Molecular characterization of haemoglobinopathies in the Surinam population

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Proefschrift

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. mr. M.J. Cohen,
volgens het besluit van het College van Dekanen
in het openbaar te verdedigen op
donderdag 26 november 1992 om 16.00 uur

door

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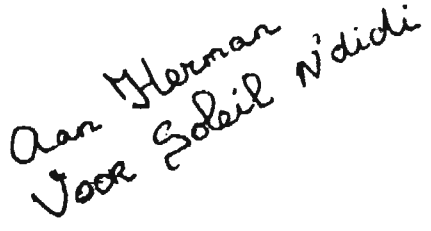

Aan Herman
Voor Soleil N'didi

CONTENTS

CONTENTS

INTRODUCTION

When the Spanish and Portuguese arrived around A.D. 1500 in what today is known as Latin America, the Caribbean area, South and Central America, and parts of North America, they found a large mongoloid population which had probably arrived 30,000 years earlier from Northeast Asia. In some countries the new invaders brought waves of violence against the native population, a very high mortality rate due to epidemic diseases, and often mixed with the native races. During the long period of colonialism, several Latin American and Caribbean countries were passed with great ease from one European power to another. It was during this period that the racial picture became further complicated by the large contribution of Africans. These were introduced to the American continent during three centuries, the West Indies being the centre of the slave trade. After the slave trade was abolished, the continent received waves of immigrants who greatly contributed to the formation of the present population. Venezuela and Argentina received important contributions of German and Italian populations; Chile of English and Italian; Brazil and Peru of Japanese, and Surinam of Javanese, Chinese, Lebanese, Dutch, and Asian Indians.

The population of Surinam (~500,000) is one of the most multi-racial populations in the world. Almost every race is represented and mixed breeds are present in all possible combinations. Almost the same number of people (~500,000) of Surinam origin live in The Netherlands. The history of the mixed population of Surinam will also be reflected in the actual picture of haemoglobinopathies in this population. The Surinam population has been the subject of several studies. The focus of these studies was primarily directed to studying the haemoglobin abnormalities in the native groups living in the interior rather than having a clear picture of the distribution of these disorders in the community at large; thus, little is known about the various haemoglobin variants and types of thalassaemia. The ethnic variability of the Surinam population will give rise to interactions of different β-thalassaemia genes which are common in certain populations (Negro, Chinese, Asian Indian, Mediterranean, and Indonesian) and with known haemoglobin variants. Identification of these interactions provides valuable information for the clinician and for the genetic counsellor because it identifies the cases in which prenatal diagnostic procedures are indicated.

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

a. To establish the types of haemoglobinopathies and thalassaemias in the Surinam population.

b. To provide data allowing the assessment of the usefulness of obligatory prenatal screening for the prevention and treatment of maternal and foetal morbidity.

CHAPTER 1

HAEMOGLOBINOPATHIES: A REVIEW

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1. HAEMOGLOBINOPATHIES: A REVIEW

1.1 Structural Variants - History. The history of the discovery of the haemoglobinopathies is related to the advances made in the basic sciences and to technical developments in the biomedical field. The same can be said for the development of treatment protocols for certain anomalies. Many of these abnormalities have only recently been described. This is probably due to the fact that in the 19th century, advances in modern medicine were made mainly in Germany, England, and France, where carriers of hereditary haemoglobin (Hb) disorders were rare, and research interest was primarily in the field of infectious diseases. The first account of an abnormal Hb in the medical literature was provided in 1910 by James B. Herrick, a Chicago physician who described a 20-year-old Black male from Grenada, West Indies, with a severe anaemia (1). Herrick described the sickle shaped cells with considerable precision and suggested that they were a manifestation of a peculiar chemical or physical condition.

Retrospective studies showed that sickle cell disease had been recognized in West Africa for several centuries (2). The disease was given specific vernacular names by different tribes, such as "chwechweechwe" (Ga tribe), "*nwiiwii*" (Faute tribe), and "*ahotutuo*" (Twi tribe). In different tribes in West Africa and in Surinam, certain tribe members were known to have the ability to forecast imminent rainfall because of sudden onset of joint pains. These tribesmen were most likely sickle cell (SS) patients. It is still customary in many of the tribes today to treat sickle cell "pain" (crisis) by applying tattoos around the painful joints involved in the crisis. Some of the traditional medicines given to SS patients by the healing doctors of the different tribes are under investigation (3). Some fractions of Fagara zanthoxyloides Lam extracts are known to reverse sickling in vitro and significantly reduce pain in SS patients (4-6). A clinical trial with a purified extract from the root of Fagara was recently performed in Nigeria with encouraging results (7).

The first practical screening test for sickling cells was done in 1917 by Victor Emmel, an anatomist at Washington University in St. Louis, Missouri, who studied the first SS patient recognized at that institution. He sealed a suspension of the patient's red cells between a microscope slide and a cover slip and noted that, with time, all the red cells assumed the peculiar sickle shape (8).

A new phase in the investigation was the study in 1949 by Linus Pauling and associates. Pauling analyzed haemolysates of SS patients by moving boundary electrophoresis and found that the Hb from a patient with sickle cell anaemia had a different mobility from that of a normal individual (9). Moreover, individuals with sickle cell trait had both Hbs in approximately equal amounts. This observation is seen as the foundation of the molecular biology field. After Pauling and his coworkers described the abnormal electrophoretic behaviour of Hb S, electrophoresis was generally adapted by researchers, and many Hbs with an abnormal electrophoretic mobility have been found in the course of the succeeding years (10-13).

The first description of a special form of Hb in newborns was given by Korber in 1866, who noticed that the Hb of adults rapidly denatured when a solution of sodium hydroxide was added, while the Hb of the newborn showed a much greater resistance to alkali denaturation (14). This investigation was extended by several investigators, and the occurrence of a special form of Hb in the foetus, newborn, and infant during the first months of life, was soon generally accepted (15-17).

The introduction of a new method, called fingerprinting, made it possible to determine the nature of the abnormality of Hb variants (18). By this means it was observed that the sixth amino acid residue from the N-terminal end of the chain, that is glutamic acid in Hb A, is substituted by valine in Hb S. The amino acid sequences in the α, β, γ, and δ chains of the normal Hbs were determined (19-21), and it became possible to compare the primary structures of these different chains. In 1968, the tertiary structure of Hb was revealed by the Nobel prizewinning X-ray crystallographer Max F. Perutz, allowing him and Hermann Lehmann to discuss the molecular pathology of Hb (22). Sophisticated high performance liquid chromatography (HPLC) equipment, in combination with automated amino acid analyzers, have been the tools of choice in analyzing new structural variants for the past decade.

The recent biotechnology era, with the use of the polymerase chain reaction (PCR) technology, and its application in related analytical procedures, is without doubt the next technological development of importance for our understanding of the haemoglobinopathies. The use of these newer techniques and some of the older ones are the basis for the research described in this thesis.

1.2 Thalassaemia - History. Like sickle cell anaemia, thalassaemia (thal) was first described in the United States, even though it is more common in Mediterranean countries (23). The reason for this is most likely the fact that the laboratory was a separate domain for the bedside-oriented European physicians, while the microscope was linked to many distinguished American clinicians in establishing their diagnoses (24). Thomas B. Cooley and Pearl Lee first described β-thal in 1925 (23). The theory that thalassaemia is a hereditary disease was first suggested in 1938 by Caminopetros (24). In 1940 Wintrobe et al (25) described the hereditary transmission of this disease in some members of a family of Italian descent living in Baltimore, and in 1956, Huisman et al (26) demonstrated that normal Hb F was present in the blood of patients with thalassaemia major. In 1957, Kulkel et al discovered Hb A_2 and showed that this Hb occurred in about twice the normal quantity in the blood of β-thal heterozygotes (27).

Pauling (28), Itano (29), and Ingram and Stretton (30) postulated the hypothesis that the abnormal expression of globin chains in thalassaemia was due to a complete or partial block of the β chain synthesis. This hypothesis was experimentally confirmed in 1965 by Weatherall et al (31) who evaluated the synthesis of Hb in vitro in reticulocytes of patients with thalassaemia using labelled amino acids. These studies showed an imbalance in the chain formation (β/α) caused by an impaired β chain synthesis. The groundwork for the in vitro Hb synthesis was laid by Dintzis in 1961, who discovered that the synthesis of Hb chains on the ribosomes in the cytoplasm was dependent upon the genetic code of the messenger RNA

(mRNA) (32). In 1964, Heywood et al (33) reported the synthesis in vitro of Hb in the reticulocytes. By adding labelled amino acids to the culture medium it became possible to study the relative rate of synthesis of the different Hb chains, a procedure that has been important in the investigation of all thalassaemia syndromes. In the early 1970's the discovery of the enzyme called reverse transcriptase in certain tumour viruses made it possible to synthesize complementary DNA (cDNA) from mRNA templates (34,35) and, because of the vast knowledge of Hb, had made it the first gene to be characterized at the molecular level.

Radiocatively labelled α- and β-globin cDNA were used as probes to demonstrate a deficiency of functional globin mRNA in thalassaemia, to demonstrate globin gene deletion in hereditary persistence of foetal Hb (HPFH), and to isolate genomic sequences responsible for the globin peptides (36,37).

In the late 1970's, the use of restriction endonucleases was introduced in the study of the thalassaemias. It was possible to obtain a picture of the physical organization of globin genes and to study the precise location and extent of some of the gene deletions that give rise to different forms of thalassaemia (38-41).

The use of PCR followed by sequencing of amplified DNA to elucidate many uncharacterized thalassaemia genes has definitely become the tool of the 1990's (42-44).

2. CLASSIFICATION OF HAEMOGLOBINOPATHIES

Haemoglobinopathies are hereditary disorders of Hb formation, which affect the function of red blood cells. The two groups of disorders with which this thesis is concerned are the Hb variants and the thalassaemias.

The chemical structure of Hb variants is abnormal and the alteration in structure may affect the rate at which Hb is synthesized in vivo or the fate of the red cells that contain abnormal pigment (45). The thalassaemias differ from the Hb variants in that no abnormal Hbs are formed (there are however a few exceptions), rather, the rate of adult Hb ($\alpha_2\beta_2$) formation is diminished, and as a consequence, various combinations of normal polypeptide chains may exist in abnormal quantities. The thalassaemias are characterized by hypochromic, microcytic red cells with an increased resistance to lysis by hypotonic solution (46).

3. NORMAL STRUCTURE OF HUMAN GLOBIN GENES

Human Hb is a tetrameric metalloprotein made up of two asymmetric dimers and four haem groups, as noted in 1930 by Anson and Mirsky (47). The iron ion associated with the haem structure normally exists in the ferrous state. The dimers of adult Hb A ($\alpha_2\beta_2$) are designated as α and β chains. The α-globin gene complex encoding the human α polypeptides (141 amino acids in length) is located on chromosome 16 (Fig. 1.1), whereas the β-like globin gene complex on chromosome 11 encodes several β-like chains. Since all the structural genes for the Hb chains are autosomal, they occur

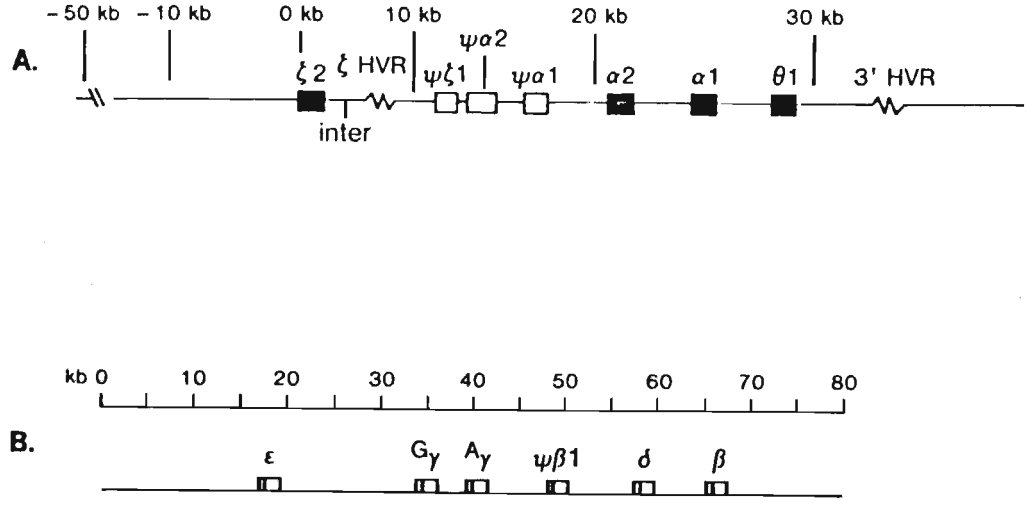

FIG. 1.1 Chromosomal Organization of the Human Globin Genes. **A** : Chromosome 16: Filled boxes represent coding genes and empty boxes the pseudo genes. **B** : Chromosome 11: Filled spaces in the boxes represent the exons and the blank spaces the IVS.

in pairs. If one of a gene pair is mutated or deleted, the individual is referred to as a heterozygote. If both genes of a pair have the same abnormality, the individual is designated as a homozygote. If both genes are affected but with different abnormalities, the individual is referred to as a compound heterozygote. The coding portion of each globin gene consists of three exons separated by two introns or intervening sequences (IVS). In the α gene, IVS-I is 117 base pairs (bp) long and separates codons 31 and 32, and IVS-II is 130 bp long and occurs between codons 30 and 31, and the IVS-II of about 850 bp divides codons 104 and 105 (Fig. 1.2). The intervening sequences are included in the precursor globin RNA transcript in the nucleus but are not present in in mature cytoplasm globin mRNA. The removal of these sequences from nuclear mRNA occurs prior to transport into the cytoplasm by a process referred to as splicing (Fig. 1.3) (i.e. introns are cut out and exons are joined together). The protein coding section of the genes are flanked on each side by untranslated sequences. At the 5' end of the gene are present promoter regions that are important for the control transcription. Also at the 5' end of the gene is found a region that is important for the correct termination of the translation. The pseudo genes, present in both the α- and β-like gene clusters, are not expressed in functional globin products, although they have great homology with the functional genes. These genes are believed to be inactivated by mutations in coding and regulatory regions through evolution.

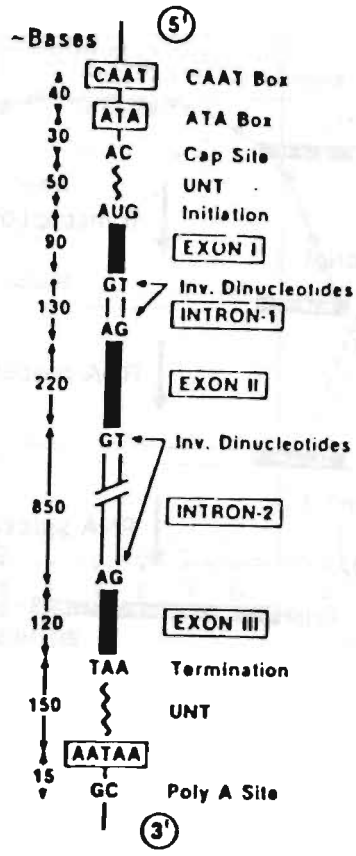

FIG. 1.2 Detailed Structure of a Globin Gene. The **CAAT** box is located approximately -72 nucleotides (nts) upstream from the Cap site. **ATA** box (Pribnow box or Goldberg-Hogness box) -26 nts upstream from the Cap site. UNT's are the untranslated regions. The **AATAA** box is located about 150 bases away from the termination codon and is the site of the polyadenylation (poly A) addition signal. The **GC** poly A site is located about 15 bases 3' from the **AATAA** box.

4. THE REGULATION OF GLOBIN GENE EXPRESSION DURING DEVELOPMENT

In early embryonic life Hb synthesis takes place in the yolk sac where ζ chains are synthesized and combine with ε or γ chains to form Hb Gower-I ($\zeta_2\varepsilon_2$) or Hb Portland-I ($\zeta_2\gamma_2$), while α and ε chains can combine to form Hb Gower-II ($\alpha_2\varepsilon_2$) (48-51). At about five weeks after conception, the switch from human embryonic to foetal Hb formation coincides with a change in the site of haematopoiesis from the yolk sac islands in the embryo to the liver in the foetus. Foetal Hb (Hb F) contains γ chains and α chains and may be designated as $\alpha_2\gamma_2$. There are two varieties of γ chains that differ in their amino acid sequence at position 136, where

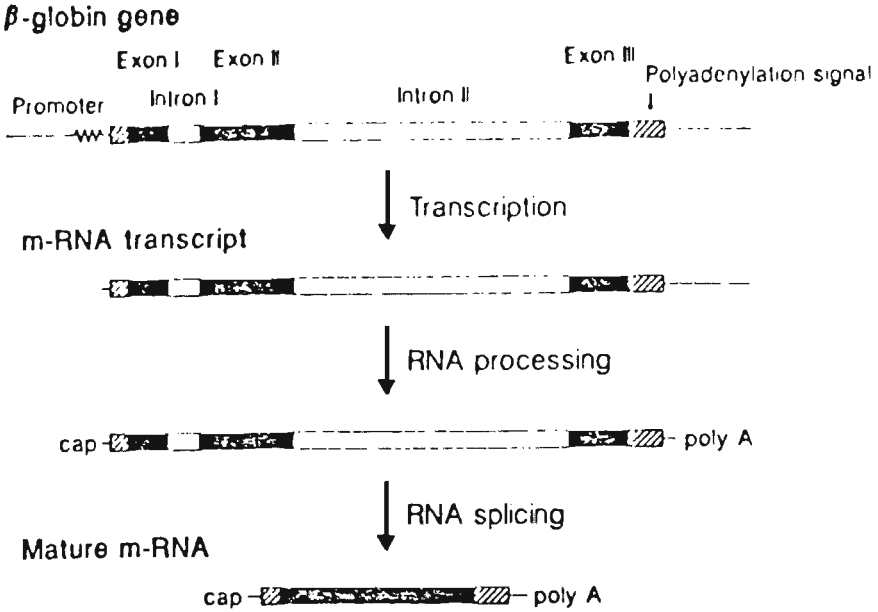

FIG. 1.3 β-Globin Structure and Expression. The hatched blocks at either end of the gene represent the 5' and 3' UNT's. The process of translation that occurs in the cytoplasm is not shown.

they have either glycine ($^{G}\gamma$ chains) or alanine ($^{A}\gamma$ chains). The relative rate of production of the $^{G}\gamma$ and $^{A}\gamma$ chains is constant throughout foetal life at a $^{G}\gamma$ to $^{A}\gamma$ ratio of approximately 3 to 1 (52). The marrow starts to produce red cells during the second trimester and becomes the major erythropoietic site during later foetal and adult life. The production of the γ chains gradually decreases during the third trimester and this is accompanied by an increase in β and δ chain production. At term birth, the ratio between the β and γ chains is about 1 to 4 (± 20% Hb A and 80% Hb F) (53). The β/δ ratio is always 40:1 (54) (Fig. 1.4). During the first few months of life the $^{G}\gamma$ to $^{A}\gamma$ ratio changes from 3:1 to 2:3 in most infants (55). Less than 1% of the total Hb in human adults cells is Hb F and is restricted to a few erythrocytes called F cells that also contain large amounts of Hb A. The relative proportion of F cells is remarkably constant in different individuals and appears to be genetically determined. Both the number of F cells and the amount of Hb F in each F cell may be increased in various acquired and genetic conditions characterized by elevated Hb F levels.

At all times, the amount of α-like and β-like globin chains are in a 1:1 ratio. The total amount of γ + β is kept relatively constant. The ζ and α chains are present early on in development. From six weeks onwards the α chains are the predominantly produced α-like chains. The α chains are encoded by two different alleles; the major α2 gene and the minor α1 gene; both produce the same α protein. The relative rate of synthesis of

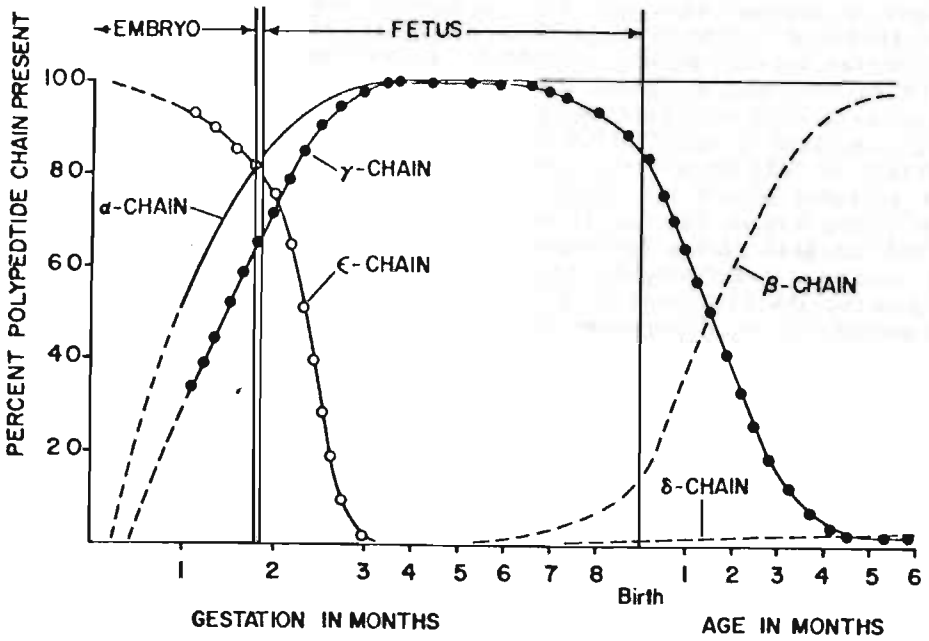

FIG. 1.4 Changes in globin chain production during the course of development.

protein by the two α genes is different. The mechanism(s) responsible for the regulation of Hb switches during foetal development is still poorly understood.

5. MECHANISM OF HUMAN GLOBIN GENE EXPRESSION

The selective expression of individual globin genes during development is regulated at the level of gene transcription by **a)** cis-acting control (regulatory) DNA sequences that are part of a globin gene or are nearly within the globin gene cluster, and **b)** trans-acting regulatory proteins with which the control sequences interact. At this level the expression of the globin genes is also regulated by the methylation stage of the genes.

The individual globin genes contain cis-acting regulatory sequences that define both tissue specific and developmentally specific patterns of expression. Cis-acting regulatory sequences include the promoter, coding sequences, enhancers, and some classes of repetitive DNA sequences.

5.1 Promoter. Promoter sequences are within 200 to 300 bp upstream or 5' to the cap site of the gene and only function in one orientation with respect to the coding portions of the gene. Sequence comparisons and functional assays have shown that promoters are composed of several functional elements or motifs, ranging from 6 to 20 nts in length. These elements

are thought to interact with specific DNA binding proteins that facilitate and regulate transcription (55,56). The most distal element of the β-globin gene is located between 90 and 105 nts 5' to the Cap site (56,57); it is designated as the CACCC or CACCCT box (58,59). The second conserved region is between 70 and 90 nts upstream from the Cap site; this has the general consensus structure 5'-GGC/TCAATCT-3' (60). This element is referred to as the CCAAT (or CAT) box and is found in the promoter region of many genes. The most proximal element is found approximately 30 bp upstream from the Cap site. This region is rich in adenine and thymine and is referred to as the ATA or TATA (Goldberg-Hogness) box. The TATA box appears to be directly involved in defining the exact transcriptional start site, presumably by binding RNA polymerase II (61). Fig. 1.5 compares the nt sequences of the promoter of the different β-like genes.

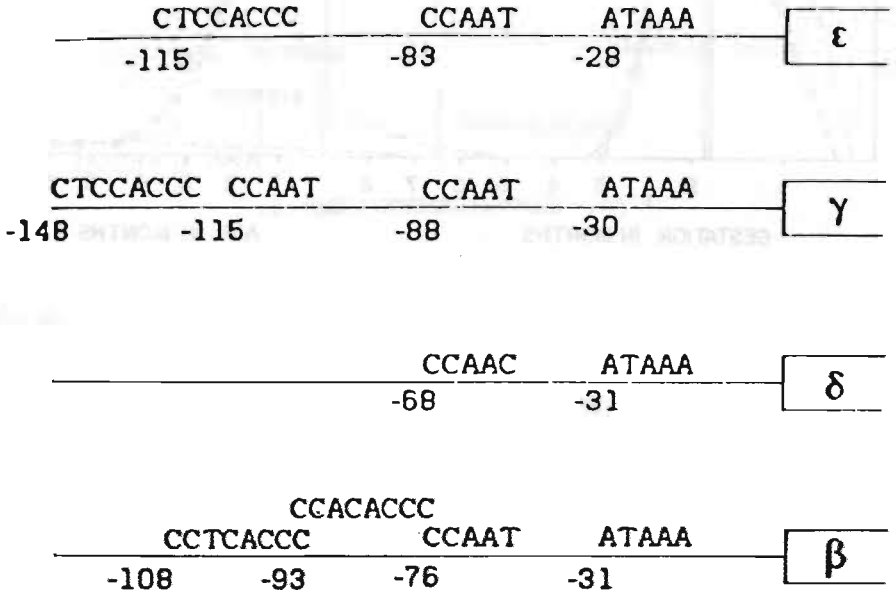

FIG. 1.5 Human β-Like Globin Gene Promoters. The conserved blocks of DNA sequence in the promoter region of several genes are shown. By convention, nts upstream from the gene are numbered as minus the number from the first transcribed nt.

5.2 Enhancers. Enhancers are segments of DNA of variable length that increase promoter function, independent of orientation and position with respect to the promoter. For example, a β-globin gene promoter is enhanced in *in vitro* experiments when the relevant DNA segment is placed on either side of the gene and in either orientation (62,63).

The immunoglobulin (Ig) heavy chain gene was the first cellular gene found to possess an active enhancer (64). A number of other cellular enhancers such as those for the Ig light chain, insulin, β-interferon, human

antithrombin III, and human placental lactogene genes, have since been identified. Cellular enhancers have been found in both the 5' and 3' flanking regions of their associated structural gene (65-69). Several functional elements analogous to the sequence "motifs" of promoters have been identified in enhancers, and they appear to interact with specific protein molecules (70-73).

The enhancer requirement of rabbit and human β-globin gene promoters is shared by δ- and γ-globin gene promoters (74-76) but the α-globin gene appears to possess enhancer activity within its coding sequences (76,77). The requirement of the β-globin gene promoter for an enhancer in an in vitro expression can be replaced by addition of the adenovirus E1a protein, a transcriptional activator that apparently interacts with the ATA box region of the β-globin gene promoter to stimulate transcription (78-80). Two points are apparent. First, the α- and β-globin genes appear to be regulated in different ways, and second, promoter activity may be achieved by different combination of linked-sequence elements and protein factors. The enhancer activity appears to be related to an increased density of RNA polymerase molecules on linked DNA (81,82).

As in the promoter, enhancer elements have been shown to bind with transcriptional factors (83). The implication for the role of transacting factors is that cooperative binding of factors may be required for the mechanism of activation. Proteins (factors) that bind specifically to enhancer elements have been identified by DNA footprinting and gel retardation assays, and in some cases these proteins have been extensively purified (84-87).

The types of regulation observed with cellular enhancers indicate that enhancers can be divided into two categories: those that respond to changes in the environment (inducible enhancers), and those that are active only at specific times during development or only in specific tissues (temporal and tissue specific enhancers) (88).

Numerous models have been proposed to account for the properties of enhancers (89). In one model, enhancers are assumed to act as entry sites for transcription factors that move to the promoter, where they have a direct effect on the initiation of transcription. This movement could occur in at least two distinct ways. The intervening DNA could loop out to bring the enhancer and associated factors into direct contact with the promoter (the looping model). Evidence supporting the loop model was obtained by examining the dependence of enhancer activity on the distance between the enhancer and the promoter (90). Rather than serving as a binding site for a factor involved directly in transcription, an enhancer could act to reorganize chromatin into a transcriptionally active conformation (the chromatin structure model). The enhancer is known to change the pattern of DNase hypersensitivity in linked DNA (1). A third possibility is that enhancers serve to modulate the superhelicity of linked DNA by providing sites for the action of topoisomerases, enzymes that can change the degree of supercoiling of DNA for transcription of a gene. In one version it was postulated that an eukaryotic type II topoisomerase (that normally will only relax DNA) gains a supercoiling activity when bound to a specific site, in this case the enhancer (92). Finally, enhancers could act by targeting the template to a particular location in the nucleus where transcription occurs (the nuclear address model) (93-94).

5.3 Methylation of Globin Genes During Development. The globin genes are hypomethylated when expressed, and they are more completely methylated during developmental periods when they are not expressed (95,96). Fig. 1.6 shows a diagram representing the relative methylation frequency of C p G residues within the cluster during various developmental stages. At all stages of ontogeny the β-like globin genes show a strong correlation between their methylation state and expression. In the case of the human globin genes, the most remarkable association between hypomethylation and gene activity can be found in the 5' flanking regions of the foetal γ-globin genes. The 5' flanking DNA's of both the $^{G}\gamma$- and $^{A}\gamma$-globin genes contain a **CCGG** sequence at position -54 from the Cap site, about midway between the **CCAAT** and **ATA** boxes. Both sites are hypomethylated in foetal liver erythroid cells that have active γ-globin genes but are fully methylated in erythroid cells of adult bone marrow where γ-globin genes are not expressed to any significant degree. Methylation in the 5' flanking region of DNA (residues -760 to +100) but not in the remainder of the γ-globin gene, prevented its expression in transfected tissue culture cells (97). Although DNA hypomethylation in 5' flanking DNA may be a prerequisite for gene expression, it is probably not the primary event responsible for the activation of the globin genes because there are many situations in which globin genes or other genes are hypomethylated but not expressed. Other factors must be responsible for triggering gene expression once the gene is in a state capable of being expressed.

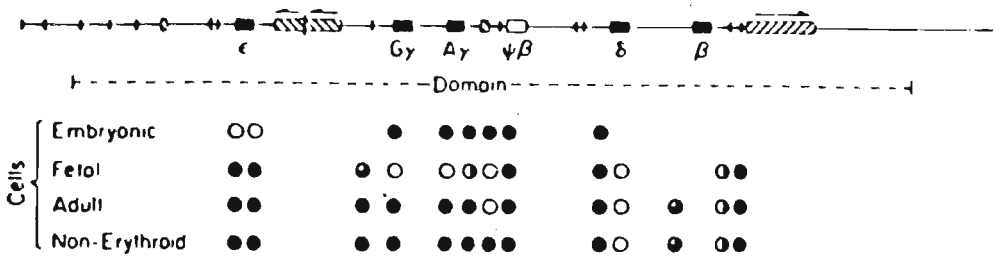

FIG. 1.6 Methylation Patterns of the Human β Locus in Various Stages of Ontogeny. Open circles show an unmethylated site, filled circles a totally methylated site, and partially filled circles show the degree of site methylation. Reproduced from The Molecular Basis of Blood Diseases, by G. Stamatoyannopoulos et al, W.B. Saunders Company, 1987, with permission.

5.4 DNase I Hypersensitive (HS) Sites. DNase I HS sites have been found 5' to the $^{G}\gamma$-, $^{A}\gamma$-, δ-, and β-globin genes in foetal liver haematopoietic tissue, but only 5' to the δ - and β-globin genes in adult haematopoietic tissue. These changes are presumably due to alterations in chromatin structure, both around the cluster reflecting its potential expression in erythroid cells, and within a cluster as each gene is activated at different times during development (98).

Several authors' reports have identified a region important for erythroid specific expression of the β-globin gene complex (99,100). These include a set of four DNase I HS sites at -18, -14.7, -10.9, and -6.1 kb 5' to the ε gene and a 3' HS site 21.9 kb 3' to the β gene (Fig. 1.7). The significance of these sites to globin gene expression has been demonstrated by Grosveld et al (101), who achieved high levels of position independent β gene expression in transgenic mice by constructing a β-globin minilocus in which the most 5' and 3' HS sequences flank a β-globin gene. These HS sites are erythroid-tissue specific, developmentally stable, and have been termed locus-activating regions (LAR) or locus controlling regions (LCR) to imply the essential nature of their presence to pois chromatin structure of the β-globin locus for developmentally regulated transcription of the globin genes (102). Three large deletions that remove variable amounts of the upstream portion of the β-globin gene cluster provided additional evidence for the potential importance of this DNA region in gene regulation. The part of the β-globin gene cluster removed by each deletion is indicated in Fig. 1.8. These deletions, which have been identified only in the heterozygous state, are characterized by a β-thal minor phenotype but with a normal Hb A_2 level (103-105). The interesting feature of these three deletions is that in each case an intact β-globin gene is present but not expressed. In the Dutch and English cases, the β-globin gene on the chromosome bearing the deletion is normal in expression studies in cultured cells and by sequence analysis (106,107). The Hispanic deletion occurs on a chromosome bearing a β^S gene that is not expressed. The 3' endpoint of this deletion has not been precisely defined but falls within the region of the upstream major HS sites (105). Thus, it appears possible that the inactivation of these intact β-globin genes might be the result of a deletion of upstream sequences essential for expression.

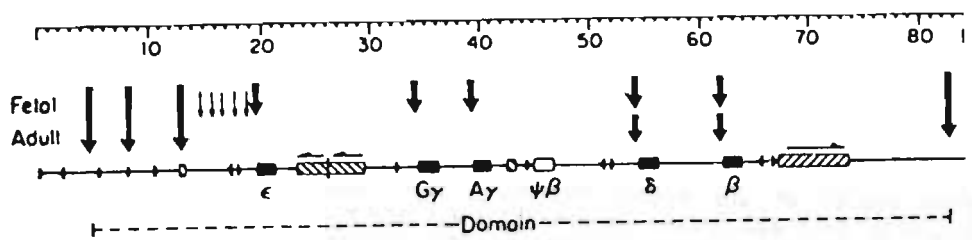

FIG. 1.7 Chromatin Structure of the Human β Locus. The arrows show the location of the DNase I HS sites. The γ-globin gene HS sites are present only in the foetus, while β and δ gene sites are present in adult as well as foetal erythroblasts. The major HS sites marking the boundaries of the β locus domain are present throughout human development.

5.5 Repetitive Sequences, KpnI and AluI. Several members of the two best characterized human repetitive DNA sequence families, the Alu and Kpn repeats, have been identified in the β-globin gene cluster (108-110). The

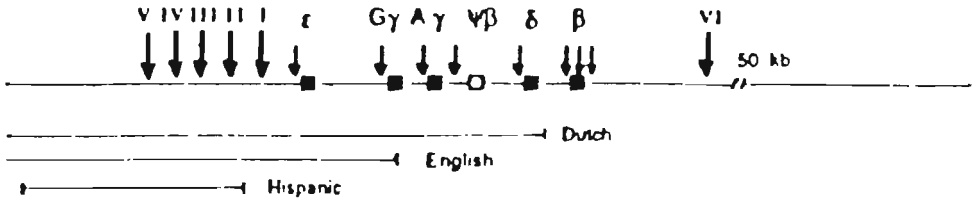

FIG. 1.8 The portion of the β-globin gene cluster deleted in three cases of δβ-thal are indicated by the lines.

Alu sequences are repetitive DNA sequences approximately 300 nts in length that are widely interspersed through all chromosomes of human DNA. The Alu family derives its name from a common AluI enzyme cleavage site found in 60% of its copies. Alu elements are thought to have been dispersed via an RNA intermediate that is converted into DNA by action of a reverse transcriptase prime off the poly A track (111).

There are eight members of the Alu repetitive sequence family in the β-globin gene cluster (48). Alu repeats appear upstream from the γ-globin genes and upstream from the ψβ gene. Inverted pairs of Alu sequences appear upstream from ε-, upstream from δ-, and downstream from the β-globin gene. Interestingly, the three inverted pairs in the β cluster are all arranged tail to tail, with about 800 bp of non-repetitive DNA between them. The Alu sequences that have attracted most interest are the inverted pair located 3 kb upstream from the δ gene because of the hypothesis that these had a role in regulation of the γ to β switch (see Section 8.2). The second major class of repeat sequence is known as the Kpn (or L1) family because of KpnI restriction endonuclease cleavage sites located at analogous positions in many copies of the sequences (68). Two copies lie downstream from the β-globin gene and another between the ε- and γ-globin genes (48,112). All three KpnI regions have been sequenced. The inter ε-γ Alu repeat that is over 6 kb in length has a strong homology with the retrovirus long-terminal repeat at the end near the γ-globin gene (113). Although RNA homologous to the human Kpn family is present in many human cells, its function and the nature of any protein that it might encode has not yet been established.

6. HAEMOGLOBIN VARIANTS

During the past 30 to 35 years the determination of the detailed chemical structures of the normal Hbs paralleled the discovery of hundreds of Hb variants. The classification of Hb variants presented here is based upon the gene anomalies; we thus distinguish γ, δ, and β variants of the β-globin gene cluster, and α variants of the α-globin gene cluster. These variants can be subdivided into different groups of abnormalities, such

as the unstable Hbs that often cause a mild-to-severe congenital haemolytic anaemia, variants with an increased affinity for oxygen, variants with a decreased affinity for oxygen, variants with special properties such as Hb S, and variants without apparent change in functional and physicochemical properties. The severity of these disorders varies, and while some cause severe symptoms, others are associated with only mild symptoms or none at all.

The majority of abnormal Hbs are found in the heterozygous state, although several have been found in homozygotes. Precise diagnosis of each abnormal state is important for the management of the individual case. Only those variants that have been reported in the Surinam population are considered here.

6.1 β Chain Variants. At present there are nearly 300 known β chain variants; the great majority (95%) are single amino acid substitutions in the β polypeptide chain. A few variants have amino acid replacements at two different sites on the same subunit (114). For example, Hb C-Harlem has a substitution as in Hb S [β6(A3)Glu→Val], and a substitution at position 73 [β73(E17)Asp→Asn] similar to Hb Korle-Bu. These variants arose by one of two mechanisms: new mutation on a variant gene or crossover between two variant genes. Other types of β variants have deletions or insertions. The quantity of β chain variants in heterozygote carriers usually varies between 25 to 45%. This range can be attributed to a number of independent factors. The mutant mRNA could be transcribed, processed or transported less efficiently, or could be unstable or degraded rapidly (115). In the case of Hb E [β26(B8)Glu→Lys] and Hb Knossos [β27(B9)Ala→Ser] the mutation causes a defect in mRNA processing (see Section 7.3a) leading to thalassaemic phenotypes. The vast majority of β gene variants are synthesized at a normal rate. The reduced amount of abnormal Hb in some cases appears to be due to increased catabolism of the variant Hb owing to abnormal subunit interactions, impaired haem binding, and decreased solubility.

In vitro experiments have shown that chain competition between normal β chains (β^A) and variant β chains (β^X) for β chains is determined in part by the surface charge of the subunits (116). Variants such as Hb E, with positively charged β chains, assemble less readily than those with negatively charged β chains such as Hb J-Baltimore (116a).

The presence of a concurrent α-thal further reduces the amount of $\alpha_2\beta^X_2$ in the red cells of the positively charged β variants (117,118). In the case of Hb J-Baltimore the amount of β^X increased when α-thal is present. There is no Hb A production in individuals with a compound heterozygosity for an abnormal β chain and a β^0-thal. This condition is, therefore, almost indistinguishable from the homozygous β^X/β^X condition, except from hypochromia, elevated Hb A_2 and high Hb F levels. Family studies and DNA analysis are often needed to establish a proper diagnosis in these cases. Hb S, Hb C, and Hb E are the most common β chain variants to occur in the Surinam population and they will be discussed further below.

6.1a Pathophysiology of the SS Disorder. Hb S is the result of a single mutation of one nucleic acid within codon 6 (GAG→GTG); it results in the replacement of a valine for a glutamic acid residue (54). Hb S ($\alpha_2\beta^S_2$) functions normally in the oxygenated state, but polymerizes in the

deoxygenated state. This polymerization of Hb S causes the red cell to be sickle-shaped. Sickling can be reversed by reoxygenation (119). The process of sickling and unsickling will ultimately lead to damage of the red cell membrane, preventing the red cell from returning to a normal shape after sickling. Such irreversible sickled cells (ISCs) are prematurely destroyed by the reticulo-endothelial system, leading to chronic haemolysis (119). The impaired red cells may become lodged in the microcirculation and initiate a sickle cell crisis due to subsequent vaso-occlusion (119). Fig. 1.9 lists the various clinical aspects of sickle cell anaemia. The vaso-occlusive crises or painful crises are characteristic of sickle cell disease. The sickling process is temperature-, pH-, and concentration-dependent. Sickling will occur when a critical deoxyHb S concentration has been reached in the cell, or when a low pH or a high temperature is present. Sickling is prevented by conditions that lower the intracellular Hb S concentration levels in the cell, e.g. extracellular hypo-osmolality, iron deficiency, and the coinheritance of an α-thal. Numerous other factors are involved in initiating sickling, such as cell age, osmotic pressure, sluggish flow, vascular damage, structure of the microcirculation, infection, pregnancy, and hypotension (119-122). X-ray analysis has shown the importance of the β121 Glu position in the crystallized Hb S polymer. The simultaneous presence of Hb D-Punjab [β121(GH4)Glu$\rightarrow$Gln] and Hb O-Arab [β121 (GH4)Glu$\rightarrow$Lys] promotes the sickling tendency, emphasizing the importance of the β121 Glu interaction in the sickling process (123,124).

The sickling process is inhibited by the presence of Hb D due to the formation of an asymmetrical hybrid $\alpha_2\beta^S\gamma$ (125,126). This result indicates that SS patients with considerable amounts of Hb F are less prone to sickling crises. Indeed, this has been confirmed for carriers of the β^S gene with a specific Saudi Arabian haplotype in which the level of Hb F is approximately 30% (127). The cause of the increased Hb F production is not known but a deletional HPFH is excluded in these cases. Based upon the above observations, reactivation of the developmentally repressed γ-globin genes has been approached as a therapeutic goal for treatment of patients with severe sickle cell anaemia. Drugs such as 5-azacytidine, hydroxyurea, and erythropoietin that increase Hb F synthesis in the red cells, are currently under investigation (128,129).

6.1b Sickle Cell Trait (AS). Sickle cell trait appears to be a benign condition. However, there are some reports indicating that some complications can occur (130). Splenic infarction during high altitude flight in non-pressurized aircraft has been reported; haematuria without any other cause occurs occasionally and is a more frequent finding than in normal (AA) subjects. Such haematuria is due to a set of local circumstances within the kidney that predisposes to *in vivo* sickling. Barbotin and Duclos showed that 25% of their hospital patients with AS in Dakar (Senegal) had recurrent symptoms, including abdominal pains, pains in the distal joints, and froto-parietal headaches (131).

6.1c Geographical Origin of Hb S. According to older theories, the β^S gene arose on the Arabic peninsula, from where it spread by trade connections to Africa, the Mediterranean, and India. The slave trade introduced Hb S to the Americas. Migration waves from the former colonies introduced the gene to Western Europe.

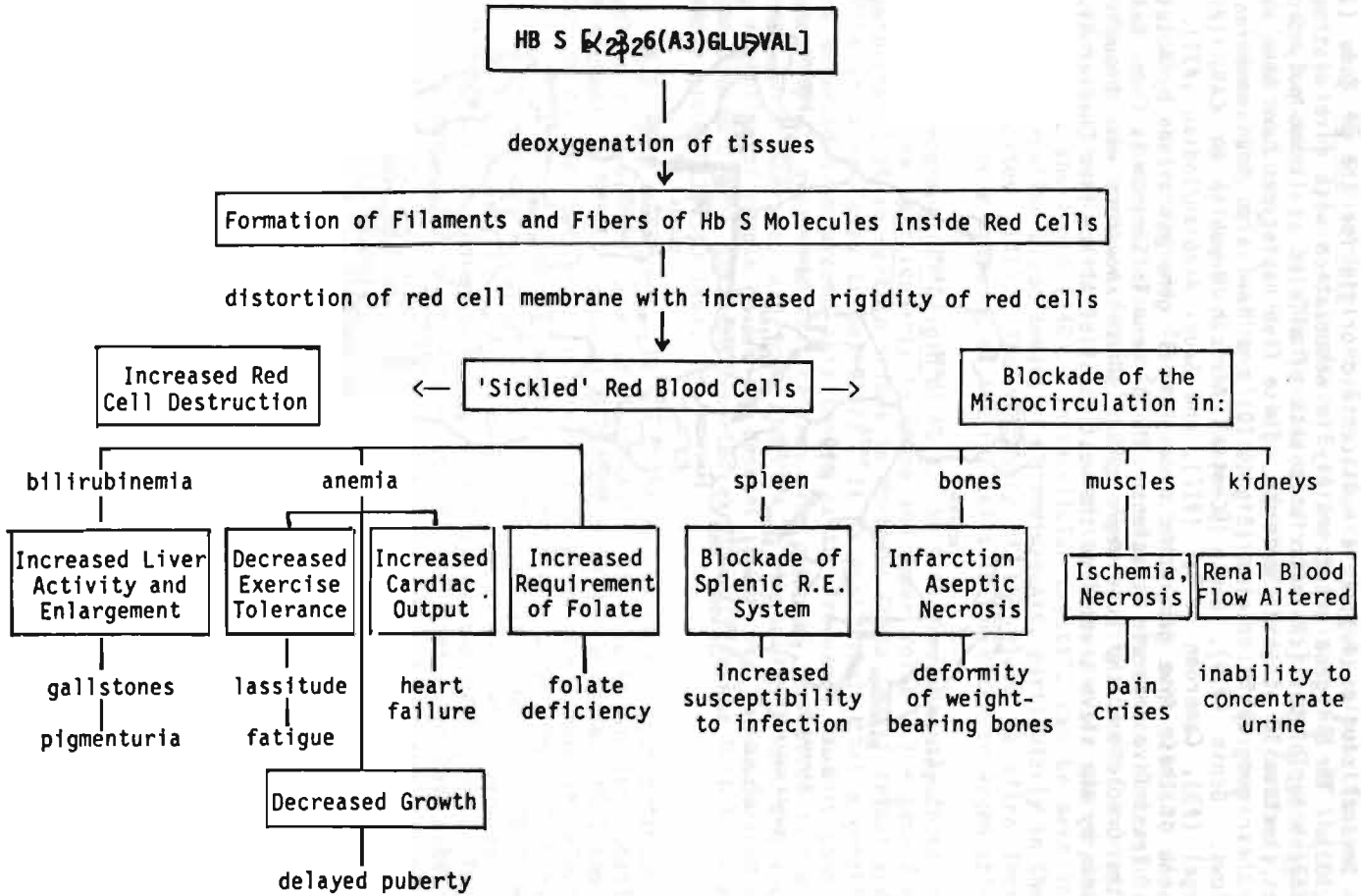

FIG. 1.9 Scheme illustrating the various clinical aspects of sickle cell anaemia.

Several studies suggest a multicentric origin for the β^S gene (127, 132,133). The β^S gene occurs mainly in association with five distinctly different haplotypes [characteristic sets of alleles at linked polymorphic sites (section 10 of this chapter)]. These five haplotypes have been named for their geographic origins (Fig. 1.10) and have also been numbered as follows: Benin (#19), Bantu (Central African Republic or CAR) (#20), Senegal (#3), Cameroon or Ant (#17), and Saudi Arabian/Indian (#31). In any one of these five geographic areas, the β^S gene has arisen by mutation on at least five separate occasions. The β^S gene in Surinam is found mainly in the Creole and the Bush Negro group, whose ancestors were brought to Surinam by the slave trade from the west coast of Africa (see Chapter 4).

FIG. 1.10 The geographical origin of β^S chromosomes with distinct haplotypes in Africa. Haplotype #31 is found exclusively in Saudi Arabia and India.

Occasionally, Hb S is observed among Hindus in Surinam; the haplotype of these cases, however, has not been determined (134). No Hb S was found in a previous study among the Trio and Wayana natives living in southern Surinam (135). Isolated cases of Hb S among natives living in the Surinam Savannah belt has been reported but were ascribed to admixtures with Negroes (136).

6.2 Hb C Disorders. Hb C is characterized by a substitution of glutamic acid by lysine at position 6 of the β chain. This is the result of a G→A mutation at the first position of codon 6 of the β gene (GAG→AAG). The substitution gives Hb C a high positive charge. Accordingly, it has a slow mobility in alkaline electrophoresis. In IEF it moves at the same position as Hb A_2 and Hb E (see Chapter 2).

Hb C heterozygosity is not associated with any clinical disability (137,138). Stained films of AC blood show an increased number of target cells and an increased density (139). Homozygosity for Hb C causes a mild haemolytic anaemia (Hb 8-10 g/dl), and the life-span of the erythrocytes is somewhat shortened (140). Intracellular crystals can be seen in small numbers in routine blood smears of CC individuals, particularly in the dense microspherocytes (141). The viscosity of the blood is often increased, which may cause a trapping of the red cells in the often enlarged spleen.

6.2a Geographical Origin of Hb C. Hb C is predominantly found in areas of West Africa, particularly in Ghana and Upper Volta. The gene frequency approaches 0.15 in certain tribes in Northern Ghana (Mossi tribe) and Upper Volta (Boukina Fasso tribe) (142). It is most likely that a genetic flow has occurred from around this centre as the frequency of this gene diminishes rapidly away from this centre. The gene was introduced in the Caribbean, North America, and South America by the slave trade, and recently, by migration of people of African descent to Europe. In the Surinam population the Hb C gene has been reported exclusively in the Bush Negro and the Creole group (134,143-145).

6.3 Hb E Disorders. Hb E is the result of a single mutation of one nucleic acid within codon 26 (GAG→AAG). It results in the substitution of glutamic acid by lysine at position 26 of the β chain. Hb E comigrates with Hb A_2 and Hb C on cellulose acetate at pH 8.6. However, electrophoresis in citrate agar at pH 6.0 can separate these Hbs. Hb E migrates toward the cathode with a mobility similar to that of Hb A and Hb D, whereas Hb C moves toward the anode. Hb E is synthesized at a reduced rate in reticulocytes due to a decreased stability of the mRNA (146). Thus, the Hb E structural gene may be viewed as a β^+-thal gene. The base substitution at codon 26 creates a new splicing sequence that causes abnormal mRNA processing as well as instability of the mRNA during erythroid maturation. Hb E heterozygotes are asymptomatic; the Hb levels are normal but the red cells are microcytic.

6.3a Geographical Origin of Hb E. Hb E is primarily found in Southeast Asia. However, it has been reported in Blacks (147,148) and Caucasians (134), and there is convincing evidence that the β^E gene, like the β^S gene, has arisen independently on a number of occasions (149). Because of the immigration of people from Southeast Asia to different parts of the world, it is encountered quite commonly in Surinam, the United States, and Europe. In Surinam, Hb E is primarily found in the Javanese population.

6.4 Hb A_2 Variants. At present, the number of discovered δ chain variants is only 20; their substitutions are listed in the 1991 issue of the International Hemoglobin Information Center variant list (114). This number is relatively small because of difficulties in identifying amino acid substitutions in a protein that occurs in low quantities (1 to 1.5%)

in heterozygotes, and in slightly higher quantities (2 to 3%) in heterozygotes who have a concomitant β-thal trait. The most common Hb A_2 variant in Surinam is Hb A_2' or Hb B_2 [$\alpha_2\delta_2$16(A13)Gly→Arg] and found among Creoles and Bush Negroes (134,150,151). Hb B_2 results from a single mutation at codon 16 (GGC→CGC), a substitution of glycine for arginine at position 16 of the δ chain. This variant can be identified by routine Hb electrophoresis. Both Hb B_2 heterozygotes and homozygotes are asymptomatic. With the development of amplification procedures of segments of DNA (144) and the availability of simple DNA sequence analysis (152), a new approach has become available for the identification of these variants using only small quantities of blood (160) and that is as reliable as classic amino acid sequencing, but less labour-intensive and, therefore, should be useful in detecting new δ chain variants.

6.5 α Chain Variants. At present there are 161 α-globin structural variants that have been described (114). These variants result primarily from single amino acid substitutions. The mutation of one of the four α genes will result in about 5-25% of the variant Hb X ($\alpha^X_2\beta_2$) in the heterozygote (154,155). The relative level of abnormal α-globin genes varies according to the degree in which the affected gene normally contributes to α-globin synthesis (155,156). As mentioned earlier in this chapter, the α2 gene encodes more protein than the α1 gene, due to a 2.6 to 1 ratio of α2:α1 mRNA levels (157). Structural mutants at the α2 locus can be expressed at higher levels due to the dominant role of the α2 gene in the α-globin expression (155). The presence of a concurrent α-thal increases the amount of $\alpha^X_2\beta_2$ in the red cell (30-35%), while in individuals with an unstable α chain variant, there is a reduction of a variant (5-10%). Several α chain variants are located on the α-thal-2 chromosomes ($-\alpha^{3.7}$ kb or $-\alpha^{4.2}$ kb). The most common is Hb G-Philadelphia [α68(E17)Asn→Lys] occurring primarily in Black populations (158,160) that is encoded at a $-\alpha^{3.7}$ locus. The G-Philadelphia mutation can also occur on a normal chromosome (αα) and is then expressed at 20-25% (161,162).

Individuals who carry an α-thal-1 (--) gene *in trans* to an unstable α chain variant ($--/-\alpha^X$) have a severe form of α-thal (Hb H disease; see Section 7.1a), that results in moderate to severe anaemia (162,163).

Hb J-Meerut is the only α chain variant reported so far in the Surinam population (134).

6.6 γ Chain Variants. γ Chain variants are the result of substitutions in the $^G\gamma$ or $^A\gamma$ chains. Sixty-two γ chain variants have been described so far (114). The relatively small number of γ chain variants is due in part to the fact that Hb F is barely detectable after the first six months of life. In addition, large scale surveys of cord blood samples have only been initiated during the past few years. No functional significance has been ascribed to any of the γ chain variants that have been reported with the exception of Hb F-Poole [$\alpha_2{}^G\gamma_2$130(H8)Trp→Gly], an unstable fetal Hb causing haemolytic anaemia in the neonatal period (164), and Hb F-M-Osaka [$\alpha_2{}^G\gamma_2$63(E7)His→Tyr], that causes neonatal cyanosis (165). Hb F-Sardinia is encountered in many populations (166-168). In this $^A\gamma$ chain variant, the isoleucyl amino acid is substituted by threonine at position 75. Carriers of this variant are clinically asymptomatic.

7. THE THALASSAEMIAS

The thalassaemias constitute a heterogenous group of inherited disorders, characterized by abnormal gene function resulting in the total absence or quantitative reduction of α- or β-globin chain synthesis in human erythroid cells. Thalassaemias are classified according to the type of chains affected, e.g. ζ-, α-, β-, δ-, $\delta\beta$-, γ-, $\gamma\delta\beta$-, or $\varepsilon\gamma\delta\beta$-thal. The imbalance of globin chain synthesis that is characteristic of thalassaemia results in an excess of α or β chains. The excess chains tend to precipitate in the red cell precursors leading to premature removal of surviving red cells by the spleen.

7.1 α-Thal. The α-thalassaemias are probably the most prevalent and most widely distributed disorders of Hb synthesis. In the foetus, a reduced synthesis in the α chains results in an excess production of γ chains. These excess γ chains will form Hb Bart's (γ_4) that is easily detectable by electrophoresis. Severely affected individuals might be identified during adulthood by the production of Hb H (β_4) that readily forms inclusion bodies in the red cells. Several authors have shown a reasonably good correlation between the level of Hb Bart's, the red cell morphology and indices, and the existence of α-thal conditions in the neonatal period (169-171).

7.1a Classification of the α-Thalassaemias. The α-thalassaemias are caused by deletion (the deletional types) or mutations (nondeletional types) of (or in) one to four of the α genes (170). The resulting disorders involve various degrees of imbalance between α and β chain synthesis, and patients can be classified using haematological, biochemical, and molecular criteria.

Fig. 1.11 shows the α-like globin gene cluster. The $\alpha 2 \alpha 1$ and ζ genes are the functional α-globin genes. As shown in Fig. 1.11 the two α-globin genes are embedded in a large region of homology that is divided by short divergent regions into three homology subsegments: X, Y, and Z (172). Non-homologous crossover between these homologous segments are responsible for the most common forms of α-thal. The crossover can occur between the two X regions or the two Z regions, resulting in the so-called "leftward" α-thal-2 genotype ($-\alpha^{4.2}$) and the "rightward" α-thal-2 genotype ($-\alpha^{3.7}$). The -3.7 kb deletion can be further subdivided into types I, II, and III, based on the exact position of the crossover within the Z box (173,174). The -3.7 kb deletion is found in Mediterranean populations and is the principal α-thal allele in Black and Asian populations. The -4.2 kb deletion has been observed in Asian populations. Fig. 1.12 shows that these crossover events can create triplications on the opposite chromosome; the anti-3.7 kb triplication ($\alpha\alpha\alpha^{+3.7}$) and the anti-4.2 kb triplication ($\alpha\alpha\alpha^{+4.2}$). Although there is a mild excess of α-mRNA, the α and β chain synthesis is essentially balanced, and individuals with a total of five α genes are clinically and haematologically normal (175,176).

In addition to the frequent $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, several deletions have been described involving both α-globin genes, thus leading to the α-thal-1 condition (--) (177-179). These deletions range in size from rather small (5.2 kb) to those that remove the entire cluster (summarized in Fig. 1.11). The two most common deletions ($--^{SEA}$) and ($--^{MED}$), occur

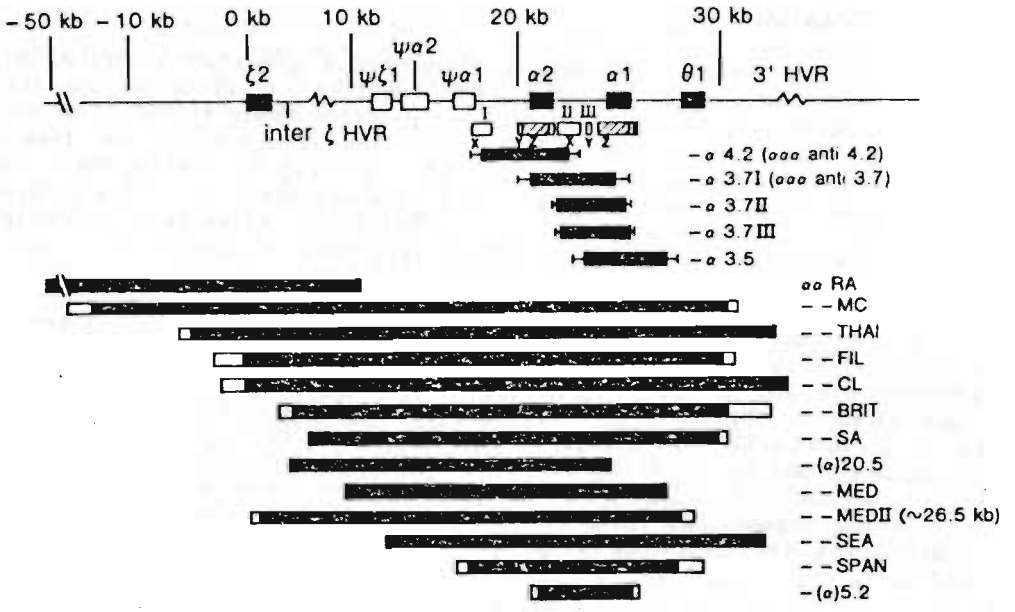

FIG. 1.11 A summary of major deletions in the α-globin gene cluster that result in the loss of expression of both α-globin genes (kindly provided by Dr. D.R. Higgs, Oxford, England). Deletions which remove both α-globin genes are denoted as -, those in which the deletion leaves part of the α-globin gene intact is denoted as -(α) [(-α)20.5 and (-α)5.2], and those in which both α-globin genes remain fully intact (although their expression is either severely reduced or abolished) are denoted as αα ($\alpha\alpha^{RA}$).

in the Southeast Asian and the Mediterranean populations, respectively. Of special interest is the $\alpha\alpha^{RA}$ deletion in which the two α-globin genes are intact but the ζ and ψζ genes are removed (179). This deletion blocks α-globin gene expression in cis to the deletion, and study of this deletion is very helpful in identifying regions critical for α-globin gene expression as is shown for similar deletions within the β-globin gene cluster (106). α-Thal-1 trait closely resembles the α-thal-2 homozygosity, in which one of the α-globin genes on each chromosome is deleted, -α/-α or --/αα or α-/α-. Individuals with either one of these conditions have a mild anaemia with microcytosis (180,181).

A more severe form of α-thal is Hb H disease in which there is only one functional α-globin gene. This syndrome occurs most commonly in Southeast Asia and the Mediterranean basin. The loss of normal α2 gene function in trans to the α-thal-1 gene appears to give a clinically more severe type of Hb H disease than that of the α1 gene, emphasizing the greater influence of a thalassaemic mutation on the α2 locus. Hb H disease leads to severe

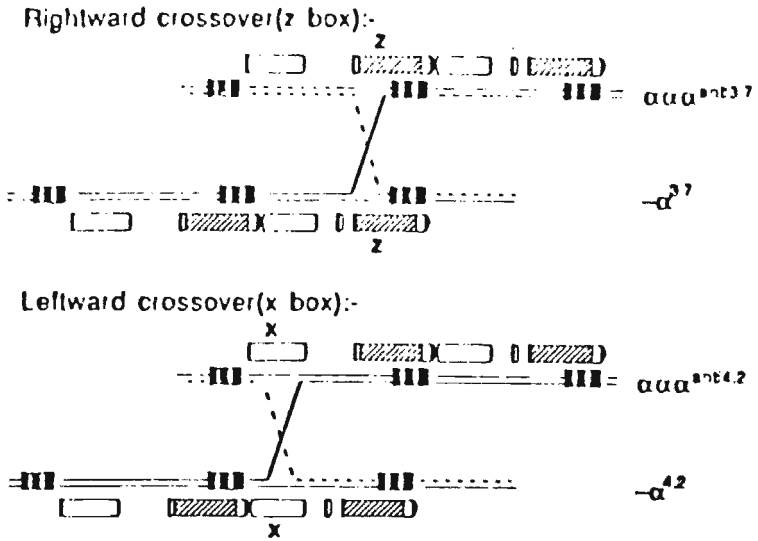

FIG. 1.12 Crossover events between the Z boxes resulting in the rightward $-\alpha^{3.7}$ or triplicated $\alpha\alpha\alpha^{anti\ 3.7}$, and between the X boxes leading to the leftward $-\alpha^{4.2}$ or triplicated $\alpha\alpha\alpha^{anti\ 4.2}$ deletions.

α chain deficiency, that in turn results in excess β chain accumulation in the form of the tetrameric Hb H. Patients with Hb H disease have a mild to moderate with sometimes severe haemolytic anaemia (187,188).

The most severe form of α-thal is the α-thal-1 homozygosity or Hb Bart's hydrops foetalis with no functional α-globin genes, --/-- (186). This condition is not compatible with life; all foetuses are hydropic stillborn or die shortly after birth (194-196).

Several forms of nondeletional α-thal have been identified (listed in Table 1.1). Such nondeletional α-thal alleles have been observed in Asian, Mediterranean, Black, and Saudi Arabian populations (189). These nondeletional mutations are found in both normal α-globin genes and in the frequently occurring $-\alpha^{3.7}$ hybrid gene. Unexpectedly, the nondeletional α-thal mutations are asymmetrically distributed between the two α-globin loci, compared with the more symmetrical distribution of the α variants. This unequal distribution is most likely due to a bias occurring because of the dominant role of the $\alpha 2$ gene in α-globin expression. Individuals with $\alpha 2$ mutations would, therefore, be more readily recognized and studied.

7.2 The β-Thalassaemias. β-Thal is an extremely heterogeneous group of disorders of Hb synthesis, and is due to a decrease or absence of β-globin chain synthesis (47). It has been divided into two types: β^0- and β^+-thal. In homozygous β^0-thal, the β chains are present for some 5-30% of normal levels. There is a proportional deficiency of β-mRNA in erythroid cells of affected individuals (47). More than 100 different β-thal genes have been identified (190,191). The heterozygous carriers can be identified by an elevated Hb A_2 level (range 3.5-8%), marked microcytosis (MCV <70 fl),

TABLE 1.1 Nondeletional α-Thal Mutations

Locus	Affected Sequence	Mutations	Geographical Origin
RNA Processing			
$\alpha 2$	IVS-I donor site	GAGGTGAGG→GAGG	Mediterranean
$\alpha 2$	Poly A signal	AATAAA→AATAAG	Middle Eastern; Mediterranean
RNA Translation			
$\alpha 2$	Initiation codon	CCACCATGG→CCACCACGG	Mediterranean
$\alpha 1$	Initiation codon	CCACCATGG→CCACCGTGG	Mediterranean
$-\alpha^{3.7}$	Initiation codon	CCACATGG→CCACCGTGG	Black
$-\alpha^{3.7}$	Initiation codon	CCACCATGG→CC-CATGG	North African; Mediterranean
$\alpha 2$	Exon 3	α116 GAC→UAG	Black
$\alpha 2$	Termination codon	α142 TAA→CAA	Southeast Asian
$\alpha 2$	Termination codon	α142 TAA→AAA	Mediterranean
$\alpha 2$	Termination codon	α142 TAA→TCA	Indian
$\alpha 2$	Termination codon	α142 TAA→GAA	Black
$-\alpha^{3.7}$	Exon 1	α30/31 GAGAGG→GAG-G	Black
Unstable Protein			
$\alpha 2$	Exon 3	α 125 Leu→Pro	Southeast Asian
$\alpha 2$	Exon 3	α 109 Leu→Arg	Southeast Asian
α	Exon 3	α 110 Ala→Asp	Middle Eastern
$-\alpha^{3.7}$	Exon 1	α 14 Trp→Arg	Black

hypochromia (MCH <25 pg), and slight anaemia (192). In about half of these cases Hb F is slightly increased (1-3%). Most individuals with homozygous β-thal manifest a chronic severe microcytic haemolytic anaemia and hepatosplenomegaly, and may have a characteristic facies due to bony distortion from expansion of the marrow. Treatment is mainly by repeated blood transfusions that relieves the chronic anaemia and prevents bony changes, combined with iron chelation therapy to remove the excess iron from the circulation. Bone marrow transplantation, of which about 1000 have been performed, is a new method that has recently been approached for therapeutic purposes (193-197).

At the molecular level, β-thal is due to deletions of part or all of the β-globin gene (deletional types), or more often to point mutations that include single base changes, and deletions and insertions of one to four nts (nondeletional types). These mutations can affect transcription of the β-globin gene, or affect the processing and translation of the β-globin mRNA.

7.3 Classification of β-Thal.

7.3a Nondeletional Types; mRNA Processing Mutants. The globin system appears to provide a good example for the requirement of splicing of RNA prior to transport into the cytoplasm. The analysis of these globin genes has been remarkably helpful in providing detailed insights about the RNA sequences required for specific selective and effective RNA splicing. Almost 50% of the naturally occurring point mutations producing β-thal have been shown to affect mRNA splicing (146).

The GT dinucleotide 5' of the intron and the dinucleotide AG 3' of the intron are known to be invariant in animal cells (198). This is defined as the consensus splice rule by Breathnach and Chambon (199). Mutations in these invariant nts result in complete splicing failure and cause β°-thal (191). A comparison of more than a hundred splice junction sequences has provided the expanded consensus splice junction sequences shown in Fig. 1.13. The splice junction consensus sequences appear to be involved in the formation of a base paired structure between U1 RNA and the precursor mRNA (199,200). U1 RNA is a small, stable, nuclear RNA species that may function as a cofactor in the splicing reaction. Adjacent portions of the 5' end of U1 RNA are complementary to the 5' and 3' splice junction sequences; by forming a base paired structure with U1 sequences, the 5' and 3' ends of an intron are juxtaposed in a way that could readily facilitate accurate splicing (201). Mutations in the consensus splice sites affect normal splicing, mostly leading to decreased splicing of mRNA, and often, milder forms of β-thal. In all cases of altered splice function, as mentioned above, the mutations lead to activation of cryptic (alternative) splice sites that are normally nonfunctional, and located in proximity to the normal splice sites (201-203). For example, inactivation of the 5' splice site of the first exon of the human β-globin gene by a base change of the first nt of intron 1 (IVS-I) leads to the utilization of GT dinucleotides at positions 105 and 127 of exon 1 and IVS-I position 13 as 5' splice sites (Fig. 1.13).

5' SPLICE SITES	
Consensus 5' splice sequence	$\frac{C}{A}$ AG ↓ GT $\frac{G}{A}$ AGT
Human β Exon 1 residue 105	A AG ↓ GT G AAC
Exon 1 residue 127	G TG ↓ GT G AGG
IVS-I residue 13	A AG ↓ GT T ACA
IVS-II residue 48	A TG ↓ GT T AAG
3' SPLICE SITES	
Consensus 3' splice sequene	YYYYYYNCAG ↓ $\frac{G}{T}$
Human β IVS-II residue 579	TTTCTTTCAG ↓ GG

FIG. 1.13 Cryptic Splice Sites in Human Genes. The splice sites shown are those used when a mutation inactivates the nearest normal splice site.

Mutations in the IVS-II region are also responsible for creating new splice sites (206,207). The base change G→G at IVS-II-745 creates a new 5' splice site (Fig. 1.14). This new splice site is joined to the normal 3' splice site of IVS-II, and the normal 5' splice site of IVS-II is joined to a cryptic 3' splice site at position 579 (Fig. 1.13) creating a new exon. In this case, however, a low level of precursor mRNA molecules is processed normally.

Consensus Splice Sequence	$\frac{C}{A}$ A G↓G T $\frac{G}{A}$ A G T
IVS-II Mutations	
654 (C→T)	C A G↓[G] T A A T A
705 (T→G)	G A [G]↓G T A A G A
745 (C→G)	C A G↓[G] T A C C A
Codon Mutations	Codon 25 Codon 26
Normal Sequence	GT'GGT'GAG'G
β^E	GTG↓GT [A] AGG
β-Knossos	GTG↓GT GAG [T]

FIG. 1.14 Thalassaemia mutations creating new 5' splice sites. The boxed sequences are sites where the single base substitution has generated a new splice site in the globin gene.

The mutations at IVS-II-654 (C→T) and IVS-II-705 (T→G) also lead to the activation of the previously mentioned cryptic acceptor site in IVS-II-579, however, in these cases no normal splicing of IVS-II sequences from precursor mRNA are transcribed leading to a β° phenotype.

Single base changes in the exons of the globin gene can also create new splice sites (208-210). This has been observed to occur with base changes in the coding region of the first exon (Fig. 1.14), e.g. Hb E, Hb Knossos, codon 24 (A→T), and Hb Malay [β19(B1)Asn→Ser]. Hb Malay is the result of a single mutation at codon 19 (AAC AGC) that activates the cryptic splice site at codons 17, 18, and 19 (AG|). The three other mutations increase the homology of the cryptic splice site at codons 24, 25, 26, and 27 with the donor consensus junction sequences, therefore producing abnormally spliced mRNA.

In all the cases mentioned above, the incorrectly processed mRNA will contain intron remnants that affect its intranuclear stability or nucleocytoplasmic transport, presumably due to the presence of in phase terminating codons.

7.3b Frameshift and Nonsense Mutations. Frameshift mutations are deletions or insertions of one to four nts in the β-globin gene, that results in a shift in the reading frame and a complete alteration of the sequence; a termination codon will usually be present rather close to the frameshift (212). Nonsense mutations are caused by a single base substitution in a

normal codon that creates a premature termination of translation by producing a premature stop codon in the reading frame (213,214). In both the frameshift and the nonsense mutations, shorter abnormal polypeptides are formed that are rapidly hydrolyzed in the red cell precursors. There is also a decreased amount of the mutated β-globin mRNA in the erythroid cells (215-217). Studies of the codon 39 C→T mutation have shown that the decreased levels of β-39 mRNA are not due to a decreased cytoplasmic stability but rather to inhibition of the nuclear to cytoplasmic transport (218,214). Both frameshift and nonsense mutations lead to the production of nonfunctional mRNA and result in a β^0 type of thalassaemia.

7.3c Transcriptional Mutations. These mutations are located in the regulatory region 5' to the β-globin gene cluster that controls transcription. The transcriptional mutation sites are located in the TATA box and in the proximal and distal CACCC boxes (Fig. 1.5). Six mutations have been found so far in the TATA box (191) that decrease the production of β-mRNA transcripts to 10-25% of normal. Five mutations are reported in the distal CACCC box with a mild β-thal gene found in the Black populations. Heterozygotes with mutations in the distal CACCC box and the Cap site have normal haematology, and normal Hb A_2 and Hb F levels, implying that these mutations cause the silent type of β-thal (219). No mutation has so far been reported for the CCAAT box. Recently, a new mutation at position +22 to the Cap site was discovered (220). This mutation reduces β-globin production either by affecting transcription or by affecting the 5' capping process, that in turn may decrease mRNA stability or its ability to initiate efficient translation.

7.3d Poly A Mutations. These mutations, located 3' to the β gene, interfere with mRNA cleavage and lead to a reduced quantity of mRNA producing mild types of β^+-thal. Five mutations have been detected so far. The mutations are located in the AATAAA poly A signal (191). For the T→C mutation (AATAAA→AACAAA), that is found in American Blacks, it was shown that the normal RNA cleavage was significantly reduced, and that a 900 bp longer transcript extended 3' from the poly A site (221).

7.4 Deletional Types of β-Thal. Although most types of β-thal are caused by single nt mutations or by frameshifts, quite a few types are the result of larger deletions affecting the β-globin gene while leaving the other genes from the β cluster intact. The deletions are considered to be caused by non-homologous breakage and reunion.

All deletional types of β-thal are β^0-thal due to the fact that mRNA synthesis is abolished or nonfunctional. The most common deletional β^0-thal observed is the 619 bp deletion that occurs as the second most prevalent deletion in the Asian population (222). The 619 bp deletion starts within the IVS-II and extends in the 3' direction 209 nts past the termination codon.

Several deletions are associated with relatively high Hb A_2 levels in the heterozygote, ranging from 7.1 to 9.6%. All these deletions remove sequences 5' to the β-globin gene, including the promoter elements. They include: a) the Black American type with a 1393 bp deletion involving the region -484 bp to the Cap site to IVS-II-415 (227); b) The Turkish 290 bp deletion extending from -125 bp to the Cap site up to nts 23, 24, or 25

of the β-IVS-I (224); c) the Czechoslovakian 4237 bp deletion extending from 3.3 kb 5' to the β-globin gene Cap site to the middle of β-IVS-II (225). Four small deletions ranging from 7 bp to 44 bp have been identified (223,226-228).

8. δβ-THALASSAEMIA

The δβ-thalassaemias are associated with extensive deletions of varying lengths involving the δ, the β, and often, one of the γ genes (Fig. 1.15). They are characterized by a normal Hb A_2 level, a markedly raised Hb F level, and varying degrees of anaemia in the heterozygous state (229). In the homozygous state of the δβ-thal there is a complete absence of Hb A_2 and Hb A; the red cells of the patients contain 100% foetal Hb with thalassaemic phenotypes such as hypochromia and microcytosis. The patients are mildly anaemic in the heterozygous state with an increase in Hb F of 5-15% that is heterogeneously distributed. Most of the deletions resulting in δβ-thal are simple deletions, but a complex rearrangement in Asian Indians is characterized by both a deletion and an inversion (230). Based upon gene mapping studies, δβ-thal can be divided into two distinct groups: a) the $^{G\gamma}(^{A\gamma})$ type or $(\delta\beta)^0$-thal in which the Hb F contains both $^{G\gamma}$ and $^{A\gamma}$, and b) the $^{G\gamma}$ type or $^{G\gamma^+}(^{A\gamma\delta\beta})^0$-thal in which there is only production of $^{G\gamma}$ chains. The deletion breakpoints of several of the δβ-thal have been characterized (112). Henthorn et al (112) showed that deletion breakpoints are more likely to occur within transcriptional units of the β-globin gene region than would be expected by chance. All of the so far characterized δβ types are the result of nonhomologous recombinations in specific parts of the genome.

9. HPFH. HPFH is a clinically benign condition characterized by the continued expression of one or both of the γ-globin genes in adult life; these genes are normally expressed at significant levels only in the foetal period (98). Molecular analysis of several HPFH genes have classified this condition into two forms, the deletional and nondeletional types. Various deletional forms in which the and genes are missing have been described (Fig. 1.15). In the nondeletional form, all globin genes in the -globin gene cluster are intact.

9.1 Deletional HPFH. It is difficult to distinctively separate the deletions causing δβ-thal from the HPFH deletions at the molecular level; however, in contrast to the situation in δβ-thal, the increased amount of Hb F (20-30%) in HPFH heterozygotes is relatively uniformly distributed among the red cells. The lack of β chains is almost fully compensated for by the increased γ chain production, and therefore, heterozygotes have a normal haematology, while the δβ-thal hetereozygote is distinctly microcytic and hypochromic. The HPFH condition is often found in Black populations and occasionally in Asian Indians and Italians (231-234).

The mechanism for the persistent γ chain synthesis in adults with these deletional types has not been completely elucidated. Several hypotheses have been proposed by which foetal Hb is elevated in these deletions. The first hypothesis was postulated by Huisman and Schroeder (235) who suggested a controlling gene in the inter $^{A\gamma}$-δ gene that regulates γ-globin gene expression (236). This hypothesis was based upon the finding that rela-

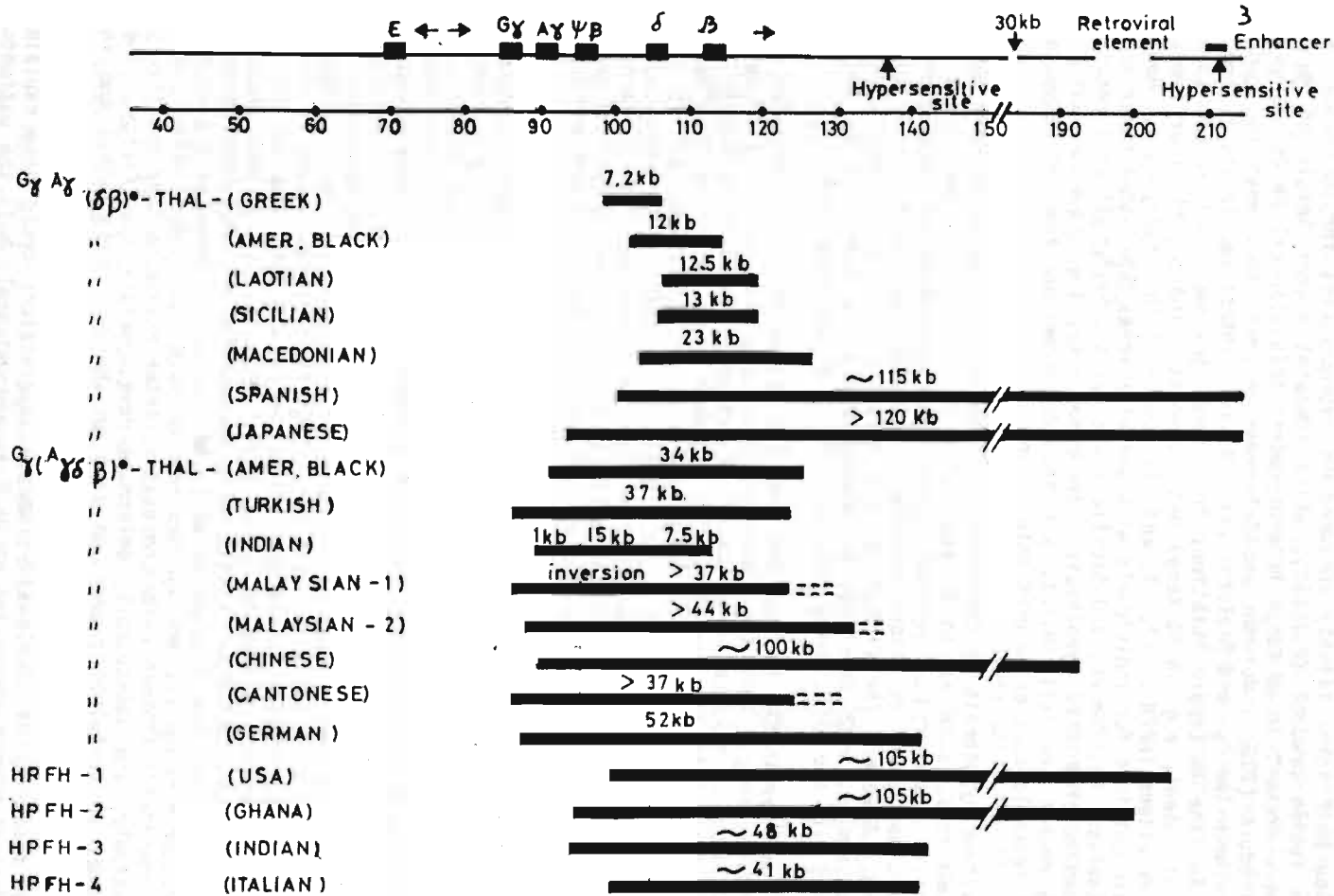

FIG. 15. Deletional types of δβ-thalassaemia and HPFH.

tively low Hb F levels (1-5%) appeared in heterozygotes for Hb Lepore, a δ-β gene fusion product (237,238), while somewhat higher levels of Hb F (5-8%) were present in Hb Kenya heterozygotes; this abnormal Hb is an $^{A}\gamma$-β fusion product (239). Huisman and Schroeder proposed that when the gene region between the $^{A}\gamma$- and β-globin genes is left intact (as in the heterozygotes for the Hb Lepore mutation) this leads to lower Hb F levels than when it is absent, e.g. in Hb Kenya heterozygotes. Indeed, all four types of HPFH deletions (HPFH-1, -2, -3, and -4) supported this hypothesis. However, data obtained for individuals with one of several $(\delta\beta)^{\circ}$-thal (American Black, Laotian, Macedonian, and Sicilian) and various $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal conditions contradicted this hypothesis. In these cases the sequences within the $^{A}\gamma$-β region are left intact, yet the phenotype for the heterozygote does not resemble that of an HPFH heterozygote.

A second hypothesis by Bernards and Flavell (240) suggested that very large deletions, such as those associated with HPFH, disrupt chromatin structure in a manner that results in continued expression of the γ-globin genes, whereas short deletions associated with $\delta\beta$-thal do not have such an effect. However, the finding of the Japanese (241) and Spanish (242) $\delta\beta$-thalassaemias, with deletions of approximately the same size as those associated with the HPFH, makes this unlikely to be the only factor.

A third hypothesis is that DNA from the region of the 3' endpoint of the deletion brought into the vicinity of the γ-globin genes may contain sequences such as enhancer elements or an actively transcribing domain of DNA that facilitate the expression of γ-globin genes to which they have become juxtaposed (241). It is suggested that sequences that act as cis enhancers are brought in from 3' to the β-globin complex. In vitro tests for enhancer activity have demonstrated that DNA sequences 2 kb 3' to the HPFH-1 are active in enhancing the activity of a bacterial gene in a gene transfer system using mammalian tissue culture cells or K562 human erythroleukemia cells (243).

Recently, a region 3' to the ε gene has been reported to play an important role in the regulation of β-globin gene expression (102,104, 244). These so-called hypersensitive sites (HS), that are also termed locus activating regions (LAR) or locus controlling regions (LCR), are erythroid specific and are present at all stages of development. Nt sequence analysis of the HS 5' to the ε gene revealed the presence of two to three enhancer core-like sequences in each of the sites, as well as long stretches of alternating purine and pyrimidine nts that are also found in transcriptional enhancers. A looping out model is proposed for the HPFH conditions in which there is a juxtaposition of the γ genes in such a way that they are influenced by the region of the HS sites (4, 3, 2, and 1). In this model, correct temporal regulation results from competition of individual globin gene family members for interaction with LAR sequences. Promoter and proximal enhancer-binding factors synthesized in yolk sac, foetal liver, and bone marrow then influence these competitive interactions, either positively or negatively, and subsequently determine developmental specificity. The differences in foetal Hb production seen in the HPFH and $\delta\beta$-thal and in the HPFH.

The necessity of stable LAR-promoter interactions could also explain the heterocellular distribution of Hb F in approximately half the patients

with nondeletional forms of HPFH. Hb F in these patients is synthesized at high levels only in a fraction (10-20%) of erythrocytes. The single base pair mutations in the γ-globin promoter that are apparently responsible for the HPFH phenotype may enhance LAR γ-globin interactions. Since stable LAR β-globin promoter interactions can also occur, individual cells may express either γ- or β-globin mRNA but not both from the mutant chromosomes.

9.2 NONDELETIONAL HPFH

The location of sites that are associated with nondeletional mutations leading to HPFH are shown in Table 1.2.

TABLE 1.2. Substitutions Associated With Nondeletional Types of HPFH

Mutation	Gene	% Hb F in Heterozygotes	G_γ %	Population/ Ethnic Group
-202 (C→G)	G_γ	10-20	95	Black
-175 (T→C)	G_γ	20-30	89	Black; Italian
-161 (G→A)	G_γ	1- 3	70	Black
-158 (C→A)	G_γ	2- 5	90	Multiple origin
-114 (C→T)	G_γ	3- 5	89	Black
-202 (C→T)	A_γ	2- 4	20	Black
-198 (T→C)	A_γ	4-12	10	British
-196 (C→T)	A_γ	10-15	10	Chinese; Italian
-195 (C→G)	A_γ	7	10	Brazilian
-175 (T→C)	A_γ	30	20	Black
-117 (G→A)	A_γ	10-15	10	Greek; Black
-114 (C→T)	A_γ	3- 5	--	Black
-114 to -102 (deletion)	A_γ	30	--	Black

Most of these mutations occur in regulatory sequences that are thought to interact with DNA binding proteins (245-247). The Hb F levels in heterozygotes vary from only a slight increase (1-3%) to a value of 30% (245). The Hb F is predominantly composed of either G_γ or A_γ chains and can be pancellularly or heterocellulary distributed. The mutations are clustered in and around the distal **CCAAT** box (positions -111 to -115), the octamer sequence **ATGCAAAT** (positions -182 to -175), and the sequence between -196 and -205 5' to the Cap site of the γ-globin gene.

An interesting finding in the nondeletional HPFH condition is that the β-globin gene *in cis* to the mutation is expressed at a lower level than normal; this was demonstrated in families in which a structurally mutant β-globin gene is *in trans* to HPFH by showing that the β^A/β^X ratio is decreased (245,248-251). The activity of the δ gene is also shown to be consistently decreased in all types of HPFH. Transfection experiments and sequence determination have shown that the β- and δ-globin genes from several nondeletional HPFH mutations are normal; this excludes β- or δ-thal as a cause of their decreased activity (252,253).

These observations suggest that decreased δ- and β-globin gene activities *in cis* to HPFH mutations may be due to reciprocal competition for interaction with a common enhancer, and that the HPFH mutations might

increase the ability of the γ genes to interact with this enhancer, that results in a decrease of the δ- and β-globin gene expression *in cis*. Another possibility is the competition for the same limited transacting factor by the promoters of the γ, δ, and β genes. Mutations in the γ promoters might enhance their affinity for binding with the transacting factors, causing a decreased availability for β- and δ-globin promoter sequences.

The most common nondeletional HPFH is the G$\rightarrow$T substitution at position -158 5' to the Cap site of $^{G}\gamma$ that causes a slight increase in the ratio of $^{G}\gamma$ to $^{A}\gamma$ chains of Hb F (254). This mutation at -158 is detectable by Xmn I digestion because of the creation of a new splice site.

10. DNA POLYMORPHISM

The use of restriction endonuclease and sequence analysis has made it possible to detect relatively common normal variations in DNA structure (polymorphism), that are inherited in a Mendelian manner and may be used as genetic markers. The first such polymorphism reported was a variation in the recognition site of the restriction endonuclease Hpa I 3' to the β-globin gene (255). In most normal human DNA samples, digested with Hpa I, the β-globin gene was located on a 7.6 kb DNA fragment. Polymorphism at this site resulted in two fragments (7.6 and 13 kb) both containing the β gene. The immediate application of this observation was in antenatal diagnosis (255), but it was also of potential value as a genetic marker in anthropological studies. A restriction site is called polymorphic in a given population if the least frequent allele is present in more than 1% of individuals (256). The pattern of combination for different polymorphic sites for any chromosome is called a haplotype. In the α-globin gene cluster, one in 60 nts is polymorphic; however, few such point mutations are responsible for loss or gain of a restriction site; instead, the polymorphisms seen in the α-globin gene cluster are primarily those of variable lengths (189).

Many polymorphic restriction sites are well defined in the β-globin gene cluster and are used as markers for the determination of the haplotype of the β-globin gene complex (257). Each polymorphic sequence can be either present (+) or absent (-) for a particular chromosome. If two polymorphic restriction enzyme recognition sequences are randomly associated with each other, the probability of the presence of both these sequences (+/+) is equal to the product of the probabilities of the presence of each individual polymorphic restriction enzyme recognition sequence.

If the two recognition sequences are not randomly associated, the probability of the presence of both recognition sequences will be significantly lower than expected. This non-randomness of association is referred to as "linkage disequilibrium". For a given number of polymorphic sites there is a maximum possible 2^n combination of recognition sequences. DNA polymorphism, present in and around the human globin genes, have been extensively studied (258). Table 1.3 shows the restriction endonucleases with their respective restriction sites, often used for haplotype studies of the β-globin gene cluster. The collection of large amounts of data on the haplotypes in Mediterraneans, Blacks, and Southeast Asians, has revealed some interesting findings. For instance, these data show that alleles at these

various polymorphic sites are not randomly associated. The $^{G}\gamma$ Hind III and the $^{G}\gamma\beta$ Hinc II sites located upstream of the δ gene are grouped in the haplotype combination [- - - -], [+ + - +], [+ - + +], [+ + - -], and [+ - + -], and are found in Mediterraneans. The other 11 possible combinations are rarely seen or not at all. Statistical tests confirm that the association of alleles at these restriction sites is non-random. The same non-random association of sites is noted for a group of restriction polymorphic sites located 3' to the δ gene. However, random association does occur between the two groups. The fact that alleles at polymorphic sites within the groups have been maintained in linkage disequilibrium suggests that recombination in these areas has been relatively infrequent, while the random association between the two groups suggests that crossover in this area is much more frequent. A possible explanation is that the region between the two groups (around the δ-β-intergenic area) might be a hot spot for recombination.

TABLE 1.3 Haplotypes in the β-Globin Gene Cluster[a]

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14
I/bl2	+	-	-	-	-	+	-	+	+	+	+	+	+	-
II/B	-	+	+	-	+	-	+	+	+	+	+	+	+	-
IIIa	-	+	-	+	+	-	+	-	+	+	+	+	-	+
IIIb/C	-	+	-	+	+	-	+	+	+	+	+	+	-	+
IV	-	+	-	+	+				-	-	+	-	+	
Va	+	-	-	-	-	+	-	+	+	+	+	+	-	+
Vb/D	+	-	-	-	-	+	-	-	+	+	+	+	-	+
VI	-	+	+	-	-	+	-	+	-	-	+	-	+	-
VIIa/Fa/ch2	+	-	-	-	-	+	-	+	-	-	+	-	+	-
Fb	+	-	-	-	-	+	-	-	-	-	+	-	+	-
VIII	-	+	-	+	-	+	-	+	+	+	+	+	-	+
IX/bl1	-	+	-	+	+	-	+	+	+	+	+	+	+	-
X	-	+	-	-	-			+	-	-	+	-	+	-
A/ch1	+	-	-	-	-	+	-	+	+	+	+	-	+	-
ch3	-	+	+	-	+	-	+	+	-	-	+	-	+	-
ch4		-	-	-	-	+	-	+	+	+	+	+	+	-
E	+	-	-	-	-	+	-	+	+	+	+	+	-	+
G	-	+	-	+	+	-	+	+	+	+	+	-	+	-
H	-	+	+	-	+	-	+	-	-	-	+	-	+	-
I	-	+	-	-	+	-	+	+	-	-	+	-	+	-
bl3		-	-	-	+			+	+	+	-	-	+	-
bl4		-	-	-	+			-	-	-	-	-	+	
bl5		+	+	-	+			+	+	+	+	-	+	
bl6		-	-	+	+			+	+	+	+	+	+	
bl7		+	-	-	+			-	+	+	+		+	

[a] The presence (+) or absence (-) of the 14 common polymorphic restriction sites along each haplotype is presented. The sites are 1 : Hinc II ; 2,3 : Hind III ; 4,5 Hinc II ; 6 : Rsa I ; 7 : Taq I ; 8 : Hinf I ; 9 : Hgi AI ; 10 : Ava II ; 11 : Hpa I ; 12 : Hind II ; 13 : Bam HI ; 14 : Rsa I

Roman numerals indicate Mediterranean haplotypes; capitals indicate Indian haplotypes; ch indicates Chinese haplotypes; bl indicates Black haplotypes.

Orkin and Kazazian and their colleagues have found that specific thalassaemia mutations are associated with particular haplotypes (257). However, coupling of a haplotype with a specific mutation is not absolute, even within a single ethnic group. Pirastu et al (259) have found that the β°-39 mutation in the Sardinian population is associated with nine different haplotypes. These findings may imply that the same mutation has arisen more than once, or more likely, that DNA sequence rearrangements in the β-globin gene cluster occur with a far higher frequency than was heretofore suspected.

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CHAPTER 2

MATERIALS AND METHODS

1. MATERIALS AND METHODS

This chapter provides details of some procedures that are not described in detail in Chapters 3 through 6. Application of the procedures to the studies involving the Hb variants and thalassaemia mutations can be found in these chapters together with the appropriate illustrations.

2. PATIENTS

2.1 Newborns. During a six months period 201 cord blood samples were collected at the Paramaribo Hospitals (Academisch Ziekenhuis, Diaconessenhuis, 's Lands Hospitaal and St.Vincentius Ziekenhuis). The samples were stored at +4°C and shipped by special air service to Augusta, GA, USA, within two weeks of collection. Each shipment consisted of approximately 40 samples and was received within three to four days after mailing. Informed consent was obtained prior to collection.

2.2 Native Indians. Blood was collected from 131 inhabitants of three native Indian villages located at the west side of Surinam (Donderskamp, Corneliskondre, and Kalebaskreek). The samples were received in Augusta within four days of collection. Informed consent was obtained prior to collection.

2.3 Patients on Record in the Local Hospitals and From Local Physicians. In order to study the different types of thalassaemias and possible abnormal Hb variants in the Surinam population, thalassaemic patients who attended the hospital clinics, and their relatives, were invited to participate in this study. From the patient records kept in the laboratory of the Academisch Ziekenhuis in Paramaribo, a selection was made of patients with high Hb A_2 values and with abnormal band patterns on cellulose acetate electrophoresis. Most of these patients and their relatives were invited to participate in this study. Samples from some members of one family (see Chapter 4) were collected in The Netherlands by Dr. K. Punt and his associates. Informed consent was obtained prior to collection.

3. BLOOD COLLECTION: SHIPMENT

Blood samples were collected in vacutainers (Becton Dickinson Vacutainer Systems, _____) with EDTA as anticoagulant. The samples were kept cold (+4°C) until the time of shipment to Augusta, GA, USA. They were shipped in wet ice, and many samples were received within four days after shipment.

4. HB ANALYSIS

In this methodology section, the procedures will be listed in the sequence they were used after we received the blood samples.

4.1 Haematological Data. These were obtained with a fully automated Sysmex CC-720 cell counter (TOA Medical Electronics Co. Ltd., Kobe, Japan). The measurement of the mean corpuscular volume (MCV) is normally a useful parameter for the assessment of the possible presence of thalassaemia and certain Hb variants (Hb E and Hb C), particularly when the MCV values are less than 80 fl. However, it must be noted that in many of our β-thal patients, the expected microcytosis was not often definite because the analyses of most of the samples took place two to three weeks after collection, and the storage conditions in Surinam were not always optimal. Several of the blood samples were partly haemolyzed upon arrival. The mean corpuscular Hb (MCH) values are probably more reliable (MCH <25 pg) for an evaluation of a possible thalassaemia as it is less variable with age or with improper storage of blood samples. It must also be emphasized that an abnormal result is non-specific and can be caused by a number of acquired conditions such as iron deficiency, and a variety of other uncommon haematological abnormalities.

4.2 IEF. Red cell lysates were analyzed by IEF which will separate variants that co-migrate in conventional procedures such as cellulose acetate and citrate agar electrophoresis. Basset et al (1) identified 70 Hb variants by IEF; 31 of these could not be separated from Hb S by cellulose acetate electrophoresis. IEF will separate Hb variants with pI's that differ by 0.02 or greater. Traditionally, IEF has been performed using polyacrylamide as the supporting matrix. The gels used in this study were made of agarose and were purchased from Isolab, Inc., Akron, OH, USA. Agarose offers a high gel strength with sufficient porosity to allow proteins with molecular weights of up to 10^6 daltons to migrate freely. The buffers used in this procedure were also obtained from the same supplier. Band identification is performed by comparing distances to known controls (see Fig. 3.1 of Chapter 3). Bands with a similar isoelectric point (Hb E, Hb O, Hb C) were characterized with citrate agar electrophoresis. The IEF procedure was used primarily for the initial screening of the samples to detect an abnormal pattern.

4.3 Citrate Agar Electrophoresis. This method is capable of resolving some variants that do not separate from Hb A, Hb S, or Hb C by cellulose acetate electrophoresis or IEF. Electrophoresis on agar gel with a citrate buffer at pH 6.1 causes most Hbs to move cathodically from the point of origin and with relative mobilities that are different from those obtained with electrophoretic techniques at alkaline pH. This method is useful in detecting haemoglobinopathies in cord blood samples because of the distinct mobility of Hb F. A major disadvantage of this procedure is that the identification of many of the common Hb variants such as Hb E or Hb G-Philadelphia cannot be made because they co-migrate with Hb A.

4.4 Fetal Hb Quantitation. Quantitation of Hb F is important in the diagnosis of the various forms of thalassaemias, sickle cell disease, the Hb E syndromes, and the HPFH syndromes, particularly in individuals aged 6 months or older. Two methods are in use in our laboratory for the quantitation of Hb F; the alkali resistance method and a recently developed HPLC method. The alkali resistance of of Hb F (% F_{AD}) provides a rapid and reasonably simple technique; there are several published variations of the alkali denaturation test (2-5). The method used in this study is a modified procedure as described by Betke et al (2). The Hb is first converted to

cyanmetHb before denaturation which prevents false-positive values, particularly in patients with increased concentrations of the alkaline-resistant carbonmonoxyHb (CO-Hb), e.g. in heavy smokers.

A recently developed HPLC procedure using a weak poly(aspartic acid) cation exchange column was also used to quantitate Hb F and other Hb fractions; the method will be discussed later in this chapter. Quantitative data obtained with this method have been compared with those by the alkali denaturation procedure (% F_{AD}) and Fig. 2.1 provides this information. The HPLC method was shown to have an overall higher accuracy rate in estimating Hb F levels, especially for values <10%.

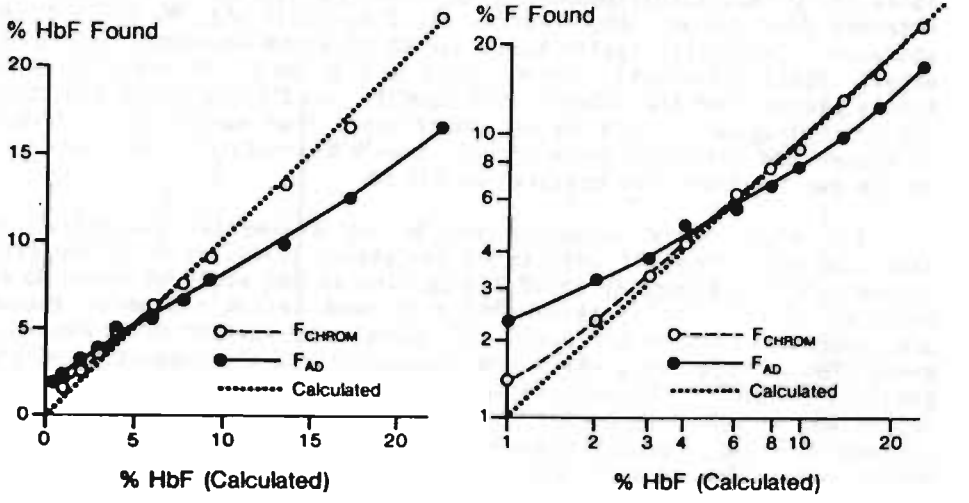

FIG. 2.1 A comparison of Hb F values determined by PolyCAT chromatography (F_{Chrom}) and by alkali denaturation (F_{AD}), presented on a linear scale (left) and logarithmic scale (right). Artificial mixtures of Hb F and Hb A were analyzed. Reproduced from Kutlar et al, Hum. Genet., 85:106-110, 1990, with permission.

4.5 DEAE-Cellulose Chromatography. In order to study the γ chain composition, Hb F was first isolated by DEAE-cellulose chromatography. Depending upon the amount of Hb F present in the red cell lysate, one of the isolation following procedures of Hb F was used: a) Hb F <3%. Isolation was on a medium sized (30 cm x 1.5 cm) column packed with DE-52 cellulose. The anion exchanger was equilibrated by repeated washings with a 0.2 M glycine + 15 mM KCN buffer, pH 7.75. About 60 mg Hb was applied to the column and the chromatogram was developed for 15 hours at a flowrate of 50 ml per hour using a 0.2 M glycine, 0.01% KCN, 0.02 M NaCl developer.

The Hb F fraction located at the top was cut from the column, poured into an analytical sized column (1 cm x 20 cm), and eluted with a stripping buffer (0.2 M NaCl, 0.01% KCN, 0.2 M glycine, pH 7.7). **b)** Three percent < Hb F <20%. Analytical sized columns (1 cm x 20 cm) were used. Column preparation and equilibration were as mentioned above. About 20-30 mg of Hb was applied to the column and chromatographed at room temperature using a 30 ml per hour flowrate for 5 hours. The Hb F fraction located at the top was cut from the column, poured into a microcolumn (0.5 cm x 7 cm) and eluted with a stripping buffer (0.2 M NaCl, 0.01% KCN, 0.2 M glycine). **c)** Hb F <20%. It is not necessary to isolate Hb F from such red cell lysates as these lysates can be applied directly to a reversed phase HPLC column.

4.6 Quantitation of Hb A_2. Elevated levels of Hb A_2 are characteristic for β-thal conditions. Column chromatography with commercial columns obtained from Isolab, Inc. (Akron, OH, USA) (Quik-Sep, Hb A_2 test system) was used. The Isolab system makes use of an anion exchanger [diethylaminoethyl) (DEAE) cellulose]. Hb A_2 (plus Hb E or Hb C, if present) is selectively eluted from the column under specific conditions of pH and chloride-ion concentration. There is no interference from Hb S. The remaining Hb is eluted and collected as a single, separate fraction. Absorbance values of the two fractions are measured at 415 nm.

4.7 HPLC. This technique has become a powerful analytical method that has influenced all aspects of analytical chemistry. In the past two decades, HPLC has been successfully applied to the study of human Hb abnormalities (1-6). Ion exchange HPLC with weak cation and anion exchangers are used for the identification of normal and variant Hbs, and reversed phase HPLC is used for globin chain separation and the separation of tryptic peptides of globin chains. Depending upon the kind of Hb quantitation or separation needed, appropriate columns and buffers are used. Analytical columns will facilitate the quantitation of Hb types in red cell lysates, while preparative columns are used to isolate Hb fractions for further studies, including structural analysis.

4.8 Cation HPLC. Quantitation of Hbs was performed on a PolyCAT A cation exchange column, 5 µm particles, 4.6 mm x 200 mm, obtained from PolyLC, Columbia, MD, USA. The chromatogram was developed with two buffers: Developer A was 35 mM Bis-Tris, 3 mM NH_4 acetate, 1.5 mM KCN, pH 6.47; developer B was 35 mM Bis-Tris, 1.5 mM KCN, 150 mM Na acetate, 16.85 mM NH_4 acetate, pH 7.0. A gradient was developed for 85 minutes from 25% B to 85% B. The column was purged for 5 minutes with 100% B and equilibrated for 10 minutes with 25% B + 75% A. Some 50-200 mg Hb in red cell lysates was applied, the absorbance of the effluent was continuously recorded at 415 nm, and peaks were quantified (as area %) by a Data Module. The flowrate was 0.8 ml/min, and the chart speed 0.25 cm/min. When using this method to isolate Hb (up to 200 mg) that are present in small amounts in red cell lysates, a preparative PolyCAT A column (21 mm x 250 mm, 12 µm particles; same manufacturer) was used. The developers were the same as for the analytical column except for the composition of developer B that contained 225 mM Na acetate instead of 150 mM. The flowrate was adjusted to 5 ml/min. The desired Hb fraction was collected and concentrated by filtration under pressure (Amicon Diaflow, Amicon, Danvers, MA, USA). The order of elution of the different Hb types is Hb Bart's and Hb H, followed by Hbs I, N, F, different types of J, A, A_2/E, G, D, S, O-Arab, C-Harlem, and C.

4.9 Reversed Phase HPLC. The γ chain composition of Hb F (i.e. % $G\gamma$, $A\gamma$, or $A\gamma^T$) was determined by reversed phase HPLC as developed by Shelton et al (12) and Kutlar et al (13) using the large-pore Vydac C_4 column and two water-acetonitrile-trifluoroacetic acid (TFA) developers. Developer A was 60% acetonitrile, 0.1% TFA, 40% water; developer B was 20% acetonitrile, 0.1% TFA, 80% water. A gradient was developed for 70 minutes from 50% A to 60% A. The column was purged with 100% A and equilibrated for 10 minutes with 50% A + 50% B. Some 50-800 µg Hb in red cell lysate was applied to the column; the absorbance of the effluent was continuously recorded at 220 nm. Fig. 2.2 provides an example of a chromatogram. The order of elution of the different Hb chains is: β^E, β^C, β^S, β^A, δ, α, $A\gamma^T$, $G\gamma$, and $A\gamma^I$. This method can also be used to isolate Hb chains for structural analyses. For this purpose, a larger column is used and a flowrate of 5 ml/min is applied. The buffers are the same as for analytical use.

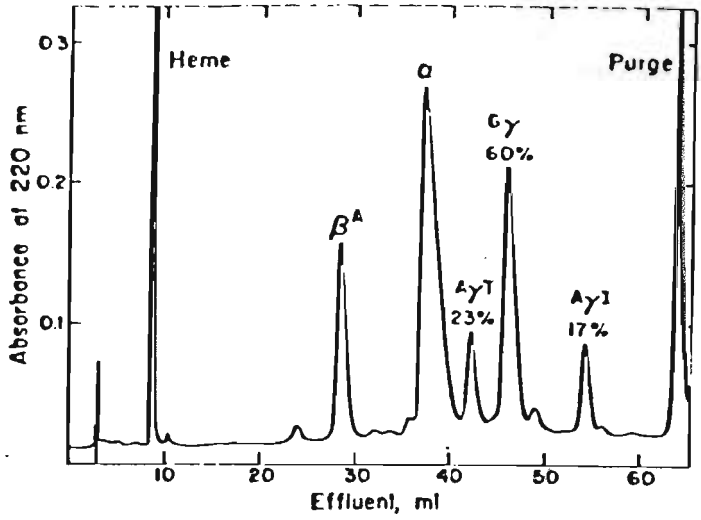

FIG. 2.2 Separation of the globin chains in the cord blood of a newborn child. Sample was 0.10 mg.

5. STRUCTURAL ANALYSES

In our survey a Hb variant with an abnormal α chain was discovered in a Creole family (see Chapter 3). The methodology used in characterizing this α chain variant is described in detail below. The first steps were the identification and quantitation of the Hb variant by IEF and HPLC, respectively. After these steps, the abnormal Hb was isolated by DEAE-cellulose chromatography (as described for Hb F isolation). The polypeptide chains of the Hb variant were separated by carboxymethylcellulose (CMC) chromatography (14). The isolated α chain was digested with trypsin, and the resulting tryptic peptides were separated by reversed phase HPLC. Finally, the isolated zones obtained by the reversed phase HPLC were analyzed with an amino acid analyzer in order to identify the amino acid substitution involved.

5.1 Tryptic Digestion; Isolation of Peptides; Amino Acid Analysis. Five to 10 mg of the α chain was dissolved in 10 ml water + 0.15 ml 10% acetic acid, and digested (under constant stirring for 6 hours at room temperature) with TPCK-trypsin (Worthington, Freehold, N.J., USA) at pH 8.5-8.9. Next, the pH of the clear supernatant of the α chain digest was adjusted to 2.5-3.0, whereafter the solution was lyophilized. Two to 3 mg digest was dissolved in 100 µl of 10% acetic acid and centrifuged for 10 minutes at 3600 rpm, and 1-1.5 mg was applied to the reversed phase HPLC columns (15). The column used was a C_{18} µBondapak (part #27324, Waters Associates, Milford, MA, USA) and the chromatogram was developed with an acetonitrile-NH_4 acetate gradient, pH 5.7, as detailed in the quoted reference. Fractions were measured at 220 nm. The reagents were analytical grade and were pre-filtered through a millipore type HA 0.45 µm filter. All solvents were degassed and sonicated before use. The isolated peptides were dried under nitrogen and part was hydrolyzed in 6 M HCl at 110°C for 24 hours. The resulting hydrolysate was analyzed with a fully automated Waters Pico-Tag amino acid analyzer.

6. DNA ANALYSES

6.1 Isolation of Human Genomic DNA From Blood. DNA was isolated from white blood cells essentially by the method of Poncz et al (16). About 5-10 ml blood was washed three times with 30-40 ml of 1X (times) reticulocyte saline (140 mM NaCl, 4 mM KCl, 6.8 mM $MgCl_2$) and centrifuged at 2500 g for 15 minutes at 4°C. The red blood cells were hemolyzed by adding 10 ml of a freshly prepared solution containing 131 mM NH_4Cl and 0.9 mM $(NH_4)_2CO_3$, pH 6.5, for 10 minutes at room temperature. The white cells were collected by centrifugation at 2500 g for 15 minutes at 4°C and the supernatant (hemolysate) was carefully removed and saved for Hb analysis (storage at -20°C). This was was repeated and the pellet (mainly white blood cells) was resuspended in 5 ml STE solution (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4, 1 mM EDTA). One ml of 10% SDS and 0.2 ml of proteinase K (100 µg in 10 mM Tris-HCl, pH 7.5) were added and the cell suspension was incubated at 37°C for 12-24 hours without shaking. Deproteination was accomplished with an equal volume of phenol (after saturation with 20 mM Tris, pH 8.0, containing 0.1% hydroxyquinoline). The mixture was shaken gently for 30 minutes at room temperature. After centrifugation at 2500 g for 15 minutes at 4°C, the aqueous phase was transferred into a new tube and the procedures were repeated. Next, an equal volume of a mixture of chloroform with isoamylalcohol (in a 24 to 1 ratio) was added and the mixture was shaken gently for 10 minutes at room temperature. After 15 minutes centrifugation at 2500 g the aqueous phase was mixed with 5 volumes of 95% ethanol in a plastic flask. After gently mixing, the DNA precipitate was recovered with the broken end of a sterile Pasteur pipette. The DNA was washed twice with 70% ethanol (to remove any residual phenol or salt) and dried in vacuum. The pellet was dissolved by incubating in 1 ml of TE (1 mM Tris-HCl, pH 7.5, 1 mM EDTA) overnight. The concentration of the DNA was determined from its extinction coefficient at a wavelength of 260 nm (A_{260}). An O.D. of 1 corresponded to approximately 50 µg/ml of DNA. The DNA was stored at 4°C.

7. GENE MAPPING

The various forms of α- and β-thal, caused by a deletion, are usually studied by gene mapping. Depending upon the type of deletion expected (α, β, $\delta\beta$, or γ, δ, β) various restriction enzymes and probes are used for the identification. The procedure of Southern (17) as modified by Blattner et al (18) was followed. Five µg of genomic DNA was digested for 3 hours at 37°C with 20 units of restriction enzyme, using buffer conditions specified by the suppliers. The DNA fragments were separated by electrophoresis on a 0.8% agarose gel using DNA digested with Hind III as a size marker. The gel buffer system (TEA; 0.04 M Tris-acetate, 0.002 M EDTA) was the same for all agarose gels. The DNA in the gel was stained with ethidium bromide (0.5 µg/ml) and photographed under UV light. The DNA was denatured by soaking the gel in 0.5 M NaOH-1.5 M NaCl solution for 1 hour and neutralized with a solution containing 3 M NaCl, 1.5 M Tris, pH 7.0, for 90 minutes. The DNA was transferred to a nitrocellulose membrane (BA 85, Schleicher and Schuell,_____) by the method of Southern (17) using 20X SSC (1X SSC: 15 mM sodium citrate, 150 mM NaCl, pH 7.0). The DNA was fixed on the nitrocellulose membrane by incubation at 80°C for 2 hours in a vacuum. In order to perform the hybridization experiments, the nitrocellulose membrane was soaked in 68°C 6X SSC plus Denhardt's solution (6X SSC containing 0.2% sterile BSA, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone (PVP), 0.5% SDS) for 10 minutes and prehybridized in about 10 ml of 1X Flavell solution (3X SSC + 0.1% SDS + 20 mM phosphate buffer, pH 6.0, 0.2% PVP) containing 0.2% nuclease-free BSA and 50 µg/ml denatured sonicated salmon sperm DNA for about 2 hours at 68°C. The membrane was hybridized by adding 1 x 10^6 cpm/ml of heat denatured probe and incubating at 68°C for 16-24 hours. The non-hybridized (unbound) probe was removed by washing the membrane twice with 3X SSC + 0.5% SDS solution at 68°C with shaking, for 30 minutes each time. The membrane was monitored with a Geiger counter to check if the washing was sufficient (few counts above background). In some instances additional washing with 1X SSC + 0.5% SDS solution was necessary. The membrane with the hybridized fragments was exposed to Kodak X-Omat film at -70°C for 72 hours. The film was developed using a Kodak X-ray film processor and the positions and sizes of particular sequences of DNA were evaluated.

8. REHYBRIDIZATION OF SOUTHERN BLOT MEMBRANES

Membrane rehybridization was accomplished by first stripping the membranes in a 2 to 3" deep plastic container with a 0.5 M NaOH-1.5 M NaCl solution under careful shaking for 4 minutes. The membranes were rinsed three times with distilled water and neutralized for 4 minutes with 3 M NaCl-1.5 M Tris-HCl, pH 7.0. Rehybridization was as described for the hybridization procedure.

9. PROBES

9.1 Gene Mapping Probes. The following probes were used to identify specific restriction fragments: **a)** the $^{G}\gamma$IVS-II probe; a 457 bp fragment obtained by a Bam HI-Pvu II double digestion of the plasmid p$^{G}\gamma$0.46pB that

contains the 5' region of the large IVS of the $^{G}\gamma$- and $^{A}\gamma$-globin gene. **b)** The βIVS-II probe; a 1.2 kb fragment obtained by a Bam HI-Eco RI double digestion of plasmid pβIVS-II that contains the large IVS of the β-globin gene. **c)** The δIVS-II probe; a 1.0 kb fragment generated by an Eco RI-Bam HI double digestion of the plasmid Pst I-2.3 kb δ that contains the δIVS-II. **d)** The pRK probe; a 0.8 kb fragment obtained by a double digestion of the plasmid pRK 28 with Eco RI-Blg II that is located 17 kb 3' to the β-globin gene. **e)** The α probe; a 1.5 kb fragment obtained by a Bam HI digestion of the pBRα1 probe. **f)** The ζ probe; a 1.9 kb fragment generated by digestion of the pBRζ plasmid with Hinf I.

10. OLIGONUCLEOTIDE DESIGN AND SYNTHESIS

Oligonucleotides employed as amplification and sequencing primers and dot-blot hybridization probes were prepared on an Applied Biosystems 380B DNA synthesizer. The primers were purified on a Sephadex G-50 column; the eluate was vacuum dried and dissolved in TE buffer (1 mM Tri-HCl, pH 7.5, 1 mM EDTA).

11. PCR

The identification of the point mutations leading to β-thal was initially achieved by the laborious procedures of cloning and sequencing. In recent years, the development of the PCR procedure and its application to the study of β-thal mutations has greatly facilitated the identification of β-thalassaemias (19-21).

11.1 Principle. PCR is an *in vitro* technique based upon the enzymatic amplification of a specific DNA fragment flanked by two synthetic oligonucleotide primers complementary to the opposite strands of the fragment of interest. Heat denaturation of double stranded DNA, followed by annealing of the primers to their complementary strands and extension of the annealed primers with DNA polymerase, leads to the amplification of the DNA sequences between the primers. Repeated cycles of denaturation, annealing, and extension results in an exponential accumulation of the target fragment (10^6-fold) in several hours. The use of heat-stable DNA polymerase from Thermus Aquaticus (Taq polymerase) greatly increases the efficiency of the procedure. The procedure can be automated by the use of a programmable Thermal Cycler (Perkin-Elmer-Cetus, Emeryville, CA, USA). The reaction is carried out in a sterile 0.5 ml Eppendorf tube.

11.2 Procedure. The mixture is first heated to 95°C to separate the strands of genomic DNA (7 minutes) and then cooled to 55°C for 5 minutes to allow the annealing of the primers to the separated strands of genomic DNA. Some 2-5 units of Taq polymerase is added, over-layered with mineral oil to prevent evaporation, and then the tubes are centrifuged. The Thermal Cycler is programmed for 30-35 cycles of denaturation at 95°C (1.5 minutes), annealing at 55°C (1.5 minutes), and extension at 72°C (3 minutes). The β-globin gene is amplified in two fragments: a 5' segment between nt 140 and IVS-II-70 (700 bp) and a 3' segment between IVS-II-547 and 200 bp 3' to the β gene (500 bp). Following amplification, an aliquot (5 µl) of the

sample is run on a 0.8% agarose minigel and stained with ethidium bromide along with Hind III digested DNA as a marker, to detect the amplified band under UV light (Fig. 2.3). Samples are kept at 4°C until analyzed.

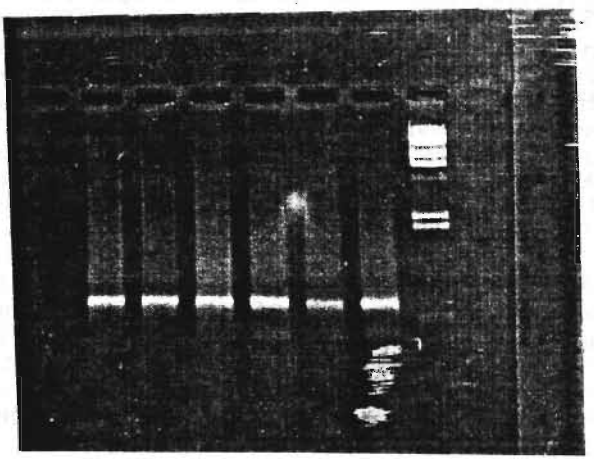

FIG. 2.3 PCR product analysis of a 700 bp fragment in the β-globin gene. A Hind III digested λ phage size marker is applied to the far right of the 1.5% agarose gel.

12. DOT-BLOT ANALYSIS

Identification of β-globin gene mutations can be made by dot-blot hybridization. A small aliquot (40 µl) of amplified DNA is alkali denatured by adding 320 µl water and 40 ml denaturing solution (0.4 N NaOH with 25 mM Na_2EDTA) and then applied in duplicate under vacuum onto a zeta probe nylon membrane using a spotting device (Bio-Dot, Bio-Rad Laboratories, _____). Allele-specific oligonucleotide probes (19-20 nt) are synthesized by an Applied Biosystems DNA synthesizer. A probe with a normal sequence and a second with the substitution (located in the centre of the probe) is required for each mutation. Both mutant and normal probes are 5' end labelled with γ-^{32}P-ATP (approximately 7000 Ci/mM, 160 mCi/ml) by T_4 polynucleotide kinase for 1.5 hours at 37°C [United States Biochemical (USB) Corporation, _____] and the radio-labelled probes are separated from free ^{32}P-ATP on either a Sephadex G-50 or a Nensorb (NEN) column (NEN DuPont, _____). The zeta probe nylon membrane is incubated for 30 minutes at 65°C in a sealed plastic bag with a prehybridization solution (pre-hybridization buffer: 5X SSPE, 5X Denhardt's, 0.5% SDS; where 20X SSPE = 3.6 M NaCl, 20 mM NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4; 50X Denhardt's = 1% Ficoll, 1% PVP, 1% BSA). Next, the radio-labelled probe is added (30-100 x 10^6 cpm/bag), and hybridization occurs for 1.5 hours in a circulating water bath. The hybridization temperature varies for each probe, depending upon the Tm of the probes, which can be calculated from the nt content according to the formula: Tm = 4 x (G + C) + 2 x (A + T). Following hybridization, the membrane is washed twice for 10 minutes with SSPE 0.1% SDS at room temperature. The membranes

are then washed at 42°C for 20 minutes in tetramethyl ammomium chloride (TMAC), followed by a second TMAC washing at 58°C for 30 minutes (22). After washing, the membranes are autoradiographed by exposure to Kodak XAR-5 X-ray film for 2-12 hours.

12.1 Interpretation. The probe will hybridize only when there is a perfect match between the sequence of amplified DNA and the probe. Single base changes will prevent hybridization and therefore, will not result in the visualization of a dark spot on autoradiography. Thus, DNA from a normal individual will only hybridize to the normal probe, while DNA from an individual homozygous for a certain mutation will only hybridize to the mutant probe and not to the normal probe. A heterozygote will show hybridization with both the normal and mutant probes. The detection of a mutation in amplified DNA by dot-blot hybridization to oligonucleotide probes is shown in different chapters of this thesis.

The choice of probes for mutant alleles is dictated by the spectrum of β-thal mutations in a given population. In some populations, a few mutations account for the majority of β-thal alleles. In other populations, β-thal is more heterogeneous and even after hybridization to a large number of mutant probes, a significant portion of β-thalassaemias remain unidentified. The identification of mutations on these unknown chromosomes can be achieved by sequencing amplified DNA (23-26).

13. DNA SEQUENCING

13.1 Synthesis of Single Strand DNA Sequencing. Amplification of single strand DNA occurred in a similar fashion as described for double strand DNA amplification, except for the use of an asymmetric ratio (50 to 1) of the two primers used to generate single strand DNA. In order to obtain optimal data it was considered advisable to limit the size of the fragments to be amplified to less than 800 bp. After amplification, the amplified DNA was purified by adding an equal volume of 2.5 M ammonium acetate and two volumes of ice-cold 100% ethanol. The mixture was left at room temperature for 5 minutes, centrifuged for 10 minutes in a microcentrifuge, and the pellet was then washed twice with 70% ethanol. The DNA precipitate was air dried and finally dissolved in 10 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). One µl of the DNA solution was electrophoresed on a 1.5% agarose gel to monitor the recovery.

13.2 Single Strand DNA Sequencing. DNA sequencing was carried out by the dideoxy method of Sanger et al (27) using a sequenase kit from USB Corporation. The dideoxy sequence analysis is a three-step procedure: **a)** annealing, **b)** labelling, and **c)** termination.

13.2a Annealing template and primer. The following reagents were combined in an Eppendorf tube: 1 µl of primer (100 pmols), 2 µl of sequencing buffer (200 mM Tris, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), and 7 µl of DNA sample obtained by the single strand DNA amplification procedure. If a smaller volume of DNA solution was used, the balance was made up with distilled water. The capped tube was incubated at 65-70°C for 3 minutes and then placed in an ice-bath.

13.2b Labelling reaction. For standard reactions (reading sequences up to 500 bp or 50 bp from the primer) the labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP) was diluted 5-fold with distilled water. The following was added to the annealed template primer: 1 µl of DTT (0.1 M), 2 µl of the diluted mix, and 1 µl of [α^{35}S] dATP (800 Ci/mmol, 10.0 mCi/ml; DuPont)]. After 1 µl of sequenase was diluted with 8 µl of TE buffer, 2 µl of the diluted sequenase was added to the reaction mixture and incubated at room temperature for 3 minutes.

13.2c Termination reaction. Two and one-half µl of each termination mix (80 µM dGTP, 80 µM dTTP, 80 µM dCTP, 80 µM dATP + 8 µM ddNTP, 50 mM NaCl) were placed in four fresh tubes labelled as A, G, T, and C. When the labelling reaction was complete, 3.5 µl of the reaction mixture was transferred to each labelled tube. They were centrifuged for a few seconds to ensure mixing, and the incubation was continued at 37°C for 5 minutes. Four µl of stop solution (9% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each of the termination mixtures. When a gel was ready for loading, the samples were heated to 80°C for 2 minutes and 3.5 µl of sample was loaded.

14. ENZYMES AND CHEMICALS

Restriction enzymes were obtained from Bethesda Research Laboratories (Bethesda, MD), Boehringer Mannheim (Indianapolis, IN), International Biotechnology, Inc. (New Haven, CT), and New England Biolabs (Beverly, MA). Nick-translation kits were purchased from Amersham (Arlington Heights, IL), and BRL (_____). Crystallized phenol for DNA isolation was obtained from Fisher (Norcross, GA), and redistilled before use. Taq DNA polymerase was obtained from Perkin Elmer Cetus (Norwalk, CT).

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CHAPTER 3

STRUCTURAL VARIANTS IN THE SURINAM POPULATION

CHAPTER 3a

HB CHAD OR $\alpha_2$23(B4)GLU→LYSβ_2 OBSERVED IN MEMBERS OF A SURINAM FAMILY IN ASSOCIATION WITH α-THALASSEMIA-2 AND WITH HB S

(Adapted from: Hemoglobin, 13:543-556, 1989)

CHAPTER 3a

HB CHAD OR $\alpha_2$23(B4)GLU→LYSβ_2 OBSERVED IN MEMBERS OF A SURINAM FAMILY IN ASSOCIATION WITH α-THALASSEMIA-2 AND WITH HB S

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(Adapted from: Hemoglobin, 13:543-556, 1989)

1. ABSTRACT

Three different haemoglobinopathies, i.e. Hb S, Hb Chad [$\alpha_2$23(B4)Glu→Lys], and α-thal-2 (-3.7 kb) have been observed in eight members of a family from Surinam. The propositus had all three abnormalities, while her mother and four of her half-brothers had Hb Chad together with an α-thal-2 heterozygosity or homozygosity. Gene mapping and dot-blot analysis of amplified DNA identified a G→A mutation in codon 23 of the $\alpha(2\alpha_1)$ hybrid gene resulting in the Glu→Lys substitution. The quantity of the α-Chad chain averaged 31.5% in its carriers with an additional α-thal-2 heterozygosity [$-\alpha^{Chad}$ (-3.7 kb)/$-\alpha$(-3.7 kb)]. These quantities are considerably higher than those reported for families from Chad, China, and Japan; the low levels of 14.5-24% Hb Chad in members of these families suggest a mutation on a chromosome with two α-globin genes [$\alpha\alpha^{Chad}/\alpha\alpha$ or $\alpha^{Chad}\alpha/\alpha\alpha$].

2. INTRODUCTION

The α chain variant Hb Chad or $\alpha_2$23(B4)Glu→Lysβ_2 was discovered in 1968 by Boyer et al (1) in a family living in the Republic of Chad, and has since been found in two Chinese families (2,3), and in a Japanese family (4). The quantity present in heterozygotes was reported to be about 16% in the Chad family, 14.5% in one Chinese family (2), and nearly 24% in a Japanese male (4). In this communication we describe the occurrence of the same variant in six members of a Surinam family including one with an additional Hb S heterozygosity; the quantity of Hb Chad in heterozygotes was considerably higher than reported for the other subjects.

3. MATERIALS AND METHODS

3.1 Blood Samples. These were collected in vacutainers with EDTA as anticoagulant and shipped by a special air delivery service from Paramaribo, Surinam to Augusta, GA; the total travel time was less than 48 hours. Informed consent was obtained.

3.2 Hb Analysis. Haematological data were collected with an automated cell counter. Red cell lysates were analyzed by IEF (5) and by cation exchange HPLC (6,7). The latter method made use of a 4.6 (ID) x 200 mm PolyCAT A WCX (5 nm) column (Custom LC, Inc., Houston, TX) and two developers. Developer A contained 35 mM Bis-Tris, 3 mM NH_4 acetate, 100 mg KCN per 100 ml (pH 6.47), and developer C contained 70 mM Bis-Tris, 33.7 mM NH_4 acetate, 300 mM Na acetate, 100 mg KCN per 1000 ml (pH 7.0). A gradient was applied between 75% developer A + 25% developer C as the initial solvent, and 100% developer C as the final solvent, at a flowrate of 0.8 ml/min over a period of 95 minutes. Quantitative data were obtained with the same HPLC procedure.

3.3 Structural Analysis. A larger quantity of Hb X (= Hb Chad) was isolated by preparative DEAE-cellulose chromatography (8). The α^X and β chains were separated on a column of CM-cellulose by the method of Clegg et al (9). Peptides from a tryptic digest of the α^X chain were separated by reversed phase HPLC (10); one isolated zone that contained two peptides was rechromatographed on a similar column but with different elution solvents (11). The determination of the amino acid composition of the isolated tryptic peptides was with a Pico-Tag amino acid analysis system (Waters; see manufacturers manual).

3.4 DNA Analysis. DNA was isolated from white cells by the method of Poncz et al (12). Gene mapping followed the same procedures used in earlier studies (13,14); hybridization was with the α probe. Amplification of the α2- and α1-globin genes with the Taq enzyme followed the procedures of Saiki et al (15,16) with primers specific for these genes (17,18). Hybridization was with ^{32}P-labelled oligonucleotides with compositions as reported elsewhere in this paper. Primers and probes were synthesized with the Applied Biosystems Model 380B DNA synthesizer.

4. RESULTS

The family has a mixed racial background, including Black. While participating in a general survey for the presence of haemoglobinopathies, the propositus, a healthy 30-year-old female, was discovered to have four Hb components. Subsequently, seven members of her immediate family were also investigated. Fig. 3a.1 gives the pedigree as well as the Hb patterns observed by IEF. A major slow-moving variant with a mobility between that of Hb A_2 and Hb S was present in six members, including the propositus. Hb S was observed in the propositus and her father, while a third abnormal band (labelled SX) was seen in the red cell lysate of the propositus. Moreover, all subjects with the X component had an additional minor Hb X_2 that suggested Hb X was an α chain variant.

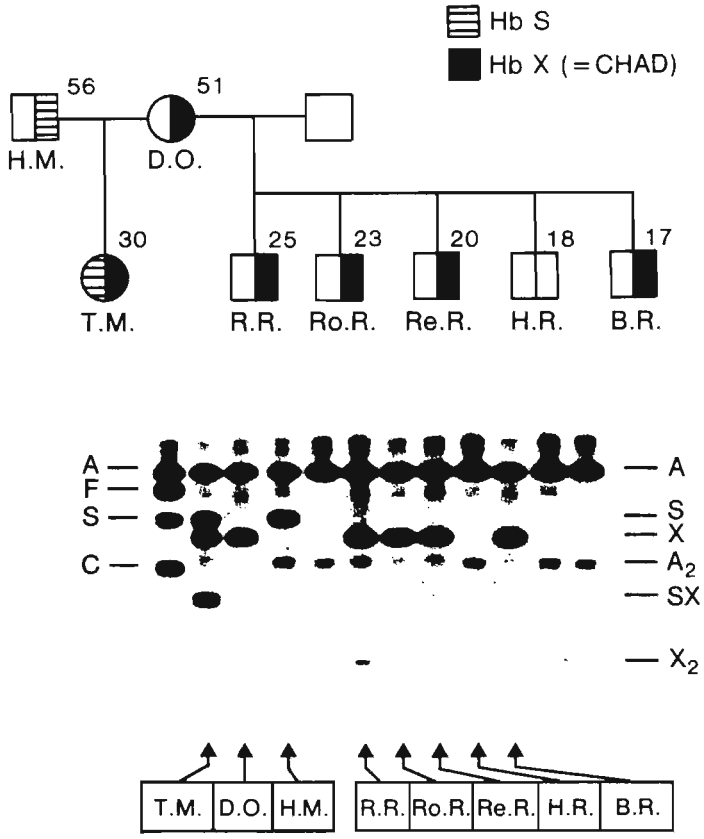

FIG. 3a.1 Pedigree of Family M with Hb S and the α chain variant Hb Chad (top). Separation of the Hb by IEF is shown at the bottom.

Chromatographic analysis by cation exchange HPLC gave a complete separation of the various Hb components (Fig. 3a.2). It also provided quantitative data for the major (A, S, X, SX) and minor (A_2, X_2) components seen in the propositus, for Hb S and Hb A_2 in her father, and for Hb X and Hb X_2 in the five Hb X heterozygotes. These data are listed in Table 3a.1 together with the results of the haematological analyses. The five Hb X heterozygotes fell into two groups: D.O. and B.R. had high Hb X levels (42.2 and 43.9%, including Hb X_2) and a distinct microcytosis and hypochromia (MCV 74-71 fl; MCH 21.7-19.4 pg), while the remaining three relatives (R.R., Ro.R, Re.R.) had lower Hb X levels (32.1, 31.8, and 31.5%, including Hb X_2) and a less distinct microcytosis and hypochromia (MCV 88-93 fl; MCH 25.3-28.5 pg). The data for the propositus were similar and her Hb X level was 31.5% (including Hb X_2) and her Hb S level was 32%; she had a more distinct microcytosis and hypochromia (MCV 68 fl; MCH 21.7 pg) than her relatives with a similar Hb X value. The father of the propositus had Hb S trait; his mild anaemia remains unexplained. One half-brother (H.R.) without Hb X, had a mild anaemia with a microcytosis and hypochromia comparable to that seen in his mother (D.O.) and his younger brother (B.R.).

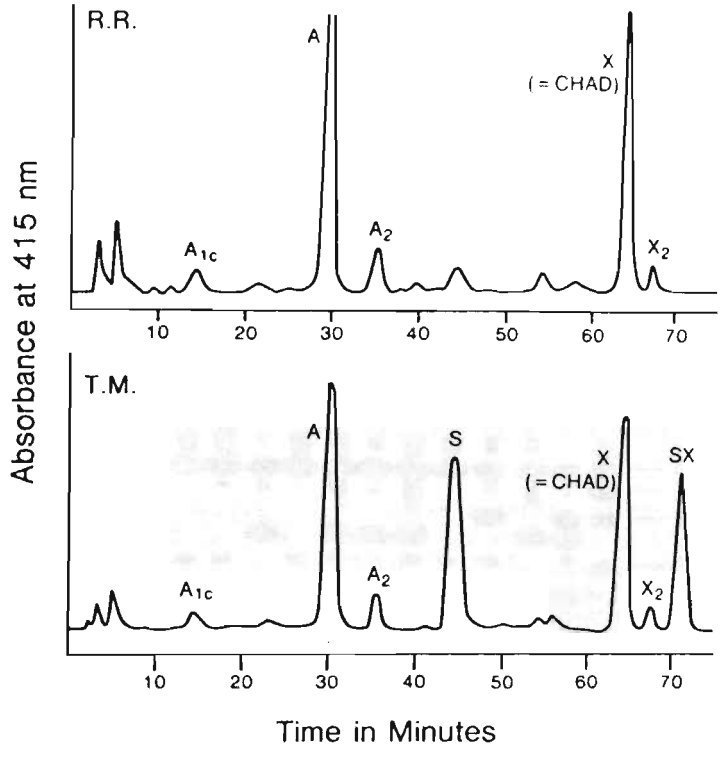

FIG. 3a.2 Separation of Hb types by cation exchange HPLC. R.R. is a simple Hb Chad heterozygote; T.M. is heterozygous for Hb S and Hb Chad. (SX = $\alpha_2^X\beta_2^S$). See text for methodology.

Structural studies were limited to the analysis of the soluble peptides in a tryptic digest of the α^X chain. Fig. 3a.3 gives an example of the chromatograms that were obtained. All peptides were recovered except αT-4 that was apparently split into two fragments. Peptide αT-4A eluted ahead of αT-1, while αT-4B eluted together with the double peptide αT-1,2; the latter two readily separated upon rechromatography (insert of Fig. 3a.3). Table 3a.2 lists the amino acid compositions of αT-4A and αT-4B. These data readily identified a Glu→Lys substitution in position 7 of this peptide or in position 23 of the intact α chain and identified the variant as Hb Chad.

Gene mapping analyses involved DNA from all eight members of this family. Data obtained with the enzymes Bam HI and Bgl II are shown in the top section of Fig. 3a.4. Abnormal fragments were observed with both enzymes; only one member (H.M., see pedigree of Fig. 3a.1) had four -globin genes due to the presence of a -3.7 kb deletion on one chromosome, while B.R., D.O., and H.R. had only two α-globin genes and were homozygous for the α-thal-2 (-3.7 kb) deletion. Subjects B.R. and D.O. are also heterozygous for Hb Chad with high levels of the α^X chain (~43%), indicating that the α-Chad mutation is present on an α-thal-2 chromosome.

TABLE 3a.1 Haematological and Hb Composition Data

Subject[a]	H.M.	T.M.	D.O.	R.R.	Ro.R.	Re.R.	B.R.	H.R.
Sex-Age	M-56	F-30	F-51	M-25	M-23	M-20	M-17	M-18
Diagnosis	AS	SX	AX	AX	AX	AX	AX	AA
Hb (g/dl)	11.6	9.2	11.8	14.9	13.2	13.7	11.3	10.9
PCV (l/l)	0.41	0.29	0.415	0.525	0.46	0.49	0.41	0.395
RBC (10^{12}/l)	4.17	4.24	5.59	6.00	5.21	5.27	5.81	5.40
MCV (fl)	98	68	74	88	93	73		71
MCH (pg)	27.8	21.7	21.1	28.4	25.3	26.0	19.4	20.2
MCHC (g/dl)	28.3	31.7	28.4	28.4	28.7	28.0	27.6	27.6
Hb A_2 (%)[b]	3.8	1.9	1.1	1.8	1.5	1.5	1.2	2.6
Hb X_2 (%)[b]	0	1.2	1.2	1.1	1.0	1.0	1.4	0
Hb S (%)[b]	37.0	21.4	0	0	0	0	0	0
Hb X (%)[b]	0	19.7	41.2	31.0	30.8	30.5	42.5	0
Hb SX (%)[b]	0	10.6	0	0	0	0	0	0
α^X (%)[b]	0	31.5	42.4	32.1	31.8	31.4	43.9	0
β^S (%)[b]	37.0	32.0	0	0	0	0	0	0
# of α genes[c]	4	3	2	3	3	3	2	2

[a] See pedigree in Fig. 3a.1.

[b] Quantitation by cation exchange HPLC; see text for details. α^X (%) = $\%X_2 + \%SX$ ($= \alpha_2^X\beta_2^S$); β_2^S (%) = %S + %SX.

[c] By gene mapping; see text for details.

TABLE 3a.2 Amino Acid Composition of the αT-4A and αT-4B Peptides of the α Chain of Hb X (= Hb Chad)[a]

Amino Acid (positions in α)	αT-4A (17-23)	αT-4B (24-31)
Glutamic acid	**0 (1)**	**1.59 (2)**
Glycine	1.97 (2)	1.04 (1)
Histidine	0.80 (1)	
Arginine		0.91 (1)
Alanine	2.12 (2)	2.06 (2)
Valine	1.05 (1)	
Tyrosine		0.94 (1)
Leucine		1.12 (1)
Lysine	**1.06 (0)**	

[a] In mole/mole; numbers expected for the α^A chain are listed between parentheses.

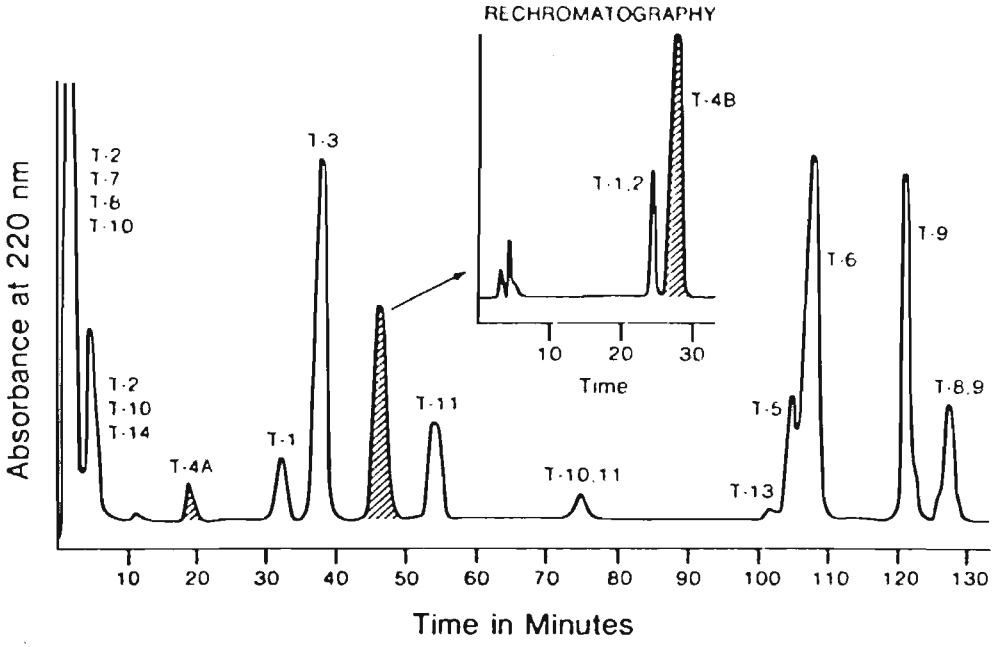

FIG. 3a.3 Separation of the soluble peptides of a tryptic digest of the α^X (= α-Chad) chain by reversed phase HPLC using the method of Wilson et al (10). Insert: Rechromatography of a mixture of the αT-1,2 and αT-4B peptides by reversed phase HPLC following the method described by Schroeder (11).

The results of the amplification-hybridization experiments are summarized in the bottom section of Fig. 3a.4. Data obtained for H.M. ($\alpha\alpha/\alpha\alpha$) show amplification of both the α2 and α1 genes, while those for control C_2 and subject H.R. [$-\alpha/-\alpha$; homozygotes for the α-thal-2 (-3.7 kb) anomaly] indicate amplification of the α1-globin gene only [or of the α2α1 hybrid gene resulting from the α-thal-2 (-3.7 kb) deletion]. DNA from five members hybridized to the mutant probe; the mutation was present on the α1-globin gene (or on the α2α1 hybrid gene of the α-thal-2 chromosome) and not on the α2-globin gene. Two of the five samples (from B.R. and D.O) showed no amplification of the α2 gene (both subjects are α-thal-2 homozygotes), while for the three remaining samples, a definite amplification of the α2 gene was detected with the normal probe (T.M., Re.R., and R.R.; all three are α-thal-2 heterozygotes).

5. DISCUSSION

Data from the structural analyses reported here identify the variant as Hb Chad with a Glu→Lys substitution at position 23 of the α chain. This is the fifth report dealing with this Hb variant; it is impossible to speculate if this abnormality is of Chinese, Japanese, or Black origin, or if it is the result of a new mutation in this population in South America. The

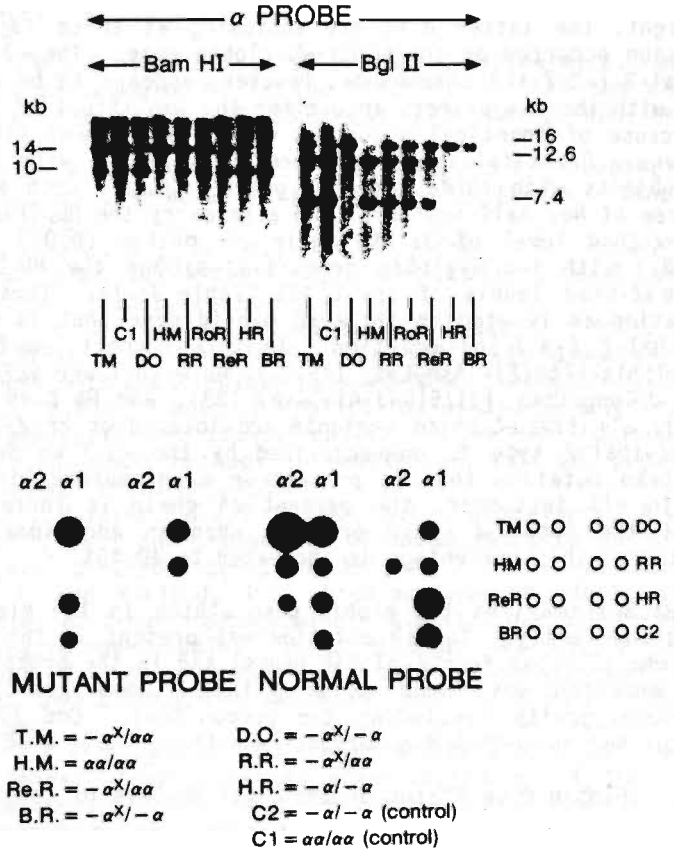

FIG. 3a.4 α-Globin gene mapping analyses for eight members of Family M. Top: Identification of the 3.7 kb deletion (α-thal-2) in Bam HI and Bgl II digests using the ^{32}P-labelled α probe for the detection of the normal and abnormal bands. Bottom: Dot-blot analyses of amplified DNA with the normal and mutant probes allowing the detection of the α-Chad mutation. Amplification was with primer sets specific for the α2- and α1-globin genes; their compositions and locations are listed in Ref. 18. The various individuals, identified by their initials, are shown in the pedigree of Fig. 3a.1.

Normal Probe:	5'	CAC	GGT	GGC	G	AG	TAT	GGT	G	3'
Mutant Probe:	5'	CAC	GGT	GGC	A	AG	TAT	GGT	G	3'
Codon #		20	21	22		23	24	25		

Surinam family is distinct from the previously reported families because: **a)** Hb Chad occurred in one member together with Hb S, resulting in the formation of the hybrid Hb S-Chad or $\alpha_2^{Chad}\beta^S$, and **b)** the Hb Chad occurred on a -α(-3.7 kb) thalassaemic chromosome. Data supporting this conclusion come from gene mapping experiments (Fig. 3a.4; top) and from dot-blot analyses after amplification of the α2- and α1-globin genes (Fig. 3a.4; bottom).

At first sight, the latter data are confusing as these suggest that the α-Chad mutation occurred on the minor α1-globin gene. The α2 1 hybrid gene of the α-thal-2 (-3.7 kb) chromosome, however, appears to be amplified perfectly well with the two primers in use for the amplification of the α1-globin gene because of identical sequences of its 3' segment with that of the α1-globin gene. Quantitative Hb data are in agreement with the above conclusions; subjects with three α-globin genes ($-\alpha/\alpha\alpha$), such as the propositus and three of her half-brothers, who also carry the Hb Chad abnormality, average an α-Chad level of 31.7%, while the mother (D.O.) and the half-brother (B.R.) with two α-globin genes ($-\alpha/-\alpha$) and the Hb Chad heterozygosity, have α-Chad levels of about 43% (Table 3a.1). Thus, the α23(B4) Gly→Lys mutation is located on the α2β1 hybrid gene that is characteristic for the α-thal-2 (-3.7 kb) deletion. In this respect, Hb Chad resembles Hb G-Philadelphia [α68(E17)Asn→Lys] (19,20), Hb Q-Thailand [α74(EF3)Asp→His] (21,22), Hb J-Tongariki [α115(GH3)Ala→Asp] (23), and Hb Duan [α75(EF4)Asp→Ala] (24,25); all five α chain variants are located on an α-thal-2 chromosome. The α-thal-2 type is characterized by the -3.7 kb deletion except for the Hb Duan mutation that is present on a chromosome with the -4.2 kb deletion. In all instances, the percent α^X chain is increased to about one-third of the total α chain present; when an additional α-thal-2 is located in trans, this percentage is increased to 40-45%.

Table 3a.3 summarizes the globin gene status in the eight members of this interesting family. The β^S mutation was present in the father of the propositus (who also had four α-globin genes) and in the propositus herself. The α-Chad mutation was found on an α-thal-2 homozygosity and for an α-thal-2 heterozygosity (including the propositus). One half-brother of the propositus had an α-thal-2 homozygosity without the α-Chad mutation.

TABLE 3a.3 Globin Gene Status of the Eight Members of Family M

Subject(s)[a]	Genes	;	Genes
H.M.	$\alpha\alpha/\alpha\alpha$	;	β^A/β^S
D.O.; B.R.	$-\alpha^X(-3.7)/-\alpha(-3.7)$	;	β^A/β^A
H.R.	$-\alpha(-3.7)/-\alpha(-3.7)$	;	β^A/β^A
R.R.; Ro.R.; Re.R.	$-\alpha^X(--3.7)/\alpha\alpha$	;	β^A/β^A
T.M.	$-\alpha^X(-3.7)/\alpha\alpha$	;	β^A/β^S

[a] See pedigree of Fig. 3a.1.

6. ACKNOWLEDGEMENTS

This research was supported in part by the United States Public Health Service Research Grants HLB-41544 and HLB-05168. This is contribution #1175 from the Department of Cell and Molecular Biology at the Medical College of Georgia in Augusta, Georgia.

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CHAPTER 3b

THE β^S GENE IN THE SURINAM POPULATION: HAEMATOLOGICAL AND GENETIC OBSERVATIONS

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HAEMATOLOGICAL AND GENETIC OBSERVATIONS

1. ABSTRACT

Haematological and genetic data have been collected for 12 SS, one SC, and four S-β-thal patients and their relatives. Haplotyping, using ^{32}P-labelled oligonucleotide specific probes, identified haplotypes #19 and #20 as the major types among the Surinam population. Both haplotypes have been reported to originate from West and Central Africa. These results suggest that the β^S gene among Surinamese was introduced by the slave trade, common in the 19th century in that part of the world. Haematological data showed that AS individuals are clinically normal, while the SS patients have a severe anaemia with a variety of complications, comparable to those observed for SS patients from Africa and the United States. The AS individuals did not have significantly increased levels of circulating foetal Hb (Hb F < 1%), while the foetal Hb level in the 12 SS patients averaged about 10%.

2. INTRODUCTION

The occurrence of the β^S gene in the Surinam population was first described in 1938 by Wolf (1) who made sickle cell preparations of the blood from people living in two Bush Negro villages. Data from several other studies have been reported (2-7) and in some, the frequencies of Hb S among the different ethnic groups were determined. Frequencies up to 0.22 were found among the different Bush Negro tribes, while the Creole group showed a frequency of about 0.10, which is comparable to that found in American Blacks (8). Some isolated cases of Hb S heterozygosities have been observed among the native Indian and Hindustani groups. The presence of Hb S among the native Indians was ascribed to admixture with Creoles (9), and in one case in which the β^S gene originated from a Hindustani male, haplotyping of the gene was not performed (10).

The clinical expression of sickle cell anaemia is severe in the Surinam population. Two factors that are considered to modify the disease are the presence of an α-thal and an increased production of Hb F (11). The variation in the level of Hb S in SS patients is related to the different genetic backgrounds on which the β^S gene occurs. There are at least five different chromosomes bearing the β^S mutation (12-18). These chromosomes have been characterized by specific haplotypes, and were identified as haplotype #19 (Benin), #20 [Bantu or CAR (Central African Republic)], #3 (Senegal), #31 (Saudi Arabia-India), and #17 (Cameroon or $^{A}\gamma^{T}\delta$). Homozygotes for haplotypes #19 or #20 have Hb F levels of about 10%, while SS patients with haplotypes #3 or #31 may have Hb F levels of 20 to 35% (19,20). Haplotype #31 has been discovered in patients from India and the eastern part of Saudi Arabia; these patients have a considerably milder disease.

Haplotypes #19, #20, and #3 are the major haplotypes present in Black SS patients from Jamaica, West and Central Africa, and the Southeastern United States (21,22). In the investigation performed by Aluoch et al (23), 16 Surinamese SS patients living in The Netherlands were studied, and their haplotypes were compared to those of Eti-Turk SS patients. The major haplotype among the 16 Surinamese SS patients were also #19, #20, and #3. Here we describe the use of haplotype-specific probes for the identification of particular haplotypes in SS patients and Hb S heterozygotes living in Surinam. A comparison will be made between the haematological data for the SS patients with the different haplotypes present in the Surinam population.

3. MATERIALS AND METHODS

3.1 Patients. Two hundred and one newborn babies were screened by IEF for the possible presence of haemoglobinopathies. Eleven individuals had the FAS phenotype and were included in this study. An additional 51 individuals (12 with SS and 39 with AS) from 35 unrelated families were selected with the help of local physicians. Data for relatives with a Hb S trait were collected so that accurate haplotypes for the SS patients could be determined. The newborn babies were included in an attempt to determine the incidence of the β^S gene among different ethnic groups. All individuals with the β^S gene were enrolled in a larger screening project, of which the haplotype data have been reported elsewhere (24).

3.2 Methods. Methodology used in this study has been described in Chapter 2. Fig. 3b.1 shows the positions of the sequence differences used to construct the specific oligonucleotide probes for the different haplotypes; this information was obtained from Refs. 25-29.

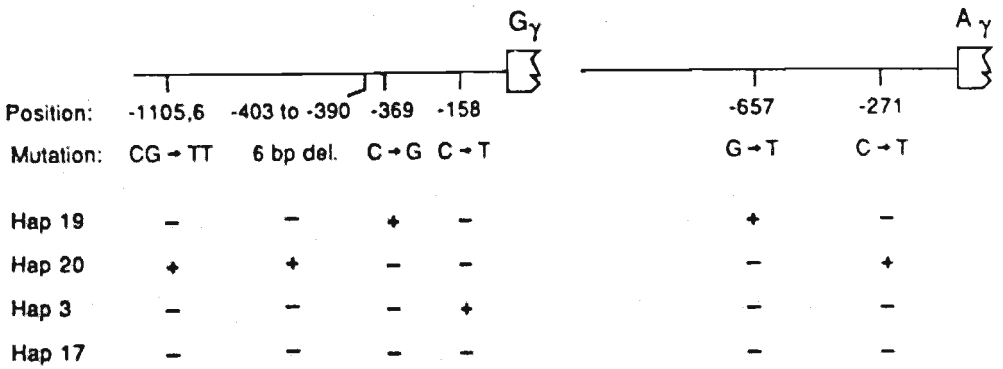


FIG. 3b.1 Haplotype-specific sequence differences in the 5' flanking region of the G_γ- and A_γ-globin genes among β^S chromosomes; + or - indicates the presence or absence of a particular mutant site.

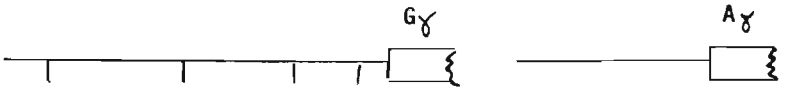
4. RESULTS

4.1 The Haplotypes. The results obtained for 62 individuals with 74 β^S chromosomes are summarized in Table 3b.1; the same table contains data for other populations which is also included in another study (24). Characterization of the β^S chromosome with haplotype #17 was based upon the presence of the $^{A\gamma T}\beta^S$ chain detected by reversed phase HPLC. The low level of Hb F in most AS subjects made it impossible to exclude haplotype #17 in these cases. The most common type was haplotype #19, that occurred on 41 of the 74 chromosomes. Haplotype #20 was observed 23 times, haplotypes #3 or #31 twice, and haplotype #17 only once; eight chromosomes were uncharacterized. Identification of haplotype #19 chromosomes was based on the presence of two specific mutations, namely a G→T mutation at position -657 relative to the Cap site of the $^{A}\gamma$-globin gene, and a C→G mutation at position -369 5' to the $^{G}\gamma$-globin gene. Haplotype #20 was determined by the presence of three specific mutations, namely C→T at position 271 5' to $^{A}\gamma$, CG→TT at positions -1105 and -1106 to $^{G}\gamma$, and a 6 bp deletion between positions -403 to -390 5' to $^{G}\gamma$ (Fig. 3b.1). Haplotypes #3 and #31 had C→T mutations at position -158.

TABLE 3b.1 Distribution of Particular Haplotypes Among Different Populations

Country	No. of β^S Chroms. Tested	Haplotypes #19	#20	#3 or #31	#17	Rare
Southeastern USA	366	205	71	58	13	19
Kenya	55	1	52	-	-	2
Turkey	179	174	-	-	-	5
Syria	13	13	-	-	-	-
Tunisia	115	109	-	-	-	6
Italy	70	70	-	-	-	-
Surinam	74	41	23	2	-	8
South Africa	5	-	-	5	-	-
Canada	14	10	-	4	-	-
India	105	-	-	94	-	11
Greece	14	13	-	1	-	-
West Nigeria	104	97	-	-	3	4

Our data also showed that some of the specific mutations occurred on a β^A chromosome. An example is the AS individual S-502, who had mutations specific for two different haplotypes on the same chromosome. This information is provided in Fig. 3b.2. The daughter of subject S-502 had severe SS disease; her two β^S chromosomes had the #20 haplotype. This information facilitated the identification of the unusual haplotype in her mother (S-502).



Position:	-1105,6	-403 to -309	-369	-158	-657	-271
Mutation:	CG→TT	6 bp del.	G→G	C→T	G→T	C→T
Haplotype #19	-	-	+	-	+	-
Haplotype #20	+	+	-	-	-	+
S-502						
Haplotype #20 (β^S)	+	+	-	-	-	-
* (β^A)	-	-	-	-	+	+

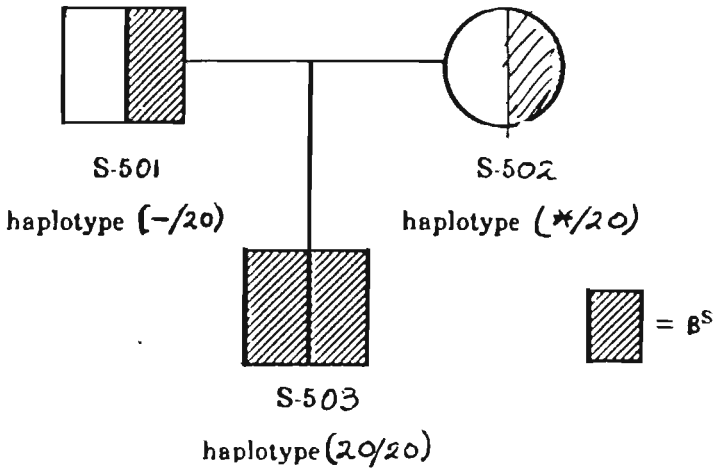
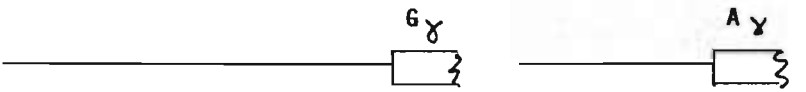


FIG. 3b.2 β^S Haplotyping for family members of a homozygous haplotype #20 subject. **Top:** The haplotype-specific mutations for the β^S chromosome of the propositus (mother S-502) and her β^A chromosome. **Bottom:** The pedigree of this family indicating the pattern of inheritance of the genes. The asterisk (*) denotes an atypical combination.

Another interesting observation was made in SS patient S-532 (Fig. 3b.3). This 7-year-old boy carried two β^S chromosomes, both with haplotype #19, except that one chromosome was also characterized by the presence of a C→T mutation at position -158 5' to $^G\gamma$. This mutation is known to increase the production of $^G\gamma$ chains, and indeed, a $^G\gamma$ to $^A\gamma$ ratio of 60

to 40 was found in patient S-502, while the Hb F of all other SS patients had a ratio of 40 to 60. Further testing of the parents indicated that this special β^S chromosome was inherited from the father (S-534). Of the several hundreds of β^S chromosomes with haplotype #19 tested so far, only the β^S chromosome of this Surinam family is know to be characterized by haplotype #19 and by the simultaneous presence of the C→T mutation at position -158.



Position:	-1105,6	-403 to -309	-369	-158	-657	-271
Mutation:	CG→TT	6 bp del.	C→G	C→T	G→T	C→T
Haplotype #19	-	-	+	-	+	-
Haplotype #3	-	-	-	+	-	-
S-532						
Haplotype #19 (β^S)	-	-	+	-	+	-
Haplotype #19 (β^S) (Xmn I +)	-	-	+	+	+	-

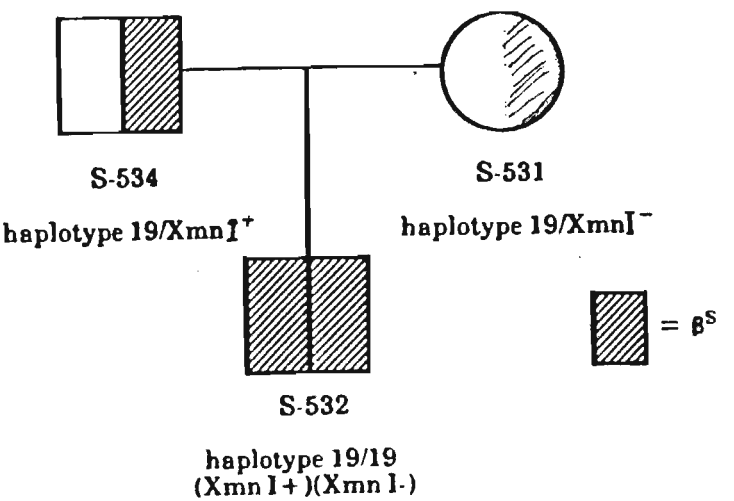


FIG. 3b.3 Top: Haplotype analyses for a family with atypical haplotype patterns. Bottom: Pedigree of this family showing the atypical combination of haplotype #19 with an Xmn I + site on the same chromosome.

4.2 Clinical Expression of the SS Disease. Haematological data and the Hb F levels in the patients homozygous for the different haplotypes are listed in Table 3b.2. Hb levels in patients S-444 and S-471 were low due to blood transfusions given prior to collection of the samples. The exact age of patient S-527 was not known but estimated at 45 years. This male individual is a fisherman and performs intensive physical labour on a daily basis. Unfortunately, we were unable to perform a detailed family study, which precluded the exclusion of a possible Hb S-HPFH combination. The high level of Hb F (22%) is likely the main reason for his well-being, as such levels probably interfere with the polymerization of deoxy Hb S molecules. It is worth noting that the haematological data for patient S-532 (see (Table 3b.2) are comparable to those of the other patients.

TABLE 3b.2 Haematological Data for 12 SS Patients From Surinam

Case	Sex-Age	RBC 10^{12}/l	Hb g/dl	MCV fl	MCH pg	Hb A_2 %[a]	Hb F %[a]	$G\gamma$ %[b]	$A\gamma$ %[b]	$A\gamma T$ %[b]
Haplotypes 19/19										
S-261	F-18	3.02	8.3	89	27.4	-	10.2	42.0	58.0	0
S-444	F-11	3.3	8.9	97	26.6	3.8	<1.0	40.0	60.0	0
S-471	M- 6	3.2	5.7	74	17.6	-	1.3	36.0	64.0	0
S-519	F-10	1.73	4.9	107	28.3	2.5	10.2	40.0	60.0	0
S-527	M-45(?)	3.5	11.1	103	31.6	3.3	21.9	48.0	52.0	0
S-532[c]	M- 7	2.17	6.8	104	31.3	2.7	10.0	60.0	40.0	0
Haplotypes 19/20										
S-254	F-18	3.79	8.6	74	22.7	-	5.9	37.0	63.0	0
S-521	F-16	2.22	6.7	106	30.0	3.1	6.0	44.0	56.0	0
Haplotypes 20/20										
S-503	F-23	1.48	4.8	105	27.7	3.6	7.8	37.0	63.0	0
S-516	F- 9	1.52	3.9	95	25.0	3.9	5.8	42.0	58.0	0
S-517	M- 6	2.58	6.4	93	24.8	3.8	3.5	37.0	63.0	0
Haplotypes 19/17										
S-447	M- 8	1.2	3.3	113	27.5	2.7	11.2	44.8	34.1	21.1

[a] By cation exchange HPLC.

[b] By reversed phase HPLC.

[c] See Fig. 3b.3 and text for further details.

All other patients with haplotypes 19/20, 20/20, and 19/17 had severe disease, low Hb F levels with a $G\gamma$ value of about 40%. The incidence of haplotype #17 was low as only one of the 12 SS patients was heterozygous for this variant. Haplotypes #3 and #31 were not found.

Analyses of the β^S haplotypes of the newborn babies with a Hb S heterozygosity showed that haplotype #19 was the most common type (nine of 11 babies had this haplotype and two had haplotype #20). The limited number

of AS newborns makes it impossible to determine the frequencies of these haplotypes.

5. CONCLUSION

Based upon population data provided by the Surinam government (Table 3b.3), it can be calculated that at least 15,000 Hb S heterozygotes are living in Surinam. This estimate is based upon the 10% frequencies expected and observed among Creoles and Bush Negroes. These data also suggest that about 500 SS patients are living in Surinam at present. The high birth rate among the Bush Negro population and their urbanization makes it likely that this number is too low.

TABLE 3b.3 Composition of the Surinam Population[a]

Racial/Ethnic Background	1950	1964	1971	1980
Creole	74,918	114,961	119,009	123,285
Hindustani	63,770	112,633	142,917	118,983
Indonesian	35,949	48,463	57,688	58,004
Chinese	2,468	5,339	6,029	5,494
European	2,626	4,322	3,999	1,558
Indian	1,846	2,979	3,929	4,664
Others	1,703	2,986	6,077	2,274
Unknown	401	522	-	710
"Bosneger in stamverb"	19,180	27,698	35,835	33,915
Native Indian	1,700	4,308	4,121	6,353
TOTAL	204,561	324,211	379,607	355,240

[a] Based on census data.

Haplotype-specific probes are valuable tools in the determination of β^S haplotypes; all 12 SS patients could be characterized by this technique. Some of the β^A chromosomes in the Surinam population may have mutations that are considered to be specific for the β^S chromosomes.

Although the group of 12 SS patients and 11 AS newborn babies is small, the data obtained indicate that most, if not all, β^S chromosomes carry a haplotype that is associated with low levels of Hb F and severe disease in the homozygote. Haplotypes #19 and #20 indeed appear to occur together in nearly all tested subjects; data from several surveys conducted in other countries have indicated that such β^S chromosomes are invariably observed in SS patients who are severely affected by this disease, as evidenced by severe haemolysis and frequent crises.

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CHAPTER 4

THALASSAEMIAS IN THE SURINAM POPULATION

CHAPTER 4a

MOLECULAR CHARACTERIZATION OF β-THALASSAEMIA AMONG THE SURINAMESE

CHAPTER 4a

MOLECULAR CHARACTERIZATION OF β-THALASSAEMIA AMONG SURINAMESE

1. ABSTRACT

Twenty Surinamese families with β-thal were screened for particular mutations using ^{32}P-labelled oligonucleotide probes, sequence analysis, and gene mapping. Thirteen different mutations were detected. The IVS-I-5 (G→C) was the most frequently observed, being present in six of the 20 families, followed by the frameshift at codons 41/42 (-TTCT) that was observed in three families. The Creole and Javanese ethnic groups were the most heterogeneous. Seven different mutations [-88 (C→T); -29 (A→G); codons 41/42 (-TTCT); codon 47 (+A); IVS-I-849 (A→G); IVS-II-1 (G→A); -28 (A→G); 1.393 kb deletion] were found in eight Creole families and four mutations in five Javanese families [IVS-I-5 (G→C); codons 8/9 (+G); codon 35 (-C); IVS-I-2 (T→C)]. Three different β-thal alleles were detected in six Hindustani families [IVS-I-5 (G→C); codons 41/42 (-TTCT), and a δβ-thal due to a 31.6 kb deletion]. One family, of Lebanese origin, had the frameshift at codons 41/42 (-TTCT). A unique frameshift mutation was detected in one family belonging to the Surinam Creole group [codon 47 (+A)].

2. INTRODUCTION

β-Thal has a worldwide distribution with high frequencies in the Mediterranean Basin, India, Southeast Asia, and the southern part of Mainland China (1); it is believed to be rare in Western Africa. The slave trade and the migration of contract labourers from these regions have contributed to the introduction of the β-thal genes in the Surinam population. Molecular characterization of β-thal alleles have been made for the populations of the Mediterranean area, China, Southeast Asia, and India. The spectrum of β-thal mutations in these populations will likely be reflected in the immigrants living in Surinam (see Table 3b.3 for the composition of the Surinam population); β-thal alleles characteristic for Indians, Indonesians, Chinese, and Blacks can be expected to be present in Surinam. All β-thal mutations have been characterized in 20 families. The analyses involved dot-blot screening, sequencing, and gene mapping using methods described in Chapter 2. Some of the families are more extensively described elsewhere.

3. MATERIALS AND METHODS

3.1 Patients. Most of the patients were contacted with the help of local physicians. Patients with β-thal major and their relatives were enrolled in this study. Patients with elevated Hb A_2 levels, who were on record at the laboratory of the Academic Hospital, were also invited to participate. Handling of blood collections and shipment was as mentioned in Chapter 2.

3.2 Methods. All the methods used for this study have been extensively described in Chapter 2.

4. RESULTS

Table 4a.1 summarizes the data obtained in this study. Thirteen different β-thal alleles were found. Two, namely the frameshift at codon 47 (+A) and the δβ-thal due to the 31.6 kb deletion, have not been previously reported and will be discussed in detail elsewhere in this dissertation.

TABLE 4a.1 β-Thal Mutations in the Surinam Population

Mutation	Type	Ethnic Group
-29 (A→G)	β^+	Creole
-28 (A→G)	β^+	Jewish/Creole mix
Codons 8/9 (+G)	β°	Javanese
Codon 35 (-C)	β°	Javanese
Codons 41/42 (-TTCT)	β°	Creole; Hindustani; Javanese; Lebanese
Codon 47 (+A)	β°	Creole
IVS-I-2 (T→C)	β°	Javanese
IVS-I-5 (G→C)	β^+	Javanese; Hindustani
IVS-II-1 (G→A)	β°	Creole
IVS-II-849 (A→G)	β°	Creole
1.393 bp deletion	β°	Creole
31.6 kb deletion	β°	Hindustani
Codon 26 (GAG→AAG)	β^+	Javanese; Hindustani

4.1 A Short Review of the Observed β-Globin Gene Defects:

-29 (A→G). This β^+-thal mutation was originally described among American Blacks (2) and Chinese (3); it was present in members of three Creole families. One member of one family had a compound heterozygosity for Hb S and this allele. The clinical symptoms in this child were mild, with microcytosis, elevated Hb A_2 (5.5%), and a Hb F level of 4.7%. A compound heterozygosity for the -29 (A→G) and the IVS-II-849 (A→G) mutations was found in two of the three families; they will be more fully reported elsewhere in this dissertation.

-28 (A→G). This β^+-thal mutation was originally found in the Chinese (4); only one Surinam family had this mutation. Two sisters in this Creole family had Jewish and Chinese admixtures. The clinical symptoms of these simple heterozygotes were as expected for promoter mutations, very mild.

Codons 8/9 (+G). This β°-thal was originally observed in the Asian Indian population and is also present in Turkish and Indonesian populations (5-7). One Javanese family with a compound heterozygosity for this allele and Hb E was found. Detailed information about this family is given elsewhere in this dissertation.

IVS-I-2 (T→C). This β°-thal was found in one Javanese family in combination with Hb E. Sequencing of amplified single strand DNA from one of the members revealed its detection.

IVS-I-5 (G→C). This β⁺-thal allele is the most common β-thal mutation in the Asian Indian population (8). It has also been reported among Chinese (9), Malay (10), and Melanesians (11). In this study this mutation was present among the Hindustani group which originated from India (four of six families). One patient was homozygous for this mutation; this child was severely affected and transfusion-dependent. Another child from a second family had a compound heterozygosity for this allele and the δβ-thal (see Chapter 6). Members of three additional families were simple heterozygotes for this mutation.

Codon 35 (-C). This β⁺-thal was first reported in 1989 among Malays (10) and was later observed in Indonesians (11). Two families, both with members having a compound heterozygosity for this allele and Hb E were detected. The patients were anaemic but were not receiving transfusions on a regular basis (discussed elsewhere in this chapter).

Codons 41/42 (-TTCT). This β°-thal which is primarily found in the Chinese (12) was surprisingly found in a Creole family, a Hindustani family, and a Lebanese family. One child of the Hindustani family had a compound heterozygosity for this frameshift and Hb E (discussed elsewhere in this chapter). The children of the Creole family were compound heterozygotes for Hb S and this frameshift. Although Hb levels in these children were low, there were never admitted to the hospital for sickle cell crises. The -thal mutation was inherited from the father. It is known from history that the first Chinese immigrants were allowed to marry Black women; thus, the introduction of this Chinese β-thal gene likely occurred through Chinese admixture. The family had no knowledge of any racial admixture. The relatively mild clinical symptoms in the two children are difficult to understand.

Codon 47 (+A). This β°-thal was unique because it occurred on a chromosome that also carried a mutation in the δ-globin gene and was the first time that it had been described. It was present in a large Creole family; a detailed description is given later in this chapter.

IVS-II-1 (G→A). This β°-thal has been reported in the Mediterranean population (13) and American Blacks (14). The patient found in our survey was first diagnosed as a homozygous SS patient with mild clinical symptoms. Our screening showed that this patient was a compound heterozygote for the IVS-II-1 (G→A) mutation and Hb S. The propositus was of Creole origin.

IVS-II-849 (A→G). The families with this condition have been described before because of their compound heterozygosities for this and the -29 (A→G) mutations.

The 1.393 bp deletion. This β°-thal has been observed among American Blacks (15). The Creole family in which this deletion occurred had a son who was a compound heterozygote for this deletion and Hb S. A detailed description of this family is given later in this chapter.

The 31.6 kb Deletion. This β^0-thal was found in a Hindustani family and is discussed in detail in Chapter 6.

Codon 26 (GAG→AAG). This substitution activates an alternative donor splice site at codon 26 resulting in a β^+-thal phenotype. The patients observed in the Surinam population were of Javanese or Hindustani origin.

5. DISCUSSION

The spectrum of β-thal mutations in the Surinam population is broad; this is not surprising because nearly every ethnic group is found in Surinam. Interestingly, the -88 (C→T) allele, a mutation that is frequently found among American Blacks, was not observed in the Creole or Bush Negro groups. Other studies, conducted in The Netherlands (16,17), also do not report this mutation. The majority of the Hindustani patients were carriers of the IVS-I-5 (G→C) mutation, which is definitely a reflection of their Asian Indian background. No Black mutations were found in this group. The Creole group is by definition a mixed ethnic group, and this is also reflected by the many different β-thal alleles that were found. Almost all β-thal mutations in the other ethnic groups could also be found in the Creole group. Prenatal diagnosis is therefore considerably more complicated for this Creole group than for Javanese or Asian Indians. Systematic screening for β-thal mutations is needed in the Surinam population because of the interaction of the mutations with the common Hb S and Hb E abnormalities; such combinations can lead to severely affected offspring. The search for β-thal alleles among the isolated inhabitants of the Bush Negro villages will be helpful in determining the original thal spectrum from the Creole group, including the newly discovered codon 47 (+A) mutation.

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CHAPTER 4b

COMPOUND HETEROZYGOSITY FOR A MILD β^+ AND RARE β°-THALASSEMIA ALLELE

(Adapted from: Acta Haematol., 84:135-138, 1990)

CHAPTER 4b

COMPOUND HETEROZYGOSITY FOR A MILD β^+ AND A RARE β°-THALASSEMIA ALLELE

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(Adapted from: Acta Haematol., 84:135-138, 1990.)

1. ABSTRACT

Haematological and Hb composition data are presented for 14 members of a Surinam family (and for one unrelated subject) with either a β-thal heterozygosity [five with the -29 (A→G) β^+ mutation and five with the IVS-II-849 (A→G) β° mutation] or a compound heterozygosity (the five remaining patients). Identification of the mutation was by hybridization of amplified DNA with ^{32}P-labelled synthetic oligonucleotides. The data indicate distinct differences between the two groups of heterozygotes, mainly in degree of microcytosis and hypochromia, in Hb A_2 levels, and in the level of $^G\gamma$ (high in the -29 heterozygotes and low in the IVS-II-849 heterozygotes). The five compound heterozygotes had a thalassaemia intermedia with high Hb F levels (high $^G\gamma$), elevated Hb A_2, and Hb A levels comparable to those seen in patients with a homozygosity for the -29 mutation or with the combination of this β^+-thal and Hb S. An α-thal-2 heterozygosity (-3.7 kb deletion) was present in two patients. Their haematological data were improved over those for the patients with four α-globin genes; one was the mother of two sets of twins. The high $^G\gamma$ value in the Hb F of the compound heterozygotes suggests that the high Hb F production in the condition is mainly directed by the chromosome with the -29 (A→G) mutation.

2. INTRODUCTION

Modern methodology has made it possible to rapidly identify the various β-thal alleles, of which more than 80 have been described thus far (1). Many surveys have been undertaken in different countries with data important for diagnosis and treatment (for review see Ref. 2). Occasionally, some interesting families participate in these studies and the data collected provide detailed information about the disease that is present. This is the case for a large Surinam family in which two different β-thal alleles interact; this report provides details of this study.

3. MATERIALS AND METHODS

Blood samples from 15 members of this family and from an unrelated double heterozygote were collected in vacutainers with EDTA as anticoagulant and shipped in wet ice by a special courier service from Paramaribo, Surinam to Augusta, GA. Informed consent was obtained.

Haematological data were obtained with an automated cell counter; the samples were 5 days old at the time of analysis. Hb F, Hb A_2, and Hb A were quantitated by cation exchange HPLC using a polyCAT column (3,4). The relative quantities of $^{G}\gamma$ and $^{A}\gamma$ in the Hb F were determined by reversed phase HPLC (5,6); DEAE-cellulose chromatography (7) was used to isolate the Hb F from the blood samples of the simple heterozygotes.

DNA was is isolated from the white cells by the method of Poncz et al (8). α-Globin gene mapping analyses used procedures that are standard in our laboratories (9,10). Identification of the β-thal mutations was by dot-blot analysis of amplified DNA with ^{32}P-labelled synthetic oligonucleotides; this procedure has been described in detail before (11,12).

4. RESULTS

4.1 The Family. Three generations of this Creole family participated in the study; the pedigree is given in Fig. 4b.1. The grandmother (I-2) has a twin sister and there are three additional sets of dizygotic twins in this family. All members are in good health except for the double heterozygotes (see below), although none received regular blood transfusions. Subject II-3, mother of two sets of twins, is a teacher and works full-time. One person (II-5) is normal.

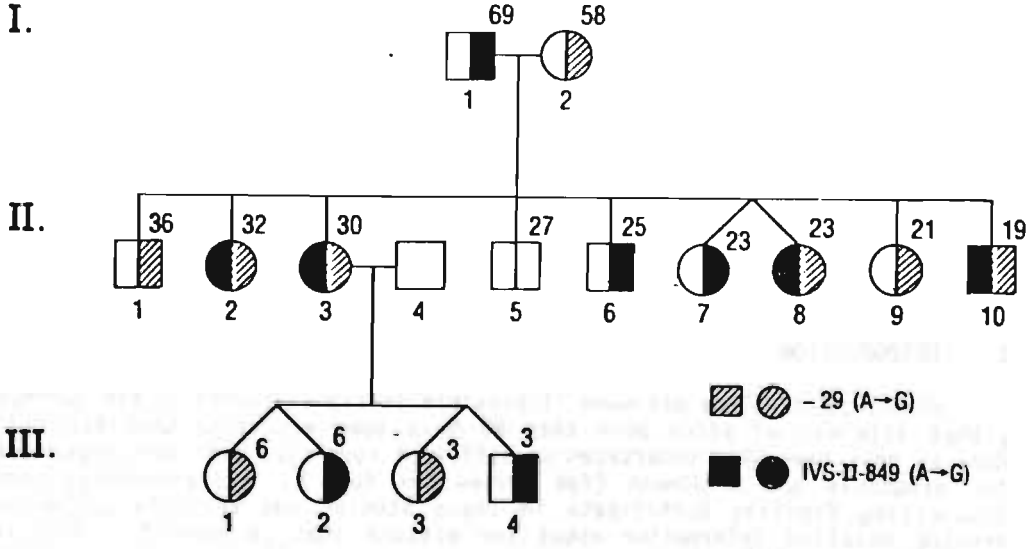

FIG. 4b.1 Pedigree of the Creole family (F) from Surinam.

4.2 Identification of the β-Thal Alleles and the Type of α-Thal. Dot-blot analyses readily identified the A→G promoter mutation at position -29 in five heterozygotes (I-2; II-1; II-9; III-1; III-3) and in five compound heterozygotes (II-2; II-3; II-8; II-10, and the unrelated adult identified by #556). This mutation is the most common transcriptional mutant observed in Blacks (13,14). The second mutation is a rare °-thal allele and is characterized by an A G mutation at position 849 of the second intron; it was present in subjects I-1; II-6; II-7; III-2; III-4, and in the five patients with a compound heterozygosity. This RNA processing mutant was first described in 1984 (13) and has been observed only a few times thereafter (15,16).

The possible presence of an α-thal was evaluated in all but two of the participants. Six subjects had a heterozygosity for α-thal-2 [-α (-3.7)/αα], while the others had four α-globin genes.

4.3 Haematological and Hb Composition Data. These results are listed in Table 4b.1 and include the Hb A_2, Hb F, and Hb A levels, the % G_γ in Hb F, and the number of α-globin genes. The data are arranged in such a way that a comparison between -29 (A→G) β^+-thal heterozygotes, IVS-II-849 (A→G) β°-thal heterozygotes, and compound heterozygotes is facilitated. The results are of some special interest because each group had the same number of patients of variable ages and of different sexes. The data do not appear to be significantly altered by the age of the samples except perhaps for the MCV values.

Discussion

The rapid advances in the methodology have made it possible to identify the precise nature of the various -thalassemic conditions, providing an insight into the clinical variations that have been observed. The data obtained in the present study are of interest because detailed comparisons between two different β-thalassemia alleles can be made among members of a large family.

As shown in table 1, tha data for the two sets of β-thalassemia heterozygotes are not very different except for the high level of $^G\gamma$ in the -29 heterozygotes (average 61.9%) and the low level in the IVS II-849 heterozygotes (average 28.4%). This difference can be explained by the presence of an Xmn I site 5' to $^G\gamma$ (T at position -158) on the chromosome carrying the A→G at mutation -29 while this is site absent (C at position -158) on the chromosome with the A→G (IVS II-849) mutation (16). Other differences include a more severe microcytosis and hyperchromia and slightly higher Hb A2 values for IVS II-849 heterozygotes (average Hb: 5.7 versus 5,05% for the -29 heterozygote). The effect of an α-thalassemia-2 heterozygosity on the data collected is negligible except perhaps for slightly higher Hb F values in the adults with four α globin genes.

The data for the five compound heterozygotes, also listed in Table 4b.1, deserved several comments. None of these adults required any transfusions; subject II-3 was the mother of two sets of twins. Subject II-10 was ill with severe infections at the time of blood collection; apparently a Hb level between 7 and 9 g/dl can be maintained. The condition and the haematological values of subjects II-2 and II-3 are considerably better than those of the other three patients. It appears likely that the coexisting α-thal-2 heterozygosity is the major contributing factor reducing the otherwise rather severe α/non-α imbalance. Except for the results obtained for patient II-8, the Hb composition data for the remaining four patients are very similar; the Hb A_2 value is elevated (average 4.8%), the Hb A values are nearly the same (average 18.5%; range 17.9-18.5%), and Hb F is the major Hb type (average 76.7%). A comparison of the data for patients II-2 and II-3 with those for patients II-10 and #556 indicate that the presence of the α-thal-2 heterozygosity does not modify these values. The synthesis of the β^A chain is regulated by the chromosome with the -29 (A→G) mutation; the average value of 18.5% observed for our patients is comparable to the values observed in patients with either a homozygosity for this mutation (12) or a heterozygosity in combination with Hb S (19). The $^G\gamma$ value in the Hb F of all five subjects was high (average 69.8%); this likely suggest that the synthesis of the $^G\gamma$ and $^A\gamma$ chains in these patients is primarily regulated by the chromosome with the -29 (A→G) mutation.

The unusual data observed for patient II-8, i.e. the low Hb A_2 and Hb A values, can at present not be explained and repeat analyses will be in order.

TABLE 4b.1 Haematological and Hb Composition Data

Case[a]	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV[b] fl	MCH pg	MCHC g/dl	A_2 %[c]	F %[c]	A %[c]	G_γ %[d]	# α Genes
A. Heterozygotes for the -29 (A→G) Mutation												
I-2	F-58	10.2	.445	4.89	91	20.9	22.9	5.0	1.1	-	70.0	4
II-1	M-36	11.5	.495	4.80	103	24.0	23.2	5.3	<1.0	-	51.7	3
II-9	F-21	9.9	.410	4.13	99	24.0	24.1	5.0	1.6	-	62.9	4
III-1[e]	F- 6	9.7	.435	5.00	87	19.4	22.3	5.3	1.9	-	54.9	-
III-3	F- 3	12.2	.510	5.42	94	22.5	23.9	4.7	11.6	-	54.9	4
B. Heterozygotes for the IVS-II-849 (A→G) Mutation												
1-1	M-69	11.0	.540	5.87	92	18.7	20.4	5.5	<1.0	-	20.0	3
II-6	M-25	12.1	.510	6.10	84	19.8	23.7	5.8	<1.0	-	30.0	3
II-7	F-23	11.0	.470	5.62	84	19.6	23.4	5.3	1.9	-	32.1	4
III-2	F- 6	10.1	.440	5.11	86	19.8	23.0	6.4	2.1	-	28.0	3
III-4	F- 3	9.9	.435	5.38	81	18.4	22.8	5.6	4.5	-	32.0	-
C. Compound Heterozygotes for the [-29(A→G)/IVS-II-849(A→G)] Mutations												
II-2	F-32	8.2	.305	4.10	74	20.0	26.9	5.1	76.9	18.0	70.6	3
II-3	F-30	9.1	.275	3.54	78	25.7	33.1	4.2	77.3	18.5	65.7	3
II-8	F-23	8.4	.330	4.11	80	20.4	25.5	2.0	86.1	11.9	67.2	4
II-10	M-19	4.7	.215	2.55	84	18.4	21.9	4.7	75.8	19.5	72.4	4
#556[f]	M-36	7.0	.285	3.54	81	19.8	24.6	5.1	77.0	17.9	73.0	4

[a] See pedigree in Fig. 4b.1.

[b] The unusually high MCV values are due in part to the age of the blood samples (5 days in transit).

[c] By polyCAT HPLC (3,4).

[d] By reversed phase HPLC (5,6).

[e] The diagnosis in this girl is based on Hb composition data only.

[f] Unrelated to the family shown in the pedigree of Fig. 4b.1.

6. ACKNOWLEDGEMENTS

This research was in part supported by the United States Public Health Service Research Grants HLB-05168 and HLB-41544. This is contribution #1236 from the Department of Cell and Molecular Biology at the Medical College of Georgia in Augusta, Georgia.

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CHAPTER 4c

OBSERVATIONS ON THE LEVELS OF HB A_2 IN PATIENTS WITH DIFFERENT β-THALASSEMIA MUTATIONS AND A δ CHAIN VARIANT

(Adapted from: Blood, 76:1246-1249, 1990)

CHAPTER 4c

OBSERVATIONS ON THE LEVELS OF HB A_2 IN PATIENTS WITH DIFFERENT β-THALASSEMIA MUTATIONS AND A δ CHAIN VARIANT

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(Adapted from: Blood, 76:1246-1249, 1990)

1. ABSTRACT

Hb A_2 and its variant Hb A_2' or B_2 [δ16(A13)Gly$\rightarrow$Arg] were quantitated in the blood of subjects with three different types of β-thal, and with the δ-B_2 anomaly *in cis* or *in trans* to the β-thal determinant. In one family, the δ-B_2 mutation was *in cis* to a newly discovered codon 47 (+A) frameshift. The levels of Hbs A_2 and B_2 were nearly the same and about 70% higher than those in simple Hb B_2 heterozygotes. In two additional families, the δ-B_2 variant was *in trans* to either a deletional β-thal (1392 bp) involving part of the β-globin gene and part of the β-globin gene promoter, or to the -88 (C$\rightarrow$T) promoter mutation. In both instances, the Hb B_2 level was increased by 80%, but the Hb A_2 level was increased by ~270 and ~200%, respectively. These data indicate two mechanisms that will cause an increase in δ chain production. One is consistent with a general mechanism concerning the relative excess of α chains in β chain deficiencies which will combine with δ chains to form variable levels of Hb A_2 dependent upon the severity of the β chain deficiency. The second concerns the loss of β-globin gene promoter activity, perhaps by an absence of (or decreased) binding of (a) specific protein(s) to this segment of DNA, and a concomitant increase in δ-globin gene promoter activity *in cis*.

2. INTRODUCTION

The increase in the level of Hb A_2 in β-thal heterozygotes was discovered in 1957 when the percentage of Hb A_2 was found to be about twice that in normal adults (1). More recent studies have indicated that the relative increase depends upon the type of β-thal that is present. For instance, carriers of some mild types of β-thal such as those caused by the C$\rightarrow$T mutation at IVS-I-6 (2) or by A$\rightarrow$G mutations in the poly A site (3), have only slight increases in their Hb A_2 levels (4). These observations are consistent with the concept that α chains combine much more readily with the β chain of Hb A than with the δ chain of Hb A_2, and that in conditions with variable β chain deficiencies, the increase in Hb A_2 level should

vary accordingly (5). However, there are exceptions to this rule; for instance, persons with a heterozygosity for either one of two mild promoter mutations, namely the C→T at position -88 or A→G at position -29, which are common among Blacks (6), have Hb A_2 values at least as high as those observed among heterozygotes for the more severe β-thal alleles like the C→T mutation at codon 39 or the G→A mutation at IVS-I-110 present in Mediterranean patients (2,6). Furthermore, β-thal heterozygotes with a δ chain variant like δ-B_2 in cis or in trans to the thalassaemia mutation have similar amounts of Hb A_2 and the variant (7-9), indicating that the increased Hb A_2 synthesis in β-thal is derived from δ genes, both in cis and in trans to the β-thal mutation.

Unusually high Hb A_2 levels of 7-9% have been found in β-thal heterozygotes with a deletional defect that involves the 5' segment of the β-globin gene and (part of) the β-globin gene promoter (10-12). The deletions concern a 292 bp deletion observed in a Turkish family (11,13), a 1392 bp deletion observed among Blacks (10,13,14), and the much larger 4237 bp deletion seen in members of a Czechoslovakian family (12); all types share the loss of the 5'β promoter. We recently detected a β-thal heterozygote from Surinam who had the 1392 bp deletion and a Hb B_2 heterozygosity, and who exhibited disproportionate levels of Hb A_2 and Hb B_2. This observation prompted us to reevaluate some members of two families with both β-thal and Hb B_2 heterozygosities who were described over 25 years ago (7,8).

3. MATERIALS AND METHODS

3.1 Blood Samples. Samples from three members of Family K (7) and from four members of a second Black family (Family M) were collected in The Netherlands (two samples) and in Surinam (five samples) and shipped by air to Augusta, GA. Blood samples from one Black male of Family R (8), his 6-year-old twin daughters, and from a fourth, unrelated adult Black female, were obtained locally. Collection was in vacutainers with EDTA as anticoagulant. Informed consent was obtained.

3.2 Methods. Haematological data were collected with an automated cell counter. Hb F was quantitated by cation exchange HPLC using the PolyCAT column system (2,15). Hb A_2 and Hb B_2 were quantitated by the same PolyCAT HPLC procedure and by the analytical DEAE-cellulose procedure with Tris-KCN-HCl developers and a pH gradient (16); a similar method was used in the earlier publication describing some of these individuals (7,8).

DNA was isolated from white blood cells with the method described by Poncz et al (17). Identification of the Hb B_2 variant was by hybridization of amplified DNA (18) with probes specific for a G→C mutation in codon 16 of the δ-globin gene. A similar procedure was used to identify one of the β-thal mutations; methodology has been described in previous publications (13). The ~1.4 kb deletion present in the third family was detected by gene mapping as shown earlier (11,13). The mutation in the third family was new and was identified through sequencing of amplified DNA (13). It was confirmed by hybridization of amplified DNA with a specific, radiolabelled, oligonucleotide probe.

4. RESULTS

4.1 The Three Families and Their β-Thal Types. Family K is of Dutch-Surinam origin and was first described in 1961 (7). Fourteen members with a β-thal heterozygosity also had a heterozygosity for Hb B_2; no normal members carried the Hb B_2 variant and no β-thal heterozygote was without it. This observation was explained by assuming that the two abnormalities were linked and occurred on the same chromosome. The average values for Hb A_2 (2%) and Hb B_2 1.9%) were only slightly less than the average Hb A_2 value (2.2%) for 12 normal family members [measured by CMcellulose chromatography (16)]. Nearly 30 years later it was possible to obtain blood samples from three β-thal heterozygotes (listed in Table 4c.1). The β-thal mutation in this family had not been observed before and was identified through sequencing as a frameshift in codon 47 (+A), which was confirmed by hybridization of amplified DNA with appropriate probes (Fig. 4c.1).

TABLE 4c.1 The Levels of Hbs A_2 and B_2 in Hb B_2 Heterozygotes With and Without an Additional β-Thal Heterozygosity

Subject[a]	Sex-Age	DEAE-Cellulose Chromatogr. B_2 %	A_2 %	Total %	$\frac{100.B_2}{\text{Total}}$	PolyCAT HPLC B_2 %	A_2 %	Total %	$\frac{100.B_2}{\text{Total}}$
Family K; Hb B_2 in cis; β-thal mutation; codon 47 (+A)									
S.K. (IV-1)	F-36	1.98	2.51	4.49	44.1	2.55	2.60	5.15	49.5
H.R. (III-4)	M-64	2.52	2.72	5.24	48.1	2.36	2.45	4.81	49.1
E.C.R. (III-2)	F-60	1.71	2.36	4.07	42.0	2.32	2.41	4.73	49.0
Average		2.07	2.53	4.60	45.0	2.41	2.49	4.90	49.2
Family R; Hb B_2 in trans; β-thal mutation; -88 (C→T)									
E.R. (II-2)	M-39	2.17	4.39	6.56	33.1	2.52	4.49	7.01	35.9
2nd collection		2.46	4.75	7.21	34:1	2.57	4.47	7.04	36.5
Family M; Hb B_2 in trans; β-thal mutation; 1392 bp deletion									
L.M.	M-54	1.77	4.75	6.52	37.3	2.51	5.47	7.98	31.5
Controls; β-thal absent									
S.B.	F- 6	1.36	1.60	2.96	45.9	1.34	1.47	2.81	47.7
L.B.	F- 6	1.29	1.50	2.79	46.2	1.45	1.50	2.95	49.2
D.C.	F-37	1.24	1.49	2.73	45.4	n.d.	n.d.	n.d.	-
Average		1.30	1.53	2.83	45.9	1.40	1.49	2.89	48.4
D.M.	F-13	1.51	1.68	3.19	47.3	1.50	1.78	3.28	45.7

[a] Numbers between parentheses identify the numbers in the pedigrees as published in 1961 (7) and 1963 (8). The controls S.B. and L.B. are the twin daughters of E.R. of Family R. The control D.M., who is the daughter of L.M, has an additional Hb S heterozygosity; the value for Hb A_2 with the PolyCAT HPLC procedure may be slightly too high because of contamination with a minute quantity of modified Hb S.

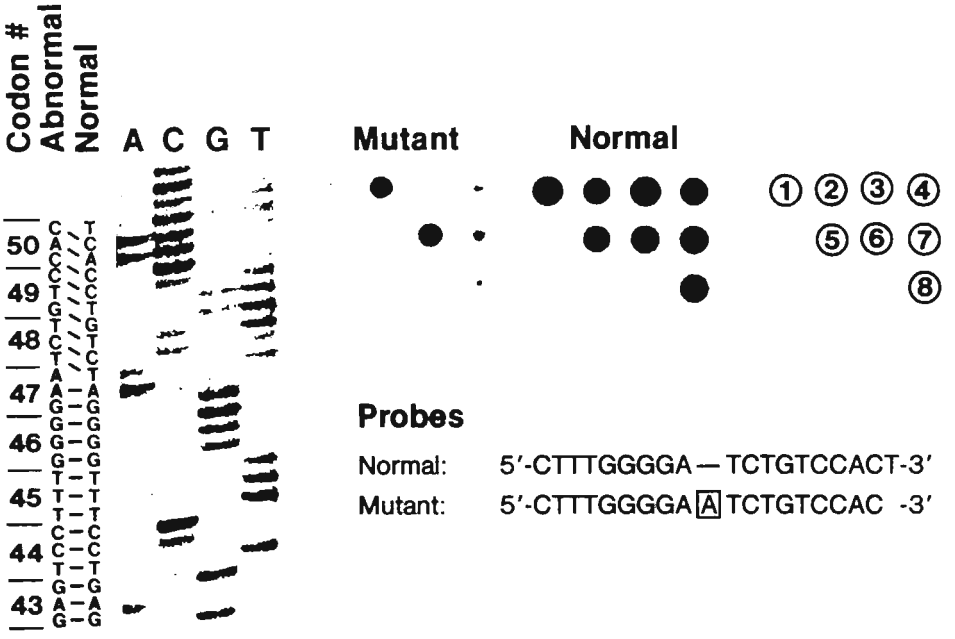

FIG. 4c.1 Identification of the frameshift at codon 47 (+A) of the β chain leading to a β°-thal. Left: Part of a sequencing gel locating the frameshift at codon 47 (+A). Right: Confirmation of the codon 47 (+A) frameshift by hybridization of the amplified DNA with ^{32}P-labelled probes.

Family R is a Black family from Central Georgia, described in 1963 (8), because Hb C [β6(A3)Glu→Lys], β-thal, and Hb B_2 occurred in different combinations. One subject, E.R., had the β-thal and Hb B_2 heterozygosities; family studies showed that the two anomalies were not linked and occurred on opposite chromosomes. Subject E.R. had 1.85% Hb B_2 and a rather high Hb A_2 level of 2.7%, while his mother with a simple Hb B_2 heterozygosity had 1.15% Hb B_2 and 1.25% Hb A_2. He was reevaluated 26 years later; his twin daughters, 6 years of age, were found to have a simple Hb B_2 heterozygosity. The β-thal mutation in this family was the second most common type found in Blacks, i.e. the C→T mutation at nt -88 of the β-globin gene promoter (11); details of this characterization are not presented.

Family M from Surinam consisted of parents and two children. The father had a Hb B_2 trait and a β-thal heterozygosity, the mother a Hb S trait, one child had Hb S-β°-thal, and the other child had Hb S trait combined with a Hb B_2 heterozygosity. The β-thal was identified as the 1392 bp deletion with a 5' end point ~600 bp 5' to the Cap site and a 3' end point ~500 bp from the 5' splice junction of IVS-II (10,13). Details of the study of Family M will not be presented.

The G→C mutation in codon 16 of the δ-globin gene leading to the synthesis of the δ-chain of Hb B_2 was confirmed for all three families through hybridization of amplified DNA with ^{32}P-labelled oligonucleotide probes.

4.2 The Percentages of Hb A_2 and Hb B_2. Quantitation was with two procedures. The DEAE-cellulose chromatographic method resembled that used in the older experiments (7,8); it had the disadvantage of a decreased recovery of Hb B_2, particularly in somewhat older red cell lysates. The data obtained with the PolyCAT HPLC method were highly reproducible, even after storage of the samples at 4°C for an extended period of time. The percentages determined with both procedures are listed in Table 4c.1; although the absolute values given for some samples in the two groups differ considerably, the relative quantities for Hb B_2 and Hb A_2 (listed as 100.B_2/total) were similar.

The percentages of Hbs A_2 and B_2 in the three members of Family K with the codon 47 (+A) frameshift mutation were nearly the same, confirming previously published results (7). The average PolyCAT values of 2.41 and 2.49% were about 70% higher than the average values of 1.40 and 1.49% observed for two Hb B_2 heterozygotes. Subject E.R. with the -88 (C→T) mutation averaged 2.55% for Hb B_2 and 4.48% for Hb A_2; this corresponds to an 80 and a 200% increase over the levels in the Hb B_2 heterozygotes. The total value of 7.03% for δ chain–containing Hb types was high; the average value for Hb A_2 in 10 β-thal heterozygotes with the same mutation was 5.4 ± 0.4% with a range of 4.5 to 6.6% (unpublished data). The level of Hb B_2 in L.M. of Family M with the 1392 bp deletion was 2.51% and that of Hb A_2 was 5.47%, in increase of ~80 and ~270% over the values for Hbs B_2 and A_2 in the Hb B_2 heterozygotes. The level of 7.98% for δ chain–containing Hb types is similar to values observed for other patients with the same deletion (6,10,13). The values for Hbs A_2 and B_2 in the one non-thalassaemic member of this family, who also had a Hb S heterozygosity, were nearly the same.

5. DISCUSSION

The results obtained in this study and summarized in Table 4c.1, suggest the following: 1) Simple Hb B_2 heterozygotes have nearly equal levels of Hb A_2 and Hb B_2, indicating that the G→C substitution at codon 16 of the δ gene (Gly→Arg in the δ chain) does not effect the relative synthesis of this variant chain, and thus, the presence of this δ gene marker offers an opportunity to study the relative formation of Hb A_2 derived from δ chains *in cis* or *in trans* to a β-thal mutation. The one child of Family M with Hb B_2 and Hb S heterozygosities also had closely similar Hb A_2 and Hb B_2 values. 2) The nearly equal values for Hb A_2 and Hb B_2 in Family K with the frameshift at codon 47 (+A) confirm earlier data (7) and suggest that mutations within the β-globin gene do not influence the relative synthesis of δ chains derived from δ genes *in cis* or *in trans* to the β-thal allele but only the total Hb A_2 formation. 3) The high level of Hb A_2 of nearly 5.5% in a subject with the deletional β-thal who has a δ-B_2 mutation *in trans* and a Hb B_2 level of 2.5%, indicates that the excess Hb A_2 is derived from δ chains *in cis* to the β-thal deletion. 4) The high level of Hb A_2 (nearly 4.5%) in a patient who had the -88 (C→T) mutation and the Hb B_2 mutation *in trans* (the Hb B_2 value was 2.5%) showed that this mutation

in the CACC box of the 5'β promoter increases Hb A_2 formation from δ chains in cis to this β-thal mutation. There are considerable differences between the Hb A_2 and Hb B_2 percentages found for this person; in 1963 at the age of 11 years, the values were Hb A_2 2.70% and Hb B_2 1.80%, and in 1990 at the age of 39 years, the average Hb A_2 and Hb B_2 values by DEAE-cellulose chromatography were 4.57 and 2.32%, respectively. The considerably lower values found in the earlier study might be due to a coexisting iron deficiency, although this possibility was not evaluated at that time. These data suggest that at least two mechanisms can be responsible for higher levels of Hb A_2 in β-thal heterozygotes. One general mechanism concerns the relative excess of α chains in β chain deficiencies which is present in all types of β-thal, albeit at different levels, dependent upon the type of β-thal. The second mechanism appears to be loss of β-globin gene promoter activity. Popovich et al (12) were the first to suggest that the transcription of the δ-globin gene is affected by loss of the 5'β promoter, perhaps because the 5'β and 5'δ promoters are influenced by the same 3'β enhancer. The data presented here support this hypothesis and suggest that a mutation in the proximal CACC has a nearly similar effect as the loss of the entire 5'β promoter. It may be that the loss of (or decreased) binding of a specific protein or a complex of proteins to this segment of DNA results in a decrease in or absence of βmRNA formation, and indirectly, in an increase in δmRNA formation because protein binding at the CACC box of the 5'δ promoter is not affected. Such a difference would not exist when the β-thal mutation is outside this promoter region, and the formation of βmRNA (although ineffective) is not influenced by this mutation. Another mechanism explaining the high δ chain production is that the deletion of the β-globin gene promoter or the mutations in the TATA [-29 (A→G)] or CACC [-88 (C→T)] boxes (partially) removes competition for limiting transcription factors, which makes these more readily available to the δ-globin gene promoter. However, this would effect equally the promoters of both δ-globin genes and not only the δ-globin gene in cis to the thalassaemia allele.

6. ACKNOWLEDGEMENTS

This research was supported by the United States Public Health Service research grants HLB-05168 and HLB-41544. The authors are indebted to Dr. K. Punt (Utrecht, The Netherlands), Dr. F. Bueno de Mesquita, Dr. J.D.G. Schaad, and to Mrs. T. Mercuur (Paramaribo, Surinam) for their help in obtaining some of the blood samples. This is contribution #1226 from the Department of Cell and Molecular Biology at the Medical College of Georgia in Augusta, Georgia.

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CHAPTER 4d

HB E-β-THALASSAEMIA IN SURINAM

HB E-βTHALASSAEMIA IN SURINAM

1. ABSTRACT

During our survey for haemoglobinopathies in the Surinam population, six families were observed with Hb E-β-thal. A detailed comparison was made of the clinical condition, haematological data, Hb composition, and types of β-thal alleles present in these Suriman E-β-thal patients and with those of 28 patients from Malaysia, China, and Georgia (USA). The majority of the 28 patients were attending a haematology clinic in Kuala Lumpur (Malaysia), five were from the southern part of China, and the one patient (D.D.) from Georgia was of mixed Korean-Italian descent. All patients from Surinam with the E-β-thal condition were descendants of immigrants from Indonesia or India. A total of 54 chromosomes from 27 individuals were analyzed, mainly by hybridization with specific oligonucleotide probes after DNA amplification. Six different β-thal mutations, including the β^E mutation, were detected in the six Suriman families; also detected was a rare splice junction mutation, IVS-I-2 (T→C), which was previously found in an American Black teenager and an Algerian child.

2. INTRODUCTION

2.1 Hb E or β26(B8)Glu→Lys, has an amino acid substitution caused by a G→A mutation in codon (CD) 26 of the β-globin gene, and is probably one of the most prevalent genetic traits in man (1). The inheritance of the β^E-globin gene is associated with hypochromia and microcytosis which is already evident for the simple Hb E heterozygote. Hb E homozygotes are slightly anaemic with a haematology resembling that of a classical β-thal heterozygosity with low MCV (<70 fl) and low MCH values (<20 pg), target cells, and an increased resistance to osmotic lysis (2-4). The synthesis of β^E chains in reticulocytes is impaired, and values of 1.2 to 2.1 have been reported for the α/β *in vitro* chain synthesis ratio in Hb E homozygotes who do not have an associated α-thal (5,6). Older studies suggested that the altered ratio could be due to an increased instability of the β^E chain (5). However, more recent investigations have shown that the decreased β^E synthesis is associated with reductions in levels of β-globin mRNA comparable to those observed for β-thal trait (6-8). The G→A mutation in CD 26 at the 3' end of the first exon results in the activation of an alternate splice site, in the formation of an abnormally spliced mRNA, and an instability of the mRNA during erythroid maturation (1). Hb E is commonly observed among the populations of Far Eastern countries such as Thailand, Vietnam, Cambodia, Laos, Malaysia, Indonesia, South China, the Philippines, and India, with frequencies up to 54% in some areas of Southeast Asia. It is also found among Southeast Asian immigrants who have been relocated in various western countries (2,3,9), including Surinam (10-13).

2.2 Hb E-β-Thal. The frequency for β-thal varies from 3 to 9% in the populations of Far Eastern countries and, thus, the occurrence of a compound heterozygosity with Hb E is high in these populations. Interaction

of the β^E-globin gene (which behaves as a mild β-thal gene) with a β^+- or β°-thal gene often results in a severe clinical condition with a profound haemolytic anaemia, splenomegaly, and in some cases growth retardation. The severe cases are completely transfusion-dependent. This condition is common among Southeast Asians because of the relatively high frequencies of different β°-thal alleles (14-18). It is somewhat difficult to understand why Hb E-β-thal is often such a severe condition; the relatively modest β chain deficit observed in Hb E heterozygotes and homozygotes would suggest that the combination of Hb E with a β-thal would lead to a thalassaemia intermedia rather than a thalassaemia major. Perhaps the relative excess of α chain in Hb E-β-thal provides an oxidative stress that will enhance the denaturation of Hb E and the haemolysis of red cells containing this denatured protein (1). Alleviation of the condition may occur if Hb E-β-thal is combined with α-thal (19). As stated before, numerous mutations leading to β-thal conditions have been discovered in the Southeast Asian populations; the most frequently occurring mutations are listed in Table 4d.1. Frameshifts or nonsense mutations such as those at CDs 8/9 (+G), CD 35 (-C), CDs 41/42 (-TTCT), CDs 71/72 (+A), CD 15 (G→A), and CD 17 (A→T), lead to a severe β°-thal. The same is the case for the mutation at IVS-I-1 (G→T) which prevents proper processing of RNA, the C→T mutation at IVS-II-654, and for the deletional β°-thal allele (619 bp) observed at a high frequency in India. Of the two β^+-thalassaemias listed in Table 4d.1, the IVS-I-5 (G→C) mutation is a severe type of β^+-thal with only a modest amount of β chains being synthesized in the homozygote (3-6% of the total Hb), while the transcriptional mutant at nt -28 (A→G) of the β-globin gene promoter, is one of the many milder types of β^+-thal. While reviewing these various β-thal alleles, it will become evident that most patients with Hb E-β-thal will have a severe disease.

TABLE 4d.1 The Most Frequently Occurring β-Thal Alleles in Some Southeast Asian Populations

India	Thailand	China	Malaysia	Indonesia
IVS-I-5 (G→C)	CDs 41/42 (-TTCT)	CDs 41/42 (-TTCT)	IVS-I-5 (G→C)	IVS-I-5 (G→C)
Deletion (-619 bp)	CD 17 (A→T)	CD 17 (A→T)	CDs 41/42 (-TTCT)	IVS-II-654 (C→T)
CDs 8/9 (+G)	IVS-II-654 (C→T)	-28 (A→G)	IVS-I-1 (G→T)	IVS-I-1 (G→T)
IVS-I-1 (G→T)	IVS-I-1 (G→C)	IVS-II-654 (C→T)	IVS-II-654 (C→T)	CD 15 (G→A)
CDs 41/42 (-TTCT)		CDs 71/72 (+A)	CD 35 (-C)	CD 35 (-C)

The transcriptional mutant -28 (A→G) and the IVS-I-5 (G→C) consensus sequence mutation cause a β^+-thal; all others result in a β°-thal. The listing is based on frequencies; the allele with the highest frequency is listed first.

3. PATIENTS AND METHODS

3.1 Patients. With the help of the local physicians and laboratory technicians in Surinam, blood samples from members of six families were collected. At least one member of each of these families had the Hb E-β-thal disease. Most of these compound heterozygote patients were given regular transfusions. The diagnosis of β-thal and of Hb E-β-thal was established by clinical and haematological examination, and by cellulose acetate electrophoresis in the Academisch Ziekenhuis in Paramaribo (Surinam). When possible, the blood of those transfusion-dependent patients was collected prior to transfusion. Collection was in vacutainers with EDTA as anticoagulant. The samples were stored at +4°C and shipped by air to Augusta, GA (USA) within one week of collection.

3.2 Haematological Procedures. Haematological data were obtained with an automated cell counter. Red cell lysates were studied by IEF and by cation exchange HPLC (20). Since Hb E and Hb A_2 are difficult to separate with this procedure, an estimate of the Hb A_2 values was determined (as % δ) by reversed phase HPLC (21,22) (Fig. 4d.1). The level of Hb F was estimated with an alkali denaturation procedure as described in Chapter 2. The composition of the Hb F was determined by reversed phase HPLC (21,22).

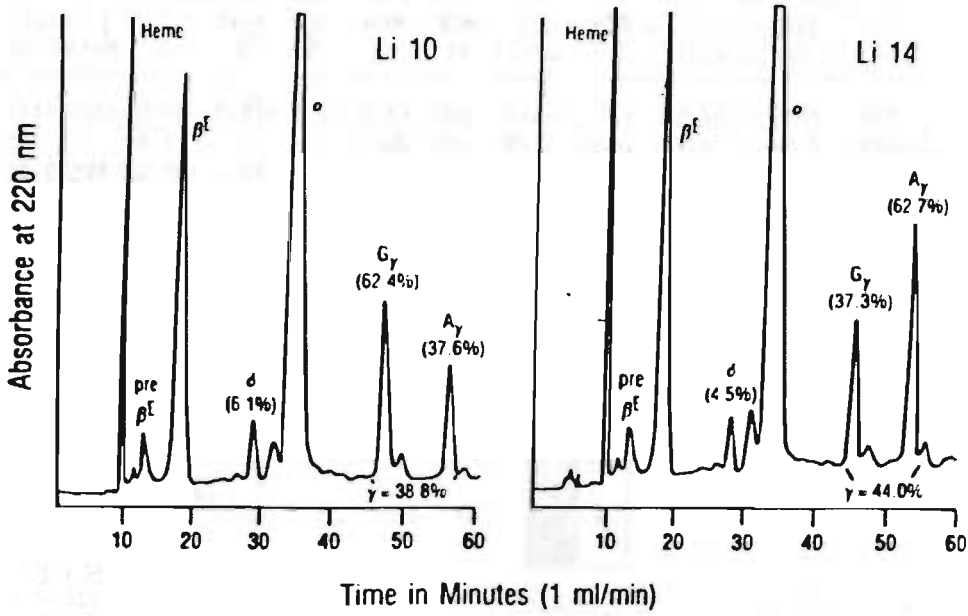


FIG. 4d.1 Separation of the β^E, ζ, α, and γ chains in red cell lysates from two Chinese patients with Hb E-β^o-thal by reversed phase HPLC. The separation of the β^E, δ, and α chains was complete. See Refs. 21 and 22 for technical details.

3.3 Analysis of α-Globin Genes. The numbers of α-globin genes and possible abnormalities in the β-globin gene arrangement were determined

in DNA digests using the restriction enzymes Bam HI and Bgl II; hybridization was with α and β probes (23,24).

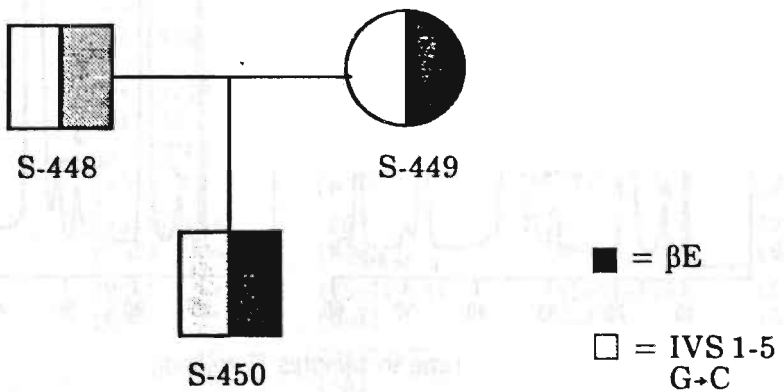
3.4 Characterization of β-Thal Mutations. In vitro DNA amplification was performed according to a modified method (described in Chapter 2). Amplified DNA was applied to nitrocellulose filters as dots and hybridized with the appropriate oligonucleotide probes. For sequencing experiments, the amplification procedure was adjusted so that single strand DNA was obtained during the amplification process (Chapter 2).

4. RESULTS

4.1 Family 1 (S-448, S-449, S-450). The propositus in this family is a 4-year-old boy who receives regular transfusions. Both parents are of Javanese descent. The IEF data showed that the mother is a Hb E heterozygote. The father has the typical characteristics of a β-thal heterozygote, namely microcytosis and an elevated Hb A_2 value of 5.6% (Table 4d.2). Dot-blot analysis revealed an IVS-I-5 (G→C) mutation for both the father and the propositus.

TABLE 4d.2 Family 1; Haematological and Hb Composition Data; Pedigree

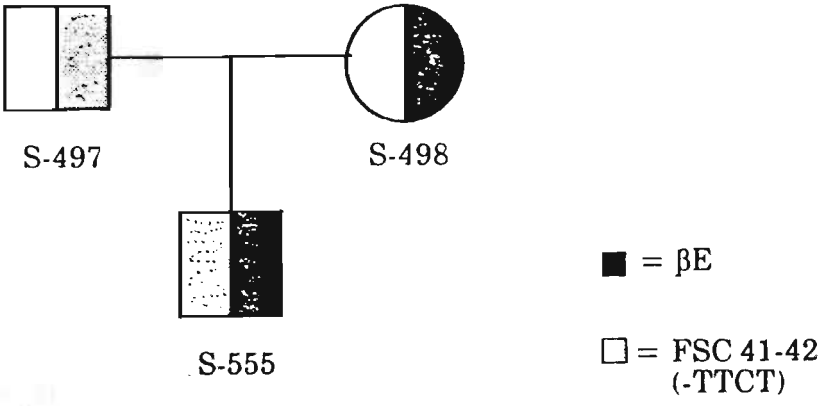
Case	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	A_2+E %	F %	β-Thal Mutation	# α Genes
S-448	M-26	13.6	.550	5.52	100	24.6	5.6	29.4	<1.0	IVS-I-5	4
S-449	F-24	10.7	.435	5.29	82	20.2		-	1.6		4
S-450	**M- 4**	**8.0**	**.310**	**3.31**	**94**	**24.2**	**4.2**	**16.9**	**21.6**	**IVS-I-5**	**4**



4.2 Family 2 (S-497, S-498, S-555). This is the only family of Asian-Indian (Hindustani) descent in this study. The propositus S-555 is a 6-year-old female who is regularly transfused. Dot-blot analysis showed that the father and the propositus have the CDs 41/42 (-TTCT) frameshift mutation. Haematological data are given in Table 4d.3; the propositus and her parents have a normal α-globin gene arrangement ($\alpha\alpha/\alpha\alpha$).

TABLE 4d.3 Family 2; Haematological and Hb Composition Data; Pedigree

Case	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	A_2+E %	F %	β-Thal Mutation	# α Genes
S-497	M-36	11.3	.46	6.04	76	18.7	5.9	-	2.8	CDs 41/42	4
S-498	F-37	11.8	.47	4.94	95	23.9	5.4	-	<1.0	-	4
S-555	**F- 6**	**5.5**	**.24**	**2.70**	**89**	**20.4**	**-**	**29.1**	**31.1**	**CDs 41/42**	**4**

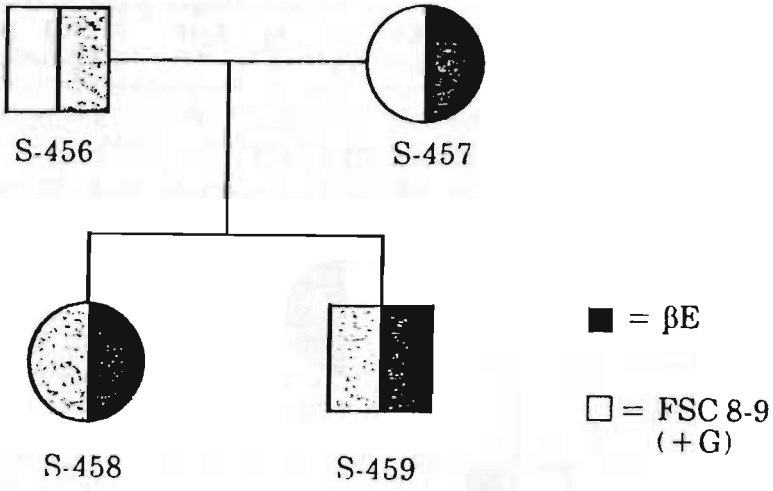


4.3 Family 3 (S-456, S-457, S-458, S-459). This family is also of Javanese origin. IEF and cation exchange HPLC showed that the two children and their mother have Hb E, while the father is a β-thal heterozygote with a Hb A_2 level of 5.9%. Additional data including the numbers of α-globin genes in all four individuals are given in Table 4d.4. Sequencing of amplified DNA from one of the children showed that the β-thal in this family is due to a frameshift at CDs 8/9 (+G). This result was confirmed by dot-blot hybridization experiments. Clinically, there were some differences between the two children. The daughter (S-458) has been transfused almost every four months, while the son (S-459) has not received any transfusions thus far. This difference is likely the result of a mild α chain deficiency in the son ($-\alpha^{3.7}$) reducing the relative imbalance between α/non-α chains.

TABLE 4d.4 Family 3; Haematological and Hb Composition Data; Pedigree

Case	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	A_2+E %	F %	β-Thal Mutation	# α Genes
S-456	M-32	12.5	.540	6.89	78	18.1	5.9	-	<1.0	CDs 8/9	4
S-457	F-28	12.8	.560	4.80	117	26.7	-	26.1	<1.0	-	3
S-458	F-13	5.9	.260	3.57	73	16.5	-	11.9	25.7	CDs 8/9	4
S-459	M- 9	7.0	.415	4.15	111	18.8	-	11.7	29.5	CDs 8/9	3

Table 4d.4 continued: Pedigree



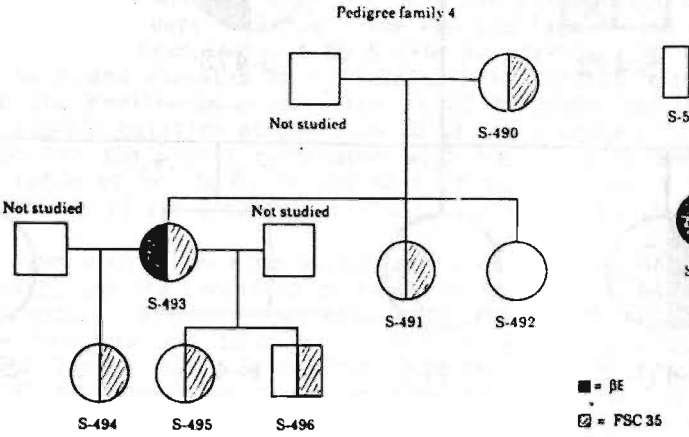
4.4 Families 4 (S-491-S-496) and 5 (S-504-S-507). Families 4 and 5 are also of Javanese descent. In Family 4, the propositus is a 35-year-old female (S-493) with three children. These three children are β-thal heterozygotes (see pedigree). The propositus has a history of severe anaemia and has taken iron for the last 15 years to treat the anaemic condition without success. She has developed a severe haemochromatosis leading to iron deposits in the liver, as was shown by liver biopsy. Dot-blot analysis indicated a frameshift at CD 35 (-C) in all β-thal carriers from both families. This thalassaemic gene was first described among Malay patients (16) and has also been observed in Indonesia (25). In Family 5, the daughter (S-506) receives blood transfusions on a regular basis (once a year), while the son (S-506), with a similar condition, has not yet been transfused. All members of Families 4 and 5 have a normal α-globin gene arrangement (αα/αα). Haematological data are listed in Table 4d.5.

TABLE 4d.5 Families 4 and 5; Haematological and Hb Composition Data; Pedigrees

Case	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	A_2+E %	F %	β-Thal Mutation	# α Genes
Family 4											
S-490	F-52	10.9	.490	5.73	86	19.0	5.0	-	0.7	CD 35	4
S-491	F-27	10.4	.455	5.40	84	19.3	5.2	-	2.9	CD 35	4
S-492	F-22	12.7	.490	4.40	111	28.9	2.5	-	<1.0	-	4
S-493	**F-35**	**6.2**	**.275**	**2.73**	**101**	**22.7**	-	**50.7**	**36.4**	**CD 35**	**4**
S-494	F-15	9.4	.405	5.48	74	17.2	5.1	-	0.5	CD 35	4
S-495	F-12	9.9	.430	5.68	76	17.4	5.4	-	0.5	CD 35	4
S-496	M- 6	9.3	.390	5.33	73	17.4	6.3	-	0.9	CD 35	4

Table 4d.5 continued: Family 5; Pedigrees

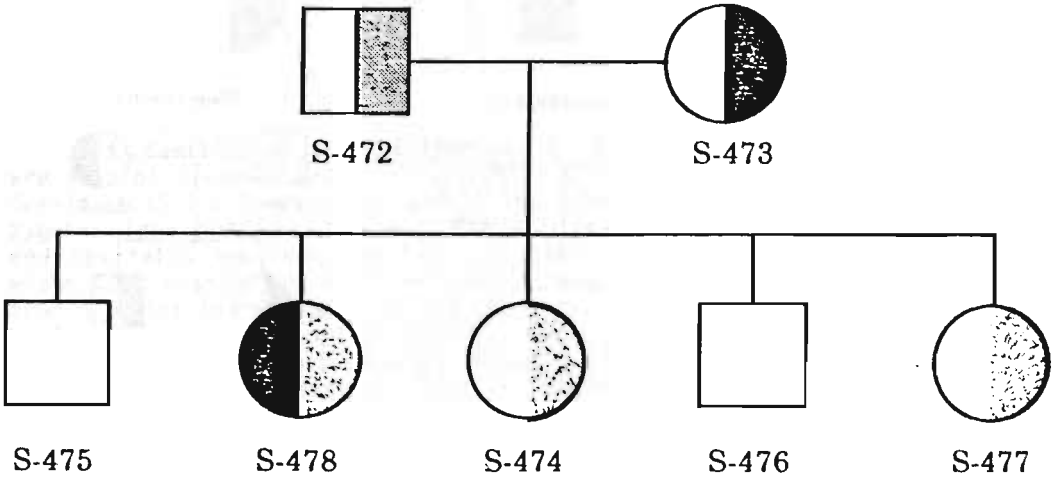
Case	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	A_2+E %	F %	β-Thal Mutation	# α Genes
Family 5											
S-504	M-52	12.3	.520	6.22	84	19.8	6.2	-	1.0	CD 35	4
S-505	F-51	12.1	.485	4.62	105	26.2	4.8	-	1.4	-	4
S-506	M-18	10.2	.400	5.07	79	20.1	-	-	52.0	CD 35	4
S-507	F-21	8.3	.340	4.25	80	19.5	-	40.6	58.6	CD 35	4



4.5 Family 6 (S-472-S-478). The propositus in this family is a 19-year-old woman (S-478), also of Javanese origin (see pedigree), who has the clinical characteristics of thalassaemia major such as growth retardation, bone malformation, severe anaemia, and a marked enlargement of liver and spleen. She has been transfused once every three weeks and has been hospitalized frequently. Her Hb F level is a low 3.7% which can be explained by the frequent blood transfusions. Haematological data are listed in Table 4d.6.

TABLE 4d.6 Family 6; Haematological and Hb Composition Data; Pedigree

Case	Sex-Age	Hb g/dl	PCV l/l	RBC 10^{12}/l	MCV fl	MCH pg	A_2 %	F %	A_2+E %	β-Thal Mutation	# α Genes
S-472	M-49	11.4	.455	5.09	89	22.4	6.0	1.9	_	IVS-I-2	4
S-473	F-42	10.6	.425	4.51	94	23.5	-	<1.0	-	-	4
S-474	F-17	9.1	.355	4.14	86	22.0	6.0	1.5	-	IVS-I-2	4
S-475	M-23	14.8	.575	5.00	115	29.6	3.0	<1.0	-	-	4
S-476	M-13	13.3	.435	4.48	97	29.7	3.1	1.4	-	-	4
S-477	F-11	10.4	.410	4.86	84	21.4	5.8	3.5	-	IVS-I-2	4
S-478	**F-19**	-	-	-	-	-	-	**3.7**	-	**IVS-I-2**	**2 4**



Sequencing of amplified DNA from the propositus showed a T→C substitution at IVS-I-2. This rare mutation was first observed in 1988 in an American Black teenager who was a compound heterozygote for Hb S and the IVS-I-2 (T→C) β°-thal allele (26). The clinical condition of the propositus (S-478) and that of the American Black teenager were similar. A second case of the IVS-I-2 (T→C) mutation was reported in a child born to Algerian parents (27). This child was homozygous for this β-thal mutation. Splenectomy was performed at the age of nine and the child has not been transfused since then.

5. DISCUSSION

In addition to the Surinam Hb E-β-thal patients, 28 patients from different countries were used to evaluate their clinical status, haematological data, Hb composition, and the types of β-thal alleles that were present (most of these data are from Ref. 28 and from personal communications). The results are listed in Table 4d.7. All 17 patients with Hb E-β-thal (IVS-I-5; G→C) were clinically severely affected; 11 were transfused at

regular intervals. The haematological data for the six untransfused patients were as expected for this haematological disease with severe microcytosis and hypochromia. The β^E haplotypes were determined in 12 patients; the three known types (a, b, c) were present (29,30). The Hb A_2 levels were elevated but rather variable. The same can be stated for the Hb F values, while the β^A chain synthesis varied between 3 and 6% only. The variation in the percentage of Hb F is most difficult to explain; the lowest level was seen in the patient with an α-thal-2 heterozygosity. The level of $G\gamma$ chains in the Hb F fell into two groups: 13 patients had high $G\gamma$ (average 62.5%) and four had low $G\gamma$ (average 38/8%), which seems to correlate with the haplotypes of the β^E chromosome (high $G\gamma$ and haplotypes E-a and E-c; low $G\gamma$ and haplotype E-b), suggesting that the synthesis of the γ chain is primarily directed by the β^E chromosome.

Eleven patients listed in Table 4d.7 had Hb E-β-thal and suffered from severe disease. Many were on a regular transfusion regimen. Three of the seven Chinese patients had the frameshift at CDs 41/42 and four that of CDs 71/72. Hb F levels were between 28 and 44% with the exception of patient Li 11 who had only 10% Hb F and a very high Hb A_2 level of 10.6%. The $G\gamma$ values were variable. The two siblings (S-458 and S-459) from Surinam with the frameshift at CD 8 (+G) maintained a Hb level of 6-7 g/dl with 30% Hb F and elevated Hb A_2 levels. The patient with Hb F in combination with the Mediterranean mutation at CD 39 (C→T) had the $A\gamma^T$ mutation, i.e. the Ile→Thr mutation at position 75 of the γ chain (31), which is characteristic for the β-thal chromosome with haplotype II and the CD 39 mutation. The ratio of $A\gamma^T$ to $A\gamma$ in the Hb F of this boy was 2 to 1, suggesting that two-thirds of the γ chain synthesis is directed by the β-thal chromosome.

The eight remaining patients listed in Table 4d.7 had a more moderate disease, and the condition of three of them should be listed as thalassaemia intermedia. All five patients with the CD 35 (-C) frameshift, i.e. two from Malaysia and three from Surinam, were not transfusion-dependent although low Hb levels were often observed. The Hb F levels were high (average 50.1%) with high $G\gamma$ values (average 62%) and low Hb E levels (average 43.2%; average δ chain values were 6.7%), and this is likely a major factor for the somewhat milder condition observed in these patients.

TABLE 4b.7 Haematological and Hb Composition Data for 36 Patients With Hb E-β-Thal

Case	Sex-Age	Hb g/dl	MCV fl	MCH pg	δ %	γ %	β^A %	β^E %	$G\gamma$ %	Origin	# α Genes
E-β^+-Thal; IVS-I-5 (G→C)											
S-450	M- 4	transfused							55.4	Surinam	4
EB-1	F-23	6.7	67	26.5	6.1	28.3	5.1	60.5	60.7	Malaysia	4
EB-5	M-41	6.3	74	23.6	4.7	45.0	3.1	47.2	61.2	Malaysia	4
EB-18	F- 2	5.8	71	22.8	4.5	56.7	3.3	35.5	65.6	Malaysia	4
EB-2	M-19	8.3	52	20.8	9.5	4.7	5.8	80.0	70.2	Malaysia	3
EB-27	M-11	5.7	54	15.5	6.3	18.3	5.4	70.0	44.8	Malaysia	4
EB-30	F-32	9.4	71	21.8	2.6	63.0	2.7	31.7	66.3	Malaysia	4
Plus 10 additional patients, all transfused										Malaysia	

Table 4d.7 continued:

Case	Sex-Age	Hb g/dl	MCV fl	MCH pg	δ %	γ %	β^A %	β^E %	G_γ %	Origin	# α Genes
E-β^o-Thal; CDs 41/42 (-TTCT)											
China 8	F-31	6.8	63	17.0	5.5	32.9	0	61.6	56.0	China	-
Li 11	M-15	4.8	74	16.4	10.6	9.8	0	79.6	31.2	China	4
S-555	F- 6	transfused							50.0	Surinam	4
E-β^o- Thal; CDs 71/72 (+A)											
Li 7	M-16	8.9	56	16.8	6.9	27.0	0	65.4	66.9	China	4
Li 10	M-12	7.3	63	19.0	6.1	38.8	0	55.1	62.4	China	4
Li 14	F-12	7.5	85	23.1	4.5	44.0	0	50.5	37.3	China	4
Li 27	F-14	4.8	70	19.1	4.5	36.5	0	59.0	39.6	China	4
E-β^o-Thal; CDs 8/9 (+G)											
S-458	F-13	5.9	73	16.5	7.1	31.5	0	61.4	62.8	Surinam	4
S-459	M- 9	7.0	111	18.8	9.0	29.3	0	61.7	57.1	Surinam	3
E-β^o-Thal; IVS-I-2 (T→C)											
S-478	F-19	transfused								Surinam	4
E-β^o-Thal; CD 39 (C→T)											
D.D.	M- 9	8.1	80	20.0	6.0	57.2	0	36.8	59.0	Georgia	-
E-β^o-Thal; CD 35 (-C)											
EB-7	F-16	7.1	80	22.0	4.4	48.7	0	47.9	69.2	Malaysia	-
EB-11	M-11	8.6	81	21.7	6.2	46.9	0	46.9	69.1	Malaysia	-
S-493	F-35	6.2	101	22.7	8.7	44.4	0	46.9	57.6	Surinam	4
S-506	M-18	10.2	79	20.1	8.8	51.9	0	39.3	58.6	Surinam	4
S-507	F-21	8.3	80	19.5	6.3	58.6	0	35.1	55.7	Surinam	4
E-β^+-Thal; Poly A (AATAAA→AATAGA)											
M.S.	M- 9	9.5	59	18.6	4.3	7.6	41.6	46.5	-	Malaysia	-
E-Malay; CD 19 (A→G)											
EB-28	F-55	10.2	58	19.4	?	3.9	0	-	72.1	Malaysia	-
EB-MB2	F-50	9.8	64	20.0	?	9.7	0	-	66.2	Malaysia	-

The three patients with a thalassaemia intermedia type disease had either Hb E in combination with the mild mutation in the poly A signal (AATAAA→AATAGA) (32), or the A→G mutation at CD 19 resulting in the synthesis of Hb Malay [β19(B1)Asn→Ser] which is a thalassaemic variant because the CD 19 mutation activates a cryptic splicing site (16). The two adults

with this condition had slightly elevated Hb F levels and no Hb A, but the normally functioning Hb Malay was present in slight excess over Hb E.

6. CONCLUSION

None of the Surinam Hb E heterozygotes had an anemia or a severe microcytic condition. The individuals with Hb E-β-thal were severely affected. The severity of the disease is determined by the type of β-thal allele that is present. Most β-thal mutations observed are nonsense mutations or frameshifts resulting in a total absence of normal β^A mRNA production (β^0-thal), while one of the few β^+-thalassaemic alleles, namely the IVS-I-5 (G→C) mutation which occurs at high frequencies in Malaysia and Indonesia, causes a severe β^+-thalassaemic condition with only a modest β^A production. It is interesting to note that two rare β-thal mutations, the IVS-I-2 (T→C) and the CDs 8/9 (+G), occur in the Surinam-Javanese ethnic group; these two mutations have never before been reported for the Indonesian populations. Although the studied Javanese group is small, the heterogeneity of the β-thal types seems to be tremendous in this ethnic group (five different types of β-thal in five families studied). An accurate diagnosis in all individuals with Hb E or Hb E-β-thal at an early age may prevent long-term iron therapy in cases such as occurred in patient S-493. Pregnancies in women with Hb E-β-thal are associated with considerable maternal and foetal risk (33), so raising the awareness of this Hb disorder can contribute to an appropriate diagnosis and proper therapy. Couples at risk of having offspring with Hb E-β-thal should be considered for genetic counselling.

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CHAPTER 5

THE SURINAM ($\delta\beta$)°-THALASSAEMIA IS DUE TO A DELETION OF 31.6 KB

CHAPTER 5

THE SURINAM ($S\beta$)°-THALASSAEMIA IS DUE TO A DELETION OF 31.6 KB

1. ABSTRACT

We describe a unique form of $\delta\beta$-thal that has been observed in two generations of a Surinam family of Asian-Indian descent. The propositus is a 19-year-old female who received monthly blood transfusions. DNA from members of this family were studied through restriction enzyme analysis. Gene mapping studies showed no abnormal α-globin gene rearrangements in any of the six family members. Studies on the β-globin gene locus using the β-IVS-II and pRK 28 probes, indicated the presence of an extensive deletion including the β, δ, and $^{A}\gamma\beta$ genes. The Stoating point of this deletion maps 1 kb 3' to the $^{A}\gamma$ gene, while the deletion ends approximately 10 kb 3' to the β gene, just outside a region of repetitive sequences named Kpn 1 or L1. The minimum size for this deletion is estimated to be 31.6 kb. An IVS-I-5 (G→C) mutation causing β-thal was present in the propositus, in trans of the $\delta\beta$° deletion. Rather high foetal Hb levels (16-29%) were observed in the Surinam ($\delta\beta$)° heterozygotes with a $^{G}\gamma$:$^{A}\gamma$ ratio averaging 85:15.

2. INTRODUCTION

The molecular mechanism that control developmentally programmed switches in gene expression have triggered the imagination of many molecular biologists. The developing human switches progressively from the embryonic Hbs ($\zeta_2\varepsilon_2$, $\alpha_2\varepsilon_2$, and $\zeta_2\gamma_2$) to the fetal Hb ($\alpha_2\gamma_2$), and finally to the adult Hbs ($\alpha_2\beta_2$ and $\alpha_2\delta_2$). In some individuals the switch is incomplete and the expression of the γ genes persists into adult life (1,2). This condition is known as HPFH, or in some cases $\delta\beta$-thal (see Chapter 1). HPFH and $\delta\beta$-thal are two phenotypically distinct syndromes that are associated with a total absence of β- and δ-globin chain synthesis (2). They are often the result of large deletions of DNA in the β-globin gene cluster (3-5).

The level of γ gene production is higher in deletional HPFH heterozygotes (15-30%) than in $\delta\beta$-thal heterozygotes (5-12%). Several ($\varepsilon\beta$)°-thalassaemic chromosomes carry deletions that vary in length from 7.2 to 120 kb (2). This report describes a Surinam family with a $^{G}\gamma^{A}\gamma(\delta\beta)$°-thal. The breakpoints were determined by restriction endonuclease mapping and by hybridization with specific probes.

3. MATERIALS AND METHODS

3.1 The Family. The propositus (II-4) is a 19-year-old female of Surinam-Asian descent who received monthly transfusions at the Academisch Ziekenhuis in Paramaribo, Surinam. She had an enlarged spleen and liver, growth retardation, and bone deformation. Blood samples were collected from the propositus and five of her relatives. The mother of the propositus had a history of multiple miscarriages. The living conditions of this uneducated family were extremely poor.

3.2 Haematological and Hb Studies. Collection of blood, preparation of DNA, restriction endonuclease digestion of DNA, and gene mapping hybridization procedures have been described previously (Chapter 2). Red cell lysates were analyzed by IEF, and by reversed phase HPLC using a Vydac C_4 column (6). This procedure allows an accurate quantitation of the two types of γ chain, $^{G}\gamma$ and $^{A}\gamma$, and the mutant $^{A}\gamma^{T}$ chain. Hb analyses included cation exchange HPLC for the quantitation of Hb A_2 (7,8) and alkali denaturation for the quantitation of Hb F (9).

3.3 DNA Analysis.

3.3a Dot-blot analysis. DNA samples from the propositus and her relatives were amplified using a primer set which allows amplification of an ~700 bp fragment that includes exon 1, intron 1, exon 2, and a small segment 3' to the second exon of 71 bp. The location of the primers is shown in Chapter 2. Amplified DNA fragments were spotted onto a nylon membrane, baked at 80°C for 1 hour, and filters were hybridized with ^{32}P-labelled probes, washed, and processed for autoradiography.

Leukocyte DNA was isolated from peripheral blood as described previously (Chapter 2). A 5 µg aliquot of DNA was digested at 37°C with 20 units each of the following restriction endonucleases: Bam HI, Hind III, Eco RI, Xmn I, Xba I, Pst I, Bgl II, Hpa I, and Bcl I, using buffer conditions specified by the suppliers. DNA fragments were separated by electrophoresis in 0.8% agarose gel, transferred to a nitrocellulose membrane, baked for 3 hours at 70°C, and filters were hybridized with ^{32}P-labelled probes, washed, and processed for autoradiography. Hybridization was performed with the γ-IVS-II, ζ-IVS-II, and pRK 28 probes, and with with α gene probes. Details about the probes are given elsewhere (Chapter 2).

4. RESULTS

The pedigree of the Surinam family is presented in Fig. 5.1 and haematological data are given in Table 5.1 (data for the propositus are not included because her blood sample was collected about three days after the last blood transfusion). HPLC studies shoed an increased Hb A_2 value for the mother of the propositus; her MCH value was decreased, while her MCV value was in the normal range. The normal MCV values is most likely due to inadequate storage conditions prior to shipment of the samples to our laboratory and a delay in the analysis. The father (I-1) and brother (II-3) of the propositus had abnormal MCH values and high Hb F values (16 and 29%, respectively). This Hb F contained $^{G}\gamma$ chains (average 83%) and $^{A}\gamma$ chains (average 17%). The Hb F in the mother consisted of $^{G}\gamma$, $^{A}\gamma$, and $^{A}\gamma^{T}$ chains; the values were 63, 16.5, and 20.5%, respectively. Two other relatives (II-1, II-2) had normal red cell indices.

Fig. 5.2 illustrates data obtained from some of the dot-blot hybridization experiments. The propositus and her mother are carriers of the IVS-I-5 (G→C) β-thal allele. A seemingly homozygous condition for this well-known β-thal mutation was detected in the propositus; this however, was excluded because the father was negative for the IVS-I-5 (G→C) mutation. An additional abnormality, inherited from the father, had to be present in trans

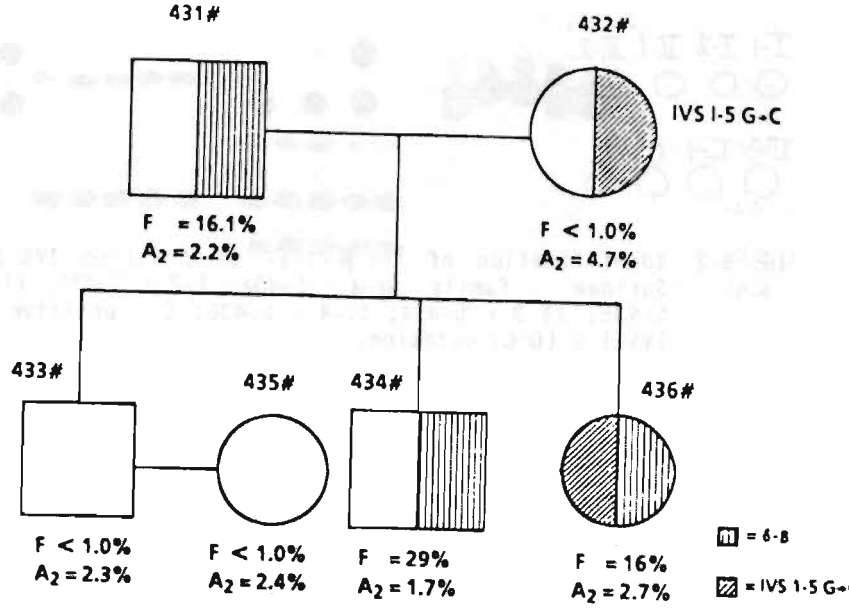

FIG. 5.1 Pedigree of the Surinam ($\delta\beta$)°-Thal Family

TABLE 5.1 Haematological and Hb Composition Data of the Surinam ($\delta\beta$)°-Thal Family

Case	Sex-Age	Hb g/dl	PCV l/l	MCV fl	MCH pg	MCHC g/dl	A_2 %	F %	$A_\gamma T$ %	G_γ %	$A_\gamma I$ %
S-431	M-58	11.5	.500	98	22.5	23.0	2.2	16.1	0	83.7	16.3
S-432	F-50	10.7	.475	92	20.7	22.3	4.7	<1.0	20.5	63.0	16.5
S-433	M-33	15.6	.575	120	32.6	27.1	2.3	<1.0	n.d.	n.d.	n.d.
S-434	M-26	13.4	.540	108	26.9	24.8	1.7	29.0	0	81.0	19.0
S-435	F-24	12.0	.430	101	28.2	27.9	2.4	<1.0	n.d	n.d	n.d.
S-436	F-19	n.d.	n.d.	n.d.	n.d.	n.d.	2.7	16.0	0	85.3	14.7

to the β-thal allele of the propositus in order to account for this observation. This abnormality was considered to be a deletion causing a $\delta\beta$°-thal which in the heterozygote, is characterized by elevated levels of Hb F and by distinct microcytosis and hypochromia (10).

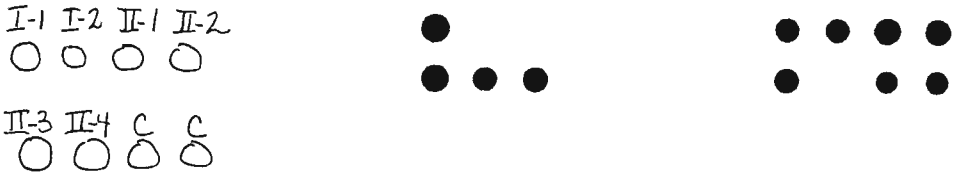

FIG. 5.2 Identification of the β-thal at position IVS-I-5 (G→C) in the Surinam family. I-1 = S-431; I-2 = S-432; II-1 =S-433; II-2 = S-435; II-3 = S-434; II-4 = S-436; C = positive controls for the IVS-I-5 (G→C) mutation.

4.1 Mapping of the 5' Breakpoint of the δβ-Thal Deletion. Digestion with Hind III, Bcl I, Bgl II, Xba I, and hybridization to a $^{G}\gamma$-IVS-II probe gave besides normal, several abnormal fragments (Fig. 5.3, Table 5.2). Digestion with Bam HI, Eco RI, and Hpa I produced normal fragments (Table 5.2). The data given in this table also show that the propositus is +/+ at the Xmn I site 5' to the $^{G}\gamma$ gene, and +/+ and -/- at the Hind III sites in the $^{G}\gamma$ and $^{A}\gamma$ genes, respectively. The Xmn I and Hind III sites are known to be polymorphic (11,12).

TABLE 5.2 Summary of Restriction Endonuclease Data for the Surinam δβ-Thal DNA Following Hybridization to the γ-IVS-II Probe

CASE / ENZYME	S-431	S-432	S-433	S-434	S-436
Bam HI	15; 5	15; 5	15; 5	15; 5	15; 5
Bgl II	12.9; **10**	12.9	12.9	12.9; **10**	12.9; **10**
Xba I	7.4; 4.9; 3.2; **2.4**	7.4; 4.9; 3.2	7.4; 4.9; 3.2	7.4; 4.9; 3.2; **2.4**	7.4; 4.9; 3.2; **2.4**
Hind III	**7.5**; 6.8; 3.3	6.8; 3.3; **2.5**	n.d.	n.d.	6.8; 3.3
Eco RI	7; 2.7	7; 2.7	n.d.	n.d.	7; 2.7
Xmn I	n.d.	**7**;	n.d.	**7**; 8	**7**
Pst I	n.d.	5.1; 4.1	n.d.	5.1; 4.1	5.1; 4.1
Bcl I	n.d.	18	n.d.	18; **15.5**	18; **15.5**
Hpa I	n.d.	4.6	n.d.	4.6	4.6

Bold figures denote a polymorphic site; underlined figures denote an abnormal fragment.

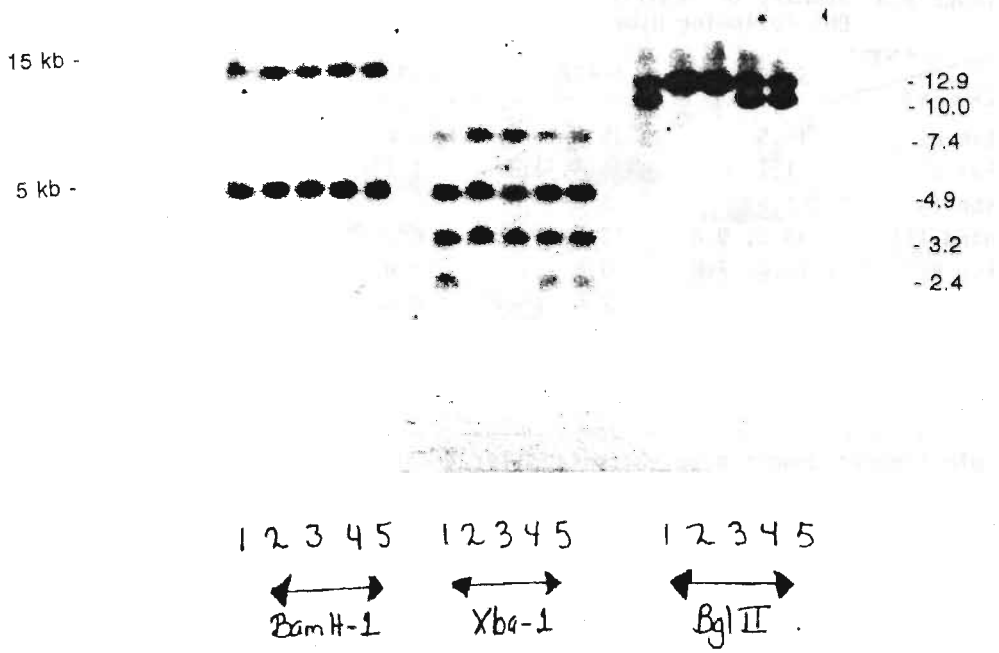

FIG. 5.3 Sizes of fragments obtained after digestion with Bam HI, Xba I, and Bgl II from members of the Surinam $\delta\beta$ family. See text for details. Lanes 1, 2, 3, 4, and 5 respectively refer to the following family members: S-431, S-432, S-433, S-434, and S-436 (see pedigree). The membrane was hybridized with the $^{A}\gamma$-IVS-II probe.

The sizes of the abnormal fragments are similar to those reported for a recently discovered Indian $\delta\beta$ deletion (13). The 5' breakpoint in this deletion was reported to be about 1 kb 3' of the $^{A}\gamma$-globin gene poly A site. By using the sequence data found 3' of the new Indian $\delta\beta$-thal (unreported data provided by one of the authors of Ref. 13) a primer set was used to amplify part of the genome containing the deletion breakpoint and the amplified DNA was in part sequenced. Our data confirmed the observation that the Surinam $\delta\beta$ deletion has the exact breakpoint as the new Indian $\delta\beta$-thal described in Ref. 13.

4.2 Mapping the 3' Endpoint of the Deletion. The 3' endpoint was mapped by hybridizing the digested genomic DNA with the pRK 28 probe that is located 17 kb 3' to the β gene. Abnormal fragments were obtained with Hind III, Eco RI, and Xmn I, but not with Bcl I, Hpa I, Pst I, Xba I, and Bam HI (Table 5.3, Fig. 5.4). The same sites are also deleted in the Black $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal and the Turkish $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal, but with different sizes of the abnormal fragments (14,15).

TABLE 5.3 Summary of Restriction Endonuclease Data for the Surinam $\delta\beta$-Thal DNA Following Hybridization to the pRK 28 Probe

Enzyme \ Case	S-431	S-432	S-433	S-434	S-436
Bam HI	15.5	15.5	15.5	15.5	15.5
Bgl II	1.1	5.4; 1.1	1.1	1.1	5.4; 1.1
Xba I	5.6	5.6	5.6	5.6	5.6
Hind III	13.5; **9.8**	13.5	n.d.	n.d.	13.5; **9.8**
Eco RI	10.8; **7.8**	10.8	n.d.	n.d.	10.8; **7.8**
Xmn I	n.d.	12.8	n.d.	12.8; **10.2**	12.8; **10.2**
Pst I	n.d.	9.5	n.d.	9.5	9.5
Bcl I	n.d.	3.9; 3.6	n.d.	3.9; 3.6	3.9; 3.6
Hpa I	n.d.	25	n.d.	25	25

Bold figures denote a polymorphic site; underlined figures denote an abnormal fragment.

The calculation of the length of the deletion has been simplified by the fact that after digestion with Bam HI no abnormal fragments were found when hybridized with the γ-IVS-II or the pRK 28 probes. Mapping the different abnormal sizes indicates that the abnormal Bam HI fragment originating from the $\delta\beta$-thal chromosome has the same size (15.5 kb) as the Bam HI fragment from the β-thal chromosome. As the exact 5' location of the breakpoint was known, the position of the intact Bam HI site 5' to the deletion could be determined in relation to the start of the deletion. The distance between this Bam HI site and the breakpoint of the deletion was 2.24 kb, indicating that at the 3' site, the deletion also extended for 2.24 kb into the 16 kb Bam HI fragment obtained from the Hind III and Eco RI fragments, that resulted in an average value of 32 kb for this deletion (Fig. 5.5). The 3' end of this deletion lies between the 3' endpoint of the Turkish $^{G}\gamma^{+}(^{A}\gamma\delta\beta)^{\circ}$-thal and that of the Black $^{G}\gamma^{+}(^{A}\gamma\delta\beta)^{\circ}$-thal (16). The Turkish deletion has its 3' endpoint 48 bp 3' to the 6.4 kb L1 repeat that is located 3' to the β-globin gene, while the 3' endpoint of the Black deletion is located with an L1 family repeat located about 3 kb outside the 6.4 kb L1 repeat (Fig. 5.6).

5. DISCUSSION

Restriction endonuclease mapping analysis made it possible to determine the breakpoints of a large deletion causing a $\delta\beta$-thal in members of a Surinam family. the 5' endpoint of the deletion is located ~1 kb 3' to the poly A site of the $^{A}\gamma$-globin gene, while the 3' endpoint was found about 0.5 kb from the L1 region, 6.4 kb 3' of the β-globin gene. Exactly the same 3' breakpoint was found in a $\delta\beta$ deletion reported by Mishima et al (13). For several reasons it was not possible for these authors to determine the exact location and size of their reported $\delta\beta$-thal (Indian $\delta\beta$-thal). It is most likely that the Suriman $(\delta\beta)^{\circ}$-thal and the Indian $\delta\beta$-thal are the same, which is supported by the fact that both families are of Surinamese descent.

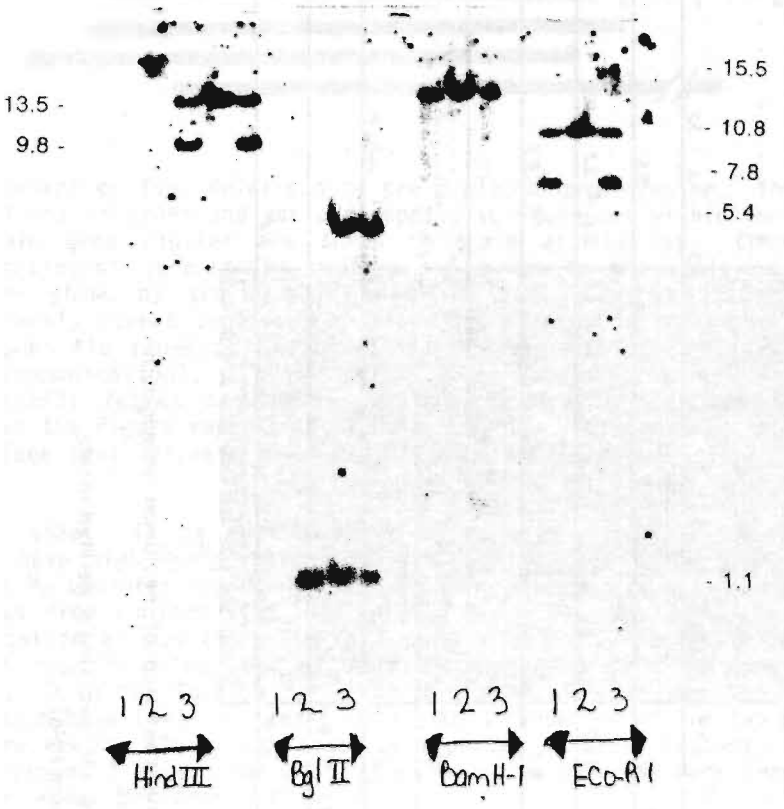

FIG. 5.4 Sizes of fragments obtained after digestion with Hind III, Bgl II, Bam HI, and Eco RI from members of the Surinam δβ family. See text for details. Lanes 1, 2, and 3 respectively refer to the following family members: S-431, S-432, and S-436 (see pedigree). The membrane was hybridized with the pRK 28 probe.

The 3' deletion endpoint of the Surinam δβ-thal is located between the 3' endpoints of the Turkish $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal (15,17,18), and the Black $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal (14,16). The Dutch β°-thal (13), the Laotian $(\delta\beta)^{\circ}$-thal (19), and several other δβ-thalassaemias, have their endpoints within a few kb 3' to the endpoint of the Surinam $(\delta\beta)^{\circ}$-thal. The clustering of these endpoints is near or in the L1, Alu, or other repetitive elements that are considered to be "hot spots" for recombination (20,21). At the 5' site, the Surinam δβ-thal falls into a cluster with the Indian $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-HPFH (20), the Japanese δβ-thal (22), the Black HPFH-2 (23), and the Black $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal (14). It is only with the Black $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thal that the Surinam $(\delta\beta)^{\circ}$-thal shares clustering of both the 3' and 5' areas. A common mechanism might be involved in generating these deletions. The Surinam δβ, however, differs from all of the above mentioned δβ-thal deletions in

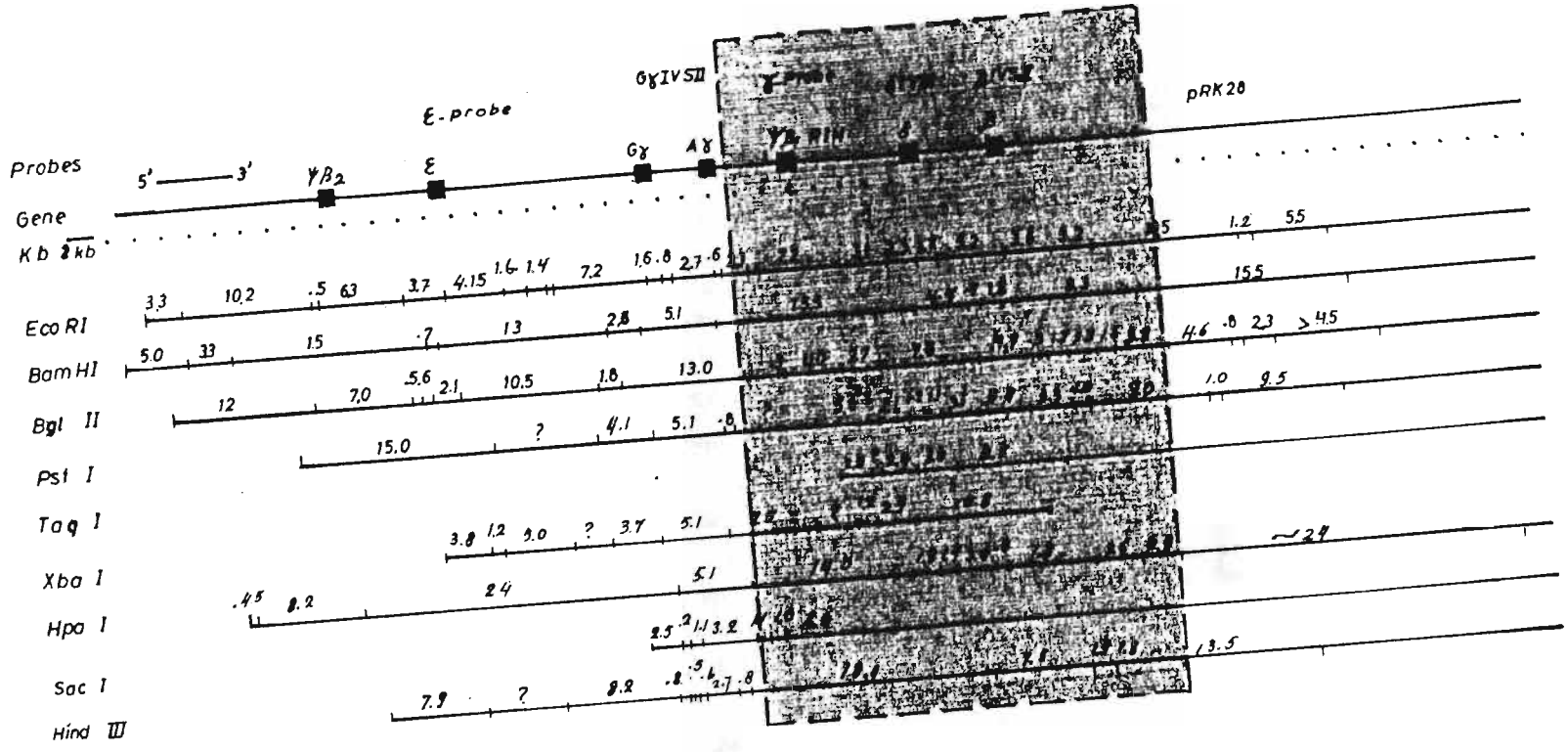

Fig. 5.5 Restriction Endonuclease Map of the Human Hemoglobin β-like Globin Genes.
(Dotted box shows the area that is deleted in this Surinam δ-β thalassaemia).

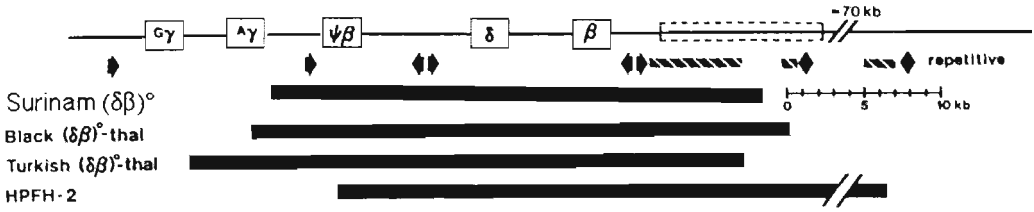

FIG. 5.6 Extent of four deletions in the β-globin gene cluster. The locations of genes and various repetitive sequences within the β-globin gene cluster are shown to scale at the top. Open boxes represent gene-coding regions. A break in the scale of 70 kb is shown by two parallel slanted lines. Arrows represent Alu family repeat sequences of known orientation, and diamonds represent Alu repeats of unknown orientation (P.S. Henthorn, personal communication). Cross-hatched bars show the locations of L1 family repeat sequences. the solid bars in the lower portion of the figure represent the extent of the four indicated deletions (see text for references) (modified from Henthorn et al, 1990).

its unique size. It is interesting that heterozygotes for the Surinam (δβ)°-thal have high G_γ:A_γ ratios (85:15). This suggests a possible mutation in the G_γ promoter region. We sequenced the promoter regions of the G_γ and A_γ genes from subject II-3, but no abnormalities were found except for the T→C mutation at position -158 of the G_γ gene that is known to be associated with high G_γ values and an increased Hb F production in many conditions (11). Both the Turkish $G_\gamma^+(A_\gamma\delta\beta)$ deletion (36.1 kb) and the Surinam (δβ)° deletion have their 5' breakpoint ~1 kb outside one of the two γ genes (16). There are no Alu or L1 sequences reported in these regions. Therefore, the regions 1 kb outside the γ genes might be involved more frequently in possible recombinations.

It is of importance to note that the Surinam δβ-thal deletes the same δ-β intergenic region as is the case for all deletional types of HPFH without leading to a phenotypically similar condition. This δ-β intergenic region was thought to be involved in contributing to the difference noted between the HPFHs and the δβ-thalassaemias in foetal Hb production and the phenotypical differences (24). It still remains unclear what is causing the differences between HPFH phenotypes and δβ-thal, but based upon our observation the δ-β intergenic region is probably not involved in regulating these differences.

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SUMMARY

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The basis of the investigation described in this thesis is the study of haemoglobinopathies in the Surinam population. Special attention has been paid to newly developed and advanced techniques, such as polymerase chain reaction (PCR) and its application to techniques, such as oligonucleotide specific probing (dot blot) and single strands' DNA sequencing (ss DNA). Were possible, clinical data of the Surinam patients with haemoglobinopathies have been compared with similar patient groups described elsewhere to investigate similarities or differences. Untill now, the lack of adequate knowledge in the field of haemoglobinopathies is responsible for a certain rigidity in the medical management of haemoglobinopathy patients. It is not excluded that this rigidity in some cases leads to excess morbidity and even premature death of these patients.
The patients' material for investigation has been collected randomly from the Paramaribo hospital clinics (Academic Hospital, Diakonessenhuis, 's Lands Hospitaal and St.Vincentius Hospital) and from patients and their relatives who were known with the disease to the medical staff and general practitioners. A total number of blood samples from over 600 individuals have been studied. It should be mentioned that most samples were obtained from the hospital where the majority of births is found ('s Lands Hospitaal).

In *chapter 1* a review is given on haemoglobinopathy. Attention has been paid to classification and expression regulation of the different globin genes. It has been explained that selective expression of individual globine genes during ontogenesis is regulated on the level of gene transcription by:
a) cis-acting regulatory control DNA sequences that are part of a globin gene or are nearly within the globin gene cluster, and
b) trans-acting regulatory proteins interacting with the control sequences.
In paragraph 5 of this review numerous models have been proposed to explain the properties of the different regulation sequences.
Several haemoglobin variants with their pathophysiological consequences, which are found in the Surinam population, have also been discussed.
In the paragraph on thalassaemia, the α-thalassaemias were discussed as the first group. They are caused by deletion (the deletion types) or mutations (non-deletion types) of (or in) one to four of the α-genes. The resulting disorders involve various degrees of imbalance between α- and β-chain synthesis, and patients can be classified using haematological, biochemical and molecular criteria.
β-Thalassaemia is an extremely heterogeneous group of disorders of haemoglobin synthesis, and is due to a decrease (β^+) or absence (β^0) of β-

globin chain synthesis. More than 100 different β-thalassaemia genes have been identified. At the molecular level, β-thalassaemia is due to deletions of part or all of the β-globin gene (deletion types) or more often to point mutations which include single base changes, and deletions and insertions of one to four nucleotides (non-deletion types). A special form of β-thalassaemia are the ∂-β-thalassaemias, which are associated with extensive deletions of varying lengths , involving the ∂-, the β- and often the γ-genes. They are characterized by a normal Hb A_2-level (1.6-3.5%), a markedly raised Hb F level and varying degrees of anaemia in the heterozygote patient. All the so far characterized ∂-β types are the result of non homologous recombinations in sopecific parts of the genome.
The last group of thalassaemias discussed in this review are the HPFH's (Hereditary Persistence of Fetal Haemoglobin). HPFH is a clinically benign condition characterized by the continued expression of one or both of the γ-genes in adult life; these genes are normally expressed at significant levels in the fetal period only. HPFH's are classified by molecular analysis in deletion- or non-deletion types.
The use of restriction endonuclease for the detection of normal variations in DNA structures (polymorphism) is described in the last paragraph of this review chapter.

In *chapter 2*, methods and techniques and also the biological specimens used in our investigations are described in detail.

Chapter 3 deals with structural haemoglobin variants found in the population of Surinam.
Chapter 3a. Three different haemoglobinopathies, i.e. HbS, Hb Chad [α23(B4)Glu→Lys], and α-thal-2(-3.7kb) have been observed in eight members of a family. The propositus had all three abnormalities, while her mother and four of her half-brothers had Hb Chad together with an α-thal-2 heterozygosity or homozygosity. Gene-mapping and dot-blot analysis of amplified DNA identified a G→A mutation in codon 23 of the α2β1 hybrid gene resulting in the Gly→Lys substitution. The quantity of α-Chad chain averaged 31.5% in its carriers with an additional α-thal-2 heterozygosity [$-\alpha^{Chad}$(-3.7 kb)/-α(-3.7 kb)]. These quantities are considerably higher than those reproted for families from Chad, China and Japan; the low levels of 14.5-24% Hb Chad in members of these familiers suggest a mutation on a chromosome with two α-globin genes [$\alpha\alpha^{Chad}/\alpha\alpha$ or $\alpha^{Chad}\alpha/\alpha\alpha$].

In *chapter 3b* the most common Hb variant in the Surinam population, i.e. HbS, is described.
Haematological and genetic data have been collected for twelve SS, one SC

and four S-β-thal patients and their relatives. Haplotyping, using ^{32}P-labelled oligonucleotide specific probes, identified haplotypes #19 and #20 as the major types among the Surinam population. Both haplotypes have been reported to originate from West and Central Africa. These results suggest that the β^S gene among Surinamese was introduced by the slave trade, common in the 19th century in that part of the world. Haematological data showed that AS individuals are clinically normal, while the SS patients have a severe anaemia with a variety of complications, comparable to those observed for SS patients from Africa and the United States of America. The AS individuals did not have significantly increased levels of circulating foetal haemoglobin (HbF < 1%), while the foetal Hb level in the twelve SS patients averaged about 10%.

Chapter 4 deals with thalassaemias in the Surinam population.
In *chapter 4a*, it is described that twenty Surinamese families with β-thal were screened for particular mutations using ^{32}P labelled oligonucleotide probes, sequence analysis and gene mapping. Thirteen different mutations were detected. The IVS-I-5 (G→C) was the most frequently observed, being present in six of the twenty families, followed by the frameshift at codons 41/42 (-TTCT) that was observed in three families. The Creole and Javanese ethnic groups were the most heterogeneous. Seven different mutations [-88 (C→T); -29 (A→G); codons 41/42 (-TTCT); codon 47 (+A); IVS-I-849 (A→G); IVS-II-1 (G→A); -28 (A→G); 1.393 kb deletion] were found in eight Creole families and four mutations in five Javanese families [IVS-I-5 (G→C); codons 8/9 (+G); codon 35 (-C); IVS-I-2 (T→C)]. Three different β-thal alleles were detected in six Hindustani families [IVS-I-5 (G→C); codons 41/42 (-TTCT), and a ∂β-thal due to a 31.6 kb deletion]. One family, of Libanese origin, had the frameshift at codons 41/42 (-TTCT). A unique frameshift mutation was detected in one family belonging to the Surinam Creole group [codon 47 (+A)].

In *chapter 4b*, haematological and Hb composition data are presented for fourteen members of a Surinam family (and for one unrelated subject) with either a β-thal heterozygosity [five with the -29 (A→G) β^+ mutation and five with the IVS-II-849 (A→G) β° mutation] or a compound heterozygosity (the five remaining patients). Identification of the mutation was by hybridization of amplified DNA with ^{32}P-labelled synthetic oligonucleotides. The data indicate distinct differences between the two groups of heterozygotes, mainly in degree of microcytosis and hypochromia, in Hb A_2 levels, and in the level of $^G\gamma$ (high in the -29 heterozygotes and low in the IVS-II-849 heterozygotes). The five compound heterozygotes had a thalassaemia intermedia with high Hb F levels (high $^G\gamma$), elevated Hb A_2, and Hb A levels comparable to those

seen in patients with a homozygosity for the -29 mutation or with the combination of this β^+-thal and Hb S. An α-thal-2 heterozygosity (-3.7 kb deletion) was present in two patients. Their haematological data were improved over those for the patients with four α-globin genes; one was the mother of two sets of twins. The high $^{G}\gamma$ value in the Hb F of the compound heterozygotes suggests that the high Hb F production in the condition is mainly directed by the chromosome with the -29 (A→G) mutation.

Chapter 4c: Hb A_2 and its variant Hb A_2' or B_2 [∂16(A13)Gly→Arg] were quantitated in the blood of subjects with three different types of β-thal, and with the ∂-B_2 anomaly *in cis* or *in trans* to the β-thal determinant. In one family, the ∂-B_2 mutation was *in cis* to a newly discovered codon 47 (+A) frameshift. The levels of Hbs A_2 and B_2 were nearly the same and about 70% higher than those in simple Hb B_2 heterozygotes. In two additional families, the ∂-B_2 variant was *in trans* to either a deletional β-thal (1392 bp) involving part of the β-globin gene and part of the β-globin gene promoter, or to the -88 (C→T) promoter mutation. In both instances, the Hb B_2 level was increased by 80%, but the Hb A_2 level was increased by ~270 and ~200% respectively. These data indicate two mechanisms that will cause an increase in ∂ chain production. One is consistent with a general mechanism concerning the relative excess of α chains in β chain deficiencies which will combine with ∂ chains to form variable levels of Hb A_2, dependent upon the severity of the β chain deficiency. The second concerns the loss of β-globin gene promoter activity, perhaps by an absence of (or decreased) binding of (a) specific protein(s) to this segment of DNA, and a concomitant increase in ∂-globin gene promoter activity *in cis*.

In *chapter 4d*, compound heterozygosity for the combination HbE and β-thalassaemia is described.
During our survey for haemoglobinopathies in the Surinam population, six families were observed with Hb E-β-thal. A detailed comparison was made of the clinical condition, haematological data, Hb composition, and types of alleles present in these Surinam E-β-thal patients and with those of 28 patients from Malaysia, China and Georgia (USA). The majority of the 28 patients were attending a haematology clinic in Kuala Lumpur (Malaysia), five were from the southern part of China, and the one patient (D.D) from Georgia was of mixed Korean-Italian descent. All patients from Surinam with the E-β-thal condition were descendants of immigrants from Indonesia or India. A total of 54 chromosomes from 27 individuals were analyzed, mainly by hybridization with specific oligonucleotide probes after DNA amplification. Six different β-thal mutations, including the β^E mutation, were detected in he six Surinam families; also detected was a rare splice junction mutation,

IVS-I-2 (T→C), which was previously found in an American Black teenager and in an Algerian child.

Chapter 5 describes a unique form of ∂β-thal that has been observed in two generations of a Surinam family of Asian-Indian descent. The propositus is a 19-year-old female who received monthly blood transfusions. DNA from members of this family were studied through restriction enzyme analysis. Gene mapping studies showed no abnormal α-globin gene rearrangements in any of the six family members. Studies on the β-globin gene locus using the β-IVS-II and pRK 28 probes, indicated the presence of an extensive deletion including the β, ∂ and ψβ genes. The 3' breakpoint of this deletion maps 1 kb 3' to the $^{A}\gamma$ gene, while the deletion ends approximately 10 kb 3' to the β gene, just outside a region of repetitive sequences named Kpn 1 or L1. The minimum size for this deletion is estimated to be 31.6 kb. An IVS-I-5 (G→C) mutation causing β-thal was present in the propositus, *in trans* of the ∂β° deletion. Rather high foetal Hb levels (16-29%) were observed in the Surinam (∂β)° heterozygotes with a $^{G}\gamma$:$^{A}\gamma$ ratio averaging 85:15.

SAMENVATTING

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De studie van haemoglobinopathieën in de Surinaamse samenleving, met behulp van nieuw ontwikkelde technieken zoals de polymerase chain reaction (PCR) en daaraan gekoppelde technieken zoals oligonucleotide specifieke probing (dot blot) en enkele strengs DNA (ss DNA) sequencing, vormen de basis van het in dit proefschrift beschreven onderzoek. Waar mogelijk zijn bij het in dit proefschrift beschreven onderzoek, klinische gegevens van Surinaamse haemoglobinopathiepatiënten vergeleken met vergelijkbare groepen in de literatuur om analogieën of verschillen aan te tonen. Het ontbreken tot nu toe van voldoende kennis op dit gebied leidt nog tot een verstarring in het nemen van beslissingen rond het medisch handelen bij haemoglobinopathie patiënten, dit kan vervolgens tot een verkorting van de levensduur van deze patiënten aanleiding geven.
Het patiëntenmateriaal is random verzameld uit de diverse ziekenhuisinstellingen (Academisch Ziekenhuis, Diakonessenhuis, 's Lands Hospitaal, Sint Vincentius Ziekenhuis) van Paramaribo en van patiënten en hun familieleden die bij de artsen bekend stonden als haemoglobinopathie patiënten. In totaal werden zo bloedmonsters van meer dan 600 individuen bestudeerd. Hierbij werden tevens 201 navelstreng-bloedmonsters bestudeerd om zodoende de frequentie van haemoglobineafwijkingen in de Surinaamse samenleving te bepalen. Dit materiaal werd v.n.l. verzameld uit het ziekenhuis met de meeste bevallingen per jaar n.l. 's Lands Hospitaal.

In *hoofdstuk 1* wordt een overzicht gegeven van de diverse haemoglobino-pathieën, waarbij tevens aandacht besteed is aan de classificatie en de expressie-regulatie van de diverse globinegenen. Aangegeven wordt dat de selectieve expressie van individuele globinegenen gedurende de ontogenese gereguleerd wordt op het niveau van gen transcriptie door:
a) cis-acterende regulatie van DNA sequenties welke weefsel specifieke en ontwikkelings specifieke patronen van expressie vertonen;
b) trans-acterende regulatie eiwitten die in interactie zijn met de regulatie-DNA sequenties.
Verschillende modellen die voorgesteld worden om de eigenschappen van de verschillende regulatiesequenties te verklaren worden in paragraaf 5 van deze review besproken. Verder worden de diverse haemoglobinevarianten die in de Surinaamse samenleving aangetoond zijn tezamen met hun pathofysiologie besproken.
Bij de thalassaemieën worden allereerst de α-thalassaemieën besproken. Deze zijn onderverdeeld in de deletie en de non-deletie typen. Het resultaat van deze deleties bestaat uit verschillende gradaties van onbalans tussen α

en β ketens. Door gebruik te maken van haematologische, biochemische en moleculaire criteria kunnen patiënten geclassificeerd worden. De β-thalassaemie is een extreem heterogene groep van Hb synthese afwijkingen en wordt veroorzaakt door een verminderde (β^+) of afwezige (β^0) produktie van het β-globine. Er zijn meer dan 100 verschillende β-thalassaemie genen geïdentificeerd. Op moleculair niveau wordt β-thalassaemie veroorzaakt door deleties van gedeelten of van alle β-globinegenen, of veel vaker door punt mutaties, wat inhoudt single base veranderingen en deleties of inserties van 1 tot 4 nucleotiden. Een bijzondere vorm van β-thalassaemie zijn de ∂-β-thalassaemieën die gekarakteriseerd worden door een normaal HbA_2 niveau (1,6 - 3,5%), een sterk verhoogd HbF gehalte, en verschillende gradaties van anaemie bij de heterozygote patiënt. Alle tot nu toe ontdekte ∂-β typen zijn het resultaat van niet homologe recombinaties in specifieke gedeelten van het genoom.
De laatste groep van thalassaemieën welke in deze review besproken worden zijn de HPFH's (Hereditary Persistence of Fetal Haemoglobin). Dit is een klinisch benigne conditie, gekarakteriseerd door de continue expressie van één of beide γ-globine genen bij volwassenen. De γ-genen worden normaliter alleen tijdens het foetale stadium in voldoende kwantiteit geproduceerd. De HPFH's worden moleculair genetisch ingedeeld in deletie en niet-deletie typen.
Haplotypering met behulp van restrictie enzym analyse voor het aantonen van normale variaties in DNA-structuren (polymorphisme) wordt beschreven in de laatste paragraaf van dit hoofdstuk.

In *hoofdstuk 2* worden de methoden, technieken en het bij het onderzoek gebruikte materiaal uitgebreid beschreven.

Hoofdstuk 3 behandelt structurele varianten van haemoglobine in de bevolking van Suriname.

In *hoofdstuk 3a* wordt een Surinaamse familie beschreven waarbij 3 verschillende haemoglobinopathieën, HbS, HbChad [α_{23} (B_4) Glu --▸ Lys], en α-thal-2 (-3,7 kb) werden gevonden. De propositus had alle 3 abnormaliteiten, terwijl haar moeder en 4 van haar half-broers het HbChad haemoglobine hadden tezamen met een α-thalassaemie-2 heterozygotie of homozygotie. Gene mapping en dot-blot analysen van geamplificeerd DNA toonden een G --▸ A mutatie in codon 23 van het $\alpha_2\alpha_1$ hybridegen welke resulteerde in een Glu --▸ Lys substitutie. Het percentage van de α-Chad keten bij de heterozygote dragers die een additionele α-thalassaemie-2 heterozygotie [-α^{Chad} (-3,7 kb/αα)] hadden, bedroeg gemiddeld 31,5%, en 43% bij de 2 heterozygote α-Chad dragers met een additionele α-thalassaemie-2-homozygotie [-α^{Chad} (-3,7 kb) / -α (3,7 kb)]. Deze percentages zijn aanzienlijk

hoger dan die gepubliceerd zijn voor families uit Chad, China en Japan. Het lage percentage van 14,5-24% Hb Chad bij leden van deze families duidde op een mutatie van een chromosoom met 2 α-globine genen [$\alpha\alpha^{Chad}/\alpha\alpha$ of $\alpha^{Chad}\ \alpha/\alpha\alpha$)].

In *hoofdstuk 3b* wordt de meest voorkomende Hb-variant in de Surinaamse samenleving besproken, nl. het HbS. Haematologische en genetische gegevens zijn verzameld van twaalf SS, één Sc en vier S-β-thalassaemie patiënten en hun families. Door middel van haplotypering, waarbij gebruik gemaakt is van ^{32}P gelabelde oligonucleotide specifieke probes, zijn de haplotypen #19 en #20 aangetoond als de meest voorkomende in de Surinaamse samenleving. Beide haplotypen zijn afkomstig uit West en Centraal Afrika. Deze resultaten geven weer dat het β^S gen welke aanwezig is in de Surinaamse bevolking, geïntroduceerd is ten tijde van de slavenhandel in de 19e eeuw. Haematologische gegevens tonen aan dat de AS individuen klinisch normaal zijn, terwijl de SS patiënten een ernstige anemie hadden met een variëteit van complicaties, vergelijkbaar met die, geobserveerd zijn voor SS patiënten uit Afrika en de Verenigde Staten van Amerika. De As individuen hadden geen significante verhoging van circulerend foetaal haemoglobine (HbF < 1%), terwijl bij de 12 SS patiënten het foetaal haemoglobine gemiddeld 10% bedroeg.
Uit bestudering van 201 navelstrengbloedjes blijkt dat 10% van de creolen drager is van het sikkelcel gen, een percentage welk elders (USA, Nederland) ook gevonden wordt voor de negroïde populatie.

Hoofdstuk 4 geeft een overzicht van de verschillende vormen van thalassaemie in Suriname.

In *hoofdstuk 4a* worden de verschillende β-thalassaemie genen die gekarakteriseerd zijn tijdens dit onderzoek weergegeven. Twintig Surinaamse families met β-thalassaemie werden gescreend, voor specifieke mutaties, waarbij gebruik gemaakt is van ^{32}P-gelabelde oligonucleotide probes, sequencing en gene mapping. Dertien verschillende mutaties werden er ontdekt.
De IVS I-5 (G --▸ C) werd het meest aangetoond, en was aanwezig in zes van de 20 families, gevolgd door de frame shift van codons 41/42 (- TTCT) welke bij 3 families werd aangetroffen. De Javaanse en Creoolse ethnische groep waren de meest heterogene. Zeven verschillende mutaties [-88 (C --▸ T); -29 (A --▸ G); codons 41/42 (-TTCT); codon 47(+A); IVS-II-849 (A --▸ G); IVS-II-1 (G --▸A); -28 (A --▸ G); 1,393 kb deletie] werden gevonden in 8 creoolse families en vier mutaties in vijf Javaanse families [IVS 1-5 (G --▸ C); codons 8/9 (+G); codon 35 (-C); IVS-I-2 (T --▸ C). Drie verschillende β-thalassaemie allelen werden ontdekt bij 6 Hindoestaanse families [IVS 1-5

(G --▸ C); codons 41/42 (-TTCT) en een ∂-β thalassaemie met een deletie van 31,6 kb].
Een familie van Libanese origine had de frameshift codon 41/42 (-TTCT). Een unieke frameshift mutatie werd ontdekt in een Surinaams Creoolse familie [codon 47 +(A)].

In *hoofdstuk 4b* wordt een grote Surinaamse familie van Creoolse origine beschreven, waarbij een dubbele heterozygotie voor een β^+ en een zeldzame β^0 thalassaemie werd gevonden. Haematologische en Hb compositie data zijn weergegeven voor 14 leden van deze Surinaamse familie (en voor één niet gerelateerd individu) met een β-thalassaemie heterozygotie [vijf met de -29 (A --▸ G) β^+ mutatie en vijf met de IVS II-849 (A --▸ G) β^0 mutatie] of een dubbele heterozygotie (de vijf resterende patiënten).
Identificatie van de mutatie werd verricht d.m.v. hybridisatie van geamplificeerd DNA met ^{32}P-gelabelde synthetische oligonucleotiden. De resultaten indiceren duidelijke verschillen tussen de 2 heterozygote groepen, voornamelijk in de mate van microcytosis en hypochromie, in HbA_2 waarden, en in de waarden van $^{G}\gamma$ (hoog bij de -29 heterozygoten en laag in de IVS II-849 heterozygoten). De vijf dubbele- heterozygoten vertoonden een thalassaemie intermediair met verhoogde HbF (hoge $^{G}\gamma$), verhoogd HbA_2 en HbA waarden vergelijkbaar met die van patiënten homozygoot voor de -29 mutatie of met de combinatie van deze β^+ thalassaemie en HbS. Een α-thalassaemie-2 heterozygotie (-3,7 kb deletie) was aanwezig bij 2 patiënten. Hun haematologische waarden waren beter t.o.v. de patiënten met vier α-globine genen; één daarvan was de moeder van twee tweelingen. De hoge $^{G}\gamma$ waarde van het HbF van de dubbele heterozygoten geeft aan dat de hoge HbF produktie bij deze conditie voornamelijk afkomstig is van het chromosoom met de -29 (A --▸ G) mutatie.

In *hoofdstuk 4c* worden o.a. twee Surinaamse families, beiden van "mixed" origine beschreven, waarbij HbA_2 en zijn variant HbA_2' of B_2 [∂ 16 (A 13) Gly --▸ Arg] werd gekwantificeerd uit het bloed van deze individuen. Verder waren er ook β-thalassaemieën genen *in cis* of *in trans* van de ∂-B_2 variant aanwezig. In één familie was ∂-B_2 mutatie *in cis* van het nieuw ontdekte codon 47 (+ A) frame shift β-thalassaemie gen. De percentages van HbA_2 en B_2 waren vrijwel identiek, maar 70% hoger dan de HbB_2 heterozygoten zonder een thalassaemie conditie. In twee andere families was de ∂-B_2 variant *in trans* van een deletie β-thal (1392 bp), waarbij een gedeelte van het β-globine gen en een gedeelte van de β-globine promotor betrokken is, en in het tweede geval de -88 (C --▸ T) promotor mutatie. In beide gevallen was het HbB_2 percentage met 80% verhoogd, terwijl het HbA_2 percentage verhoogd was met respectievelijk 270 en 200%. Deze

gegevens indiceren 2 mechanismen welke betrokken zijn bij de verhoging van de ∂-keten produktie. Het ene is consistent met het algemene mechanisme, dat ervan uitgaat dat de relatieve overproduktie van α-ketens bij β-keten deficiënties zal combineren met ∂ ketens om zodoende variërende percentages van HbA_2 te vormen, afhankelijk van de ernst van de β-keten deficiëntie. Het tweede mechanisme neemt het verlies van het β-globine gen in aanmerking, veroorzaakt door de afwezigheid of verminderde binding van (een) specifieke eiwit(ten) aan dit DNA segment, met een gelijktijdig optreden van een verhoging van de promotor activiteit van het ∂-globine gen *in cis*.

In *hoofdstuk 4d* wordt de dubbel heterozygotie voor de combinatie HbE en een β-thalassaemie gen beschreven. Gedurende het onderzoek naar haemoglobinopathieën in de Surinaamse populatie zijn 6 families gevonden met de combinatie HbE-β-thalassaemie. Een uitgebreide vergelijking werd gemaakt van de klinische condities, haematologische data, Hb compositie en de typen β-thalassaemie allelen, aanwezig in deze Surinaamse E-β-thalassaemie patiënten met 28 patiënten van Malaysia, China en Georgia (USA). De meerderheid van de 28 patiënten bezochten een haematologische kliniek in Kuala Lumpur, 5 waren afkomstig van het zuidelijk gedeelte van China en één patiënt (D.D) van Georgia was van gemengd Koreaans-Italiaans afkomst. Alle patiënten van Suriname met de E-β-thalassaemie conditie waren van Indonesische of Indiase afkomst. Een totaal van 54 chromosomen van 27 individuen uit Suriname werden geanalyseerd, voornamelijk m.b.v. hybridisatie van specifieke oligonucleotide probes, na DNA amplificatie. Zes verschillende β-thalassaemie mutaties, inclusief de β^E mutatie werden aangetoond in deze 6 Surinaamse families. Ook werd een zeldzame splice function mutatie aangetoond [IVS 1-2 (T --▸ C), welke eerder was gevonden in een zwarte Amerikaanse tiener en een Algerijns kind.

In *hoofdstuk 5* wordt een unieke vorm van ∂-β-thalassaemie beschreven welke is aangetoond in twee generaties van een Hidoestaans-Surinaamse familie. De propositus is een 19 jaar oude vrouw die maandelijks bloedtransfusies ontving. DNA van leden van deze familie is bestudeerd m.b.v. restrictie enzym analyse. Gene mapping studies toonden aan dat er geen abnormale α-gen veranderingen waren in de zes bestudeerde individuen van de familie. Onderzoek van het β-globine gen, met gebruikmaking van de βIVSII en pRK 28 probes, indiceerden de aanwezigheid van een grootte deletie inhoudende de β, ∂ en ψβ genen.
Het 3' breekpunt van deze deletie lag 1 kb 3' van het $^A\gamma$ gen terwijl de deletie eindigt 10 kb 3' van het β gen, net buiten het gebied van repeterende

sequenties genaamd Kpn 1 of L1. De minimale lengte van deze deletie wordt geschat op 31,6 kb. Een IVS 1-5 (G --▸ C) mutatie was tevens aanwezig in de propositus (*in trans*) van de ∂-β^0 deletie. Vrij hoge foetaal Hb percentages (16-29%) werden waargenomen in de heterozygoten van de Surinaamse $(\partial\text{-}\beta)^0$-thalassaemie met een gemiddelde $^G\gamma$:$^A\gamma$ ratio van 85:15.

EPILOOG

EPILOOG

Het onderzoek naar de moleculaire karakterisatie van abnormale haemoglobines in de bevolking van Suriname werd om een aantal redenen ondernomen. In eerste instantie is een nadere studie welhaast een morele verplichting voor een uit deze populatie voortkomende onderzoeker, gezien de frequentie van met deze afwijkingen samenhangende ziektebeelden en de ernst van de aandoeningen, waarmee de patienten geconfronteerd worden. Deze morbiditeit trok dan ook reeds eerder de aandacht. Uit literatuuronderzoek is gebleken, dat er op zeer beperkte schaal een poging was ondernomen om de verschillende haemoglobine afwijkingen, voorkomend binnen onze multi-raciale gemeenschap, in kaart te brengen. Deze eerdere onderzoekingen concentreerden zich voornamelijk op de inheemse bevolking van Suriname, te weten de indianen en de boslandcreolen, en dateren van de jaren veertig en vijftig. De beperktheid van deze studies lag ten dele aan de in die vroegere dagen beschikbare technieken, waarbij het in principe alleen maar mogelijk was om met behulp van elctroforese op celluloseacetaat naar Hb-varianten te zoeken.

Al in 1966 is echter door een werkgroep van de World Health Organisation vastgesteld (WHO Bulletin, 1966) dat er een nationaal gezondheidszorgprogramma met betrekking tot haemoglobinopathieën in Suriname noodzakelijk was.

De beschikbaarheid van moderne technieken zoals de Polymerase Chain Reaction (PCR), sequencing van geamplificeerd single strand DNA en dot blotting technieken, maken het thans mogelijk om zowel voor de Hb-varianten als ook voor de thalassaemieën tot precieze karakterisering te komen.

Met gebruikmaking van deze technieken is onderzoek gedaan naar de moleculaire compositie van genetische afwijkingen bij klinisch bekende haemoglobinopathie patienten en hun verwanten. Het nut van zodanig basaal onderzoek staat voor een Derde Wereld land als Suriname zeker ter discussie. Gelet echter op ook de sociale aspekten hiervan kan het nuttig aspect worden samengevat als hieronder geformuleerd.

Door middel van moleculair-genetische karakterisering van afwijkende haemoglobines is het mogelijk om:

1. de ernst van de diverse Hb-pathieën bij patienten tijdig te voorspellen, waardoor geantecipeerd kan worden op adequate behandeling.
2. genetisch inzicht te verwerven in de onderhavige problematiek en de verworven kennis omtrent deze veel voorkomende ziekte beschikbaar te doen komen aan medici, studenten en aan de gehele samenleving.

Uit ons onderzoek is komen vast te staan dat het ontbreken van kennis op dit gebied in enkele gevallen heeft geleid tot de premature dood van haemoglobinopathie patienten ten gevolge van onjuiste behandeling, waarbij gedacht moet worden aan onnodige en schadelijke toediening van ijzerpraeparaten. Ook wordt ten onrechte aan heterozygote dragers van o.a. het HbS gen meegedeeld, dat zij klachten vertonen, die gerelateerd worden aan het dragerschap.

Het grote aantal mensen (circa 75000 personen ofwel 1 van elke 4 Surinamers) in de Surinaamse bevolking dat drager iis van een haemoglobinopathie, vereist dat de gehele samenleving voor deze problematiek gesensibiliseerd moet worden. Hierbij gelden de volgende aanbevelingen:

* verplichte screening van pasgeborenen, om zodoende tijdig patienten op te sporen;
* opzetten van algemeen toegankelijke faciliteiten voor genetic counseling.

Het geheel aan maatregelen zal er dan toe moeten leiden dat er geen, op grond van de nieuwe inzichten vermijdbare, onjuiste behandeling van patienten meer plaats vindt. Ook zal het aantal hospitalisaties van patienten met haemoglobinopathie verminderen. In het kader van kosten/baten van de te nemen maatregelen moet onder de aandacht van de Surinaamse overheid gebracht worden dat, naast de winst aan menselijk geluk en welzijn, er van uit gegaan moet worden dat de vele dragers/patienten met haemoglobinopathie een langer en productiever leven beschoren zal zijn.

Zonder de medewerking van de vele door mij onderzochte patienten en hun verwanten zou het onderhavige onderzoek nimmer zijn voltooid; ik ben hun allen zeer erkentelijk voor hun vrijwillige medewerking. Mijn hoop is ook daarom, dat dit onderzoek een beter inzicht in en gewaarwording van de problematiek van de haemoglobinopathie met zich meebrengt, waarvan de patienten profijt mogen hebben.

De uitwerking van mijn in dit proefschrift beschreven onderzoek is voornamelijk tot stand gekomen door de kennis en ervaring welke ik tijdens mijn twee-en-half jarige opleiding in de Afdeling 'Cell and Molecular Biology' (Hoofd: Prof. Dr. T.H.J. Huisman) van het Medical College of Georgia in Augusta/USA heb opgedaan. Ik wil dan ook op deze plaats alle medewerkers van deze Afdeling bedanken voor de fijne tijd, die ik tijdens mijn verblijf in de U.S.A. heb gehad, maar in het bijzonder Mrs. Cox, die gedeelten van mijn proefschrift van vele engelse taalfouten heeft ontdaan, en Mrs. Carver voor het typen van het manuscript.

Alle goede dingen bestaan uit drie. Dit geldt zeker voor de drie geestesvaders zonder wier medewerking dit werk niet mogelijk was, te weten,

- Dr. J.H. Wisse, hoofd van het klinisch-chemisch laboratorium van het Academisch Ziekenhuis in Paramaribo/Suriname, mijn eerste opleider in de klinische chemie. Hij was de ontdekker van één der haemoglobinevarianten en heeft het mij mogelijk gemaakt om het spoor uit te zetten bij mijn introductie in de haemoglobineresearch. Ik ben hem veel dank verschuldigd.
- Prof. Dr. P.J. Brombacher, mijn promotor, die mede door zijn rust en zakelijke manier van organiseren de laatste loodjes minder zwaar heeft gemaakt. De warmte van U en Uw wederhelft, ook tijdens de periode dat ik in Uw laboratorium werkzaam was, heb ik zeer op prijs gesteld.
- Prof. Dr. T.H.J. Huisman. De goeroe achter dit onderzoek.

Beste Professor Huisman, ik ben U zeer erkentelijk voor Uw kritische begeleiding bij het tot stand komen van dit proefschrift en voor de prettige en leerzame periode, die ik onder Uw supervisie heb mogen doorbrengen. U heeft mij doen inzien dat zelfs de zon niet altijd voor niets op gaat.

Een bijzonder woord van dank gaat uit naar wijlen Herman Harris. Herman, jouw liefde voor de medemens zal mij altijd ten voorbeeld zijn in de rest van mijn leven. Dank zij jou heb ik mijn eerste succes op het gebied van de haemoglobine-afwijkingen geboekt, namelijk de ontdekking van het Haemoglobine-Wrens. Dit proefschrift draag ik mede ter nagedachtenis aan jou op.

De laboratoriummedewerkers van het Academisch Ziekenhuis te Paramaribo/Suriname en van het De Wever-Ziekenhuis te Heerlen/Nederland, die op enigerlei wijze hebben bijgedragen aan dit proefschrift, wil ik hierbij volgaarne dank zeggen.

Een woord van dank gaat uit naar iemand, die mij verzocht heeft geen gewag te maken van haar medewerking, maar waarvan ik het toch mijn plicht acht om dit tenminste incognito te doen: K.M.E., jouw steun en inspiraties tijdens mijn middernachtelijke expedities in het laboratorium van het Medical College of Georgia waren van onschatbare waarde. Hartelijk dank daarvoor.

Ongedeeld, maar toch in drieën gaat mijn dank tenslotte uit naar mijn Schepper, God de Almachtige, naar mijn ouders Frits en Hermien, en naar mijn dochter Soleil N'didi; zonder jullie was er nooit een begin en ook geen einde gekomen. Bedankt voor het geduld en ik zal altijd van jullie blijven houden.

Curriculum vitae

Onder het sterrebeeld *Vissen* werd John Frits Codrington in 1958 in Paramaribo/Suriname geboren.

In 1975, na het behalen van het Atheneum-B diploma aan de Algemene Middelbare School (AMS) in Paramaribo, vertrok hij naar Nederland om zich aldaar aan de Technische Universiteit te Delft in te schrijven voor een studie in de Chemische Technologie. In 1978 werd deze opleiding verruild voor de studie Scheikunde aan de Rijksuniversiteit te Leiden, waar in september 1983 het candidaatsexamen met goede resultaten werd afgelegd. In januari 1986 slaagde hij voor het doctoraalexamen met hoofdvak Biochemie.

In februari 1986 begon hij met de opleiding tot klinisch chemicus in het De Wever-Ziekenhuis te Heerlen (opleider Prof.Dr. P.J. Brombacher), gevolgd van december 1986 t/m maart 1987 door een stage in het laboratorium van het Wilhelmina Kinderziekenhuis te Utrecht onder supervisie van Dr. G. van Stekelenburg. De opleiding werd voortgezet, van april 1987 t/m juli 1988, in het laboratorium van het Academisch Ziekenhuis te Paramaribo (opleider Dr. J.H. Wisse) en afgesloten met een stage van augustus 1988 t/m december 1990 in de het laboratorium van de Faculty of Cell and Molecular Biology (opleider Prof.Dr. T.H.J. Huisman), Medical College of Georgia, Augusta/Georgia/U.S.A. In deze periode werd ook het materiaal verzameld en bewerkt met betrekking tot het in dit proefschrift beschreven onderzoek.

In mei 1991 volgde de registratie als Klinisch Chemicus in het Specialisten Register van de Vereniging van Medici in Suriname.

Vanaf de registratie als specialist is John Frits Codrington werkzaam als klinisch chemicus in het Academisch Ziekenhuis te Paramaribo/Suriname. Tevens is hij vanaf april 1991 Commissaris van de Bloedtransfusiedienst van het Surinaamse Rode Kruis en Lid van het Nationale Aids Comité van Suriname.

Van zijn hand verschenen tot nu toe een twaalftal artikelen in internationale wetenschappelijke tijdschriften op klinisch-chemisch en haematologisch gebied.

Met dank wordt vermeld dat de uitgave van dit proefschrift is mogelijk gemaakt door financiële steun van Roche Nederland B.V. te Mijdrecht en van de Stichting Dr.Ir. J.H.J. van der Laar te Heerlen