THE MOLECULAR BASIS OF ALPHA-THALASSEMIA IN TURKEY:ESTABLISHMENT AND APPLICATION OF PCR-BASED METHODS

by

A. Özlem Taştan

B.S. in Physics, Boğaziçi University, 1993

Submitted to the Institute for Graduate Studies in Science and Engineering in partial fullfilment of the requirements for the degree of Master of Science in Molecular Biology and Genetics

Boğaziçi University

1996

Sevgili Pan,
İçimi güzelleştir; çünkü dışımın güzelleşeceği yok nasılsa.
Asıl zenginlerin bilgeler olduğunu düşüneyim,
Tanrılara en çok benzeyenlerin, en azla yetinen olduğunu.
Elimde ne varsa, onunla yetineyim,
Ama kendimi yeterli bulmayayım.
Aldığımdan fazla vereyim; nefret ettiğimden fazla seveyim.
Yaşamış olduğumdan çok şimdi yaşadığımı düşüneyim.

Maxwell Anderson
(Sokrates'ın Duası)

TO MY MOTHER, BROTHER

AND

TO THE MEMORY OF MY FATHER

ACKNOWLEDGEMENTS

The work presented here was performed under the auspices of the Department of Molecular Biology and Genetics at Boğaziçi University between September 1993 and February 1996, supervised by Prof. A. Nazlı Başak and supported by Boğaziçi University Research Funds.

I would like to express my sincere gratitude to my thesis advisor Prof. A. Nazlı Başak for her continuous supervision and valuable criticisms throughout this work.

I would like to thank Assoc. Prof. Hande Çağlayan, Assoc. Prof. Lebriz Sağlamer and Ass. Prof. Nalan Gökgöz for their contribution to participate in my thesis presentation as Committee Members.

I would like to thank Ass. Prof. Erol Baysal for his guidance in the establishment of the methods.

Appreciation is also extended to my graduate teachers Prof. A. Nazlı Başak, Prof. Betül Kırdar, Prof. Aslı Tolun, Assoc. Prof. Hande Çağlayan, Assoc. Prof. Kuyaş Buğra for their help in building up my present background.

I wish to thank Assoc. Prof. Duran Canatan, Antalya State Hospital, and Prof. Nejat Akar and Hakkı Taştan, Ankara Medical School, for providing the blood samples.

I am grateful to the teachers of the Physics Department for their continuous support to force me to find a new area of science to study.

I would like to thank Assoc. Prof. Yağmur Denizhan for extending my scope and knowledge in different aspects.

I would like to say thank you to all my friends in the Department of Molecular Biology and Genetics, for their continuous help and constructive criticisms as well as their endless cheerful talks. I would also like to thank Fatma Yıldız, Ümit Bayraktar, Cenan Özbakır and Fulya Bergin for their support.

I am indepted to my family for their patience and moral support.

Last but not least I would like to say an enourmous thank to my special friend Metin Sitti for his continuous will to type this thesis, for the excellent preparation of the figures, for his patience and for his supply of love.

ABSTRACT

As in many other Mediterranean countries, hemoglobinopathies, especially thalassemias pose a major health concern in the Turkish population. Alpha-thalassemias, which may be the most common genetic disorders of human being, result from underproduction of the α-globin chains. Reviewing the thalassemia studies at molecular level, it is observed that β-thalassemia has attracted much more attention than α-thalassemia in Turkey. The lack of essential molecular studies in α-thalassemia may likely be due to diagnosis difficulty of the α-thalassemia symptoms; furthermore, a variety of technical and economical reasons may have prevented the establishment of large-scale population screening programs to detect the α-thalassemia mutations in Turkey thus far.

A breakthrough in the molecular diagnosis of α-thalassemia mutations occured with the advent of PCR technology. The present thesis is focused on the establishment of PCR-based methods and their applications to the detection of the four most common α-thalassemia determinants in the Turkish population. The introduction of PCR-based methods for direct and specific detection of the most frequently encountered α-thalassemia determinants are very promising in screening programs; because PCR-based methods are faster, more specific, cheaper and simpler than other methods that are used to detect the α-thalassemia deletions.

Making use of two different PCR strategies, 32 Turkish Hb H patients and α-thalassemia carriers were studied in the framework of this thesis. It was observed that the most common genotype in Hb H disease was associated with a combination of 20.5 kb and 3.7 kb deletions. Moreover, the systematic investigation of cord-blood samples from newborn babies revealed the presence of a potential α-thal-2 carrier population in Antalya. Due to their relatively common presence in the world, both α- and β-thalassemia mutations can be coinherited in the same individual. In this thesis, such a case was observed in a family.

ÖZET

Diğer birçok Akdeniz ülkesinde olduğu gibi, hemoglobinopatiler, özellikle talasemiler, Türkiye'de önemli bir sağlık problemi oluşturmaktadır. İnsanoğlunda en sık görülen genetik hastalık olan α-talasemiler, α-globin zincirlerinin yetersiz üretiminden ortaya çıkmaktadır. Türkiye'de moleküler düzeyde yapılan talasemi çalışmaları incelendiğinde, β-talaseminin α-talasemiden daha fazla çalışılmış olduğu görülmektedir. Bunun bir nedeni α-talasemi semptomlarının tanısındaki zorluklardır. Ayrıca, ülkemizde çeşitli teknik ve ekonomik nedenlerden dolayı α-talasemi mutasyonlarını belirlemek amacıyla büyük ölçekli nüfus tarama programları henüz gerçekleştirilmemiştir.

PCR yönteminin geliştirilmesi α-talasemi mutasyonlarının moleküler düzeyde incelenmesinde bir devrim yaratmıştır. Bu tez, α-talasemide sıklıkla görülen dört mutasyonu incelemekte kullanılan PCR yöntemlerinin kurulması ve uygulanmasını amaçlamaktadır. PCR'a dayalı yöntemlerin sık rastlanan α-talasemi mutasyonlarına uygulanması tarama programları açısından çok yararlıdır; PCR yöntemleri bundan önce α-talasemi tanısında kullanılan metodlardan daha hızlı, daha özgün, daha ucuz ve daha direkttir.

Bu çalışmada, iki farklı PCR yöntemi kullanılarak Türkiye'deki Hb H ve α-talasemi hastaları taranmıştır. Hb H hastalığında en sık rastlanan genotipi 20.5 kb ve 3.7 kb'lık delesyonların oluşturduğu gözlenmiştir. Bunun dışında yeni doğan bebeklerin kordon kanında yapılan bir çalışma, Antalya bölgesinde α-talasemi-2 mutasyonu taşıyan geniş bir nüfusun yer aldığını göstermiştir. Türkiye'de oldukça yaygın olan α ve β talasemilerin aynı kişide görülme olasılığı mevcuttur; bu tez çerçevesinde hem α, hem β talasemi mutasyonu taşıyan bir ailede incelenmiştir.

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LIST OFABBREVIATIONS

A_{260}	absorption at 260 nm
A_{280}	absorption at 280 nm
BSA	bovine serum albumin
BPB	bromophenol blue
bp	basepair
DMSO	dimethyl-sulfoxide
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenedinitrilo-tetraacetate
EtBr	ethidiumbromide
Hb	hemoglobin
Htc	hematocrite
kb	kilobase
Ma	marker
MCV	mean cell volume
PCR	polymerase chain reaction
RBC	red blood cell
rpm	revolution per minute
SDS	sodium dodecyl sulphate
UV	ultraviolet

I. INTRODUCTION

A. Why Human Globin Genes?

Studies on globin genes have contributed to the understanding of many basic principles of human molecular genetics since 1950 (Weatherall and Clegg, 1981; Bunn and Forget, 1986). Initial landmarks in both molecular and population genetics have been derived from the analysis of globin genes, and hemoglobinopathies mostly arose from the mutations of these genes.

The importance of globin genes in molecular biology studies comes from their properties such as unusual features of terminally differentiated red blood cells which synthesize huge amounts of hemoglobin due to selective expression of the globin genes and switching mechanisms of the α-like and β-like globin genes during the developmental stages of the hemoglobin (Higgs and Weatherall, 1993).

The study of human globin genes has provided excellent ways of how new methods can be applied to analyze genetic diseases such as thalassemias and the use of molecular genetic techniques has made possible new approaches to the question of why genetic diseases exist in a population or how the geographical and ethnic distribution of a genetic disease is (Flint et al. 1993; Higgs and Weatherall, 1993; Baysal, 1995).

In the introduction part of this thesis, in which one of the most common genetic disorders worldwide, α-thalassemia, is studied, in Turkey, the structure of the α or α-like globin genes and the properties of α-thalassemia are presented in more detail than the other hemoglobin abnormalities.

1. Alpha-Globin Genes

a. Localization and Cloning: Historical Background

Genetic studies in a family with α and β-globin variants performed in the late 1950s showed that these globins are encoded by genetically distinct loci (Higgs et al., 1989). In 1976, Deisseroth et al. confirmed that the α and β loci are on different chromosomes by studying somatic cell hybrids.

One year later Gandini et al. (1977) proposed that α loci are on the long arm of chromosome 4 (4q28-q34) by means of a finding of excess α-chain synthesis in patients with duplication of this region. In the same year, Deisseroth et al. (1977) established the assignment of the α-globin locus to chromosome 16 by combining the methods of somatic cell hybridization and DNA-cDNA hybridization. They used 16 human x mouse somatic cell hybrids containing a variable number of human chromosomes. Globin gene sequences were detected by annealing purified human α-globin cDNA to DNA extracted from hybrid cells. The cDNA probes for human globin genes did not react with mouse globin DNA sequences. The chromosomal composition of hybridization was also characterised by treating different types of staining techniques such as Giemsa trypsin, Hoechst 33258 and alkaline Giemsa stain (Giemsa 11). Comparison of the chromosomal composition of the hybrid clones with the presence or absence of the human α-globin gene permitted identification of human chromosome 16 as the one which contains the human α-globin locus. In a final test of the association of human chromosome 16 and the α-globin structural gene, hybrid clones were grown in selective media designed to promote the retention or elimination of this chromosome. The analysis of these clones confirmed that the presence of the human α-globin gene correlates with the presence of human chromosome 16.

To define more precisely the number and arrangement of the α-globin gene, Orkin (1978) performed a series of restriction endonuclease mapping experiments. In these experiments, α-specific fragments, in restriction enzyme digests of total genomic DNA, were identified after electrophoresis by hybridization with [^{32}P] cDNA probes followed by

Southern blotting. The data indicated that the α genes occur in duplicate and that the two copies lie close together, about 3.7 kb apart.

In 1980, Liebhaber et al. cloned one of the two α-globin genes and reported its complete nucleotide sequence. For this purpose, they digested genomic DNA isolated from liver tissue of aborted fetuses which was then fractionated by discontinuous horizontal gel electrophoresis followed by Southern blotting with a cDNA probe. Then, the DNA from the 3 to 4.5 kb digest was inserted and cloning was performed. Positive plaques were purified and DNA from each positive clone was digested with EcoRI and analysed by Southern hybridization using cDNA probes specific for the α-globin gene. According to the results, the α globin gene was 832 base pairs long, from the 5'-cap site to the 3'-polyadenylylation site. The amino acid coding sequences were separated into 3 segments (exons) by two short (117 and 140 bp) intervening sequences. Highly conserved regions were identified in the 5'-flanking region, the intron-exon junctions and the 3'-noncoding regions.

In the same year Lauer et al. (1980) studied the chromosomal arrangement of the human α-like globin genes. They used molecular cloning procedures to demonstrate physical linkage between the two α-globin genes and the two α-like sequences (ζ_1 and $\psi_{\alpha 1}$); they were able to present an analysis of homologous regions within the coding, intervening and flanking sequences of the two adult α-globin genes and characterized two types of deletions which occur in recombinant phage DNA during propagation in E.coli., as a consequence of unequal crossing over between homologous sequences.

Finally by combining somatic cell hybridization with a cDNA probe in the study of a cell line with reciprocal translocation between 16q and 11q, it was showed that the α-globin genes are in the short arm of chromosome 16 (Koeffler et al., 1981).

In 1981 Gerhard et al. used an improved method of *in situ* hybridization to confirm the assignment of the α-globin cluster to chromosome 16p. This method involved the use of a labeled hybrid cDNA plasmid for direct hybridization in situ to metaphase cell spreads from the organism under investigation. They employed a human α-globin cDNA plasmid (JW101) to localize the corresponding gene cluster; to obtain a sufficiently large autoradiographic signal, they labeled this plasmid with ^{125}I to a high specific activity and took advantage of the ability of a double-stranded probe to form networks.

By a combination of *in situ* hybridization, Southern blot analysis and linkage analysis, using the fragile site 16p12.3 and translocation breakpoints within band 16p13.1, Simmers et al. (1987) mapped the α-globin gene complex to 16pter-p13.2. Again recently, two independent studies of individuals with unbalanced karyotypes have unequivocally located the α locus to the Giemsa negative band 16p13.3 at the very tip of chromosome 16 (Breuning et al., 1987; Buckle et al., 1988) (Figure I.1). Buckle described a child in whom cytogenetic analysis indicated monosomy for 16pter-p13.3. DNA studies showed that the patient had not inherited either maternal α-globin allele. DNA obtained from peripheral blood mononuclear cells from mother, father and the child was cut in single digest with a variety of restriction enzymes. The DNA was then Southern blotted and hybridized on separate occasions with α-globin 3'HVR and α-globin 5'HVR probes. Using these probes, the proband was shown to have inherited one paternal allele at each locus, however she did not inherit a maternal allele at either locus. Thus the proband, who is monosomic for 16p13.3 $\rightarrow$ pter, failed to inherit the α-globin complex and at least 100 kb of the surrounding DNA from her mother. This child had α-thalassemia trait as well as a moderate mental retardation and dysmorphic features.

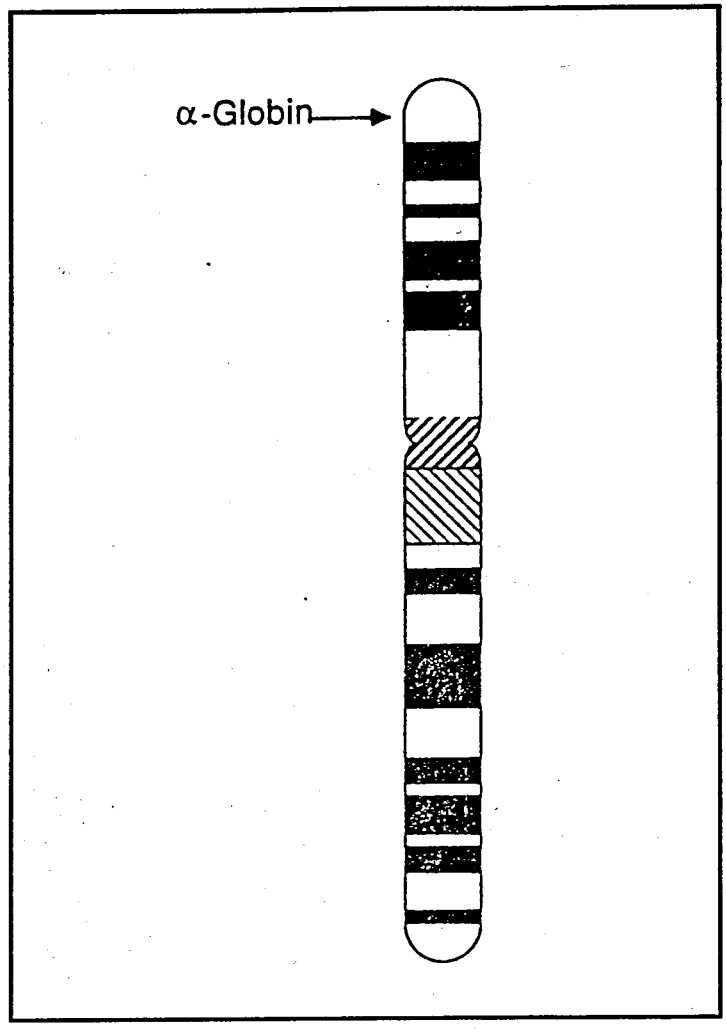


Figure I.1. The α-globin gene cluster at 16p13.3 (Higgs et al., 1989).

b. Structural Features of the α-Globin Gene Cluster

The human α-globin gene cluster which is located near the telomere of the short arm of chromosome 16, encompasses approximately 29 kb and consists of four genes and three pseudogenes arranged in the order 5'-ζ-Ψζ-Ψα$_2$-Ψα$_1$-α$_2$-α$_1$-θ$_1$-3' (Nicholls et al., 1987; Higgs et al., 1989) (Figure I.2). Each of these genes has been fully sequenced, and the functions of all but one (θ$_1$) is clearly defined (Liebhaber, 1989; Fortina, 1991). DNA

sequence analysis of the α-globin gene cluster has shown that the α_2 and α_1 genes are embedded with two highly homologous four kb duplicated segments which can be divided into homologous subsegments (X, Y, Z) by nonhomologous elements (I, II, III) (Higgs et al., 1989; Baysal and Huisman, 1994) (Figure I.3). The α-globin genes which are composed of three exons interrupted by two short introns are highly similar due to these homologous subsegments. The structural divergence is limited to two single base substitutions and a seven bp deletion in the second intron of α_2 relative to α_1, and eighteen base substitutions and a single base deletion in exon three of α_2 relative to α_1, all located within the 3'-untranslated region. Since the structural divergence of the two mature α-globin mRNAs is in the 3'-untranslated region, they encode identical α-globin proteins (Liebhaber, 1989; Albitar et al., 1992). It is thought that the high degree of sequence similarity between the two α-globin genes is due to the α-globin gene duplication, occurred over 300 million years ago (Liebhaber, 1989).

Another interesting hypothesis is about the positions of the introns conserved in all globin genes. This may reflect the early (more than 500 million years ago) evolutionary events that brought together the functional domains of the protein. For instance, exon 2 encodes the entire heme binding region and $\alpha_1\beta_2$ contacts whereas exon 3 encodes $\alpha_1\beta_1$ contacts (Higgs et al., 1993). The α-globin cluster contains three pseudogenes: one of them, pseudo ζ, differs from the functional ζ gene by only three base substitutions in the protein coding region, the other two are $\Psi\alpha_1$ and $\Psi\alpha_2$. $\Psi\alpha_2$ is probably the oldest of the three pseudogenes in the cluster and has diverged far from the normal gene (Liebhaber, 1989).

There are separate regions within or adjacent to the α-globin cluster which are structurally hypervariable. Each of these regions is composed of multiple tandem repeats of a short GC rich sequence (Liebhaber, 1989). These hypervariable regions (HVRs) are located at the 3' end of the complex (α-globin 3' HVR) between the ζ_2 and $\Psi\zeta_1$ genes (interzeta-HVR) and within the introns (IVS1 and IVS2) of the ζ-like genes (ζ-intron HVRs) and a region approximately 70 kb upstream of ζ_2 (α-globin 5' HVR) (Higgs et al., 1981; Proudfoot et al., 1982; Jarman et al., 1986; Jarman and Higgs, 1988).

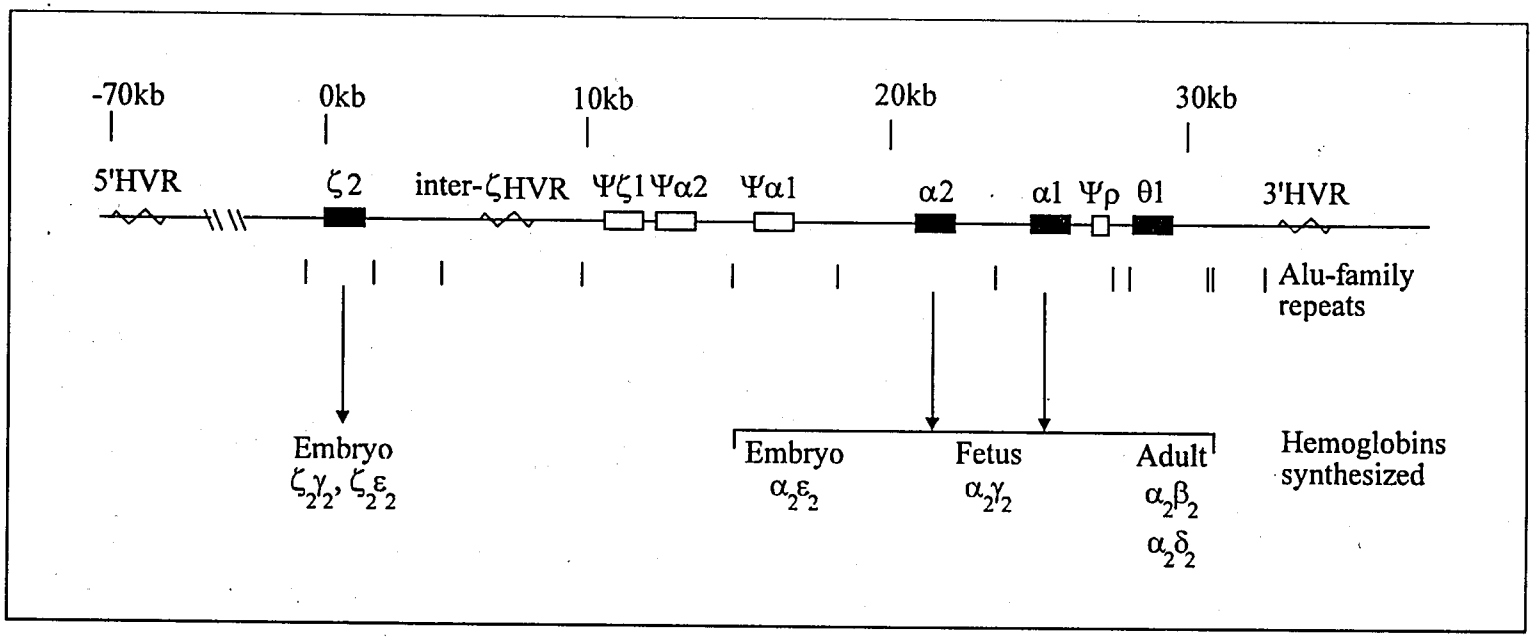


Figure I.2. The organization of the α-globin complex (Higgs, 1989). Filled boxes indicate functional genes and open boxes pseudogenes. The positions of Alu family repeats are shown below the complex. The hemoglobins synthesized at each stage of development are indicated below the α complex.

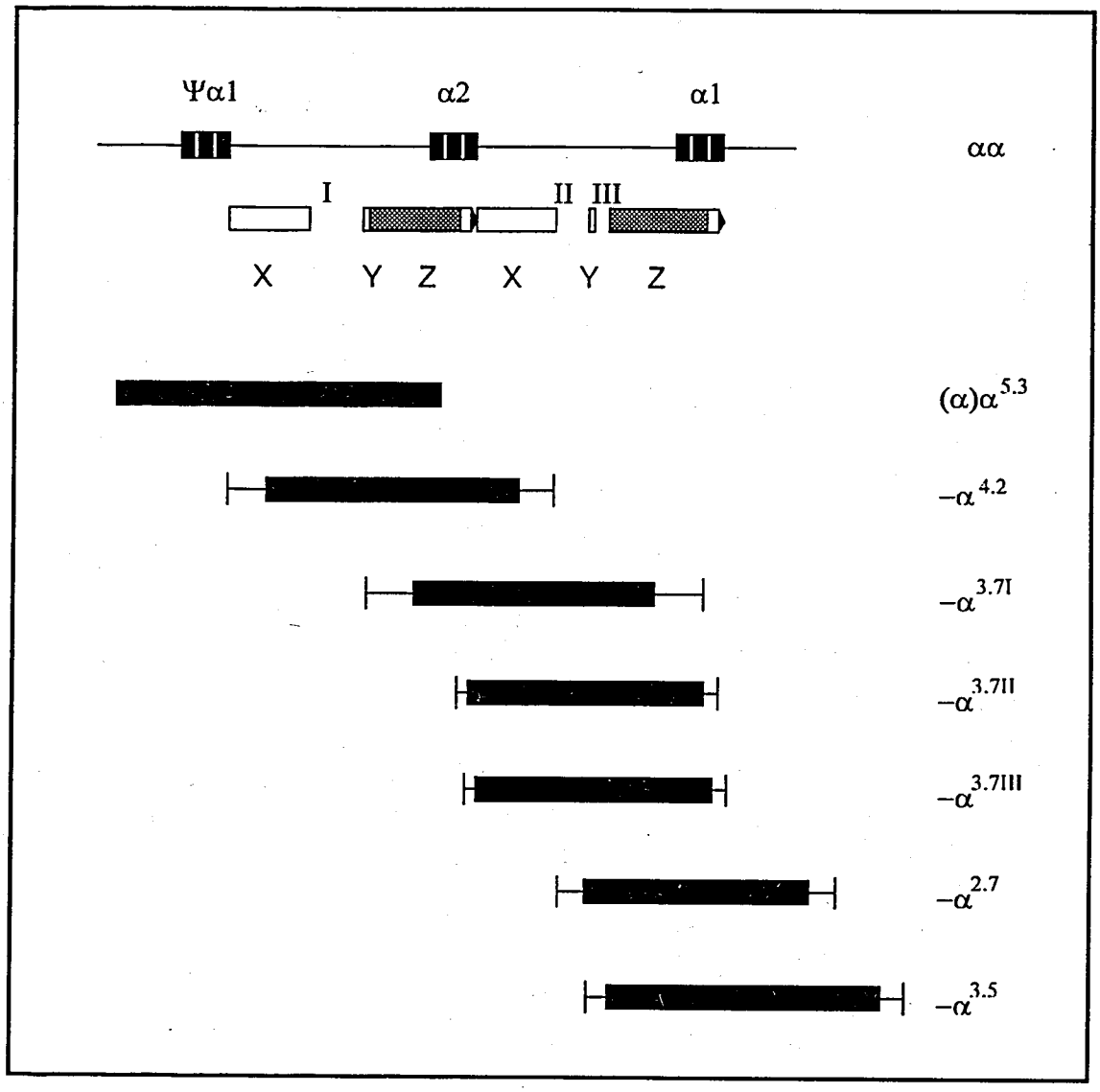


Figure I.3. α-globin gene: duplication units with regions of non-homology and deletions that cause α-thal-2. The extend of each deletion described in the text is indicated by the solid blocks and the limits of the breakpoints are represented by solid lines.

2. Molecular Regulation of the α-Globin Gene Expression

Expression of globin genes is restricted to erythroid cell-lines by a mechanism which has not been elucidated precisely yet. But it is known that expression of both α (or α-like) and β (or β-like) globin genes is controlled by a complex interaction between regulation sequences within the genes and their control elements which are located far upstream of the structural genes, with the help of trans-acting proteins. However, α- and β-globin complexes lie in remarkably different chromosomal environments implying that this complex interaction somehow activates these two globin gene clusters in different ways (Vyas et al., 1992). For instance, the β cluster is contained within a segment of chromatin which is early replicating, DNase I sensitive and transcriptionally active with the influence of a remote locus control region in erythroid cells, whereas the opposite is observed in other cell types. On the other hand, each of the α-genes is associated with CpG-rich islands located in a constitutively "open", transcriptionally active chromatin domain that is early-replicating in all cell types. Additionally, the expressed α genes are found to be little or non-methylated in any tissue in contrast to the β-globin gene cluster, showing that the α-globin gene complex has a more open conformation in non-erythroid cells than the β genes.

As in the β cluster, the expression of genes within the α complex is dependent on a remote regulatory sequence named HS-40 because it is located 40 kb upstream of the ζ_2 mRNA cap site (Higgs et al., 1990; Jarman et al., 1991). Although there are other identified erythroid-specific DNase I hypersensitive sites between HS-40 and the embryonic ζ-gene, HS-40 is the major α-globin regulatory element. The α genes are adjacent to widely expressed genes, and HS-40 is within an intron of one of these genes. This means that if the chromatin containing the α genes and their cis-acting regulatory sequences were to be closed in nonerythroid cells, this would prevent the expression of the widely expressed genes (Vyas et al., 1992). This constitutively open chromatin structure may show us that activation of the α-globin gene cluster simply depends on the interaction with HS-40, whereas β-LCR can establish an open chromatin environment besides its transcription enhancing role (Gourdan et al., 1994).

HS-40 was first identified by DNA analysis of a thalassemic individual with a 62 kb deletion removing HS-40 and leaving both structural genes (Hatton et al., 1990). Transgenic mice studies showed that although the α genes are not expressed in the absence of HS-40, high levels of human globin expression are seen in the constructs with HS-40, α and/or ζ regardless of the position of integration of the construct in the host genome (Jarman et al., 1991; Sharpe et al., 1992). The most important dissimilarity between HS-40/α-gene and β-LCR/β-gene constructs in transgenic mice is that the human α gene expression is not copy number dependent and decreases by 1.5-9.0 fold during development (Sharpe et al., 1992; Gourdan et al., 1994). The reasons of these differences are not yet clear.

It has been shown that both β-LCR elements and the α (HS-40) consist of 200-300 bp segments of DNA containing binding sites for erythroid and ubiquitous transcription factors in vivo and in vitro (Grosveld et al., 1993). The segment of HS-40 contains binding sites for GATA-1, NF-E2 and CACC box proteins (Jarman et al., 1991; Strauss et al., 1992). The pattern of HS-40 protein binding in vivo and in vitro resembles that of β-LCR HS2, and both regions behave as classical erythroid-specific enhancers in transient assay systems (Grosveld et al., 1993). Although the activation of the α- and β-globin genes appears to involve the common group of DNA-binding proteins, the difference in their chromosomal environments influence their activations differently. For instance, mutations of XH2 gene which is located on the X chromosome cause α-thalassemia (ATR-X syndrome). This specific effect of XH2 mutations on the α- rather than β-globin gene expression may indicate that XH2 is a transcriptional activator that works via an interaction with chromatin (Gibbons et al., 1995).

3. Relationship Between Structural Organization and Expression of the α- and β-Globin Gene Clusters

The genes of both the α and β clusters are arranged along the chromosome so that they are expressed in an order during developmental stages. In embryonic life, the dublicated α-genes are expressed in equivalent amounts. However, despite the remarkable structural similarity between the two α-globin genes, from about the eighth week of gestation onwards, expression of the α_2-gene exceeds that of the α_1-gene by a factor of 2.6 (Albitar et al., 1992). In all species so far studied, the more 5' α-globin gene is, the major locus in the adult, contributing two to three times more α-globin than the more 3' locus.

There is an important difference between switching mechanisms of the α-like and β-like genes. While the α-like genes undergo a single developmental switch (embryonic → fetal/adult), β-like genes take an additional switch after three-six months from the birth (embryonic → fetal → adult).

These two globin clusters are co-ordinately but independently regulated. There are evidences from α- and β-thalassemia so that excess globin chains of one type continue to be synthesized despite a deficit of the other (Weatherall and Clegg, 1981; Higgs et al., 1989). This means that there is apparently no active mechanism that feeds back from either gene locus to the other to maintain balanced globin production (Grosveld et al.,1993)

B. Hemoglobin Synthesis in Erythroid Cells with the Selective Expression of Globin Genes

A self-renewing population of pluripotential (common) stem cell gives rise to a series of progenitor cells for different types of marrow cell lines (Hoffbrand and Pettit, 1985). One of them, erythroid stem cells, result after terminal differentiation along the erythroid

pathway in mature red blood cells which almost exclusively synthesize hemoglobin due both to selective expression of the globin genes and differential stability of globin as opposed to non-globin mRNA (Grosveld, 1993). Each red cell contains approximately 640 million hemoglobin molecules and each molecule of human hemoglobin consists of four polypeptide chains, two of one kind and two of another. The four chains, each of which contains a heme group and a single oxygen-binding site, are held together by noncovalent attractions (Stryer, 1988).

There are different types of hemoglobin during developmental stages (Figure I.2). Following activation of the α and β loci toward the end of the third week of gestation, the yolk-sac, the main site of hemopoiesis produces embryonic hemoglobins. Major types of embryonic hemoglobins are Hb Gower-I ($\zeta_2\varepsilon_2$), Hb Gower-II ($\alpha_2\varepsilon_2$), Hb Portland-I ($\zeta_2\gamma_2$), and minor types are Hb Portland-II ($\zeta_2\beta_2$) and Hb Portland-III ($\zeta_2\delta_2$). From six weeks until six-seven months of fetal life the liver and the spleen are the main organs involved in hemopoiesis, and they continue to produce blood cells until about two weeks after birth. The bone marrow is the most important site of blood formation from six-seven months of fetal life. Erythroid cells which are either derived from a second set of hemopoietic stem cells or from stem cells that have migrated from the yolk-sac to the liver, synthesize two fetal hemoglobins ($\alpha_2{}^G\gamma_2$ and $\alpha_2{}^A\gamma_2$). The major switch from fetal to adult hemoglobin occurs three to six months after birth. The major site of erythropoiesis is the bone marrow in which definitive-line erythroblasts synthesize two adult Hbs which are Hb A ($\alpha_2\beta_2$) and Hb A_2 ($\alpha_2\delta_2$) (Figure I.4). In the healty adult the ratio of Hb A and Hb A_2 is constant at 40:1.

In the normal assembly of hemoglobin, the α- or α-like and the β- or β-like globins are synthesized by genes located on different chromosomes whereas heme synthesis occurs in mitochondria by combination of protoporphyrin with iron. The imbalance in globin chain production results in lesser amounts of normal hemoglobin per cell, which partially accounts for well-described hypochromia and microcytosis.

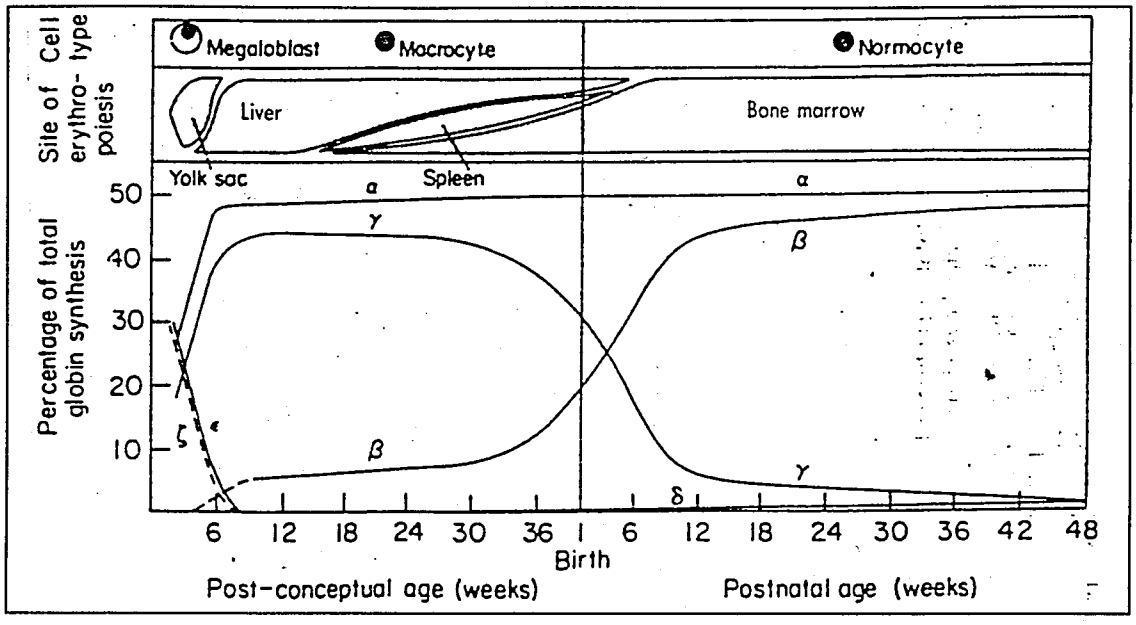


Figure I.4. Sites of erythropoiesis (Weatherall et al., 1989).

C. Hemoglobinopathies

Inherited disorders of hemoglobin, hemoglobinopathies, are the most common single gene disorders in the world. Although hemoglobinopathies involve either the heme or the globin parts of the molecule, this term is mostly used for the inherited disorders of the structure and synthesis of globin chains. There are four groups of hemoglobinopathies: The first group constitutes the structural hemoglobin variants which are due to inherited changes in the structure of the globin chains and are also called abnormal hemoglobins (Weatherall and Clegg, 1981). As a second group, thalassemias are characterized by a reduced rate of synthesis of one or more of globin chains of hemoglobin (Weatherall and Clegg, 1981; Weatherall et al., 1989). In addition to the qualitative (structural variants) and quantitative (thalassemias) classification, there is another group that possesses both types of

abnormalities. These disorders are called thalassemic hemoglobinopathies. Finally, there are conditions in which fetal hemoglobin synthesis persists beyond the neonatal period, known as hereditary persistance of fetal hemoglobin (HPFH) which overlaps with thalassemias (Weatherall and Clegg, 1981; Embury and Mentzer, 1989).

1. Abnormal Hemoglobins

Starting from the date of description of sickle cell hemoglobin by Pauling and his colleagues in 1949, more than 800 human hemoglobin variants have been discovered mostly by electrophoretic techniques which depend on an altered charge of abnormal hemoglobins (Weatherall and Clegg, 1981; Carver and Huisman, 1993; Huisman 1993).

The majority of the abnormal hemoglobins results from single aminoacid substitutions in the α- or β-globin chains, e.g. Hb C, Hb D, Hb E and Hb S. Additionally, there are other structural hemoglobin variants owing to deletions, insertions or arrangements of nucleotides in variable sizes (Bunn and Forget, 1986). Amino acid substitutions may cause little or no alteration in the structure or function of the hemoglobin molecule or may alter its physical properties resulting in abnormal function or instability. This gives rise to clinical abnormalities of varying severity depending on the site of substitution; the best example to latter is sickle cell anemia.

a. Sickle Cell Anemia

Hemoglobin S, the first discovered hemoglobin variant, differs from Hb A by the substitution of valine for glutamic acid at position six of the β-globin chain (Ingram, 1956). The sickling disorders can be in the heterozygous state [sickle cell trait (AS)], the homozygous state [sickle cell anemia (SS)], or the compound heterozygous states with other hemoglobin variants; β-thalassemia or α-thalassemia.

Sickle cell anemia (SS) is one of the most common genetic diseases worldwide. The disease is widely-spread in Africa and in every population where there has been migration from Africa, in parts of Middle East, India, Southern countries and blacks in the United States, South America and Canada (Weatherall et al., 1989; Adekile, 1992). In homozygous state, Hb S is insoluble and forms crystals when exposed to low oxygen tension, the red cells form sickling shape and may block different areas of microcirculation resulting in infarction and destruction of tissues (Hoffbrand and Pettit, 1985).

The hematological characteristics and clinical severity of sickle cell anemia are variable and influenced by a number of factors. These include the coinheritance of α-thalassemia, the Hb F level and the β-globin haplotype (Ballas, 1991; Öner, 1992). There are distinct haplotypes that are linked to the β^s gene; these are numbered as 17, 19, 20, 3 and 31 and are also known as Cameroon, Benin, Bantu, Senegal and Saudi Arabian-Indian haplotypes, respectively.

2. Thalassemias

The thalassemias, the most common single gene disorders in the world population, are extremely heterogeneous in terms of their clinical severity and their pathophysiology related directly to the degree of imbalance in the globin chain production.

The term thalassemia is derived from the Greek word for sea (thalassa) due to the first observation in Mediterranean originated patients. However, these inherited forms of anemia are not found only in the Mediterranean basin but they have developed in multiple geographic regions, probably because they provided partial protection against malaria (Higgs 1993; Stanley, 1994).

The various thalassemia disorders are named according to the chain whose synthesis is reduced. The most common types are α-thalassemia and β-thalassemia:

α-Thalassemias, which may be the most common genetic disorders of human-being, result from underproduction of the α-globin chains of fetal ($\alpha_2\gamma_2$) and adult ($\alpha_2\beta_2$) hemoglobin due to inherited deficits in the synthesis of the α-globin chains. The presence

of α-thalassemia results in the production of β-like globin tetramers (Hb Bart's γ_4, Hb H β_4 and δ_4 tetramers).

β-thalassemias are a heterogeneous group of autosomal recessive disorders characterized by reduced (β^+) or absent (β^0) production of the β-chains due to mutations in or around the β-globin gene. This leads to imbalance between the α and β-globin chains. Unpaired α chains precipitate in the red cell precursors forming inclusion bodies which are responsible for the intramedullary destruction of the erythroid precursors and hence the ineffective erythropoesis that characterizes all β-thalassemias (Thein, 1993).

Up to now, more than 180 different molecular defects, most of which are single nucleotide substitutions affecting critical areas for the function of the β-globin gene, have been defined (Baysal, 1995). The nature of point mutations causing β-thalassemia can be considered in terms of their effects on globin gene expression e.g., transcriptional mutants, RNA processing mutants (splice junction, consensus sequence, cryptic splice sites in introns, cryptic splice sites in exons) nonfunctional mRNA (nonsense mutants, frameshift mutants, initiator codon mutations), RNA cleavage and polyadenylation mutants (Huisman, 1992). Besides the large group of point mutations, the β-globin gene is also silenced by a small number of deletions involving the β-globin gene cluster.

According to clinical pictures, β-thalassemia can be divided into four categories, namely the silent carrier state, β-thalassemia trait, β-thalassemia intermedia and β-thalassemia major.

Silent β-thalassemias are thalassemic disorders characterized by normal red cell indices and normal Hb A_2 and Hb F levels; they are defined only by imbalanced α/β-chain synthesis. Because of these characteristics this determinant may be missed by the procedures commonly used for carrier identification (Cao et al., 1994).

Individuals with *β-thalassemia trait* (minor) are mildly anemic. The red blood cell count is elevated, the MCV is lowered to 60 fl to 75 fl, the MHC is reduced to 20 pg to 25 pg and the Hb A_2 concentration is increased to 3.5 per cent to 7 per cent (Kazazian, 1990).

Thalassemia intermedia is used to define a syndrome between β-thalassemia minor and major. This form of thalassemia includes patients with clinical phenotypes which are more severe than its heterozygous state but milder than homozygous β-thalassemia (Todd, 1984; Thein, 1993).A number of molecular mechanisms are able to produce thalassemia

intermedia which is a-nontransfusion dependent form of β-thalassemia. The most common are homozygosity for mild β-thalassemia mutations, coinheritance with α-thalassemia or factors which may modify the expression of Hb F (Thein, 1993; Cao et al., 1994). The patients with thalassemia intermedia show bone deformity, enlarged liver and spleen, extramedullary erythropoiesis and features of iron overload in adulthood (Hoffbrand and Pettit, 1985).

β-thalassemia major, also known as Mediterranean or Cooley's anemia, results when both β globin genes are defective. This anemia becomes apparent at three to six months after birth. It is characterized by dependence on blood transfusion in every three to four weeks. The most characteristic clinical features are enlargement of the liver and spleen due to excessive red cell destruction, extramedullary hemopoesis and iron overload; expansion of bones due to intense marrow hyperplasia results in marked skeletal deformities with frontal bossing, cheek bone and jaw protrusion, distortion of ribs and vertebrae and pathological features of lay bones (Todd 1984, Thein, 1993).

D. Alpha-Thalassemia

1. Mutations Giving Rise to α-Thalassemia and The Mechanisms Underlying Them

a. Deletional α-Thal-2 Determinants

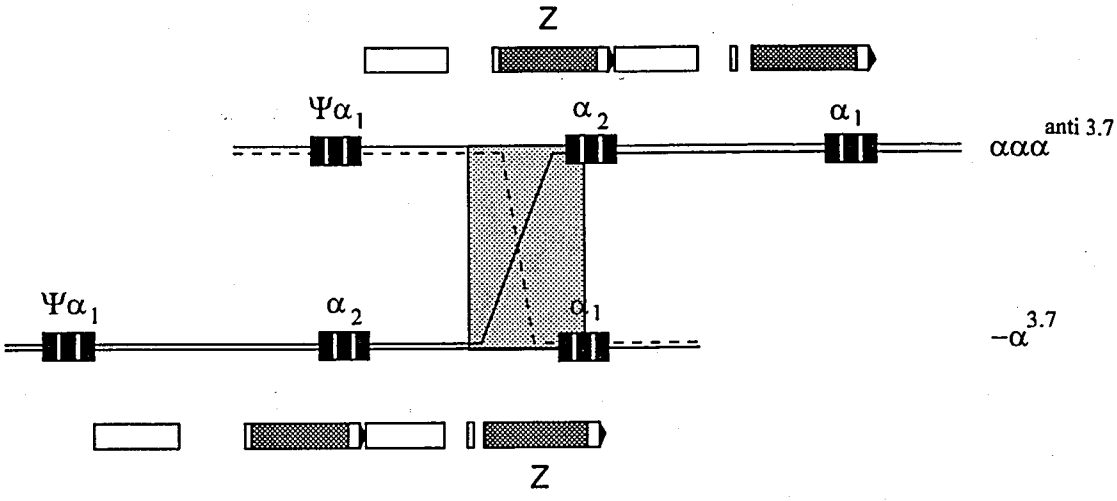
The presence of homologous duplication units in and around the α-globin genes can mediate homologous unequal recombination events that lead to a single α-globin gene deletion and duplications. The loss of one of the two linked α-genes has been called α^+-

thalassemia or α- thal-2 and the duplications are defined as the "anti" since they are complementary to deletions of the same length.

The two most common subtypes of the single α-globin gene deletions are the 4.2 kb ($-\alpha^{4.2}$) and the 3.7 kb ($-\alpha^{3.7}$) deletions. Recombination between homologous X boxes, which are 4.2 kb apart, results in α-thalassemia determinants by removing the entire α2 gene and leaving the α_1 gene intact (Embury et al., 1980, Baysal 1993) and a $\alpha\alpha\alpha^{anti\ 4.2}$ chromosome (Trent et al., 1981). The crossover between Z segments which are 3.7 kb apart produces chromosomes with only one α-gene (Embury et al., 1980) that leads to α-thalassemia and another chromosome with three α-genes ($\alpha\alpha\alpha^{anti\ 3.7}$). The 3.7 kb deletion yields a fusion gene derived from the α_2 and α_1 loci (Baysal, 1993). The 3.7 kb rearrangements may be type I, II or III, depending on the Z subsegments in which recombination has taken place (Michelson and Orkin 1983; Higgs et al., 1984). Because of the relative posions of the deleted DNA fragments, the 3.7 and 4.2 kb deletions are also known as the rightward and leftward deletions, respectively (Figure I.3, Figure I.5). Although in both leftward and rightward deletions, the remaining α-globin gene is identical to the α_1 globin gene, the expression level of it is not same as the native one, since each of these deletions result in a major structural change in the cluster (Liebhaber, 1989). This is also valid in other -α's. In all cases, the remaining α gene behaves like neither α_2 nor α_1 (Higgs, 1993). For example, homozygotes for the $-\alpha^{4.2}$ determinant ($-\alpha^{4.2}/-\alpha^{4.2}$) appear to express more α-globin than the predicted 25 per cent of normal. Additionally, studies about the expression of $\alpha^{3.7}$ deletion showed that it was expressed in 1.8 fold higher levels than the α_1 gene. In other words, it is expressed at a level roughly half way between that of a normal α_2- and a normal α_1-gene (Liebhaber, 1989; Higgs, 1993).

There are other known but rare deletions that produce α^+ thalassemia. One of them, $-\alpha^{3.5}$ described in an Indian family, removes the entire α_1-gene and its flanking DNA (Kulozik et al., 1988). The second rare deletion $(\alpha)\alpha^{5.3}$ was observed in a family from Southern Italy (Lacerra et al., 1991). It removes the 5' end of the α_2 globin gene, causing an α^+ thalassemia defect. The 5' breakpoint is located 822 bp upstream of the cap site of the $\Psi\alpha_1$ gene and about 150 bp upstream of a 300 nucleotide Alu family member. The 3' breakpoint is located in the IVS-1 nucleotide 58 of the α_2 globin gene. In 1991, the third rare α-thalassemia mutation was described in an adult Chinese patient with Hb H disease

Rightward Crossover (Z box):



Leftward Crossover (X box):

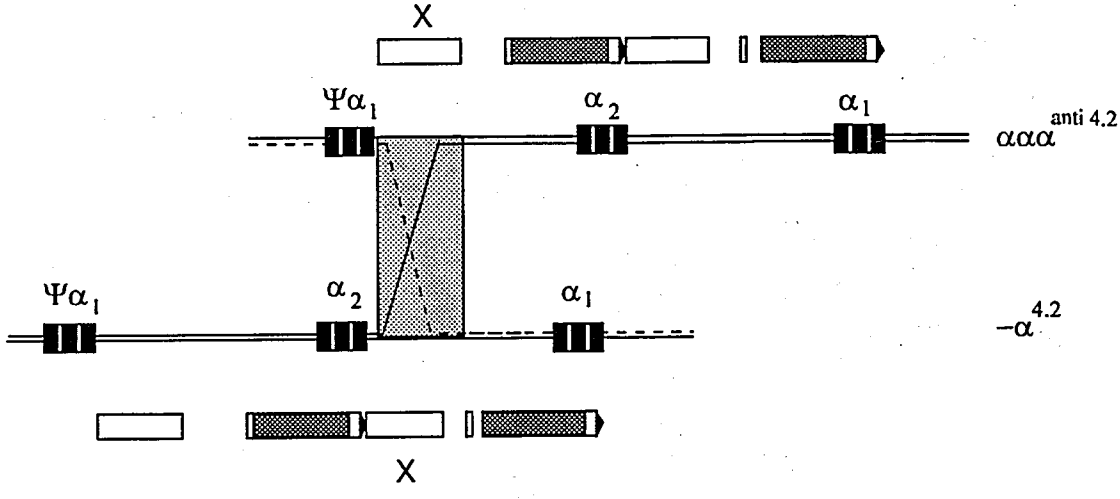


Figure I.5. Mechanism by which deletions underlying α-thal-2 occurs (Higgs, 1993; Weatherall et al., 1989).

(Zhao et al., 1991). This 2.7 kb deletion was involving the α_1 globin gene. More recently a novel α-thal-2 with a large (18+kb) deletion involving the α_1 and θ_1 globin genes and the 3' HVR sequence has been detected (Indrak et al., 1993). In contrast to all known deletions involving the α_1 globin gene and which result in a mild α-chain deficiency with nondetectable hematological differences, heterozygous individuals for this new deletion have a marked microcytosis and hypochromia, thus its absence results in a down-regulation of the α_2-globin gene *in cis* of the deletion.

b. Deletional α-Thal-1 Determinants

The α-thal-1 deletions are caused by either removal of rather extensive DNA segments which partially or fully include both the α_1 and α_2 genes or removal of the regulatory region (HS-40) leaving both α-globin genes intact and normal; in all cases no α-chain synthesis is directed by these chromosomes (Figure I.6). The sizes of these deletions vary widely from 5.2 kb to 62 kb or even larger as seen in $--^{TAI}$ and $--^{FIL}$ deletions which remove the entire gene cluster (Winichagoon et al., 1984). So far, several deletions that remove both α-genes and the ζ globin gene (Higgs, 1993) have been described but nondeletions from the α-globin gene cluster that encompass the 3' HVR are very limited. They are the 47 kb deletion in a German family (Fortina et al., 1988), the 27 kb deletion in a Spanish family (Gonzalez-Redondo et al., 1988) and the 37 kb deletion found in British patients (Higgs et al., 1989). Additionally, a very new deletion has been defined in Yemenite patients (Shalmon, 1994). It involves at least 39 kb and includes the two α-globin genes, θ_1 gene, all the pseudogenes, and the two HVRs, interzeta-HVR and α-globin 3'-HVR. The conserved ζ globin gene has been identified in an abnormal size. Another deletion ($--^{MA}$) that causes a triplication of the ζ gene cluster which is formed by $\Psi\zeta_1$ gene, the interzeta region and possibly the insertion of the $\Psi\alpha_2$ fragments has been found in a Spanish family in 1995 (Villegas et al., 1995).

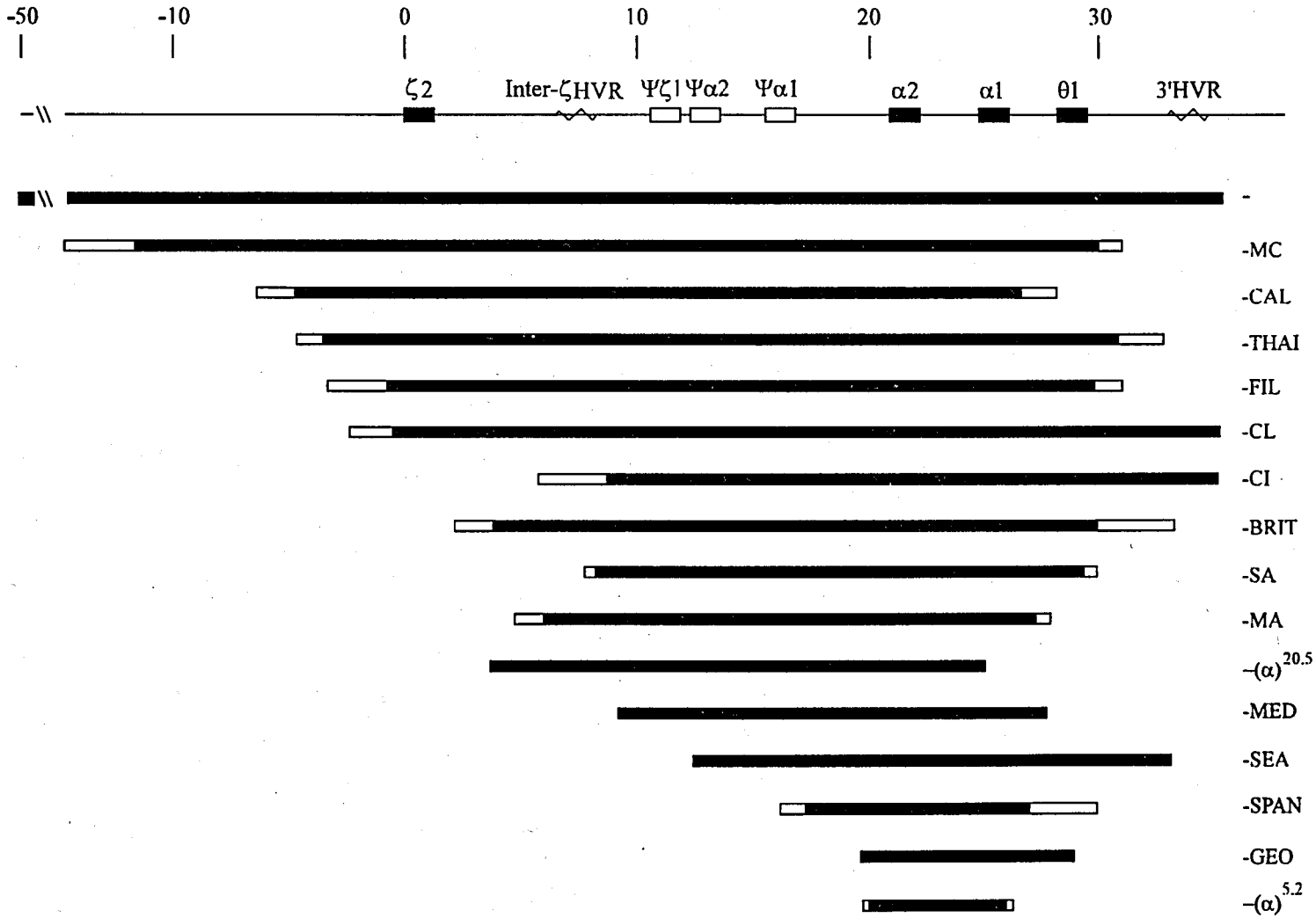


Figure I.6. Deletions that cause α-thal-1 (Higgs, 1993).

The most common α-thal-1 deletions are $--^{SEA}$, $--^{MED}$ and $-\alpha^{20.5}$. Another deletion $--^{SA}$ is approximately same size with $-\alpha^{20.5}$ (20-30 kb) and all four remove both α-globin genes but spare the functional ζ_2 gene (Liebhaber, 1989).

Some of the deletions in which the embryonic gene remains intact are accompanied by the continued expression of very small amounts of ζ-globin in both fetal and adult life (Higgs, 1993). This may be analogues to the persistent γ-globin production. However, the amount of ζ-globin present in adults with these types of α-thalassemia is considerably less than the level of γ-globin present in individuals with comparable deletions of the β complex.

The mechanisms underlying the large deletions in the α-globin gene cluster are usually illegitimate or non-homologous recombinations (Nicholls et al., 1987). Sequence analysis has shown that sequence features at recombination junctions in these α-globin deletions are direct repeats, palindromic repeats and the presence of members of interspersed repetitive DNA families. Alu-family repeats which are common in and around the α-globin cluster can provide appropriate sites for recombinations (Figure I.2). For example, the large $(\alpha\alpha)^{RA}$ deletion results from a simple crossover event between the Alu sequences at coordinate -52 and coordinate +10. $--^{MED}$ deletion is an example to complex rearrangement. A new piece of DNA from upstream of the α cluster is inserted to the two breakpoints of the deletion, suggesting that the upstream segment lies close to the breakpoint region during replication.

c. Alpha$^+$ Thalassemia due to Nondeletional Defects

α-Thalassemia which results from point mutations or small oligonucleotide insertions and deletions within the α globin genes or in the upstream or downstream regulatory sequences is much less common than α-thalassemia due to deletions.

These nondeletion defects effect a variety of critical steps in gene expression such as RNA processing, transcription initiation and termination or give rise to posttranscriptional instability (Weatherall et al.,1989; Higgs et al., 1989; Higgs 1993). Since 1977, which is the date of the first description of nondeletional α thalassemia (Kan et al., 1977), many non-

deletional α thalassemia determinants have been characterized as shown in Table I.1. These mutations which can occur in an otherwise normally αα cluster or on the common $-\alpha^{3.7}$ gene are assymetrically distributed between the two α globin loci (Liebhaber, 1989). Nearly, all of the mutations occur within the dominant α_2 globin gene by causing a more severe α thalassemia phenotype than mutations at the α_1 locus. Additionally, there appears to be no increase in expression of the remaning functional α_1 gene as it occurs in the -α determinants (Liebhaber, 1985; Higgs, 1993).

The most common nondeletional α thalassemia mutation, $\alpha_2^{\text{constant spring}}$ (α^{CS}), is due to a single-base substitution at the termination codon of the α_2 globin gene (UAA → CAA). Since this mutation converts the stop codon into glutamine (Gln), it allows the ribosome to read into the 3'-untranslated region; thus the encoded α^{CS} protein contains an additional 31 amino acids at its carboxyl-terminus (Liebhaber, 1989).

2. The Clinical Syndromes of α-Thalassemia

It is possible to subdivide the clinical syndromes of α-thalassemia into four main categories: silent carriers, α-thal-trait, Hb H disease and Hb Bart's hydrops fetalis (Table I.2).

Silent carriers with a single α-globin gene deletion due to α-thal-2 mutations have no significant phenotypical difference with normal individuals. The hematological indices (Hb, MCV, MCHC, PCV, etc.) are within normal limits (Fucharoen et al., 1988; Liebhaber, 1989; Baysal, 1993). Only one α-thal-2 heterozygote identified by Indrak et al. in 1993 has a different hematological parameters. The patient with the 18^{+}kb deletion has a mild anemia with a marked microcytosis and hypochromia and a significant decrease in the in vitro α/β chain ratio (see also Section I.D.1.a).

Table I.1. Non-deletional mutants that cause α-thalassemia.

	Affected Gene	Affected Sequence	Mutation	Nomenclature	Geografic Distribution	References
RNA Proscessing	α_2	IVS-I donor site	GAGGTGAGG→GAGG	$\alpha^{Hph}\alpha$	Mediterranean	Higgs (1993) Weatherall et al (1989)
	α_2	Poly(A) signal	AATAAA→AATAAG	$\alpha\alpha^{TSaudi}$	Mediterranean Middle East	Higgs (1993) Weatherall et al (1989)
	α_2	Poly(A) signal	AATAAA→AATGAA		Mediterranean	Higgs (1993)
	α_2	Poly(A) signal	AATAAA→ATA		Indian	Harteveld et al (1994)
	α_1	Acceptor splice site	IVS-I-117(G→A)		Indian	Çürük (1993)
RNA Translation	α_2	Initiation codon	CCACCATGG→CCACCACGG	$\alpha^{Nco}\alpha$	Mediterranean	Higgs (1993) Weatherall et al (1989)
	α_1	Initiation codon	CCACCATGG→CCACCGTGG		Mediterranean	Higgs (1993)
	-α	Initiation codon	CCACCATGG→CCACCGTGG		Black	Higgs (1993)
	$-\alpha^{3.7II}$	Initiation codon	CCACCATGG→CC--CATGG	$-\alpha^{3.7T}$	Mediterranean North African	Higgs (1993) Weatherall et al (1989)
	α_2	Exon III	α116 GAC→TAG		Black	Higgs (1993)
	α_2	Termination Codon	α142 TAA→CAA	$\alpha^{CS}\alpha$	Southeast Asian	Higgs (1993) Weatherall et al (1989)
	α_2	Termination Codon	α142 TAA→AAA	$\alpha^{ic}\alpha$	Mediterranean	Higgs (1993) Weatherall et al (1989)
	α_2	Termination Codon	α142 TAA→TCA	$\alpha^{kD}\alpha$	Indian	Higgs (1993) Weatherall et al (1989)
	α_2	Termination Codon	α142 TAA→GAA	$\alpha^{SR}\alpha$	Black	Higgs (1993) Weatherall et al (1989)
	-α	Exon I	α30/31 GAGAGG →GAG--G		Black	Higgs (1993)
Post-Translational Instability	α_2	Exon III	α125 Leu→Pro	$\alpha^{QS}\alpha$	Southeast Asian	Higgs (1993) Weatherall et al (1989)
	α_2	Exon III	α109 Leu→Arg	Suan Dok	Southeast Asian	Higgs (1993) Weatherall et al (1989)
	α_2	Exon I	α29 Leu→Pro	$\alpha^{Agr}\alpha$		Hall (1993)
	α_1	Exon II	α59 Gly→Asp	Hb Adana		Çürük (1992)
	α	Exon III	α110 Ala→Asp	Petah Tikvah	Middle Eastern	Higgs (1993) Weatherall et al (1989)
	-α	Exon I	α14 Trp→Arg	Evanston	Black	Higgs (1993) Weatherall et al (1989)

Table I.2. Clinical phenotypes and genotypes of α-thalassemia (Weatherall et al., 1989).

Phenotype	*Equivalent number of functional α-genes*	*Level of Hb Bart's at birth, %*	*% Hb H (inclusions)*	*MCV (fl)*	*MCH (pg)*	*α/β-globin chain synthesis ratio*	*Interacting haplotypes*	*Most frequently encountered genotypes*
Normal	4	0	0 (none)	85-100	~28	~1.0	α / α	$\alpha\alpha/\alpha\alpha$
α-thal-2	3	0-2	0 (rare)	75-85	~24	~0.8	α^{+}/α	- $\alpha/\alpha\alpha$
α -thal-1	2	2-8	0 (occasional)	65-75	~20	~0.6	α^{0}/α or α^{+}/α^{+}	--/$\alpha\alpha$ $\alpha\alpha^{T}/\alpha\alpha$ -α/-α
Hb H	1	10-40	2-40 (many)	55-65	~20	~0.3	α^{0}/α^{+} or α^{+}/α^{+}	--/α- --/$\alpha\alpha^{T}$ $\alpha\alpha^{T}/\alpha\alpha^{T}$
Hb Bart's hydrops fetalis	0	~80	Present (present)	110-120	Reduced	0.0	α^{0}/α^{0}	--/-- --/$\alpha\alpha^{T}$

The genotype of *α-thal-trait* can be (--/αα), (-α/-α) or $\alpha\alpha^T/\alpha\alpha$. These are significantly different from normal persons and from α-thal-2 heterozygotes. Comparison of hematological data in the adults with two defective α-globin genes show similar values such as a significant microcytosis (MCV 70-80 fl), hypochromia (MCH 22-24 pg), elevated RBC and an α/β ratio of ~0.7 (Liebhaber, 1989). However, there is the evidence from the amount of Hb Bart's in cord blood, which is a sensitive index of α-thalassemia, that --/αα is more severe than -α/-α (Trent et al., 1984). Additionally, the two types of the latter which are $-\alpha^{4.2}/-\alpha^{4.2}$ and $-\alpha^{3.7}/-\alpha^{3.7}$ differ in severity. It is observed that homozygotes for 4.2 kb deletion have significantly higher levels of Hb Bart's at birth than homozygotes for the 3.7 kb deletion, meaningly homozygotes for the leftward deletion have a more marked phenotype than the homozygotes for the rightward deletion with compound heterozygotes, $-\alpha^{3.7}/-\alpha^{4.2}$, intermediate in severity (Bowden et al., 1987).

Hb H is the most severe form of the α-thalassemia syndromes compatible with life. The heterogeneity in the genetic basis of Hb H disease which mostly results from the interaction of α-thal-2 and α-thal-1 leading to (--/-α) or ($--/\alpha\alpha^T$) genotype or which may also result from the inheritance of two of the more severe α-thal-2 determinants ($\alpha^T\alpha/\alpha^T\alpha$ or $-\alpha^T/-\alpha^T$), appears to correlate with its clinical phenotype (Liebhaber 1989, Higgs 1993). The severity of Hb H disease is proportional to the degree of α-chain deficiency. Although the common deletional form of Hb H (--/-α) is rarely symptomatic and is usually not severe enough to interfere with daily activities of the individual, Hb H disease involving nondeletional α-thalassemia determinants tends to be more severe. Additionally, the loss of the α_2 gene by nondeletion mutations ($--/\alpha^T\alpha$) results in a more severe phenotype than the loss of α_1 globin gene expression by a nondeletion defect ($--/\alpha\alpha^T$). It means that the latter which is quite mild is inbetween the (--/-α) form and the ($--/\alpha^T\alpha$) form of Hb H.

The predominant clinical features of Hb H disease are anemia (2.6-13.3 g/dl), which results from a deficiency in Hb A and the formation of unstable Hb H homotetramers (γ_4) (0.8-40 per cent), jaundice and hepatosplenomegaly (Weatherall and Clegg, 1981). The most common complication of Hb H disease is the development of severe splenomegaly. Splenectomy can be therapeutic in slowing down the rate of hemolysis and ameliorating the symptoms, since premature removal of red cells containing Hb H precipitates occurs in the spleen (Liebhaber, 1989). Other complications of this disease are infections, leg ulcers, gall

stones, folic acid deficiency and acute hemolytic episodes in response to drugs (Weatherall and Clegg, 1981).

The *Hb Bart's hydrops fetalis* syndrome which is nearly always due to the coinheritance of two α-thal-1 determinants cause either the death of infants in utero (23-38 weeks) or soon after birth (Pootrakul et al., 1967; Weatherall et al., 1970). On the other hand some cases appear to survive for a few days (Isarangkura et al., 1988). Additionally, two infants with this syndrome were delivered at 28 and 32-34 weeks by intensive life-support therapy and blood transfusion (Beaudry et al., 1986; Bianchi et al., 1986), and normal development was observed in them.

Although this syndrome is almost always deletional in origin (--/--), there have been some reports about hydrops fetalis with very low levels of α-chain synthesis (Sharma et al., 1979; Chan et al., 1985; Trend et al., 1986; Ko et al., 1991) resulting from the interaction of common α^0 determinants with nondeletional mutations ($\alpha\alpha^T$).

Infants with signs of cardiac failure and prolonged intrauterine anemia are the clinical pictures (Baysal, 1993; Higgs, 1993; Weatherall et al., 1989). Hepatosplenomegaly is always present with other congenital abnormalities, particularly of the skeletal and cardiovascular system (Liang et al., 1985). The peripheral blood demonstrates severe erythroblastosis with accompanying reticulocytosis, target cells, hypochromia with fragmentation and decreased osmotic fragility (Liebhaber, 1989). The MCV level is increased owing to the large number of circulating nucleated red cells, and the hemoglobin levels are between 3 to 10 g/dl (Weatherall et al., 1989). The hemoglobin consists of ~80 per cent Hb Bart's which is nonphysiological and which does not function in O_2 transport, the remainder being Hb H and Portland. Since Hb H is also nonphysiologic, only Hb Portland is efficient in the transport of oxygen, resulting in the survival of the fetus to late pregnancy. Pathological findings in the affected fetuses include gross enlargement of the placenta, heart, liver, spleen, adrenal glands with hypoplasia of the lungs and retardation of brain growth (Higgs 1993).

During the pregnancy, obstetric care is necessary, otherwise mothers might suffer lethal complications such as pre-eclampsia, antepartum haemorrhage and post-partum haemorrhage.

3. Coinheritance of α-Thalassemias with Other Abnormalities

a. ATR-X Syndrome

Although α-thalassemia is mostly due to deletions involving one or both of α-globin genes and lessly due to point mutations, there are other mechanisms that give rise to different forms of this disease. One of them, the ATR-X syndrome, in which α- thalassemia is associated with mental retardation, is due to trans-acting abnormality.

This syndrome was first recognized by Weatherall et al. (1981a). Three males were described with severe mental retardation and Hb H disease which was different from the common forms of it. In 1990, Wilkie et al. identified other cases and grouped them according to their distinct syndromes (Wilkie et al., 1990a; Wilkie et al., 1990b). Although the first group with eight patients had large (1 to 2 mb) deletions at the tip of chromosome 16p, the second group of patients had no deletions or other abnormalities in the α-globin gene complex. The clinical features of the deletion cases were variable, whereas the non-deletion cases showed a strikingly uniform phenotype. Further studies suggested that the nondeletion types of α-thalassemia condition could be called X-linked α-thal/mental retardation (ATR-X) to distinguish it from the deletion form (ATR-16) (Gibbons et al., 1991; Wilkie et al., 1991; Donnai et al., 1991). Studying seven pedigrees that included individuals with the ATR-X syndrome, Gibbons et al. (1992) concluded that intellectually normal female carriers could be identified by the presence of rare cells containing Hb H inclusions in their peripheral blood and by an extremely skewed pattern of X inactivation in cells from a variety of tissues (OMIM). Finally, linkage analysis has localized the ATR-X disease locus to an interval of approximately 11 cM between DXS 106 and DXYS1X, with a peak lod score of 5.4 at $\theta=0$ at DXS 72 (Xq12-q21.31) (Gibbons, 1992). By linkage analysis in nine further families, Gibbons et al. (1995) identified key recombinants that reduce the area of interest to 1.4 cM between DXS 453 and DXS 72, within Xq13.1-q21.1.

In this syndrome, although the cis-acting regulatory sequences of the α-globin gene cluster are intact and retain their normal pattern of methylation, the expression of α-genes is

down-regulated by a trans-acting factor which is encoded on the X-chromosome and which interacts with the regulatory sequences of the α-globin cluster (Higgs, 1993; Gibbons et al., 1995). It has been shown that the ATR-X syndrome results from mutations involving the XH2, a gene that lies in the region Xq13.3. A wide variety of mutations distributed throughout the central portion of the XH2 protein give rise to a surprisingly uniform phenotype (Gibbons et al., 1995).

The most common clinical features of this syndrome for affected boys are mental retardation which is severe, global and apparent from the early age, dysmorphic facial features, genital abnormalities, hypotonia and feeding difficulty which leads to developmental delay such as delayed sitting and walking and skeletal abnormalities (Figure I.7). Carrier females may have very mild hematologic changes but are otherwise normal (Gibbons et al., 1991; Cole et al., 1991; Wilkie et al., 1991; Donnai et al., 1991; Gibbons et al., 1995).

Although there is some overlap of clinical pictures with other syndromes like Coffin-Lowry, Angelman, FG and Smith-Lemli-Opitz syndromes (Gibbons et al., 1991), the diagnosis of the ATR-X syndrome can be confirmed or refuted by demonstrating the associated hematological features of α-thalassemia. Patients have a milder type of Hb H disease with less hypochromia and a lesser level of Hb H inclusions which range from 0 per cent to 6.7 per cent (Gibbons et al., 1991). Therefore, when there is a high index of suspicion from the family history and phenotype, a careful search for Hb H inclusions should be made and repeated, if necessary (Logie et al., 1994).

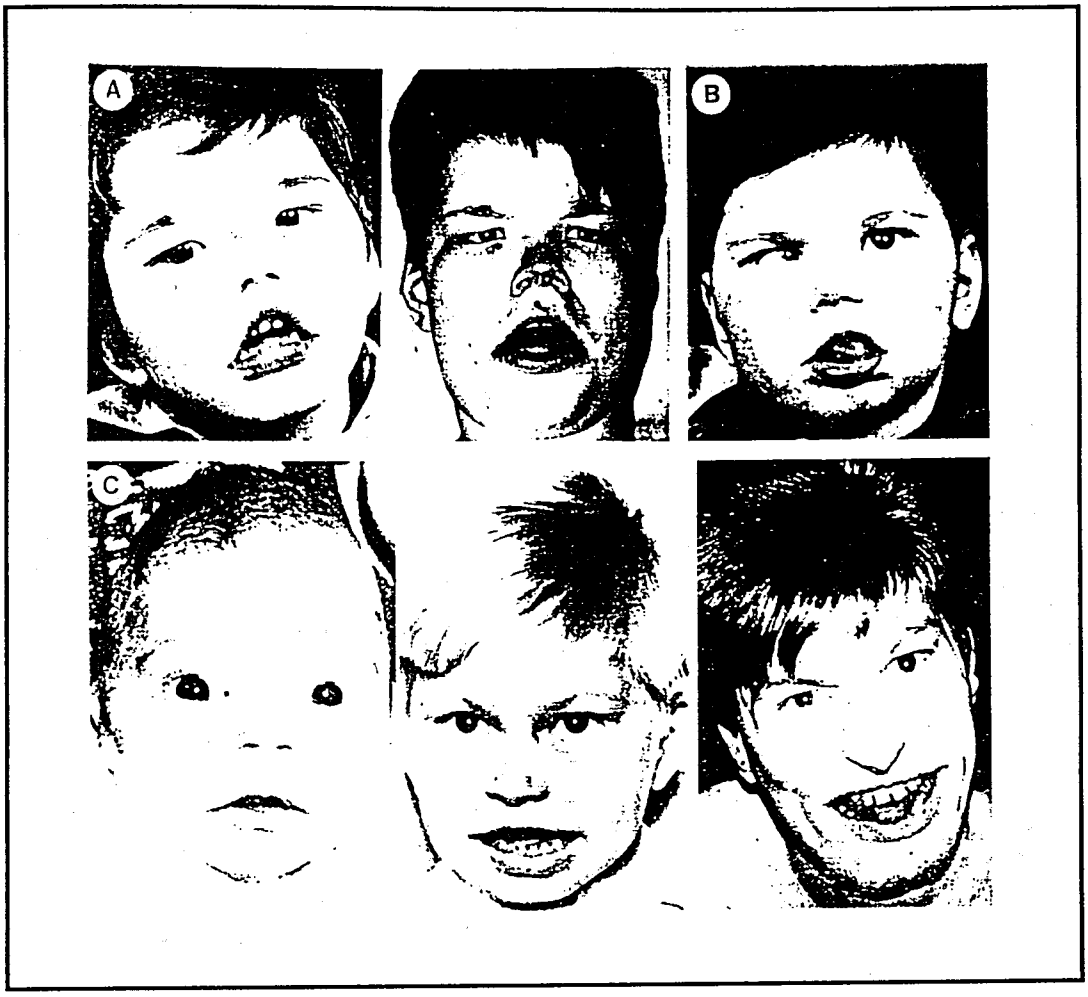


Figure I.7. Facial features of ATR-X affected boys (Wilkie et al., 1991). (A) case 1 aged 3 years (left) and 15 years (right); (B) case 2 aged 15 months; (C) case 3 aged one month (left), 4 years (center) , and 23 years (right).

b. Coinheritance of α-Thalassemia with β-Thalassemia

By diminishing the over-abundance of the α-chains, α-thalassemia lessens the globin chain imbalance which is a major factor in determining the severity of anemia in β-thalassemia and may ameliorate the clinical and hematological picture.

This partnership may make heterozygous β-thalassemia difficult to detect by increasing Hb levels, MCV and MCH across the series αα/αα, α-/αα and α-/α-. In β-thalassemia heterozygotes with an α-/α- genotype, there is considerably overlap with normal values (Weatherall et al., 1989).

Co-inheritance of α-thalassemia can transform the homozygous β-thalassemia from its severe phenotype into thalassemia intermedia phenotype (Steinberg, 1991). It depends on the deleted gene number and type of β-mutations. Although homozygous β^0- thalassemia patients with a single α-gene deletion have little effect on their phenotype, patients who co-inherited Hb H and homozygous β-thalassemia (β^+/β^+ or β^0/β^+) have thalassemia intermedia (Thein, 1993).

In contrast, the co-inheritance of extra α-globin genes with β-thalassemia may result in more severe clinical pictures especially in heterozygous β-thalassemia forms. Triplicated or quadruplicated (ααα/, αααα/) α-globin gene arrangements occur in most populations at a low frequency. But the cases show us that homozygosity for α-globin gene triplication with heterozygosity for β-thalassemia results in thalassemia intermedia (Galanello, 1983; Thein, 1984; Kulozik et al., 1987; Steinberg, 1991; Oron et al., 1994; Altay and Başak 1995).

c. Coinheritance of α-Thalassemia with Sickle Cell Anemia

α-Thalassemia is an important genetic factor among the criteria that have been proposed to be a potential modulator of sickling hemoglobinopathies.

i. Alpha-Thalassemia and Sickle Cell Trait

Coinheritance of α-thalassemia with sickle cell trait (Hb AS) causes distinct hematological alterations such as reduction of the Hb S level.

The decrease in the Hb S level can be due to either the number of α-globin genes deleted or to the type of the α gene deletion. (Kulozik et al., 1987; Steinberg, 1991). As the α_2 globin gene is normally expressed at approximately two to three times the rate of the α_1 globin gene, the lesions deleting α_1 or α_2 globin gene may be expected to have different phenotypic effects (Kulozik et al., 1987). The small number of individuals studied with Hb AS and the $-\alpha^{4.2}$ deletion which causes greater reduction of the α-globin synthesis than the $-\alpha^{3.7}$ deletion, appear to have lower Hb S levels than those with the $-\alpha^{3.7}$ deletion; this was supported by an umbilical cord blood analysis in Malanasians (Kulozik et al., 1987). However, studies performed in Indian AS persons who were heterozygous for either of the $-\alpha^{4.2}$, $-\alpha^{3.7}$ or $-\alpha^{3.5}$ deletions, showed that Hb S levels did not differ in relation to the deletion type. It is certain that in sickle cell trait , the Hb S levels varied according to the α-globin gene number; as the α-globin deficit increases, the level of Hb S falls. Because the β^A chains compete more effectively than the β^S chains for the α chains in limited supply (Kulozik et al., 1987; Steinberg, 1991). Microcytosis and a fall in the Hb concentration also reflect the deficit in α-chain synthesis.

To examine the role of α-thalassemia in patients with AS, 355 black men were divided into four groups on the basis of their Hb S levels according to their mean concentration of Hb S (26,33,37 and 43 per cent). Both hemoglobin levels and MCV fell with Hb S level. Thus, it was concluded that the presence of Hb S levels of less than 35 per cent in all Hb AS strongly suggests the coexistance of α-thalassemia (Steinberg, 1991).

ii. Alpha-Thalassemia and Sickle Cell Anemia

Although the clinical manifestation of sickle cell disease (Hb SS) is very heterogeneous and considerably varies from one patient to another, it can be characterized by following three major set of signs and syndroms: chronic hemolytic anemia and its sequelae, recurrent painful crises, and tissue damage such as aseptic necrosis, retinopathy leg ulcers, pulmonary infarcts and cerebrovascular accidents (Ballas, 1991).

Inheritance of α-thalassemia in Hb SS disease ameliorates hematologic features. α-Thalassemia decreases the rate of hemolysis by decreasing the MCHC, results in higher Hb, HCT and RBC values, and lowers reticulocyte counts (Adams, 1994). Alpha-thalassemia also modifies the hematologic expression of Hb SS resulting in increased total Hb A_2 level due to a greater affinity of the δ chains than the β chains for the available α chains (Stevens, 1986; Steinberg, 1991).

The study performed in Jamaican SS children and based in three different categories consisting of a two-gene group (homozygous for α-thal-2), a three-gene group (heterozygous for α-thal-2) and a four-gene group (normal α-globin gene composition) showed that the most characteristic differences of the two-gene group, as compared with the four-gene group, are raised Hb A_2 and lowered MCV levels (Stevens, 1986). The presence of elevated Hb A_2 and microcytosis seen in Hb SS patients with homozygous α-thal-2, makes them a phenocopy of Hb S-β^0-thal (Steinberg, 1991). This situation was also observed in Jamaican children with SS and α-thal-2 (Stevens, 1986).

The α-globin genotype may influence Hb F levels in patients with thalassemia owing to increased affinity of the β chains as compared to the γ chain for the diminished quantity of α chains (Steinberg 1991). For instance, 125 Indian SS patients with different α-globin genotypes were studied and Hb F levels were found to be lower in the group with the -α/-α genotype than in the groups with the -α/αα and αα/αα genotypes (Kulozik, 1988).

It is thought that α-thalassemia has a possible favorable effect on the survival of patients with sickle cell disease (Kulozik, 1988 and Steinberg, 1991). According to a study in India, a higher prevalence of α-thalassemia was found in patients elder than 10 years of age than in the younger group. Other studies done in West African, Equatorial African and American populations approve this fact by showing that the frequency of α-thalassemia in SS patients is increased with age. The reason may be either due to the positive effect of α-thalassemia on the survival of SS patients or to a notional effect of α-thalassemia delaying the onset of symptoms (Kulozik, 1988).

4. The World Distribution of α–Thalassemia

Alpha-thal-2, possibly the most common single gene disorder in the world, is spread throughout Africa, the Mediterranean, the Middle East, Southeast Asia and the Pacific island populations (Weatherall et al.,1989). The distribution of α-thal-2 is likely to follow the prevalence of malaria so that its frequency is rarely less than 20 per cent in these regions and is over 80 per cent in some populations (Flint et al., 1993). However, the distribution of α-thal-2 does not always coincide with that of malaria (Trent et al; 1985). For instance, although there has never been endemic malaria in Pacific islands; the occurance frequency of α-thal-2 there reaches 12 per cent.

Studies show that two different deletions, $-\alpha^{3.7}$ and $-\alpha^{4.2}$, which commonly cause α-thal-2 have occured in different populations, even the subtypes of $-\alpha^{3.7}$ differ in occurance frequency. For example, the $-\alpha^{3.7I}$ deletion is found in all populations which have been studied and $-\alpha^{3.7III}$ is bordered to in parts of the Oceania; on the other hand $-\alpha^{4.2}$ reaches its highest frequencies in Southeast Asia and Pacific. This gives us an idea that besides a certain amount of drift effect, the different types of α-thal-2 have arisen independently in different populations (Weatherall et al., 1989).

Additionally, α-thal-2 occurs sporadically outside tropical and subtropical regions of the world such as Britain, Iceland and Japan with a frequency less than 1 per cent (Flint, 1993).

In contrast to the high occurance frequency of α-thal-2, both nondeletional and α-thal-1 determinants have limited geographical distributions. α-Thal-1 is restricted to the Mediterranean island populations and to Southeast Asia. While one of the most common α-thal-1 deletion ($--^{SEA}$) occurs in Thai, Filipino, Vietnamese and Chinese populations, the other ($--^{MED}$) is limited to the Mediterranean basin, primarily Italy, Greece and Sardinia (Figure I.8).

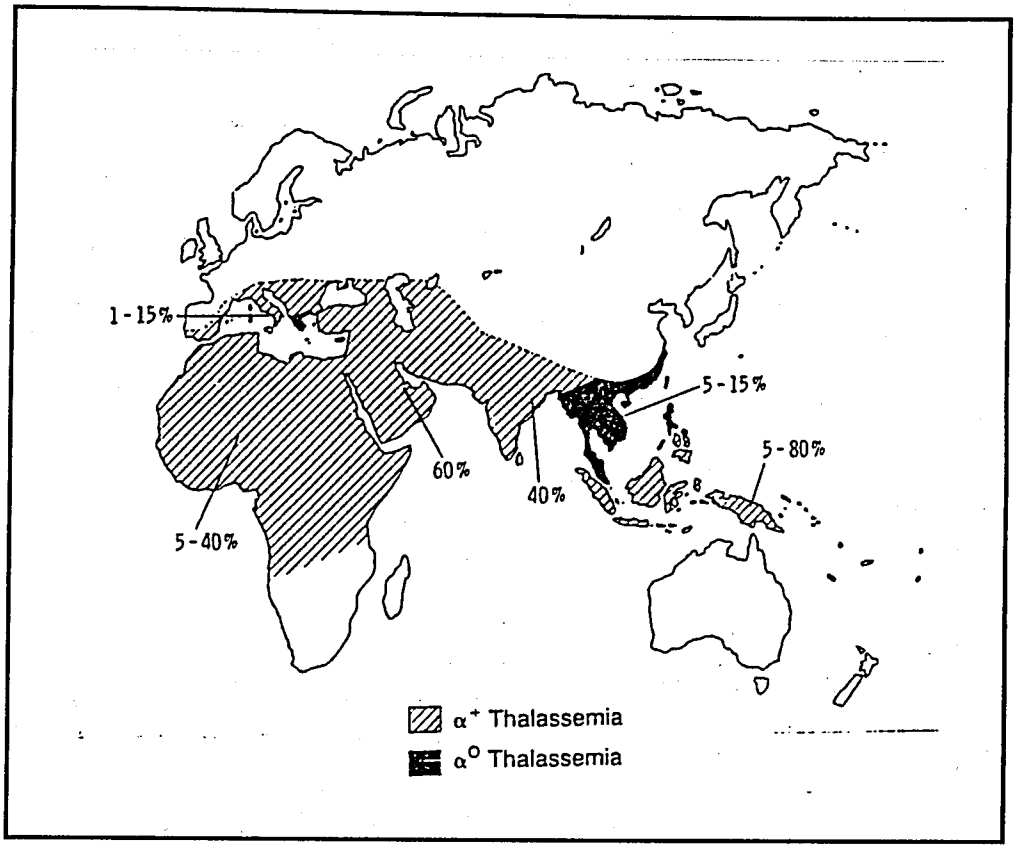


Figure I.8. The world distribution of α-thalassemia (Weatherall et al., 1991).

5. Prenatal Diagnosis

Since the couples who are carriers of a double α-gene deletion chromosome have always the risk of getting on infant with Hb Bart's, prenatal diagnosis of α-thalassemia is crucial for them.So in a population with high frequency of hydrops fetalis, a deletional α-thalassemia screening program should be essential.

Until 1991, methods used for prenatal diagnosis were mostly expensive, time-consuming and involved radioactive isotopes. Strategies to screen for carriers of the severe determinants of α-thalassemia (--/αα) have relied on either restriction mapping or use of radioactively labeled probes (Bowden et al., 1992).

In most laboratories carrying out prenatal diagnosis of α-thal-1, Southern blot analysis has been used. But this analysis has several drawbacks: At first, nondeletion defects which have rarely been associated with Bart's hydrops, are not observed. Secondly, the two most common forms of the double α-globin gene deletion ($--^{SEA}$) and ($--^{MED}$) can be detected by Southern blot analysis (Liebhaber, 1989), but the Filipino deletion that eliminates the entire α gene cluster presents a problem for diagnosis, since the band pattern obtained by Southern blot is identical to the normal pattern (Kazazian, 1990).

Prenatal diagnosis of the Hb Bart's syndrome has either relied on time consuming DNA analysis by radioactive methods in the early stages of the gestation, or ultrasonography in the later stages. In addition to this, electrophoretic methods have been used to detect small amounts of Hb Bart's in newborn babies, and immunological methods have been developed for the same purpose. Unfortunately, the detection of Hb Bart's in cord blood samples underestimates the frequency of α-thal-2 determinants (Baysal and Huisman, 1994).

Due to limitations of all these approaches, rapid, inexpensive and nonisotopic PCR-based methods for direct and specific detection of the most frequently encountered α-thal-2 and α-thal-1 determinants have been recently developed (Bowden et al., 1992; Baysal, 1993).

6. Alpha-Thalassemia in Turkey

As in many other Mediterranean countries, hemoglobinopathies, especially thalassemias are a major health concern in the Turkish population. Reviewing the thalassemia studies at molecular level, it is observed that β-thalassemia which is very heterogenous concerning its population set up has attracted much more attention than

α-thalassemia in Turkey. The lack of essential studies done at molecular level in α-thalassemia in Turkey may likely be due to the observation difficulty of α-thalassemia symptoms.

In 1989, a cord blood study of 138 Turkish newborns performed, by Southern blot analysis, indicated that the frequency of the 3.7 kb deletion was the same as the gene triplication which is 3.6 per cent (Fei et al., 1989). According to other studies, the most frequently observed large deletion in Turkey is the 20.5 deletion (Altay et al., 1980), and the most common α-thal-2 determinants in Hb H disease are the 3.7 kb deletion (41 per cent) followed by a non-deletional α-thalassemia, the 5 nucleotide deletion (33 per cent) (Altay and Başak 1995).

Hb Bart's hydrops fetalis resulting from a homozygosity for α-thal-1, namely the type 20.5 deletion was first reported by Gürgey et al. (1989). This was the first observation of hydrops fetalis in a Turkish family. Characterization of the abnormality was based on data from family studies and from α-globin gene mapping. One year later Öner et al. (1990) studied a small Turkish family with $(\gamma\beta)^0$- thalassemia. They showed the relationship between hemoglobin F level and the amount of α-globin genes such that the heterozygous father with five α-globin genes (αα/ααα) had twice the hemoglobin F level as the heterozygous mother with three α-globin genes (-α/αα). In 1992, Yüregir et al. described data for 36 members of a large family from Southern Turkey with four different types of α-thalassemia in six combinations, including Hb H disease. In the framework of the latter study, a new mutation in the polyA signal of the α_2-globin gene was discovered. This non-deletional α-thalassemia was detected through sequencing and confirmed by hybridization of amplified DNA with ^{32}P-labeled synthetic oligonucleotides. Another novel mutation leading to a severely unstable hemoglobin variant was identified by sequencing of amplified DNA involving the α_1-globin gene (Çürük et al., 1993). It was recognized also that this variant was present in association with a common α-thal-1 deletion, $-\alpha^{20.5}$, in two adults of a small family causing a severe type of Hb H disease. Characterization of the abnormality was based on data from α-globin gene mapping, sequencing and dot blot analysis. In 1994, another unstable variant with an A→G mutation at codon 94 of the α_1-globin gene was discovered in a family from Kars in East Turkey (Dinçol et al., 1994).

Coinheritance of α-thalassemia with β-thalassemia intermedia (B-TI) was observed in two patients (Altay and Başak 1995). One of them was homozygous for the IVS-I-6 mutation with milder anemia and lower Hb F value than expected, the other one was compound heterozygous for IVS-I-6 and IVS-II-848. In the same study, triplication of the α-gene was observed in β-thalassemia carriers belonging to three different families, resulting in all cases in a β-TI-like picture.

II. AIM OF THE STUDY

According to the literature, it is considered that α-thalassemia is the most wide-spread hemoglobin disorder all around the world. Also high frequencies are expected to occur in Turkey. However, until two to three years ago, a large scale population screening program to detect the α-thalassemia mutations has not been performed in Turkey; this was largely due to a variety of technical and economical problems. After the introduction of PCR-based methods for direct and specific detection of the most frequently encountered α-thalassemia determinants, studies have gained speed. PCR-based strategies are very promising in screening programs and in identification of fetuses at risk for Hb H (β_4) and Hb Bart's (γ_4) disease for many countries with limited resources on one hand, and with high incidence of α-thalassemia on the other. The present thesis, focused on PCR-based methods and their applications to the detection of common α-thalassemia determinants in the Turkish population, was designed to include the achievement of the following goals:

1. To establish the PCR-based methods of DNA analysis and DNA diagnosis for the identification of the four most common α-thalassemia deletions in Turkey;
2. To identify the different types of α-thalassemia in a large number of Turkish patients and to determine their frequencies by:
    i. identifying α-thalassemia determinants in silent carriers, carriers and Hb H patients,
    ii. screening for common α-thal-2 deletions in newborn babies,
    iii. studying compound heterozygotes for α-thalassemia and other hemoglobin disorders;

The long-term goal is the development of a logical strategy for the molecular diagnosis of α-thalassemia carriers and Hb H patients in Turkey and to prevent the birth of severely affected Hb Bart's infants.

III. MATERIALS

A. Equipment

Autoclave : Medexport, Former USSR
Eyela Autoclave, MAC-601, JAPAN

Balances : Electronic Balance Type 1574, SARTORIUS, GERMANY
Electronic Balance Libror EB-3200H, SHIMADZU, JAPAN
Presicion Balance H72, METTLER, GERMANY

Cameras : MAMIYA, RB 67, JAPAN
POLAROID, DS 34, USA
BioDoc Video Documentation System, BIOMETRA, GERMANY

Centrifuges : SORVALL RC-5B Refrigirated Superspeed Centrifuge, DuPont, USA
Eppendorf, Centrifuge 5415C, GERMANY

Deepfreezes : -20 °C, BOSCH, GERMANY
-20 °C, AEG, TURKEY
-70 °C, GFL, GERMANY
-70 °C, SANYO, JAPAN

Electrophoresis Equipment : Horizon 58, Model 200, BRL, USA

Incubators : Oven 300, Plus Series, GALLENKAMP, GERMANY
Incubator, Plus Series, GALLENKAMP, GERMANY

Magnetic Stirrers : Mini mag, Iled DK-4000 Roskilde, Intermed, DENMARK
Morat-Magnetruhrer, M10, NETHERLANDS

Oven : Microwave Oven, VESTEL, TURKEY

Spectrophotometers : Lambda 3 UV/VIS, PERKIN ELMER, USA
UV/Visible Spectrophotometer, BIO-PROJECTS GmbH, GERMANY

Thermocycler : Model-480, PERKIN ELMER CETUS, USA

Transilluminators : Reprostar II, CAMAG, SWITZERLAND
CONSORT, B-2300, BELGIUM

B. Buffers and Solutions

1. Solutions Used for DNA Extraction from Whole Blood

RBC Lysis Buffer : 155 mM NH_4Cl
10 mM $KHCO_3$
0.1 mM Na_2EDTA

Nuclei Lysis Buffer : 10 mM Tris (pH 8.0)
400 mM NaCl
2 mM Na_2EDTA

SE Buffer : 75 mM NaCl
25 mM Na_2EDTA
30 mM Tris-HCl (pH 8.0)

Proteinase K : 20 mg/ml in H_2O

Sodiumdodecyl Sulphate (SDS) : 10% stock solution

Phenol/Chloroform-Isoamylalcohol : 50 mM TE saturated phenol and chloroform with isoamylalcohol (25:24:1)

Sodium Acetate (NaAc) : 3 M

Sodium Chloride (NaCl) : 5 M saturated stock solution

TE Buffer : 20 mM Tris (pH 8.0)
0.1 mM Na_2EDTA

Alcohol : Absolute ethanol

2. Solutions Used for DNA Purification

Phenol/Chloroform-Isoamylalcohol : 50 mM TE saturated phenol and chloroform with isoamylalcohol (25:24:1)

Sodium Acetate (NaAc) : 3 M

Amonium Acetate (NH_4Ac) : 3 M

Alcohol : Isopropanol

3. Gel Electrophoresis Buffers and Solutions

10X Loading Buffer : 2.5 mg/ml BPB
1% SDS in glycerol

Ethidium Bromide : 10 mg/ml

20X TEA (Tris Acetate) : 800 mM Tris, 20 mM Na_2EDTA
400 mM NaAc (pH 8.0)

4. Agarose Gels

1.0-1.5% agarose (w/v) in 1X TEA buffer containing ethidium bromide (final concentration 0.5 μg/ml).

5. Polymerase Chain Reaction Buffer

1X Buffer	: 67 mM Tris-HCl (pH 8.8) 16.6 mM $(NH_4)_2SO_4$ 10 mM β-Mercaptoethanol 0.1 mg/ml BSA
$MgCl_2$	: 4 mM
dNTPs	: 0.2 mM of each dNTP
Primers	: 12.5 pmoles of each primer
DMSO	: 7.5%

All chemicals and solutions used in this study were purchased from MERCK (GERMANY) and SIGMA (USA) unless stated otherwise in the text. Absolute ethanol was from TEKEL (TURKEY).

C. Fine Chemicals

1. Enzymes

The enzyme Taq DNA polymerase was purchased from MBI Fermentas (LITHVANIA) and PERKIN ELMER CETUS (USA).

2. DNA Molecular Weight Standards

ϕX 174 DNA / Bsu RI (Hae III) Marker

Band Number	Band Size (bp)
1	1353
2	1078
3	872
4	603
5	310
6	281
7	271
8	234
9	194
10	118
11	72

λ-Hind III: (Hind III digested λ-phage DNA)

Band Number	Band Size (bp)
1	23,130
2	9,420
3	6,560
4	4,360
5	2,320
6	2,020
7	560
8	100

3. Primers

a. Primers Used for the Detection of Common α-Thal-1 Deletions

Determinant	Primer	Sequence (5' → 3')
$-\alpha^{20.5}$	1	GGCAAGCTGGTGGTGTTACACA
	2	TGGAGGGTGGAGACGTCCTG
	3	CCATGCTGGCACGTTTCTGAGG
$--^{MED}$	4	ACAGTCACTCCTGAGGCCAGTC
	5	TACAGCAGAGTGAGTGCTGCAT
	6	GGAGAAGTAGGTCTTCGTGGC

b. Primers Used for the Detection of Common α-Thal-2 Deletions

Determinant	Primer	Sequence (5' → 3')
$-(\alpha)^{3.7}$	A	CTTTCCCTACCCAGAGCCAGGTT
	B	CCCATGCTGGCACGTTTCTGAGG
	C	CCATTGTTGGCACATTCCGGGACA
$-(\alpha)^{4.2}$	E	CCCTGGGTGTCCAGGAGCAAGCC
	F	GGCACATTCCGGGACAGAGAGAA
	G	CCGGTTTACCCATGTGGTGCCTC

D. Blood Samples

Blood samples of α-thalassemia patiens and their family members were provided by Medical School of Ankara, ANKARA and Antalya State Hospital, ANTALYA. Cord-blood samples of newborn babies and blood samples of patients with other hemoglobin disorders, used in this study, were also sent to our laboratory from Antalya State Hospital.

IV. METHODS

A. DNA Extraction From Whole Blood

Peripheral blood samples are collected from individuals in K_2EDTA containing tubes to prevent coagulation and kept at 4 °C if DNA is to be extracted immediately, otherwise they are stored at -20 °C.

For DNA extraction, blood samples are thawed and transferred to 50 ml Sorvall centrifuge tubes. After adding ice-cold RBC lysis buffer to the blood samples (30 ml/10 ml of blood) and mixing thoroughly, the samples are kept at 4 °C for 15 minutes to allow lysis of erythrocyte (RBC) membranes. For colling the nuclei of the leukocytes, the samples are centrifuged at 5000 rpm (5K) and 4 °C for 10 minutes. The supernatant with the lysed erythrocyte cell contents is then discarded, and the pellet is resuspended in 10 ml lysis buffer by vortexing. At this step, the nuclei may be stored at -70 °C until DNA isolation. The suspension is centrifuged again at 5000 rpm and 4 °C for 10 minutes. The supernatant is discarded and the nuclear pellet is treated either with phenol/chloroform or according to salting out (NaCl) method (Poncz et al., 1982; Miller et al., 1988).

Phenol/chloroform extraction method: After the second lysis step, the pellet is resuspended with 4.5 ml SE buffer, proteinase K (final concentration 150 µg/ml) and SDS (final concentration 0.14 per cent) and incubated at 37 °C for overnight or at 56 °C for 2-4 hours. After the addition of 5 ml of SE buffer, one volume of a phenol/chloroform mixture is added to each sample. Then the samples are shaken vigorously for removing the protein debris. Centrifugation at 5000 rpm for 5 minutes is repeated two times; thi is followed by taking the aqueous upper phase (contains the DNA) and treating it with one volume of phenol/chloroform. The upper phase is then transferred into a sterile Falcon tube, and DNA is precipitated by addition of 1:30 volume of sodium acetate (final concentration 0.1 M) and one volume of isopropanol.

Salting out (NaCl extraction) method: It is simpler and faster than the phenol/chloroform method. It also avoids the use of hazardous phenol and recovers larger amounts of DNA from cells.

In NaCl method, after the second RBC lysis buffer step, the nuclear pellet is resuspended in 3 ml of nuclei lysis buffer to lyse the nuclear envelope of leukocytes. The suspension is vortexed until all the clumps are broken, then proteinase K (final concentration 150 μg/ml) and SDS (final concentration 0.14 per cent) are added, and the mixture is incubated at 37 °C for overnight or at 56 °C for 2-4 hours in order to degrade the nuclear proteins. After the incubation equal amounts of sterile distilled water and saturated NaCl are added (5 ml each) the tubes are shaken vigorously to precipitate the proteins, and then centrifuged at 10,000 rpm (10K), at room temperature for 20 minutes.

In both methods, the precipitated DNA is fished out, dried and dissolved in 500-1000 μl of TE buffer in an Eppendorf tube. The broken DNA threads, which cannot be fished out, are recovered by centrifugation.

After DNA extraction with NaCl method, if DNA samples are impure, thet can be re-extracted with phenol/chloroform method in order to get purified DNAs.

B. Quantitative and Qualitative Analysis of DNA

Two methods used for the quantitative and qualitative determination of DNA are the spectrophotometric measurement and the agarose gel electrophoresis. In both methods, DNA should be homogenously dissolved in TE buffer.

1. Spectrophotometric Measurement Method

To estimate the concentration and the purity of the isolated DNA by spectrophotometry, DNA is diluted in a ratio of 1:50 or 1:100, depending on its concentration and measured at 260 and 280 nm.

The formula below is used to calculate the concentration of DNA. This formula is based on the fact that 50 μg of dsDNA has an absorbance of 1.0 at 260 nm:

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

The purity of the DNA is estimated by taking the ratios between the spectrophotometric measurements at 260 nm and 280 nm (OD_{260}/OD_{280}). If the ratio gives 1.8, then the DNA is considered to be pure. Values greater than 1.8 indicate RNA, and values less than 1.8 protein contamination.

2. Agarose Gel Electrophoresis

A more qualitative evaluation of the isolated DNA can be done by agarose gel electrophoresis, which is a standard method for separating and identifying DNA fragments. In this method, the separation of DNA fragments can be performed by running them from cathode to anode by the aid of a power supply providing the required current. DNA molecules run towards the positively charged electrode, due to the presence of negatively charged phosphate groups at the DNA backbone. The bands can be visualized in the UV light when the gel is stained with the fluorescent dye EtBr, which intercalates within the stacked DNA bases.

One per cent agarose gel is prepared by boiling 1 g of agarose in 100 µl of 1X TEA buffer. After the solution is cooled down to 55 °C, EtBr is added to a final concentration of 0.5 µg/ml (this is done to prevent the dye from degradation). The gel solution is poured onto the plate and combs are positioned. The gel is left at room temperature to polymerize. 1-2 µl from each DNA sample is mixed with 1X loading buffer and put into the slots of the gel with a micropipette. The gel is run at 100 V for 15 minutes in 1X TAE. The amount of DNA is then estimated by comparing its intensity with known amounts of DNA.

C. Polymerase Chain Reaction (PCR) for the Analysis of α-Thalassemia Deletions

Since its first discovery by K. Mullis in the mid-1980s, the Polymerase Chain Reaction (PCR) technique has taken an important place in molecular biology (Saiki et al., 1985; Mullis et al., 1987; Saiki et al., 1988; Mullis, 1990). The PCR is an in vitro method for the enzymatic synthesis of specific DNA fragments over a period of a few hours.

The idea of PCR is to hybridize two synthetic oligonucleotide primers to opposite strands which 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 the Taq polymerase enzyme (a heat-stable DNA polymerase) result in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Because the primer extension products, synthesized in one cycle, can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 30 cycles of PCR yields about a million-fold amplification (Saiki et al., 1988).

The simplicity of PCR have stimulated many scientists to develop modifications of the original protocol. Some of these were realized by Bowden et al. in 1992 and Baysal and Huisman in 1994 in order to overcome the technical problems in the detection of α-thalassemia determinants. These scientists introduced for the first time PCR-based protocols for determining common α-thalassemia mutations.

In both protocols, specific primers are designed, such that they are adjacent to the 5' and 3' breakpoints of each deletion. To demonstrate the presence of the deletion, two separate reactions are carried out simultenously for each DNA sample. One reaction contains a pair of primers designed to amplify a segment of the affected chromosome, and the other contains a pair of primers to amplify a comparable segment of the normal gene (Figure IV.1 and Figure IV.2).

Approximately 0.4-0.8 μg of genomic DNA is used for each PCR reaction (50 μl). The amplification buffer contains 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$, 0.10 mg/ml BSA, 10 mM β-mercaptoethanol, 4.0 mM $MgCl_2$, 7.5 per cent DMSO, and 200 μM dNTPs. 12.5 pmoles of each of the primers are used per tube. The amplification reactions are carried out with 1.0-1.6 U Taq polymerase (MBI or PERKIN ELMER-CETUS) in a thermal cycler (Perkin Elmer Cetus). A total of 25 cycles are performed under the following conditions:

i) for $-\alpha^{20.5}$, $--^{MEDI}$, $-\alpha^{3.7}$ determinants

Temperature (°C)	Time (minutes)
99	6 (hot start)
94	1
55	1
72	3

ii) for $-\alpha^{4.2}$ determinant

Temperature (°C)	Time (minutes)
99	6 (hot start)
94	1
60	1
72	3

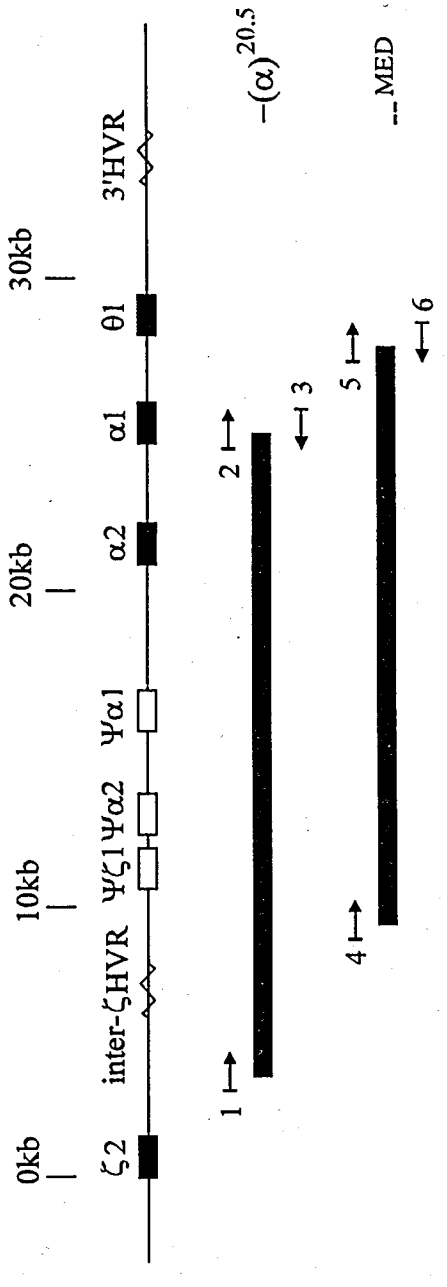


Figure IV.1. Localization of the oligonucleotide primers for α-thal-1 deletions (Bowden et al., 1992).

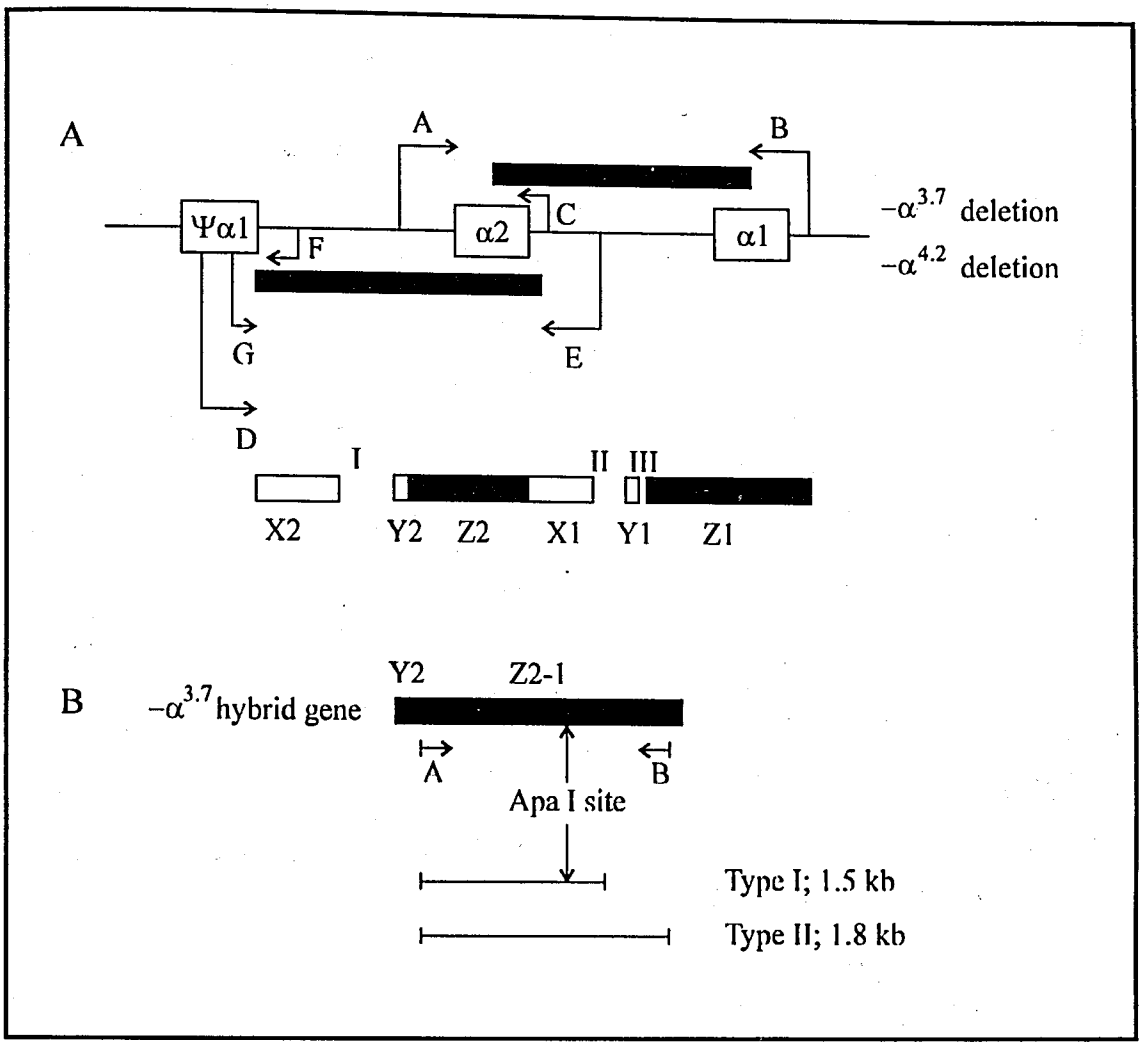


Figure IV.2. Localization of oligonucleotide primers for the α-thal-2 deletions (Baysal and Huisman, 1994).

V. RESULTS

A. Identification of α-Thalassemia Mutations in Hb H Patients and Their Families

1. Patients

A total of 32 Turkish α-thalassemia silent carriers, carriers and Hb H patients were investigated in the framework of this study; their blood samples were sent to our laboratory from hospitals in Ankara and Antalya. These include eight families with Hb H off-springs in addition to 12 single Hb H patients. Genomic DNA was extracted from blood samples of the probands as described in Section IV and run on agarose gels to test the quality and quantity of DNA (Figure V.1).

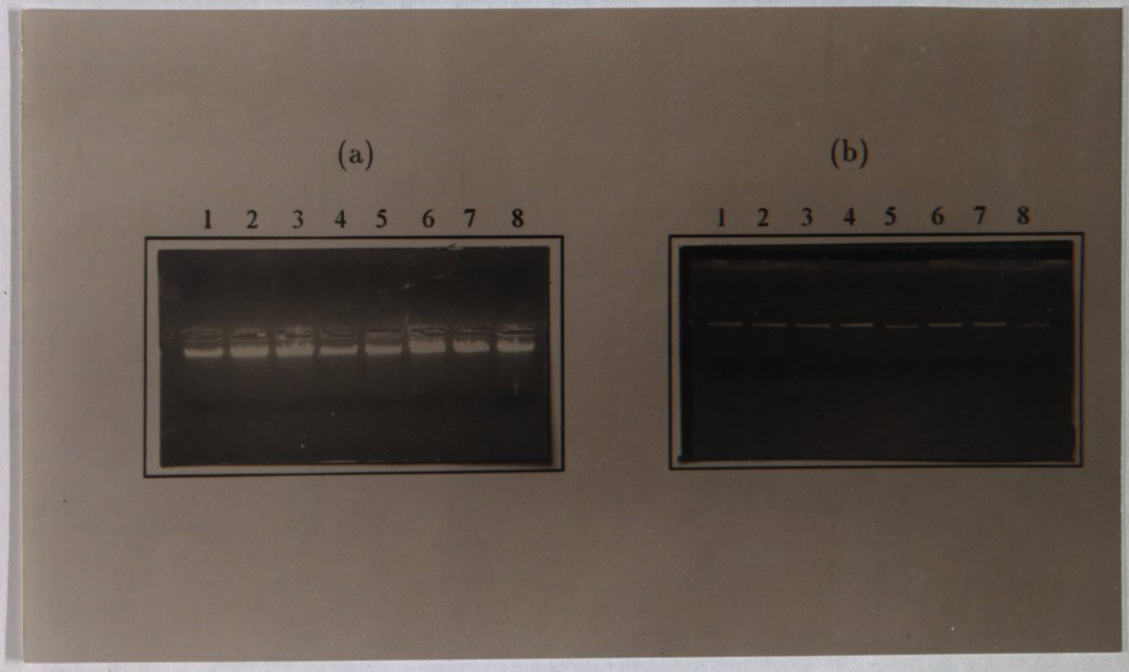


Figure V.1. (a) Genomic DNA samples of various α-thalassemia cases run on a one per cent agarose gel. (b) The same DNA samples in 1/10 to 1/20 dilutions.

2. Screening for α-Thal-1 Determinants: $-\alpha^{20.5}$ and $--^{MEDI}$

In order to identify the genotypes of the Hb H patients and their families, two common α-thal-1 deletions were searched for, utilizing the PCR-based strategy described in Section IV. First, the patients were analyzed for the $-\alpha^{20.5}$ deletion. For this purpose two amplification reactions were carried out for each DNA sample: Primers 1 and 3 were used for mutant and primers 2 and 3 for normal amplification (Figure V.2).

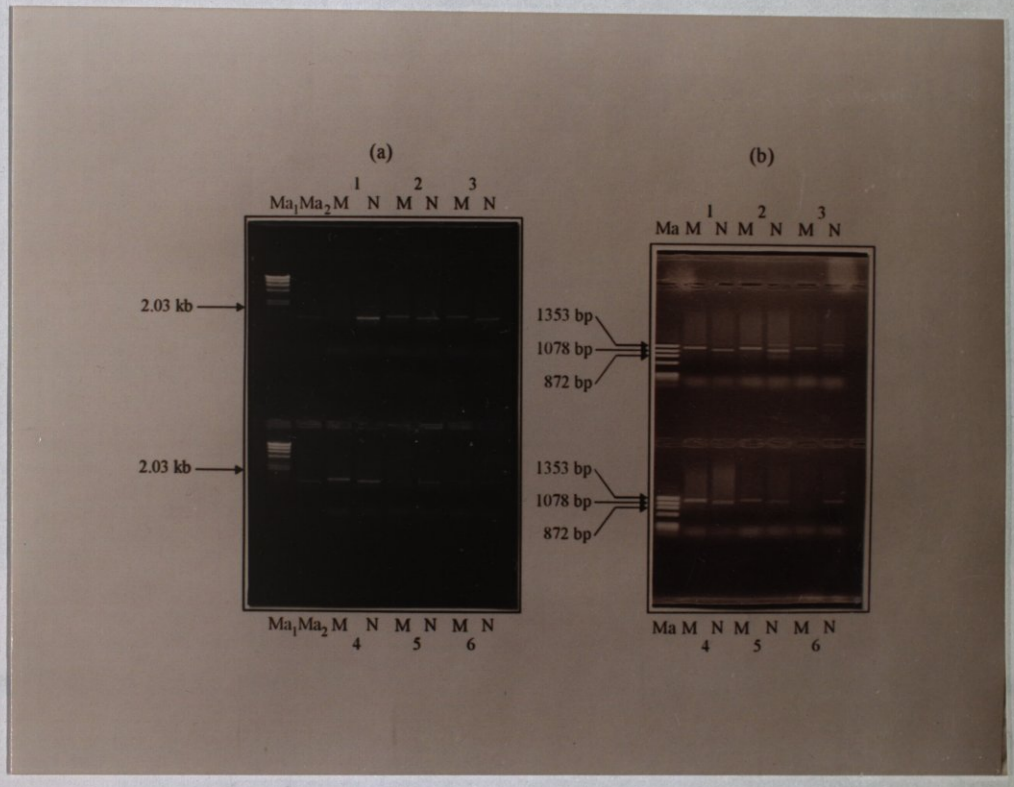


Figure V.2. Identification of the $-\alpha^{20.5}$ deletion. Amplifications with primers 1 and 3 (mutant) and 2 and 3 (normal) were performed in separate tubes since the size of the products are very close to each other. Primers 1 and 3 generate a specific fragment in the presence of the $-\alpha^{20.5}$ determinant. (a) Patients 2, 3, 4 and 6 are carriers for the $-\alpha^{20.5}$ mutation ($-\alpha^{20.5}/\alpha\alpha$), whereas Patients 1 and 5 are normal for this mutation. Ma_1 contains λ-HindIII weight standard and Ma_2 a PCR product of known size. (b) Patients 1, 2, 3, 4 and 5 are carriers ($-\alpha^{20.5}/\alpha\alpha$) and Patient 6 is normal for the $-\alpha^{20.5}$ mutation. Ma: ΦX174/HaeIII weight marker.

The cases that were normal for the $-\alpha^{20.5}$ deletion, were analyzed for the $--^{MEDI}$ mutation (Figure V.3).

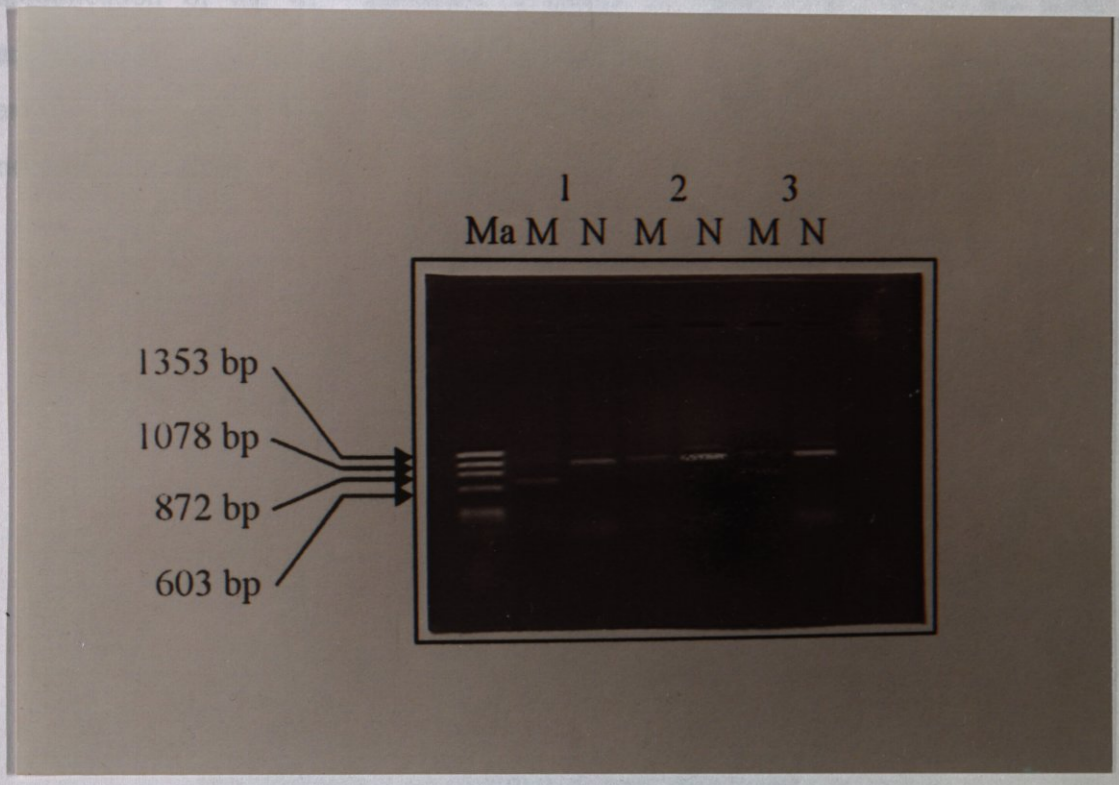


Figure V.3. Agarose gel electrophoresis of PCR products amplified with primers 5 and 6 (normal) and 4 and 6 (mutant). In the presence of the $--^{MEDI}$ determinant, primers 4 and 6 result in the amplification of a specific fragment. Patients 1 and 3 are carriers for the $--^{MEDI}$ mutation ($--^{MEDI}/\alpha\alpha$), whereas Patient 2 is normal for $--^{MEDI}$. Ma is ΦX174/HaeIII digest.

The total number of cases that were studied in the framework of this thesis for common α-thalassemia deletions was 32. 17 out of these 32 cases (~50 per cent) turned out to be carriers for the $-\alpha^{20.5}$ deletion. The $--^{MEDI}$ deletion was detected only in one family, in which the father and his son were carrires, whereas the mother was normal for this mutation.

3. Screening for Common α-Thal-2 Determinants: $-\alpha^{3.7}$ and $-\alpha^{4.2}$

The 32 cases of the previous investigation were studied for the $-\alpha^{3.7}$ mutation. The identification of the $-\alpha^{3.7}$ genotypes is shown in Figure V.4. As in the other amplifications, two different PCRs were carried out for each DNA sample, one to amplify the abnormal chromosome carrying the deletion, and the other to amplify the normal chromosome.

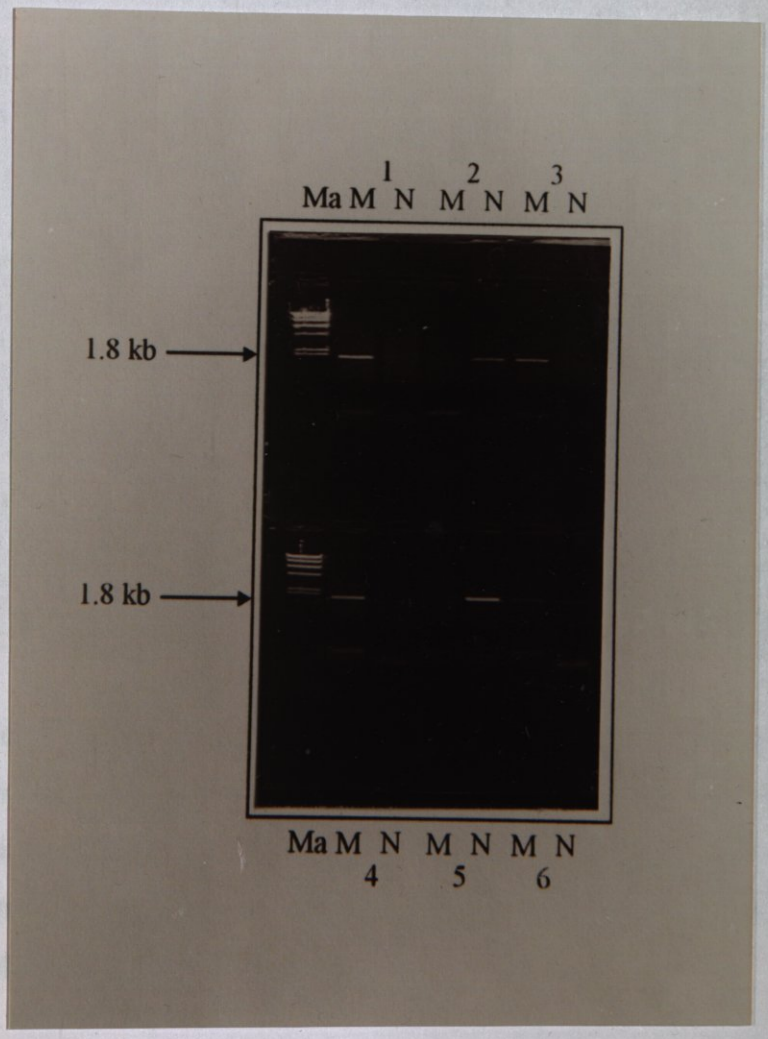


Figure V.4. Agarose gel electrophoresis of amplification products for $-\alpha^{3.7}$. Amplification with primers A and C (normal) and A and B (mutant) were performed in separate tubes since their products have the same size of 1.8 kb. Patients 1, 3, 4 and 6 are carriers ($-\alpha^{3.7}/\alpha\alpha$), Patients 2 and 5 are normal for $-\alpha^{3.7}$. Ma: λ-HindIII-digest.

The cases in whom the $-\alpha^{3.7}$ mutation was not revealed, were subjected to a second amplification using the $-\alpha^{4.2}$ set of primers (Figure V.5).

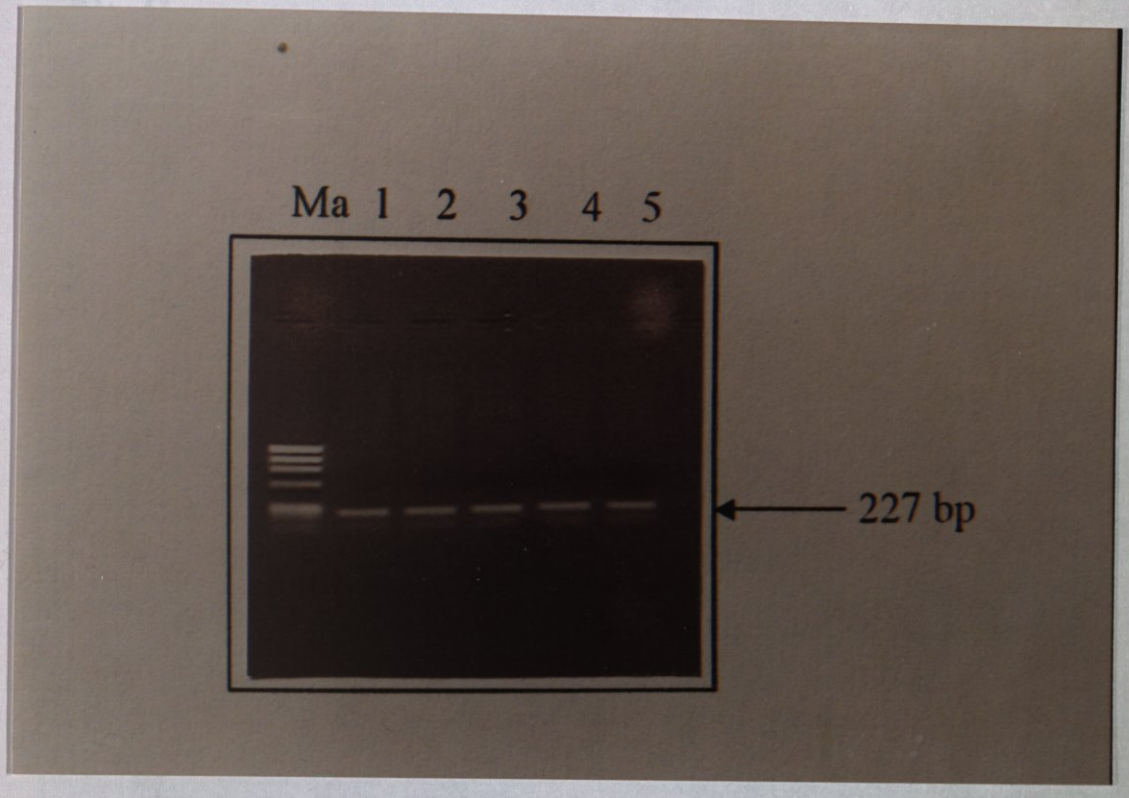


Figure V.5. Identification of the amplification products ($-\alpha^{4.2}$) by gel electrophoresis. Primers G, E and F are put together in a single reaction mixture. The expected fragments with these primers are 1761 bp (mutant) and 227 bp (normal), respectively. All individuals on this gel are normal for $-\alpha^{4.2}$. Ma is ΦX174/HaeIII digest.

In the framework of this investigation, 15 out of 32 cases were identified as carriers of an abnormal chromosome for the $-\alpha^{3.7}$ mutation; the $-\alpha^{4.2}$ deletion was not found at all.

4. Combinations of α-Thal-1 and α-Thal-2 Determinants

By combining the PCR protocols of Bowden et al. (1992) and Baysal and Huisman (1994), we have been able to identify the genotypes of 11 Hb H patients: All were having $-\alpha^{20.5}/-\alpha^{3.7}$ (Figure V.6). Compound heterozygotes for $--^{MEDI}/-\alpha^{3.7}$, $--^{MEDI}/-\alpha^{4.2}$ and $-\alpha^{20.5}/-\alpha^{4.2}$ were not detected at all.

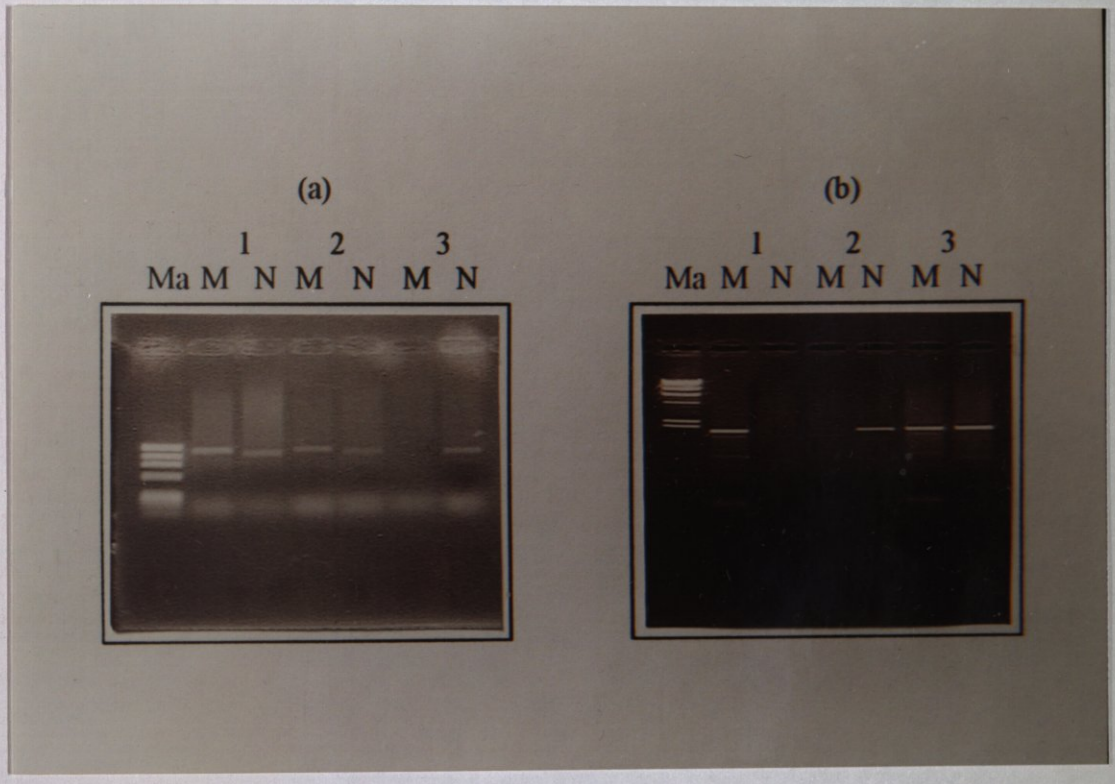
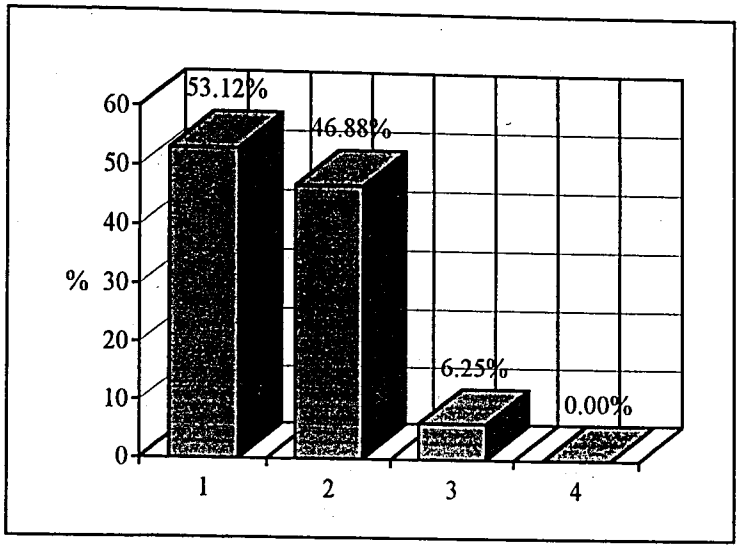


Figure V.6. Identification of the genotype of a family: (a) Screening for the $-\alpha^{20.5}$ deletion. Ma is ΦX174/HaeIII digest. (b) Screening for the $-\alpha^{3.7}$ deletion. Ma is λ-HindIII weight standard. The absence of the normal band in Patient 1 (sick child), who is not homozygous for $-\alpha^{3.7}$ is the result of the large deletion of 20.5 kb on his other chromosome. 1) sick child: $-\alpha^{3.7}/-\alpha^{20.5}$, 2) mother: $\alpha\alpha/-\alpha^{20.5}$, 3) father: $-\alpha^{3.7}/\alpha\alpha$.

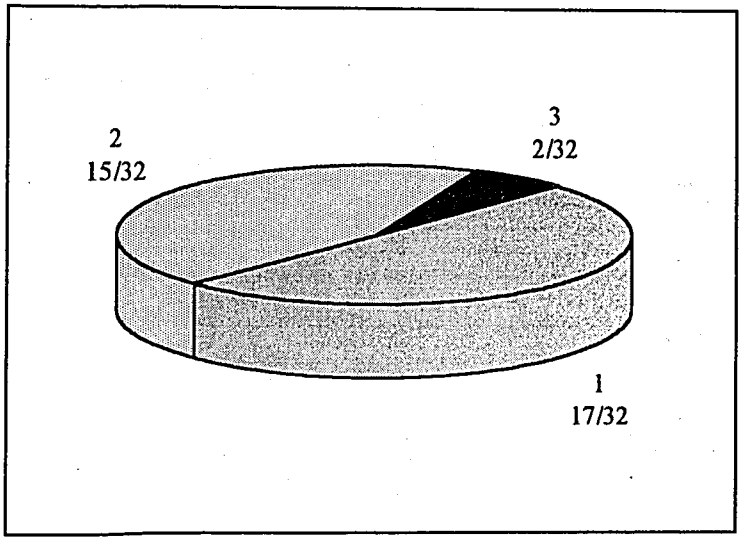
The genotypes of the α-thalassemia families, that were detected in the framework of this study are shown in Table V.1; the distribution pattern of mutations is illustrated in Figure V.7 a and b, in form of a bar and a pie chart, respectively.

Table V.1. Genotypes of patients investigated in this study.

Genotype	*Number of Cases*	*Percentage*
$-\alpha^{3.7}/-\alpha^{20.5}$	11	34.375
$\alpha\alpha/-\alpha^{20.5}$	3	9.375
$?/-\alpha^{20.5}$	3	9.375
$\alpha\alpha/-\alpha^{3.7}$	2	6.250
$?/-\alpha^{3.7}$	2	6.250
$?/--^{MEDI}$	2	6.250
$-\alpha^{4.2}/\alpha\alpha$	0	0.000
unknown	9	28.125



(a)



(b)

Figure V.7. Frequencies of the $-\alpha^{20.5}$ (1), $-\alpha^{3.7}$ (2), $--^{MEDI}$ (3) and $-\alpha^{4.2}$ (4) mutations as percentages in (a) and fractions in (b).

B. Cord Blood Survey Utilizing the PCR Approach

1. Samples

A total of 80 randomly selected newborn babies, whose cord blood samples were sent to our laboratory from Antalya State Hospital, participated in this study. Genomic DNA was extracted from blood samples of these babies with the salting out method as described in Section IV, and run on one per cent agarose gels to test the DNA samples for their quality and quantity (Figure V.8).

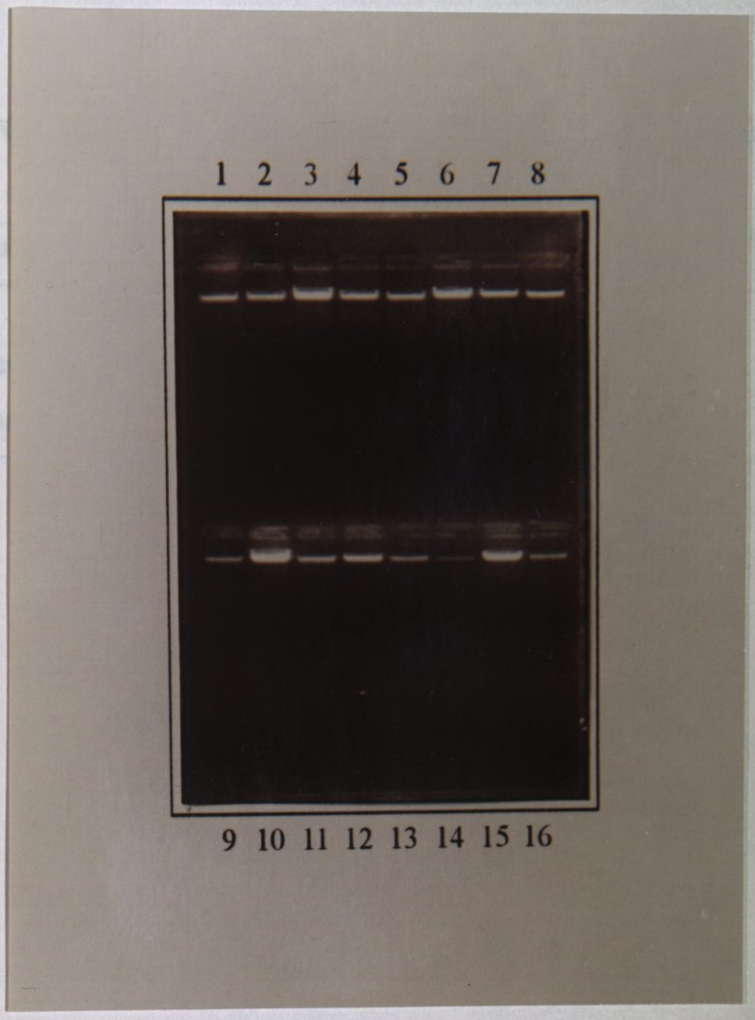


Figure V.8. Genomic DNA samples of 16 babies run on a one per cent agarose gel.

2. Screening for Common α-Thal-2 Determinants

The samples were analyzed for the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ determinants by using the PCR approach described in Section IV (Figure V.9 and Figure V.10). In four out of 80 babies, we have been able to detect the $-\alpha^{3.7}$ determinant, who were heterozygous for this mutation. Heterozygous α^{+} thalassemia for the $-\alpha^{4.2}$ determinant and homozygous α^{+} thalassemia ($-\alpha^{3.7}/-\alpha^{3.7}$) or ($-\alpha^{4.2}/-\alpha^{4.2}$) were not detected in any of these 80 specimens (Table V.2).

Table V.2. PCR-based detection of common α-thal-2 determinants in newborn babies.

Subjects	N	$-\alpha^{3.7}/\alpha\alpha$	$-\alpha^{4.2}/\alpha\alpha$	$-\alpha/-\alpha$	% ($-\alpha^{3.7}$)
Newborn babies	80	4	0	0	5.0

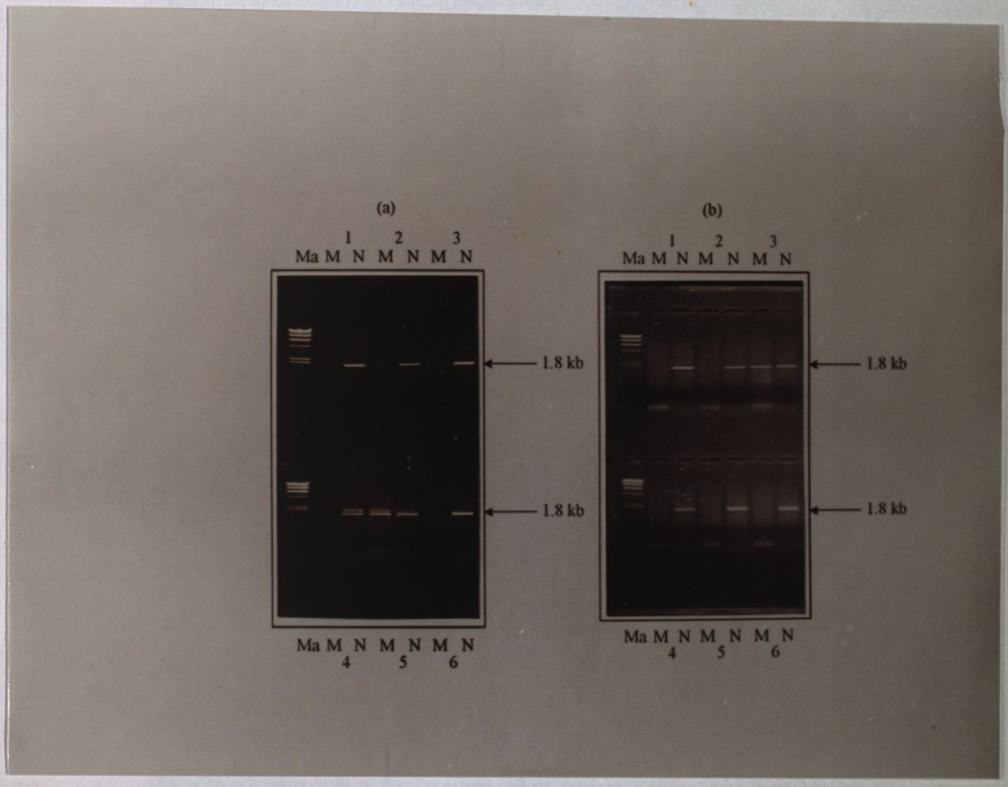


Figure V.9. Identification of the $-\alpha^{3.7}$ deletion in cord blood samples: Agarose gel electrophoresis of PCR products amplified with primers A and C (normal) and primers A and B (mutant). Since the normal and mutant PCR products have the same size of 1.8 kb, amplifications were carried out in separate tubes. (a) Baby 5 is a carrier for $-\alpha^{3.7}$, all other babies do not carry this mutation. Ma: λ-HindIII marker. (b) Baby 3 is a carrier for the $-\alpha^{3.7}$ deletion, whereas all others are normal for this mutation. Ma: λ-HindIII marker.

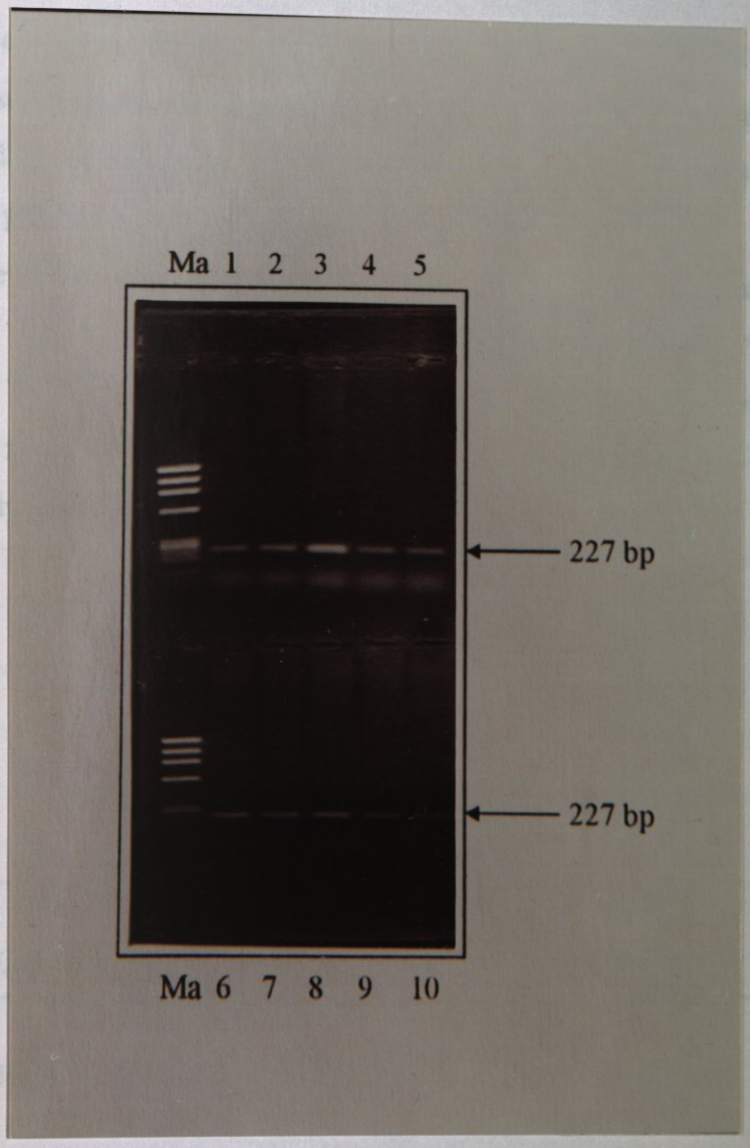


Figure V.10. Analysis of the $-\alpha^{4.2}$ deletion in cord blood samples: Amplifications with primers G, E and F are performed in a single reaction mixture. The expected fragments with these primers are 1761 bp (mutant) and 227 bp (normal), respectively. All individuals (lanes 1-10) are normal for the $-\alpha^{4.2}$ deletion. Ma: ΦX174/HaeIII digest.

C. Search for Coinheritance of α-Thalassemia with Other Hemoglobin Abnormalities

Three cases who were suspected for the possible presence of an α-thalassemia determinant were selected out of 50 β-thalassemia and/or abnormal hemoglobin patients. All three had Hb H inclusions, in addition to their abnormal hematological data (Table V.3). These patients were screened for $-\alpha^{3.7}$, $-\alpha^{4.2}$, $-\alpha^{20.5}$ and $--^{MEDI}$.

In case 3, we were able to detect the $-\alpha^{3.7}$ determinant in heterozygous form. Interestingly, this individual was also a compound heterozygote for Hb D and β-thalassemia. To confirm the result, the patient's father and mother were analyzed. The mother was found to be the Hb D carrier, whereas β-thalassemia and α-thalassemia heterozygosity were shown to be present in the father.

Table V.3. Hematological data of the cases.

Cases	*Hb (g/dl)*	*Htc (%)*	*RBC ($x10^{12}$/l)*	*MCV (fl)*	*Hb A_2 (%)*	*Hb H (%)*	*Diagnosis*
1	10.60	33.40		60.20	2.08	2.13	Thal. Major
2	10.50	29.50	3.10		2.70	69.60*	S&S
3	9.60	27.20	3.19	77.50	2.40	57.00*	Hb D & β-Thal

*: This high value of Hb H may be due to the determination of basophilic stippling.

VI. DISCUSSION

Since its introduction in 1985, the PCR procedure has become one of the most important tools in molecular biology for the characterization of mutations, and it has revolutionized the diagnosis of genetic diseases in a short time. The PCR technique can be applied to various molecular defects including point mutations and deletions for diagnosis and carrier detection.

The simplicity of this reliable technique has motivated many researchers to develop modifications of the original protocol in order to apply the procedure to their field of study. In α-thalassemia research, some modifications had to be realized to overcome the technical problems encountered in the determination of the α-thalassemia mutations. Previous methods used to detect α-thalassemia determinants have relied on DNA analysis by gene mapping using the Southern Blot technique, while other less specific and less sensitive screening methods have been applied for the evaluation of the possible presence of an α-thalassemia mutation. Although the gene mapping procedure has been the only available way to identify the α-thalassemia mutations correctly up to the introduction of the PCR technique, this method is cumbersome, labor-intensive, expensive and not applicable in a large population (Bowden et al., 1992; Baysal and Huisman, 1994).

It is known that PCR-based methods are fast, specific, low in cost and relatively simple. Another important property is the safety of PCR which does not include the use of a radioactive isotope. Presently, there are several studies utilizing different PCR approaches to detect α-thalassemia mutations (Hsia et al. 1989; Kropp et al., 1989; Dide et al., 1990; Lebo et al., 1990; Chang et al., 1991; Bowden et al., 1992; Chen et al., 1993; Baysal and Huisman, 1994; Chang et al., 1994). However, the application of PCR techniques to identify common α-thalassemia mutations are relatively late with respect to other diseases, i.e. β-thalassemia, because of the complexity of the α-globin genes. Additionally, α-globin genes are embedded into two homologous regions, and these regions have resulted in difficulty of primer design. Most of the present techniques for primer selection rely on the usage of non-homologous regions which are very short between homologous regions.

In the framework of this thesis, two PCR-based strategies were applied. One of them was developed by Bowden et al. (1992) to detect the most common α-thal-1 determinants, and the other was modified by Baysal and Huisman (1994) to diagnose the most common α-thal-2 deletions. These two methods have the same basic principle. They both depend on the deletion event bringing 5' and 3' primers, which are widely separated on the normal chromosome, close enough for amplification. Bowden's method is a rapid way of screening the $--^{SEA}$, $--^{MEDI}$ and $-\alpha^{20.5}$ determinants. It allows specific detection of carriers of α-thal-1 and fetuses at risk of the Hb Bart's hydrops fetalis syndrome. On the other hand, Baysal's protocol is useful in identifying the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ carriers. A combination of these two protocols provides an excellent method for the identification of fetuses at risk for the Hb H (β_4) disease. Thus, the application of these PCR-based strategies, separately or in a combined way, is very promising in screening programs and in the identification of fetuses at risk for Hb H or Hb Bart's hydrops fetalis in populations where each of these determinants are maintained at high levels.

However, these methods have also some limitations. The drawback of Baysal's method is its inability to distinguish between large deletions and the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ genotypes. For instance, a compound heterozygote for the $-\alpha^{20.5}$ and $-\alpha^{3.7}$ deletions will be identified as a homozygote for the $-\alpha^{3.7}$ deletion if the PCR is only performed for the $-\alpha^{3.7}$ deletion but not for $-\alpha^{20.5}$ (Figure V.6). In Bowden's method, the determination of the $-\alpha^{20.5}$ mutation by amplification of the α_1 gene shows the presence of the normal chromosome without excluding the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ determinants. For this reason, the possible presence of the α-thal-2 determinants should always be checked, when applying this method.

Reviewing the thalassemia studies at molecular level, it is observed that studies on β-thalassemia, in which country-scale frequencies have already been determined few years ago (Başak et al., 1992), are much more advanced than α-thalassemia investigations thus far performed in Turkey. One of the major reasons for the lack of essential molecular studies in α-thalassemia is the mild clinical picture of the disease. Except Hb Bart's hydrops fetalis and some severe forms of Hb H cases, all other α-thalassemia patients and carriers live with normal daily activities. They are non-transfusion dependent and do not have the bone deformities seen in most β-thalassemia patients. Although being advantageous for the

patient, this results in the unawareness of a potential threat of α-thalassemia on public health. The other important reason is related to technical and economical problems. Until recently, the complexity of the application of Southern Blot technique and the absence of sufficient molecular know-how have prevented to pay enough attention to the α-thalassemia studies in Turkey.

The deletions $-\alpha^{20.5}$, $--^{MEDI}$ and $-\alpha^{3.7}$ and to a lesser extent $-\alpha^{4.2}$ are the most commonly observed mutations in the Mediterranean region; hence it seemed very suitable to establish the above mentioned methods in our laboratory and apply them in the framework of a systematic study to the Turkish population. For this purpose, three different groups of probands, which will be discussed separately, have been chosen as subjects of this thesis.

Identification of α-Thalassemia Mutations in Hb H Patients and Their Families: A total of 32 Turkish α-thalassemia carriers and Hb H patients were studied for the α-thal-1 and α-thal-2 determinants. In the framework of this thesis, the most common α-thal-2 determinant was found to be the 3.7 kb deletion. 15 out of 32 cases were identified as carriers of this mutation, corresponding to a frequency of 46.88 per cent (Figure V.7). In contrast, the other α-thal-2 determinant, $-\alpha^{4.2}$, was not encountered at all in the 32 patients investigated.

When the same patients were screened for the α-thal-1 determinants, the most frequent large deletion was found to be the 20.5 kb deletion. 17 out of the 32 cases turned out to be a carrier for the $-\alpha^{20.5}$deletion, which corresponds to a gene frequency of 53.12 per cent. On the other hand, the second type of large Mediterranean deletion, $--^{MEDI}$ was detected only in one family, in which the father and his son were carriers. The frequency of this mutation is considerably low (6.25 per cent) when compared to Cyprus, where the MedI deletion is the most common α-thal-1 determinant (37.8) (Baysal et al.,, 1994).). According to our results the most common genotype in Hb H disease was associated with a combination of 20.5 kb and 3.7 kb deletions. These data are in good accordance with the results of other studies reviewed by Altay and Başak (1995).

Although screened for the most common four deletions, approximately half of the chromosomes analyzed in this study, remained undefined. This may have different explanations: The combination of α-thal-2 with α-thal-1 genes is only one of the genotypes giving rise to Hb H disease (Weatherall and Clegg, 1981; Bunn and Forget, 1986; Higgs et al., 1989). Thus, other less common α-thal-2 and α-thal-1 determinants should be studied

for their possible presence. Furthermore, another common genotype of Hb H disease is due to the combination of α-thal-1 with a non-deletional type of mutation. A highly possible candidate may be the 5nt deletion, which is the second most common mutation in Hb H disease (33 per cent), following the 3.7 kb deletion (Altay and Başak, 1995). Other point mutations which should be considered are the three Poly A mutations (Gürgey et al., 1985; Yüregir et al., 1992).

Cord Blood Studies: 80 randomly selected babies from Antalya were analyzed for the presence of the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ determinants and four out of 80 were detected as $-\alpha^{3.7}$ carriers. This corresponds to a frequency of five per cent. Heterozygous α^{+} thalassemia for the $-\alpha^{4.2}$ determinant and homozygous α^{+} thalassemia ($-\alpha^{3.7}/-\alpha^{3.7}$) or ($-\alpha^{4.2}/-\alpha^{4.2}$) were not encountered in any of these 80 babies. However, more accurate results are expected to be obtained when the more than 300 cord-blood samples, planned to be analyzed, are screened for all four deletions, including the $-\alpha^{20.5}$ and $--^{MEDI}$ mutations. The relatively high incidence of the $-\alpha^{3.7}$ genotype is important in many aspects: Firstly, it may pose a problem for couples at risk, especially if the frequencies of the α-thal-1 determinants are also high. Secondly, β-thalassemia is a very important health problem and occurs everywhere in Turkey. Thus, there is a high possibility for the coexistance of α-thalassemia and β-thalassemia or α-thalassemia with sickle cell anemia which is also very common in this part of Turkey. Since the decrease in α-globin gene number may ameliorate β-thalassemia or β-thal/Hb S disease, this situation may become crucial in prenatal diagnosis.

Co-inheritance of α-Thalassemia with other Hemoglobin Abnormalities: As mentioned above, due to their relatively common presence, both α- and β-thalassemia mutations can be coinherited by the same individual. Aiming the detection of such a case, three patients with β-thalassemia and an abnormal hemoglobin, who were suspected for the possible presence of an α-thalassemia determinant, were analyzed. In one of them, the $-\alpha^{3.7}$ mutation was detected in heterozygous form. This individual is a compound heterozygote for Hb D in cis to a β-thalassemia mutation under investigation yet.

Since there is no treatment for α-thalassemia presently, carrier identification and prenatal diagnosis are important to decrease the number of affected individuals in a population. The couples who are carriers of a double α-gene deleted chromosome have always the risk of getting an infant with Hb Bart's, thus, carrier detection and prenatal

diagnosis of α-thalassemia become very important for such families. We strongly believe that the PCR-based methods, established and introduced in the framework of this thesis, can be widely applied to the identification of α-thalassemia in individuals at risk. We furthermore hope that this study will initiate the foundation of a large-scale screening program for the identification of α-thalassemia mutations in Turkey and will thus help to evaluate the distribution and frequency of α-thalassemia in the Turkish population.

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