A MOLECULAR INVESTIGATION OF β-THALASSEMIA IN THE AEGEAN AND MEDITERRANEAN COASTS OF TURKEY: IS THERE A REGION-DEPENDENT SPECIFICITY?

by
Edward Bisweck Senga

B.Sc. in Biology and Organic Chemistry,
The University of Malawi, Chancellor College, 1988

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APPROVED BY:

Prof. Dr. A. Nazlı Başak :
(Thesis Supervisor)

Assoc. Prof. Dr. Hande Çağlayan :

Assoc. Prof. Dr. Dilek Çalgan :

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DEDICATED TO MY DEAR WIFE MERVEE

"The greater the values, the more are the mere chance effects eliminated."

Gregor Mendel

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ABSTRACT

As in many other Mediterranean countries, β-Thalassemia is also a major public health concern in Turkey. The average gene frequency.is estimated to be two per cent, and regions with higher figures are known to exist.

Country scale frequencies of β-thalassemia mutations have been established in Turkey by several investigators in the past, but mutational data maps at regional scale are not available yet. The present study was designed to investigate the presence of a possible locus-specific heterogeneity of β-thalassemia mutations at the Aegean and the Mediterranean coasts of Turkey. Blood samples of patients sent to our laboratory from the Medical Schools in İzmir, Antalya and Adana were chosen as being representative for the Western and Southern parts of the country.

The method of choice for screening a large number of chromosomes for point mutations, involves the PCR amplification of the gene under investigation, followed by hybridization of the amplified DNA to Allele Specific Oligonucleotide (ASO) probes.

A total of 191 chromosomes were analyzed in the framework of this thesis using 19 oligonucleotide probes specific for the Mediterranean countries. The results obtained do confirm the remarkable molecular heterogeneity of β-thalassemia in all three districts investigated. Although a marked locus-specific heterogeneity of mutations was not observed, different patterns of mutational distribution were obvious, which may help in the elucidation of the molecular heterogeneity of Turkey in certain cases.

ÖZET

Beta-talasemi diğer Akdeniz Ülkelerinde olduğu gibi, Türkiye'de de önemli bir sağlık sorunu oluşturmaktadır. Genin, Türkiye genelinde görülme sıklığı %2 olmakla birlikte, bu sayı bazı yörelerde oldukça daha yüksektir.

Hastalığa neden olan mutasyonların ülke çapındaki sıklıkları tanımlanmış olmakla birlikte, bu mutasyonların bölgelere özgün dağılımını gösteren bir mutasyon haritası henüz mevcut değildir. Bu çalışma, Türkiye'nin Ege ve Akdeniz kıyılarında β-talasemi mutasyonları açısından olası bir bölgeye özgün heterojeniteyi araştırmayı hedeflemektedir. İzmir, Antalya ve Adana Tıp Fakültelerinden Bölümümüze gönderilen hasta örneklerinin, Türkiye'nin Batı ve Güney Kıyılarındaki mutasyon dağılımını aksettirdiği varsayılmaktadır.

Günümüzde nokta mutasyonlarının tanımında kullanılan en güncel yöntem, incelenen genin PCR metodu ile çoğaltılmasını, amplifikasyonunu, ve amplifiye edilmiş gen bölgesinin mutasyona özgü oligonükleotid probları ile hibridizasyonunun içermektedir.

Bu tez çalışması çerçevesinde 191 β-talasemi kromozomu Akdeniz Ülkelerine özgü 19 oligonükleotid probu kullanılarak analiz edilmiştir. Elde edilen sonuçlar incelenen her üç bölgede de Türkiye geneli ile kıyaslanabilecek bir moleküler heterojenite olduğunu ortaya koymuştur. Bölgeye özgün bir mutasyon çeşitliliği bulunmamış olmakla birlikte, incelenen bölgelerdeki mutasyon dağılımının ve sıralamasının birbirinden farklı olması, Türkiye'deki moleküler heterojeniteye bir ölçüde çözüm getirecektir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A	adenine
ARMS	amplification refractory mutation system
ASO	allele specific oligonucleotide
A_{260}	absorption at 260 nm
A_{280}	absorption at 280 nm
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
C	cytosine
Ci	Curie
Cd	codon
cpm	counts per minute
DGGE	denaturing gradient gel electrophoresis
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenedinitrilotetraacetic acid
EtBr	ethidium bromide
G	guanine
IVS	intervening sequence
o/n	over night
PCR	polymerase chain reaction
PNK	polynucleotide kinase
PVP	polyvinylpyrrolidone
rpm	revolution per minute
r.t.	room temperature
SDS	sodium dodecyl sulphate
T	thymidine
TCA	trichloroacetic acid
TEA	triethylamine
Tm	melting temperature
Tris	tris (hydroxymethyl)-aminomethan
UV	ultraviolet

I. INTRODUCTION

A. Hemoglobin Structure

The human hemoglobin is a red blood cell (erythrocyte) component consisting of tetramers of globin chains and four heme molecules. The primary function of the erythrocyte is the transport of oxygen from the lungs to the peripheral tissues. This function is dependent upon hemoglobin which normally constitutes over 90% of the soluble protein in the red cell. The hemoglobin molecule consists of two α- or α-like globin chains and two β- or β-like globin chains, each containing a heme Fe^{++} prosthetic group which can reversibly bind to O_2. Allosteric interactions among the four globin chains result in the physiologically appropriate sigmoid oxygen binding curve. Allosteric behaviour also adjusts oxygen binding affinity to local tissue pH (Bohr effect),temperature, pCO_2 and levels of erythrocyte organic phosphates (2,3DPG). Thus the erythrocyte function is dependent upon balanced synthesis of alpha and beta globin chains and their subsequent assembly into the functional hemoglobin tetramers (Liebhaber, 1989). Adult human hemoglobin molecules are principally composed of hemoglobin A ($\alpha_2\beta_2$) with a minor hemoglobin A_2 ($\alpha_2\delta_2$) component; between three and nine months of gestation, the hemoglobin present is principally hemoglobin F ($\alpha_2\gamma_2$) (Lebo et al., 1979). In addition to the fetal hemoglobin, there are also several embryonic hemoglobins which are present during embryonic and fetal life. These include Hbs Gower 1($\zeta_2\varepsilon_2$), Gower 2($\alpha_2\varepsilon_2$) and Portland($\zeta_2\gamma_2$) (Bank, 1985). Thus during ontogenetic differentiation of red blood cells, production of β-like globin undergoes two switches (embryonic to fetal to adult) while that of α-like globin undergoes only a single switch (embryonic to adult) (Embury and Mentzer, 1983).

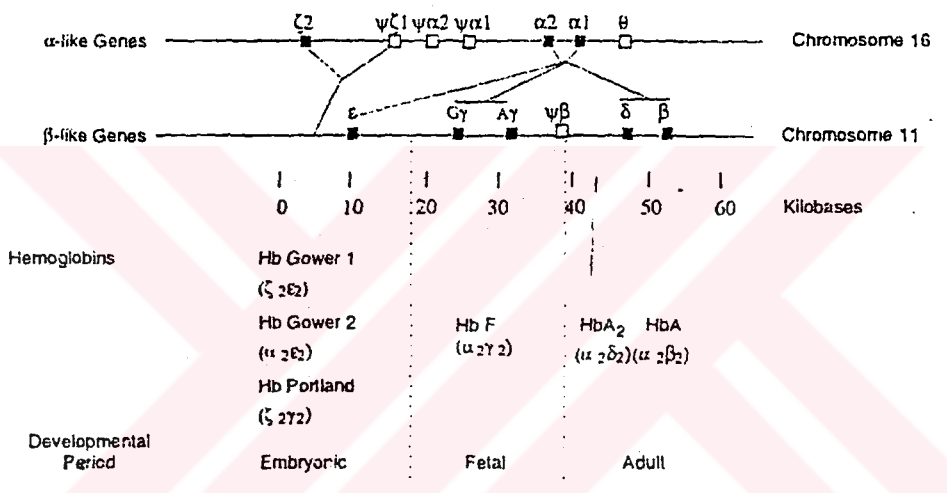


Figure I.1 Human hemoglobins. The α- and β-like genes are shown. The embryonic, fetal, and adult hemoglobins are also indicated. The distances between the β-like genes are given in kilobases. (Bank, 1985)

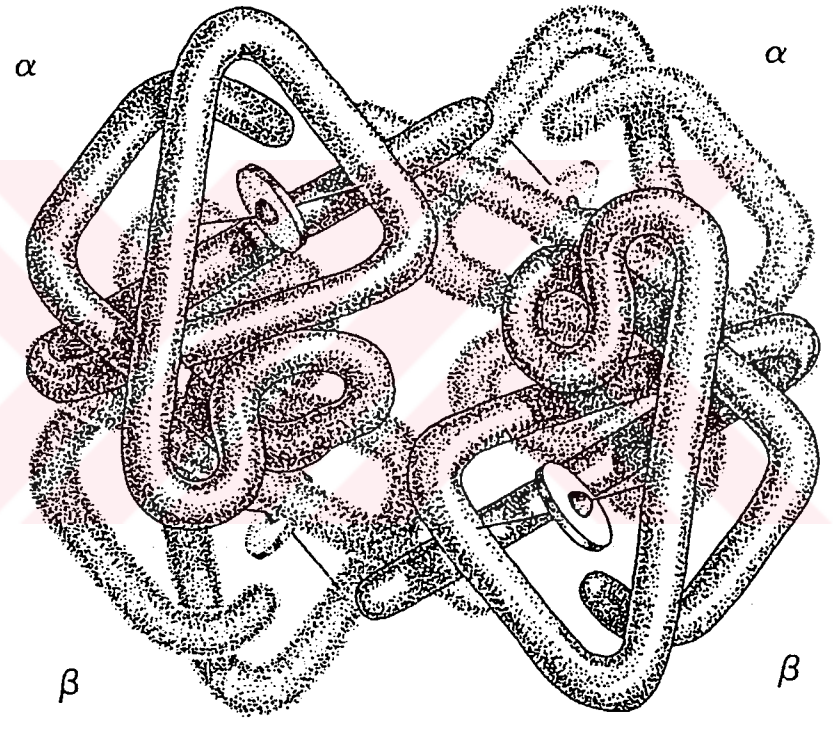


Figure I.2 A schematic representation of the hemoglobin molecule. (Mange and Mange, 1990)

B. Hemoglobinopathies

Hemoglobinopathies are inherited disorders of hemoglobin (Hb) and involve either the heme, or the globin part of the molecule. Very little is known about the genetic defects of the heme hence the term hemoglobinopathy usually refers to inherited disorders of the structure and synthesis of globin (Weatherall and Clegg, 1981). The knowledge of the abnormal hemoglobin proteins, was obtained by employing electrophoretic techniques in which separation of different proteins is based on the net charge. Thus the pathology of hemoglobin disorders started with the elucidation of sickle cell anemia, and it was shown that sickle cell hemoglobin (HbS) moved in an electric field at a rate different from that of normal hemoglobin (Pauling et al., 1949).

The hemoglobinopathies are categorized into four overlapping groups:

(a) an inherited change in the structure of globin chains giving rise to an alteration in Hb stucture and resulting in abnormal function or instability; this may cause a significant defect in oxygen transport.

(b) the thalassemias, being the second group, are characterized by inherited defects in the rate of synthesis of one or more globin chains.

(c) the third group consists of inherited disorders of both structure and rate of synthesis of globin chains giving rise to also a clinical picture of thalassemia.

(d) and finally a diverse condition which comes about as a result of a genetic failure of the normal neonatal switch from fetal to adult hemoglobin production, causing little or no clinical or hematological abnormality. This is referred to as the Hereditary Persistence of Fetal Hemoglobin (Weatherall et al., 1981).

Advances in recombinant DNA technology and especially the new advent of the polymerase chain reaction (PCR) technique, have led to the elucidation of the molecular etiology of the human hemoglobins (Huang et al., 1990). With rare exceptions, the more than 600 human hemoglobin variants described, are caused by a single point mutation. Other abnormal features such as unequal crossing-over, frameshift mutagenesis or double mutations in the same polypeptide chain, have seldom been encountered (Wajcman et al., 1992).

1. Sickle Cell Anemia

The most studied mutation in the beta globin gene is the one that leads to the sickle cell disease. Luzzatto and Goodfellow, (1989) reported : "No genetic disease could be simpler than sickle cell anemia but though simple, as it is caused by just a single base change from adenine to thymine at position six of β-chain, thereby changing glutamic acid to valine, there is literally no known cure for the disease." Sickle cell anemia is an autosomal recessively inherited blood disorder. It is found primarily in African populations and is caused by homozygosity (HbS/HbS) for a unique DNA base pair substitution in the sixth codon of the gene (Saiki et al., 1988). Heterozygotes (HbS/HbA) are referred to as sickle cell carriers. In venous blood the altered molecules tend to stack into narrow crystals, distorting the small contour of the red blood cells (RBCs). These abnormal RBCs sickle out and have a very short life span of a few weeks instead of the normal life span of four months. There are a lot of life threatening effects, crises, painful tissue damage, and infections due to trapped sickle cells. Heterozygotes tend to express some resistance to malaria, an advantage not expressed in normal homozygotes (HbA/HbA), hence in regions where malaria exists, sickle cell allele is maintained by selection at relatively high frequencies. In non-malarial regions such as in the USA, it is found that carriers have no advantage over the normal homozygotes thus, the frequency of the sickle cell genotype is decreasing slowly over successive generations (Friedman and Trager, 1982).

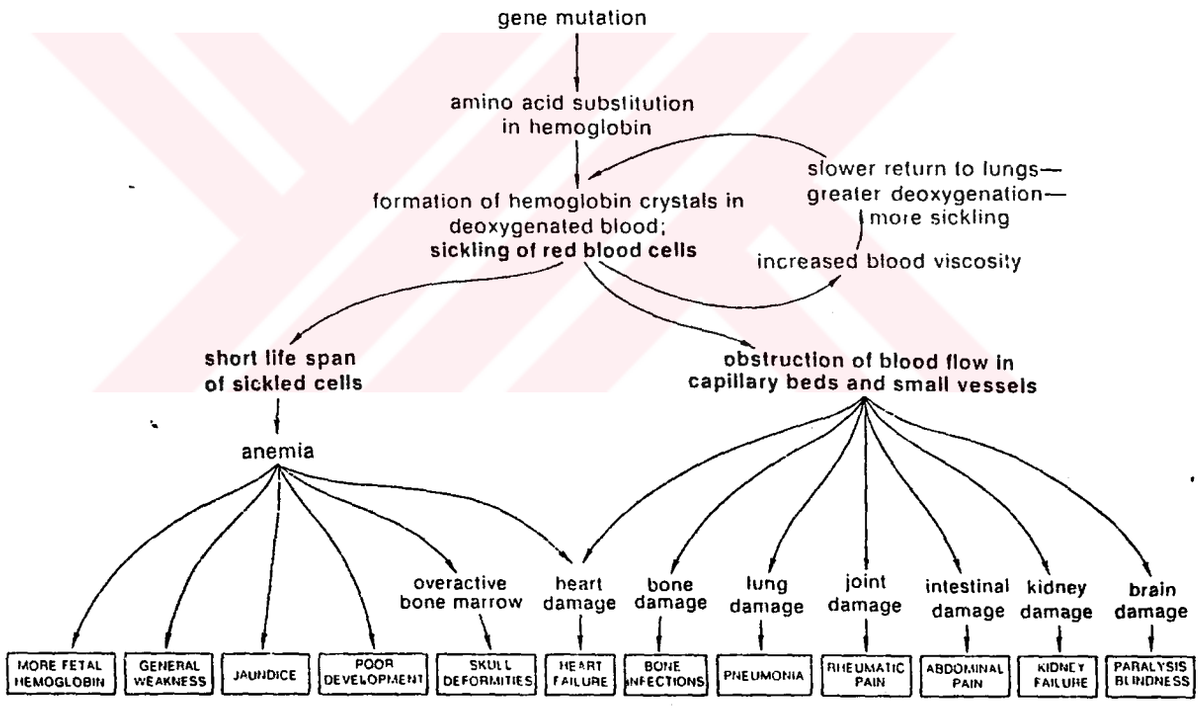


Figure I.3 A pedigree of effects in sickle cell anemia (HbS/HbS). (Mange and Mange, 1990)

2. Thalassemias

The term thalassemia, derived from the Greek word for sea, (thalassa) was introduced in the USA to high-light the Mediterranean origin of the first group of patients described. However, these inherited forms of anemia are found not only in Mediterranean regions but also in many other areas where malaria exists or existed previously (Embury and Mentzer, 1983).

The thalassemias are said to be a diverse group of microcytic hemolytic anemias that are characterized by defective synthesis of one or another globin chain. Alpha thalassemia is characterized by defective α-globin production while beta thalassemia by reduced β–globin synthesis (Kazazian, 1990). The expression of the human α-and β-globin genes, require the interaction of various transcription factors, including the LCR (locus control region) upstream of the epsilon gene in the β-globin gene cluster and the HS-40 element, upstream of the alpha gene complex as well as the promoters of the various individual genes (Thein et al., 1993).

a. Alpha Thalassemia

Alpha thalassemia is a hemolytic anemia resulting from deficient synthesis of alpha globin. The alpha globin gene is localized to chromosome 16; this was arrived at by observing the specific pattern of cDNA-DNA hybridization to human-mouse somatic cell hybrids. Subsequently the localization of the alpha gene has been refined to the distal segment (p13.1-pter) of the short arm of chromosome 16 by a variety of methods. Two independent studies of individuals with unbalanced karyotypes have unequivocally located the alpha locus to the Giemsa negative band 16p13.3 at the very tip of chromosome 16 (Higgs et al., 1989).

The deficiency of α-globin results in insufficient production of functional hemoglobin and in the accumulation of unstable γ_4 (Hb Bart's) or β_4 (HbH) tetramers with consequent accelerated red cell destruction. Alpha thalassemia can be inherited or acquired and can result from defects in or deletion(s) of one or more of the four α-globin genes (Liebhaber, 1989). Thus four different clinical syndromes may occur. Deletion of a single α-globin gene (heterozygous α-thalassemia-2 or the silent carrier state) produces no clinical abnormality.

Deletion of two α-globin genes is termed as α–thalassemia trait. It may result from

the inheritance of two α-thalassemia–2 deletions in trans (homozygous α-thalassemia–2) or from the inheritance of two adjacent α-globin gene deletions on the same chromosome. Mild anemia may be present but clinical symptoms are uncommon (Embury and Mentzer, 1983).

Deletion of three alpha globin genes produces HbH (β_4) disease, a life long microcytic anemia of moderate severity. As with hemolytic anemias, jaundice and reticulocytosis are usually present. Splenomegaly may develop and may produce hypersplenism if pronounced.

Deletion of all four α-globin genes (homozygous α-thalassemia–1) produces severe intra-uterine hemolytic anemia and high levels of Hb Barts (γ_4). Furthermore, inability to form fetal or adult hemoglobin because of the absence of α-globin chains, greatly limits O_2 transport. As a consequence, fetuses with homozygous α-thalassemia-1 develop hydrops fetalis and are nearly always stillborn (Embury and Mentzer, 1983).

b. β-Thalassemia

β-thalassemia is a common single gene disorder, and it has been estimated that three per cent of the world's population or 150 million people carry a β-thalassemia gene (Huang et al., 1990).

More than 180 β-thalassemia alleles have been characterized by DNA sequence analysis and all are the result of mutations within the β-globin gene itself or in its flanking regions. In rare instances sequencing of the beta globin gene has failed to identify a mutation responsible for the beta thalassemia phenotype. Thus, it has been suggested that the defect may lie in the β-globin locus control region (LCR), the major regulator of the β-globin cluster lying 50 to 60 kb upstream of the gene itself (Thein et al., 1993). β-thalassemia is an autosomal recessive disease characterized by hypochromic, hemolytic anemia, an increase in the proportion of the minor adult hemoglobin (HbA_2) and an imbalanced ratio of alpha to beta globin chain synthesis. There is a dependence on blood transfusions to sustain life (Kazazian et al., 1988; Thein et al., 1993). Life expectancy in classic β-thalassemia major is shortened to 30 years on average, even with the use of chelation therapy to remove excess iron stores. Those individuals who carry a single β-thalassemia gene (β-thal trait) are thought to have a selective advantage over normal individuals in malarial infections. Thus the disorder and β-thalassemia genes are concentrated in peoples residing in regions of the world endemic for malaria, including Mediterraneans, North Africans, Middle Eastern populations, Asian Indians, Chinese and

Southeast Asians, (Chan et al., 1986; Weatherall et al., 1981; Kazazian and Boehm, 1988).

Although there are many different β-thalassemia genes, there are basically two phenotypes: those associated with the production of some β-globin are called β^+-thalassemia and those which produce no β-globin are called β^0-thalassemia genes. The spectrum of clinical findings in β-thalassemia can be conveniently divided into four categories. Two syndromes, the silent carrier state and β-thalassemia trait are due to abnormalities of a single β-globin gene. The other two, thalassemia intermedia and β-thalassemia major or Cooley's anemia are due to abnormalities of both β-globin genes (Embury and Mentzer, 1983). Individual homozygotes for β-thalassemia have either two β^+- or two β^0-or one β^+- and one β^0-thalassemia genes. When two β^0-thalassemia genes are present, the patient has β^0-thalassemia. and no β-globin is produced. When at least one β^+-thalassemia gene is present, then the individual continues to produce small amounts of β-globin and has the β^+-thalassemia phenotype. While the fundamental defect in β–thalassemia is decreased or no β-globin synthesis, the pathogenesis of the disease is largely due to the continued normal production of α-globin chains. The large amounts of α-globin chains produced in the bone marrow have no significant amounts of non alpha chains (β– chains) with which to combine to form stable hemoglobins. Because of this situation α–globin chains aggregate and precipitate in the earliest erythroid precursors in the bone marrow. These α-chains disrupt the normal metabolism of nucleated red cells and cause their premature destruction. Thus although the production of erythroid cells in the bone marrow in β-thalassemia is remarkably increased, the number of functional erythroid precursors which mature normally and produce hemoglobin is markedly diminished. In addition, cells that do produce enough HbA and HbF to survive and reach the peripheral blood also contain excess α-globin. This excess α-globin material in the circulating erythroid cells causes increased destruction of these cells by the spleen and other phagocytic organs. The production of γ-globin chains and fetal hemoglobin has two effects:

(i) increased γ-chains lead to increased hemoglobin F, producing more viable oxygen-carrying red cells. (ii) the increased γ-globin production leads to less accumulation of excess α–globin chains decreasing the severity of the thalassemia (Bank, 1985).

Thalassemia intermedia is a clinical term used to describe a syndrome falling between β-thalassemia minor and major in severity. Moderately severe anemia (Hb 6 to 9 g/dl), splenomegaly and often hepatomegaly are characteristic features. Patients are only occassionally transfusion dependent (Embury and Mentzer, 1983).

β–thalassemia major produces anemia of such severity that lifelong treatment with blood transfusions is mandatory. Affected individuals appear normal at birth but during the first year of life as the normal developmental decline in γ-globin synthesis occurs without an

equivalent increase in β-chain, anemia gradually becomes evident (Embury and Mentzer, 1983). Three separate effects produce the anemia of the disease: (i) ineffective erythropoiesis (ii) hemolysis and (iii) reduced hemoglobinization of red cells. Splenomegaly may be extreme and splenectomy is often carried out to relieve abdominal discomfort and hypersplenism. Because of the rapid rate of hemolysis, there is an increased frequency of pigment gallstones. Most untransfused children die in the first decade of life. Bone marrow transplantion as a cure for beta thalassemia has been used with considerable success, especially in Italy but mortality post-transplant from graft versus host disease remains a problem (Kazazian, 1990). The major goal of transfusion is therefore to suppress the endogenous erythropoiesis so as to minimize complications such as hepatosplenomegaly or marrow hypertrophy that are caused by accelerated erythropoiesis. Thus blood transfusion is followed by iron chelation to remove excess iron, the cumulative effects of which are toxic to the most susceptible parts of the body, e.g. the liver, heart, pancreas and endocrine glands. It is reported that 3.28 mg of iron is found in the normal RBCs and that in each cubic centimeter of packed RBCs there is 0.76 mg of iron. This therefore indicates that thalassemic transfusion dependent individuals who receive 24 to 36 units of blood per year have an annual transfusion iron load of approximately 5 g. However in the body with its natural excretory pathways, only 1 mg of iron can be eliminated per day. Thus the parenteral administration of desferrioxamine, a potent iron chelator, has been effective in reducing the risk of iron toxicity and extending survival. Given as a daily intramuscular injection (0.5 g), the drug can retard the hepatic complications but has little or no effect on endocrine or cardiac complications. If given by a continuous intravenous or subcutaneous infusion, there is more effective chelation (Embury and Mentzer, 1983).

Since the treatment of individuals with β-thalassemia major which entails regular blood transfusions and extensive iron chelation regimes, is not yet satisfactory, the disease causes significant morbidity and mortality in affected individuals making prenatal diagnosis an important option for couples at risk of having a thalassemia major offspring, (Varawalla et al., 1991).

C. Molecular Analysis of Hemoglobinopathies

The rapid development of laboratory techniques has greatly aided the characterization of β-globin defects. Firstly, a reliable transient expression system was

developed in HeLa cells for the study of β-globin gene expression. Secondly, oligonucleotide hybridization allowed the rapid assay of β-thalassemia genes in genomic DNA for previously characterized alleles. Thirdly, the polymerase chain reaction technique has permitted genomic sequencing of amplified β-globin genes and rapid characterization of uncommon alleles (Kazazian and Boehm, 1988; Saiki et al., 1985; Wong et al., 1987; Wrischnik et al., 1987; Diaz -Chico et al., 1988; Gonzalez-Redando et al., 1988). This new methodology is suitable for screening rather large groups of patients with β–thalassemia, and the results of such studies made in various countries have been published during the past few years (Huisman, 1990).

Today, in the carrier identification and prenatal diagnosis of β–thalassemia alleles, the following laboratory techniques are commonly used : (i) Restriction Endonuclease digestion; (ii) Dot-Blot hybridization; (iii) ARMS - Amplification Refractory Mutation System; (iv) DGGE - Denaturing Gradient Gel Electrophoresis and (v) Direct Sequencing of the PCR product of the region of interest.

In Restriction Endonuclease digestion the amplified gene product of the area of interest in which the mutation resides, is digested by a restriction enzyme which recognizes a particular sequence. Thus a mutation in this particular sequence either creates or abolishes a cutting site for the enzyme. It is, for instance, possible in sickle cell anemia to directly diagnose a mutation by the use of a restriction enzyme called *Dde* I, the recognition sequence (C-T-N-A-G) of which in the β-globin gene is abolished by the sickle cell mutation. In contrast, the detection of HbC which involves a mutation (G to A) at codon 6 is more difficult than that of sickle cell anemia because no known restriction site is abolished or created by the mutation.

Direct detection of the mutations involved in β-thalassemia have been used for prenatal diagnosis in Sardinia for several years. The development of Allele Specific Oligonucleotide (ASO) hybridization technique for genomic DNA and the fact that one mutation, nonsense codon 39, is found in 95 per cent of β-thalassemia genes facilitated this development (Kazazian and Boehm, 1988). Improvements and modifications were instituted over the conventional dot-blot procedure (in which PCR products of the β-globin gene are blotted onto a nylon membrane and hybridized with synthetic ASO probes) that requires multiple hybridization to screen for large numbers of mutations. Thus the newly modified technique, the so called Reverse Dot-Blot approach, obviates the need for multiple probings as the oligonucleotide probes are immobilized onto one membrane strip. A PCR product encompassing the region(s) where the mutations are located is hybridized to the membrane and a non-radioactive system is used to visualize the hybridization products (Maggio et al., 1993).

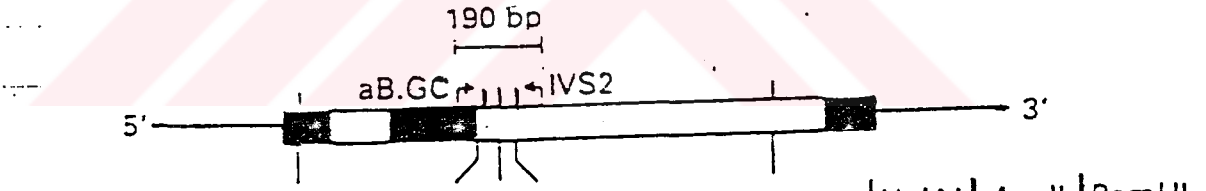
Techniques for prenatal diagnosis of monogenic disorders by DNA analysis have

progressed such that today following DNA amplification by the PCR technique, prenatal diagnosis by the direct detection of the relevant mutations , is possible in most cases in the first trimester of pregnancy. The ARMS (Amplification Refractory Mutation System) technique used for carrier screening, has been successfully applied to prenatal diagnosis (Old et al., 1990). This system was first introduced in 1989 for the analysis of a1-antitrypsin allele. Since then it has been developed for the detection of common cystic fibrosis and β-thalassemia mutations (Old, 1981). The principle of ARMS is that oligonucleotides with a mismatched 3' residue will not function as PCR primers under appropriate conditions. As DNA synthesis takes place in the 3' end of the primer, the mismatch will not permit extension of the primer on the DNA being amplified (Miedzybrodzka et al., 1992). Since ARMS detects directly the mutations, this method overcomes the labour-intensive process of studying the DNA polymorphisms in family members. Furthermore, unlike described techniques of dot-blot analysis of PCR products where radioactively labelled oligonucleotides are used (Saiki et al., 1986), ARMS is nonradioactive, rapid and relatively simple once the reaction conditions have been determined, more so if all the allele specific primers in use are designed to work at uniform PCR conditions. Thus this method is particularly relevant for the prenatal diagnosis of β-thalassemia in the Indian subcontinent considering the heterogeneous nature of the disease in this population and the scarcity of resources available for more expensive and tedious methods (Varawalla et al., 1991).

The DGGE (Denaturing Gradient Gel Electrophoresis) technique, has proven particularly useful in the detection of mutations responsible for genetic diseases and also in oncogene and tumour suppressor gene mutations. This technique is used to predetermine the area in which the mutation is located, and this same region can be amplified and sequenced without necessarily cloning or sequencing the whole gene (Losekoot et al., 1992). In this system, dsDNA is run on a linearly increasing gradient of denaturants. The dsDNA, runs along this gel until it reaches the denaturant which is equal to the melting temperature of the melting domain of the fragment. Thus this partial melting, causes the molecule to branch, the result of which decreases the mobility of the fragment in the gel. For this reason, DNA fragments differing just by a single base, melt at slightly different denaturant concentrations because of differences in the stacking interactions between adjacent bases. These differences in melting causes two DNA fragments, to slow down at different locations in the gel thereby separating from each other. However, DGGE is not able to separate DNA fragments which differ by a base substitution in the highest melting domain, as the sequence-dependent gel migration is lost because of complete strand separation (Myers et al., 1985). A β-globin framework is introduced rapidly from the analysis of a PCR product by this DGGE technique by employing two amplification primers

(aB.GC) and IVS-II, designed to span a region enveloping the nucleotides at positions 16, 74, and 81 in the second intron in a 190 bp fragment. There are four frameworks available (Figure I.4), and the nucleotides present at the three positions (16, 74, 81), when electrophoresed by the DGGE technique, provide different patterns, and every pattern, is specific to a particular framework which is specific to a particular ethnic group.

When a mutation cannot be characterized by the application of all the methods mentioned above, it is indicative of the possibility of a rare mutation. In such cases Direct Sequencing of the amplified genomic DNA is carried out. A part of the globin gene where most of the mutations producing β-thalassemia (in the Mediterranean populations) are clustered, is amplified (Antonarakis et al., 1985). Thereafter, a second amplification in which the primers are in ratio 100 : 1, which leads to preferential single strand amplification is carried out. In this way the two strands, forward and reverse are both obtained separately for sequencing (Gyllenstein and Erlich, 1988; Spiegelberg et al., 1989).



DGGE type	Frame-work	6	16	74	81	666	HgiAI pos 6	AvaII pos 16	BamHI
A	1	C	C	G	C	T	+	+	+
B	2	C	C	T	C	T	+	+	−
C	3	T	G	T	T	C	−	−	+
D	3 Asian	T	G	T	C	C	−	−	+

Figure I.4 A schematic representation of the β-globin gene and its polymorphisms. Exons and introns are represented as filled and open boxes, respectively. The positions and orientations of the amplification primers, aB.GC and IVS II, are shown. (Losekoot et al., 1992)

D. Molecular Basis of β-Thalassemia

1. A Brief Survey on the Localization of the β-Globin Gene

The β-globin gene was localized by cytogenetic experiments to be contained in the short arm of chromosome 11 and on band p15. Additional genetic evidences favouring this position rely on linkage to the insulin gene and other nearby loci (Gerhard et al., 1987).

Four techniques were employed: (a) hybrid cells with a translocated chromosome; (b) three human cell lines with naturally occurring deletions, (c) fluorescence-sorted cells having a translocated chromosome and (d) 'in situ' hybridization with beta globin gene probe (Mange and Mange, 1990).

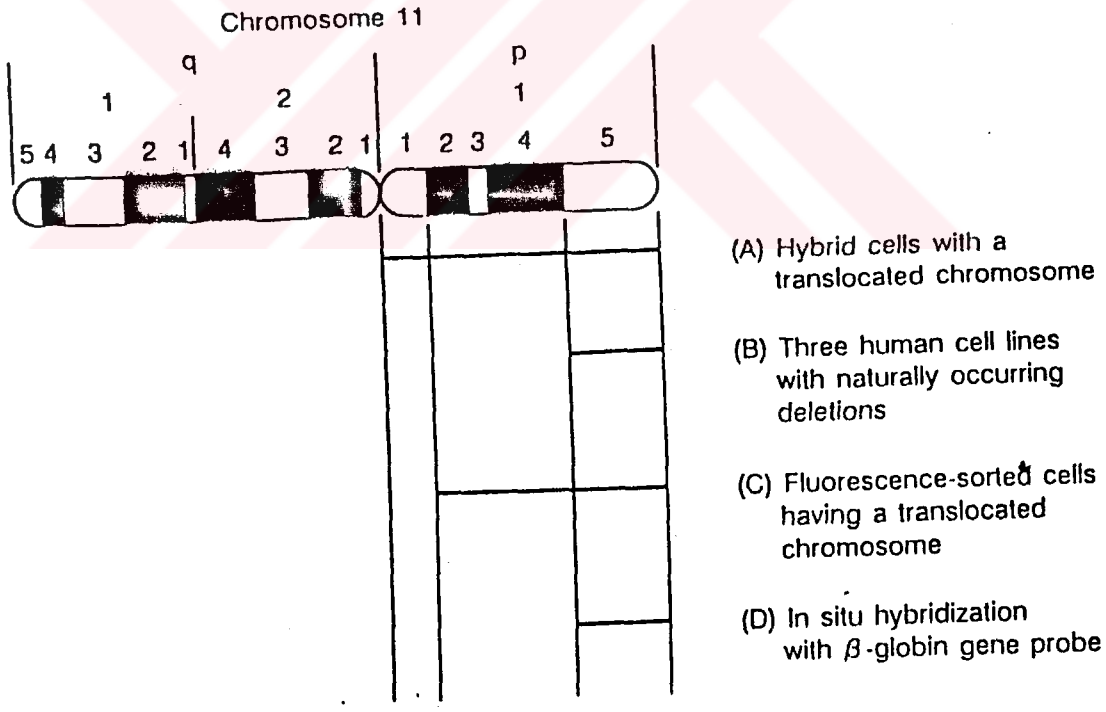


Figure I.5 Regional localization of the β-globin genes to band p15 of chromosome 11 by four techniques (A-D) (Mange and Mange, 1990).

The cytogenetic localization, (Figure I.5), involved the following steps: researchers examined mouse-human cell hybrids whose human donor had a reciprocal translocation between chromosome 17 and 11 where the break point in chromosome 11 was at the centromere. A radioactive cDNA probe was prepared from an mRNA from immature RBCs by the use of reverse transcriptase. In a Southern blot procedure this probe hybridized to the filter only when the short arm of chromosome 11 was present and not when it was absent. Secondly, a finer localization, (Figure I.5, step B), was done with deletions that cut out all of the short arm of chromosome 11 except band p15. Thirdly, by the use of a fluorescence detecting cell sorter, chromosome 11 by itself or translocations involving parts of chromosome 11 were isolated directly from human mitotic metaphase cells and run through a Southern blotting protocol. This method, allowed the researchers to locate the beta globin gene to the region depicted in Figure I.5 step C, a result consistent with band p15. The fourth technique employed was the 'in situ' hybridization in which the gene was localized to band p15 (Figure I.5, step D). In this technique human metaphase chromosomes were prepared on a microscope slide in the standard way and stained and photographed to reveal the banding patterns then the RNA within the chromosomes was digested away and the DNA remaining in place (in situ) was denatured to single strands and then bathed with a radioactive β-globin probe and autoradiographed. Thus from in situ hybridization studies, it was concluded that the beta globin gene is situated at band p15 of chromosome 11 (Morton et al., 1984).

The human β-globin gene complex is composed of five linked genes which code for one embryonic (ε), two fetal (Gγ and Aγ) and two adult (δ and β) globin polypeptides. These genes are located within a 60kb pair region on the short arm of chromosome 11 and are arranged in order of their developmental expression (5' ε – Gγ – Aγ – δ – β 3'). Interspaced between these genes are one pseudogene (ψβ) located between Aγ and δ and repeat sequence elements of varying repetition frequency (Poncz et al., 1983; Kaufman et al., 1980). The close linkage of the δ- and β-globin genes was initally suggested by the finding that the non-α -chain of hemoglobin Lepore appeared to be a hybrid product containing δ-globin amino-terminal sequences and β-globin carboxy-terminal sequences (Embury and Mentzer, 1983). These different genes are expressed in a developmental stage and tissue specific manner that is the embryonic epsilon (ε) gene is expressed in the yolk sac; the fetal Gγ and Aγ in the fetal liver while the adult delta (δ) and beta (β) in the bone marrow (Grosveld et al., 1987). The fact that β-globin production is determined by a pair of alleles at a single genetic locus, was demonstrated by the absence of HbA in individuals homozygous for mutations of the β-globin gene. In contrast, the γ-globin genes are duplicated as two forms of γ-globin, having either glycine (Gγ) or alanine (Aγ) at position 136, and only one of these variants is present in any single mutant form of hemoglobin F

(Embury and Mentzer, 1983). The two γ-, the δ-and the β-globin genes have been shown to be closely linked on a 30kb segment, and each globin gene has two introns (Kaufman et al., 1980). Each of the globin genes has a cap site approximately 50 to 60 nucleotides 5' to the initiation codon. There are three coding regions called Exons (I, II, and III, consisting of 30, 74, and 42 codons respectively) which are separated by two non-coding regions called Introns (IVS I and IVS II) which are approximately 120 and 900 nucleotides long, respectively. All these together, form the whole β-globin gene which is approximately 1600 base pairs long. The terminal end of the β-globin gene has a 3' untranslated region called a polyadenylation (poly-A) signal, AATAAA. A total of 146 amino acids are coded on these three exons. The β-globin LCR (Locus Control Region) located 6 to 22kb upstream of the ε-globin gene is the major regulatory region that controls transcription of the β-globin domain (Thein et al., 1993). Some other very important regions 5' to the β-globin gene are available for optimal function in initiation of globin mRNA transcription. These regions are approximately 90 to approximately 80 nucleotides 5' to the cap site (CCC); similarly another region at approximately 50-60 nucleotides 5' to the cap site (-50) and (-60), the so called CAAT box, appears to be important in optimal function. A third region is the so called TATA or ATA box (the Goldberg-Hogness box) at -30 to -40, 5' to the β–globin gene which is also required for the transcription of the gene. Thus, all these three 5' flanking sequences are considered to be the "promoter" sequences important in the regulation of transcription of the globin genes (Bank, 1985).

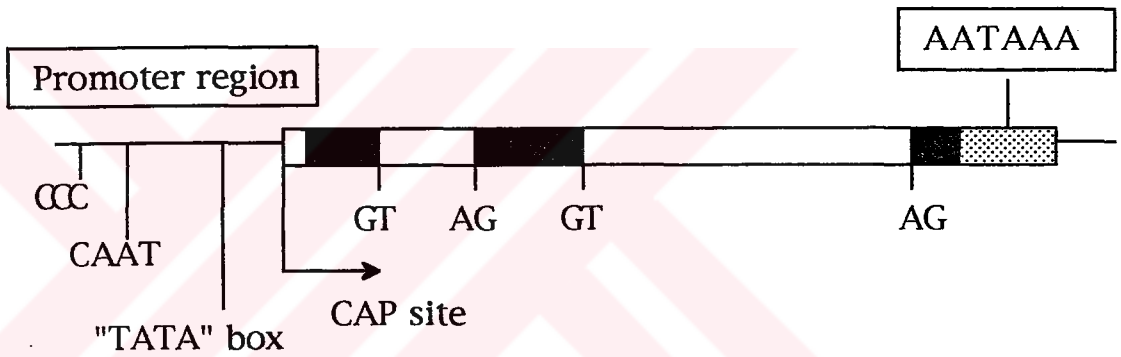


Figure I.6 Schematic representation of the structure of the β-globin gene and its 5' and 3' flanking sequences. Closed boxes are exons; open boxes introns; The stippled boxes indicate the untranslated region. The splice junction and CAP site are indicated at the lower side of the figure. The conserved sequences of the promoter region of the β-globin gene are shown above. The CCC sequence, "CAT box" and "TATA the box" are indicated.

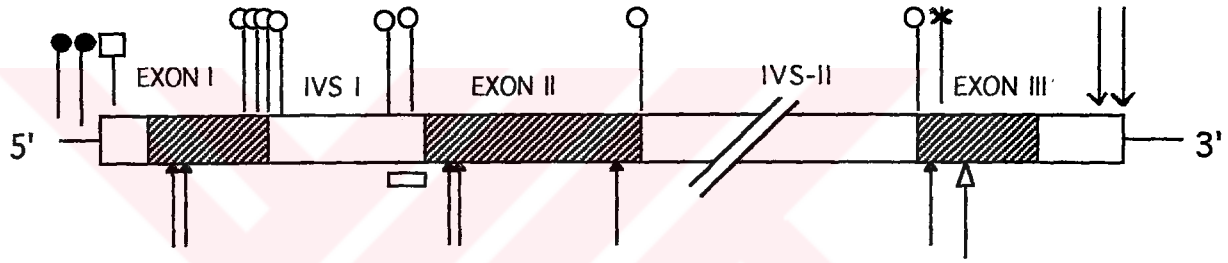


Figure I.7 Point mutations in β-thalassemia. The various types of mutations are depicted by different symbols. RNA splicing mutations (), transcription (), cap site (), RNA cleavage (), frameshift (), nonsense codon (), unstable globin (), small deletion (). (Adapted from Kazazian and Boehm, 1988)

2. β-Thalassemia Mutations

Most of the mutations are single nucleotide substitutions which produce defects in transcription, RNA splicing, RNA modification, transcription via frame-shifts and nonsense codons or they produce highly unstable β-globin (Kazazian and Boehm, 1988). Deletions are a rare cause of β–thalassemia . One class of mutations involves mutations in the coding regions within the β-globin gene and usually either Exon I or Exon II is involved (Bank, 1985).

a. 5'-Promoter Mutations

A large number of alleles that affect transcription have been observed. They are known as promoter mutations in the region 5' to the β–globin gene and they cause only a moderate impairment in globin synthesis. These mutations are commonly though not exclusively observed in β-thalassemia intermedia (Kazazian and Boehm, 1988). In particular a single change has been detected in the middle cystidine residue of a CCC residue at position -87 of the β-globin gene. Other single base mutations 5' to the β-globin gene in the region of great significance is the so called ATA box (the sequence CATAAAA located roughly 30 nucleotides (nts) upstream of the cap site). This TATA region is important for proper efficiency of transcription and the location of the start site of transcription. Mutations are now known at the ATAA residues at nts -31, -30, -29 and -28 (Kazazian, 1990)

b. Coding Region Mutations

Frameshift mutations are deletions or additions of one, two or four nucleotides that change the ribosome reading frame and cause premature termination of translation. An example of frameshift mutations is that in codon 8. There is a deletion of two A residues (-AA) giving rise to a β^0-thalassemia. Mutations that produce a nonsense codon, directly stop translation. The most common example of termination or nonsense mutations is the first and most extensively studied of all the β-thalassemia mutations due to single base changes, codon 39. This is a common cause of β-thalassemia in the Sardinian population. It is also found in other individuals of Mediterranean and Asiatic origins. In codon 39, there

is a single base change in which CAG coding for glutamic acid is changed to TAG, a stop codon thereby causing termination of globin chain synthesis.

c. Intervening Sequence Mutations

Mutations that affect RNA splicing occur at a variety of locations within the gene: (i) at splice junctions; substitutions in the variant GT and AG dinucleotides destroy the essential sequence for normal splicing to occur (Kazazian and Boehm, 1988). Nearly all eukaryotic genes have the GT as donor site at the 5' end and the AG as the acceptor site at the 3' end of every intron. The splicing process to produce normal mRNA, begins with cleavage of the 5' splice junction and a looping back of the 5' end of the intron to form a covalent 5'-2' phosphodiester bond with an A residue 30 or so nucleotides 5' to the 3' splice junction. All mutations affecting these GT or AG residues produce β^o-thalassemia alleles e.g. IVS I-1 and IVS II-1 mutations; (ii) substitutions around splice junctions destroy consensus sequences that are important but not essential for splicing, leading to β^+-thalassemia because some normal splicing occurs : for example mutations at positions 5 and 6 in the IVS I:

(a) position 5 : AG/GTTGGT to AG/GTTGCT (β^+ mild)

(b) position 6 : AG/GTTGGT to AG/GTTGGC (β^+ very mild)

The mildness in the phenotypic effects between IVS I-5 and IVS I-6 appears to be related to the relative distance of the mutations from the 5' IVS splice junction; thus the mutation at position 6 of IVS I is milder than that at position 5 (Bank, 1985).

A cryptic splice site is a sequence which mimics the consensus sequence for a splice but is never used under normal circumstances. In IVS II, there is a known splice site at position 580. Three unique genes in the IVS II at positions 654, 705 and 745 of the β-globin have been described to give rise to new 5' splice sites; e.g. for the mutation at position 705 in which there is a change from ATGT to AGGT , it is found that there is no normal splicing of the gene from its 5' to the 3' end instead a cryptic splice site at position 580 of IVS II is used. Position 580 is said to contain an ideal sequence for a 3' splice junction with 12 pyrimidines followed by an AG (Bank, 1985; Kazazian, 1990)

d. Mutations in the 3'-Untranslated (UTR) Region

The highly conserved sequence AATAAA in the 3' untranslated region is required for optimal polyadenylation of RNA, which occurs as an early event of RNA biosynthesis in the nucleus. One of the mutations in this region at the 3' end of the transcript markedly reduces RNA cleavage and leads to elongated mRNA molecules, resulting in β^{+}-thalassemia with the loss of the appropriate polyadenylation signal (Bank, 1985).

e. Deletion Mutations

A number of deletions affecting much of the β-globin gene cluster have been observed in which both the δ and β -globin genes are deleted giving rise to hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia. Only a few deletions all but one of which is rare, affect the β-globin gene and produce β-thalassemia. The most common deletion removes 619bp of the IVS II, Exon III and sequences 3' of the β-globin gene. This deletion causes mutational effects by deleting a " locus activating region". Three other deletions of particular interest are the ones which leave the β-globin gene intact yet silence its expression. They are all rare and have been reported in heterozygotes (Kazazian, 1990).

Other examples reported are : $G\gamma A\gamma(\delta\beta)^{0}$-thalassemia in Sicilians and other Mediterranean populations in which an about 13kb deletion in the β-globin gene cluster is involved. The 5' end point of the deletion is in the IVS II of the δ-gene between nucleotides 659 and 766 and its 3' end is about 5.0 - 5.5kb 3' to the β-globin gene (Ottolenghi et al., 1979; Fritsch et al., 1979). Another deletion is the $(A\gamma\delta\beta)^{0}$-thalassemia reported in Blacks and is due to a deletion of 34kbp of DNA (Henthorn et al., 1984).

E. β-Thalassemia In Turkey

Turkey is situated at the meeting point of three continents, Asia, Europe and Africa. It therefore acts as a crossroad and throughout history it has been the site of interaction of various civilizations.

Just as the first case of hemoglobin disorders started from the analysis of an abnormal sickle cell gene by Pauling et al., (1949), likewise the history of abnormal hemoglobins in Turkey started with the description of a sporadic case of sickle cell anemia in a Greek woman living on a small Turkish island. This was reported by Egeli and Ergun in 1946. Later, population surveys and research on thalassemia and abnormal hemoglobins were embarked on by Aksoy et al., (1955). Abnormal hemoglobins such as HbD-like variants were found in 1956. Thus HbD Los Angeles in Southest Anatolia was found at a frequency of 0.3 per cent and with a frequency of 0.2 per cent among Turks, according to Dincol et al.; HbE in Eti-Turks was found in frequency ranges between 0.16 to 2.4 per cent. Some of the other abnormal hemoglobins reported are : Hb Istanbul, Hb Ankara, Hb Antakya and HbF Baskent (Arcasoy, 1991).

1. Studies at Country Level

As in many Mediterranean countries, β-thalassemia is said to be a major public health concern in Turkey and has been studied for several decades by different investigators (Çavdar and Arcasoy, 1971; Arcasoy et al., 1978 ; Öner et al., 1990; Başak et al., 1992). According to WHO, β-thalassemia in Turkey has the frequency ranges between 0.2 per cent to 6 per cent (Holzgreve et al., 1990). The average gene frequency is now estimated to be 2 per cent and regions with higher figures are known to exist (Aksoy et al., 1985).

Thalassemia centers have been established at medical faculties of different universities for the treatment and investigation, the largest being at the Childrens Hospital of Hacettepe University in Ankara (Öner et al., 1990).

Previous studies dealing with β-thalassemia in Mediterranean countries have indicated that in most of these populations only a few mutations (six or less) are prevalent, accounting for more than 90 per cent of the β-thalassemia genes (Chehab et al.,1987; Rosatelli et al., 1992; Amselem et al., 1988; Dimovski et al., 1990; Petkov et al., 1990). In contrast to most of these countries and in accordance with the neighbouring Balkan

populations (Huisman, 1990), β-thalassemia in Turkey does not seem to be associated with a few predominant lesions. Studies presently conducted concerning the molecular heterogeneity of β-thalassemia in Turkey (Öner et al., 1990; Başak et al., 1992), do confirm the remarkable heterogeneity of β-thalassemia among Turks. These studies demonstrate that β–thalassemia in Turkey is not only represented by a few Mediterranean-specific mutations. The marked heterogeneity is probably the result of at least two different events in the evolution of β-thalassemia in Turkey : genetic admixture with populations in peripheral areas and immigration of chromosomes from distant regions (Başak et al., 1992).

2. Studies at Regional Scale

Mutational data maps at a regional basis are presently still unavailable. The first attempt on the regional study has been allocated to four main regions of Turkey. In this study Atalay et al., (1993) have come up with a regional distribution of β–thalassemia in Turkey and it is observed that the most frequent mutation at country level, IVS I-110, appears to be the most frequent at regional scale level. Likewise the other mutations such as IVS I-6, IVS I-1, Codon 39, FSC-8, are also reflected at regional basis in particular in the Western and Southern Anatolia. Thus from this study it was deduced that these results appear to provide implications, that there is lack of a locus-specificity concerning these mutations. Since only a few studies dealing with the molecular characterization of β-thalassemia in the Turkish population have been undertaken (Akar et al., 1987; Diaz-Chico et al., 1988), an elucidation of the data with respect to possible region-dependent mutational differences should contribute to a better understanding of these mechanisms thus helping to elucidate the populational mosaic that has been formed in the course of history (Başak et al., 1992; Atalay et al., 1993).

3. Prenatal Diagnosis

Since the treatment of β-thalassemia is still unsatisfactory, prenatal diagnosis is the only option for couples at risk. Prenatal diagnosis programs in countries such as Cyprus (Angastiniotis and Hadjimanis, 1981), and Sardinia (Cao et al., 1984) have been highly

effective in reducing the incidence of β-thalassemia major. In Sardinia, with the recent application of chorionic villi sampling instead of amniocentesis, the acceptance rate of prenatal diagnosis had been risen from 97.4 per cent to 99.1 per cent (Holzgreve et al., 1990). Thus, characterization of the molecular basis of β-thalassemia in Turkey and the successful application of chorion villus sampling in the first trimester of the pregnancy, is a step towards achieving a similar goal (Başak et al., 1992).

II. AIM OF THE STUDY

Beta-thalassemia in Turkey is reflected by a wide spectrum of clinical manifestations ranging from mild β-thalassemia intermedia to severe, transfusion-dependent β-thalassemia major(Aksoy et al., 1978). Mutational data maps at country scale indicate a marked molecular heterogeneity of β-thalassemia mutations in Turkey (Başak et al., 1992), whereas maps at regional basis are restricted to two studies thus far (Atalay et al., 1993; Tadmouri, 1994). Considering the fact that in Turkey there is a frequent occurrence of consanguineous marriages, which leads to a high frequency of homozygosity for a clinically severe β-thalassemia allele (Öner et al., 1990), and also taking into account that preventive medical services such as genetic counselling and prenatal diagnosis are greatly improved by the detailed knowledge of the molecular pathology of β-thalassemia (Rosatelli et al, 1987), the aim of this study is as follows:

(i) Adapting the most recent analytical and diagnostic DNA methodologies, used in the molecular investigation and prenatal diagnosis of β-thalassemia, to our laboratory; this will enable us to realize the long-term goal of establishing a nation-wide strategy for the early prenatal diagnosis of β-thalassemia major in Turkey.

(ii) Investigating the frequency and distribution pattern of β-thalassemia mutations in three distinct districts of Turkey, Izmir, Antalya and Adana, which are believed to be representative for the coastal regions of the country. An elucidation of the data with respect to possible region-dependent differences is expected to simplify the molecular heterogeneity of Turkey to a certain extent.

III. MATERIALS

A. Buffers and Solutions

1. Solutions Used in DNA Isolation

a. Whole Blood

RBC Lysis Buffer	:	155 mM NH_4Cl 10 mM $KHCO_3$ 1mM EDTA (pH 7.4)
Nuclei Lysis Buffer	:	10 mM Tris (pH 8.0) 400 mM NaCl 2 mM EDTA (pH 8.2)
SDS	:	10% stock solution
Proteinase K	:	20 mg/ml in H_2O
NaCl	:	5 M saturated stock solution

b. Chorionic Villi Sample

NaCl/EDTA Lysis Buffer	:	100 mM NaCl 125 mM Na_2EDTA
SDS	:	10% stock solution
Proteinase K	:	20 mg/ml in H_2O

Chloroform/Isoamylalcohol	:	24:1 chloroform/isoamylalcohol
Ammonium Acetate	:	7.5 M stock solution

2. Gel Electrophoresis Buffers and Solutions

10X Loading Buffer	:	2.5 mg/ml BPB 1% SDS in glycerol
Ethidium Bromide	:	10 mg/ml
5X TBE Buffer	:	89 mM Tris 89 mM Boric acid 2 mM EDTA

3. Agarose Gels:

0.8-2.0% agarose (w/v) in 0.5X TBE buffer, containing ethidium bromide (0.5 μg/ml).

4. Polymerase Chain Reaction: Buffers and Solutions

DNA polymerase Buffer 1 X		10 mM Tris-HCl (pH 9.0)
(Mg-free)	:	50 mM KCl
		0.1% Triton X-100
$MgCl_2$	:	1.5 mM $MgCl_2$
dNTPs	:	20 mM of each dNTP
Primers	:	50 pmoles of each primer

5. Solutions Used in Dot-Blot Analysis

a. Labeling of ASO Probes

Spermidine	:	10 mM
10X PNK	:	700 mM Tris (pH 7.6) 100 mM $MgCl_2$ 50 mM DTT 1 mM KCl

b. TCA Precipitation

500 g of TCA, dissolved in 227 ml H_2O

c. Probe Purification by Nensorb Column Chromatography

Reagent A	:	100 mM Tris (pH 7.7) 1 mM EDTA 10 mM TEA

d. Hybridization Buffers

50X Denhart's Solution	:	1% Ficoll 1% PVP 1% BSA
20X SSPE Solution	:	3.6 M NaCl 0.2 M NaH_2PO_4 20 mM EDTA (pH 7.4) adjusted with NaOH
Prehybridization/Hybridization		5X SSPE

Solution	:	5X Denhart's solution 0.5% SDS
Denaturation Solution	:	4 N NaOH 250 mM EDTA (pH 8.0)
First Wash Solution	:	2X SSPE 0.2% SDS
Last Wash Solution	:	5X SSPE 0.2% SDS
Stripping Solution	:	0.1X SSC 0.5% SDS

B. Chemicals and Enzymes

Chemicals and Solutions used were mostly purchased from MERCK (GERMANY) or SIGMA (USA) unless stated otherwise in the text. Absolute alcohol was from TEKEL (TURKEY).

The radionucleotide, γ-[^{32}P]-ATP, was purchased from NEN-DUPONT (USA) and AMERSHAM (USA).

Developing Solutions were from KODAK (USA) and FOFIMA (TURKEY).

The enzymes, Taq DNA polymerase and T4 polynucleotide kinase, were purchased from PROMEGA (USA)

C. Equipment

Autoclave	:	Medexport, Former USSR Eyela Autoclave, MAC-601, JAPAN
Balances	:	Electronic Balance Type 1574, SARTORIUS, GERMANY Electronic Balance Libror EB-3200H, SHIMADZU, JAPAN Precision Balance H72, METTLER, GERMANY
Camera	:	MAMIYA, RB 67, JAPAN POLAROID, DS 34, USA
Centrifuges	:	Biofuge A, HERAUS CHRIST, GERMANY SORVALL RC-5B Refrigerated Superspeed Centrifuge, DuPont, USA Hettich EBA 35, GERMANY Eppendorf, Centrifuge 5415C, GERMANY
Deepfreezes	:	-20°C, BOSCH, GERMANY -20°C, AEG, TURKEY -70°C, GFL, GERMANY -70°C,CFC Free Sanyo, JAPAN
Dot-Blot Apparatus	:	Bio-Dot Apparatus, BIORAD, USA
NEN-SORB prepacked columns	:	NEN-DUPONT, USA
ZETA-Probe nylon membranes	:	BIORAD, USA
GF/C filters	:	Whatman LTD, UK
X-ray films	:	X-OMAT AR5, X-OMAT RP and Agfa Curix RP1, KODAK, USA

Instant films	:	Polaroid 667, USA
Electrophoresis Equipment	:	Horizon 58, Model 200
Incubators	:	Shaking Incubator, GFL, GERMANY Orbital Shaking Incubator Oven 300, Plus Series, GALLENKAMP, GERMANY Oven MAXO/156/EC/DGW, UK Incubator, Plus Series, GALLENKAMP, GERMANY
Shaker	:	VIB, InterMed, DENMARK
Scintillation Counter	:	TRI-CARB 4530, United Technologies, PACKARD, USA
Spectrophotometers	:	Lambda 3 UV/VIS, PERKIN ELMER, USA UV/Visible Spectrophotometer, BIO-PROJECTS GmbH, GERMANY
Thermocyclers	:	Model 480, PERKIN ELMER CETUS, USA UNO-Thermoblock, BIOMETRA, GERMANY
Transilluminators	:	Reprostar II, CAMAG, SWITZERLAND CONSORT B-2300, BELGIUM
Vacuum Oven	:	Model 6002, COLE PARMER, USA
Water Baths	:	Thermomix, BU, BRAUN, GERMANY Thermomix, 1441, BRAUN, GERMANY KÖTTERMANN Labortechnik, GERMANY

D. Oligonucleotide Primers and Probes

1. Amplification Primers

a. Forward Primers

KM 29	:	5' GGTTGGCCAATCTACTCCCAGG 3'
TCG1	:	5' CAATGTATCATGCCTCTTTGCACC 3'
108	:	5' CAAGGACAGGTACGGCTGTCAT 3'
Common C	:	5'TCACTTAGACCTCACCCTGTGGAGCCCA 3'
CD 1	:	5' CTGGGTTAAGGCAATAGCAAT 3'

b. Reverse Primers

RS 43	:	5' GTGAGTCACACCGTTTC 3'
TCG2	:	5' TCCTGCATCTCTCAGCCTTGACTC 3'
109	:	5' ATGGTTAAGTTCATGTCATAGGAAGGGGAG 3'
#31	:	5'TTAAGTTCATGTCATAGGAAGGGG 3'
CD 2	:	5' AAAAGGGAATGTGGGAGGTC 3'

2. ASO Probes Used in this Study for Dot-Blot Hybridization

ASO probe sets specific for Mediterranean countries were used in the framework of this thesis. The sequences, melting temperatures and hybridization conditions for these probes are listed in Table III.1.

Table III.1 The melting (Tm), hybridization, and stringent washing temperatures of the 19 ASO probes used in the study.

Probes		Sequences	Hybridization Temperatures (°C)	Last Wash Temperatures (°C)
IVS I-110 (G-A)	Mt	5'-CTGCCTATTAGTCTATTTT-3'	50	54
	N	5'-CTGCCTATTGGTCTATTTT-3'	50	54
IVS-I-1 (G-A)	Mt	5'-GCAGATTGGTATCAAGGTT-3'	57	63
	N	5'-GCAGGTTGGTATCAAGGTT-3'	57	63
IVS I-1-6 (T-C)	Mt	5'-GCAGGTTGGCATCAAGGTT-3'	56	63
	N	5'-GCAGGTTGGTATCAAGGTT-3'	56	63
FSC-8 (-AA)	Mt	5'-TGAGGAGGTCTGCCGTTAC-3'	58	62
	N	5'-TGAGGAGAAGTCTGCCGTT-3'	58	62
FSC-5 (-TC)	Mt	5'-CTGACTCGAGGAGAAGTCT-3'	57	61
	N	5'-CTGACTCCTGAGGAGAAGT-3	57	61
CD.39 (C-T)	Mt	5'-CCTTGGACCTAGAGGTTCT-3'	57	63
	N	5'-CCTTGGACCCAGAGGTTCT-3'	57	63
CD.44 (-C)	Mt	5'-TTTGAGTCTTTGGGGATCTG-3'	59	61
	N	5'-TTGAGTCCTTTGGGGATCTG-3'	59	61
-30 (T-A)	Mt	5'-GGCTGGGCAAAAAAGTCA-3'	57	63
-29	N	5'-GGCTGGGCATAAAAGTCAG-3'	57	63
IVS-I-5 (G-A)	Mt	5'-CAGGTTGATATCAAGGTTA-3'	55	62
IVS-I-6	N	5'-GCAGGTTGGTATCAAGGTT-3'	55	62
IVS-I-5 (G-C)	Mt	5'-CAGGTTGCTATCAAGGTTA-3'	55	62
IVS-I-6	N	5'-GCAGGTTGGTATCAAGGTT-3'	55	64

(Table III.1 continued)

IVS-I-116 (T-G)	Mt	5'-ATTGGTCTAGTTTCCCACC-3'	55	59
	N	5'-ATTGGTCTATTTTCCCACC-3'	55	59
IVS-II-1(G-A)	Mt	5'-GAACTTCAGGATGAGTCTA-3'	54	58
	N	5'-GAACTTCAGGGTGAGTCTA-3'	54	58
IVS-II-745 (C-G)	Mt	5'-AATCCAGGTACCATTCTGC-3'	55	65
	N	5'-AATCCAGCTACCATTCTGC-3'	55	65
IVS-II-848 (C-A)	Mt	5'-TTCCTCCCAAAGCTCCTGG-3'	59	61
	N	5'-TTCCTCCCACAGCTCCTGG-3'	61	63
FSC-8/9 (+G)	Mt	5'-GAGGAGAAGGTCTGCCGTT-3	58	64
FSC-8	N	5'-TGAGGAGAAGTCTGCCGTT-3'	58	62
FSC 6 (-A)	Mt	5'-TGACTCCTGGAGAAGTCT-3'	57	62
FSC-8	N	5'-TGAGGAGAAGTCTGCCGTT-3'	58	62
-87 (C-G)	Mt	5'-GAGCCACACGCTAGGGTTG-3'	62	65
-88	N	5'-GGAGCCACACCCTAGGGTT-3'	62	65
Cd 121 (G-T)	Mt	5'-TTTGGCAAATAATTCACC-3'	47	52
	N	5'-TTTGGCAAAGAATTCACCC-3'	49	56
Poly A (TAA-TGA)	Mt	5'-TCTGCCTAACAAAAAACAT-3'	49	53
	N	5'-TCTGCCTAATAAAAAACAT-3'	49	53

IV. METHODS

A. DNA Extraction

1. DNA Extraction from Blood By NaCl Method

Peripheral blood samples of the probands are collected into vacutainer tubes coated with EDTA. They are stored at -4°C if to be extracted immediately or the next day, otherwise they are frozen at -20°C or -70°C until the extraction is done.

Blood samples are allowed to thaw and are transferred to 50 ml polypropylene centrifuge tubes (Sorvall). To 10 ml of blood sample 30 ml of RBC lysis buffer (stored at 4°C) is added and shaken well. The samples are then kept at 4°C for 15 minutes to allow lysis of the erythrocyte membranes. They are then centrifuged at 5000 rpm at 4°C for 10 minutes using an SS-34 rotor in Sorvall RC-5B superspeed centrifuge. This centrifugation collects the leukocyte nuclei which are resuspended in 10ml RBC lysis buffer by vortexing. At this step, the nuclei may be stored at -70°C until further extraction. Otherwise the resuspended leukocyte nuclei are recentrifuged at 5000 rpm at 4°C for again 10 minutes. If the RBC debris still appears to be too much, this step may be repeated for a second or third time to remove it as quantitatively as possible. The less RBC debris in the reaction mix, the easier it is to isolate the DNA. After discarding the supernatant containing the RBC debris, the pellet is resuspended in 3 ml of nuclei lysis buffer and vortexed. A strong detergent SDS (Sodium Dodecyl Sulphate) with a final concentration of 0.14 per cent, is added in order to dismantle the tough nuclear membrane for the release of DNA. Proteinase K with a final concentration of 150 µg/ml is also added to help the degradation of the proteins. The mixture is gently mixed and incubated for three hours at 56°C or for overnight at 37°C. Then 5 ml of sterile H_2O and 5 ml of NaCl are added and the mixture is shaken gently to precipitate out the proteins and dehydrate the DNA before centrifugation. Centrifugation at this step which is the final step before the actual DNA isolation, is performed at 10,000 rpm for 20 minutes in an SS-34 rotor and at room temperature. At this final stage the supernatant is very important, for it contains the DNA, and it is transferred into a clean Falcon tube. The DNA is precipitated out by adding two volumes of absolute ethanol, the tube is gently inverted for several times until DNA threads become visible floating slowly to the top. The

DNA is then fished out into a clean Eppendorf tube containing 250 - 1000 µl of TE buffer and allowed to dissolve by gently shaking and tapping the tube with a finger.

2. DNA Extraction from Chorionic Villi

If there are enough chorionic villi cells (~40 mg), they are immediately fished out by a Pasteur pipette and transferred into a clean 1.5 ml Eppendorf tube. If there are only a few cells, the sample is centrifuged to separate the cells from the supernatant. Then 0.5 ml NaCl/EDTA lysis buffer, 0.1% SDS and Proteinase K (50 μg/ml) are added and mixed well to lyse the cells. The suspension is incubated at 56°C for 3 hr or at 37°C for o/n. After incubation, the nuclei are treated twice with a 1:1 mixture of phenol/chloroform. In order to remove phenol, two additional extractions are performed with chloroform, mixed with isoamylalcohol in a ratio of 24:1. DNA is precipitated by adding 0.4 M ammonium acetate and two volumes of ethanol; then it is dissolved, depending on the yield, in 10 - 100 μl of sterile distilled water.

3. Qualification and Quantification Analysis

a. Spectrophotometry

The amount and concentration of the isolated DNA is determined by spectrophotometry. The formula below is used, which is based on the fact that 50μg of dsDNA has an absorbance of 1.0 at 260 nm (OD_{260}):

$$50\mu g/ml \times OD_{260} \times \text{Dilution Factor} = \text{Concentration in } \mu g/ml$$

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, and values less than 1.8 protein contamination.

b. Gel Electrophoresis

Gel electrophoresis is a standard method for the separation and identification of DNA fragments according to their molecular weights. This separation is done, by loading the DNAs onto an agarose gel, and running them from cathode to anode. DNA molecules run towards the positively charged electrode, because of the presence of negatively charged phosphate groups at the backbone of the DNA. The presence of the fluorescent dye EtBr in the agarose gel, enables the DNA bands to be observed under UV light. This is possible because EtBr, intercalates between the stacked DNA bases and when exposed to UV-light, the dye, emmits a fluorescent light, thereby showing the DNA bands. A polaroid photograph of the gel is then taken by using transmitted UV-light.

B. Polymerase Chain Reaction (PCR)

The PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, which uses two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by DNA polymerase result in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. This method was invented by Kary Mullis in the mid 1980s and like DNA sequencing, it has revolutionized molecular genetics by making possible a whole new approach to the study and analysis of genes. The technique was originally applied to the amplification of the β-globin DNA and the prenatal diagnosis of sickle cell anemia by scientists at Cetus (Erlich, 1992).

In the PCR, initially the Klenow fragment of the E. coli DNA polymerase I enzyme was being employed. The draw-back, however, was that this enzyme being heat-sensitive is destroyed at temperatures required for the separation of the double-stranded genomic

DNA. Consequently, fresh enzyme had to be added manually in each step, thereby making the whole process laborious.

The discovery of a hot spring bacterium, the *Thermus aquaticus* that lives in water at high temperatures as far as 75°C, transformed the PCR into a single and robust reaction. This is so, because the DNA polymerase from this bacterium has a temperature optimum activity at 72°C and besides, it is reasonably stable at 94°C (Watson et al., 1992), a temperature which is very important for the separation of the double-stranded genomic DNA. Because of this capability of the*Taq* polymerase enzyme, as it is normally abbreviated, it was thought worthwhile to transform the originally manual exercise into an automated process by a thermo-cycling device. Thus the *Taq* polymerase and the reaction components (template, primers, dNTPs, buffer and $MgCl_2$) can be placed in a thermo-cycler with the *Taq* polymerase remaining active throughout the complete set of cycles without any manual intervention.

Prior to amplification, the genomic DNA samples always undergo a series of dilutions since the amount of DNA needed for PCR is very small; usually less than a microgram of the total genomic DNA is sufficient for the reaction. In the framework of this study, the DNA amount employed for PCR, is between 0.1-0.5 µg. Two oligonucleotide primers (50-100 pmole each) directing the starting points for DNA synthesis and 1-2 units of Taq polymerase, a mixture of all the four deoxynucleotide precursors and $MgCl_2$ are added to the tube containing the genomic DNA. The total volume is usually 50 µl. Normally one master mix is prepared in which all the PCR components except the genomic DNA samples are present. The master mix is then divided into PCR tubes each containing a different genomic DNA. To prevent evaporation during the reaction, 2-3 drops of mineral oil are added on top and the samples are centrifuged briefly. Then they are put in the thermo-cycler in which the tubes go through an initial denaturation step and then through a repeated number of cycles of primer annealing, DNA synthesis and denaturation. The target sequence doubles in concentration in each cycle (Figure IV.1).

Temperature (°C)	Time	
94	7 min	
94	30 sec	} 30 cycles
55	30 sec	
72	2 min	
72	7 min	

To check the success of amplification, a 5 μl aliquot of the PCR product is mixed with loading buffer to a final concentration of 1X and electrophoresed on a 1 per cent agarose gel.

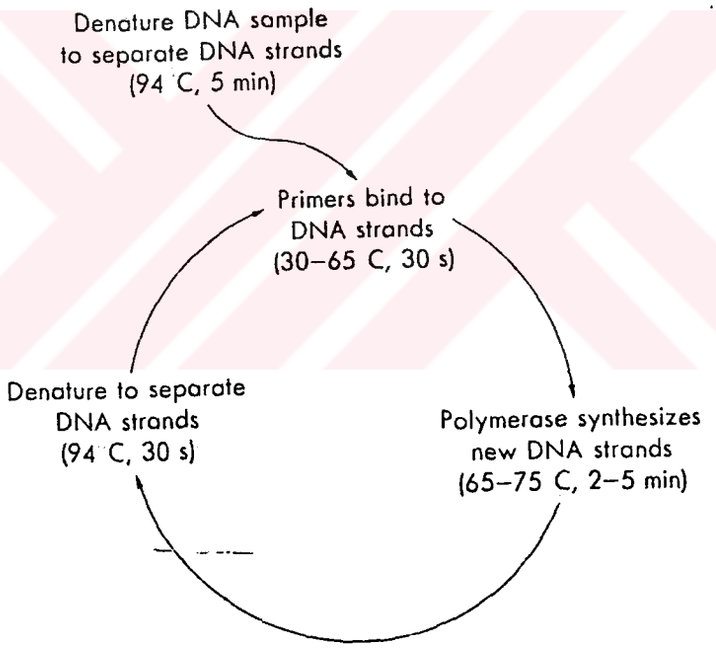


Figure IV.1 The PCR cycle. (Watson et al., 1992).

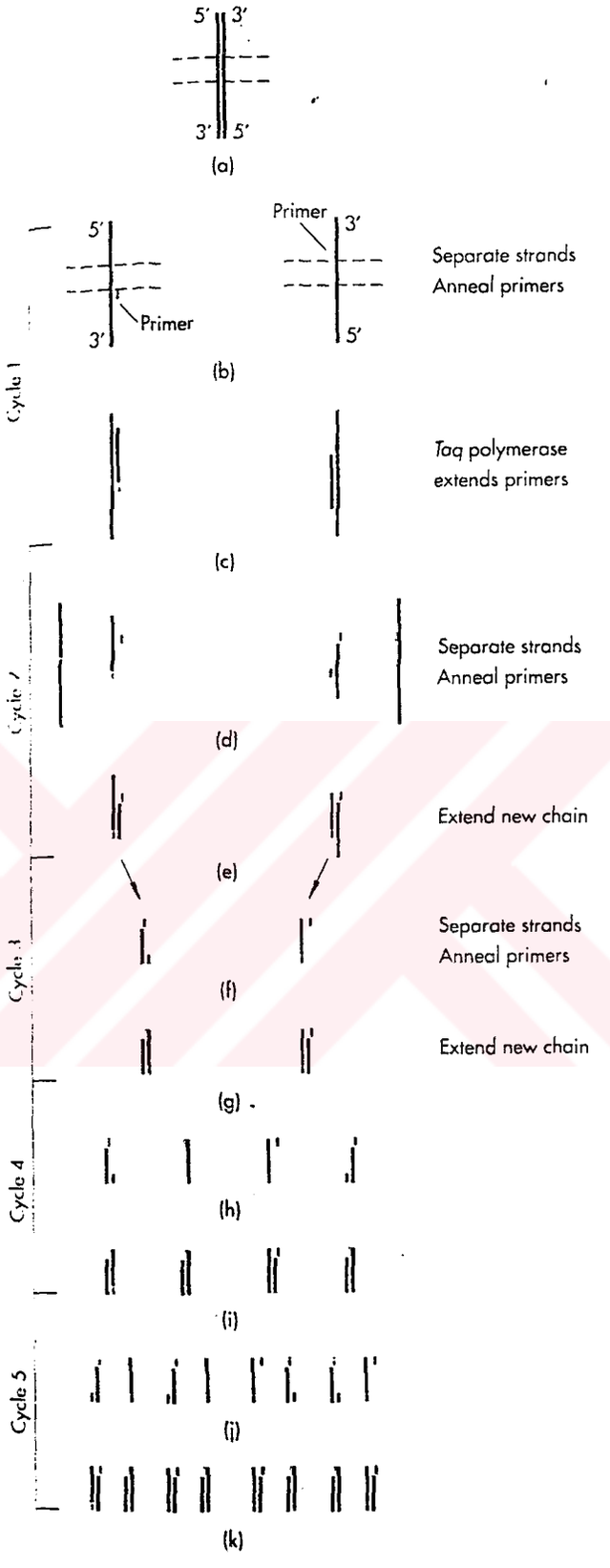


Figure IV.2 A schematic representation of the Polymerase Chain Reaction showing how the number of amplified target sequence fragments subsequently double for each cycle of the reaction. (Watson et al., 1992)

C. Analysis of Known β-Thalassemia Mutations

1. Dot-Blot Analysis

An important advance in the ability to directly detect small genetic lesions was made a few years ago when a technique based on the differential hybridization of short, radioactively labelled oligonucleotides was introduced (ASO hybridization). This highly sensitive and simple method allows the rapid analysis of allelic variation on minute amounts of DNA, the sensitivity of oligomer hybridization assays being significantly enhanced through the selective amplification of short genomic DNA fragments containing the target sequences. Synthetic DNA probes, typically 19 bases in length are designed to span the region of the gene where the aberrant sequence is located. When used under appropriate conditions, such probes will anneal only to sequences with which they are perfectly matched; the mismatch of a single base pair is sufficiently destabilizing to prevent hybridization. Thus two of these alleles-specific oligonucleotide probes, one specific for the normal sequence and the other for its mutant counterpart, can directly determine the genotype of a DNA sample at that locus. However because of the complexity of human genomic DNA and inefficiencies in hybridization that are inherent in short oligonucleotide probes, microgram quantities of sample DNA, gel electrophoresis and highly radioactive DNA probes are required for this approach (Saiki et al., 1988).

a. Preparation of the Blots

The blotting membranes are cut according to the size of the dot-blot apparatus, that is 8.5 cm by 12.5 cm. Before the membrane is fixed into the apparatus, it is soaked for 10 minutes in distilled water. Then the apparatus is tightly secured to ensure that there is no cross-contamination of the samples from well to well. Once tightly secured, it is then connected to a vacuum pump. The dot-blot membrane is divided into two equal parts whereby, the left hand side is considered as the mutant side and the right hand side as the normal. Thus half of the PCR product is applied into the mutant side and the other half into the normal side.

The amount of PCR product used in dot-blot hybridization is dependent upon the strength of the PCR band showing in the EtBr stained agarose gels. The amplified DNA

samples are transferred into clean Eppendorf tubes and the volumes are adjusted to 360 µl with distilled sterile water and finally 40 µl of a denaturant (4N NaOH and 250 mM EDTA) is added. The reactions are then centrifuged and denaturation is enhanced by incubating the tubes at 95°C for 10 minutes. Prior to application of the samples into the wells, the latter are tested with 50 µl of distilled H_2O or TE buffer, then the samples are carefully loaded (200 µl) into each well and vacuum is applied. When all samples are blotted, the wells are rinsed by adding 50 µl of 0.4N NaOH. This again enhances further denaturation of the dsDNA transferred onto the membrane which are then removed from the blotting apparatus and briefly rinsed in 2xSSC and dried. Then they are put between Whatman paper and baked at 80°C in a vacuum oven for 1 hour in order to firmly fix the DNA samples onto the membranes.

b. Labeling of the Oligonucleotide Probes

Synthetic oligonucleotide probes (ASO probes), each specific for a different β-thalassemia mutation are labeled at their 5' ends with T_4-Polynucleotide Kinase (T_4-PNK) and γ-[^{32}P]-ATP. The enzyme catalyses the transfer of the γ-phosphate of ATP to the 5'-OH terminus of the probe.

$$\text{5' OH-DNA} \xrightarrow[\gamma\text{-}[^{32}\text{P}]\text{-ATP}\ \text{Mg-ions}]{T_4\text{-PNK}} \text{5' } [^{32}\text{P}]\text{-DNA} + \text{ADP}$$

The labeling reaction is performed in a total volume of 20 µl, containing 100 pmoles of oligonucleotide probe, 1X PNK buffer, 0.5 mM spermidine, 20-30 µCi of γ-[^{32}P]-ATP and 12 units of T_4-PNK enzyme. The reaction is incubated at 37°C for 1.5 hours.

c. Probe Purification by Nensorb Chromatography

After labelling of the synthetic ASO probe, proteins, salts, free γ-[^{32}P]-ATP as well as unincorporated nucleotides and other low molecular weight materials have to be removed. Purification is performed by means of prepacked Nensorb-20 cartridges according to the following procedure: The loose resin inside the cartridge is washed with 100 per cent methanol applying a constant gentle pressure with a plastic syringe. The column is then primed with 2 ml of reagent A and it is ready for sample application. Prior to sample loading, 400 μl of reagent A is added to 20 μl of labeling reaction, and the sample is then applied directly onto the column bed. In this step, all nucleic acids and proteins bind tightly to the column. By rinsing the column with 2 ml of reagent A, the sample is then washed clean from the impurities and then it is thoroughly rinsed by adding 2 ml of distilled sterile water. Probe elution is performed by passing 500 μl of 20 per cent n-propanol, a nonpolar solvent, through the column which elutes the oligonucleotides off the column. The final product is collected into a 1.5 ml Eppendorf tube and 1/100th of the total sample volume is counted in the scintillation counter. The counts of both probes are compared, and if they are not labeled in the same amount, they are "balanced"; this means, care is taken that both mutant and normal probes have exactly the same amount of radioactivity, when they are used for hybridization. The probe which has counts in excess, is decreased in its volume according to the following example:

Mutant probe: 10 million cpm/500 µl
Normal probe: 12 million cpm/500 µl

In this case, all mutant probe is used, whereas only 416 µl, corresponding to 10 million cpm, is taken from the normal probe for hybridization.

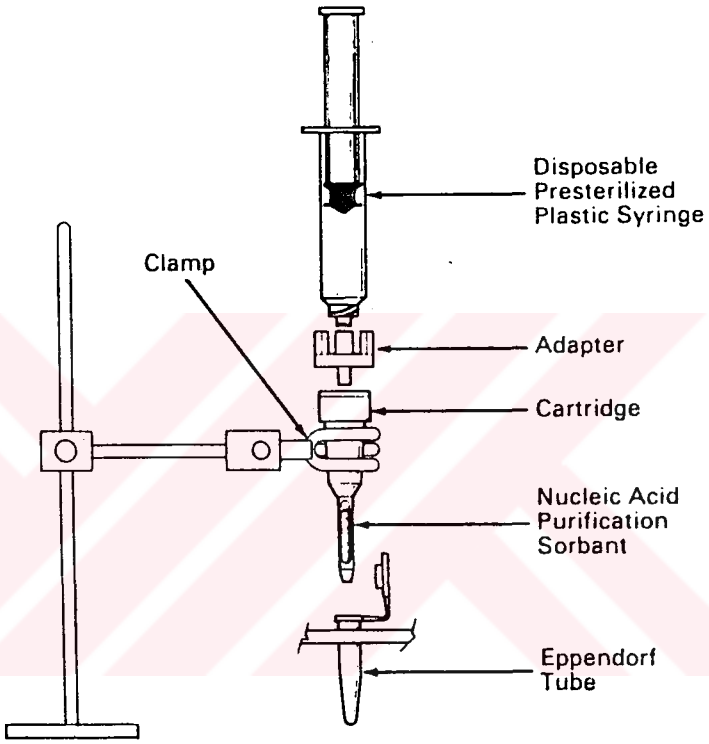


Figure IV.3 Manual use of the DuPont NENSORB™ 20 nucleic acid purification cartridge.

d. Trichloroacetic Acid (TCA) Precipitation

TCA precipitation is carried out in cases, when column chromatography is not available, to determine how much of the radioactivity is incorporated into the probe. Thus 1 μl of the labelled probe (mutant and normal separately) is mixed with 99 μl of sterile water. From this mixture, 5 μl is pipetted onto a GF/C filter and allowed to dry. Four filters, two for each probe are prepared, one of each to be TCA precipitated, the other serving as a reference.

The two filters for TCA precipitation are placed in 10-15 ml of a 10 per cent TCA solution at 4°C for 15 minutes and are shaken at intervals. After 15 minutes, the solution is replaced by a fresh one and the procedure is then continued for another five minutes. The filters are then washed in 70 per cent EtOH and dried. After this step the probe, labelled with γ–[^{32}P]-ATP, is supposed to bind to the filter, whereas the excess radioactivity, not bound to the probe, is washed away. Counting of the filters is done in the scintillation counter (i.e. mutant filter with TCA ppt., mutant filter without TCA ppt.; normal filter with TCA ppt., and normal filter without TCA ppt.) and the difference between incorporated and unincorporated γ-[^{32}P]-ATP is calculated.

e. Hybridization and Autoradiography

The procedure employs synthetic oligonucleotides as hybridization probes which identify homologous DNA sequences fixed onto the membranes. Hybridization occurs when there is base pairing between the synthetic ASO probes and the genomic DNA, governed by the complementary hydrogen bonding of the nucleic acids, giving rise to DNA duplexes. Thus if upon hybridization of the DNA of a patient to a normal probe, a DNA duplex is formed then the DNA is normal, and no duplex formation occurs if it is mutated. Similarly if the corresponding DNA is having a sequence complementary to the mutant probe, there is hybridization, giving rise to a DNA duplex formation. The stability and stringency of binding of the mutant ASO probe to the DNA on the membrane is enhanced by fixing the mutation at the middle of the 20 mer synthetic ASO probes. The procedure consists of four main steps:

Prehybridization: This is the step in which the two identical membranes are primed in the prehybridization/hybridization buffer for the main hybridization step. This step is carried out at a temperature, which is usually 1°C below the Tm of the probe in use

(T_m-1), for 30 minutes. The Tm for each probe is determined according to the formula:

$$T_m = 4 (G + C) + 2 (A + T)$$

Hybridization: Prior to hybridization, both mutant and normal probes, are re-counted in the scintillation counter and care is taken that they are properly "balanced". After pipetting each probe into the corresponding container, hybridization starts. It is given a period of 1.5 hours and the same temperatures used for prehybridization are applied.

First washing: Before starting the washing procedure, the purpose of which is to wash away the excess counts, caution is taken to put back the probes into their original tubes in which they are stored for other hybridizations. While still in their hybridization containers, the membranes are rinsed with 100 ml of "first wash solution" and this solution is put into the ^{32}P-containing radioactive waste. Both membranes are then transferred into the same washing container and are washed with 200 ml of "first wash solution" at r.t. using a shaking platform. Since these washes are not as hot as the first rinsing, the solutions can be poured into the sink; however, they have to be diluted away with plenty of tap water. The washing is repeated for four times, each for 10 minutes, and the washing solution is replenished every time.

Stringent washing: This is a very important step in which the nonspecifically bound probes are washed away so that only the perfectly matched probe remains bound to the DNA on the membrane. The stringent washing is carried out in the "last wash solution" at temperatures 1-4°C above the Tm of the probes, for 15 minutes.

After the last wash, the membranes are allowed to dry at r.t. or blow-dried with a hair drier prior to autoradiography. The dried membranes are then packed in saran-wrap, placed in a film cassette and an X-ray film is put on top. The cassette is stored at -70°C usually for o/n, depending on the activity of the probe. Radioactivity causes darkening of the film, indicating the position of homologous sequences as dark spots. The X-ray film is developed manually for 1.5 minutes until the dark dots appear. After water rinsing for 20 seconds, the film is fixed for 1-5 minutes and then extensively washed under tap water.

After the autoradiograms are evaluated, the membranes are "stripped," that is the radioactivity is removed by washing the membranes in "stripping solution" at 95°C for 20 minutes. Then the membranes are rinsed with distilled water and screened with the Geiger counter for the absence of counts. If necessary, the procedure is repeated. When they are devoid of radioactivity, the membranes are rinsed with distilled water, dried at r.t., and then stored between two layers of Whatman paper until the next hybridization.

V. RESULTS

A. Screening for β-thalassemia Mutations and Evaluation of the Data

1. Patients

A total of 113 β-thalassemia patients and their family members sent to our laboratory from various hematological centers in Izmir, Antalya and Adana were investigated in this study. Out of these 113 subjects, 62 were from Izmir (112 β-thal chromosomes), 32 from Antalya (58 β-thal chromosomes) and 19 from Adana (21 β-thal chromosomes) (Table V.1). Thus a total number of 191 chromosomes were investigated in the framework of this study.

Table V.1 The total number of subjects and chromosomes studied in the three distinct districts of Izmir, Antalya and Adana.

	Izmir	**Antalya**	**Adana**
β-thalassemia patients	50	26	2
carriers	12	6	17
# chromosomes	112	58	21

2. Amplification of the β-Globin Gene

The β-globin gene is a relatively small gene consisting of a promoter region, three exons, two intervening sequences and a 3' terminal end, known as the poly A tail, all of which span together a region of approximately 1.8 kilobases. In the 5' end of the gene, which includes the promoter, Exon I, IVS I and Exon II, there is a hot spot of recombination, where the majority of the mutations are located. In the framework of this study, in which the 5' region of the gene was investigated prior to the 3' region, the amplification primers KM29, F108 and Common C were used as forward, and RS43, R109 and #31 as reverse primers. For the 3' region, the primers TCG1 and CD1 were used as forward and TCG2 and CD2 as reverse primers (Figure V.1).

Approximately 1 μg of DNA was amplified with the first set of primers, which was then subjected to dot-blot hybridization, using ASO probes complementary to known mutations in this region. The 3' end of the gene was only amplified in those samples, in which the mutations could not be revealed by the above approach. These samples were analyzed with an additional set of oligonucleotide probes, specific for the IVS-II, Exon 3 and the Poly A region of the gene.

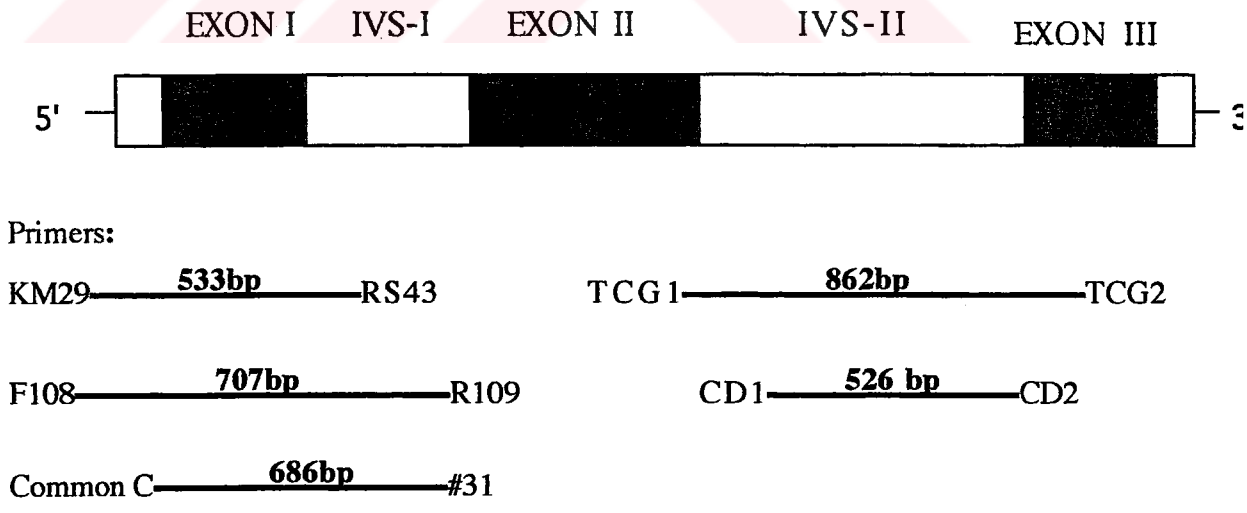


Figure V.1 A schematic diagram representing the β-globin gene and the location of the amplification primers.

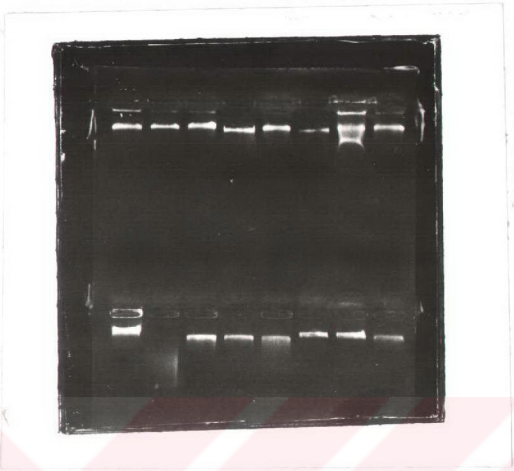


Figure V.2 Quantification of genomic DNA samples run on a 1 per cent agarose gel.

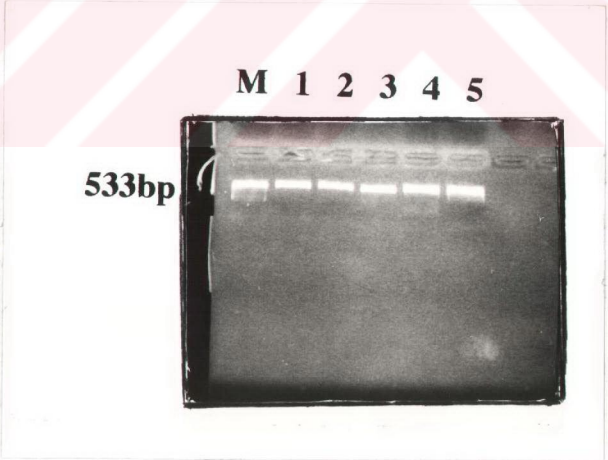


Figure V.3 Agarose gel electrophoresis of PCR products amplified with primers KM29 and RS43: 1/10 of each PCR product is run on a 1 per cent agarose gel containing EtBr; the target sequence in this case is 533bp long.

3. Dot-Blot Analysis

A total of 113 subjects including β-thalassemia patients and their heterozygous relatives, were analyzed in the framework of this study using 19 different ASO probes, specific to the Mediterranean populations. For each mutation, two probes, one complementary to the defective and the other to the normal allele, were used. After autoradiography, samples that hybridized only to the normal probe were considered as not having a mutation at that locus, while samples that hybridized only to the mutant probe as having the particular mutation on both chromosomes and those that hybridized to both probes as having the mutation only on one chromosome.

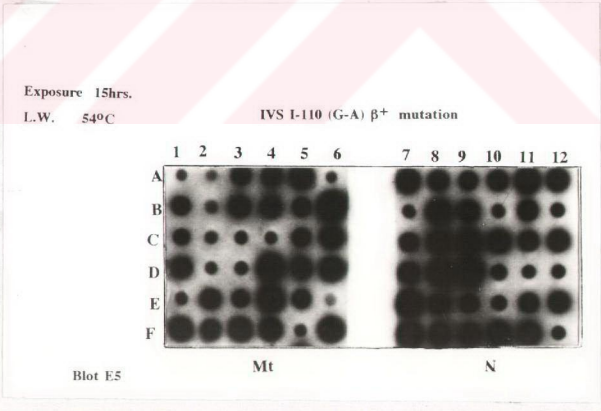


Figure V.4 Identification of the IVS-I-110 mutation by dot-blot analysis; on this blot sixteen patients are heterozygous and eight are homozygous for this mutation, which is very predominant in Turkey.

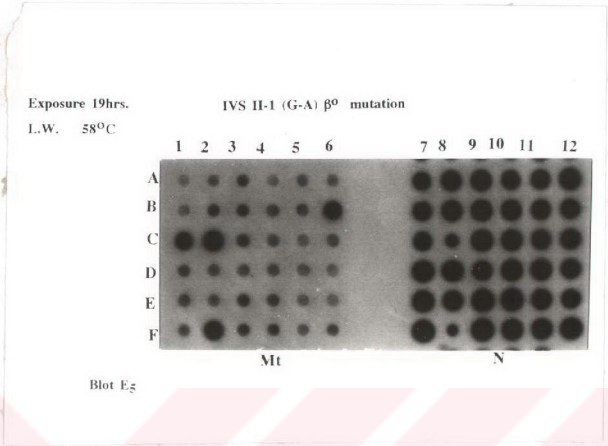


Figure V.5 Identification of IVS II-I by dot-blot hybridization. B6/B12 and C1/C7 are carriers, while C2/C8 and F2/F8 are homozygous for this mutation.

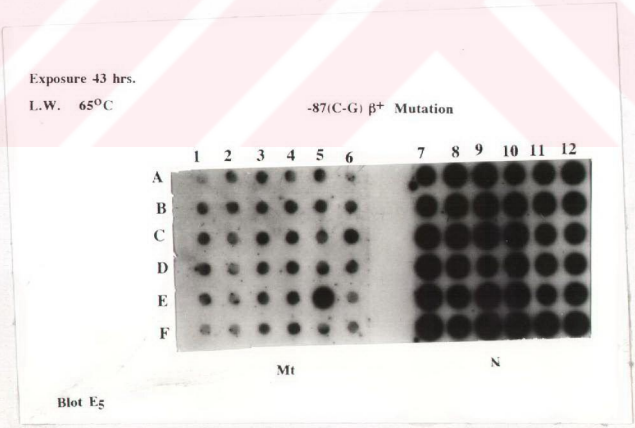


Figure V.6 Identification of the rare mutation -87 by the same procedure. Only one patient, E5/E11 is a carrier for -87; all the other patients on this blot do not carry this mutation.

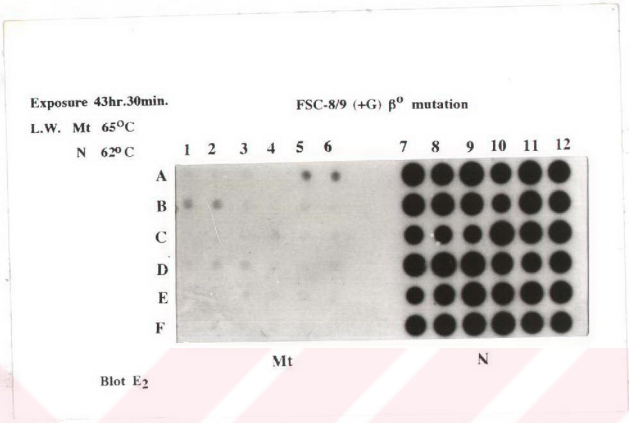


Figure V.7 Dot-blot analysis for the FSC-8/9 mutation; all patients on this blot are negative for this rare mutation.

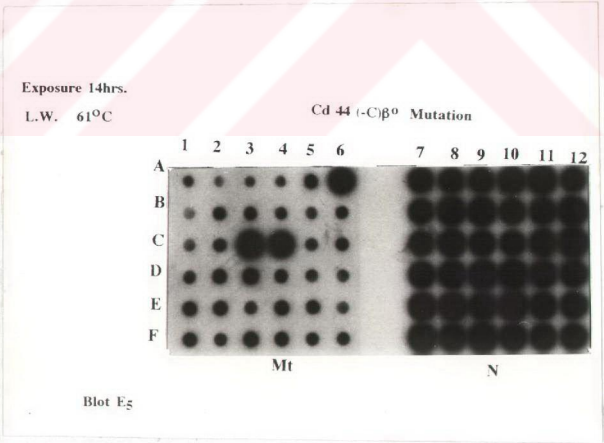


Figure V.8 Identification of Cd44: A6/A12 and C3/C9, C4/C10 (husband and wife) are carriers for this mutation, which until recently was thought to be exclusive for the Kurdish Jewish population living in Israel.

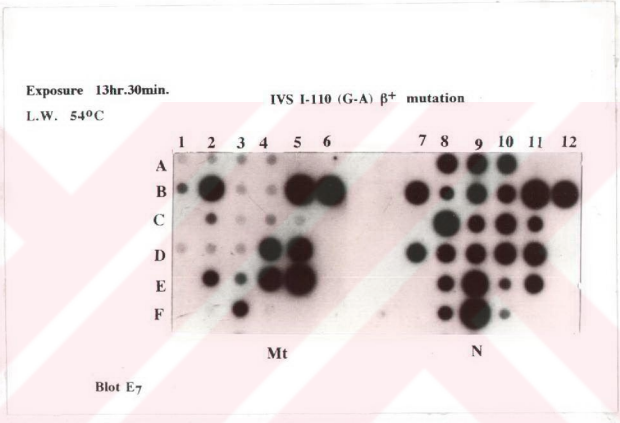


Figure V.9 Prenatal diagnosis by ASO hybridization : B2/B8 is a child who is homozygous for IVS-I-110. The fetus, B1/B7, of the same family does not carry the mutation, which means that the pregnancy may continue.

In the framework of this thesis, 191 chromosomes from three distinct districts of Turkey, Izmir, Antalya and Adana, which as large cities, are thought to be representative for their close environments, were investigated, using 19 different oligonucleotide probes specific for Mediterranean countries. The distribution pattern of these mutations in these three regions are compiled in Tables V.2, V.3, V.4 and will be discussed here in more detail.

In the patients from Izmir, who were screened for a total of 19 mutations, nine different lesions (HbS not included) were found; this accounts for 70.6 per cent of the diseased gene (Table V.2). The results obtained show, that IVS-I-110 is the most common mutation, with a frequency of 33.9 per cent, followed by IVS-I-6 (14.3 per cent); IVS-I-1 comes on third position with 9.8 per cent, while IVS-II-745 ranges at position four (4.5 per cent) and Cd 39 at position five (2.7 per cent). The remaining four mutations, -30, IVS-II-1, FSC-8 and -87, all of which are represented with only one or two chromosomes, have frequencies below 2 per cent and HbS an occurrence of 2.7 per cent.

Seven different mutations were found in the patients from Antalya, accounting for 82.8 per cent of the 58 diseased chromosomes studied (Table V.3). The results obtained show that IVS-I-110 is by far the most predominant mutation with a frequency of 48.3 per cent. It is followed by IVS-I-6 (12.1 per cent), IVS-II-1 (6.9 per cent) and IVS-II-745 (5.2 per cent). The other three mutations, Cd 39, -30 and Cd 44, were observed in only two chromosomes each in Antalya.

The data from Adana may not be as representative as it is the case for Izmir and Antalya, since the chromosome number analyzed in patients from this district, is only 21 (Table V.4). In total, six different β-thalassemia and the HbS mutations were found in these patients, accounting for 52.4 and 28.6 per cent of the diseased genes, respectively. IVS-I-110 is also here the most common mutation with a frequency of 23.7 per cent. The other five β-thalassemia mutations encountered in this district are, IVS-II-745, IVS-I-6, IVS-I-1, Cd 39 and Cd 44. The high incidence of HbS in Adana is not surprising.

Table V.5 compiles the mutations found in the three districts. This table again emphasizes the predominance of IVS-I-110 in Turkey with a frequency of 37.2 per cent in the patients studied in the framework of this thesis. IVS-I-110 is followed by IVS-I-6 with 12.6, IVS-I-1 with 6.3 and IVS-II-745 with 5.2 per cent. IVS-II-1 and Cd 39 are both ranging at position five (3.1 per cent). The remaining four mutations, -30, Cd 44, FSC-8 and -87 are only represented in frequencies of 2.1, 1.6 and 0.5 per cent, respectively. Although screened with 19 β-thalassemia probes, only 10 mutations were found in the three districts studied and the mutations FSC-6, FSC-5, FSC-8, IVS-I-5 (G-A), IVS-I-5 (G-C), IVS-I-116, IVS-II-848, Cd 121 and Poly A were not observed in any of them.

Figure V.10, is a bar chart representation of Tables V.2, V.3 and V.4, showing the frequencies of the mutations studied. This figure clearly demonstrates that IVS-I-110 is by far the most frequently occurring mutation in all the three districts. It also indicates that

Antalya has the highest frequency of IVS-I-110 (48.3 per cent), followed by Izmir (33.9 per cent). IVS-I-6 ranges at position two, with the highest frequency observed in Izmir, seconded by Antalya. On the other hand IVS-I-1, represented with almost 10 per cent in Izmir, is not present at all in the patients from Antalya. IVS-II-1 in contrast, seems to be common in Antalya and very lowly represented in Izmir. IVS-II-745, as shown by the chart, is found in all three districts and has a high frequency (9.5 per cent) in Adana, where it ranges at position two. The presence of Cd 44 mutation in Antalya and Adana (3.4 per cent) which was reported to be exclusive for the Kurdish Jewish population, living in Izrael, until recently, seems to be interesting indicating the complex admixture of several ethnic groups in this region. According to our results and as expected this mutation is not represented at all in Izmir.

As many as 50 per cent of the patients studied are homozygous for one mutation ("true homozygotes"), whereas approximately half of them are homozygous for the IVS-I-110 mutation. Among the compound heterozygotes, there are many combinations involving IVS-I-110 with IVS-I-6, IVS-I-1 and IVS-II-745. In general, it is observed that, there is a greater number of β^{+}-chromosomes in all districts investigated, as opposed to β^{o}-chromosomes.

Table V.2 The distribution pattern of β-thalassemia mutations in patients from Izmir (112 chromosomes)

Mutation*	Type	# Chromosomes	Frequency %	
IVS I-110 (G-A)	β^+	38	33.9	
IVS I-6 (T-C)	β^+	16	14.3	
IVS I-1 (G-A)	β^o	11	9.8	
IVS II-745 (C-G)	β^+	5	4.5	
Cd 39 (C-T)	β^o	3	2.7	
-30 (T-A)	β^+	2	1.8	
IVS II-1 (G-A)	β^o	2	1.8	
FSC-8 (-AA)	β^o	1	0.9	
-87(C-G)	β^+	1	0.9	Σ 70.6%
HbS		3	2.7	
Unknown		30	26.7	
Total		112	100	

*The mutations which were looked for but not found in these patients are :

IVS-I-5 (G-A)
IVS -I-5 (G-C)
IVS-I-116 (T-G)
FSC-5 (-CT)
FSC-6 (-A)
FSC-8/9 (+G)
Cd 44 (-C)
IVS-I-848 (C-A)
Cd 121 (G-T)
Poly A (AATAAA-AATGAA)

Table V.3 The distribution pattern of β-thalassemia mutations in patients from Antalya (58 chromosomes).

Mutations*	**Type**	**# chromosomes**	**Frequency %**	
IVS I-110 (G-A)	β^+	28	48.3	
IVS I-6 (T-C)	β^+	7	12.1	
IVS II-1 (G-A)	β^o	4	6.9	
IVS II-745 (C-G)	β^+	3	5.2	
Cd 39 (C-T)	β^o	2	3.4	
-30 (T-A)	β^+	2	3.4	
Cd 44 (-C)	β^o	2	3.4	Σ 82.8 %
Unknown		10	17.2	
Total		58	100	

*The mutations which were looked for but not found in these patients are :

-87 (C-G)
IVS-I-1 (G-A)
FSC-8 (-AA)
FSC-8/9 (+G)
FSC-5 (-CT)
FSC-6 (-A)
IVS-I-5 (G-A)
IVS-I-5 (G-C)
IVS-I-116 (T-G)
IVS-II-848 (C-A)
Cd 121 (G-T)
Poly A (AATAAA-AATGAA)

Table V.4 The distribution pattern of β-thalassemia mutations in patients from Adana (21 chromosomes).

Mutation*	Type	# Chromosomes	Frequency %	
IVS I-110 (G-A)	β^+	5	23.7	
IVS II-745 (C-G)	β^+	2	9.5	
IVS I-6 (T-C)	β^+	1	4.8	
IVS I-1 (G-A)	β^o	1	4.8	
Cd 39 (C-T)	β^o	1	4.8	
Cd 44 (-C)	β^o	1	4.8	Σ 52,4%
HbS		6	28.6	
Unknown		4	19.0	
Total		21	100	

*The mutations which were looked for but not found in these patients are :

-30 (T-A)
-87 (C-G)
IVS-II-1 (G-A)
IVS-I-5 (G-A)
IVS-I-5 (G-C)
FSC-5 (-CT)
FSC-6 (-A)
FSC-8 (-AA)
FSC-8/9 (+G)
IVS-I-116 (T-G)
IVS-II-848 (C-A)
Cd 121 (G-T)
Poly A (AATAAA-AATGAA)

Table V.5 Distribution of mutations in three distinct districts of Turkey, Izmir, Antalya, and Adana.

Mutation	Type	# Chromosomes	Frequency %	
IVS I-110 (G-A)	β^+	71	37.2	
IVS I-6 (T-C)	β^+	24	12.6	
IVS I-1 (G-A)	β^o	12	6.3	
IVS II-745 (C-G)	β^+	10	5.2	
IVS II-1 (G-A)	β^o	6	3.1	
Cd 39 (-C)	β^o	6	3.1	
-30 (T-A)	β^+	4	2.1	
Cd 44 (-C)	β^o	3	1.6	
FSC-8 (-AA)	β^o	1	0.5	
-87 (C-G)	β^+	1	0.5	Σ 72.3
IVS I-5 (G-A)	β^+	0	0	
IVS I-5 (G-C)	β^+	0	0	
IVS I-116 (T-G)	β^o	0	0	
FSC-5 (-CT)	β^o	0	0	
FSC-6 (-A)	β^o	0	0	
FSC-8/9 (+G)	β^o	0	0	
IVS II-848 (C-A)	β^+	0	0	
Cd 121 (G-T)	β^o	0	0	
Poly A (AATAAA- AATGAA)	β^+	0	0	
HbS		9	4.7	
Unknown		44	23.0	
Total		191	100	

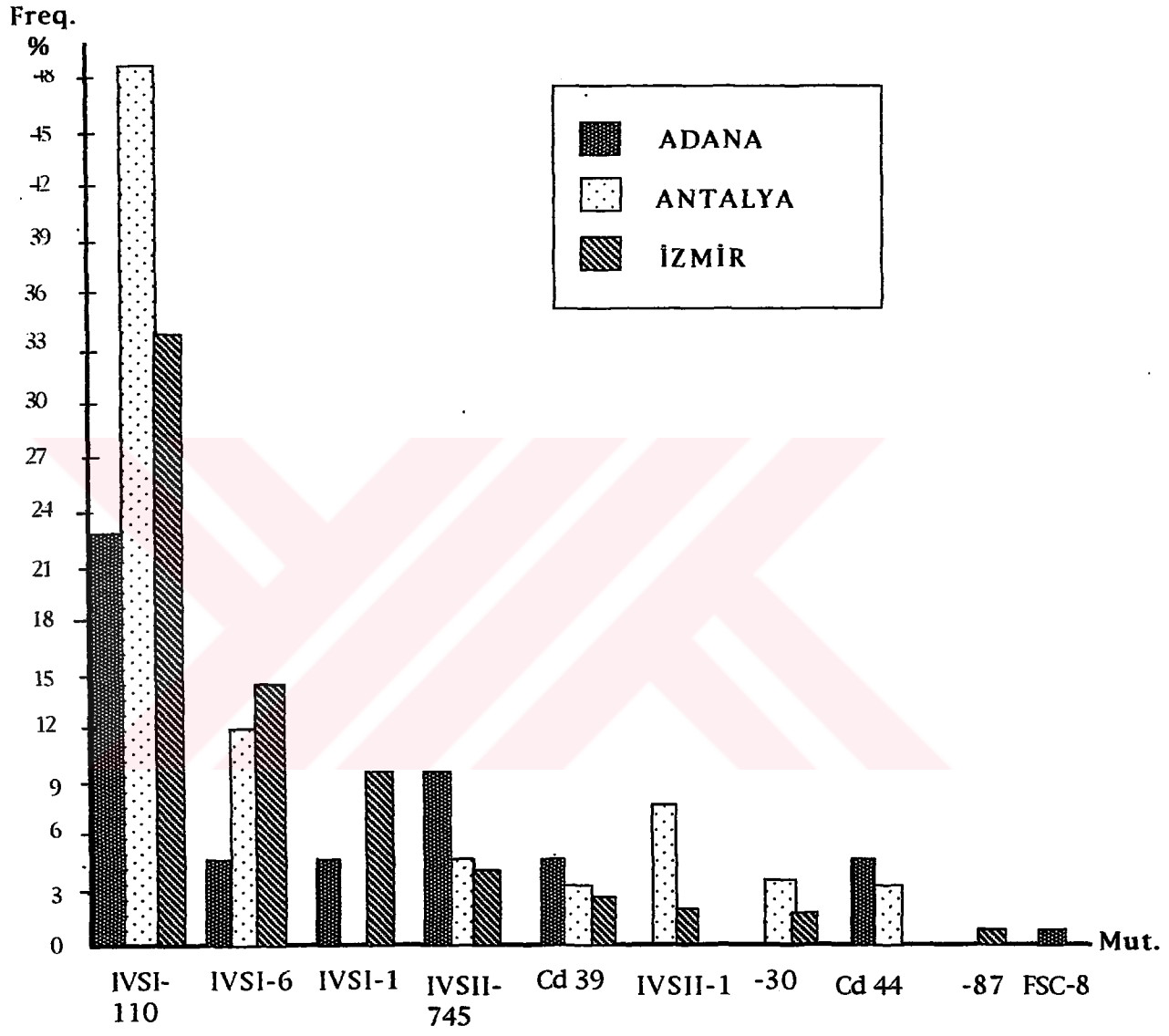


Figure V.10. Bar chart representation of Tables V.2, V.3 and V.4

VI. DISCUSSION

The high incidence rate, broad geographic distribution and clinical severity of the hemoglobinopathies make testing for carriers and prenatal diagnosis a clinical public health issue in the areas affected. Current hematological tests are adequate for the screening of carriers, but the identification of specific mutations and prenatal diagnosis during the first trimester can be accomplished only with a DNA based genetic test (Saiki et al., 1988).

Long before scientists realised the extreme genetic heterogeneity, clinicians recognized the wide variety of phenotypic expressions of the hemoglobinopathies, and they applied the terms thalassemia "major" and "intermedia" to define the existing differences in the severity of clinical manifestations (Chini and Valeri, 1949). At present, clinicians are greatly interested in exploring the patients' genotype since there is strong evidence that the phenotype of the thalassemia syndrome is basically related to the genotype. Certain genotypes are most commonly associated with the severe manifestations of thalassemia major while other genotypes are correlated with mild manifestations of thalassemia intermedia, and others possess even both severe and mild phenotypes. The variations in clinical severity are also associated with the presence of β^o or β^+ genes (Kattamis et al., 1982).

According to several studies performed by different research groups, β-thalassemia in most Mediterranean countries, is mainly caused by a few, six or less, prevalent mutations which account for more than 90 per cent of the β-thalassemia chromosomes in these countries (Wainscoat et al., 1983; Milland et al., 1987; Chehab et al., 1987; Rosatelli et al., 1987; Amselem et al., 1988; Cao et al., 1989; Kattamis et al., 1990 and Petkov et al., 1990). However, the Turkish population appears to display a relatively high degree of heterogeneity concerning β-thalassemia mutations (Diaz-Chico et al., 1988; Öner et al., 1990; Başak et al., 1991 and Başak et al., 1992). This study is in accordance with the above results.

The mutations prevalent in Izmir, Antalya and Adana, accounting for 73.3, 82.8 and 81.0 per cent of the diseased genes, respectively, do exhibit the presence of heterogeneity in these three districts. These findings are in total agreement with those of Atalay et al., (1993). In their research article, four regions of Turkey (Western, Northern, Southern and Eastern Anatolia) were investigated, and it was found that particularly Western and Southern Anatolia, reflected the country scale frequencies. Similarly, the findings in this study indicate that Izmir and Antalya, do exhibit an apparent reflection of the distribution pattern described before (Başak et al., 1992).

Consistent with the findings of Başak et al., (1992) and Atalay et al., (1993), IVS-I-110 and IVS I-6, which were found to be the two most frequent mutations in the Turkish population, have been shown to follow the same trend in this study. According to this thesis, the highest frequency of IVS I-110 is in Antalya with 48.3 per cent, followed by Izmir with 33.9 per cent. Although the number of patients from Adana is much less, compared to the other two districts, IVS I-110 is also found to be the most frequent mutation in this city.

IVS I-1, IVS I-6 and IVS II-745 are all Mediterranean specific mutations (Orkin et al. 1982a); IVS I-1 is seen mainly in Greece, Cyprus and Yugoslavia, while IVS II-1 is found mostly among Turkish patients (Huisman, 1990). The results obtained in this study demonstrate that IVS II-1 ranges at third position in Antalya (6.9 %), while in Izmir the same mutation comes on position seven, with a frequency of only 1.8 per cent. On the other hand, the mutation IVS I-1, found mainly in Greece and Yugoslavia, is fairly high in Izmir (9.8 %), coming on third position after IVS I-110 and IVS I-6, while IVS I-1 is not represented at all in Antalya. The severe β^+ mutation at IVS II-745, which has a high frequency in Bulgaria (Petkov et al., 1990) is reported to be present at rather low frequency in populations of the East Mediterranean coast (Huisman 1990). In this study IVS II-745 is represented in all the three districts, with a frequency of about 4.5 per cent.

In the past, there have been two generations of immigrants from Greece and Bulgaria to Turkey (Atalay et al., 1993); according to the findings in this study, the origin of IVS I-1, the incidence of which is significant in Izmir (9.8%), can be traced as being from these immigrants from Greece and Bulgaria, whereas the IVS-II-1 mutation seems to be mainly confined to Antalya.

In contrast to some findings by other researchers, where the FSC-8 mutation has been shown to be the third most frequent mutation in Turkey (Diaz-Chico et al., 1988; Başak et al., 1992), the results of this study are in agreement with those of Atalay et al., (1993), in which FSC-8 was found not to be represented at all in Western and Southern Anatolia. Similarly, in this study it is observed that in the districts of Adana and Antalya FSC-8 is not present at all while only one chromosome is observed in Izmir.

The FSC-44 had been reported to be an exclusive Kurdish-Jewish lesion (Rund et al., 1991 and Kinniburgh et al., 1982), until it was shown by Başak et al. (1992), that it is also present in Turkey. In this study, this mutation has been detected in three patients from Adana and Antalya.

The results obtained in the framework of this thesis indicate that the IVS I-110 mutation has the highest frequency of "true" homozygosity among the patients investigated; 64.5 per cent of the patients from Izmir and 50 per cent of the patients from Antalya have the IVS I-110 mutation on both chromosomes. These elevated figures can be explained by the high frequency of this mutation in Turkey, as well as by the frequent occurrence of

consanguineous marriages in the Turkish population (Başaran et al., 1988; Öner et al., 1990). The reason for the relatively high number of unknown chromosomes in all three districts may be caused by the fact that some of the DNA samples were too old, thus difficult to extract and to amplify, as a result of which the dots obtained in the autoradiography were too ambiguous to be interpreted. These cases have to be re-analyzed.

In their attempt to determine the regional distribution of the common β-thalassemia mutations in Turkey, Atalay et al., (1993), have concluded that there is no locus-specific heterogeneity in Turkey. It also appears from the results of this study, that no marked locus-specificity concerning the β-thalassemia mutations in the three districts of Izmir, Antalya and Adana is observed. However, some results in this thesis indicate an exception from the general trend, particularly in Izmir and Antalya, where IVS I-1 may be confined to Izmir and IVS II-1 to Antalya.

It may be concluded from the results of this study that :

i. β-thalassemia in Turkey is very heterogeneous at clinical as well as at molecular level;

ii. There is not an apparent locus-specific heterogeneity of β-thalassemia mutations in three distinct districts of Turkey, investigated in the framework of this thesis;

iii. An exception from the general rule concerning some mutations is observed in some areas; this may help to solve the molecular heterogeneity of Turkey to a certain extent.

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