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THE SPECTRUM OF β-THALASSEMIA MUTATIONS AMONG PALESTINIANS IN THE WEST BANK

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CHAPTER I

INTRODUCTION

Amazingly, in the language of numbers $3x10^{-9}$ could be meaningless, whereas regarding the DNA; a change in a single nucleotide among the 3 billion base pairs found in each cell can lead to detrimental consequences.

I.1 THE THALASSEMIAS

Thalassemia can be defined as a condition in which there is a reduced rate of synthesis of one or more of the globin chains leading to imbalanced globin chain synthesis, defective hemoglobin production, and damage to the red cells or their precursors from the effects of the globin subunits that are produced in excess [Weatherall, 1994]. Thalassemias can be classified according to the globin chain or chains the synthesis of which is deficient. The best characterized disorders are α-, β-, δ-, δβ-, γδβ-, εγδβ-thalassemia [Lee *et al.*, 1993; Weatherall, 1994].

I.2 THE EARLY HISTORY OF β-THALASSEMIA

Thalassemia is such a common condition, particularly in the Mediterranean region and Southeast Asia, that it must have been frequently seen by doctors before the first clinical descriptions in 1925 [Weatherall and Clegg, 1981]. However, there is very little evidence in the literature that

the condition was recognized as a specific entity before that time. Another interesting approach to the question of whether thalassemia occurred in historic or prehistoric times is the study of peculiar thalassemia-like bone changes in skulls found in ancient burial places, or mummies in a good state of preservation. There is a condition well-known to anthropologists called 'porotic hyperostosis' in which the radiological changes in the skull are very similar to those of homozygous thalassemia [Weatherall and Clegg, 1981]. Bone changes of this type have been found in skulls excavated in Sicily and Sardinia as well as in those of the ancient native populations of America, the Incas of Peru, Indians from Colombia, Aztecs from Mexico, Mayan Indians from the Yucatan, and from many other sites [Weatherall and Clegg, 1981].

I.3 THE FIRST DESCRIPTION OF β-THALASSEMIA

The first description of what became known as thalassemia by Thomas. B. Cooley of Detroit was written with his collaborator, Dr. Pearl Lee, and appeared on a single page [Cooley and Lee, 1925], entitled as "*A series of cases of splenomegaly in children, with anemia and peculiar bone changes*". The term 'thalassemia' was first used by Whipple and Bradford in 1932 in their classical paper on the pathology of the condition [Whipple and Bradford, 1932]. The word is taken from the Greek 'θαλασσα' meaning 'the sea', since all early cases were reported in children of Mediterranean origin. Over the next 20 years, it became apparent that Cooley and Lee had described the homozygous or compound heterozygous state for a recessive Mendelian disorder not confined to the Mediterranean but occurring widely throughout tropical countries [Olivieri, 1999]. By the late 1930's, the clinical syndrome of

thalassemia was well described. However, it seems likely that the first definite evidence that Cooley's anemia is a genetically determined disorder was provided by the Greek clinician Caminopetros in his important papers in 1936 and 1938 [Caminopetros, 1936, 1938]. Valentine and Neel named the mild form of Cooley's anemia 'thalassemia minor' [Valentine and Neel, 1944, 1948], while Gatto called it 'thalassemia minima' [Gatto, 1947, 1948], and Silvestroni and Bianco used the term 'microcytemia' [Silvestroni and Bianco, 1944, 1946]. Indeed, by the year 1949, it was already apparent that thalassemia is not a single disorder but a complex syndrome resulting from the interaction of more than one, and probably many, genetic factors [Weatherall and Clegg, 1981]. Hence, the stage was set for further analysis of thalassemia by studies of the hemoglobins of affected patients. The period following the late 1940's was of rapid progress in all aspects of the human hemoglobin field so that by 1959 it was possible for Ingram and Stretton to set out their famous theoretical model for the genetic basis of thalassemia [Ingram and Stretton, 1959].

I.4 THE β-GLOBIN GENE

The globin genes occur in clusters, where the α- and α-like genes, are located on the short arm of chromosome 16, and the β- and β-like genes are located on the short arm of chromosome 11 [Fritsch *et al.*, 1980]. The β-gene complex comprises 6 genes, physically linked within a 65 kilobase segment of human DNA, and arranged in the following 5′ to 3′ order according to their developmental expression: a single embryonic gene (ε), 2 fetal genes ($^{G}\gamma$

and $^{A}\gamma$), pseudo β-gene (ψβ), δ-gene and the β-gene [Lawn *et al.*, 1980; Lee *et al.*, 1993]. Because of the extensive homology between them, the δ- and β-genes are thought to have arisen from duplication of a single gene. Subsequent mutations in the regulatory region of the δ-gene have rendered it functionally insignificant [Lee *et al.*, 1993].

All of the functional globin genes share a common general structure, consisting of three exons and two introns or intervening sequences [Lee *et al.*, 1993]. Sequences in the 5´ upstream region, as well as those that are 3´ downstream to the gene are important for gene expression [Lee *et al.*, 1993]. The β-globin gene has been one of the most intensively studied of all human loci, due to its association with severe inherited hemoglobinopathies such as sickle cell anemia and β-thalassemia that represent some of the most common genetic diseases in the world [Fullerton *et al.*, 1994].

The complete sequence of the β-globin mRNA was determined in 1977 and 1978 [Marrotta *et. al*, 1977; Wilson *et al.*, 1978]. Shortly after, and by 1980, the sequence information was extended to include the intervening and the flanking regions of the gene [Lawn *et al.*, 1980]. The sequenced region stretches between 103 base pair (bp) 5´ to the capping site [Baralle, 1977], extends for 2060 bp, and ends 343 bp 3´ to the poly-adenylation signal sequence [Proudfoot, 1977].

I.5 HEMOGLOBIN PATTERN IN PATIENTS WITH β-THALASSEMIA

In 1946 the Italian worker Vecchio noted that the hemoglobin of patients with Cooley's anemia was more alkali resistant than normal adult

hemoglobin [Vecchio, 1946]. This suggested that these patients have more fetal hemoglobin than is usually found after the first year of life. Eventually, Rich in 1952 suggested that thalassemia results from a defect in hemoglobin A synthesis with persistent production of hemoglobin F [Rich, 1952]. Another key observation on the alteration of the hemoglobin patterns in patients with thalassemia was made in 1955 when Kunkel and Wallenius showed that levels of hemoglobin A_2 were elevated in thalassemia heterozygotes [Kunkel and Wallenius, 1955]. This was confirmed by Gerald and Diamond in 1958 [Gerald and Diamond, 1958], and by many other workers subsequently. However, in a later paper Kunkel and his colleagues noted that in two out of thirty four parents of children with thalassemia major there were normal levels of hemoglobin A_2 [Kunkel *et al.*, 1957]. Again this suggested heterogeneity of thalassemia and indicated that at least two types must exist [Weatherall and Clegg, 1981].

I.6 HEMOGLOBIN SYNTHESIS IN β-THALASSEMIA

In 1964, Heywood and his colleagues showed for the first time unequal labeling of the α- and β-chains of hemoglobin after incubation of thalassemic reticulocytes with radioactive amino acids. These workers separated the globin chains chromatographically but the method they employed made it impossible to assess the relative rates of production of the α- and β-chains [Heywood *et al.*, 1964]. The technique which enabled further progress to be made in the field was developed by Clegg and coworkers, who showed that the peptide chains of globin could be quantitatively fractionated by

chromatography on CM-cellulose in 8M urea [Clegg *et al.*, 1965, 1966]. Furthermore, it was established that in some forms of thalassemia there is a complete suppression of β-chain synthesis (β^0), while in others the defect was only partially effective (β^+) [Weatherall and Clegg, 1981]. Hence, those biosynthesis studies demonstrated unequivocally that β-thalassemia is a disorder of imbalanced globin chain production: the problem was thus to elucidate the cause of the defective production of β-chains [Weatherall and Clegg, 1981]. Normal human hemoglobin A consists of two pairs of globin chains, $\alpha_2\beta_2$ whose synthesis is normally tightly coordinated to ensure equal production. In the β-thalassemias, the synthesis of β-globin chains is either partially or completely abolished, the consequence is a deficiency of hemoglobin per cell, which produces hypochromia and microcytosis [Schrier, 1997].

I.7 PREVALENCE AND GEOGRAPHIC DISTRIBUTION

The β-thalassemias are widespread throughout the Mediterranean region, Africa, the Middle East, the Indian subcontinent and Burma, Southeast Asia including southern China, the Malay Peninsula, and Indonesia [Weatherall, 1994]. About 3% of the world's population carry β-thalassemia genes. These genes are particularly prevalent in inhabitants of Italy and Greece. On the other hand, β-thalassemia is encountered less frequently in the northern and western parts of Africa [Weatherall, 1981; Ibrahim and Barakat, 1970], Turkey [Aksoy, 1959], Iran, and Syria [Chernoff, 1959; Ibrahim and Barakat, 1970]. It has been described in Indian and

Kurdish Jews [Horowitz *et al.*, 1966; Ramot *et al.*, 1964], in Arabs (especially those in Saudi Arabia) [Weatherall, 1981], Pakistanis, and Indians [Chatterjea, 1966]. Documentation of natives in Southeast Asia and southern China show that β-thalassemia is far less prevalent in these regions than is α-thalassemia [Flatz *et al.*, 1965; Minnich *et al.*, 1954; Wasi *et al.*, 1969]. In North America, thalassemia is noted primarily in persons of Italian and Greek descent [Neel and Valentine, 1945, 1947] and in Blacks [Weatherall, 1963]. In sporadic cases, β-thalassemia has been noted in north Europeans with no apparent Mediterranean or Oriental ancestry [Weatherall, 1981]. Migration, changing marriage patterns among ethnic groups, and differences in the relative growth of populations can be expected to change the distribution and prevalence of thalassemia [Lee *et al.*, 1993].

I.8 CLASSIFICATION AND CLINICAL FORMS OF β-THALASSEMIA SYNDROMES

Before the genetic basis for the disorder was appreciated, the thalassemias were classified on the basis of clinical severity: two conditions are generally asymptomatic; the silent carrier state (thalassemia minima) and β-thalassemia trait (thalassemia minor), both result from the inheritance of one mutant β-globin gene, and two require medical management; thalassemia intermedia and thalassemia major (Cooley's anemia) [Lee *et al.*, 1993]. The more severe forms most often result from homozygosity or compound heterozygosity for a mutant β-globin allele [Lee *et al.*, 1993], and occasionally, from heterozygosity for dominant mutations [Thein *et al.*, 1990].

I.9 COMPLICATIONS OF THE DISEASE

Iron overload, is the most important complication of β-thalassemia and represents a major focus of management [Olivieri and Brittenham, 1997]. After approximately one year of blood transfusion, iron begins to be deposited in parenchymal tissues [Risdon *et al.*, 1973], where it may cause substantial toxicity compared to that within reticuloendothelial cells [Hershko and Weatherall, 1988; Hershko *et al.*, 1998]. As iron loading progresses, the capacity of serum transferrin, the main transport protein of iron, may be exceeded and a non-transferrin-bound fraction of plasma iron may promote the generation of free hydroxyl radicals, propagators of oxygen-related damage [Hershko and Weatherall, 1988; Hershko *et al.*, 1998]. Although the body maintains a number of antioxidant mechanisms against damage induced by free radicals, including superoxide dismutases, catalase, and glutathione peroxidase, in patients with large iron burdens these mechanisms will not prevent oxidative damage [Hershko and Weatherall, 1988; Hershko *et al.*, 1998]. In the absence of chelating therapy, the accumulation of iron results in progressive dysfunction of the heart, liver, and endocrine glands [Olivieri and Brittenham, 1997]. Iron loading within the anterior pituitary is the primary cause of disturbed sexual maturation [Italian working group, 1995]. Even in the modern era of iron chelating therapy, diabetes millitus is observed in about 5% of adults [Italian working group, 1995]. Studies reporting reduced serum concentrations of trypsin and lipase [Gullo *et al.*, 1993], suggest that the exocrine pancreas is also damaged

by iron loading. Over the long term, iron deposition also damages the thyroid, parathyroid, and adrenal glands [Sklar *et al.*, 1987].

I.10 DIAGNOSIS

I.10.1 *Clinical Features*

β-Thalassemia major is not expressed clinically during the first months of life. Thereafter, progressive pallor develops, the abdominal girth expands, and patients fail to thrive. In the absence of transfusion therapy, a clinical picture emerges; characterized by small stature, a relatively large head, and protuberance of the abdomen. Prominence of the cheekbones tends to obscure the base of the nose and to expose the upper teeth. Thickening of the cranial bones produces frontal bossing. These disturbances of craniofacial growth constitute the "thalassemic facies" [Logothetis *et al.*, 1971].

Signs and symptoms of thalassemia intermedia are comparable to those of thalassemia major but are of lesser magnitude [Aksoy, 1970; Erlandson *et al.*, 1964; Heller *et al.*, 1966; Pearson, 1964]. Although chronically anemic, individuals with thalassemia intermedia do not require transfusions, except in association with intercurrent illness. Growth and development during childhood is relatively uncompromised, pubescence takes place normally, and fertility is preserved [Erlandson *et al.*, 1964; Meital *et al.*, 1961; Pearson, 1964]. Survival into adulthood is the rule [Farhangi *et al.*, 1970], and patients typically enjoy a full life span [Schokker *et al.*, 1966].

With respect to thalassemia minor, the syndrome is almost always discovered accidentally during examination for unrelated symptoms or as a consequence of a study designed to characterize better the nature of

symptomatic anemia in a family member. Affected women are more anemic than non-thalassemic women during pregnancy, but blood transfusion is not required [Schuman *et al.*, 1973].

I.10.2 *Hematologic Features*

Anemia in thalassemia major is usually pronounced when first documented. Before blood transfusion, the hemoglobin concentration is 2.5 to 6.5 g/dl and the packed cell volume is 11 to 24%. The anemia is microcytic (MCV ~ 48 to 72 fl) and hypochromic (MCHC ~ 23 to 32 g/dl) [Cooley and Lee, 1925; Smith, 1943, 1948]. Anisocytosis is significant, with cells ranging from 3 to 15 μm in diameter. Target cells are numerous and basophilic stippling is prominent. After splenectomy, inclusion bodies composed of denatured α-chain aggregates can be demonstrated with methyl violet staining [Fessas, 1966]. Leukocytes characteristically are increased in number and platelet numbers are normal. Leukocytosis results from an increase in neutrophils and (to a lesser extent) from the presence of myelocytes and metamyelocytes [Kato and Downey, 1933]. Other laboratory features include; increased unconjugated bilirubin and serum iron levels. Serum transferrin is often fully saturated, and a non-transferrin-bound iron fraction may be present.

The hemoglobin concentration in thalassemia intermedia is maintained in the range of 6 to 9 g/dl without transfusion. Peripheral blood erythrocytes show changes comparable to those of thalassemia major; significant anisocytosis, hypochromia, target cells, basophilic stippling, and numerous nucleated forms. Bone marrow hyperplasia is prominent and

inclusions of denatured α-chains can be demonstrated in late normoblasts by using supravital stains [Yataganas and Fessas, 1969, 1972]. In contrast, anemia is mild or absent in thalassemia minor. The red cell count is elevated, and the MCV and MCH values are reduced [Hammond *et al.*, 1964]. The MCHC is normal or only slightly decreased. The reticulocyte count and serum bilirubin concentration may be slightly increased. In contrast to the minor degree of anemia, morphologic alterations of peripheral blood erythrocytes are prominent. These changes include microcytosis, hypochromia, anisocytosis, and basophilic stippling. The bone marrow is normal except for mild erythroid hyperplasia. No inclusions of precipitated α-chains can be identified in red cell precursors by methyl violet staining. The most consistent feature of β° and β^{+} thalassemia is an increase in HbA_2, levels in persons with heterozygous β^{+} thalassemia tend to be somewhat lower than those in persons with β° thalassemia and, at least in Black patients, may fall within the normal range [Millard *et al.*, 1977]. Levels of HbA_2 appear normal in individuals with Mediterranean β-thalassemia mutations when they are co-inherited with a δ-thalassemia gene mutation(s) [Moi *et al.*, 1988]. Infants destined to develop thalassemia minor are hematologically normal at birth. By 4 months, the hemoglobin concentration, MCV, MCH, HbA_2, and HbF levels are all outside normal ranges [Galanello *et al.*, 1981].

Interestingly, thalassemia minima refers to a condition in which the impairment of β-chain synthesis is so subtle as to escape attention by clinical evaluation or conventional hematological assessment. In this case the MCV

and MCH values are normal or only minimally decreased and the hemoglobin pattern is normal [Lee *et al.*, 1993].

I.10.3 *Measurement of Globin Synthesis*

Measurement of the rate of globin chain synthesis can be a useful way of characterizing the thalassemias. In normal subjects, values for the β:α ratio lie near 1.0. They are decreased in patients with β-thalassemia and increased in those with α-thalassemia in proportion to the severity of the defect [Nathan, 1972; Schwartz, 1974].

I.10.4 *Molecular Diagnosis*

The use of molecular techniques has been widely documented to confirm the diagnosis of β-thalassemia and, more importantly, for prenatal diagnosis. Polymerase chain reaction (PCR) coupled with a great variety of approaches such as restriction endonuclease analysis, dot-blot analysis with allele specific oligonucleotide (ASO) probes, allele-specific PCR, reverse dot-blot, competitive oligopriming, and direct DNA sequencing, is now widely used to determine the presence of known β-thalassemia mutations on a DNA template [Ugozzoli *et al.*, 1998].

I.11 PATHOPHYSIOLOGY OF RED BLOOD CELLS

Sturgeon and Finch in Seattle were the first workers to carry out careful erythrokinetic studies on patients with thalassemia major. Their studies showed that there was a marked degree of ineffective erythropoiesis in this condition of a very similar pattern to that found in pernicious anemia

[Sturgeon and Finch, 1957]. This was later confirmed by *in vivo* studies of hemoglobin turnover by Grinstein, Bannerman and others [Bannerman *et al.*, 1959]. This implies that there is extensive intramedullary destruction of red-cell precursors as well as a shortened survival in the peripheral red blood cells. A clue to the basis for the destruction of the red cells in thalassemia came in 1963 following the elegant studies of Fessas in Athens [Fessas, 1963]. Evidence was presented to show that there are large, ragged inclusion bodies in the red-cell precursors of patients with β-thalassemia. These structures were also detected in peripheral blood but only after splenectomy. Fessas suggested that these inclusions are precipitated α-chains, which might interfere with erythroid maturation and so cause ineffective erythropoiesis [Fessas, 1963].

The idea that thalassemia is primarily a disease of imbalanced globin chain production was extended in 1966 by Nathan and Gunn who suggested that the abnormalities of red-cell maturation and survival could be ascribed entirely to the effects of unbalanced globin production with precipitation of the particular chains which are produced in excess [Nathan and Gunn, 1966]. They argued that in β-thalassemia those cells which continue to produce γ-chains would be relatively protected from the deleterious effects of α-chain precipitation as in these cells some of the excess α-chains could combine with γ-chains to make hemoglobin F ($\alpha_2\gamma_2$) [Nathan and Gunn, 1966]. Several other workers showed that hemoglobin F-containing cells survive relatively longer than hemoglobin A-containing cells in the blood of patients with β-thalassemia. Nathan and Gunn further suggested, that in addition to causing

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abnormalities of cellular maturation the precipitating globin chains might interfere with membrane function and cause 'leakiness' of the red-cell membrane [Nathan and Gunn, 1966]. This might contribute to the hemolysis observed in all the thalassemia syndromes [Weatherall and Clegg, 1981].

I.11.1 *Excess α-Chains*

In normal assembly of adult hemoglobin (HbA- $\alpha_2\beta_2$), α and β-globin chains are synthesized by genes on different chromosomes, whereas heme is synthesized primarily in the mitochondria. The synthesis of these chains is very tightly coordinated so that the ratio of α-globin to β-globin (β in this case includes the β-like globins δ and γ) is normally 1 ± 0.05. Furthermore, specific erythroid proteases [Testa *et al.*, 1981] are designed to attack and destroy excess α- or β-globin chains, demonstrating the deleterious impact of the accumulation of excess unmatched globin chains. In β-thalassemia, production of β-globin decreases and excess α-globin accumulates. The imbalance in globin chain production results in lesser amounts of normal hemoglobin per cell, which partially accounts for the well-described hypochromia and microcytosis. However, these characteristics are nevertheless insufficient to explain why in homozygous β-thalassemia major 80% of the erythroid progenitors die in the bone marrow before the reticulocytes are delivered to the peripheral blood in a process known as ineffective erythropoiesis or intramedullary hemolysis [Finch *et al.*, 1970]. In addition, these characteristics do not also explain the relatively few red blood

cells that reach the peripheral blood and are rapidly removed through hemolysis [Schrier, 1994].

I.11.2 *Oxygen Reactive Species*

While no single mechanism is likely to account for the complex pathophysiology of the β-thalassemic erythrocyte, oxidation of cellular components has been implicated as an important factor. Previous studies have demonstrated that excess α-hemoglobin chains autoxidize, release their heme, and mediate the production of reactive oxygen species (O_2^-, H_2O_2) [Bunn *et al.*, 1967; Brunori *et al.*, 1975; Joshi *et al.*, 1983; Scott *et al.*, 1990]. The released heme has been shown to directly inhibit a number of cytoplasmic enzymes, further disrupting normal cellular homeostasis and predisposing the cell to additional injury [Zerez *et al.*, 1988]. Interaction between these reactive oxygen species and the heme or heme derived iron [Scott *et al.*, 1991a; Scott *et al.*, 1991b] has been shown to result in a "biologic Fenton reaction that catalyses the production of the hydroxyl radical (•OH) and reactive lipid radicals "[Sadrzadeh *et al.*, 1984]. These species are believed to mediate much of the damage seen in thalassemic erythrocytes [Scott *et al.*, 1993]. Hydroxyl radical generation in biological systems is suggested to be induced by $O_2^{\bullet -}$ and H_2O_2 interaction with iron or copper [Miller and Aust, 1989; Saltman, 1989], according to the following Haber-Weiss mechanism [Grinberg *et al.*, 1995]:

$$O_2^{\bullet -} + Fe\ (III) \longrightarrow O_2 + Fe(II) \qquad (1)$$

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (2)$$

$$Fe\ (II) + H_2O_2 \longrightarrow Fe(III) + OH^- + {}^{\bullet}OH \qquad (3)$$

Hence, while the reducing capacity of the normal erythrocyte is >250 times its oxidizing potential [Scott, 1968], excess α-hemoglobin chains can circumvent and overwhelm the normal protective mechanisms of the cell [Scott *et al.*, 1993].

I.11.3 *Cytoskeletal Membrane Proteins*

Membrane stability is thought to be controlled by the content and function of its skeleton, which is principally composed of ternary complexes of spectrin, actin, and band 4.1 arranged in apparent hexagonal arrays [Chasis and Mohandas, 1986; Fortier *et al.*, 1988; Liu *et al.*, 1991; Liu and Derick, 1992]. Another partial explanation for the hemolysis seen in β-thalassemia came out from studies performed on β-thalassemia intermedia red blood cells, which provided evidence that excess unmatched α-globin chains accumulate at the membrane and its skeleton where they probably lead to alterations in membrane deformability, stability, and cellular hydration [Schrier *et al.*, 1989]. These cellular abnormalities, in turn, are associated with membrane changes consisting of a varying degree of unstable attachment of the three major membrane skeletal proteins to the cell membrane [Yuan *et al.*, 1995], reduced spectrin/band 3 ratio [Shinar *et al.*, 1987], partial oxidation of band 4.1 [Advani *et al.*, 1992], defective band 4.1 function [Shinar *et al.*, 1989], in addition to clustering of band 3 with binding of IgG and complement over the band 3 clusters [Yuan *et al.*, 1992]. However, it is not clear how this membrane instability could contribute to the peripheral hemolysis seen in thalassemia. It is likely that the accumulation of the α-globin chains at the

membrane or their oxidation products produce derangement sufficient to account for as much as 10% of spectrin dissociation [Yuan *et al.*, 1995].

I.11.4 *Apoptosis*

Based on prior morphologic data showing nuclear abnormalities, it was hypothesized that accelerated apoptosis account for some of the intramedullary cell death seen in β-thalassemia patients [Yuan *et al.*, 1993]. Programmed cell death (apoptosis) is a selective process of physiologic cell deletion, its occurrence plays a major role in the control of normal and abnormal processes. The role of apoptosis has been shown for many tissues and cell lines [Koury, 1992; Zychlinsky *et al.*, 1992; Manabe *et al.*, 1992]. Apoptosis has been described in erythroid precursors in *in vitro* systems. Although there is presently no evidence for programmed cell death in normal erythropoiesis, the late or orthochromic erythroblast with a shrunken pyknotic nucleus being extruded from the cells looks morphologically like apoptosis. The cause of intramedullary erythroid cell death in the marrow has never been fully identified, but there are morphologic clues showing that erythroid precursors in β-thalassemia major show evidence of α-globin chain deposition [Fessas, 1963], cytoplasmic vacuolization [Polliack and Rachmilewitz, 1973], and abnormalities of the nuclear membrane [Wickramasinghe and Bush, 1975]. The hypothesis [Yuan *et al.*, 1993] is that unmatched excess α-globin chain accumulation, somehow causes these defects and ultimately the death in marrow of about 80% of the affected erythroid precursors. The α-globin chains accumulate progressively as the erythroid cell undergoes progressive differentiation. Although the late stage

precursors have the most α-globin chain accumulation, α-globin chain deposition can be seen as early as the proerythroblastic stage [Yuan *et al.*, 1993]. Evidence indicate, the presence of ladder patterns of DNA breakdown products in β-thalassemia major samples, with less occurring in β-thalassemia trait [Yuan *et al.*, 1993]. This finding might partially explain why most of the erythroblasts never survive to become mature erythrocytes. The mechanism by which α-globin chain accumulation could lead to apoptosis is still under investigation [Yuan *et al.*, 1993].

I.12 MOLECULAR PATHOLOGY

In the early 1970's, the discovery of the enzyme called reverse transcriptase in certain tumor viruses made it possible to synthesize complementary DNA (cDNA) from messanger RNA (mRNA) templates [Temin and Mizutani, 1970; Baltimore, 1970]. Radioactively labeled α- and β-globin cDNAs were thus obtained which could then be used to analyze the mRNA of patients with thalassemia by molecular hybridization. Several groups applied this approach to the study of both α- and β-thalassemia, and showed that these disorders usually result from the decreased production of globin mRNA [Kacian *et al.*, 1973; Housman *et al.*, 1973]. The initial problem in trying to characterize the defect in thalassemia disorders was to decide at which level the protein synthesis is abnormal. It was apparent that the abnormality could be anywhere between the structural gene and the globin chain. For example, it was possible that in those forms of thalassemia in which there is no globin synthesis, the genes might have been deleted. On

the other hand the genes might be present but there might be a defect in the mechanism of transcription or of processing of the RNA transcript [Weatherall and Clegg, 1981].

The development of techniques for gene cloning, DNA sequencing, and assessment of gene function have been exploited to define the molecular pathology of the thalassemias in remarkable detail [Modell and Berdoukas, 1984; Nienhuis *et al.*, 1984; Higgs *et al.*, 1989]. Most of the α-thalassemia syndromes result from gene deletions. The α-globin genes appear to be predisposed to deletions because of tandemly duplicated sequences in the α-gene cluster [Lee *et al.*, 1993]. In contrast, most of the β-thalassemia syndromes result from one or more nucleotide substitutions or deletions in genes that are otherwise intact [Lee *et al.*, 1993]. The well-known clinical heterogeneity of the thalassemia syndromes is a reflection of the great heterogeneity of mutations affecting the globin genes [Lee *et al.*, 1993]. Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders [Olivieri, 1999]. Although most are small nucleotide substitutions within the cluster, deletions may also cause β-thalassemia [Weatherall, 1994].

I.13 GENETIC MECHANISMS

I.13.1 *Promoter Region Mutations*

These mutations reduce binding of RNA polymerase, thereby reducing the rate of mRNA transcription to 20 - 30% of normal. The β-globin synthesis,

even in homozygotes and doubly heterozygotes, is sufficient to preclude thalassemia major [Rosatelli *et al.*, 1989].

I.13.2 *Chain Terminator Mutations*

These are caused by two types of mutations. Nonsense mutations, where a single nucleotide substitution alters a codon that normally is decoded into an amino acid to one that stops translation. Frameshift mutations, where a nucleotide deletion or insertion changes the grouping of nucleotide triplets, or codons, thereby garbling the gene's message downstream from the mutation. The mRNA produced by both types of mutations is incapable of being translated into full-length globin chains, resulting in the β° thalassemia phenotype [Lee *et al.*, 1993].

I.13.3 *Splice Junction Mutations*

Point mutations involving the donor (GT) or acceptor (AG) dinucleotides at either end of the intervening sequences result in abnormal splicing. Base substitutions at the donor sites lead to complete loss of splicing at the mutation site [Chibani *et al.*, 1988]. Deletions [Orkin *et al.*, 1983b], and point mutations [Antonarakis *et al.*, 1984; Pandanilam and Huisman, 1986] at the 3′ end of the introns render these acceptor sites inoperative. In addition to these dinucleotides, certain preferred nucleotide sequences adjacent to them are required for accurate and efficient splicing. These sequences are referred to as *consensus* sites. The consensus sequences for donor sites include the three nucleotides 5′ and the four nucleotides 3′ to the dinucleotide GT. In at least some of the consensus mutations, cryptic

donor sites are utilized to process mRNA transcripts [Treisman *et al.*, 1983]. These cryptic sites resemble normal splice sites but are not recognized unless the normal sites are altered. Consensus sequence mutations reduce the efficiency of splicing but do not abrogate it. Consequently, they give rise to the β^+ thalassemia phenotype [Lee *et al.*, 1993].

I.13.4 *Mutations Creating New Splice Signals*

Several nucleotide substitutions within introns involve sequences that differ only slightly from normal splice sites. These mutations have the effect of changing a cryptic splice sight to a legitimate one. The new splice site competes with normal splice sequences. The mutation GG > AG at position 110 of the first intron creates an acceptor splice site in a sequence that resembles the normal acceptor splice site [Spritz *et al.*, 1981; Westaway and Williamson, 1981]. The new site is used preferentially by the normal donor site [Fukamaki *et al.*, 1982]. Because some normal mRNA is generated, this mutation is associated with the β^+ thalassemia phenotype [Lee *et al.*, 1993].

I.13.5 *Enhanced Cryptic Splice Sites*

Codons 24 through 27 in the first exon contain a nucleotide sequence that resembles the normal donor sequence at the 5´ end of the first intron. Mutations involving these sites lead to a competition between the new splice sequence and the normal sequence. A portion of mRNA is processed from the mutated cryptic donor to the normal acceptor at the 3´ end of the first intron [Lee *et al.*, 1993].

I.13.6 *RNA Cleavage Defect*

The six nucleotides at the end of the third exon (AATAAA) trigger an enzymatic process that cuts the growing mRNA at the appropriate point and releases it for further processing. Base substitutions that change this signal permit transcription to continue to the next cleavage signal approximately 900 nucleotides farther downstream. The added material more than doubles the length of pre-mRNA and renders it unstable [Lee *et al.*, 1993].

I.13.7 *Cap Site Mutant*

A single cap site variant has been described in association with the β^+ thalassemia phenotype. Substitution of C for A in the first position may reduce transcription or slow the 5´ capping process, thereby reducing mRNA stability [Lee *et al.*, 1993].

I.13.8 *Deletion Defects*

Several deletion mutants have been characterized in individuals with the β° thalassemia phenotype. The most prevalent of these relatively rare alleles is characterized by a deletion that begins in the second intervening sequence and extends beyond the 3´ end of the β-globin gene [Orkin *et al.*, 1979; Spritz and Orkin, 1982; Kazazian *et al.*, 1984]. Some authors suggest that deletion of the DNA immediately 5´ to the β-globin gene may produce changes in the chromatin structure of the remaining β-globin cluster, thereby activating the γ- and δ-globin genes [Anand *et al.*, 1988].

I.14 DNA POLYMORPHISMS

It has been well known that individuals are genetically different, yet that most gene products are highly conserved from one person to another. Some of the differences between individuals represent pathologic DNA changes. However, most probably represent "silent" variation in DNA. DNA polymorphism is a direct consequence of these differences [Orkin *et al.*, 1983a].

I.14.1 *DNA Polymorphisms in the β-Globin Gene Cluster*

Most polymorphism is found in intergenic and intervening sequences and results most often from single nucleotide substitutions, although insertions and deletions of DNA have occasionally been described [Orkin and Kazazian, 1984]. DNA polymorphism in man was first observed independently by Lawn *et al.* [Lawn *et al.*, 1978] and by Kan and Dozy [Kan and Dozy, 1978]. Lawn and colleagues found a P*st* I site polymorphism in the second intron (IVS-II) of the δ-globin gene, while Kan and Dozy found polymorphism of an H*pa* I site 5 Kb 3′ to the β-globin gene. Both polymorphisms were produced by single nucleotide substitutions. The H*pa* I site, was clinically important because it was frequently absent downstream from the β^S globin gene, while it was nearly always present on β^A bearing chromosomes [Kan and Dozy, 1978]. Thus, the presence or absence of the H*pa* I site was used as the first DNA-based prenatal diagnostic test for sickle cell anemia [Kan and Dozy, 1978]. Soon thereafter, after surveying 60 Caucasian samples, Jeffreys located two polymorphisms for H*ind* III cleavage [Jeffreys,

1979]. Subsequent to this work, intensive searches for restriction endonuclease site polymorphisms led to the recognition of 17 common polymorphisms [Antonarakis *et al.*, 1982a; Orkin and Kazazian, 1984]. Of these, 14 are found in all racial groups and are termed public, while three are found in one racial group termed private. Twelve are in flanking DNA, three are in introns, one is in a pseudogene, and one is in the first exon of the β-globin gene. Three other nucleotide polymorphisms that can be detected by DNA sequence analysis, but not by restriction endonuclease analysis, have also been observed in the second intron of the β-globin gene [Orkin *et al.*, 1982]. Other polymorphism has been seen rarely upon direct gene analysis. Jeffreys originally estimated that about one in every 100 nucleotides was polymorphic [Jeffreys, 1979]. Subsequent estimates have largely confirmed this [Chakravarti *et al.*, 1984; Kazazian *et al.*, 1983a]. As mentioned above, this sequence variation is almost entirely limited to flanking and intron DNA [Orkin and Kazazian, 1984].

I.14.2 *Polymorphism of the β-Globin Structural Gene*

The β-globin gene exhibits polymorphism limited almost exclusively to common sequence types that have been designated β-gene frameworks [Orkin *et al.*, 1982; Orkin *et al.*, 1983a]. In each racial group three β-gene frameworks are observed. Frameworks 1 and 2 differ by a single nucleotide and are found in all groups studied to date. Framework 3 has four additional substitutions in Mediterraneans, but only three in Asians and Blacks [Orkin *et al.*, 1982; Antonarakis *et al.*, 1984; Orkin *et al.*, 1983a]. Except for the

polymorphic site at codon 2 (C > T), the polymorphisms lie in the second intervening sequence [nucleotide positions; 16 (C > G), 74 (G > T), 81 (C > T), and 666 (C >T)]. These fixed sequence variants of the β-gene are termed frameworks because they are the backgrounds upon which mutations leading to hemoglobinopathies and thalassemia have occurred [Orkin *et al.*, 1982]. In cloned and sequenced β-genes, only two additional rare nucleotide substitutions, both in exon-1, have been observed [Orkin *et al.*, 1982; Kazazian *et al.*, 1984]. The rare DNA polymorphism, C > T in the second codon of the β-globin, with no alteration in the amino acid is present in 13% of the Mediterranean β-thalassemia chromosomes [Filon *et al.*, 1995a]. Recently, two additional polymorphic sites have been described, both of them are located at the first intervening sequence; one, at nucleotide position 91 (C > T) [Primignani *et al.*, 1999], and the other at position 108 (T > C) [Badens *et al.*, 1999].

I.15 CONTROL AND MANAGEMENT

I.15.1 *Prevention Programs and Prenatal Diagnosis*

Although bone marrow transplantation has been used in the past with success in a limited number of patients and increasing success recently, still the most applicable approach is primarily supportive management. Therefore, at present, prevention of the birth of affected children by heterozygous screening, genetic counseling, and prenatal diagnosis have proven to be the most effective management for thalassemia disorders [Cao and Rosatelli, 1993]. Previous screening programs, aimed at prevention of the

disease, and prenatal diagnosis have resulted in a marked reduction in the birth rate of affected children in Greece, Cyprus, continental Italy, and Sardinia [Cao *et al.*, 1998]. Widespread use of similar programs in other areas of the world has not yet been possible [Olivieri, 1999]. Screening for carriers (β-thalassemia trait) is performed most efficiently by measurement of the red cell indexes, and estimation of the HbA_2 concentration. Prenatal diagnosis, first carried out by fetal-blood sampling and assessment of globin-chain synthesis in fetal blood, more recently has involved direct analysis of fetal DNA obtained by chorionic villus sampling [Embury, 1995]. This approach is associated with a very slightly increased risk of fetal loss and an error rate in experienced laboratories of less than 1 percent [Cao *et al.*, 1998; Weatherall and Clegg, 2001].

Prediction of the phenotype from the genotype is an important parameter in genetic analysis as it provides guidance for genetic counseling. It has been suggested that genetic counseling and termination of pregnancy is justified for thalassemia intermedia [Kollia *et al.*, 1990] since in some patients, morbidity can be severe even if mortality is not high. It was shown that the very variable and at times quite severe phenotype of thalassemia intermedia warrants consideration of prenatal diagnosis in selected families [Rund *et al.*, 1997]. Clearly and evidently, genetic counseling that is based only on genotypic information is likely to be inadequate for many families. Further study to define the nature of genetic modifiers, which interact with β-thalassemia will provide the basis for more accurate genetic counseling as well as enabling a better understanding of the disease process [Rund *et al.*,

1997]. Since the treatment of thalassemia major is still unsatisfactory and the disease is ultimately fatal, prenatal diagnosis has become an important option for couples at risk of producing an affected fetus. Therefore, in addition to providing valuable insights into the pathophysiology of thalassemia, determination of the molecular basis of the severe and mild forms of homozygous β-thalassemia in each population group is of increasing importance for planning prenatal diagnosis programs [Thein *et al.*, 1988].

I.15.2 *Therapy*

Primary management of patients with thalassemia major and other transfusion-dependent thalassemic syndromes can be considered under four major headings: transfusion therapy, splenectomy, iron chelation, and bone marrow transplantation. Several therapeutic strategies are in the developmental stages, including pharmacologic activation of γ-globin synthesis and genetic engineering [Lee *et al.*, 1993].

I.15.2.a Transfusion Therapy

A decision to initiate regular transfusions in patients with β-thalassemia may be difficult and should be based on the presence and severity of the symptoms and signs of anemia, including failure of growth and development. Only rarely is genotyping helpful in this decision. The goals of transfusion include correction of anemia, suppression of erythropoiesis, and inhibition of increased gastrointestinal absorption of iron [Olivieri, 1999].

I.15.2.b Splenectomy

Good transfusion programs retard the development of splenomegaly [Beard *et al.*, 1969]. Progressive splenomegaly expands the blood volume, shortens red cell survival, increases the transfusion requirement, and accelerates iron loading [Blendis *et al.*, 1974]. Occasionally, splenic trapping of platelets and leukocytes is sufficient to produce thrombocytopenia and leukopenia [Rowley and Jacobs, 1972]. The main indication for splenectomy is an increasing transfusion requirement. Findings of recent studies have prompted the somewhat simpler recommendation to remove the spleen when the red cell transfusion requirement exceeds 200 [Cohen *et al.*, 1980] to 250 [Graziano *et al.*, 1981] ml packed red cells/kg/year. The procedure should be postponed until late childhood or adolescence if possible because of the exaggerated risk of fatal sepsis in thalassemic children whose spleens have been removed during the first years of life [Diamond, 1969; Singer, 1973]. Pneumococcal immunization is recommended before splenectomy [Lee *et al.*, 1993].

I.15.2.c Iron Chelation

Most patients with thalassemia major die between 16 and 24 years of age from complications of iron overload. Nearly all deaths ultimately result from myocardial hemosiderosis. At a total body iron burden of 40 g, organ function begins to fail, and at 60 g or more, intractable cardiac failure has its onset. The most effective iron chelating agent currently available is desferrioxamine, a siderophore produced by *Streptomyces pilosus*

[McDonald, 1966]. Iron excretion after the administration of desferrioxamine is proportional to body iron stores. The requisite amount of drug is given most conveniently with a small portable infusion pump that permits delivery of microliter quantities of the drug subcutaneously from a standard syringe through a butterfly needle placed by the patient in the anterior abdominal wall [Propper *et al.*, 1977]. Ascorbic acid appears to render tissue iron more accessible to desferrioxamine, thereby enhancing total iron excretion without increasing iron absorption [Hussain *et al.*, 1977]. Unfortunately, ascorbate also increases the toxicity of tissue iron. Sudden deterioration of heavily iron-loaded patients given both desferrioxamine and ascorbic acid is well documented [Nienhuis *et al.*, 1976; Cohen and Schwartz, 1980]. Even in the absence of clinical worsening, serial echocardiographic studies may show a fall in the ejection fraction and dilatation of the heart after the introduction of ascorbic acid [Nienhuis *et al.*, 1979]. For this reason, ascorbic acid probably should be reserved for use in individuals who are not severely iron overloaded [Lee *et al.*, 1993]. In clinical practice, the serum ferritin concentration is commonly used to assess the effectiveness of treatment. It is increasingly recognized that reliance on this test may lead to errors in management; changes in body iron account for little more than half the variation in serum ferritin concentrations [Brittenham *et al.*, 1993]. By contrast, the measurement of hepatic iron stores, whose concentrations are highly correlated with total body iron stores [Angelucci *et al.*, 1997], provides the most quantitative, specific, and sensitive method for evaluating iron burden in patients with thalassemia. Determination of hepatic iron

concentrations in liver biopsy specimens obtained with ultrasonographic guidance is safe and permits rational adjustments in iron-chelating therapy [Olivieri and Brittenham, 1997]. Magnetic susceptometry provides a direct measure of hepatic iron stores that is quantitatively equivalent to that determined by biopsy. However, magnetic susceptometry is currently available in only two centers worldwide [Olivieri, 1999].

I.15.2.d Bone Marrow Transplantation

Bone marrow transplantation from HLA identical donors has been successfully performed worldwide in over 1000 patients with severe β-thalassemia [Giardini, 1997]. The outcome after transplantation is greatly influenced by the presence of hepatomegaly, portal fibrosis, and ineffective chelating therapy before transplantation [Lucarelli *et al.*, 1997]. Bone marrow transplantation is attended by many of the same problems as experienced by patients with neoplastic disease who undergo transplantation, including death from opportunistic infections and chronic graft-versus-host disease [Lee *et al.*, 1993]. There is increased interest in experimental approaches to bone marrow replacement in patients with thalassemia, including cord-blood transplantation [Issaragrisil *et al.*, 1995], the use of unrelated phenotypically matched donors [Contu *et al.*, 1994], and *in utero* transplantation [Westgren *et al.*, 1996].

I.15.3 *Experimental Therapies*

I.15.3.a Chelators Other than Desferrioxamine

The current standard iron chelator desferrioxamine requires intravenous or subcutaneous injection and does not easily penetrate cell membranes. Deferiprone (L-1), on the other hand, has a nine fold greater capacity to enter cells through the membrane and complex with chelatable iron in both cytosol and membrane compartments [Schrier, 1997]. The administration of deferiprone, was reported to have a favorable short-term effect on body iron in the one study in which serial systematic determinations of hepatic iron were obtained [Olivieri *et al.*, 1995]. Two subsequent long term studies have suggested that hepatic iron may stabilize at or increase to concentrations associated with an increased risk of cardiac disease and early death [Brittenham *et al.*, 1994] in approximately half of patients [Hoffbrand *et al.*, 1998]. Previously recognized adverse effects of deferiprone included embryotoxicity, teratogenicity, neutropenia, and agranulocytosis [Hoffbrand, 1996]. Long-term treatment has been reported to be associated with progression of hepatic fibrosis [Carthew *et al.*, 1994]. Taken together, these data suggest that deferiprone does not adequately control body iron in a substantial proportion of patients and may promote worsening of hepatic fibrosis. The results of long-term follow-up of the effectiveness of other modes of administration of desferrioxamine are awaited. These include desferrioxamine attached to high molecular starch [Olivieri *et al.*, 1996], administered in twice-daily subcutaneous boluses [Borgna-Pignatti and Cohen, 1997], and given in a lipid vehicle, permitting

slow release [Porter *et al.*, 1997]. Additional efforts to produce oral desferrioxamine tablets are currently in the experimental and developmental stages [NOVARTIS].

I.15.3.b Augmentation of Fetal-Hemoglobin Synthesis

Several trials have attempted to augment the synthesis of fetal hemoglobin (HbF) in an effort to ameliorate the severity of β-thalassemia [Olivieri, 1996] by reactivation of γ-gene expression. Based on the finding of relative hypomethylation of the 5′-flanking region of the γ-genes in fetal erythroid cells compared to adult erythroid cells [van der Ploeg and Flavell, 1980; Busslinger *et al.*, 1983], DeSimone and colleagues [DeSimone *et al.*, 1982] used baboons to explore the action of 5-azacytidine, a drug known to lead to extensive demethylation [Jones and Taylor, 1980; Creusot *et al.*, 1982]. They observed a remarkable increment in HbF levels of bled baboons, giving some credence to the notion that drug treatment might be a means of modulating the switch of fetal hemoglobin experimentally. Ley and associates [Ley *et al.*, 1983], and Charache and coworkers [Charache *et al.*, 1983] cautiously treated a small number of β-thalassemia and sickle cell anemia patients with this agent and observed dramatic increases in HbF expression. Concomitant with HbF expression was the apparent hypomethylation of the γ-globin gene 5′-flanking region. A most curious observation was the rapidity of the general response to the drug: within about two days reticulocytes capable of synthesizing HbF emerged from the marrow. The potential toxicity of the drug later shifted interest to less toxic alternatives. Therapy with hydroxyurea [Arruda *et al.*, 1997], butyric acid

compounds [Perrine *et al.*, 1993], or a mixture of these agents [Olivieri *et al.*, 1997] has reduced or eliminated transfusion requirements in some thalassemic patients. Although they address a highly desirable, cost-effective goal in β-thalassemia, therapies to increase the synthesis of fetal hemoglobin in the disorder have, with few exceptions, proved disappointing to date. Nonetheless, important avenues to be pursued in further studies include the identification of specific mutations that may respond to therapy, particularly with specific combinations of agents [Olivieri, 1999].

I.15.3.c Gene Therapy

In light of the severe consequences of β-thalassemia and the increasing base of information regarding the β-gene locus, considerable discussion and effort have been directed toward the possible permanent correction of the genetic deficit of the hematopoietic system. This requires the transfer of genes into stem cells and long-term, high level, lineage-specific expression of these cells. Over the past decade, there has been progress in the development of transduction methods and vectors [Verma and Somia, 1997], other vectors include; Oncoretroviruses which do not inegrate in the host genome, Foamy virus which is known to be efficient in stem cells, Lentivirus which has a less efficiency in integration, AAV. is not very efficient and cannot infect stem cells, and deleted Adenovirus which is an improved virus that can infect stem cells. Remaining problems include the identification of all sequences required for stable, high-level expression of the genes and the development of more effective and safe vectors for the transfer of genes [Higgs *et al.*, 1998]. Another approach, correction of the defective gene by site directed

recombination, is feasible, but current methods lack the degree of efficiency required [Shesely *et al.*, 1991].

I.16 GENOTYPE-PHENOTYPE RELATIONSHIP

Several genetic factors may influence the clinical course of β-thalassemia. These include; the nature of β-thalassemia mutations that vary widely in their effect on the synthesis of the β-globin chains [Huisman *et al.*, 1997], co-inheriting factors that affect either α-globin [Thein *et al.*, 1984], or γ-globin gene expression [Camaschella *et al.* 1995], and many different interactions with structural hemoglobin variants may also result in a complex series of clinical phenotypes [Weatherall and Clegg, 2001]. In contrast, a number of acquired and environmental factors, including progressive splenomegaly, exposure to infections, socioeconomic factors, and the availability of medical care, may also modify the severity of the disease [Olivieri, 1999]. However, it is still speculative whether the genotype may be predictive of the expected phenotype. In general, the severity of thalassemia is usually assessed according to the age at presentation, which may vary according to awareness of the family and economic status, hemoglobin level, age at first blood transfusion, and the frequency of blood transfusion. Transfusions are usually administered for indications such as severe anemia or congestive heart failure. Hypertransfusion is usually performed with thalassemia major. However, in a number of patients with moderate severity, their hemoglobin levels may decrease after 3 to 4 years of age and they will require regular transfusion [Winichagoon *et al.*, 2000]. Accumulating

evidence points to the presence of additional unlinked, some as yet unknown, genetic factors influencing the severity of the anemia [Rund *et al.*, 1997].

I.16.1 *Association of β-Thalassemia with Increased Hemoglobin F Production*

The regulation of Hb F expression is multifactorial. Elevated HbF production has been known, for some time, to ameliorate the severity of β-thalassemia [Weatherall and Clegg, 1981; Rund *et al.*, 1997; Winichagoon *et al.*, 2000]. However, elevated HbF may also reflect anemic stress [Rochette *et al.*, 1994], and is therefore not always an indicator of the presence of mild disease. Increased hemoglobin F levels have been associated with certain β-globin gene promoter mutations such as those in the proximal CACCC box [Gonzalez-Redondo *et al.*, 1988; Camaschella *et al.*, 1990], co-inheritance of a gene for hereditary persistence of fetal hemoglobin [Thein and Weatherall, 1988; Cappellini *et al.*, 1981], or co-inheritance of β-thalassemia haplotypes associated with a determinant that produces high fetal hemoglobin [Thein *et al.*, 1988; Diaz-Chico *et al.*, 1988], and most frequently the -158 (C > T) substitution at the promoter region in the $^{G}\gamma$ globin-gene [Wood, 1993], which represents a cleavage site for X*mn* I that has been previously shown to be strongly linked to the (- + - + +) and (+ + - + +) haplotypes in sickle cell anemia and β-thalassemia [Thein *et al.*, 1987]. Sequence variations located in the β-locus control region (LCR; the main control region of the entire β-globin gene cluster), the 5′ hypersensitive site 2 (HS-2) [Öner *et al.*, 1992; Camaschella *et al.* 1995], the $^{A}\gamma$ IVS-II [Ragusa *et al.*, 1992a], and the 5′

flanking region of the β-globin gene [Ragusa *et al.*, 1992b] have also been implicated in the regulation of HbF expression, mainly in patients with sickle cell anemia (SS) and β-thalassemia. An X-linked determinant has also been associated with elevated HbF levels and F-cell production in normal adults and SS patients [Miyoshi *et al.*, 1988; Dover *et al.*, 1992]. HbF levels are also influenced by unlinked genetic modifiers of which there are likely to be multiple factors [Oppenheim *et al.*, 1990; Thein *et al.*, 1994].

I.16.2 *Interaction of β-Thalassemia with α-Globin Genotypes*

Heterozygosity for α-thalassemia in conjunction with β-thalassemia ameliorates the clinical condition of the disease [Weatherall and Clegg, 1981]. Conversely, excess α-globin genes have been implicated in increasing the severity of β-thalassemia [Sampietro *et al.*, 1983; Thein *et al.*, 1984]. Despite many advances that have been made in the molecular characterization of the thalassemias, some aspects of the genotype-phenotype relationships of this disease are still puzzling. One such aspect is the interaction of heterozygous β-thalassemia with α-globin triplication. This combination can cause thalassemia intermedia of mild to moderate severity [Sampietro *et al.*, 1983; Thein *et al.*, 1984; Camaschella *et al.*, 1987; Kulozik *et al.*, 1987] or, alternatively, simple β-thalassemia trait [Galanello *et al.*, 1983; Kanavakis *et al.*, 1983]. Previous studies [Galanello *et al.*, 1983; Camaschella *et al.*, 1987; Kulozik *et al.*, 1987; Camaschella *et al.*, 1995; Rund *et al.*, 1997], have shown that the presence of five α-globin genes could cause a heterozygote for β-thalassemia to develop a clinical picture compatible with mild thalassemia

intermedia. Whereas, the presence of six α-globin genes is associated with a more severe clinical manifestation [Rund *et al.*, 1997].

The co-inheritance of α-thalassemia is the only well-known unlinked factor that ameliorates the clinical course of β-thalassemia [Wainscoat *et al.*, 1983; Camaschella *et al.*, 1995]. The inheritance of three rather than four functional α-genes is capable of converting a severe transfusion-dependent form of homozygous β-thalassemia into thalassemia intermedia. [Wainscoat *et al.*, 1983]. The loss of two α-genes was reported to ameliorate the clinical course of homozygotes for β^{o} thalassemia [Galanello *et al.*, 1989]. The ameliorating effect of α-thalassemia probably results from a reduction in the excess of α-globin chains produced, leading to a lesser degree of ineffective erythropoiesis and hence to a milder clinical picture [Wainscoat *et al.*, 1983].

I.17 β-THALASSEMIA IN THE WEST BANK

As in other Mediterranean countries, β-thalassemia among Palestinians in the West Bank is widespread. Several studies have been conducted between the years 1994 to 1996 with the aim to screen for β-thalassemia carriers in the West Bank. These studies included 9734 university students. Findings of these studies indicate that the frequency of β-thalassemia trait among Palestinians was estimated to be 3.13% [Younis *et al.* 1994, 1996]. Currently, the total number of β-thala
intermedia patients, officially registered in the W
416 cases [Thalassemia Patients' Friends Society /
The West Bank, with an area of 5,500 square kilom

density of 200 persons per square kilometer. Approximately 30% of the population live in 12 urban areas, 60% in over 500 villages ranging in size from several hundred persons to over 25,000 with some 10% living in 19 refugee camps. Births in the West Bank have increased over the years, from 30.4 thousand total births in 1980 to 42.7 thousand in 1993. Based on these data, and the fact that consanguineous marriages are high among our population, it became very necessary to establish a population screening program aiming to control and prevent the occurrence of thalassemia major. Thalassemia major as an inherited disease will precipitate a socio-economical disaster in our country if left uncontrolled. Therefore, like in other similar societies where thalassemia is prevalent, it is extremely important to develop a national plan to counter this socio-medical problem.

I.18 OBJECTIVES OF THE STUDY

1. Analysis of β-Globin Gene Mutations Among Thalassemia Patients in the West Bank.

2. Distribution and Frequency of the Various Mutations and Genotypes in Several Districts of the West Bank.

3. Development of Prenatal Testing for Identification of Thalassemia Major Patients *in utero*.

4. Polymorphism Linkage Analysis and its Potential Implication on the Severity of the Disease.

5. Genotype – Phenotype Relationship.

6. Implications of the Genetic Data on the Social Structure and Behavior in the Various Districts.

CHAPTER II

MATERIALS AND METHODS

II.1 SUBJECTS AND PATIENT REFERRAL

A total of 131 patients identified on the basis of their hematological and clinical features from 115 families aged 3 - 65 years, and from seven different districts in the West Bank were subjected for genetic analysis in the β-globin gene. In all cases, informed consent was obtained. The complete description and related information about the patients is summarized in Table 1. Eight of these patients came from Jericho (6 families), 20 from Ramallah (18 families), 27 from Nablus (24 families), 27 from Qalqilia (19 families), 14 from Tulkarem (14 families), 7 from Jenin (7 families), and 28 from Hebron (27 families). In 17 families, there was more than one patient in the family (range 2 - 4), and 68.7% of the patients were below the age of 15 years.

II.2 SAMPLE PROCESSING

Five ml of blood specimens were collected in vacutainers with EDTA as anticoagulant, and transported in an ice box to the molecular genetic laboratory at Makassed Hospital.

Table 1. Patients' Profile

Patient code	Age (year)	Gender	Age at presentation	Age at first blood transfusion	Desferal administration	Blood transfusion intervals	Splenectomy	Phenotype
RM1	13	F	2 y	3 y	N	--	N	Intermedia
RM 2	16	M	3 y	3 y	Y	4 wk	Y	Major
RM 3	6	M	4 m	4 m	Y	5 wk	N	Major
RM 4	27	M	8 m	--	Y	4 wk	Y	Major
RM 5	12	M	4 m	4 m	N	--	N	Major
RM 6	33	F	10 y	10 y	N	4 m	Y	Major
RM 7	19	M	1 m	--	Y	2 wk	Y	Major
RM 8	8	F	7 m	1 y	Y	4 wk	Y	Major
RM 9	9	M	6 m	6 m	Y	2 wk	N	Major
RM 10	20	M	1 y	5 y	N	8 wk	N	Intermedia
RM 11	21	F	3 y	3 y	Y	4 wk	N	Major
RM 12	5	F	6 m	9 m	--	--	N	--
RM 13	6	M	7 m	7 m	Y	2 wk	Y	Major
RM 14	18	M	2 y	2 y	N	4 wk	N	Major
RM 15	15	M	2 y	2 y	Y	5 wk	N	Major
RM 16	16	F	4.5 y	4.5 y	Y	3.5 wk	Y	Major
RM 17	9	M	8 m	8 m	N	> 6 wk	N	Minor
RM 18	3	M	prenatal	1 m	Y	2 wk	Y	Major
RM 19	8	M	< 1 y	1 y	Y	4 wk	N	Major
RM 20	4	F	10 m	10 m	N	> 6 m	N	Intermedia
QL 1	11	F	2 m	2 m	N	6 wk	Y	Major
QL 2	9	F	3 y	3 y	N	> 6 wk	Y	Intermedia
QL 3	19	M	2 y	2 y	N	4 wk	N	Intermedia
QL 4	23	F	2 y	2 y	Y	6 wk	Y	Intermedia
QL 5	33	F	27 y	27 y	N	rare	N	Intermedia
QL 6	10	F	4 y	--	N	N	N	Intermedia
QL 7	9	M	3 y	1 wk	N	4 m	N	Intermedia
QL 8	10	F	1 y	1 y	Y	--	Y	Major
QL 11	40	M	37 y	37 y	N	6 wk	N	Intermedia
QL 12	6	M	2 m	2 m	Y	4 wk	N	Major
QL 13	15	F	2 m	2 m	Y	4 wk	Y	Major
QL 14	13	M	1 wk	1 m	Y	4 wk	N	Major
QL 15	10	M	1 y	1 y	Y	4 wk	Y	Major
QL 16	4	F	6 m	6 m	Y	4 wk	N	Major
QL 17	22	M	12 y	12 y	N	> 6 wk	N	Intermedia
QL 18	15	M	5 m	5 m	N	4 wk	Y	Major
QL 19	21	F	3 m	3 m	Y	4 wk	Y	Major
QL 20	4	F	2 y	--	N	N	N	Intermedia
QL 21	21	M	2 m	2 m	Y	6 wk	Y	Major
QL 22	11	M	--	--	--	--	--	--
QL 23	6	M	7 m	7 m	N	4 wk	N	Major
QL 24	7	F	9 m	9 m	N	4 wk	N	Major
QL 26	11	F	5 m	5 m	Y	4 wk	Y	Major
QL 27	19	F	2 y	4 y	N	--	Y	Intermedia

Patient code	Age (year)	Gender	Age at presentation	Age at first blood transfusion	Desferal administration	Blood transfusion intervals	Splenectomy	Phenotype
QL 28	21	F	4 y	4 y	N	--	Y	--
QL 29	20	M	4 y	4 y	N	> 6 wk	Y	Intermedia
QL 30	8	M	--	--	--	--	N	Intermedia
JR 1	17	F	3 y	3 y	Y	4 wk	Y	Major
JR 2	23	F	6 y	6 y	Y	> 6 wk	Y	Major
JR 3	12	F	3 m	5 m	Y	4 wk	Y	Major
JR 4	7	F	4 m	4 m	Y	4 wk	N	Major
JR 5	12	M	5 m	5 m	Y	2 wk	N	Major
JR 6	13	M	2 m	2 m	Y	2 wk	N	Major
JR 7	6	M	5 m	5 m	Y	4 wk	N	Major
JR 8	5	M	6 m	8 m	Y	4 wk	N	Major
NB 1	--	F	--	--	--	--	--	--
NB 2	60	M	30 y	24 y	N	--	--	Intermedia
NB 3	--	F	--	--	--	--	--	--
NB 4	7	M	--	--	--	--	--	--
NB 5	12	F	--	--	--	--	--	--
NB 6	14	M	--	--	--	--	--	--
NB 7	15	M	3 y	3 y	N	6 wk	--	Intermedia
NB 8	10	M	1 y	1 y	N	4 wk	N	Intermedia
NB 9	6	F	7 m	7 m	N	6 wk	N	Intermedia
NB 10	13	M	4 y	4 y	N	4 wk	N	Major
NB 11	11	M	2 y	2 y	--	4 wk	N	--
NB 12	7	F	--	--	--	--	--	--
NB 13	40	F	13 y	20 y	--	--	--	--
NB 14	6	F	2 y	1.5 y	N	3 wk	N	Intermedia
NB 15	6	F	-	-	-	-	-	--
NB 16	7	M	6 m	6 m	Y	4 wk	N	Major
NB 17	3	M	6 m	7 m	--	--	--	--
NB 18	9	M	1 y	1 y	--	4 wk	--	Major
NB 19	14	M	1 day	6 m	Y	2 wk	N	--
NB 20	20	F	4 y	--	--	--	--	--
NB 21	18	F	1 y	1 y	--	4 wk	--	Major
NB 22	6	F	--	--	--	--	--	--
NB 23	8	M	7 m	--	--	--	--	--
NB 24	5	F	--	--	--	--	--	--
NB 25	12	M	--	--	--	--	--	--
NB 26	3	M	--	--	--	--	--	--
NB 27	9	F	10 m	--	Y	--	N	Major
TK 1	9	M	1 y	1 y	Y	6 wk	N	Major
TK 2	7	F	4 m	4 m	Y	8 wk	N	Major
TK 3	10	M	3 m	3 m	Y	1 wk	Y	Major
TK 4	12	F	3 m	3 m	Y	4 wk	N	Major
TK 5	7	M	6 m	--	N	--	N	Major
TK 6	18	F	1 y	1 y	Y	4 wk	N	Major
TK 7	5	F	1 y	1 y	N	> 6 wk	N	Major
TK 8	24	F	2 y	2 y	Y	--	Y	Major
TK 9	10	M	6 y	6 y	N	8 wk	N	Intermedia

Patient code	Age (year)	Gender	Age at presentation	Age at first blood transfusion	Desferal administration	Blood transfusion intervals	Splenectomy	Phenotype
TK 10	10	F	4 y	4 y	N	4 wk	N	Major
TK 11	21	F	2 y	2 y	N	8 wk	N	Intermedia
TK 12	17	M	10 m	10 m	N	8 wk	N	--
TK 13	13	M	1.5 m	1.5 m	N	4 wk	N	Major
TK 14	15	M	2 y	2 y	--	--	N	Major
JN 2	8	M	6 m	6 m	Y	2 wk	N	Major
JN 3	5	F	1 m	2 m	Y	4 wk	N	Intermedia
JN 4	6	F	--	--	Y	6 wk	Y	--
JN 5	4	F	--	--	N	4 wk	N	Major
JN6	5	F	--	--	Y	--	N	Major
JN 7	5	M	6 m	6 m	Y	4 wk	N	Major
JN 8	4	F	3 m	3 m	Y	2 wk	N	Major
HE 1	34	F	20 y	20 y	N	4 wk	Y	Intermedia
HE 2	11	M	2 y	2 y	N	> 6 wk	Y	Intermedia
HE 3	9	M	7 m	7 m	Y	4 wk	N	Major
HE 4	5	M	6 m	6 m	Y	6 wk	N	Major
HE 5	6	F	9 m	--	N	> 6 wk	N	Intermedia
HE 6	7	M	2 y	3.5 y	Y	4 wk	Y	Major
HE 7	6	F	8 m	8 m	Y	4 wk	N	Major
HE 8	18	M	5 y	5 y	N	12 wk	N	Intermedia
HE 9	18	F	3.5 y	3.5 y	N	> 6 wk	Y	Intermedia
HE 10	20	M	6 m	6 m	Y	4 wk	Y	Major
HE 11	4	F	--	--	--	--	--	--
HE 12	23	M	8 m	8 m	Y	4 wk	N	Major
HE 13	4	M	8 m	8 m	N	6 wk	N	Major
HE 14	10	F	8 m	8 m	Y	4 wk	Y	Major
HE 15	5	F	3 m	3 m	Y	6 wk	N	Major
HE 16	10	F	8 m	8 m	Y	4 wk	N	Major
HE 17	26	M	4 y	4 y	N	18 wk	Y	Intermedia
HE 18	6	F	--	--	--	Y	--	--
HE 19	8	F	5 m	5 m	N	10 wk	N	Intermedia
HE 20	14	M	3 m	3 m	N	> 6 wk	N	Intermedia
HE 21	8	M	--	--	N	> 1 y	--	Intermedia
HE 22	8	F	4 m	4 m	Y	--	N	Major
HE 23	20	M	3.5 y	3.5 y	N	10 wk	Y	Intermedia
HE 24	12	M	9 y	9 y	N	> 6 wk	N	Intermedia
HE 25	15	M	1 y	2 y	Y	> 6 wk	Y	Major
HE 26	5	F	1.5 y	1.5 y	Y	4 wk	N	Major
HE 27	6	M	2 wk	2 wk	Y	4 wk	N	Major
HE 28	65	M	10 y	10 y	Y	6 wk	Y	Intermedia

Note: RM: Ramallah, QL: Qalqilia, JR: Jericho, NB: Nablus, TK: Tulkarem, JN: Jenin, HE: Hebron, Y: yes, N: no, M: male, F: female, y: years, m: months, wk: weeks, the dash represents not available data.

Source: These data were obtained from the Thalassemia Patients' Friends Society/West Bank.

II.2.1 *Buffy Coat DNA Purification*

Genomic DNA was extracted (within 48 hours of blood collection) using the MasterPure™ Genomic DNA Purification Kit (Epicentre Technologies) based on the method of Miller *et al.* [Miller *et al.*, 1988], and according to the protocol outlined below.

Draw 5 ml of EDTA blood

↓

Centrifugation at 1,000 xg for 15 minutes

↓ Transfer 600 µl of the buffy coat to a 1.5 ml microcentrifuge tube

Vortex mix and transfer 300 µl to two 1.5 ml microcentrifuge tubes

↓ Add 1.2 ml of Lysis Buffer 1 (10mM Tris-HCl, 400 mM NaCl and 2 mM Na_2EDTA, pH 8.2)

Invert 3 times to mix and then flick the bottom of the tubes to suspend any remaining material

↓

Incubate at room temperature for 5 minutes, Invert 3 times to mix and then flick the tubes (repeat this step twice)

↓ Centrifuge for 25 seconds at ≥10,000 xg

Pull off most of the supernatant, leaving approximately 25 µl of liquid

↓ Vortex mix to suspend the pellet

Add 600 µl of Lysis Buffer 2 (10% SDS, and protease K solution [1 mg protease K in 1% SDS and 2 mM Na_2EDTA]), then pipette the cells up and down 5-7 times

↓ Add 250 µl of the Precipitation Solution (6M NaCl)

Vortex mix vigorously for at least 30 seconds

↓ Centrifuge for 10 minutes at ≥10,000 xg

Pour the supernatant into a clean microcentrifuge tube, add 700 µl of isopropanol. Invert the tube several (30 – 40) times; a stringy precipitate should be visible

↓ Centrifugation at 4°C for 10 minutes

Carefully pour off the supernatant without dislodging the pellet. Rinse twice with 75% ethanol. Centrifuge briefly if the pellet is dislodged. Remove all *the residual ethanol with a pipette*

↓ Add 200 µl of Tris-EDTA (TE) Buffer (10 mM Tris-HCl, 0.2 mM Na_2EDTA, pH 7.5)

Incubate overnight at room temperature, or suspend the DNA by pipetting repeatedly followed by vortex mixing for 10 seconds

↓

Quantify the DNA by following the procedure described below

↓

Store the purified DNA at –20°C until required for analysis

II.2.2 *DNA Quantitation*

DNA quantitation was performed according to the following procedure; purified DNA was diluted 1:100 (5 μl of DNA) in 500 μl of TE buffer (10 mM Tris-HCl, and 1 mM Na_2EDTA, pH 8). Absorbance was measured at 260 nm and 280 nm, using a photometer adapted for microsamples (Gene Quant II-Pharmacia Biotech). The ratio of 260/280 was calculated to evaluate the purity of DNA in each sample. The DNA concentration was calculated according to the following formula; 1A unit = 50 μg double-stranded DNA. The DNA concentration obtained for all samples was in the range of 0.112 – 1.62 μg/μl.

II.3 IDENTIFICATION OF MUTATIONS

In order to identify the various mutations in the β-globin gene, the polymerase chain reaction (PCR) method was utilized combined with the amplification refractory mutation system (ARMS) using the MiniCycler™ (MJ Research, Inc.). Direct sequence analysis was performed according to the dideoxynucleotide method [Sanger *et al.*, 1977] utilizing a commercially available sequencer (ABI PRISM™ Model 3700 version 3.3 DNA Sequencer) and ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, with DNA Sequenase.

Initially, mutation screening using the ARMS method focused on the five most common β-thalassemia mutations previously described in the Mediterranean region namely the mutations at IVS-I position 110 (G > A), Codon 39 (C > T), IVS-I position 6 (T > C), IVS-I position 1 (G > A), and IVS-

II position 1 (G > A) [Orkin and Kazazian, 1984]. The amplification refractory mutation system (ARMS) is an amplification strategy in which a polymerase chain reaction (PCR) primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue. Thus, amplifying a specific member of a multi-allelic system while remaining refractory to amplification of another allele that may differ by as little as a single base from the former. Hence, the presence of an amplified product indicates the presence of a particular allele and vice versa. ARMS is based on the principle that the *Thermus aquaticus* (*Taq*) polymerase, the DNA polymerase commonly used in PCR, lacks a 3′ to 5′ exonuclease activity and thus a mismatch between the 3′ end of the PCR primers and the template will result in greatly reduced amplification efficiency. The principle of this method is illustrated in Figure 1. Three primers are utilized in ARMS: Common primer that is chosen to flank the site of the suspected mutation, Normal primer, in which the last nucleotide at the 3′ end is chosen to match the corresponding nucleotide in the normal sequence, Mutant primer, in which the last nucleotide at the 3′ end is chosen to match the mutation point and to mismatch the normal sequence under appropriate specific conditions. The main advantage of ARMS is that the amplification step and the diagnostic steps are combined, in addition to being an accurate, rapid, and a simple method [Lo, 1998; Tan *et al.*, 1994]. The samples were then analyzed by direct sequence analysis. Consequently, and based on the sequencing results, other mutations could be identified and eventually screened for by ARMS.

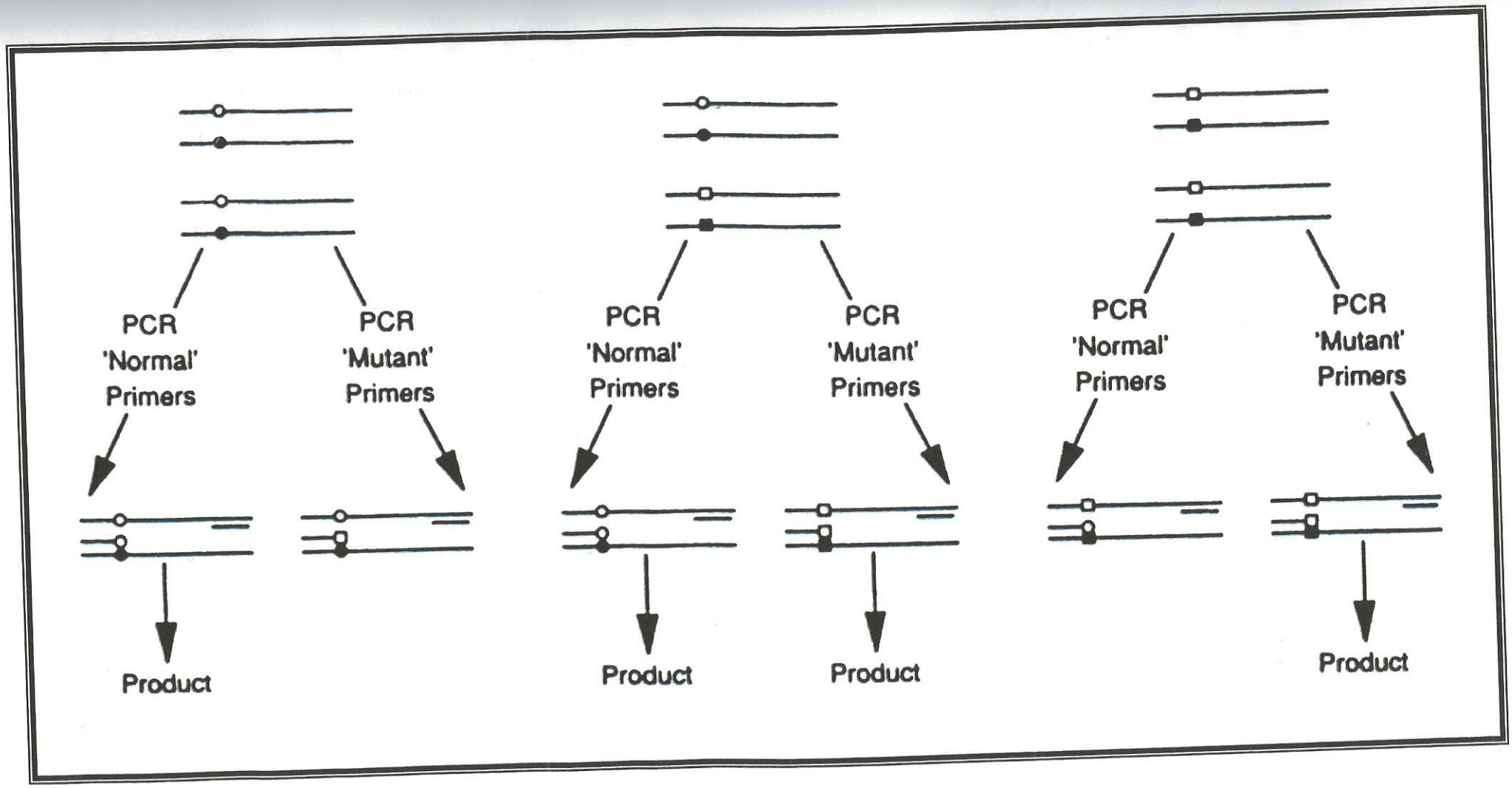

Figure 1. The Amplification Refractory Mutation System (ARMS)

The list of commercially synthesized oligonucleotide primers (GibcoBRL Custom Primers-Life Technologies, Inc.) used to detect the various β-globin gene mutations, is described in Table 2. These include primers used in ARMS, direct sequence analysis primers, in addition to a set of internal control primers that amplify an 861 bp fragment (IVS-II-593 to 361 nucleotides downstream of the polyadenylation signal) within the β-globin gene that were included to demonstrate the efficacy of the PCR protocol. Primer stock solutions were prepared as follows; the lyophilized primers were first carefully centrifuged for few seconds to collect the DNA in the bottom of the tubes. The DNA was then reconstituted with an appropriate volume of TE buffer to a final concentration final concentration of 0.2 μg/μl.

II.3.1 ARMS-PCR PROTOCOL

The ARMS reaction was conducted in a total volume of 25 μl reaction mixture containing 1X TaKaRa PCR buffer (50 mM KCl, 10mM Tris HCl pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM dNTPs (TaKaRa Biomedicals), 0.625 Unit (U) of recombinant of 10 μg/μl. Primers were then diluted for PCR in sterile distilled water to a *TaKaRa Taq*™ DNA polymerase, 0.5 – 0.7 μg of template DNA, and 0.2 μg each primer. The mixture was then overlaid with 30 μl of mineral oil. All PCR experiments included a negative control sample using DNA from a non- thalassemic person, and an internal control to ensure the efficiency and specificity of the reactions. The detailed procedure for the ARMS-PCR is shown in Table 3. The PCR conditions for the ARMS reactions were as follows; one cycle of initial denaturation at 94°C for 4 minutes, a

Table 2. Primers Utilized in PCR Assays

Mutation	5′ – 3′ Primer Sequence	Primer Type	Size product, bp	Reference
	ARMS PRIMERS			
-30 [T>A]	GCAGGGAGGGCAGGAGCCAGGGCTGGGGAT GCAGGGAGGGCAGGAGCCAGGGCTGGGGAA **CCCCTTCCTATGACATGAACTTAA**	Normal (F) Mutated (F) **Common (R)**	626	[Elles, 1996]
Cd 5 [-CT]	CAAACAGACACCATGGTGCACCTGACTCCT TCAAACAGACACCATGGTGCACCTGAGTCG **CCCCTTCCTATGACATGAACTTAA**	Normal (F) Mutated (F) **Common (R)**	528	[Elles, 1996]
Cds 8/9 [+G]	CCTTGCCCCACAGGGCAGTAACGGCACACT CCTTGCCCCACAGGGCAGTAACGGCACACC **ACCTCACCCTGTGGAGCCAC**	Normal (R) Mutated (R) **Common (F)**	225	[Elles, 1996]
IVS-I-1 [G>A]	TTAAACCTGTCTTGTAACCTTGATACCCAC TTAAACCTGTCTTGTAACCTTGATACCGAT **ACCTCACCCTGTGGAGCCAC**	Normal (R) Mutated (R) **Common (F)**	281	[Elles, 1996]
IVS-I-5 [G>C]	CTCCTTAAACCTGTCTTGTAACCTTGTTAC CTCCTTAAACCTGTCTTGTAACCTTGTTAG **ACCTCACCCTGTGGAGCCAC**	Normal (R) Mutated (R) **Common (F)**	285	[Elles, 1996]
IVSI-110 [G>A]	ACCAGCAGCCTAAGGGTGGGAAAATACACC ACCAGCAGCCTAAGGGTGGGAAAATAGAGT **ACCTCACCCTGTGGAGCCAC**	Normal (R) Mutated (R) **Common (F)**	390	[Elles, 1996]
Cd 37 [G>A]	TCCCCAAAGGACTCAAAGAACCTCTGGGTC TCCCCAAAGGACTCAAAGAACCTCTGGGTT **ACCTCACCCTGTGGAGCCAC**	Normal (R) Mutated (R) **Common (F)**	406	[Present study]
Cd 39 [C>T]	TTAGGCTGCTGGTGGTCTACCCTTGGTCCC **CTGAGACTTCCACACTGATGC** CAGATCCCCAAAGGACTCAAAGAACCTGTA **ACCTCACCCTGTGGAGCCAC**	Normal (F) **Common (R)** Mutated (R) **Common (F)**	365 436	[Elles, 1996]
IVS-II-1 [G>A]	AAGAAAACATCAAGGGTCCCATAGACTGAC AAGAAAACATCAAGGGTCCCATAGACTGAT **ACCTCACCCTGTGGAGCCAC**	Normal (R) Mutated (R) **Common (F)**	634	[Elles, 1996]
IVSII-848 [C>A]	TCATGTTCATACCTCTTATCTTCCTCCCAC TCATGTTCATACCTCTTATCTTCCTCCCAA **GAGTCAAGGCTGAGAGATGCAGGA**	Normal (F) Mutated (F) **Common (R)**	635	[Present study]
IVS-I-6 [T>C]	TCTCCTTAAACCTGTCTTGTAACCTTCATA TCTCCTTAAACCTGTCTTGTAACCTTCATG **TACGGCTGTCATCACTTAGACCTCACCCTG**	Normal (R) Mutated (R) **Common (F)**	305	[Cyprus, 1999]
	DNA SEQUENCING PRIMERS			
P5 P12	CCAACTCCTAAGCCAGTGCC CTGAGACTTCCACACTGATGC	**Forward** **Reverse**	799	[Rund *et al*, 1991]
P7 P10	CTTTCCCTAATCTCTTTCTTTCAGGGC CACTGACCTCCCACATTCCC	**Forward** **Reverse**	616	[Rund *et al*, 1991]
	INTERNAL CONTROL PRIMERS			
P15 P14	CAATGTATCATGCCTCTTTGCACC GAGACAAGGCTGAGAGATGCAGGA	**Forward** **Reverse**	861	[Elles, 1996]

Note: F: Forward, R: Reverse

Table 3. PCR Procedure for ARMS and β-Globin Gene Amplification for Sequence Analysis

REAGENTS		ARMS		β-Globin Gene Amplification
		Tube # 1	Tube # 2	Tube
Buffer (10 X) (final concentration)		2.5 µl (1X)	2.5 µl (1X)	10.0 µl (1X)
dNTP mixture (2.5 mM each) (final concentration)		2.0 µl (0.2 mM)	2.0 µl (0.2 mM)	8.0 µl (0.2 mM)
Primers (0.2µg/µl)	Normal (final quantity)	1.0 µl (0.2 µg)	—	—
	Mutated (final quantity)	—	1.0 µl (0.2 µg)	—
	Common (final quantity)	1.0 µl (0.2 µg)	1.0 µl (0.2 µg)	—
	P5, P7 (Forward) (final quantity)	—	—	4.0 µl (0.8 µg)
	P12, P10 (Reverse) (final quantity)	—	—	4.0 µl (0.8 µg)
Enzyme (5 U/µl) (final quantity)		0.125 µl (0.625 U)	0.125 µl (0.625 U)	0.5 µl (2.5 U)
DNA (0.5 - 0.7 µg/µl) (final quantity)		1.0 µl (0.5 - 0.7 µg)	1.0 µl (0.5 - 0.7 µg)	4.0 µl (2.0 - 2.8 µg)
Sterile Water		Up to 25 µl	Up to 25 µl	Up to 100 µl
Total Volume		25 µl	25 µl	100 µl

second denaturation step at 93°C for one minute (the number of cycles and annealing temperatures for the different alleles are outlined in Table 4), extension at 72°C for 1 minute and 30 seconds, and a final extension cycle at 72°C for 3 minutes.

II.3.2 SAMPLE PREPARATION FOR DIRECT DNA SEQUENCING

Two DNA fragments of the β-globin gene were amplified for direct sequencing analysis between nucleotides -166 to nucleotide 60 downstream from the polyadenylation signal site, as described by Rund *et al.* [1991]. The first fragment was amplified by the P5 and P12 primers, and extends between nucleotide -166 upstream of the cap site to nucleotide +633 resulting in a 799 base pair fragment. The second fragment was amplified by the P7 and P10 primers, and extends between nucleotides +1050 to +1666 resulting in a 616 base pair fragment. A fragment extending between nucleotides 139 to 555 in the second intervening sequence was not included in the sequencing analysis [Rund *et al.*, 1991]. The PCR amplification was performed in a 100 µl reaction volume containing the following mixture; 1X TaKaRa PCR buffer (50 mM KCl, 10mM Tris HCl pH 8.3, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP (TaKaRa Biomedicals), 2.5 U of recombinant *TaKaRa Taq*™ DNA polymerase, 2.0 - 2.8 µg of template DNA, and 0.8 µg of each primer. The mixture was then overlaid with 100 µl of mineral oil. The amplification program was as follows; initial denaturation at 98°C for 2 minutes, followed by 35 cycles of denaturation at 98°C for 15 seconds, annealing at 62°C for 45 seconds, extension at 72°C for 1 minute, followed by a final extension cycle at 72°C for 5 minutes.

Table 4. PCR - ARMS Conditions for Various Mutations

Mutation	DNA (µg)	Annealing Temp	# of cycles	Additives
Promoter – 30 (T > A)	0.7	65°C	26	1% Formamide + 1.5% DMSO*
Cd 5 (- CT)	0.5	65°C	27	4% Formamide
Cds 8/9 (+ G)	0.5	65°C	25	—
IVS-I-1 (G > A)	*0.5*	*65°C*	*26*	—
IVS-I-5 (G > C)	0.5	65°C	26	—
IVS-I-6 (T > C)	0.5	62°C	25	1% DMSO
IVS-I-110 (G > A)	0.5	65°C	24	—
Cd 37 (G > A)	0.5	67°C	25	4% Formamide
Cd 39 (C > T)	0.7	65°C	22	—
IVS-II-1 (G > A)	0.5	65°C	26	—
IVS-II-848 (C > A)	0.7	65°C	23	—

* DMSO: Dymethyl Sulfoxide

II.4 DNA POLYMORPHISM ANALYSIS

Linked sequence variations within the β-globin gene were examined for 210 chromosomes by direct sequence analysis of the anti-sense strand for the 799 base pair fragment amplified by the P5 and P12 primers as explained above.

II.5 AGAROSE GEL ELECTROPHORESIS

II.5.1 *ARMS Products Separation*

The PCR products (10 µl of 25 µl) were separated on a 4-mm-thick horizontal 2% (w/v) agarose (Techcomp, Ltd.) gels prepared in Tris-Acetate EDTA (TAE) buffer (0.04 M Tris-Base, 0.04 M glacial acetic acid, 0.001 M Na_2 EDTA, pH 8.0) as previously described [FMC®], with the addition of 0.5 µg ethidium bromide/ml. Three µl of glycerol loading buffer (6x recipe; 30% Glycerol in distilled water, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF)[FMC®] were added to the PCR product prior to loading on the gel. The TAE running buffer contained 0.125 µg/ml of ethidium bromide. Each gel was allowed to run for 1 hour at 100 – 110 volts, using a standard 100 bp DNA ladder size marker (CibcoBRL®-Life Technologies), and later visualized under UV light.

II.5.2 *β-Globin Fragments Purification For Sequence Analysis*

The PCR products (100 µl) were mixed with 12 µl of glycerol loading buffer and separated on 2% agarose gels (w/v), according to the same protocol used for ARMS analysis as described above. The gel segments containing the amplified β-globin gene fragments were excised from the gel and the DNA

was purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech.) according to the protocol described below. Direct genomic sequencing of the purified DNA fragments was performed on either the sense and/or the anti-sense strand(s), using the automated sequencer in the sequencing facilities at the Hadassah Hospital in Jerusalem.

PURIFICATION OF DNA FROM GEL BANDS

Weigh an empty 1.5 ml microcentrifuge tube to the nearest 10 mg and record the weight

↓

Using a clean scalpel, excise the slice of agarose containing the DNA band to be purified. Cut as close to the DNA band as possible. Cut the slice into several smaller pieces and transfer them to the pre-weighed 1.5 ml microcentrifuge tube

↓

Weigh the tube containing the agarose slice to the nearest 10 mg, and subtract the weight of the empty tube to determine the weight of the slice

↓

Add 10 µl of Capture Buffer (Buffered solution containing acetate and chaotrope) for each 10 mg of gel slice

↓

Close the tube and mix by vortexing vigorously. Incubate at 60°C until the agarose is completely dissolved (5-15 minutes)

↓

During the incubation, place one GFX Column (MicroSpin™ columns pre-packed with a glass fiber matrix) in a Collection Tube for each purification to be performed

↓

After the agarose is completely dissolved, centrifuge briefly to collect the sample at the bottom of the tube

↓

Transfer the sample to the GFX Column. Incubate at room temperature for 1 minute and centrifuge at full speed (10,000 to 16,000xg) for 30 seconds

↓

Discard the flow-through by emptying the Collection Tube (2 ml capless microcentrifuge tubes). Place the GFX Column back inside the Collection Tube

↓

Add 500 µl of Wash Buffer (TE buffer, and absolute ethanol to a final concentration of 80%) and centrifuge at full speed for 30 seconds

↓

Discard the Collection Tube and transfer the GFX Column to a fresh 1.5 ml microcentrifuge tube (i.e. not a Collection Tube)

↓

Apply 35 µl of autoclaved double-distilled water directly to the top of the glass fiber matrix in the GFX Column

↓

Incubate the sample at room temperature for 1 minute

↓

Centrifuge at full speed for 1 minute to recover the purified DNA