-ATG)**	β^+	-	-	4	0.5
15.	**FSC-6 (-A)**	β^0	2	0.5	3	0.4
16.	**IVS-II-848 (C-A)**	β^+	-	-	3	0.4
17.	**IVS-I-116 (T-G)**	β^0	1	0.2	2	0.2
18.	**FSC-74/75 (-C)**	β^0	-	-	1	0.1
19.	**-101 (C-T)**	β^+	1	0.2	1	0.1
20.	**-28 (A-C)**	β^+	1	0.2	1	0.1
21.	**Cd 15 (G-A)**	β^0	-	-	1	0.1
22.	**Cd 27 (G-T)**	$\beta^{Knossos}$	1	0.2	1	0.1
23.	**3'-UTR (-13 bp)**	β^+	-	-	1	0.1
24.	**FSC 22/23/24 (-7 bp)**	β^0	-	-	1	0.1
25.	**FSC-36/37 (-T)**	β^0	-	-	1	0.1
26.	**IVS-I-130 (G-A)**	β^0	-	-	1	0.1
27.	**290 bp deletion**	β^0	-	-	1	0.1
28.	**IVS-II-654 (C-T)**	β^+	-	-	1	0.1
29.	**Cd 6 (A-T) HbS**	β^S	-	-	39	4.9
30.	**Cd 121 (G-C)**	$\beta^{D\ L.A.}$	-	-	1	0.1
31.	**Cd 22 (G-A)**	$\beta^{E\ Saskatoon}$	-	-	1	0.1
32.	**δβ-Thalassemia**	δβ	-	-	1	0.1
	Unknown		61	14.2	71	9.0
	Total		429	100.0	795	100

*Number of chromosomes.

TABLE V.8. Regional distribution of β-globin gene mutations in Turkey.*

Mutation	MR	AM	CA	SEA	BSR	EA	BLK	Unknown
IVS-I-110 (G-A)	30 (34.1)	84 (42.4)	**58 (52.3)**	31 (26.4)	17 (31.0)	16 (27.1)	29 (47.7)	47 (44.4)
IVS-I-6 (T-C)	**13 (14.8)**	25 (12.6)	8 (7.2)	10 (8.5)	6 (10.9)	6 (10.2)	6 (9.8)	6 (5.7)
FSC-8 (-AA)	7 (8.0)	9 (4.6)	7 (6.3)	5 (4.3)	1 (1.8)	**5 (8.4)**	4 (6.6)	5 (4.7)
IVS-I-1 (G-A)	8 (9.1)	13 (6.6)	5 (4.5)	1 (0.9)	1 (1.8)	3 (5.1)	**6 (9.8)**	3 (2.8)
IVS-II-745 (C-G)	4 (4.6)	9 (4.6)	8 (7.2)	7 (6.0)	**6 (10.9)**	1 (1.7)	4 (6.6)	1 (0.9)
IVS-II-1 (G-A)	3 (3.4)	**11 (5.6)**	6 (5.4)	3 (2.6)	3 (5.5)	2 (3.4)	-	9 (8.5)
Cd 39 (C-T)	4 (4.6)	5 (2.5)	4 (3.6)	1 (0.9)	2 (3.6)	2 (3.4)	**4 (6.6)**	8 (7.6)
-30 (T-A)	-	5 (2.5)	2 (1.8)	8 (6.7)	**5 (9.1)**	5 (8.5)	-	-
FSC-5 (-CT)	**3 (3.4)**	3 (1.5)	2 (1.8)	**4 (3.4)**	1 (1.8)	-	-	4 (3.8)
FSC-8/9 (+G)	2 (2.3)	-	2 (1.8)	-	1 (1.8)	**3 (5.1)**	-	2 (1.9)
FSC-44 (-C)	1 (1.1)	-	2 (1.8)	**3 (2.6)**	-	1 (1.7)	-	3 (2.8)
IVS-I-5 (G-C)	-	4 (2.0)	**3 (2.7)**	1 (0.9)	-	1 (1.7)	-	-
-87 (C-G)	1 (1.1)	1 (0.5)	-	-	**4 (7.3)**	-	-	-
Poly A (TA-TG)	-	-	-	1 (0.9)	**1 (1.8)**	1 (1.7)	-	1 (0.9)
FSC-6 (-A)	**1 (1.1)**	-	-	1 (0.9)	-	-	-	1 (0.9)
IVS-II-848 (C-A)	1 (1.1)	-	-	1 (0.9)	-	-	**1 (1.6)**	-
IVS-I-116 (T-G)	1 (1.1)	-	-	-	-	-	**1 (1.6)**	-
Cd 15 (G-A)^{R}	-	-	-	-	**1 (1.8)**	-	-	-
FSC-74/75 (-C)^{N}	-	-	-	-	-	**1 (1.7)**	-	-
FSC-36/37 (-T)^{R}	-	-	-	-	-	**1 (1.7)**	-	-
290 bp deletion^{R}	-	-	-	-	-	**1 (1.7)**	-	-
Cd 27 (G-T)^{R}	-	-	-	-	-	-	**1 (1.6)**	-
IVS-I-130 (G-A)^{R}	-	-	-	-	-	-	**1 (1.6)**	-
IVS-II-654^{R}	-	-	-	-	-	-	**1 (1.6)**	-
-101 (C-T)^{R}	**1 (1.1)**	-	-	-	-	-	-	-
3'-UTR (-13 bp)^{N}	-	-	1 (0.9)	-	-	-	-	-
FSC22-24 (-7 bp)^{N}	-	-	-	**1 (0.9)**	-	-	-	-
Cd 121 (G-C)^{R}	-	-	-	**1 (0.9)**	-	-	-	-
-28 (A-C)^{R}	-	**1 (0.5)**	-	-	-	-	-	-
Cd 22 (G-A)^{R}	-	**1 (0.5)**	-	-	-	-	-	-
HbS	-	5 (2.5)	-	**28 (23.8)**	1 (1.8)	1 (1.7)	1 (1.6)	3 (2.8)
δβ-Thalassemia	-	-	-	-	-	-	**1 (1.6)**	-
Unknown	8 (9.1)	22 (11.1)	3 (2.7)	10 (8.5)	5 (9.1)	9 (15.2)	1 (1.7)	13 (12.3)
Total	88 (11.1)	198 (24.9)	111 (13.9)	117 (14.7)	55 (6.9)	59 (7.5)	61 (7.7)	106 (13.3)

*Values indicate the number of chromosomes and values in parentheses indicate percentages. Mutations followed by (R) are rare and by (N) are novel.

TABLE V.9. Frequency distribution of β-globin gene mutations in 44 towns of Turkey.*

	N	IVSI-110 (G-A)	IVSI-6 (T-C)	FSC-8 (-AA)	IVSI-1 (G-A)	IVS-II-745 (C-G)	IVS-II-1 (G-A)	Cd 39 (C-T)	-30 (T-A)	FSC-5 (-CT)	FSC-8/9 (+G)	Others
Adana	54	20.4	9.3	9.3	1.9	-	-	-	3.7	-	-	55.4
Izmir	48	47.9	14.6	2.1	-	12.5	6.2	2.1	-	-	-	14.6
Antalya	34	50.0	11.8	5.9	2.9	-	5.9	-	5.9	2.9	-	14.7
Konya	25	48.0	2.1	4.0	4.0	-	8.0	8.0	8.0	8.0	-	9.9
Ankara	25	44.0	2.3	12.0	8.0	8.0	8.0	4.0	-	-	8.0	5.7
Istanbul	20	30.0	3.3	25.0	10.0	5.0	-	5.0	-	-	-	21.7
Denizli	18	33.3	11.1	11.1	11.1	-	16.7	-	-	-	-	16.7
Malatya	15	40.0	13.3	-	13.3	-	6.7	-	-	-	-	26.7
Antakya	13	23.1	4.3	-	7.7	-	15.4	-	-	23.1	-	26.4
Mersin	12	33.3	3.0	-	-	25.0	-	-	8.3	-	-	30.4
Muğla	11	54.6	-	-	-	-	-	-	-	-	-	45.4
Aydın	11	9.1	36.7	-	9.1	-	9.1	-	18.2	-	-	17.8
Sivas	10	40.0	20.0	-	-	-	-	-	-	-	20.0	20.0
Kayseri	10	80.0	20.0	-	-	-	-	-	-	-	-	-
Uşak	9	44.4	-	-	-	-	-	11.1	-	-	-	44.5
Tokat	9	11.1	-	-	11.1	22.2	-	11.1	11.1	-	-	33.4
Yozgat	9	22.2	-	-	11.1	33.3	11.1	-	-	-	-	22.3
Samsun	9	33.3	33.3	-	-	33.3	-	-	-	-	-	-
Erzincan	8	12.5	25.0	-	12.5	-	-	25.0	-	-	25.0	-
Siirt	7	14.3	-	-	-	14.3	-	-	14.3	-	-	57.1
Bursa	7	28.6	28.6	-	-	14.3	-	-	-	-	-	28.5
Çanakkale	7	57.1	-	-	-	14.3	-	-	-	14.3	-	14.3
Tekirdağ	7	57.1	1.8	-	14.3	-	-	-	-	14.3	-	12.5
Isparta	7	43.0	28.6	-	-	-	-	-	14.3	14.3	-	-
Diyarbakır	7	71.4	-	-	-	-	-	14.3	14.3	-	-	-
Manisa	7	43.0	-	28.6	-	-	28.6	-	-	-	-	-
Tarsus	6	33.3	-	-	-	-	-	-	-	-	-	66.7
Salihli	6	50.0	-	-	-	-	-	-	-	16.7	-	33.3
Batman	6	50.0	-	-	-	-	-	-	16.7	-	-	33.3
Luleburgaz	6	33.3	16.7	-	16.7	-	-	-	-	-	-	33.3
Mardin	6	16.7	-	-	-	33.3	-	-	33.3	-	-	16.7
Ordu	6	16.7	-	-	-	-	-	-	66.7	-	-	16.6
Kars	6	-	-	50.0	-	-	16.7	-	16.7	-	-	16.6
Çorum	6	66.7	1.5	-	-	16.7	-	-	-	-	-	15.1
Edirne	6	66.7	33.3	-	-	-	-	-	-	-	-	-
Çankırı	5	20.0	-	-	-	-	-	-	-	-	-	80.0
Iskenderun	5	-	-	-	-	40.0	-	-	20.0	-	-	40.0
Merzifon	5	-	20.0	-	-	-	40.0	-	20.0	-	-	20.0
Tunceli	5	60.0	-	-	-	-	-	-	-	-	40.0	-
Gaziantep	5	60.0	-	-	-	-	20.0	-	-	20.0	-	-
Çorlu	5	40.0	40.0	-	-	-	-	20.0	-	-	-	-
Zonguldak	5	80.0	-	-	-	-	-	20.0	-	-	-	-
Fethiye	5	40.0	20.0	-	20.0	20.0	-	-	-	-	-	-
Niğde	5	100	-	-	-	-	-	-	-	-	-	-

*Values indicate percentages. N indicates number of chromosomes.

There is an increasing heterogeneity of β-globin gene mutations from towns of the Southwest to those of the Northeast of Turkey (Tables V.8 and V.9). This is similarly paralleled in the same direction by an increasing percentage of genes with unidentified mutations with maximums encountered the Black Sea region, eastern, and southeastern Turkey (Table V.8).

C. Analysis of Some Factors Modulating the Expression of Globin Genes

1. Polymorphic Pattern of the $(AT)_xT_y$ Motif at -530 bp 5' of the β-Globin Gene

The $(AT)_xT_y$ sequence, located at -530 bp upstream of the β-globin gene was analyzed in 229 Turkish chromosomes carrying 24 mutations. Four types of this motif were observed (Table V.10, Figure V.7), of which the $(AT)_7T_7$ pattern is the most common (90.7%; Table V.11). This is followed in decreasing order by $(AT)_9T_5$, $(AT)_{11}T_3$, and $(AT)_8T_4$. Of the 24 mutations analyzed, only the IVS-I-110 (G-A), IVS-I-6 (T-C), and FSC-5 (-CT) mutations are each related to two types of motifs. The rest of the mutations are linked to only a single $(AT)_xT_y$ pattern (Table V.10). Regional distribution of β-globin $(AT)_xT_y$ sequences in Turkey reveals a considerable homogeneity in samples from the BLK group that carries only the $(AT)_7T_7$ motif (Table V.11). Samples from the AM, CA, and BSR groups each present three types of $(AT)_xT_y$ motifs. However, it is only in the BSR group that the $(AT)_9T_5$ and $(AT)_{11}T_3$ motifs occur in high frequencies (20.8% and 8.4%, respectively) not met in the other groups.

The analysis of 17 wild-type β-globin genes from Turkey and 12 wild-type β-globin genes from the Balkan countries (BLK), Pakistan (PKN), Lebanon and Syria (LBNSYR) demonstrates the association of these chromosomes with five $(AT)_xT_y$ patterns, $(AT)_7T_7$, $(AT)_9T_5$, $(AT)_8T_5$, $(AT)_{11}T_3$, and $(AT)_8T_7$, of which $(AT)_7T_7$ is the most common form

(60.9%). In Turkey, only three of these patterns are present ($(AT)_7T_7$, $(AT)_9T_5$, and $(AT)_8T_7$). Despite the limited sampling, Lebanese and Syrian chromosomes are as heterogeneous since they exhibit three $(AT)_xT_y$ patterns ($(AT)_7T_7$, $(AT)_9T_5$, and $(AT)_8T_5$). Chromosomes from the Balkan are considerably less heterogeneous since they are associated with only two $(AT)_xT_y$ forms ($(AT)_7T_7$ and $(AT)_{11}T_3$). The two chromosomes analyzed from Pakistan did not display the common $(AT)_7T_7$ motif at all (Table V.12).

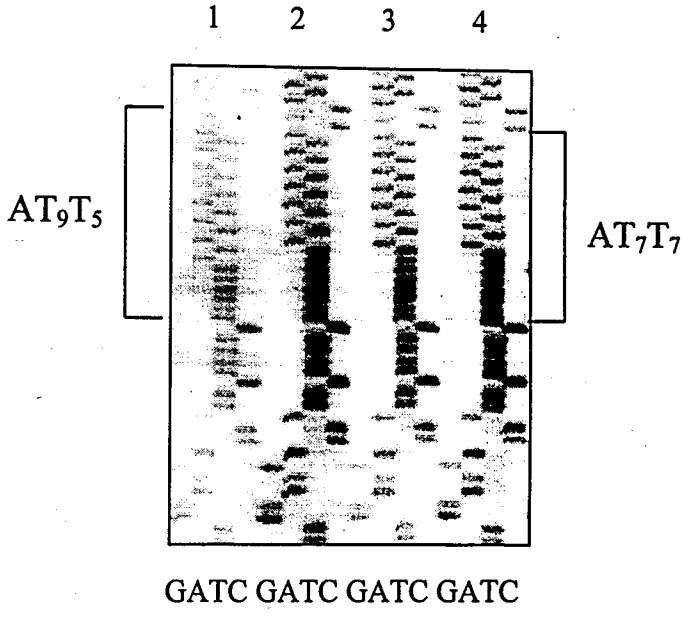


FIGURE V.7. Sequencing of the polymorphic $(AT)_xT_y$ pattern located at -530 bp upstream of the β-globin gene. Lane 1 indicates the homozygous inheritance of the $(AT)_9T_5$ pattern, whereas lanes 2-4 indicate the homozygous inheritance of the $(AT)_7T_7$ pattern.

TABLE V.10. $(AT)_xT_y$ pattern associations to different β-globin gene mutations in Turkey.

Mutation	$(AT)_7T_7$	$(AT)_9T_5$	$(AT)_8T_4$	$(AT)_{11}T_3$	Total
IVS-I-110 (G-A)	100			2	102
IVS-I-6 (T-C)	24		2		26
Cd 39 (C-T)	18				18
-30 (T-A)	12				12
IVS-II-745 (C-G)	9				9
IVS-II-1 (G-A)		8			8
IVS-I-1 (G-A)	6				6
FSC-8 (-AA)	5				5
FSC-8/9 (+G)	3				3
-87 (C-G)		3			3
FSC-5 (-CT)	1	1			2
IVS-I-116 (T-G)	2				2
HbS	2				2
IVS-I-5 (G-C)			1		1
Poly A (TA-TG)	1				1
IVS-II-848 (C-A)	1				1
Cd 15 (G-A)	1				1
FSC-74/75 (-C)	1				1
290 bp deletion	1				1
Cd 27 (G-T)	1				1
IVS-I-130 (G-A)	1				1
IVS-II-654 (C-T)		1			1
Cd 121 (G-C)	1				1
Cd 22 (G-A)	1				1
Total	191 (91.4)	13 (6.2)	3 (1.4)	2 (1.0)	209 (100)
Uncharacterized	18	2			20

TABLE V.11. Regional distribution of $(AT)_xT_y$ patterns among mutant β-globin genes from Turkey (the highest χ^2 value is calculated to be 1.14; P=0.286).

Motif	MR	AM	CA	SEA	BSR	EA	BLK	Unknown	Total
$(AT)_7T_7$	17 (94.4)	31 (94.0)	41 (95.4)	27 (93.1)	17 (70.8)	20 (95.2)	**18 (100)**	37 (86.1)	208 (90.7)
$(AT)_9T_5$	-	1 (3.0)	1 (2.3)	2 (6.9)	**5 (20.8)**	1 (4.8)	-	5 (11.6)	15 (6.6)
$(AT)_{11}T_3$	-	-	1 (2.3)	-	**2 (8.4)**	-	-	1 (2.3)	4 (1.8)
$(AT)_8T_4$	1 (5.6)	1 (3.0)	-	-	-	-	-	-	2 (0.9)
Total	18 (7.9)	33 (14.4)	43 (18.7)	29 (12.7)	24 (10.5)	21 (9.2)	18 (7.9)	43 (18.7)	229 (100)

TABLE V.12. $(AT)_xT_y$ patterns in some wild-type β-globin genes from Turkey and neighboring countries.

Motif	MR	AM	BSR	EA	Unknown	Turkey	BLK	LBNSYR	PKN	Total
$(AT)_7T_7$	1	1		2	9	13 (76.5)	4 (80)	3 (60)		20 (69.0)
$(AT)_9T_5$	1		1		1	3 (17.6)		1 (20)	1 (50)	5 (17.3)
$(AT)_8T_5$								1 (20)	1 (50)	2 (6.9)
$(AT)_{11}T_3$							1 (20)			1 (3.4)
$(AT)_8T_7$					1	1 (5.9)				1 (3.4)
Total	2	1	1	2	11	17 (58.5)	5 (17.3)	5 (17.3)	2 (6.9)	29 (100)

2. The -158 (C-T) *XmnI* Gγ-Globin Gene Polymorphism in Turkish β-Thalassemia Patients

Results of the analysis of the -158 (C-T) polymorphism of the Gγ-globin gene in 31 Turkish β-thalassemia homozygotes are presented in Tables V.13 and V.14 and in Figure V.8. Out of the 62 β-thalassemia chromosomes analyzed, 58 carry 12 mutations, one had the sickle cell gene, and three had uncharacterized mutations. Of these chromosomes only 10 (16.1 per cent) carry the -158 C-T polymorphism, which is only associated with the FSC-8 (-AA) deletion and the IVS-II-1 (G-A) mutation (100 per cent of cases). On the other hand, only six chromosomes carry the $(AT)_9T_5$ pattern, which is mainly associated with the IVS-II-1 mutation as presented in section V.D.

TABLE V.13. Genotypes, HbF levels, geographical origins, the -158 Gγ-globin status, and $(AT)_xT_y$ β-globin gene polymorphism in 31 Turkish β-thalassemia patients. The decreased HbF values in β^0-thalassemia patients (marked by †) may be attributed to transfusion.

Sample No.	Genotype	HbF (%)	Region	-158 (C-T)*	$(AT)_xT_y$
T-249	IVS-I-110/IVS-I-110	98.0	EA	-/-	7*7/7*7
T-466	FSC-8/FSC-22-24†	96.0	SEA	+/-	7*7/8*5
T-459	IVS-I-110/Cd 39	94.5	BSR	-/-	7*7/7*7
T-522	IVS-I-110/IVS-II-745	94.0	BSR	-/-	7*7/7*7
T-080	IVS-II-1/IVS-II-1†	92.6	CA	+/+	9*5/9*5
T-462	IVS-I-110/IVS-I-110	90.2	SEA	-/-	?*?/?*?
T-513	FSC-44/?	90.0	SEA	-/-	?*?/?*?
T-567	IVS-I-110/Poly A	82.0	BSR	-/-	7*7/7*7
T-108	IVS-I-1/IVS-I-1†	81.0	CA	-/-	7*7/7*7
T-109	IVS-I-110/IVS-I-110	77.0	SEA	-/-	7*7/7*7
T-237	IVS-II-745/IVS-II-745	77.0	CA	-/-	7*7/7*7
T-555	FSC-8/FSC-8†	76.8	EA	+/+	7*7/7*7
T-526	IVS-I-110/IVS-I-110	74.7	CA	-/-	?*?/?*?
T-328	IVS-I-110/IVS-I-110	74.0	AM	-/-	7*7/7*7
T-107	IVS-II-745/IVS-II-745	73.8	CA	-/-	7*7/7*7
TI-139	IVS-I-110/?	72.2	SEA	-/-	?*?/?*?
T-067	IVS-I-110/IVS-II-745	72.0	CA	-/-	7*7/7*7
T-408	IVS-I-110/IVS-I-110	71.0	EA	-/-	7*7/7*7
T-066	IVS-I-110/IVS-I-110	70.0	CA	-/-	7*7/7*7
T-518	IVS-I-110/IVS-I-110	69.7	CA	-/-	11*3/11*3
T-323	IVS-I-1/?	69.0	CA	-/-	7*7/7*7
T-575	IVS-I-110/IVS-II-1	66.0	SEA	-/+	7*7/9*5
T-046	FSC-8/9/FSC-8/9†	65.0	EA	-/-	7*7/7*7
TI-009	FSC-8/IVS-II-1†	63.8	EA	+/+	7*7/9*5
T-399	-30/Poly A	62.9	EA	-/-	?*?/?*?
T-096	IVS-I-110/IVS-I-110	62.3	CA	-/-	7*7/7*7
TI-133	290 bp deletion/HbS†	60.4	EA	-/-	7*7/7*7
T-403	IVS-II-745/IVS-II-745	60.0	SEA	-/-	7*7/7*7
T-228	IVS-I-110/IVS-I-110	59.7	AM	-/-	7*7/7*7
T-331	IVS-I-110/IVS-I-110	58.3	CA	-/-	7*7/7*7
T-660	IVS-II-1/IVS-II-1†	55.0	BSR	+/+	9*5/9*5

* The (-) and (+) signs indicate the absence or presence of the -158 polymorphism, respectively. The (?) marks indicate unsolved cases.

TABLE V.14. β-Globin mutations identified in the 62 chromosomes studied. Only 10 are found to carry the -158 polymorphism.

Mutation	Type	-158 (C-T)	N*	$(AT)_7T_7$	$(AT)_8T_5$	$(AT)_9T_5$	$(AT)_{11}T_3$	$(AT)_7T_7$
IVS-I-110	β^+	-	28	21 (75%)	-	-	2 (7.1%)	5 (17.9%)
IVS-II-745	β^+	-	8	8 (100%)	-	-	-	-
IVS-II-1	β^0	6 (100%)	6	-	-	6 (100%)	-	-
FSC-8	β^0	4 (100%)	4	4 (100%)	-	-	-	-
IVS-I-1	β^0	-	3	3 (100%)	-	-	-	-
FSC-8/9	β^0	-	2	2 (100%)	-	-	-	-
Poly A	β^+	-	2	1 (50%)	-	-	-	1 (50%)
290 bp del.	β^0	-	1	1 (100%)	-	-	-	-
-30	β^+	-	1	-	-	-	-	1 (100%)
Cd 39	β^0	-	1	1 (100%)	-	-	-	-
FSC-22-24	β^0	-	1	-	1 (100%)	-	-	-
FSC-44	β^0	-	1	-	-	-	-	1 (100%)
HbS	β^S	-	1	1 (100%)	-	-	-	-
Unknown		-	3	1 (33.3%)	-	-	-	2 (66.7%)
Total		**10 (16.1%)**	**62**	**43 (69.4%)**	**1 (1.6%)**	**6 (9.7%)**	**2 (3.2%)**	**10 (16.1%)**

* (N) indicates number of chromosomes.

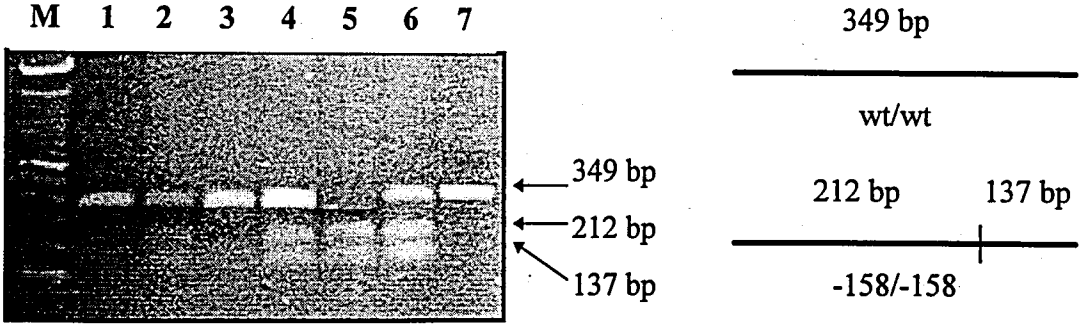


FIGURE V.8. *XmnI* digestion and band patterns demonstrating variations at the -158 C-T polymorphism of the Gγ-globin gene. Genotype of samples in lanes 1-3 is wt/wt, in lanes 4 and 6 is -158/wt, and in lane 5 is -158/-158. Lane 7 demonstrates an undigested PCR fragment amplified, using primers G5 and G4. M is the 100 bp ladder DNA marker.

D. Sequence Haplotype Diversity of β-Globin Genes in Turkey and Neighboring Countries

Analysis of the polymorphic region spanning the region between positions -1069 and -490 bp relative to the 5' Cap site of the β-globin gene demonstrates the presence of 12 sequence haplotypes (HTs) in the 204 β-thalassemia and wild-type chromosomes investigated in this study (Figures V.9 and V.10; Table V.15).

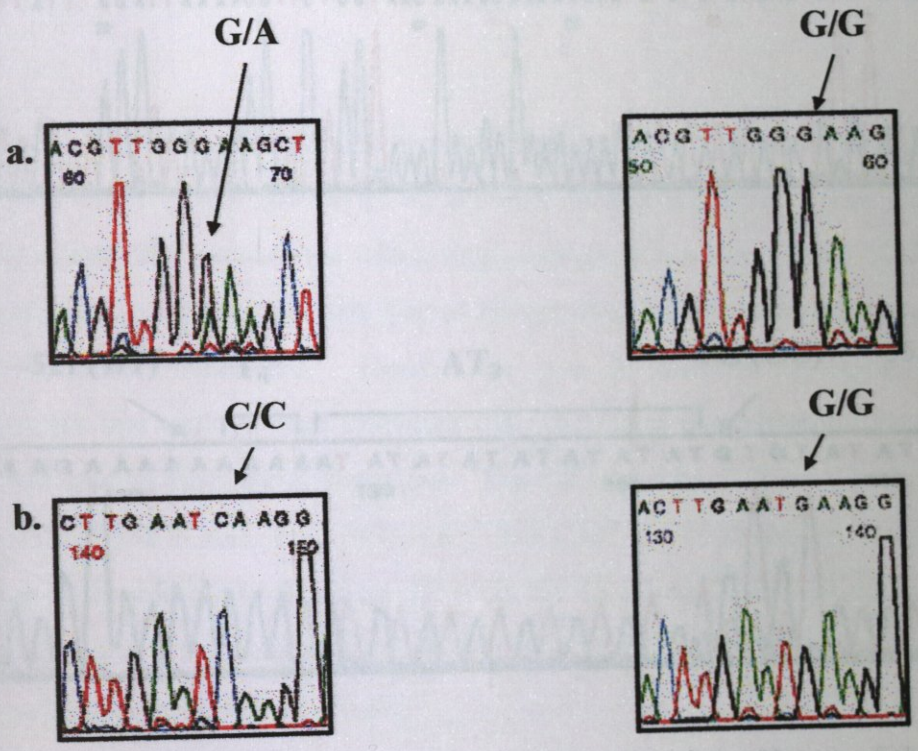


FIGURE V.9. Non-radioactive DNA sequencing results of the 5' part of the β-globin gene sequence polymorphisms of the β-globin gene (stated 5' to 3'). (a) Two patterns at the -1069 polymorphism: G/A on the left (the patient is IVS-I-110/IVS-I-110) and G/G on the right (the patient is IVS-II-1/IVS-II-1). (b) Two patterns at the -969 polymorphism for the same two patients as in (a).

D. Sequence Haplotype Diversity of β-Globin Genes in Turkey and Neighboring Countries

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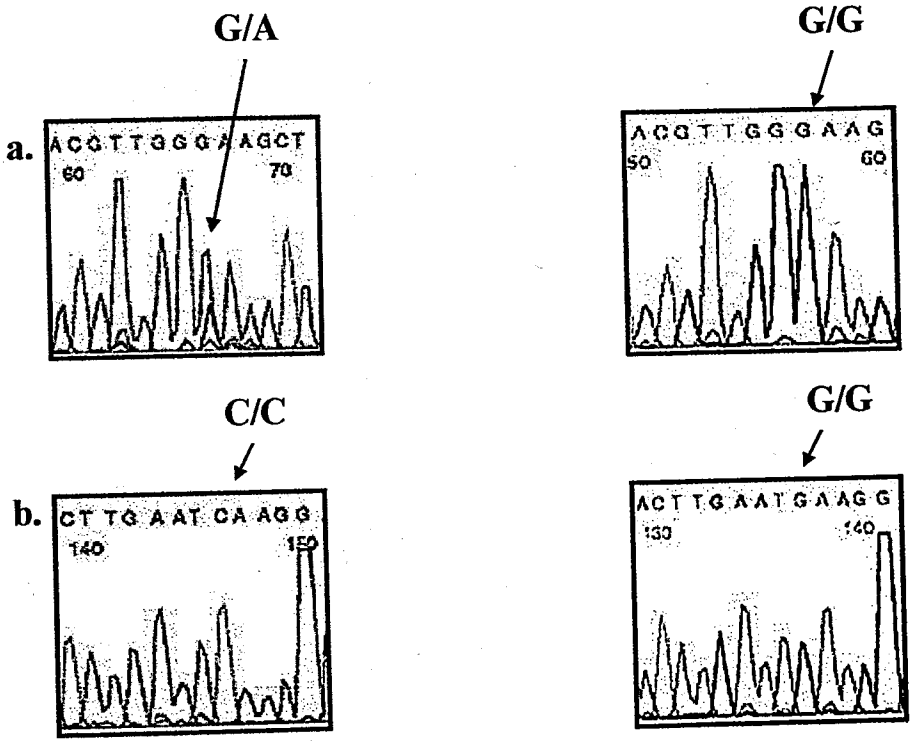


FIGURE V.9. Non-radioactive DNA sequencing results of the 5' part of the β-globin gene sequence polymorphisms of the β-globin gene (stated 5' to 3'). (a) Two patterns at the -1069 polymorphism: G/A on the left (the patient is IVS-I-110/IVS-I-110) and G/G on the right (the patient is IVS-II-1/IVS-II-1). (b) Two patterns at the -969 polymorphism for the same two patients as in (a).

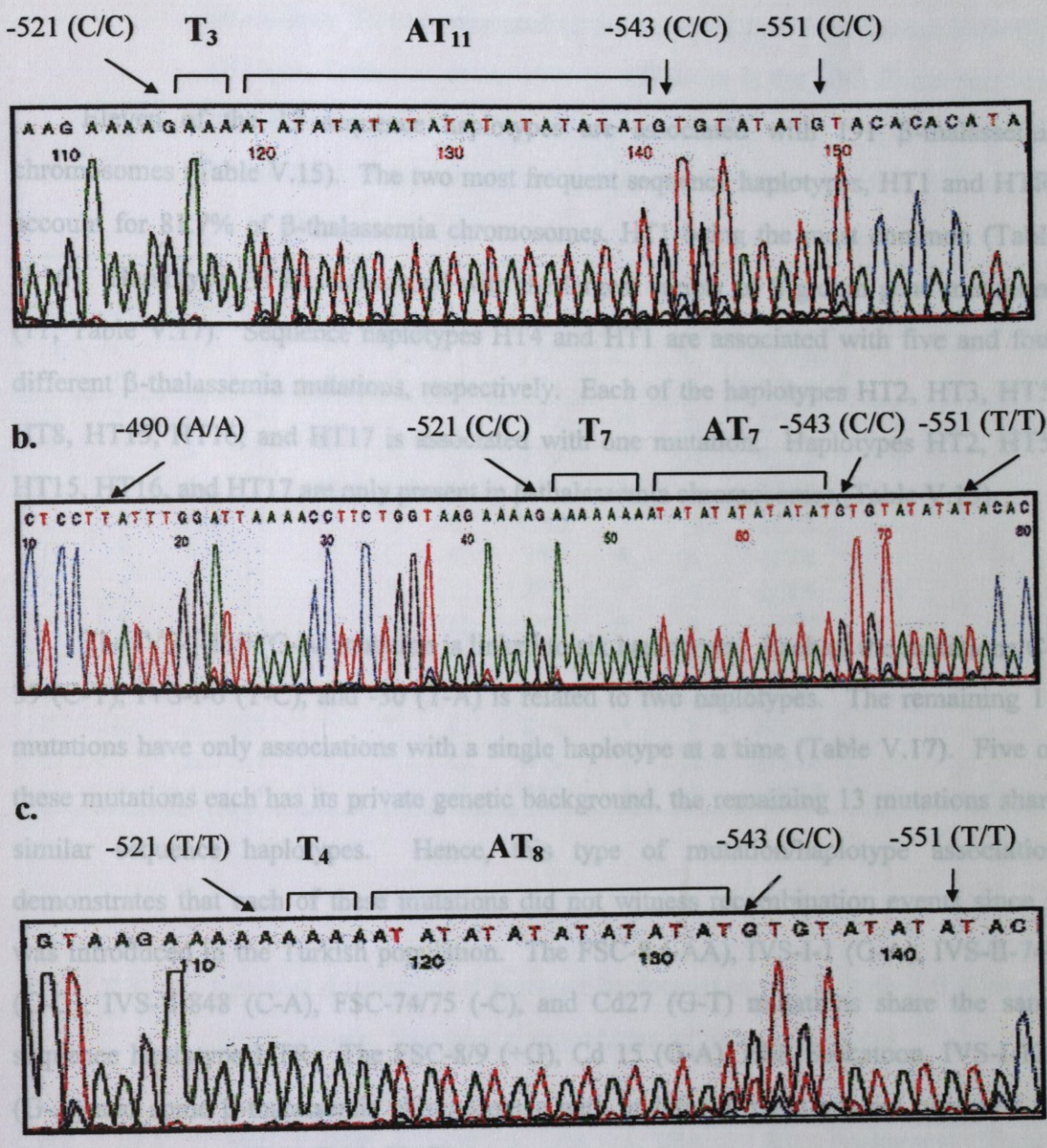


FIGURE V.10. Non-radioactive DNA sequencing results of the 3' part of the sequence polymorphisms of the β-globin gene (stated 3' to 5', and, thus, should be read in the counter sense). (a) An IVS-I-110/IVS-I-110 patient homozygous for the partial sequence haplotype $CC(AT)_{11}T_3C$. (b) An IVS-I-110/IVS-I-110 patient homozygous for the partial sequence haplotype $TC(AT)_7T_7CA$. (c) An IVS-I-5/IVS-I-5 patient homozygous for the partial haplotype $TC(AT)_8T_4T$.

1. Sequence Haplotypes Associated With β-Thalassemia Chromosomes

Eleven of the 12 sequence haplotypes are associated with 191 β-thalassemia chromosomes (Table V.15). The two most frequent sequence haplotypes, HT1 and HTR, account for 81.7% of β-thalassemia chromosomes, HT1 being the most common (Table V.16). Haplotype HTR is associated with the largest variety of β-globin gene mutations (11; Table V.17). Sequence haplotypes HT4 and HT1 are associated with five and four different β-thalassemia mutations, respectively. Each of the haplotypes HT2, HT3, HT5, HT8, HT15, HT16, and HT17 is associated with one mutation. Haplotypes HT2, HT5, HT15, HT16, and HT17 are only present in β-thalassemia chromosomes (Table V.15).

The IVS-I-110 (G-A) mutation is linked to six haplotypes. Each of the mutations Cd 39 (C-T), IVS-I-6 (T-C), and -30 (T-A) is related to two haplotypes. The remaining 18 mutations have only associations with a single haplotype at a time (Table V.17). Five of these mutations each has its private genetic background, the remaining 13 mutations share similar sequence haplotypes. Hence, this type of mutation/haplotype association demonstrates that each of these mutations did not witness recombination events since it was introduced in the Turkish population. The FSC-8 (-AA), IVS-I-1 (G-A), IVS-II-745 (C-G), IVS-II-848 (C-A), FSC-74/75 (-C), and Cd27 (G-T) mutations share the same sequence haplotype HTR. The FSC-8/9 (+G), Cd 15 (G-A), HbE Saskatoon, IVS-I-130 (G-A) and some β-thalassemia chromosomes with undefined mutations are observed on sequence haplotype HT4 (Table V.17).

TABLE V.15. DNA sequence haplotypes observed in 204 mutant and wild type β-globin genes from Turkey compared to the ancestral human sequence haplotype (HTAnc; Trabuchet et al., 1991a). HTBenin is the HbS Benin sequence haplotype described by Trabuchet et al. (1991a). The * symbol indicates homology with the corresponding ancestral haplotype position (HTAnc).

-1069	-989	-780	-710	-703	-551	-543	$(AT)_yT_x$	-521	-491	HT	Occurrence	
											β-thal	Wild-type
A	*C*	*T*	*T*	*T*	*T*	*T*	*?*?*	*C*	*C*	***HTAnc***		
*	*G*	*A*	*	*	*	*	*8*4*	*	*	***HTBenin***		
G	*	A	*	*	*	C	7*7	*	A	**HTR**	+	+
*	*	A	*	*	*	C	7*7	*	A	**HT1**	+	+
*	*	A	*	C	C	C	9*5	*	A	**HT2**	+	-
G	*	A	*	C	C	C	11*3	*	A	**HT3**	+	+
G	*	A	*	C	C	C	7*7	*	A	**HT4**	+	+
G	*	A	*	C	*	C	7*7	*	A	**HT5**	+	-
*	*	A	*	*	C	C	7*7	*	A	**HT7**	+	+
G	*	A	*	C	C	C	9*5	*	A	**HT8**	+	+
G	*	A	*	*	C	C	9*5	*	A	**HT9**	-	+
*	G	A	*	*	*	C	8*4	*	*	**HT15**	+	-
G	*	A	*	*	*	C	8*4	T	A	**HT16**	+	-
G	G	A	*	C	C	C	9*5	*	A	**HT17**	+	-

TABLE V.16. Regional distribution of sequence haplotypes in 191 Turkish β-thalassemia chromosomes. Numbers in parentheses indicate percentages.

HT	BLK	MR	BSR	AM	CA	SEA	EA	Unknown	Total
HT1	11 (52.3)	5 (33.3)	5 (26.3)	16 (53.3)	22 (66.7)	11 (44)	7 (38.9)	13 (43.3)	90 (47.1)
HTR	7 (33.3)	6 (40)	6 (31.6)	10 (33.3)	9 (27.3)	11 (44)	6 (33.3)	11 (36.7)	66 (34.6)
HT4	1 (4.8)	2 (13.3)	1 (5.3)				4 (22.2)	4 (13.3)	12 (6.3)
HT17			2 (10.5)	1 (3.3)	1 (3)	1 (4)	1 (5.6)	2 (6.7)	8 (4.2)
HT3			2 (10.5)		1 (3)				3 (1.6)
HT7	1 (4.8)			1 (3.3)		1 (4)			3 (1.6)
HT8			3 (15.8)						3 (1.6)
HT15		2 (13.3)							2 (1.0)
HT16				2 (6.7)					2 (1.0)
HT2	1 (4.8)								1 (0.5)
HT5						1 (4)			1 (0.5)
	21 (11.0)	15 (7.9)	19 (10.0)	30 (15.7)	33 (17.2)	25 (13.1)	18 (9.4)	30 (15.7)	191 (100)

TABLE V.17. β-Thalassemia mutations in Turkey and their corresponding sequence haplotypes. Numbers in parentheses indicate percentages.

Mutation	HT	BLK	MR	BSR	AM	CA	SEA	EA	Unknown	Total
IVS-I-110 (G-A)	HT1	8	3	4	16	20	9	7	10	77 (87.5)
	HTR			1	1	1	1			4 (4.6)
	HT3			2		1				3 (3.4)
	HT4								2	2 (2.3)
	HT5						1			1 (1.1)
	HT7	1								1 (1.1)
IVS-I-6 (T-C)	HTR	3	4		4	3	4	2	1	21 (91.3)
	HT15		2							2 (8.7)
Cd39 (C-T)	HT1	1	2			2	1		3	9 (56.2)
	HTR	2		1					4	7 (43.8)
-30 (T-A)	HTR			4	2			2	1	9 (90)
	HT1						1			1 (10)
IVS-II-1 (G-A)	HT17			2	1	1	1	1	2	8 (100)
IVS-II-745 (C-G)	HTR					2	4			6 (100)
FSC-8 (-AA)	HTR				2	2		1		5 (100)
IVS-I-1 (G-A)	HTR	1	1			1			2	5 (100)
FSC-8/9 (+G)	HT4		2					1		3 (100)
-87 (C-G)	HT8			3						3 (100)
IVS-I-116 (T-G)	HT1	2								2 (100)
IVS-I-5 (G-C)	HT16				2					2 (100)
FSC-5 (-CT)	HT7				1					1 (100)
HbD Los Angeles	HT7						1			1 (100)
Cd 15 (G-A)	HT4			1						1 (100)
HbE Saskatoon	HT4								1	1 (100)
IVS-I-130 (G-A)	HT4	1								1 (100)
IVS-II-654 (T-C)	HT2	1								1 (100)
IVS-II-848 (C-A)	HTR								1	1 (100)
FSC-74/75 (-C)	HTR							1		1 (100)
Cd 27 (G-T)	HTR	1								1 (100)
HbS	HTR						1			1 (100)
Unknown	HTR		1		1		1		2	5 (50)
	HT4							3	1	4 (40)
	HT1			1						1 (10)
Total		21	15	19	30	33	25	18	30	191

2. Sequence Haplotypes Associated With Wild-Type Chromosomes

The 13 wild-type chromosomes analyzed in this study exhibit seven sequence haplotypes (Tables V.15 and V.18). The four most frequent sequence haplotypes (HTR, HT1, HT4 and HT7) account for 77.1% of wild-type chromosomes, HTR being the most common with 30.9%. Sequence haplotype HT9 is exclusively observed in wild-type β-globin chromosomes (Table V.15).

3. Geographical Distribution of Sequence Haplotypes

Geographically speaking, the Black Sea Region presents seven sequence haplotypes. Turkish chromosomes from the Balkan countries and Cyprus exhibit six sequence haplotypes, whereas, the Marmara region, the Aegean-Mediterranean region, Southeast Anatolia, and samples of unknown origin each exhibit five sequence haplotypes; samples from East and Central Anatolia present four sequence haplotypes each (Tables V.16 and V.18).

TABLE V.18. Regional distribution of sequence haplotypes present in 13 Turkish wild-type β-globin chromosomes. Numbers in parentheses indicate percentages.

HT	BLK	MR	BSR	EA	Unknown	Total
HTR	3 (60)				1 (20)	4 (30.9)
HT1	1 (20)				1 (20)	2 (15.4)
HT4				1 (50)	1 (20)	2 (15.4)
HT7					2 (40)	2 (15.4)
HT9			1 (100)			1 (7.7)
HT3	1 (20)					1 (7.7)
HT8		1 (100)				1 (7.7)
Total	5 (38.5)	1 (7.7)	1 (7.7)	1 (7.7)	5 (38.5)	13 (100)

VI. DISCUSSION

A. Analysis of Some Factors Modulating the Expression of Globin Genes

β-Thalassemia is a severe transfusion-dependent anemia that exhibits itself starting at the first year of life. The clinical course of the disease varies widely. Clinical studies have shown an inverse relationship between the severity of β-thalassemia and the level of fetal hemoglobin (HbF) encoded by the two γ-globin genes (Gγ and Aγ). Considerable effort has been made to identify the genetic determinants of γ-globin chain expression among SCD and β-thalassemia patients because higher levels of HbF ameliorate the phenotypic expression of these diseases. Several putative *cis*-acting genetic determinants are said to enhance γ-globin chain synthesis. Among these, the *XmnI* polymorphism at nucleotide -158 upstream of the Gγ-globin gene (Gilman and Huisman, 1985) appears to be the most consistently associated with increased Gγ-globin gene expression in normal, SCD, and β-thalassemia individuals (and also other states of erythropoietic stress). SCD patients carrying the Senegal or the Saudi/Indian haplotypes, either homozygous or heterozygous for an *XmnI* site, display increased levels of (Nagel et al., 1991; Chang et al., 1995). Some reports, however, argue that the observed phenotype may not be directly linked to the -158 itself, which may only be a marker for other unknown genetic factors (reviewed by Steinberg, 1996).

Negative regulatory elements in the β-globin gene promoter that bind BP1 protein have attracted considerable attention. The $(AT)_9T_5$ motif, in particular, has been studied with varying implications. While this motif has been associated with silent β-thalassemia, and with mild phenotype and higher levels of HbF in some homozygous β-thalassemia patients (Semenza et al., 1984; Murru et al., 1990; Elion et al., 1992; Ragusa et al., 1992), others have not found this association (Dover et al., 1987; Galanello et al., 1993). It is noteworthy to mention that a definite haplotype-linked polymorphism of this motif exists

among SCD patients (Elion et al., 1992). Only the Saudi/Indian haplotype is associated with the $(AT)_9T_5$ rearrangement (Zeng et al., 1994), while the Senegal haplotype, which as the Saudi/Indian haplotype is *XmnI*-positive, has the $(AT)_8T_4$ motif. The Benin haplotype, which is *XmnI*-negative, also has the $(AT)_8T_4$ arrangement (Trabuchet et al., 1991a; Trabuchet et al., 1991b).

1. Polymorphic Patterns of the $(AT)_xT_y$ and *XmnI* polymorphisms in β-Thalassemia Patients

Studying of 229 Turkish β-thalassemia chromosomes with 24 mutations in the β-globin gene shows that the $(AT)_xT_y$ motif appears to have a specific polymorphic pattern. Thus, chromosomes with the Cd39 (C-T), -30 (T-A), IVS-II-745 (C-G), IVS-I-1 (G-A), FSC-8 (-AA), FSC-8/9 (+G), IVS-I-116 (T-G), HbS, Poly A (TA-TG), IVS-I-848 (C-A), Cd15 (G-A), FSC-74/75 (-C), 290 bp deletion, Cd27 (G-T), IVS-I-130 (G-A), Cd121 (G-C), and Cd22 (G-A) mutations are associated with the $(AT)_7T_7$ motif, the IVS-II-1 (G-A), -87 (C-G), and IVS-II-654 (C-T) mutations with the $(AT)_9T_5$ patterns, while the IVS-I-5 (G-C) mutation is on a chromosome with the $(AT)_8T_4$ arrangement. The IVS-I-110 (G-A), IVS-I-6 (T-C), and FSC-5 (-CT) mutations, on the other hand, appear to be associated with various patterns (Table V.10).

In this study, we examined 31 Turkish β-thalassemia patients with variable levels of HbF for the occurrence of the Gγ -158 C-T substitution. The polymorphism is present in 10 of the studied patients and is only associated with the FSC-8 (-AA) and IVS-II-1 (G-A) mutations, both of which are said to cause a β^0-thalassemia type. This result may explain the findings of Diaz-Chico et al. (1988), Gürgey et al. (1989), and Altay and Başak (1995) in which most of the thalassemia intermedia patients carried these two mutations as well as other mild lesions. Our data also demonstrate a strong linkage between the $(AT)_xT_y$ motif and the Gγ-globin *XmnI* polymorphism. All $(AT)_9T_5$ chromosomes analyzed for the -158 polymorphism were *XmnI*-positive for the polymorphism, while $(AT)_7T_7$ chromosomes

were not associated with it, except in three FSC-8 chromosomes (Table V.13 and Table V.14).

The fact that all homozygous β-thalassemia patients with the $(AT)_9T_5$ motif were also *XmnI*-positive, makes the assessment of the relative contributions of these two variations to any elevated HbF levels observed difficult. However, IVS-II-1 and FSC-8 patients offer a unique opportunity for comparison. In homozygous patients, both groups are *XmnI*-positive (+/+) but the former have the $(AT)_9T_5$ motif, while the latter is associated with the $(AT)_7T_7$ arrangement. The HbF levels in both groups are similar with an average of 73.8 and 76.8%, respectively (Table V.13). However, the coinheritance of the IVS-II-1 and FSC-8 mutations, along with their respective *XmnI* and $(AT)_xT_y$ motifs, exhibited a lower value for HbF in one patient, thus pointing to a probable role of the mutations and not the polymorphisms in this case (Table V.13). Moreover, a patient, heterozygous for the *XmnI* polymorphism, not having the $(AT)_9T_5$ pattern, exhibited a much higher HbF value than a similar patient heterozygous for the $(AT)_9T_5$ pattern (Table V.13). This comparison indeed suggests that the *XmnI* site has a dominant contributory factor to elevated HbF levels in β-thalassemia patients. In individuals with the same mutation, *XmnI* and $(AT)_xT_y$ status, the variability in HbF values may be attributed to differences in the transfusion regimen and/or sex and/or age of the patient.

Therefore, while the $(AT)_xT_y$ motif might indeed have a negative regulatory role on β-globin gene expression, as shown by *in vitro* binding studies (Berg et al., 1989), there is no evidence that it plays an important regulatory role in γ-gene expression among β-thalassemia patients analyzed in this study. Although the *XmnI* polymorphism at -158 5' to the β-globin gene shows a more positive association with γ-gene expression, its presence does not account for all the variations in HbF levels observed. Two of our patients (Table V.13) seem to have a mild course of the disease with increased HbF because of the types of mutations they carry and not to the presence or absence of the -158 polymorphism (i.e., -30 T-A, and 290 bp deletion). An additional patient had a modified phenotype because of the coinheritance of β-thalassemia with another genetic disorder.

In an extensive review about β-thalassemia in Turkey, Altay and Başak (1995) reported the positive presence of the C-T change at -158 Gγ-globin gene in a homozygous β-thalassemia patient for the IVS-I-110 mutation. This indicates that the -158 (C-T) polymorphism is not only linked to FSC-8 and IVS-II-1 mutations. Further analysis including a larger number of chromosomes may indicate other associations between the -158 polymorphism and any other mutation(s).

In 22 of our patients the genetic evidence behind the increased levels of HbF remains unanswered (Table V.13). Several other genetic and non-genetic variables have been proposed to influence peripheral HbF levels in patients with hematopoietic stress through one or more mechanisms. These variables are age, sex, α-globin gene number, genetic factors linked to the β-globin gene region on chromosome 11, and genetic factors not linked to the β-globin gene complex (Chang et al., 1995; reviewed in Steinberg, 1996). Several reports discussed the effect of a newly characterized dominant X-linked determinant, associated with elevated HbF levels and F-cells, in normal adults and SCD patients. The gene was localized to the chromosomal region Xp22.2-22.3 and is termed the F-cell production (FCP) locus (Dover et al., 1992). This locus is believed to cause the increase in the level of HbF in 35-40 per cent of sickle cell patients (Chang et al., 1997) and, thus, it may be a good candidate for a future investigation to explain increased HbF levels in many of our patients. The results that are presented here should be considered as preliminary; however, with the relatively small number of chromosomes that are positive for the -158 C-T polymorphism, we can conclude that the polymorphism seems to be present homogeneously in Turkey with no specific concentrations in certain geographical regions (Table V.13).

With the successful implementation of the methods to detect the C-T change at position -158 upstream of the Gγ-globin gene in our laboratory, it is recommended to conduct regular screening for this substitution in any coming prenatal diagnosis case to

help in understanding the clinical phenotype of the fetus. This information may even allow parents, in mild β-thalassemia cases, to avoid the hard decision of abortion, if necessary. On the other hand, hydroxyurea (HU) has been shown to increase HbF levels in SCD as well as β-thalassemia patients. The amplitude and rapidity of the HbF response to HU were found to be enhanced, to a certain extent, by the presence of the T at position -158 5' of the Gγ-globin gene (reviewed in Steinberg, 1996). Thus, our results may also give those patients hope for this newly developed treatment.

2. β-Thalassemia Trait Associated With Fanconi Aplastic Anemia in a Turkish Patient

The patient had Fanconi anemia and β-thalassemia trait associated with the IVS-I-5 (G-C) mutation in the β-globin gene. Despite the severe anemia episode caused by Fanconi anemia, microcytosis was not observed in this patient. This may indicate that the majority of her red blood cells were produced by precursor cells of a fetal line in which hemoglobin synthesis was probably unimpaired because of the active synthesis of the γ-chains of globin (Betke et al., 1959). This observation supports the hypothesis that fetal red cell precursors are the most resistant cell lines of the marrow precursor cells to abnormalities causing bone marrow aplasia (Alter et al., 1991). However, the presence of microcytosis in remission of Fanconi anemia indicated that the effect of thalassemia on red cell volume overwhelms the effect of Fanconi anemia on the same parameter. This observation conflicts with the previous knowledge that during the remission or before the anemic episode in Fanconi anemia macrocytosis would be present and that this assumption may only be valid in Fanconi anemia patients without a coexistent thalassemic determinant (Altay et al., 1996a).

This example indicates the importance of detailed hematological evaluation in Fanconi anemia, not only in the anemic period, but during remission as well. Such a detailed study will not only help detect the presence of another genetically transmitted

hematological abnormality, but will also aid in understanding the counter-effects of different genetic disorders when coexisting in a patient (Altay et al., 1996a).

3. HbS/β^{del}-Thalassemia Associated With High Levels of HbA_2 and HbF in a Turkish Family

Several reports have described β^0-thalassemia mutations coexisting with HbS in patients from various populations. However, only four reports have recorded the association of a deletional type of β-thalassemia (1.35 kbp or 532 bp deletions) with HbS in the same patient (Padanilam et al., 1984; Gonzalez-Redondo et al., 1991; Waye et al., 1991a; Waye et al., 1991b). The 290 bp deletion causing β-thalassemia was first observed in a Turkish patient (Diaz-Chico et al., 1987) and then in several others (Spielberg et al., 1989; Aulehla-Scholz et al., 1989; Thein et al., 1992). In our present investigation, we describe the first occurrence of the 290 bp deletion along with HbS in a sister and brother. Although carrying two deleterious types of mutations, our patients have mild symptoms because of their increased levels of HbF (18.1 and 33.6%) and HbA_2 (6.7 and 6%). Under conditions of erythropoietic stress, it is known that, HbF might be synthesized at a slightly higher rate in early childhood, but the hemoglobin levels of our patients are far too high for this, and their ages are already 10 and five.

We tested our two β^S/β^{del}-thalassemia patients for the *XmnI* polymorphism and the $(AT)_xT_y$ microsatellite sequence. Our results showed no changes from the reference sequences at these two positions; i.e., C at position -158 5' to the Gγ-globin gene and $(AT)_7T_7$ at -530 bp 5' to the β-globin gene. Since the hematology of the parents did not show any change from the normal, the dominant X-linked FCP determinant is not expected to be the reason behind the observed phenotype (Boyer et al., 1984; Myoshi et al., 1988; Dover et al., 1992; Chang et al., 1997).

Because of their severe mutations, the majority of patients with β^S/β^0-thalassemia had severe disease with many clinical complications similar to those of severe β^S/β^S patients (Steinberg, 1996). However, all β^S/β^{del}-thalassemia patients, reported so far, exhibited a mild phenotype with increased levels of HbA_2 and HbF (Padanilam et al., 1984; Gonzalez-Redondo et al., 1991; Waye et al., 1991a; Waye et al., 1991b). The reason for this observed phenotype is the β^{del}-thalassemia gene (i.e., the 290 bp deletion) and not the β^S-chromosome. The common outcome between these β-globin gene deletions, is the removal of the region between positions -125 to +78 upstream from the β-globin gene mRNA cap site (Figure I.6). Some of the elements deleted in this region are the CAC (-90), CAAT (-70), and TATA (-30) boxes. The absence of these elements is thought to be of great functional significance in eliciting the unusually high HbA_2 and HbF phenotypes. Two principal hypotheses may explain the effect of the 290 bp deletion on the expression of δ- and γ-globin genes:

A) The deletion of sequences around the β-globin gene decreases the distance separating the δ- and γ-globin genes from the enhancer sequences located 3' to the β-globin gene. On a chromosomal-scale, the 290 bp deletion is not expected to significantly translocate the enhancer element closer to the δ- and γ-globin genes. However, it may have a relevant effect at the three-dimensional level of the chromatin structure in that chromosomal location (Wood, 1993).

B) The second and more probable hypothesis involves a competition between the fetal and the adult globin genes for the hypersensitive sequence in the upstream locus control region (LCR). Without the 5' β-globin gene promoter, the LCR would interact with either the δ- or γ-genes enhancing their expression (Codrington et al., 1990; Hanscombe et al., 1991).

Molecular examination of these two mechanisms will shed light to the interactions causing the increase in HbA_2 and HbF levels in our patients.

B. RFLP Haplotypes Versus Sequence Haplotypes

Since their first description in 1982, many haplotype polymorphisms detectable with restriction endonucleases have been observed in the ~60 kbp DNA fragment spanning the β-globin gene cluster (Antonarakis et al., 1982; Antonarakis et al., 1985). By calculating linkage disequilibrium in population data, Chakravarti et al. (1984) estimated that most of the recombination events in the β-gene cluster occur within a 9.1 kbp DNA sequence present between the δ- and β-globin genes and that the recombination rate in this segment is about 30 times the average expected rate for a DNA stretch of this length. This increased rate of recombination is believed to be the reason behind the subdivision of the full RFLP haplotypes into 5' and 3' sub-haplotypes. By comparing data from the present study about the sequence haplotypes associated with common β-thalassemia mutations and data published in the literature about RFLP haplotypes for these mutations in Turkey (Figure VI.1), several conclusions can be drawn:

A) Each RFLP haplotype might be associated with multiple sequence haplotypes.
B) There is a tight, but not exclusive, linkage between each sequence haplotype with a 3' RFLP haplotype (Figure VI.1).
C) Unequal crossing-over alternatively occurs upstream and/or downstream of the variable sequence 400 bp 5' to the β-globin gene and leads to the subdivision of the full RFLP haplotype into 5' and 3' sub-haplotypes.

A detailed sequence analysis for the stretch of human DNA sequence present between the δ- and β-globin genes is a necessity to understand the processes leading to the proposed recombinations. The study may include computer-assisted characterization of the purine/pyrimidine content, the presence of repetitive elements that might have effects on the topology of the DNA sequence, and their possible role in causing any recombinational activities.

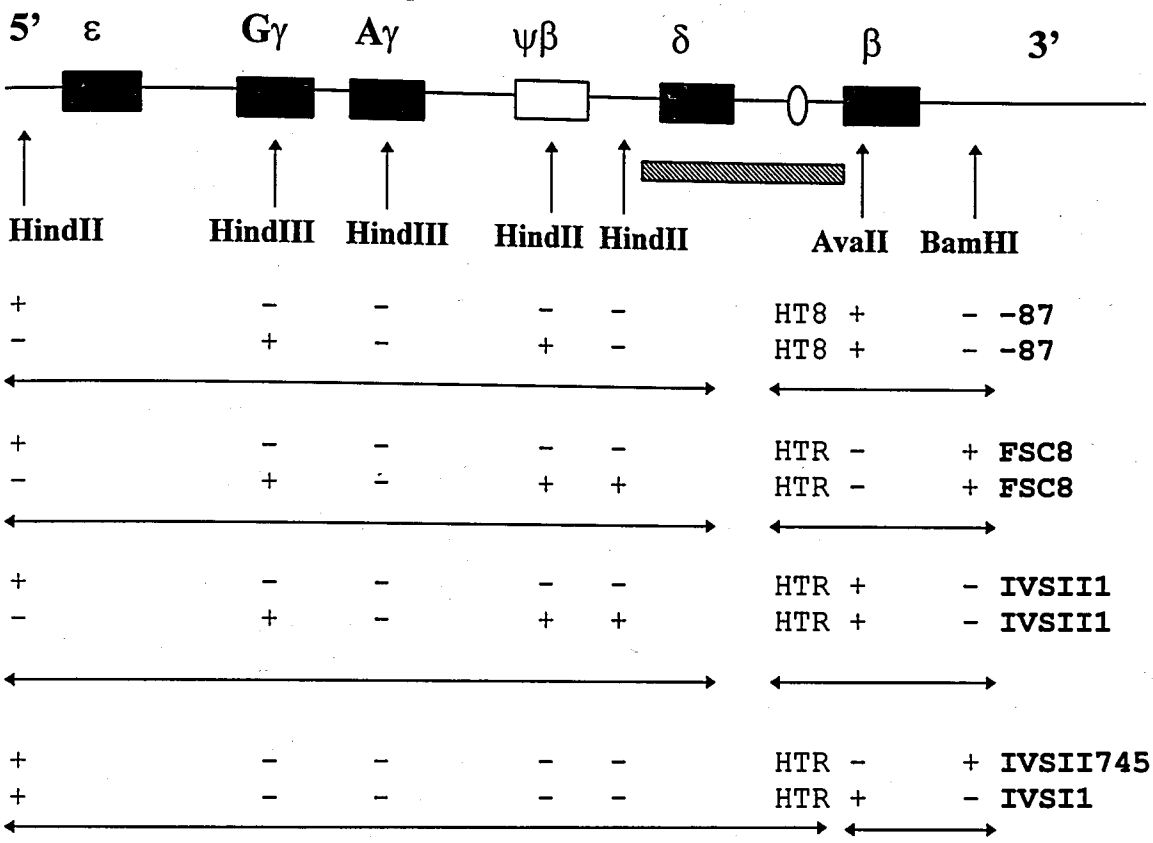


FIGURE VI.1. The β-globin cluster with some of its RFLP markers (map not to scale). The empty circle indicates the polymorphic sequence analyzed in this study. Below the map are comparative data of RFLP haplotypes (Flint et al., 1993) and sequence haplotypes (present study) associated with some β-thalassemia mutations in Turkey. The region indicated by a hatched rectangle represents a hot-spot area of recombinations that may have taken place between 5' and 3' sub-haplotypes.

C. Population Genetics of Turkish People

The genetic heterogeneity of Turkish people was analyzed in this study using various genetic tools such as the $(AT)_xT_y$ motif, sequence haplotypes, and β-thalassemia mutation distributions. Turkish people exhibit a genetic heterogeneity that is unparalleled in any other country irrespective of the genetic tool implemented. These findings are consistent with the history of Turkey, which is situated at the meeting point of three continents and stands in the center of the Old World.

1. $(AT)_xT_y$ Patterns in Turkish People

By comparing data of the $(AT)_xT_y$ motif presented in this study with those published by other groups in the world, it is easily observed that Turkish people stand at a mid-point between Europeans and Asians (Table VI.1); this is in accordance with the conclusion drawn from the analysis of data of genetic variation at the apoB 3' hypervariable region in 80 chromosomes from Turkey (unpublished observations). The frequencies of the $(AT)_7T_7$ type of motif have an increasing gradient when moving from western to eastern Mediterranean; the highest values being recorded in the Balkan and in the eastern regions of Turkey (Table VI.1, Table V.12). A decline in the frequencies of this motif is observed when moving to eastern Asia. On the opposite, the $(AT)_9T_5$ motif exhibits a different picture since it occurs mostly in chromosomes of western Mediterranean ancestry (Table VI.1). Low frequencies for this motif are recorded elsewhere. In Turkey, the $(AT)_9T_5$ pattern was encountered in three individuals, two of whom, expectedly, are from the western and northern parts of the country.

Despite the limited sampling, the presence of a chromosome (1/5) carrying the $(AT)_{11}T_3$ arrangement in the Balkan area hints to a probable common presence of this motif in the region. This is further supported by the observation of this motif which occurs

in 50% of β-thalassemia chromosomes from the Black Sea region (Table V.11), close to the Balkan in terms of history and geography.

TABLE VI.1. $(AT)_xT_y$ patterns in randomly selected wild-type β-globin genes from various populations of the world (per cent of genes).

	UK[1]	France[2]	Italy[3]	Balkan	Turkey	Lebanon/Syria	Pakistan	China[4]
$(AT)_7T_7$	58	50	48.5	80	76.5	60	-	30
$(AT)_9T_5$	16	50	48.5	-	17.6	20	50	13
$(AT)_{11}T_3$	3	-	3	20	-	-	-	1
Others	23	-	-	-	5.9	20	50	56
n	48	12	64	10	13	7	2	83

*1: (Harding et al., 1997a), 2: (Perrin et al., 1998), 3: (Galanello et al., 1993), 4: (Zhou et al., 1995). **n**: indicates the number of chromosomes investigated.

2. β-Globin Mutations and Sequence Haplotypes in Turkish People

World data indicate that in regions where malaria and β-thalassemia are less common, a large diversity of β-thalassemia mutations is found. In contrast, in regions with long histories of exposure to malaria, only a small number of alleles account for most of the β-thalassemia genes. A comparison of the different regions of Turkey shows that the distribution of β-thalassemia mutant alleles differs within each geographical area with marked local variations in their prevalence and a decreased gradient of mutation numbers when moving from the East to the West of Anatolia (Figure VI.2). Eastern Anatolia is mainly a rugged region with many lofty ranges of altitudes higher than 5,000 meters, not helpful for the proper development of the malarial parasite. On the opposite, the landscape of Thrace and Western Anatolia is simpler and more suitable for the transmission of malaria. Thrace is composed of several low plateaus, and Western Anatolia includes many

depressed floors with many valleys leading from the Aegean Sea up to the borders of Central Anatolia (The New Encyclopaedia Britannica, 1991). Interestingly, this mutation/malaria gradient is also mirrored by the results of surveys conducted in Turkey during the past 20 years and that noted a regional β-thalassemia trait frequency gradient increasing from Eastern (3.4%) to Western Anatolia and Thrace (11%; Aksoy et al., 1980; Aksoy et al., 1985; Kürkçüoğlu et al., 1986; Bircan et al., 1993; Kocak et al., 1995).

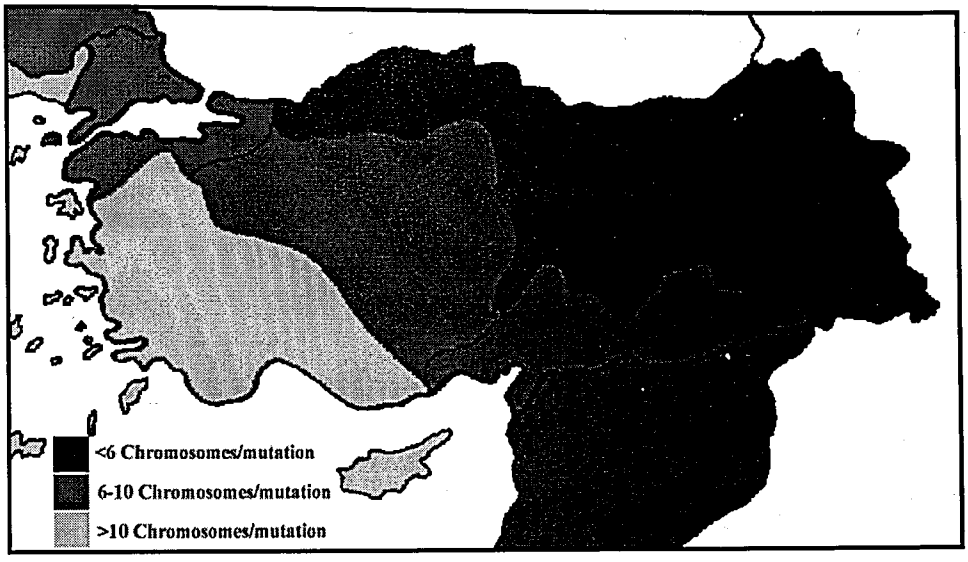


FIGURE VI.2. Differences in β-thalassemia mutation heterogeneity within Turkey and neighboring countries (adapted from data of: Kattamis et al., 1990; Petkov et al., 1990; Baysal et al., 1992; Adekile et al., 1994; El-Hazmi et al., 1995a; Traeger-Synodinos et al., 1998; Tadmouri, 1998; Tadmouri et al., 1998a).

At a detailed mutational level, several β-globin alleles described in Eastern Turkey are thought to be of Asian Indian origin (e.g., FSC-8/9, IVS-I-5, Cd15, and others). Through history this area acted as a spongy barrier separating the Caucasoid and Indian groups since their divergence 10,000-35,000 years ago (Cavalli-Sforza et al., 1996). When these groups witnessed the appearance of their specific types of β-thalassemia mutations,

these alleles leaked slowly from east and west through the rugged land of Eastern Anatolia to form the presently observed mosaic. Another notable feature of this region, especially in the towns of Tarsus, Siirt, Adana, Iskenderun, and Tokat, is the relatively high number of chromosomes with unidentified mutations. This may be explained by the possible presence of rare and novel lesions in these isolated areas. In contrast, some towns of the west, such as Çorlu, Edirne, Isparta, Fethiye, Manisa, and Zonguldak, mostly exhibited common β-thalassemia mutants.

Besides the $(AT)_xT_y$ motif, nine neighboring polymorphic nucleotides were investigated in this study by direct DNA sequencing. Different combinations of these variables make β-globin sequence haplotypes. Haplotype analysis in the present study exhibits a discontinuous gradient of sequence haplotype diversity observed in the regions of the country (Figure VI.3). Central Anatolia demonstrates a relative haplotypic homogeneity (Table V.11, Table V.12). This region is mainly a plateau that is bordered on the north, south, and east by high mountains that may have isolated Central Anatolians and conserved them from admixture with different migratory groups that passed by the coastal areas of Turkey (The New Encyclopaedia Britannica, 1991). On the other hand, the heterogeneous composition of subjects originating from the Black Sea region (Table V.11, Table V.12) may be explained by the fact that since 1849 AD an increasing flow of people from different parts of Anatolia took part towards mining centers in the area. This flow was fortified by the introduction of genetic elements from former Turkish territories around the Black Sea during the decline of the Ottoman Empire early in this century (The New Encyclopaedia Britannica, 1991). In between these two extremes lies the rest of the Turkish groups residents of Thrace, Marmara, Eastern, Southeastern, and Western Anatolia.

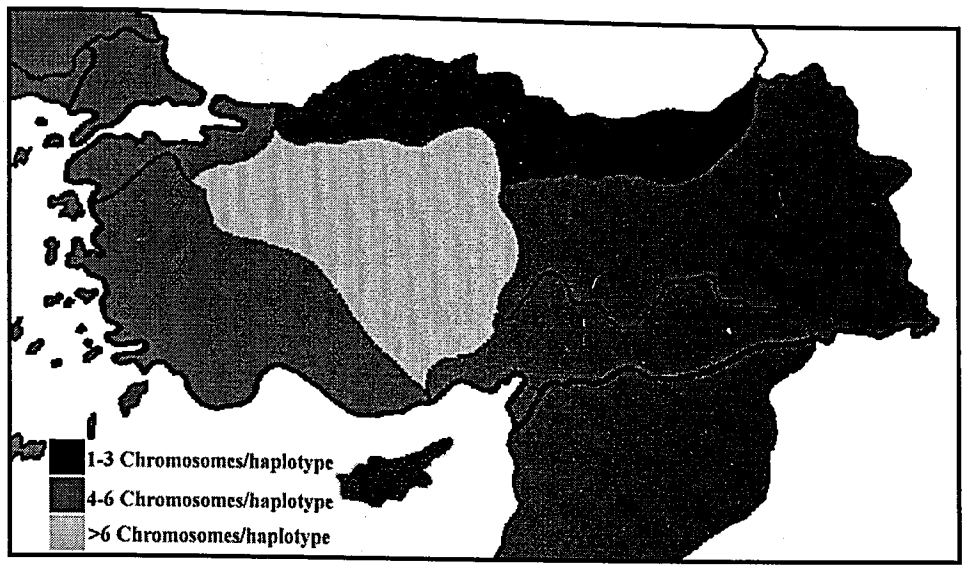


FIGURE VI.3. Differences in sequence haplotype heterogeneity in β-globin chromosomes from Turkey and neighboring countries.

D. History and Origin of Common β-Globin Alleles in Turkey

During the last 12 years numerous point mutations, including frameshifts and short deletions, have been identified in more than 19,459 chromosomes representing 47 nations of the world (Table VI.2). In Turkey, the application of gene cloning techniques, DNA sequencing, and southern blot analysis enabled the characterization of many of the mutations causing β-thalassemia (reviewed in Lanclos and Kutlar, 1986; Akar et al., 1987; Diaz-Chico et al., 1987; reviewed in Kazazian, 1990). However, the number of known mutations has almost doubled over the last few years because of the implementation of PCR to amplify DNA for direct sequence analysis (Diaz-Chico et al., 1988; Gürgey et al., 1989; Gonzalez-Redondo et al., 1989a; Gonzalez-Redondo et al., 1989b; Schnee et al., 1989; Aulehla-Scholz et al., 1990; Öner et al., 1990; Öner et al., 1991a; Başak et al., 1992a; Başak et al., 1992b; Özçelik et al., 1993; Başak et al., 1993; Atalay, et al., 1993; Jankovic et al., 1994; Altay and Başak, 1995; Nişli et al., 1997; Tüzmen et al., 1997). Research on β-globin mutations in Turkey at the Department of Molecular Biology and

Genetics at the Bogaziçi University went through two main periods. The first, extending from 1988 to 1992, characterized by the description of all the common β-globin gene mutations in Turkey along with a limited number of rare ones (Table V.7). The second period is characterized by the implementation of new PCR-based molecular techniques in the determination of gene mutations, such as DGGE followed by DNA sequencing (Tüzmen, 1995). This in turn limited the chances for an allele to remain undiagnosed and allowed the description of some rare and novel mutations; some of these rare mutations were described in the framework of this study (FSC-36/37, IVS-I-130, 290 bp deletion, IVS-II-654). Another achievement of this thesis is the compilation of all β-globin gene data collected since 1988 in a single, regularly, updated digital form. This allowed an easy means to investigate the distribution of β-globin gene mutations in various regions and towns of Turkey, thus helping to understand the possible population dynamics that formed the present picture of β-thalassemia in the country.

Along with the geographic distribution, haplotype analysis was conducted to allow the construction of the history of β-globin mutations, particularly how often their frequencies have been independently elevated and in which population they first occurred. Because the majority of Turkish β-thalassemia chromosomes are expected to occur on RFLP haplotype I (reviewed by Flint et al., 1993), the investigation of the RFLP system seemed unsatisfactory and analysis of β-globin sequence haplotypes was preferred since they (a) contain hypervariable genetic markers and (b) lie at the proximity of the β-globin gene (-530 bp from the 5' end), thus, being more informative for determining the chromosomal origin of a particular mutation.

1. Mutations Associated With Multiple Sequence Haplotypes

IVS-I-110 (G-A): In the analysis performed in the framework of this thesis, the IVS-I-110 (G-A) substitution, a severe β^+ mutation, is shown to be the most frequent allele in all regions of Turkey. However, notable differences in the frequency clines of this

mutation in the various regions investigated are observed (Table V.8). The highest frequencies were recorded in the central (52%) and western regions of Turkey (34-42%), whereas these figures decline to their lowest values in eastern Turkey (26-27%), sometimes absent in some towns such as Kars, Iskenderun, and Merzifon (Table V.7). The IVS-I-110 mutation is known to be an eastern Mediterranean defect (Figure VI.1); this fact may explain why the frequency of this mutation tends to be low in the eastern inland of Turkey.

TABLE VI.2. β-Globin mutation frequencies in some world populations.

(A) β-Globin mutations in Americans (compiled from: Economou et al., 1991; Gonzalez-Redondo et al., 1991; Roldan et al., 1997; Fonseca et al., 1998).

(B) β-Globin mutations in Europeans (compiled from: Milland et al., 1987; Amselem et al., 1988; Petkov et al., 1990; Laig et al., 1990; Kattamis et al., 1990; Dimovski et al., 1990; Oner et al., 1991b; Baysal et al., 1992; Ribeiro et al., 1992; Rosatelli et al., 1992; Faustino et al., 1992; Indrak et al., 1992; Ringelhann et al., 1993; Tamagnini et al., 1993; Coutinho-Gomes et al., 1993; Pagano et al., 1993; Boletini et al., 1994; Magro et al., 1995; Schiliro et al., 1995; Camaschella et al., 1995; Giambona et al., 1995; Benito et al., 1996; Rady et al., 1997; Vetter et al., 1997; Traeger-Synodinos et al., 1998).

(C) β-Globin mutations in Arabs (compiled from: Chehab et al., 1987; Rouabhi et al., 1988; Chibani et al., 1988; Novelletto et al., 1990; Fattoum et al., 1991; Nadifi et al., 1993; El-Kalla and Mathews, 1993; Hussein et al., 1993; Adekile et al., 1994; Bennani et al., 1994; Sadiq and Huisman, 1994; Waye et al., 1994; El-Hazmi et al., 1995a; El-Hazmi et al., 1995b; Hasounah et al., 1995; Filon et al., 1995b; El-Hashemite et al., 1997; Zahed et al., 1997; Tadmouri, 1998).

(D) β-Globin mutations in Western Asians (compiled from: Thein et al., 1988; Rund et al., 1991; Curuk et al., 1992; Merat et al., 1993; Varnavides et al., 1993; Tagiev et al., 1993; Adekile et al., 1994; Kuliev et al., 1994; Noori-Daloii et al.; 1994; Curuk et al., 1994; Filon et al., 1994; Filon et al.,

1995b; Nozari et al., 1995; Ahmed et al., 1996; Furuumi et al., 1998; Tadmouri et al., 1998a; Khan and Riazuddin, 1998).

(E) β-Globin mutations in Eastern Asians (compiled from: Chan et al., 1987; Thein et al., 1988; Zhang et al., 1988; Brown et al., 1989; Liu et al., 1989; Lie-Injo et al., 1989; Fucharoen et al., 1989; Yang et al., 1989; Laig et al., 1989; Huang et al., 1990; Thein et al., 1990; Lin et al., 1991; Varawalla et al., 1991; Brown et al., 1992; George et al., 1992; Tan et al., 1993; Chiou et al., 1993; Baysal et al., 1994; Adekile et al., 1994; Liang et al., 1994; Ng et al., 1994; Wakamatsu et al., 1994; Ohba et al., 1997; Verma et al., 1997).

(A)

Mutation	Type	Argentina	Brazil	Mexico	American Blacks
-88 (C-T)	+				21.1
-87 (C-G)	+	2.4			
-29 (A-G)	+				59.4
-28 (A-C)	+			10.5	
FSC-6 (-A)	0	1.2		10.5	0.8
FSC-11 (-T)	0			5.3	
Cd 24 (T-A)	+				2.3
IVS-I- -1/Cd 30 (G-C)/Hb Monroe	0				0.8
IVS-I-1 (G-A)	0	9.4		5.3	
IVS-I-1 (G-T)	0		4.3		
IVS-I-2 (T-C)	0				1.6
IVS-I-5 (G-C)	+			10.5	
IVS-I-5 (G-T)	+				1.6
IVS-I-6 (T-C)	+	5.9	18.6		
IVS-I-110 (G-A)	+	22.4	18.6	5.3	
Cd 39 (C-T)	0	47	54.3	26.3	
Cd 61 (AAG-TAG)	0				0.8
IVS-II-1 (G-A)	0	3.5			0.8
IVS-II-745 (C-G)	+	2.4			
IVS-II-848 (C-A)	+				0.8
IVS-II-849 (A-C)	0				1.6
IVS-II-849 (A-G)	0				2.3
FSC-106/107 (+G)	0				1.6
Poly A (AATAAA-AACAAA)	+				0.8
619 bp deletion	0			10.5	
1,393 bp deletion	0				1.6
Unknown and/or Others		5.9	4.3	15.8	3.1
Chromosomes studied		85	70	19	128

*+: β^+-thalassemia, 0: β^0-thalassemia.

(B)

Mutation	Type	POR	SPA	FRA	SAR	SIC	ITA	GER	CZE	HUN	YUG	ALB	GRE	BUL	CYP
-101 (C-T)	+					0.1	1.2							0.8	
-92 (C-T)	+					0.1									
-90 (C-T)	+	0.9													
-87 (C-G)	+				0.2	2.2	2.1	2.4			0.9		1.6	3.9	
-87 (C-T)	+							1.4							
-30 (T-A)	+												0.3		
-28 (A-G)	+												0.3		
5'-UTR +33 (C-G)	+												0.3		
Init. Cd (ATG-GTG)	0							0.7		12.5					
Init. Cd (ATG-ACG)	0							0.7			0.5				
Init. Cd (ATG-ATA)	0							0.7							
FSC-1 (-G)	0				0.1										
FSC-5 (-CT)	0					0.04	0.1				0.9	1.7	1.1	4.7	
FSC-6 (-A)	0		1.2		2.2	0.9	2.4	1.4			1.4		2.7	4.7	0.1
FSC-8 (-AA)	0		0.4			0.2	0.1				0.5		0.5	5.5	0.2
FSC-8/9 (+G)	0												0.3	5.5	
Cd15 (TGG-TGA)	0	10.8													
IVS-I- -3/Cd29 (C-T)	+										0.9				
IVS-I- -1/Cd30 (G-C)	0					0.4							0.5		
IVS-I-1 (G-A)	0	21.4	29.6	10.5	0.03	8.6	6.1	6.8	52.9	28.1	10.6	5.2	12.6	3.1	5.8
IVS-I-2 (T-A)	0					0.6	0.1								
IVS-I-2 (T-C)	0							1.4							
IVS-I-2 (T-G)	0					0.1									
IVS-I-5 (G-A)	+					0.1	0.2						0.3		
IVS-I-5 (G-C)	+					0.3	0.1	2				1.7	0.8		
IVS-I-5 (G-T)	+							3							
IVS-I-6 (T-C)	+	19.2	8.3	8.6	0.1	13.8	17.6	1		6.2	23.2	17.2	6.6	10.2	6.1
IVS-I-110 (G-A)	+	9.6	11.5	25.7	0.5	23.9	19.9	20	5.9		45.8	43.1	39.5	24.2	78.4
IVS-I-116 (T-G)	0					0.1									
IVS-I-130 (G-C)	0	0.6				0.1		1.7					0.3		
FSC-35 (-C)	0						0.1								
FSC-38/39 (-C)	0								2.9						
Cd39 (C-T)	0	36.8	36	41.9	95.7	34.4	38	34.8	2.9	31.2	3.7	22.4	15.6	21.9	2.4
FSC-44 (-C)	0					0.04	0.1					3.4	0.3		
FSC-51 (-C)	0									3.1					
FSC-76 (-C)	0				0.7	0.1									
FSC-82/83 (-G)	0							4.4	5.9						
IVS-II-1 (G-A)	0			1	0.03	1.7	4.2	0.7	11.8	6.2	0.9	1.7	1.9	1.6	
IVS-II-4/5 (-AG)	0?	0.3													
IVS-II-705 (T-G)	+		0.4					1.4							
IVS-II-745 (C-G)	+			2.9	0.4	6.1	4.3	3	5.9	3.1	1.4		6.3	10.2	5.4
IVS-II-844 (C-G)	+						0.1								
IVS-II-848 (C-A)	+										0.9		0.5		
Cd121 (G-T)/HbD$^{L.A.}$	0 dom.	0.6						0.7	8.8						
Cd126 (GTG-GGG)	0 dom.					0.04	0.2								
3'UTR +6 +1,480 C-G	+												2.5		
Poly A (TAA-TGA)	+										4.2	1.7	0.3	0.8	
25 bp (IVS-I 3') del.	0												0.3		
44 bp (Greek) deletion	0												0.5		
Unknown/Others		?	?	9.5		5.8	3.2	12.2	2.9	9.4	6.9	1.7	1.1	3.1	1.8
Chromosomes studied		323	253	105	3000	2274	1080	296	34	32	216	58	365	128	937

*POR: Portugal, SPA: Spain, FRA: France, SAR: Sardinia, SIC: Sicily, ITA: Italy, GER: Germany, CZE: The Czech Republic, HUN: Hungary, YUG: Former Yugoslavia, ALB: Albania, GRE: Greece, BUL: Bulgaria, CYP: Cyprus, Init.: Initiation, +: β^+-thalassemia, 0: β^0-thalassemia, Dom.: Dominant.

(C)

Mutation	Type	MOR	ALG	TUN	EGY	PAL	JOR	LEB	SYR	BED	KUW	SAR	UAE	YEM
-88 (C-T)	+							1.4						
-87 (C-A)	+				0.4									
-87 (C-G)	+						1.5	0.5						
-86 (C-G)	+							0.5						
-30 (T-A)	+		0.4	2.7				0.5						
-29 (A-C)	?		3.2											
Cap +1 (A-C)	+											1		
FSC-5 (-CT)	0			1.8	1.5	9.5	2.3	3.4					4.3	
FSC-6 (-A)	0	11.1	19	10.7	1.1	4.8	0.8					4.5		
FSC-8 (-AA)	0			0.9	0.7			3.4			7.3		4.3	
FSC-8/9 (+G)	0				0.4		2.3				3.1	1	8.6	
Cd 15 (TGG-TAG)	0												2.2	
Cd 22 (A-C)	?				5.5									
FSC-25/26 (+T)	0			0.9										
Cd 27 (G-T)/HbKnossos	+		0.4		0.4	1.2	2.3							
FSC-28 (-C)	0				0.4									
FSC-28/29 (-G)	0				1.5									
IVS-I- -3 or Cd 29 (C-T)	+							3.9						
IVS-I- -1/Cd 30 (G-A)	0		0.7					1						
IVS-I- -1/Cd 30 (G-C)	0			1.8				0.5					3.8	
IVS-I-1 (G-A)	0		11.3	0.9	12.4	19	6.2	9.7	16.7		7.3			
IVS-I-2 (T-A)	0		1.1											
IVS-I-2 (T-C)	0		2.8											
IVS-I-2 (T-G)	0			0.9										
IVS-I-5 (G-A)	+		0.7	0.9										
IVS-I-5 (G-C)	+		0.4				3.8	2.4			18.8	15.5	54	
IVS-I-6 (T-C)	+		3.5	6.2	15.8	7.1	6.2	6.8			7.3		3.8	
IVS-I-110 (G-A)	+		26.8	12.5	35.2	35.7	26.9	48.8	44.4	16.7		28.2	1.6	40
FSC-36/37 (-T)	0										11.5			
Cd 37 (G-A)	0				0.7	1.2	6.2							
FSC-37 (-G)	?												1.1	
Cd 39 (C-T)	0	16.7	26.4	27.7	1.1	15.5	1.5	1.4	11.1	66.7	7.3	12.4	5.4	
FSC-44 (-C)	0			3.6				1			1		1.1	
IVS-II-1 (G-A)	0				3.7	1.2	16.2	5.8	2.8		29.2	15.8	3.2	26.7
IVS-II-1 (G-T)	?							1						
IVS-II-745 (C-G)	+		0.7	4.7	5.5		10	3.4	16.7					
IVS-II-843 (T-G)	+		0.4											
IVS-II-848 (C-A)	+		0.4	0.9	4.4		1.5							
FSC-106/107 (+G)	0				0.7									
Poly A [AATAAA-A]	+					2.4								
25 bp (IVS-I 3') deletion	0							0.5		16.7	7.3	13.4	6.5	
290 bp deletion	0							1.4						
Unknown/Others		72.2	2.1	23.2	9.2	2.4	12.3	2.9	8.3			16.8		33.3
Chromosomes studied		72	284	112	273	84	130	207	36	12	96	291	185	15

*MOR: Morocco, ALG: Algeria, TUN: Tunisia, EGY: Egypt, PAL: Palestine, JOR: Jordan, LEB: Lebanon, SYR: Syria, BED: Bedouins of Arabia, KUW: Kuwait, SAR: Saudi Arabia, UAE: United Arab Emirates, YEM: Yemen, +: β^{+}-thalassemia, 0: β^{0}-thalassemia.

(D)

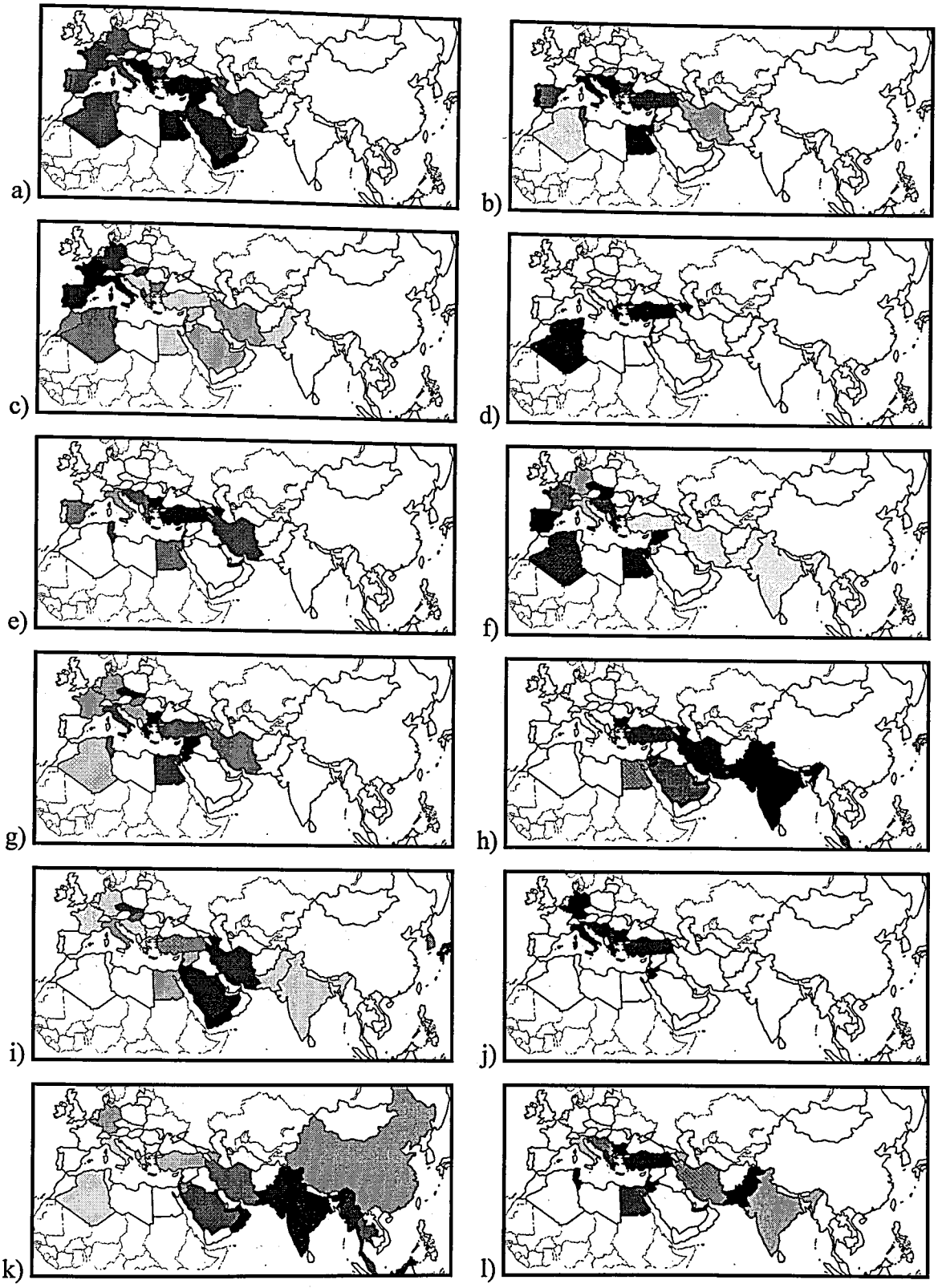
Mutation	Type	Israel	Kurds	Turkey	Azerbaijan	Russia	Iran	Pakistan	Maldives
-101 (C-T)	+	0.8	1.2	0.1			0.4		
-90 (C-T)	+	0.1							
-88 (C-A)	+		1.2		0.6		0.4		
-88 (C-T)	+	0.1					0.4	0.3	
-87 (C-G)	+			0.8					
-30 (T-A)	+	0.4		3.1	1.2				
-29 (A-C)	?				0.3				
-28 (A-C)	+	3.8	22.2	0.1	0.3				
Cap +1 (A-C)	+							1.5	
5'-UTR +22 (G-A)	+				0.9				
Initiation Cd (ATG-ATT)	0						0.4		
FSC-5 (-CT)	0	1.3		2.1	0.3		0.4	2.7	
FSC-6 (-A)	0	0.3		0.4					
FSC-8 (-AA)	0	3.1		5.4	29.4	11.1	2.5		
FSC-8/9 (+G)	0			1.3	6.2		4.2	24.3	
FSC-14 (+T)	?				0.3				
Cd 15 (TGG-TAG)	0	0.4		0.1	0.9			3.7	
Cd 15 (TGG-TGA)	0				0.9	11.1			
FSC-15 (-T)	0							0.1	
FSC-16 (-C)	0				0.6		0.4	2.3	
Cd 19 (A-G) Hb Malay	+						0.4		
FSC-20/21 (+G)	?	0.1							
FSC-22/23/24 (-AAGTTGG)	0			0.1	0.3				
Cd 26 (G-A) Hb E	+						0.4	0.9	
Cd 27 (G-T) Hb Knossos	+	0.3		0.1					
IVS-I- -3 or Cd 29 (C-T)	+				0.9				
IVS-I- -1/Cd 30 (G-A)/Hb Kairouan	0							0.7	
IVS-I- -1 or Cd 30 (G-C) Hb Monroe	0	0.3	1.2		0.6		1.7	2.3	
IVS-I-1 (G-A)	0	7		5	2.1	14.8	3	0.7	9
IVS-I-1 (G-T)	0							6.7	
IVS-I-1 (G-C)	?						0.4		
IVS-I-2 (T-C)	0					11.1			
IVS-I-5 (G-C)	+	1.5	1.2	1.1	1.8		6.8	26.5	74.3
IVS-I-5 (G-T)	+				0.3				
IVS-I-6 (T-C)	+	14.9	1.2	10	4.7		6.4		
IVS-I-110 (G-A)	+	26.8	11.1	39.2	12.4	3.7	5.1		
IVS-I-116 (T-G)	0			0.2					
IVS-I-128 (T-G)	+				0.3				
IVS-I-130 (G-A)	0			0.1					
IVS-I-130 (G-C)	0					7.4			
Cd 30 (G-C) [IVS-I-130 (+1)]	0							0.7	15.4
FSC-36/37 (-T)	0	0.7	2.5	0.1	1.2		0.8		
Cd 37 (G-A)	0	6.6							
Cd 39 (C-T)	0	11.2	9.9	3.8	1.8		14.8	0.1	
FSC-41/42 (-TTCT)	0							5.4	1.3
FSC-44 (-C)	0	5.9	29.6	1.3	1.8		1.3		
FSC-47/48 (+ATCT)	0							0.2	
FSC-74/75 (-C)	0			0.1					
FSC-82/83 (-G)	0				3.2				
FSC-88 (+T)	0							0.1	
IVS-II-1 (G-A)	0	7.4	2.5	4.6	17		15.7	0.8	
IVS-II-1 (G-C)	0						0.8		
IVS-II-654 (C-T)	+			0.1		3.7	0.4		
IVS-II-745 (C-G)	+	2.7	1.2	5	0.9		3.4		
IVS-II-848 (C-A)	+			0.4					
FSC-106/107 (+G)	0	0.1							
Cd 114 (T-C) or $Hb^{DurhamN.C.}$/$Hb^{Brescia}$	0 do					22.2			
Cd 121 (G-T) HbD L.A	0 do	0.1							
FSC-124 (-A)	0 do					3.7			
FSC-124...126 (+CCA)	0 do					3.7			
FSC-126...131 (-17 bp)	0 do							0.1	
3'-UTR +1,565 to +1,577 (-13 bp)	+?			0.1					
Poly A (AATAAA-AATGAA)	+			0.5					
Poly A (AATAAA-AATAAG)	+	1.7	12.4						
25 bp (IVS-I 3') deletion	0						2.1	0.1	
290 bp deletion	0			0.1					
619 bp deletion	0							8.8	
Unknown and/or Others		2.4	2.5	14.3	9.4	7.4	27.1	0.3	
Chromosomes Studied		713	81	796	340	27	236	1818	78

*Kurds: Kurdish Jews in Israel, +: β^{+}-thalassemia, 0: β^{0}-thalassemia, Do.: Dominant.

(E)

Mutation	Type	INDI	CHI	BUR	THA	MAL	SIN	INDO	TAI	KOR	JAP
-88 (C-T)	+	0.6				0.4					
-86 (C-G)	+				0.3						
-31 (A-G)	+										15.6
-29 (A-G)	+		0.6		3.9	0.4			0.6		
-28 (A-G)	+		10.8	3.1	1.9	4.2	8.2		9.6		0.3
Cap +1 (A-C)	0	0.8				1.1					
Initiation Cd (ATG-GTG)	0										3.8
Initiation Cd (ATG-ACG)	0										0.7
Initiation Cd (ATG-AGG)	0						0.8			38.9	1.7
Initiation Cd (ATG-ATA)	0										1.7
Initiation Cd (ATG-ATC)	0										0.7
FSC-5 (-CT)	0	0.2									
FSC-8/9 (+G)	0	14					0.8				0.7
FSC-14/15 (+G)	0		0.1								
Cd 15 (TGG-TAG)	0	0.6				0.8		6.7			1.7
Cd 15 (TGG-TGA)	0										1
FSC-15 (-T)	0	0.05									
FSC-16 (-C)	0	1.2					0.8				
Cd 17 (A-T)	0		14.6	4.4	24.5	3.8	9	1.7	14.7	22.2	0.3
Cd 19 (A-G) Hb Malay	+				1.6	7.2	0.8				
Cd 24 (T-A)	+										0.7
Cd 26 (G-A) Hb E	+					17.7					
FSC-27/28 (+C)	0						1.5		2.6		
FSC-28/29 (-G)	0										0.3
IVS-I- -1/Cd 30 (G-A)/Hb Kairouan	0	1									
IVS-I- -1/Cd 30 (G-C)/Hb Monroe	0	0.6						1.7			
IVS-I-1 (G-A)	0	0.05						1.7			
IVS-I-1 (G-T)	0	13.7	1.4	25.8	1	4.9		10.2	1.3		1
IVS-I-1 (G-C)	?						0.8	54.2			
IVS-I-2 (T-C)	0					0.4					
IVS-I-5 (G-C)	+	34.8	2	20.4	5.2	24.9	9				
IVS-I-128 (T-G)	+								0.6		
IVS-I-130 (G-A)	0									5.6	
IVS-I-130 (G-C)	0									5.6	0.3
FSC-33/34 (-GTG) Hb Korea	0 dom									5.6	
Cd 35 (C-A)	0										0.3
FSC-35 (+A)	?				1.3						
FSC-35 (-C)	0					2.3	0.8	1.7			
FSC-36/37 (-T)	0	0.05									0.3
Cd 39 (C-T)	0										0.3
FSC-40 (-G)	0										6.9
FSC-41/42 (-TTCT)	0	9.2	43	15.1	44.8	18.9	37.3	1.7	30.1	5.6	0.3
FSC-42/43 (+G)	0										0.3
FSC-42/43 (+T)	0										
Cd 43 (G-T)	0		0.4				0.8				
FSC-44 (-C)	0	0.1									
FSC-47/48 (+ATCT)	0	0.4									
FSC-53/54 (+G)	0										0.7
FSC-71/72 (+A)	0		6		1	1.1			0.6		
FSC-84/85 (+C)	0										1
FSC-84/85/86 (+T)	0										2.1
FSC-89/90 (-GT)	0									5.6	
Cd 90 (G-T)	0										16.7
IVS-II-1 (G-A)	0	0.2								5.6	11.5
IVS-II-5 (G-C)	+		0.1								
IVS-II-654 (C-T)	+		15.2	1.8	7.7	7.9	25.4	11.9	46.2		13.9
IVS-II-848 (C-G)	+										1
IVS-II-850 (G-A)	0										0.7
IVS-II-850 (G-T)	0										0.3
Cd 121 (G-T) HbD L A	0 dom									5.6	3.1
FSC-123 (-A) Hb Makabe	0 dom										0.3
FSC-125 (-A)	0 dom										0.3
FSC-127/128 (-AGG) Hb Gunma	0 dom										3.8
Poly A (AATAAA-AATAGA)	+	.				0.4	1.5				
619 bp deletion	0	20.1									
3.485 bp (Thai) deletion	0				0.3						
Unknown and/or Others		2	5.6	29.3	6.1	3.8	3	8.5			4.5
Chromosomes Studied		1983	732	225	310	265	134	59	156	18	288

*INDI: India, CHI: China, BUR: Burma, THA: Thailand, MAL: Malaysia, SIN: Singapore, INDO: Indonesia, TAI: Taiwan, KOR: Korea, JAP: Japan, Init.: Initiation, +: β^+-thalassemia, 0: β^0-thalassemia, Dom.: Dominant.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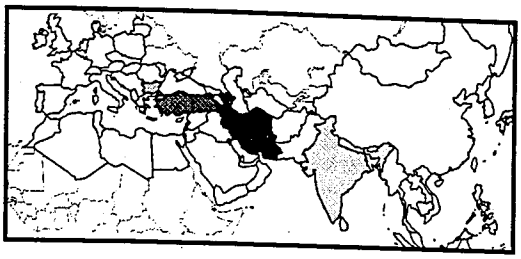
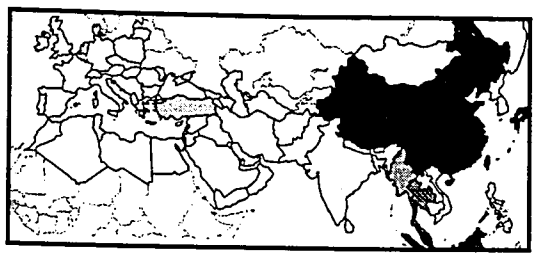
m)  n) 

FIGURE VI.4. World distribution of some β-thalassemia mutations. (a) IVS-I-110 (G-A), (b) IVS-I-6 (T-C), (c) Cd39 (C-T), (d) -30 (T-A), (e) FSC-8 (-AA), (f) IVS-I-1 (G-A), (g) IVS-II-745 (C-G), (h) FSC-8/9 (+G), (i) IVS-II-1 (G-A), (j) -87 (C-G), (k) IVS-I-5 (G-C), (l) FSC-5 (-CT), (m) FSC-36/37 (-T), and (n) IVS-II-654 (C-T). Darker regions indicate higher frequencies for the allele in the corresponding countries (adapted from data in Table VI.2).

In contrast to the relative homogeneous mutation/haplotype associations present in many Mediterranean and European populations (Table VI.3), the IVS-I-110 (G-A) mutation is related to six sequence haplotypes in Turkish people (Table V.17). At first sight, this result may infer that the mutation could have multiple origins. However, the contiguous distribution of the mutation in the Mediterranean area (Figure VI.1) points to a relatively recent origin postdating the racial divergence between Indian and Caucasoid groups (10,000-30,000 years; Cavalli-Sforza et al., 1996). In the Eastern Mediterranean, six haplotypes are associated with the IVS-I-110 (G-A) mutation (Table VI.3). The chances for the mutation to recur on six haplotypes during such a relatively short evolutionary scale and in a confined area are very little. Approximately 87% of the Turkish IVS-I-110 chromosomes occur on sequence haplotype HT1, parsimonially very close to the ancestral haplotype suggested by Trabuchet and colleagues (1991a; Table V.15) and possibly present in the area since a long time. That Turkey and Lebanon show a variety of sequence haplotypes for IVS-I-110 (G-A) chromosomes (Table VI.3), may be an indication for a founder effect and selection of the mutation in the Eastern Mediterranean.

TABLE VI.3. Percentages of β-globin sequence haplotypes associated with IVS-I-110 (G-A) β-thalassemia chromosomes in 12 countries (Perrin et al., 1998; Tadmouri et al., 1999c).

Haplotype	Spain	Algeria	Sardinia	Czech	Macedonia Greece Cyprus	Bulgaria	Turkey	Syria	Lebanon	Jordan	Total
HT1	88.9	70	100	100	100	87.5	87.7	80	70	100	85.1
HTR	11.1						4.9	20	25		7.8
HT2		30									2.0
HT3							3.7				2.0
HT4							2.5				1.3
HT5							1.2				0.6
HT6									5		0.6
HT7						12.5					0.6
n	9	10	4	3	5	8	81	10	20	4	154

*n: Number of chromosomes

Both, frequency and haplotype data hint for a probable origin of the IVS-I-110 mutation somewhere in the inner Anatolia on haplotype HT1, probably commonly found in the ancestral population that inhabited this area. When the inhabitants of the most western lobe of the Fertile Crescent in the south of Asia Minor started practicing agriculture in the early Neolithic ages (8,000-9,000 years ago; Diamond, 1997), thus hosting malaria, the mutant gene was selected and reached significant frequencies. Subsequently, the massive migrations out of Anatolia scattered the gene in Eastern Europe and some parts of the Mediterranean. Later, some other populations such as the Phoenicians (2500-332 BC), Greeks (1100-215 BC), Carthaginians (800-146 BC), Persians (558 BC-330 AD), Romans (27 BC-491 AD), Seljuks (900-1200 AD), Mamlukes (1250-1517 AD), and Ottomans (1300-1923 AD), may have taken over the task of further spreading and increasing the frequency of the gene and its haplotype (HT1) in the Mediterranean basin through increases in population densities, migrations, and further malarial selection (Cavalli-Sforza et al., 1996; Bjorklund et al., 1980). This might explain why HT1 is tightly linked (85.1%) to the IVS-I-110 mutation in the 12 populations analyzed (Table VI.2). At this time, the IVS-I-110 mutation introduced by immigrants may have spread by recombination and gene conversion mechanisms, that occur more frequently than mutations, onto new locally common, haplotypes. This mechanism may explain the shift of the β-thalassemia IVS-I-110 mutation from a chromosome carrying haplotype HT1 to another carrying HTR. HTR

is a commonly observed haplotype in association with rare mutations (Table V.17), thus recent ones, and many wild-type β-globin alleles (27.9%) from 12 populations analyzed (χ^2=0.08, P=77.7; Tadmouri et al., 1999c). IVS-I-110 β-globin genes present on the HTR haplotype are only seen in Lebanon, Syria, Turkey, and Spain. The distribution pattern of this association may indicate that it first occurred on a Middle Eastern chromosome and spread to other countries by migrations. Turkish IVS-I-110 chromosomes, carrying this haplotype, are mostly seen in Southeast Anatolia, neighboring Syria. The presence of IVS-I-110 alleles carried on haplotype HTR in Spain may be explained by the fact that genetic elements, mainly of Eastern Mediterranean origin (i.e., the Phoenicians-Carthaginians and Umayyads), ruled the Iberian Peninsula for ~1000 years (Bjorklund et al., 1980) and this may have contributed to the gene pool of the Spanish population. Later, IVS-I-110 genes, carried on haplotypes HT1 and HTR, were transferred by similar recombination and/or microsatellite slippage events to other more rarely encountered haplotypes, such as HT3 and HT4, common in Lebanon, Turkey, Cyprus, Macedonia and Spain. The association with haplotype HT7 (common in normal individuals from Greece, Turkey, and Cyprus) in an immigrant Turk from Bulgaria also refers to the same mechanism.

IVS-I-6 (T-C): The IVS-I-6 mutation is equally distributed in the different Turkish regions (Table V.8, Table V.9) and mostly occurs on sequence haplotype HTR (Table V.17) as in the case of all IVS-I-6 chromosomes from Algeria and Greece (Perrin et al., 1998). The mutation occurs almost at equal frequencies in the Mediterranean (Table VI.1). The highest frequencies for this allele are recorded in Italy, Yugoslavia, and Portugal. This might explain the slight impact of this mutation in western regions of Turkey. Six RFLP haplotypes are associated with this mutation in different Mediterranean countries, five of which are described in Lebanese and Turkish people (Flint et al., 1993), hence demonstrating an older origin for this mild mutation in the Eastern Mediterranean. Besides HTR, two IVS-I-6 chromosomes from Turkey are associated with an exclusive type of sequence haplotype (HT15; Table V.17), that is only at one parsimonial distance from the 'ancestral' HbS Benin sequence haplotype (Trabuchet et al., 1991a; Table V.15). Analysis of more IVS-I-6 chromosomes from other Mediterranean populations is required to clarify the probably long and complex history of this mutation.

Cd39 (C-T): High frequencies for the Cd 39 (C-T) mutation are observed in Turkish citizens living in the Marmara region as well as in those originating from the Balkan countries, namely Greece, Bulgaria, Romania, former Yugoslavia, and Albania. Less frequent incidences were observed elsewhere in Turkey (Table V.8). This may be explained by the fact that this mutation is primarily a western Mediterranean abnormality (Figure VI.1). Chromosomes carrying the Cd 39 mutation in Turkey equally presented two sequence haplotypes (HT1 and HTR), similar to what is seen in other Mediterranean populations (Perrin et al., 1998). Analysis of 22 Cd39 chromosomes from Algeria demonstrated the association of this mutation with four sequence haplotypes (Perrin et al., 1998). Studies of RFLP haplotypes associated with this allele showed its association with 14 haplotypes, six of which were described in Tunisians (Flint et al., 1993). These two independent observations are strong arguments for a Northwestern African origin of this mutation.

-30 (T-A): The rare β^+ promoter T-A mutation at position -30 is mostly seen in the eastern and northern regions of Turkey (7-9%; Ordu, Mardin, Iskenderun, Batman, Kars, Siirt, Diyarbakır, and Tokat) and is associated with two sequence haplotypes: HTR (90%) and HT1 (10%; Table V.17). The association of the -30 mutation with the 'ancestral' sequence haplotype HT1 in combination with its high frequency in Turkey, might favor a considerably old age for this allele as well as a Turkish origin. Low frequencies for this mutation in the Mediterranean may be explained by a recent spread out of Anatolia (Figure V.1). The presence of this mutation in North Africa (Tunisia: 6.8%) could be linked to the Ottoman influence in the region during the 16^{th}-19^{th} centuries.

2. Mutations Associated With Single Sequence Haplotypes

Of the 22 mutations analyzed, 18 mutations are each associated with single sequence haplotypes (Table V.17). Five of these mutations each has its private genetic background, the remaining 13 mutations share similar sequence haplotypes. Hence, this type of mutation/haplotype association demonstrates that each of these mutations did not witness recombination events since it was introduced in the Turkish population. For that reason, the origin of these mutations can be assigned by (a) comparing their frequencies in Turkey with those in other countries, and/or (b) the world distribution of the haplotypes carrying them.

The FSC-8 (-AA), IVS-I-1 (G-A), IVS-II-745 (C-G), IVS-II-848 (C-A), FSC-74/75 (-C), and Cd27 (G-T) mutations share the same sequence haplotype HTR. As in Turkey, HTR is the most common sequence haplotype in many wild-type chromosomes of Mediterranean origin (Tadmouri et al., 1999c). The restricted geographical distributions of the above mutations mostly to the Mediterranean favor a recent unicentric origin for each of them, the origins being confined to the areas where they occur most frequently:

FSC-8 (-AA): The dinucleotide deletion at codon 8, originally detected in a Turkish patient (Orkin and Goff, 1981), occurs mostly in Eastern Anatolia (8.3%), mainly because of the presence of this mutation in Azerbaijan at high frequencies, too (Table VI.1, Figure, V.1). This distribution argues for a probable Seljuk origin of this mutation. In 1071 A. D., the Seljuk Turks opened Anatolia and today's Azerbaijan to four million Turkish settlers, hence pushing the Byzantines to the western coastal areas and assimilating those who remained (The New Encyclopaedia Britannica, 1991). The importance of the FSC-8 (-AA) lies in the fact that it was the first mutation the occurrence of which in Ottomans was directly proven through the molecular analysis of the archeological remains of an Anatolian child (16^{th}-19^{th} centuries; Filon et al., 1995a). The recent general pattern of migration from the rural eastern provinces towards the coal and metallurgical districts in the Northwest

may explain the relatively high presence of this mutation in the Marmara region (Table V.8).

IVS-I-1 (G-A): The G-A substitution at IVS-I-1 is mainly observed in Western Mediterranean and Central Europeans and is less encountered in Africans and Asians (Figure VI.1). This distribution pattern explains the occurrence of this allele in immigrant Turks from the Balkan area and in the western and southern regions of Turkey (Table V.8).

IVS-II-745 (C-G): The severe β^+ mutation at IVS-II-745 (C-G) is mainly confined to the Turkish coastal areas extending from the Black Sea, Marmara Sea, Aegean Sea to the Mediterranean Sea (Table V.8). The presence of this allele in Bulgaria, Syria, and Jordan (Figure VI.1) reflects the route that was followed during the 12^{th} and 13^{th} centuries by the large troops of Crusaders heading to Jerusalem, who started in Eastern Europe and passed by the coastal areas in Turkey and may have introduced new genetic elements in these parts of the country (Tadmouri 1998a).

IVS-II-848 (C-A), Cd 27 (G-T), and FSC-74/75 (-C): The occurrence of the IVS-II-848 (C-A) mutation in Tunisia, Egypt, and Jordan (Table VI.1) points to a Northeastern African origin. This could explain the limited presence of this mutation in Cyprus and Turkey (Tadmouri et al., 1998a). The restriction of the Cd 27 (G-T) mutation (Hb Knossos) to Turkey, Israel, Palestine, Jordan, and Egypt (Table VI.1) reflects an Eastern Mediterranean origin for this mutation. However, the novel FSC-74/75 (-C) mutation, reported once in a single Turkish family (Basak et al., 1992b), is most probably autochtonous for Turkey.

The FSC-8/9 (+G), Cd15 (G-A), HbE Saskatoon, IVS-I-130 (G-A), two IVS-I-110 chromosomes, and some β-thalassemia chromosomes with undefined mutations are observed on sequence haplotype HT4 (Table V.17). This haplotype is observed in β-

thalassemia and wild-type chromosomes from Spain, Algeria, Cyprus, Turkey, and Lebanon (Tadmouri et al., 1999c). World distribution of the FSC 8/9 (+G) mutation (Figure VI.1) mirrors the Great Silk trade route that extends from Xian in China to Iran. For this reason, the FSC-8/9 allele is restrictedly present in East Anatolia (Table V.8). The same path might have been followed by the Cd 15 (G-A) mutation. Two reasons support this assumption: (a) this mutation shares the same sequence haplotype with FSC-8/9, and (b) it occurs in patients of Eastern Asian ancestry, mainly Indonesians and Pakistanis (Table VI.1). However, the rare HbE Saskatoon occurs in a number of individuals from spatially remote countries (Vella et al., 1967; Gonzalez-Redondo et al., 1987; Gürgey et al., 1990; Igarashi et al., 1995; Tadmouri et al., 1998a), hence multicentric origins for this mutation are a more probable option. As for the IVS-I-130 (G-A) mutation, a Northeastern African descent for this allele was recently suggested (Tadmouri et al., 1999b).

IVS-II-1 (G-A): The IVS-II-1 allele occurs on the 'private' sequence haplotype HT17 (Table V.17). In Turkey, this mutation occurs in all regions with a slightly increased impact in the southern coastal areas. In the world, this allele is observed at variable frequencies in many countries. High frequencies are recorded in the Czech Republic, Jordan, Saudi Arabia, Yemen, Kuwait, Azerbaijan, Iran, and Japan (Table VI.1, Figure VI.1). Local sequence haplotypes from most of the countries where the mutation occurs are not known. Data from RFLP haplotypes indicate the association of this mutation with four backgrounds in various populations. This observation lead Chifu et al. (1992) to suggest multiple origins for this mutation. Analysis of sequence haplotypes for this allele in other world populations should shed more light on this subject.

-87 (C-G): The highest frequencies for the -87 (C-G) promoter mutation are recorded in Northern Mediterranean countries (Figure VI.1), especially in Bulgaria where 4% of β-thalassemia chromosomes carry this mutation (Table VI.1). In Turkey, this mutation is almost confined to the Black Sea region, specifically to the town of Cankiri (data not shown). The sequence haplotype associated with the -87 (C-G) mutation is restricted to normal β-globin chromosomes from Turkey, Cyprus, Algeria, and Spain

(Tadmouri et al., 1999c). Both sequence haplotype and frequency distribution data of this mutation favor a local origin for the mutation in Black Sea populations.

IVS-I-5 (G-C): The IVS-I-5 (G-C) mutation is one of the most common β-thalassemia alleles in Southern Asia especially in the region extending from Saudi Arabia to Malaysia (Figure VI.1). World data indicate the association of this allele with nearly a dozen of RFLP haplotypes, each of which is dominant in various populations (Flint et al., 1993), thus demonstrating a long and complex history for this allele. Turkish IVS-I-5 (G-C) chromosomes occur on the 'private' sequence haplotype HT16 (Tadmouri et al., 1999d). Analysis of Southern Asian chromosomes should shed more light on the sequence haplotype background(s) of this common β-thalassemia allele.

IVS-I-116 (T-G): The IVS-I-116 mutation is a rare β-thalassemia allele that is confined to some families in Turkey, Greece, and Sicily (Huisman et al., 1997). This allele is associated with the sequence haplotype HT1. These two observations may be indications for a probable recent eastern Mediterranean origin of this mutation.

The micro-deletion of the CT nucleotides at codon 5 (frequent in Palestine, Turkey, Bulgaria, Tunisia, and UAE) and HbD Los Angeles (seen in sporadic cases in the world) occur at haplotype HT7. This sequence haplotype is observed in Bulgarian and Algerian β-thalassemia alleles (Perrin et al., 1998), and is restricted to Greek, Cypriot, and Turkish wild-type chromosomes (Table V.18). These data might favor an Eastern Mediterranean origin for the FSC-5 and HbD Los Angeles mutations. The presence of these mutations in patients originating from Southeastern Anatolia (e.g., FSC-5 in Antakya and Gaziantep) can be a further indication for its introduction to Turkey through the southern parts of the country.

HbS: Most of the HbS genes in Turkey occur in an Arab-speaking group who lives in Southeastern Anatolia (Koçak et al., 1995). It is commonly known that HbS genes in Turkey are of the 'Benin' type that is associated with specific RFLP (Benin) and sequence haplotypes (Table V.15; Aluoch et al., 1986; Trabuchet et al., 1991a). However, the uncommon presence of a Turkish HbS chromosome on haplotype HTR (Table V.17) and another carrying the $(AT)_7T_7$ motif (Table V.10) might demonstrate a new variant for this hemoglobinopathy that is specific for Turkey. Extensive analysis of Turkish HbS chromosomes and those from neighboring countries would clarify this issue.

3. Rare Mutations First Described in Turkey

a. FSC-36/37 (-T)

Of the 180 β-thalassemia mutations described in the literature so far, some are said to be unique for Kurdish Jews (Rund et al., 1991; Huisman et al., 1997). However, subsequent molecular analysis of β-thalassemia patients from different countries revealed the presence of some of these mutations elsewhere as well. For example, the FSC-44 (-C), the Poly A (AATAAA-AATAAG), and the TATA box A-C mutation at position -28 have been described in 0.3-1.2%, 0.5%, and 0.1-0.4% of β-thalassemia cases in Turkey, respectively (Tadmouri et al., 1998a). To these we added the additional finding of the FSC-36/37 (-T) mutation in a Turkish β-thalassemia patient originating from the city of Adana.

To define the possible origin of the FSC-36/37 mutation in the family analyzed, we did a thorough investigation of the origin of the heterozygous mother (Table V.3). This revealed that at least three of her grandparents, deceased at the time of sampling, were from the city of Muş (in Eastern Anatolia) and had a Kurdish origin. A search of the literature for other records of the FSC-36/37 mutation uncovered its first description in an Iranian

individual from Masjed Solaiman in the northwestern province of Khuzistan in Iran (Kazazian, 1990). Later, the mutation was described in, at least, five Kurdish Jews (Rund et al., 1991; Filon et al., 1994), four chromosomes from Azerbaijan (Çürük et al., 1992; Tagiev et al., 1993; Kuliev et al., 1994), 11 chromosomes from Kuwait, a single Indian individual (Adekile et al., 1994), and in some Iranian families (Kim et al., 1994; Nozari et al., 1995). Recently, Jankovic and colleagues (1994) reported the same mutation heterozygously inherited along with the rare Cd30 (G-C) mutation in a thalassemic child of Turkish nationality who lived in southern Bulgaria (Figure VI.1). In some of these studies, haplotype analysis was performed and the FSC-36/37 mutation was found exclusively on haplotype I, reflecting the same ancestral background for patients carrying this mutation (Kazazian, 1990; Rund et al., 1991; Jankovic et al., 1994).

From these results it can be concluded that the FSC-36/37 (-T) mutation could have arisen somewhere in the region, including northern Iran and the remote mountainous region of Eastern Anatolia. These regions are mainly inhabited by a rural population of nomadic Kurdish tribes related to the Persians by language and genotype. Thus the mutation might have followed two migration routes during the time of the Ottoman Era, the first being to Azerbaijan and the second, probably a more recent one, passing through southeastern Anatolia and reaching southern Bulgaria (Tadmouri et al., 1997).

b. IVS-II-654 (C-T)

Analysis of a great number of β-globin genes from various countries of the world demonstrated that nearly 180 alleles are responsible for the occurrence of β-thalassemia worldwide (Huisman et al., 1997). Yet population studies indicate that probably only 13 alleles account for more than 80% of the β-thalassemic chromosomes in the world. The most common mutations tend to be geographically the most widespread, and presumably also the oldest. Of these mutations, the substitution of C-T at position IVS-II-654 of the β-globin gene (Cheng et al., 1984) is described as one of the most common molecular lesions

leading to β-thalassemia in Chinese populations (Figure VI.1). While screening for rare mutations in some of the Turkish chromosomes with uncharacterized molecular defects, we came across the IVS-II-654 (C-T) mutation in three generations of a family who had recently migrated from Xanthe, Greece (western Thrace), to Turkey.

To identify the origin and the chromosomal background of the IVS-II-654 mutation, a detailed family history was collected and analysis of polymorphic nucleotides located near the 5' end of the β-globin gene was undertaken. The Balkan (western Thrace) origin of the studied family was confirmed in at least five generations (about 200-250 years) with no known record of migration or consanguinity. Thus, if migration was to explain the occurrence of this mutation, this event would have taken place a long time ago (>250 years ago) and would be expected to result in a wider distribution of the allele in at least several other families in western Thrace. However, this is not the case because extensive analyses of β-thalassemia genes from Balkan countries have not revealed the presence of the IVS-II-654 mutation (Huisman, 1990). In addition, this mutation has not been reported in the region between the Mediterranean and China (Akbari, personal communication, 1997; Mehdi, personal communication, 1997; Varawalla et al., 1991; Figure VI.20).

Chromosomes bearing the IVS-II-654 mutation in the studied Turkish family were shown to carry the $(AT)_9T_5$ type of microsatellite and the ACATCCCCA arrangement (i.e., HT2). Chinese IVS-II-654 chromosomes, however, are strongly linked to the $(AT)_8T_5$ motif; the remaining elements of the haplotype were not reported (Zhou et al., 1995). To the best of our knowledge, sequence haplotype HT2 has thus far been described in only three IVS-I-110 (G-A) β-thalassemia patients from the Oran region of Algeria (Perrin et al., 1998), in one individual from western Thrace, and in several β-globin genes from the United Kingdom (Fullerton et al., 1994; Harding et al., 1997a), Spain, Algeria, and France (Perrin, personal communication, 1998). This line of evidence suggests a western Mediterranean and thus an independent origin for the IVS-II-654 mutation occurring in our study family (Tadmouri et al., 1999a). Confirmation and refinement of this finding would

need a screening of β-globin chromosomal backgrounds in countries extending from the Balkan region to East Asia.

c. IVS-I-130 (G-A)

Of the 180 β-thalassemia mutations identified so far, only three occur at the acceptor site of the IVS-I of the β-globin gene; namely, IVS-I-128 (T-G; Wong et al., 1989), IVS-I-130 (G-C; Öner et al., 1990; Yamamoto et al., 1992), and IVS-I-130 (G-A; Deidda et al., 1990). To our knowledge, the latter mutation was described only once in an Egyptian patient from the city of Mansoura (Deidda et al., 1990). This patient carries the IVS-I-130 (G-A) lesion along with the Mediterranean IVS-I-6 (T-C) β-thalassemia mutation. The patient we describe in the present report has also heterozygously inherited the same two mutations. Such a coincidence allowed us to compare the phenotypes of the two patients and conclude that their hematological data were almost similar, with the exception that the Egyptian patient (Deidda et al., 1990) had an increased level of HbA_2 (4.2%) whereas our patient's HbA_2 is 1.8%. Both patients, however, had remarkably increased levels of HbF (40 and 59%).

To deduce a possible origin for the IVS-I-130 (G-A) mutation, we have characterized sequence polymorphisms in the DNA of our patient and her family. The IVS-I-6 (T-C) mutation was found to be associated with the expected reference sequence haplotype (HTR) of Poncz et al. (1983), frequently observed in wild-type β-globin chromosomes from the Mediterranean (Perrin et al., 1998). The IVS-I-130 (G-A) mutation, on the other hand, was present on the less common sequence haplotype HT4 (Table V.16, Table V.18). The haplotype HT4 seems to be mainly associated with the Algerian IVS-I-2 (T-C) mutation (Bouhass et al., 1994; Perrin et al., 1998). The description of the IVS-I-130 mutation in an Egyptian patient (Deidda et al., 1990) could be a good indication favoring a Northeastern African origin. It is known that many Turkish families originating from Albania settled and ruled in Egypt until the end of the Ottoman domination (1805-1882

AD; The New Encyclopaedia Britannica, 1991). The mutation may have well migrated into Albania during this time.

E. Nuclear Autosomal DNA Markers Versus Others

Over the last few decades, human ancestry studies relied on the analysis of nuclear RFLPS, microsatellite changes, mitochondrial DNA (mtDNA), and Y chromosome (Y-DNA) markers. Most of the published reports focused more on the initial events during human evolution, that is, the origin of modern humans (Jorde et al., 1998). Although the period directly preceding classical historical recordings is thought to have contributed most to the present variability observed among world populations, a limited number of molecular-based studies discussed this issue (Cavalli-Sforza et al., 1996; Cavalli-Sforza, 1998). Some of these studies demonstrate that the portion of genetic variation of Y-DNAs within populations is significantly smaller than in nuclear autosomal and mtDNA (Cavalli-Sforza, 1998). These markers cannot be applied at a sub-regional level since preliminary data demonstrate that Turkish people are rather a homogenous group (Jarjanazi, unpublished observations). On the contrary, the high mutation rates of mtDNA exhibit a flattened geographic distribution of mtDNA variants (Horai et al., 1995). The variable sequence 5' to the β-globin gene overcomes all these obstacles since it combines the characteristics of different sequence variation mechanisms. The sequence includes single nucleotide polymorphisms, informative about long historical events, and microsatellite changes and recombination mechanisms, informative about more recent evolutionary events. In contrast to separately analyze the maternal (mtDNA) or the paternal (Y-DNA) lineages, which disperse at different rates (Cavalli-Sforza, 1998), the nuclear autosomal DNA-based analysis reported in this dissertation, provides information about the contributions of both ancestries, naturally coexisting together, in the populations analyzed. This demonstrates the potentiality of nuclear autosomal DNA sequence variation analysis in delineating some important events in the history of modern humans when combined with data drawn from historical records (Tadmouri et al., 1999c; Tadmouri et al., 1999d).

F. A Brief History of Anatolia and β-Thalassemia

Anatolia has a very long and complex history. Its position as a cross-road between Asia and Europe allowed extensive intermixing of racially and culturally separate populations since early prehistoric times. The early inhabitants of Anatolia were physically very similar to the Alpine sub-race of Europe, akin to the Sumerians of Mesopotamia and the Turanian Turks of Central Asia (The New Encyclopaedia Britannica, 1991). These inhabitants made Anatolia one of the major regions of agricultural development in the Neolithic time (9,000-7000 BC; Scarre, 1998). When Anatolian farmers settled in mosquito-infested soft and marshy soil (6500-2000 BC), large malarial outbreaks occurred in Anatolia (Angel, 1966; de Zulueta, 1994) and imposed a positive selection effect on heterozygous carriers of the oldest β-thalassemia alleles in the region (e.g., IVS-I-110; Tadmouri et al., 1999c). At 2000 BC, a group of Indo-Europeans, probably Nordic, settled in Central Anatolia and reorganized the population under the Hittite Empire (The New Encyclopaedia Britannica, 1991). By 1500 BC, this dominion expanded to cover Anatolia, upper Mesopotamia, present day Armenia, Syria, and Lebanon carrying the selected β-thalassemia mutations out of Anatolia (e.g., IVS-I-110; Tadmouri et al., 1999c). From that moment up to 1100 BC, tight relationships developed among Hittites and their neighboring Phoenicians. This latter group carried on much of the remaining task of further spreading β-thalassemia allele(s) out of the region (that is, North Africa, South Europe, and Sicily) until the decline of their influence some ten centuries later (Tadmouri et al., 1999c). By 546 BC, Anatolia felt under Persian domination, and it was to remain as such until 334 BC (The New Encyclopaedia Britannica, 1991). It is at this time that most of the β-thalassemia mutations common in Persian and Indian populations might have been introduced to Anatolia through its Eastern parts (e.g., FSC-8/9, IVS-I-5, Cd15). Through the Greek, Roman, and Byzantine occupations, the racial make-up of the original population inhabiting Anatolia was only slightly altered (e.g., the introduction of Cd 39). The arrival of the Turks of the Western-Central Asiatic steppe at 1070 AD, who were physically similar to the early inhabitants (The New Encyclopaedia Britannica, 1991), served to strengthen the original stock in Anatolia and might have introduced newer β-thalassemia

mutations uncommon to the region (e.g., FSC-8). In the 13th century AD, Anatolia witnessed an incomparable spread of agricultural prosperity that led to a major malarial recrudescence (Angel, 1966; Grmek, 1994). It is at this time, probably, that all β-thalassemia mutations present in Anatolia were selected and brought to frequencies close to what is observed at present. The expansion of the Ottoman Empire towards Eastern Europe, Northern Africa, and Central Asia permitted further spread of β-thalassemia mutations in and out of Anatolia making it a melting-pot of a large number of alleles. The migration of many Muslim groups living in former Turkish territories in Southeastern Europe during the decline of the Ottoman Empire (starting from 1914 AD) contributed more to the racial mixture (i.e., the introduction of IVS-II-745, -87, IVS-II-654). As a whole, all these episodes have genetically influenced the resident population of today's Turkey; molecular analyses conducted on several other genes in Turkey are unceasingly proving the validity of this assumption (Onay et al., 1998).

G. Future Perspectives

In addition to macroscopic examination of ancient and fossil bone specimens and the analysis of genetic polymorphisms in living populations, a more recent way of analyzing the migration patterns of human populations is based on the sequencing of ancient DNA (aDNA) preserved in ancient bones. The interest in aDNA has grown considerably since it was first demonstrated that mitochondrial DNA (mtDNA) could be recovered from dried muscles of animals (Higuchi, 1984). Molecular biology gave a fresh impetus to this new field of research and made it evolve from the inefficient and error-prone cloning of multi-copy DNA some hundreds of years old, to sophisticated PCR-based studies of genetic material deposited well before the last Ice-Age. Different research groups have succeeded in recovering DNA from animal bones and have resolved important questions by introducing extinct species into molecular phylogenies. In the field of archeology, one such problem was approached by introducing mtDNA sequences from ancient human bones (up to 6000 years old) into a genealogical tree showing their relatedness to mtDNA

sequences from modern individuals (reviewed by Audic and Beraud-Colomb, 1997). Although the work of aDNA analysis is still in its infancy, major directions for the applications of this research can be noticed. At present, one important application of aDNA analysis in the field of archeology involves the accumulation of data on DNA polymorphisms to investigate genetic variation in historically key populations.

Since early Anatolian civilizations are considered as vital links in human evolution by evolving into Western Asians and Europeans, we would like to use the approach of ancient DNA analysis in the identification of genetically inherited anemias in human skeletal remains excavated from different archeological sites in Turkey dating to different historical episodes. Much importance will be given to Neolithic and post-Neolithic sites in which there is a higher probability to uncover human bone remains suggestive of genetically inherited anemias. Results of this research will be considered as the first definitive clue for the presence of these disorders in early inhabitants of Anatolia and exclusion of anemia caused by malnutrition. Conclusions about the distribution of thalassemia mutations in past communities will also be drawn and events that affected their migrations in and out of Anatolia will be discussed. This will be important both for tracing the evolution and spread of this disease and for understanding environmental pressures experienced by past populations which caused their divergence.

VII. PRODUCTS OF THE THESIS

The research performed in the framework of this thesis comprised several major achievements concerning improvement of currently used methodologies in our laboratory:

A) Simplification of the protocol followed in DNA sequencing.

B) Establishment of methods to test for the presence or absence of the *XmnI* polymorphism.

C) Investigation of the origin of individual β-globin gene mutations by means of frequency and haplotype distributions.

D) Compilation of β-globin gene data in a single digital repository.

The work resulted in six original publications shown below and in the Appendix; two more papers on sequence haplotype studies are in preparation.

Studies on the clinical variations in β-thalassemia:

A) Altay Ç, Gürgey A, Başak AN, Tadmouri GO, Schroeder-Kurt T, "Fanconi Aplastic Anemia Associated with β-Thalassemia Trait," *American Journal of Hematology*, 52:239-240, 1996.

B) Tadmouri GO, Sağlamer L, Başak AN, "HbS/β^{del}-Thalassemia Associated with High Levels of Hemoglobins A_2 and F in a Turkish Family," *American Journal of Hematology*, 59:83-86, 1998.

Characterization of rare β-globin mutations:

A) Tadmouri GO, Tüzmen Ş, Başak AN, "A Rare β-Thalassemia Mutation in a Turkish Patient: FSC-36/37 (-T)," *Human Biology*, 69:263-267, 1997.

B) Tadmouri GO, Bilenoğlu O, Kutlar F, Markowitz RB, Kutlar A, Başak AN, "Identification of the 'Chinese' IVS-II-654 (C-T) β-Thalassemia Mutation in an Immigrant Turkish Family: Recurrence or Migration?," *Human Biology*, 71:297-304, 1999.

C) Tadmouri GO, Bilenoğlu O, Kantarci S, Kayserili H, Perrin P, Başak AN, "A Rare Mutation [IVS-I-130 (G-A)] in a Turkish β-Thalassemia Major Patient," *American Journal of Hematology*, submitted, 1999.

A general update for the β-globin mutation data in Turkey:

Tadmouri GO, Tüzmen Ş, Özçelik H, Özer A, Baig SM, Senga EB, Başak AN, "Molecular and Population Genetic Analyses of β-Thalassemia in Turkey," *American Journal of Hematology*, 57:215-220, 1998.

Studies on sequence haplotypes of β-globin genes in Turkey:

A) Tadmouri GO, Garguier N, Demont J, Perrin P, Başak AN, "History and Origin of β-Thalassemia in Turkey: Sequence Haplotype Diversity of β-Globin Genes," manuscript in preparation for the *American Journal of Human Genetics*.

B) Tadmouri GO, Bouhass R, Demont J, Garguier N, Trabuchet G, Zahed L, Başak AN, Perrin P, "The Origin and History of the IVS-I-110 (G-A) β-Thalassemia Mutation," manuscript in preparation for the *Proceedings of the National Academy of Sciences of the USA*.

APPENDIX

PRENATAL DIAGNOSIS, VOL. 16: 252-258 (1996)

SHORT COMMUNICATION

PRENATAL DIAGNOSIS OF β-THALASSAEMIA AND SICKLE CELL ANAEMIA IN TURKEY

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SUMMARY

This paper reports our experience of molecular analysis and diagnosis of β-thalassaemia and sickle cell anaemia (HbS) in 70 prospective parents of Turkish descent and their fetuses. Molecular screening was carried out by allele-specific oligonucleotide (ASO) hybridization of amplified DNA to the most common mutations in the Turkish population. By using this approach, we were able to define the mutation in 95 per cent of chromosomes investigated. Genomic sequencing led to the additional detection of three rare mutations: Cd 44 (-C), IVS-I-5 (G-C), and IVS-I-116 (T-G). All diagnoses were successfully accomplished and no misdiagnosis occurred. Consanguineous marriage appears to contribute significantly to the frequency of affected births in Turkey. Out of the 14 homozygous fetuses, six were the result of close consanguinity. This study indicates that fetal diagnosis of β-thalassaemia and HbS may be obtained in practically all cases, even in a heterogeneous population like the Turkish population, when early methods of fetal sampling are combined with polymerase chain reaction (PCR)-based techniques. Until gene therapy becomes a reality, the only approaches to the control of haemoglobinopathies are prevention and avoidance. The most relevant and common aspects of the programmes, which have been very effective in reducing the birthrate of β-thalassaemia major in several at-risk areas of the Mediterranean basin, are the continuous educational campaigns directed at the population at large, the voluntary basis, and non-directive counselling. The most important challenge for the eradication of the haemoglobinopathies in Turkey is the organization of a nation-wide and comprehensive genetic preventive programme based on DNA technology.

.. [This paper] is an excellent account of five years experience and contains all the relevant data on mutations screened for, strategy of diagnosis and diagnostic outcome.

Anonymous reviewer

American Journal of Hematology 52:239-240 (1996)
Letters and Correspondence

Fanconi Aplastic Anemia Associated With β-Thalassemia Trait

To the Editor: The coexistence of two genetic defects both associated with anemia may cause some clinical and hematological abnormalities, different from those found when they are present separately. In populations with a high incidence of β-thalassemia, such as Turkey, the combination of thalassemia mutation and another congenital hematological disorder may occur [1-2]. In Turkey, where the incidence of Fanconi's anemia (FA) also seems high, the coexistence of β-thalassemia trait and Fanconi's anemia in a patient was not surprising [3]. Examination of this child indicated changes in some of her hematological parameters during the severe anemia period, caused by FA and during the remission period which gave us some clues about the counter effects of both abnormalities on these hematological parameters.

This study indicates the importance of detailed hematological evaluation in FA, not only in the anemic period, but during remission as well. Such a detailed study will not only help detect the presence of another genetically transmitted hematological abnormality, but will also aid in understanding the countereffects of different genetic disorders when present in a patient.

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Rare β-Thalassemia Mutation in a Turkish Patient: FSC-36/37 (-T)

GHAZI OMAR TADMOURI,[1] ŞÜKRÜ TÜZMEN,[1] AND A. NAZLI BAŞAK[1]

Abstract We describe the rare β-thalassemia mutation at codons 36/37 (-T) for the first time in Turkey. The propositus is a Turkish patient with β-thalassemia major who originated in Adana but now resides in Istanbul. Molecular analysis revealed a compound heterozygosity for the common eastern Mediterranean mutation IVS-I-10 (G-A) along with mutation FSC-36/37 (-T). The FSC-36/37 (-T) could have arisen somewhere in the region, including northern Iran and the inaccessible mountainous region of eastern Anatolia. The mutation could have followed two migration routes during the time of Ottoman rule, the first being to Azerbaijan and the second, probably a more recent one, passing through southeastern Anatolia and reaching southern Bulgaria.

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Human Biology, April 1997, v. 69, no. 2, pp. 263-267.

.. Although we do not normally publish descriptive case reports, this Brief Communication has broader implications concerning the spread of rare mutations and its underlying molecular basis.

Prof. M.H. Crawford, Editor-in-Chief of 'Human Biology'

.. [This paper] has anthropogenetic significance in that it shows how one mutant can spread and it may be able to determine its origin and age. It is very well reviewed, well written, and terse to the point..

Anonymous reviewer

American Journal of Hematology 57:215-220 (1998)

Molecular and Population Genetic Analyses of β-Thalassemia in Turkey

G.O. Tadmouri,[1] Ş . Tüzmen,[2*] H. Özçelik,[3] A. Özer,[4] S.M. Baig,[5] E.B. Senga,[6] and A.N. Başak[1]

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In this report we describe the molecular analysis of 795 chromosomes derived from unrelated Turkish β-thalassemia and sickle cell anemia carriers identified in hematology clinics in Istanbul, Ankara, Izmir, Adana, and Antalya. The determination of the molecular pathology of 754 β-thalassemia and 42 abnormal hemoglobin genes and analysis of the frequency distribution in six distinct regions of Turkey was accomplished. The experimental strategy, based on PCR amplification of the β-globin gene, included dot-blot hybridization With 18 probes specific for the Mediterranean populations, denaturing gradient gel electrophoresis, and genomic sequencing. When the regional results are compared With the overall frequency of mutations in the country, it is observed that the frequencies in the western and southern parts of Turkey are in good accordance With the overall distribution, whereas the northern and eastern parts have a more region/population-specific profile With some rare mutations having a significantly high occurrence in these regions. Further evaluation of the data With respect to region- or population-dependent differences will contribute to a better understanding of the mechanisms leading to the marked genetic heterogeneity in Turkey, but could also be extremely valuable in facilitating rapid identification of mutations in families at risk for different hemoglobinopathies. Am. J. Hematol. 57:215-220, 1998.

.. [This paper] is of great interest to individuals .. concerned with antenatal screening and people involved in the anthropology of this region.

Anonymous reviewer

.. It is plain to see that the authors spent considerable time in the research involved..

Anonymous reviewer

American Journal of Hematology 59:83-86 (1998)

HbS/β^{del}-Thalassemia Associated With High Levels of Hemoglobins A_2 and F in a Turkish Family

G.O. Tadmouri,[1] L. Yüksel,[2] and A.N. Başak[1]

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β-Thalassemia and sickle cell disease (SCD) are common disorders in Turkey. Compound heterozygosity for these two disorders (β^S/β-thalassemia) is encountered frequently. In this report we present hematological and molecular data of two Turkish siblings With β^S/β^{del}-thalassemia caused by a 290 base pair (bp) deletion and associated With increased levels of hemoglobin A_2 (HbA_2) and hemoglobin F (HbF). Clinical analysis of the two patients showed a mild course of the disease. Haplotypic factors involved in increasing the levels of HbF were analyzed. The two patients showed no changes from the normal sequences at the XmnI site of Gγ-globin promoter and the $(AT)_xT_y$ microsatellite 5' to the β-globin mRNA cap site. The removal of the region between positions -125 to +78 relative to the β-globin gene mRNA cap site by the 290 bp deletion is thought to allow the β-locus control region to interact With the promoters of the δ- and γ-globin genes, leading to increased HbA_2 and HbF levels. Am. J. Hematol. 59:83-86, 1998.

Brief Communication

Identification of the Chinese IVS-II-654 (C-T) β-Thalassemia Mutation in an Immigrant Turkish Family: Recurrence or Migration?

GHAZI OMAR TADMOURI,[1] ONUR BILENOGLU,[1] FERDANE KUTLAR,[2] RHEA-BETH MARKOWITZ,[2] ABDULLAH KUTLAR,[2] AND A. NAZLI BAŞAK[1]

Abstract In this study we describe the Chinese IVS-II-654 (C-T) β-thalassemia mutation for the first time in an immigrant Turkish family living in Istanbul and originating from Xanthe, Greece. Four members of the family, representing 3 generations, are heterozygous for this mutation. A detailed family history demonstrated a Greek origin for members of 5 generations with no records of migration or consanguineous marriages. Analysis of polymorphic nucleotides located at the 5' end of the β-globin chromosomes bearing the IVS-II-654 mutation in the family described carried the $(AT)_9$ $(T)_5$ type of microsatellite sequence and the ACATCCCCA haplotype. These two haplotype components favor a non-Eastern Asian origin for this chromosome, hence suggesting an independent origin for the IVS-II-654 mutation described in this family.

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[2]Sickle Cell Center and Institute for Molecular Medicine and Genetics, Department of Medicine, Medical College of Georgia, Augusta, GA.

Human Biology, April 1999, v. 71, no. 2, pp. 297-304.

.. This is an important and well-written manuscript.. This is a solid contribution to the literature on beta-globin mutations.

Prof. M.H. Crawford, Editor-in-Chief of 'Human Biology'

.. It is noticeable .. that the work was done in its major part in Turkey, and only confirmed in Augusta. This is the right way collaboration has to be organized. This paper surely deserves publication, since it is together a molecular study, and a population approach.

Anonymous reviewer

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