MOLECULAR GENETICS OF THALASSEMIAS IN PAPUA NEW GUINEA AND NEIGHBOURING REGIONS

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by

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STATEMENT

This thesis describes the results of research carried out in the Department of Human Genetics, John Curtin School of Medical Research, Australian National University, Canberra, between February 1983 and August 1986, during the tenure of an Australian National University Research Scholarship.

The results embodied in this thesis are my own work accomplished under the supervision of Dr Kim M Summers and Dr Philip G Board, unless otherwise acknowledged in the text.

Pa-thai Yenchitsomanus.

Pa-thai Yenchitsomanus

25 August 1986

DEDICATION

All my effort which appears in this thesis is dedicated to Dr Robert L Kirk, D.Sc., Head of the Department of Human Genetics, JCSMR, ANU, on the occasion of his retirement, December 1986.

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ABSTRACT

The aims of study presented in this thesis were to examine the incidence and types of thalassemia found in the populations of Papua New Guinea (PNG) and to evaluate their distribution in relation to the endemicity of malaria. α-Thalassemia 2, which is caused by a single α-globin gene deletion (-α/) and can only be detected accurately by DNA analysis, was found to be the most common form of thalassemia in the populations of lowland PNG where malaria has been highly endemic. In some groups of the PNG population, the frequency of α-thalassemia 2 is at fixation or nearly so. By contrast, this defect is rare or absent in several groups of highland PNG where malaria is only sporadic. A remarkable correlation between the distribution of α-thalassemia 2 and the endemicity of malaria supports the role of malaria as the selective factor for this defect.

α-Thalassemia 1, a more severe form of α-thalassemia caused by dysfunction or deletion of both α-globin genes on one chromosome (--/), has not been detected by DNA analysis in the populations studied although some form of double α-globin gene dysfunction or deletion is known to be present in these populations from the reported cases of hemoglobin H disease. The extreme rarity of α-thalassemia 1, of which heterozygotes (--/αα) should have an advantage similar to that of α-thalassemia-2 homozygotes (-α/-α), may be due to the loss of this defective allele when it produces lethal homozygotes (--/--) and diseased heterozygotes with α-thalassemia 2 (--/-α).

Three subtypes of the -α/ deletion ($-\alpha^{4.2}$, $-\alpha^{3.7}$I, and $-\alpha^{3.7}$III) were observed in PNG. The $-\alpha^{4.2}$ deletion is predominant in many regions particularly in the north while the $-\alpha^{3.7}$I deletion is common in some areas in the south. The $-\alpha^{3.7}$III deletion, previously reported to be unique in Melanesians and Polynesians, was detected on an offshore island of PNG but was extremely rare on the mainland. This subtype may thus have been introduced from island Melanesia or Polynesia to the PNG region.

β-Thalassemia is also present in PNG but in a much lower frequency than α-thalassemia 2 and its correlation with the endemicity of malaria is less obvious. It was found to be patchily increased in frequency in some eastern areas, which is most likely to result from random effects. Apart from its homozygous disadvantage, the spread of β-thalassemia may also be restricted in the populations by the presence of α-thalassemia 2, which is more common and has a greater fitness. Co-inheritance of the two conditions results in less globin-chain imbalance. If globin chain imbalance gives protection against malaria, then, co-inheritance of α-thalassemia 2 and β-thalassemia may reduce the protective effect. The common form of this defect seems to be β^{+}-thalassemia. Analysis of the β-globin genes has shown that β-thalassemia genes in PNG are associated with two β-globin gene haplotypes, indicating different origins of mutations which have occurred at least twice.

The widespread distribution of α-thalassemia 2 in Southeast Asians, Melanesians, and Polynesians raised the question of whether this defect could be detected in other neighbouring groups. This investigation has also demonstrated for the first time that α-thalassemia 2 caused by the -α/ deletion is indeed

present in some groups of Australian Aborigines. The -α/ deletion in these groups consists of two subtypes, $-\alpha^{3.7}$I and $-\alpha^{3.7}$II. The former seems to be similar to that found in southern PNG while the latter, which has been found as a rare subtype in Southeast Asians, has not been observed in Melanesians and Polynesians. The deletion form of α-thalassemia 2 (the $-\alpha^{3.7}$ I subtype) was also detected in a low frequency in a group of Indonesians. However, the origin of the defects and the role of malaria selection in these groups are not known.

Microgeographic correlation between α-thalassemia 2 and the endemicity of malaria in PNG has provided strong evidence in support of malarial selection for this defect. If α-thalassemia 2, which produces only minimal phenotypic changes in red blood cells, really confers an advantage in protecting against malaria, this advantage must be remarkably small. However, the apparent absence of homozygous disadvantage may allow it to displace the normal haplotype (αα/) and to compete with the more severe defects that confer a similar advantage. This may, thus, be an example of how allelic replacement and genetic interaction and divergence occur in human populations.

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CHAPTER 1

GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1 Human globin genes and natural selection

Genetic diversity between populations can arise through directed or random processes. Natural selection is probably the main process directing the evolution of organisms by promoting their adaptation to the environment where they live. Selection occurs when individuals of different genotypes differ in their reproductive fitness. Genotypes favoured by the environment tend to increase in frequency as individuals carrying these genotypes tend to have greater reproductive success; those that are not favoured tend to decrease in frequency. If environmental conditions do not change, eventually the favoured genotype may be the only one that remains. In reality, reproductive fitness depends upon a complex interaction of total genotypes with changing and varied environments.

That selection occurs in nonhuman species is well documented. When species are exposed to new environmental conditions, the increased reproduction of the more fit genotype has led to new genetic strains: antibiotic-resistant bacteria, DDT-resistant mosquitos, and poison-resistant rats. Industrial melanism in peppered moths, in which pale-coloured moths have evolved to dark strains less likely to be predated by birds in industrial areas, is another example.

To demonstrate natural selection in human populations has been more difficult. Genetic variation in specific populations has been investigated in an attempt to identify environmental, historical or other explanations for gene frequency differences. Where a potential selective factor is identified, further studies may be performed to ascertain its validity.

The human hemoglobinopathies have been investigated by such an approach, and considerable progress has been made towards an explanation of their frequencies and distribution. Haldane (1949), from the observation of the geographic correlation between malaria and thalassemia (a defect in globin synthesis), first suggested an interaction between the infectious disease and the genetic one, to be known as the "malaria hypothesis". The hypothesis proposes that interpopulational differences in frequencies of the defective globin genes are the product of natural selection through the agency of malaria. This hypothesis has been widely examined and expanded to explain the prevalence of many variant hemoglobin genes and red cell abnormalities. The sickle-cell defect was the first well-illustrated case, and provides the best evidence of polymorphism and natural selection in man (Allison 1954). The sickle-cell gene occurs in high frequency in Africans despite the fact that the homozygous sickle-cell condition is virtually lethal. Balanced polymorphism has resulted because the sickle-cell heterozygote is at an advantage over normal homozygotes, as a consequence of protection against malaria. The evidence suggesting this advantage (which includes results of epidemiological as well as laboratory studies) is considerably greater than that for selection operating on other hemoglobinopathies and red cell defects.

Malaria has been and continues to be a selective force in many human populations, since it has not been successfully eradicated from their habitats (WHO 1983). Some human populations have undergone severe selection by malaria for countless generations for they live in highly malarious environments, and

the disease has never been brought under control. These include groups who reside in holoendemic coastal and lowland areas of the Papua New Guinea (PNG) mainland (section 1.6). Unlike African populations, the sickle-cell gene is absent in the population of PNG (Walsh and Cotter 1955; Swindler 1955; Jonxis et al 1958). It is thus interesting to study other defective genes that may confer protection against malaria in this population. This thesis reports a survey and characterization of globin gene defects in populations in various parts of PNG where malaria is highly endemic and non-endemic, and an investigation of gene migration and population relationships in PNG and surrounding regions.

Many excellent reviews on the molecular genetics of hemoglobin and hemoglobinopathies have been published in the last six years (Maniatis et al 1980; Weatherall and Clegg 1981 and 1982; Orkin and Nathan 1981; Benz and Forget 1982; Kan 1982, 1983, and 1986; Dickerson and Geis 1983; Higgs and Weatherall 1983; Orkin et al 1983a; Spritz and Forget 1983; Wood and Weatherall 1983; Collins and Weissman 1984; Nienhuis et al 1984; Orkin and Kazazian 1984; Todd 1984; Antonarakis et al 1985; Karlsson and Nienhuis 1985; Kazazian 1985). The remainder of this introduction thus presents a summary of current understanding which provides the framework for the studies outlined in the thesis.

1.2 Synthesis of hemoglobin during development

Human hemoglobin (Hb) is a tetrameric metalloprotein with a molecular weight of 64,650 (Dickerson and Geis 1983). It consists

A variant of A_γ chain known as $A_\gamma T$ ($A_\gamma 75$ Ile→Thr) has been described and occurs in varying frequencies in different populations (Huisman et al 1985).

of two α-like and two β-like globin polypeptide chains complexed with four molecules of heme, one per globin chain. The α-like globin polypeptides are 141 amino acids in length, and the β-like globin chains are 146 amino acids long. At all times, the amounts of α-like and β-like globin chains in normal individuals are present in a 1:1 ratio. The first globin chains synthesized in embryonic life are the α-like ζ-globin chain and the β-like ε-globin chain (Huehns et al 1969). These, in combination with the α and γ chains synthesized later in this period, form Hb Gower I $\zeta_2\varepsilon_2$(Huehns and Farooqui 1975), Hb Portland, $\zeta_2\gamma_2$ (Capp et al 1967), and Hb Gower II, $\alpha_2\varepsilon_2$(Huehns 1974). Zeta and ε chain production ceases before the tenth week of gestation, and Hb F ($\alpha_2\gamma_2$) predominates throughout most of intrauterine life (Wood 1976). Hb F continues to be a major hemoglobin component at birth constituting from 50 to 90% of the total hemoglobin. It decreases progressively to less than 1% by the age of one year, and this is associated with a reciprocal rise in Hb A ($\alpha_2\beta_2$) and Hb A_2 ($\alpha_2\delta_2$), which constitutes approximately 97% and 2.5% respectively, of hemoglobin in adult blood. There are 2 types of γ chains, $^{G}\gamma$ and $^{A}\gamma$, which differ in having glycine or alanine at amino acid position 136. The ratio of $^{G}\gamma$ chains to $^{A}\gamma$ chains is about 3:1 in the fetus and 2:3 after birth (Schroeder et al 1971).

Thus, there are two developmental switches in hemoglobin phenotype, the embryonic to fetal switch that occurs very early in gestation, and the fetal to adult hemoglobin switch that occurs around the time of birth (Karlsson and Nienhuis 1985). The mechanism of these switches is still unknown and is the focus of intense investigation.

1.3 Molecular structure and arrangement of human globin genes

Prior to the molecular cloning of the globin genes, it had been known for some time from traditional genetic studies that human α- and β-like globin genes were unlinked and their arrangement was understood to some degree. For example, duplication of the α-globin gene was first suggested by Lehmann and Carrell (1968) and supported by others (Hollan et al 1972; Wasi 1973; Nute 1974; Kan et al 1975). Furthermore, the linkage of $^{A}\gamma$-δ-β globin genes was predicted from genetic recombinations that created the fused genes producing Hb Lepore (δβ-chain fusion; Baglioni 1962), Hb anti-Lepore (βδ-chain fusion; Lehmann and Charlesworth 1970; Ohta et al 1971), and Hb Kenya ($^{A}\gamma$β-chain fusion; Kendall et al 1973; Smith et al 1973). This understanding of the α- and β-globin gene arrangements has now been confirmed and extended, and the structure of human globin genes and gene clusters has been precisely defined by use of techniques of gene cloning, restriction endonuclease analysis, and DNA sequencing (Maniatis et al 1980; Orkin and Nathan 1981; Collins and Weissman 1984).

Comparison of nucleotide sequences of the globin genes within the human genome and with those in other species suggests that all were derived by duplication from a common ancestral gene and a description of the evolution of the human globin genes can be compiled. An initial duplication leading to the α-like and β-like lineages is thought to have occurred 450-500 million years (MY) ago with further duplication events leading to the formation of the α-like and β-like globin gene clusters as shown in Figure 1.1.

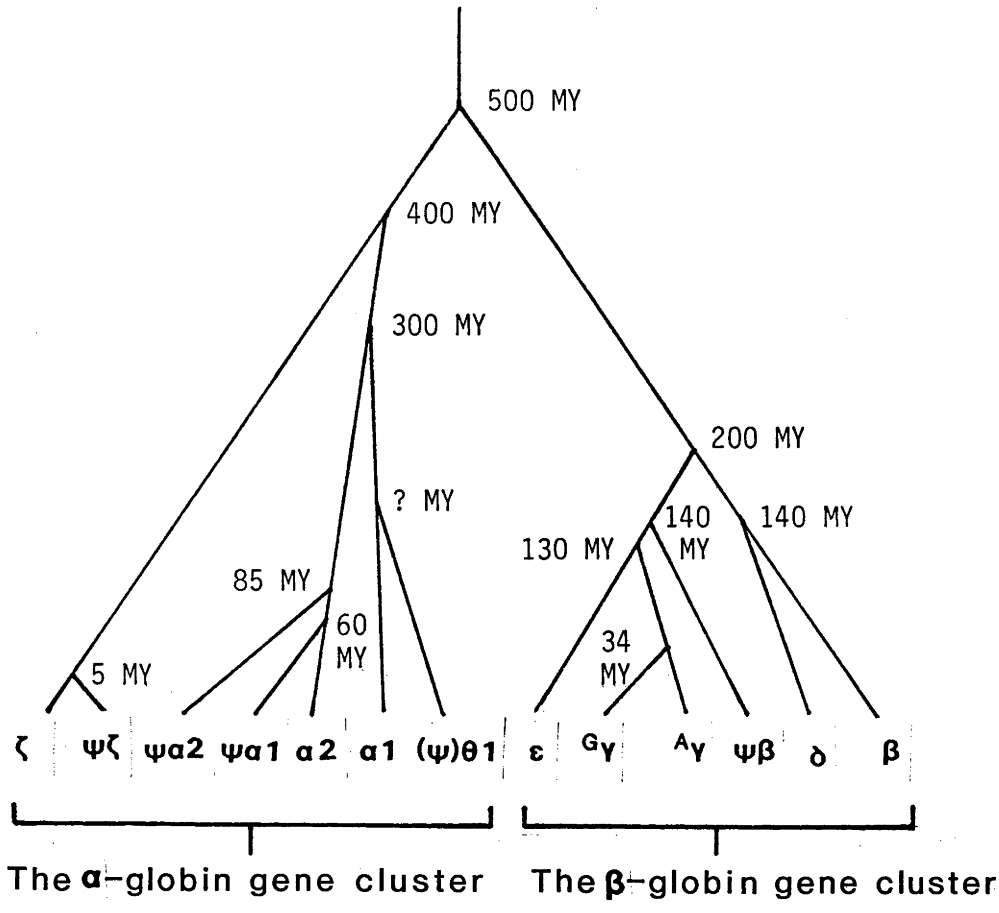


Figure 1.1 Schematic evolutionary tree for human globin genes (drawn from information in Efstratiadis et al 1980; Proudfoot and Maniatis 1980; Zimmer et al 1980; Barrie et al 1981; Goodman 1981; Shen et al 1981; Czelusniak et al 1982; Proudfoot et al 1982; Jeffreys et al 1982; Martin et al 1983; Collins and Weissman 1984; Goodman et al 1984; Hardies et al 1984; Hardison 1984; Harris et al 1984; Scott et al 1984; Hardison et al 1986; Marks et al 1986).

1.3.1. General structure of human globin genes

Human and other vertebrate globin genes consist of 5' and 3' conserved sequences and the coding region of three exons separated by two introns (intervening sequences).

The 5' conserved sequences contain three elements thought to be responsible for RNA polymerase binding and for accurate and efficient initiation of RNA transcription (Efstratiadis et al 1980; Dierks et al 1983; Collins and Weissman 1984). The first is the ATA sequence (the "Hogness-Goldberg box") 25-30 nucleotides upstream from the transcription initiation site. Approximately 80 nucleotides 5' from the site of transcription initiation is the sequence CCAAT, which is duplicated in $^{G}\gamma$ and $^{A}\gamma$ genes and corrupted in the δ gene (which may explain the inefficient transcription of the δ gene). A final conserved component found in the 5' region of the β-globin gene system is the repeated sequence CACCC which is a potential analogue of the sequence GGGGYG (or CRCCCC), known to be critical in transcription of SV40 genes (Collins and Weissman 1984). Mutations in these three regions of the promoter of the mouse β-major globin gene result in a significant decrease in the level of transcription (Myers et al 1986).

The transcribed region of all human globin genes contains three coding regions (exons) separated by two non-coding intervening sequences (IVS or introns) which are not present in mature mRNA. IVS-1 is located between the codons for the amino acids at positions 31 and 32, and 30 and 31 in the α- and β-like globin genes, respectively. IVS-2 is situated between positions 99 and 100 in the α1, α2 and ψα genes, 100 and 101 in

Table 1.1 The lengths of exons and introns in all the known human globin genes[a].

Genes	Exon-1 (Codon no.)	IVS-1 (Base-pairs)	Exon-2 (Codon no.)	IVS-2 (Base-pairs)	Exon-3 (Codon no.)
$\alpha 1$	1-31	117	32-99	149	100-141
$\alpha 2$	1-31	117	32-99	140,142	100-141
$\psi\alpha 1$	1-31	127	32-99[b]	134	100-141[c]
$\psi\zeta$	1-31	1264[d]	32-100	341	101-142
ζ	1-31	886	32-100	239	101-142
β	1-30	130	31-104	850	105-146
δ	1-30	128	31-104	889	105-146
$\psi\beta$	1-30[e]	121	31-104	840-844	104-146[f]
$A\gamma$	1-30	122	31-104	866,876	105-146
$G\gamma$	1-30	122	31-104	866,904	105-146
ε	1-30	122	31-104	850	105-146

a Two newly described genes, $\psi\alpha 2$ and $\theta 1$ (Hardison et al 1986; Marks et al 1986) are not included.

b Codons 38-45 are deleted from exon 2 of the $\psi\alpha$ gene.

c The middle nucleotide of codon 116, the last nucleotide of codon 121, and all three nucleotides of codon 122 are absent.

d IVS-1 of the $\psi\zeta$ gene is variable in length.

e,f There are two 1 nucleotide deletions, at codons 20 and 145, in the $\psi\beta$ gene.

(Efstratiadis et al 1980; Liebhaber et al 1980 and 1981; Proudfoot and Maniatis 1980; Proudfoot et al 1982; Michelson and Orkin 1983; Chang and Slightom 1984).

the ψζ and ζ genes, and 104 and 105 in all the β-like globin genes. Both IVS-1 and IVS-2 are variable in length (Table 1.1). Generally, in all globin genes, the length of the IVS-1 is shorter than that of the IVS-2, except for ψζ and ζ genes in which the length of the IVS-1 is much longer. The exon/intron junctions of the globin genes follow the Breathnach-Chambon rule of consensus nucleotides that an IVS generally begins with GT and ends with AG (Breathnach et al 1978; Breathnach and Chambon 1981). The consensus junction sequences are necessary for normal RNA splicing.

Although the function of introns is still unknown, it has been proposed that they play a role in evolution by joining different combinations of DNA sequences encoding protein structural domains (Gilbert 1978 and 1979). Most striking is the example of the immunoglobulin genes where introns separate the three functional domains of the constant regions within the heavy chain gene (Sakano et al 1979; Early et al 1979; Gough et al 1980). Although less clear in the case of globin genes, the arrangement of exon and intron sequences can also be correlated with the segmentation of the α- and β-globin polypeptides in a functional sense (Gilbert 1978 and 1979; Blake 1979; Eaton 1980). The central exon encodes the region of the polypeptide containing most of the heme contacts. The $\alpha_1\beta_2$ contacts that are responsible for the formation of the cooperative dimer are concentrated in the region encoded by exon-2; the $\alpha_1\beta_1$ contacts that are responsible for the formation of cooperative tetramer map largely in the region encoded by exon-3; and the groups giving rise to the Bohr effect and those that bind the effector

2,3-diphosphoglycerate, both involved in the regulation of hemoglobin function, are located in the regions encoded by exon-1 and -3.

The 3' untranslated sequences of the human globin genes vary in length, but most fall in the range 80 to 150 nucleotides. The only obvious conserved sequence, AATAAA in the DNA (corresponding to AAUAAA in the RNA transcript), is thought to act as a recognition site for cutting the primary transcript some 20 nucleotides downstream from the signal, thereby allowing addition of the poly A tract (Proudfoot and Brownlee 1976; Fitzgerald and Shenk 1981; Montell et al 1983; Proudfoot 1984; Orkin et al 1985). No other specific functional sequences have yet been demonstrated in this region of the gene.

1.3.2 The α-globin gene cluster

The human α-globin genes have been mapped to the short arm of chromosome 16 in the 16p12-16pter region (Deisseroth and Hendrick 1978; Koeffler et al 1981; Barton et al 1982). The α-globin gene cluster spans 30 kilobases (kb) and includes a single functional embryonic ζ gene at the 5' end of the cluster and two functional adult α genes, α2 and α1, that encode identical polypeptides (Földi et al 1980) at the 3' end. Three pseudogenes, ψζ, ψα2 and ψα1, are situated between the ζ and α2 genes (Lauer et al 1980; Proudfoot and Maniatis 1980; Proudfoot et al 1982; Hardison et al 1986). Another α-globin-like sequence, designated as θ1, has recently been detected immediately downstream from the α1 gene in this cluster in primates as well as humans (Marks et al

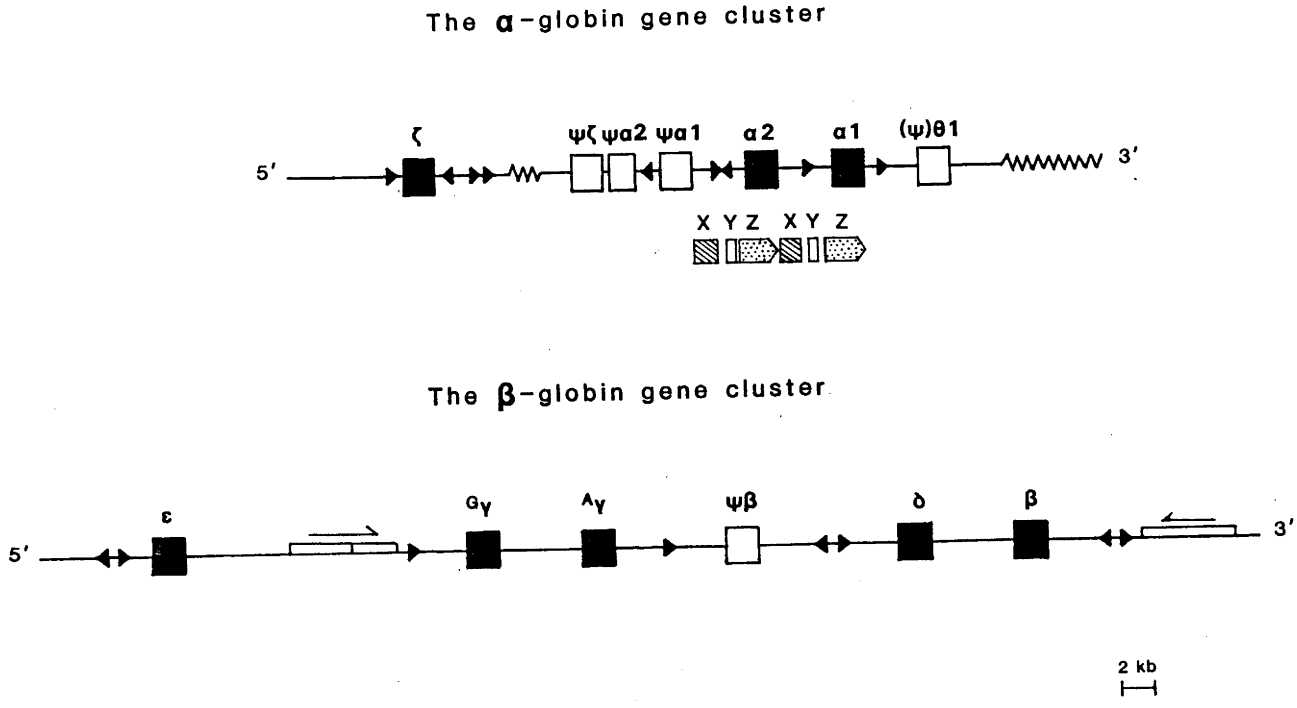


Figure 1.2 Organization of the α- and β-globin gene clusters. Filled-in boxes are coding genes; open boxes are pseudogenes. The triangles (◀) indicate Alu repetitive sequences and their orientation. In the α-globin gene cluster, ⁓⁓ indicates the region of hypervariable DNA, and blocks of sequence homology designated as X,Y, and Z are shown under the cluster. Two long open boxes above the line in the β-globin gene cluster indicate Kpn repeat sequences and their orientations (modified from Collins and Weissman 1984; Karlsson and Nienhuis 1985; Proudfoot 1986).

1986). The genes in this cluster, as in the β-globin gene cluster, are arranged in the 5' to 3' direction in order of their activation (Figure 1.2).

Electron microscopic heteroduplex studies and DNA sequence analyses have demonstrated that the α genes, each of which spans only 850 base pairs, are embedded within two homologous DNA segments of 4 kb long (Laurer et al 1980; Michelson and Orkin 1983; Hess et al 1983). In each of these 4 kb segments, there are three blocks of sequence homology (denoted as X, Y, and Z) and the α1 and α2 genes are located in the Zα1 and Zα2 blocks, respectively (Figure 1.2).

Although duplication of the α genes has been estimated to have occurred before the time of primate divergence, at least 300 MY ago (Zimmer et al 1980), the homology of nucleotide sequences in the X, Y, and Z blocks (including the α gene in the Z block) has been well preserved (Lauer et al 1980; Liebhaber et al 1981; Michelson and Orkin 1983; Hess et al 1983 and 1984). The maintenance of such sequence homology among nonallelic members of a multigene family within a single species has been termed concerted evolution (Zimmer et al 1980). Gene conversion and expansion/contraction of gene number by homologous but unequal crossing over have been proposed to account for this phenomenon (Smith 1973 and 1976; Black and Gibson 1974; Tartoff 1975; Hood et al 1975; Zimmer et al 1980; Slightom et al 1980; Baltimore 1981). Evidence of gene conversion has been shown by nucleotide sequence analysis in the X-homology block (Hess et al 1984). Strong evidence for unequal crossing over in the α-globin gene complex

has been obtained from the identification of individuals possessing one (Orkin et al 1979a; Embury et al 1979 and 1980) or three (Goossens et al 1980; Higgs et al 1980a; Lie-Injo et al 1981; Trent et al 1981) α-globin genes on a single chromosome instead of the normal two genes. In addition, deletions of an α-globin gene, which probably resulted from unequal crossing over and which have locations of the breakpoints indistinguishable from those occurring in humans, have been observed in recombinant phage DNA during propagation in E.coli (Lauer et al 1980).

The α1 and α2 genes are very similar and their structure complies with the general pattern observed for all globin genes (Liebhaber et al 1981; Michelson and Orkin 1983). They consist of three exons and two introns (Table 1.1). The three exons and IVS-1 of the α1 and α2 genes are identical. IVS-2 of the α1 and α2 genes differs by two base substitutions and a seven base-pair (bp) deletion. Homology of the two α genes extends about 870 bp in the 5' untranslated portion. However, their 3' untranslated regions are more divergent, differing by 19 of 113 nucleotides, a total of 17% divergence (Michelson and Orkin 1980 and 1983; Liebhaber et al 1980 and 1981). This has permitted quantitation of α1- and α2-specific mRNA transcripts in human erythroid cells (Orkin and Goff 1981a; Liebhaber and Kan 1981). The ratio of α1 to α2 mRNA is approximately 1:3 to 1:2 while the translational efficiency of α1 is higher, so that the globin production from the α1 and α2 genes is very nearly equal (Nute 1974; Liebhaber and Kan 1982).

The ψα1 gene is approximately 73% homologous to α2 and α1, with many single-base changes and small frameshifts, as well as a 20 bp deletion from codons 38-45 that results in UGA terminations at codon 75, 79, and 83 (Proudfoot and Maniatis 1980). The newly identified ψα2 pseudogene is located very close to the ψζ gene, beginning only 65 bp 3' to the polyadenylation site of the ψζ gene. This pseudogene has suffered many deleterious mutations and seems to be older than the ψα1 gene. The ψα2 is so extremely diverged from the α genes that it was only detected by a computer search for α-like sequences in the regions between the ψζ and α2 genes (Hardison et al 1986). Comparison of ψα2 with the coding regions in the three exons of the α2- and ζ-globin genes reveals only 42% and 38% matching of amino acids.

The ζ and ψζ genes have also been analysed by DNA sequencing (Proudfoot et al 1982). Both contain larger introns than the α genes, due to the presence of varying numbers of homologous simple repeat sequences. Excluding these, extremely close homology extends between nucleotides -148 and 2032. There are only six nucleotide differences within a 2 kb region, three in noncoding sequences, two resulting in amino acid replacements, and one that gives rise to termination at codon 6 of the ψζ gene. Thus, the ψζ gene is unlike other globin pseudogenes (Little 1982) in that it apparently contains all the necessary sequences for transcription initiation, splicing, and polyadenylation.

Three variable-length regions are found in the α-globin gene cluster. These regions are situated between the ζ and ψζ genes, in IVS-1 of the ψζ gene, and about 8 kb beyond the 3' end

of the α1 gene. The region between the ζ and ψζ gene has been sequenced (Goodbourn et al 1983) and has been shown to vary in length due to the presence of differing numbers of a tandemly repeated sequence. The variable length region in IVS-1 of the ψζ gene is likely also to represent differing copy numbers of a repeat sequence although this has not yet been confirmed by sequencing. There is no evidence of variation within IVS-1 of the ζ gene. The variable-length region 3' to the α1 locus has been cloned and partially sequenced (as mentioned in Reeders et al 1985). It consists of a tandem-repeat sequence, and polymorphism at this locus is attributed to variation in the copy number of the elements of the repeat. However, no details of the nucleotide sequence in this region are so far reported. In addition to these short tandemly repeated sequences, the α-globin gene cluster includes several members of the Alu family of interspersed sequences (Hess et al 1983; Sawada et al 1983; Nicholls et al 1985; Figure 1.2) present in multiple copies in the human genome (Singer and Skowronski 1985).

A new α-like globin sequence, the θ1 gene, has recently been discovered 3' to the α1 gene in orang-utans. A similar α2-α1-θ1 linkage was also demonstrated in the human genome using the cloned orang-utan θ1-gene sequence as probe. Furthermore, multiple copies of a sequence homologous to the θ1 gene were detected in both humans and orang-utans (Marks et al 1986). The orang-utan θ1 gene, like the adult α1 gene, contains three exons and two introns and their exons are about 75% identical. The human θ1 gene has not been studied in detail. The recent

discoveries of the $\psi\alpha 2$ and $\theta 1$ genes reveal the posibility that other globin-like sequences may have escaped notice in humans.

1.3.3 The β-globin gene cluster

The human β-globin gene cluster is located on the short arm of chromosome 11 (Deisseroth et al 1978; Jeffreys et al 1979; Lebo et al 1979). Using radiation induced deletions of 11p in somatic cell hybrids, the β-globin gene was originally mapped close to the centromere in band 11p11-11p1208 (Gusella et al 1979, 1980 and 1982). However, more recent data suggest that the β-globin locus is in fact located at 11p15 (de Martinville and Francke 1983; Junien et al 1984; Morton et al 1984; Magenis et al 1985). Other genes have also been localized on 11p, including the insulin gene (Harper et al 1981), c-Ha-Ras 1 oncogene (de Martinville et al 1983), the parathyroid hormone gene (Gerhard et al 1984), and the insulin-like growth factor II gene (Brissenden et al 1984; Tricoli et al 1984). The probable gene order suggested by recent linkage studies is centromere/parathyroid hormone/β-globin/c-Ha-ras1/insulin (Antonarakis et al 1983; Fearon et al 1984).

The β-globin gene cluster, which spans 50 kb of DNA, is more complex than the α-globin gene cluster. The nucleotide sequences of all functional genes in this cluster (the ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β genes), the $\psi\beta$ pseudogene and intergenic DNA regions have been determined (Baralle et al 1980; Efstratiadis et al 1980; Fritsch et al 1980; Kaufman et al 1980; Lawn et al 1980; Slightom et al 1980; Spritz et al 1980; Shen et al 1981; Jagadeeswaran

et al 1983; Poncz et al 1983; Chang and Slightom 1984; Collins and Weissman 1984). Sequence analysis has also been extended approximately 20 kb 5' to the ε gene (Li et al 1985) and about 9 kb 3' to the β gene (Collins and Weissman 1984; Hattori et al 1985), altogether covering a region of DNA over 70 kb long.

The linkage arrangement of the β-globin gene cluster is shown in Figure 1.2. The β gene lies at the 3' end of the cluster. The δ gene is situated approximately 5 kb 5' from the β gene. Further upstream, a ψβ gene separates the δ gene from the $^{A}\gamma$ and $^{G}\gamma$ loci. The distance between the $^{A}\gamma$ and δ genes is 14 kb and it is about 3.5 kb between the $^{A}\gamma$ and $^{G}\gamma$ genes. The ε gene is situated about 13 kb upstream from the $^{G}\gamma$ locus. Another β-like pseudogene, ψβ2, lying 5' to the ε gene was originally described (Maniatis et al 1980) but DNA sequence analysis has failed to confirm the existence of ψβ2 (Shen and Smithies 1982). All functional human β-like globin genes studied contain three exons and two introns (Lawn et al 1980; Spritz et al 1980; Slightom et al 1980; Baralle et al 1980; Table 1.1). The basic structure of the ψβ is consistent with that found for the functional β-globin gene. However, it carries several defects in its coding sequence that completely inactivate the gene. These include the non-functional initiation codon (GTA instead of the normal ATG), a termination signal at codon 15 and two 1 bp deletions at positions 20 and 145. The deletion in codon 20 is the most serious because the resulting frameshift introduces many termination signals in exons 2 and 3.

Restriction endonuclease analysis of the β-globin gene cluster has demonstrated that there are two DNA regions where linkage disequilibrium of polymorphic restriction enzyme sites is maintained (Antonarakis et al 1985). These two regions are a 34 kb DNA portion including the ε, $^{G}\gamma$, $^{A}\gamma$, and ψβ genes (5' to the β gene) and an 18 kb region comprising the β gene and extending beyond it in the 3' direction. The 9 kb DNA segment containing the δ gene between these two clusters is presumed to be an area in which the recombination rate must be relatively high compared with surrounding DNA segments. The recombination rate in this region has been calculated to be up to 30 times greater than the average recombination rate (Chakravarti et al 1984). The precise area where frequent recombination occurs is not known. However, there is an area of 4-6 tandem repeats of ATTTT, 1.3 kb 5' to the β gene, and a sequence of GT dinucleotides repeated 16 times about 1.2 kb 5' to the ATTTT repeats (Spritz 1981; Moschonas et al 1982a). These areas may participate at a high frequency in recombination events.

The β-globin gene cluster contains eight Alu family members (Fritsch et al 1980; Coggins et al 1980). In addition, there is a full-length member of the Kpn family (6125 bp) located about 3 kb downstream from the β gene (Hattori et al 1985). Between the ε and $^{G}\gamma$ genes are two Kpn family members of 3 and 4 kb in length in tandem array and in an inverted orientation compared with the complete Kpn sequence downstream from the β gene. There are 70-bp segments of DNA, homologous to the 3' end of the consensus Kpn

sequence, in the γ-globin gene duplication units. One such segment is found at the 5' end of the duplication unit, one is between, and the third at the 3' end (Karlsson and Nienhuis 1985).

The β gene itself also exhibits polymorphism limited to common sequence types that have been designated β-gene frameworks (Orkin et al 1982a and 1983a). Sequence analysis has revealed that there are five polymorphic sites within the β gene (Figure 1.3). Two of these sites are recognized by restriction enzymes Hgi AI and Ava II, whereas the three other sites in the IVS-2 do not alter known restriction sites. The β-gene frameworks in individuals can be identified by gene mapping of uncloned DNA (Antonarakis et al 1985). In each racial group, three β-gene frameworks are observed. Since these frameworks predate racial divergence, they are presumably of ancient origin.

1.4 Defects of the human globin genes

A large number of heritable abnormalities in hemoglobin can be traced to defects in one or more of the globin genes. Hemoglobinopathies can broadly be divided on the basis of their phenotypic expression into three groups: structural hemoglobin variants, thalassemias (α-like and β-like globin chain imbalances), and hereditary persistence of fetal hemoglobin (HPFH). At the DNA level, several types of mutations have been found to be responsible for the three phenotypes. Point mutations are the most common cause of structural variants and β-thalassemia, while gross deletions of the globin genes are the

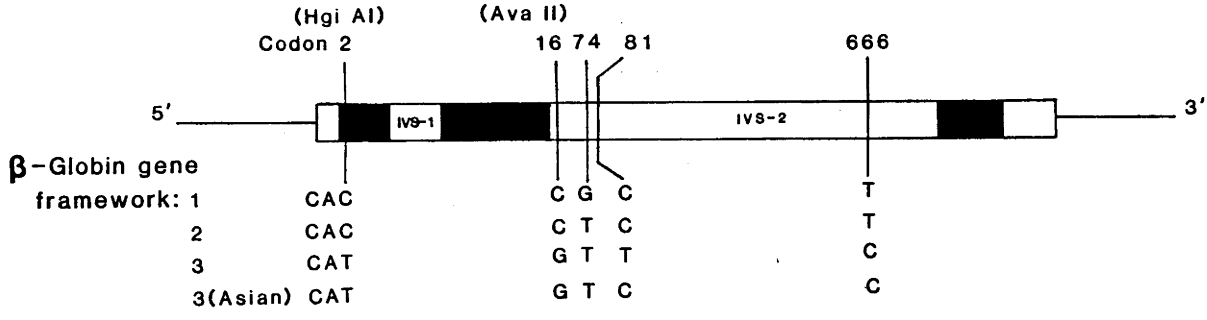


Figure 1.3 Polymorphisms within the human β-globin gene defining the specific gene frameworks (Orkin et al 1983a; Collins and Weissman 1984).

major determinants of α-thalassemia, $\delta\beta$-thalassemia and HPFH. Other mutations (frameshift deletions, frameshift insertions, gene fusions, gene inversions etc) have also been described.

More than 470 hemoglobin variants have been found (IHIC 1986) and have provided important insight into the structure-function relationship of the hemoglobin tetramer. These have been reviewed extensively (Lehmann and Huntsman 1974; Bunn et al 1977; Lehmann and Casey 1982; Dickerson and Geis 1983) and will be considered only briefly in the following sections which will be focussed on thalassemia and related conditions.

The thalassemias are hereditary hemolytic anemias characterized by decreased or absent synthesis of one or more of the globin subunits of the hemoglobin molecule (Weatherall and Clegg 1981). Normally, globin chain production is regulated so that the α- and β-like globin chains are produced in equimolar amounts. In thalassemia, the impaired production of one or more of these globin components causes a deficiency of functional hemoglobin in erythroid cells. The unaffected chain continues to be produced in normal amounts, and in the homozygous state excessive accumulation of the unaffected chain may disrupt erythroid cell maturation and function, causing premature destruction of the cell in the bone marrow and peripheral circulation. The anemia is, thus, the result of defective globin chain production, ineffective erythropoiesis and a hemolytic state. Thalassemic red blood cells show varying degrees of hypochromia, microcytosis, anisopoikilocytosis, target-formation, polychromasia, and basophilic stippling. Untreated severe anemia

results in tissue hypoxia, which is probably one of the major causes of retardation of growth and development. Gross bone marrow hyperplasia may lead to skeletal deformities and increased iron absorption, which is followed by iron overload. The chronic hemolytic state results in hepatosplenomegaly, and hypersplenism may occur. Other anatomical, physiological and biochemical changes subsequently develop.

Thalassemias are among the most common genetic defects in man and occur with high frequency in a belt extending from the Mediterranean littoral and Africa, through the Middle East, India, and South-East Asia to Melanesia. The molecular lesions in thalassemia are extremely diverse. Although defective synthesis of one or more of the α-, β-, γ-, and δ-globin chains has been described, the frequency and clinical severity of α- and β-thalassemias make them the most significant defects.

1.4.1 The α-thalassemias

The α-thalassemias are characterized by partial or complete deficiency of α-globin chain synthesis in the erythroid cells of affected individuals (Weatherall and Clegg 1981). Four clinical syndromes of increasing severity are recognized: the silent carrier state, α-thalassemia trait, Hb H disease, and Hb Bart's hydrops fetalis due to homozygous α-thalassemia. These four clinical syndromes were originally explained on the basis of two different genetic defects, the α-thalassemia-1 genotype, which severely depresses α-globin chain synthesis, and the

α-thalassemia-2 genotype, which mildly affects α-globin chain synthesis (Wasi et al 1964). It is now known that α-thalassemia 2 (silent carrier, or α^+-thalassemia) is caused by deletion or dysfunction of one of the four α genes (-α/αα) (Dozy et al 1979; Higgs et al 1979 and 1980b; Embury et al 1979 and 1980; Pressley et al 1980a; Sancar et al 1980a). α-Thalassemia 1 (severe α-thalassemia or α^0-thalassemia) is caused by deletion or dysfunction of two α genes on one chromosome (--/αα) (Ottolenghi et al 1974; Taylor et al 1974; Pressley et al 1980b,c; Orkin and Michelson 1980). In a more severe form of α-thalassemia, Hb H disease, there is deletion or dysfunction of three of the four α genes due to compound heterozygosity for α-thalassemia-2 and α-thalassemia-1 determinants (-α/--) (Kan et al 1975, 1977 and 1979; Embury et al 1974; Orkin et al 1974a; Phillips et al 1979; Sancar et al 1980a; Tabone et al 1981). In this condition, α-globin synthesis is markedly reduced; the excess β chains accumulate and form Hb H (β_4). Patients with Hb H disease have a mild to moderate hemolytic anemia. In the most severe form of α-thalassemia, Hb Bart's hydrops fetalis, there is complete absence of α-chain synthesis due to deletion or dysfunction of all four α genes (--/--) (Ottolenghi et al 1974; Taylor et al 1974; Surrey et al 1978; Kattamis et al 1980; Ohene-Frempong et al 1980; Pressley et al 1980c; Sophocleous et al 1981). This leads to the formation of Hb Bart's (γ_4), which has a very high oxygen affinity and is

almost unable to release oxygen to the tissues. The resultant severe tissue hypoxia leads to hydropic infants who are stillborn or die shortly after birth.

Detection of α-thalassemia in adults has previously encountered many difficulties. Although Hb H disease can easily be diagnosed by examination of the presence of Hb H in red blood cells, diagnosis of non-disease forms of α-thalassemia on the basis of hematological findings is not reliable. However, it is now possible to accurately detect almost all of the deletion forms of α-thalassemia in adults by the DNA mapping technique.

In α-thalassemias, most of the mutations hitherto described are deletions of relatively large (>1 kb) segments of DNA. Figure 1.4 shows a number of different specific deletions that have been characterized. There are two deletions that result in the -α/ haplotype. The $-\alpha^{3.7}$ deletion (Embury et al 1979 and 1980; Higgs et al 1980b; Phillips et al 1980), also known as the "rightward deletion", seems to have arisen from misalignment and crossover within or near to the Z boxes (Figure 1.2), which are 3.7 kb apart, giving rise to a fusion product between the two α genes. Sequence analysis of this fusion α gene in a Chinese patient shows that the region just 5' to the Z block is clearly derived from α2, while the region for IVS-2 to the end is from α1 (Michelson and Orkin 1983). Subsequent restriction mapping and DNA sequence analysis of the $-\alpha^{3.7}$ deletion indicate that different $-\alpha^{3.7}$ chromosomes are the result of at least three independent recombination events. The $-\alpha^{3.7}$ deletion is, therefore, divided into subtypes I, II, and III, corresponding to the areas where

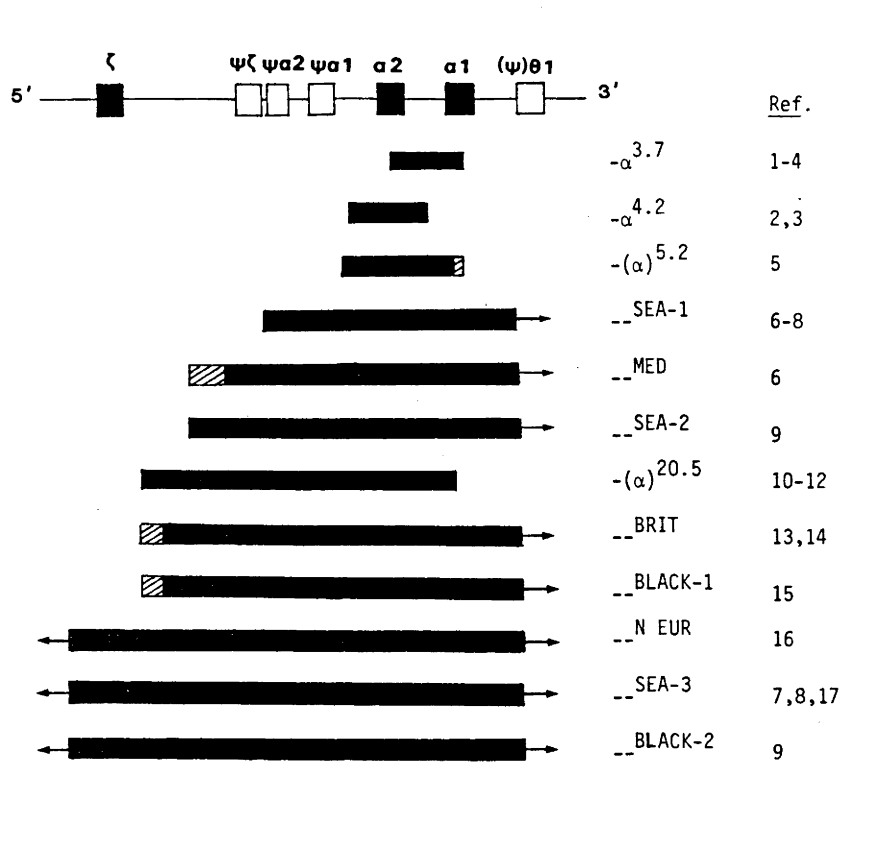


Figure 1.4 Extent of various deletions within the α-globin gene cluster causing α-thalassemias (shown as solid bars below the map); hatched bars indicate regions where the precise endpoint of the deletion is indeterminate, and arrows indicate unmapped endpoints of the deletions. The $-(\alpha)^{20.5}$ deletion was formerly known as the Mediterranean $-(\alpha)^{2.6}$ type due to the presence of a 2.6 kb Eco RI fragment. A recent finding has shown that the ζ is still present in this deletion. (References 1: Embury et al 1979; 2: Embury et al 1980; 3: Higgs et al 1980b; 4: Phillips et al 1980; 5: Pressley et al 1980b; 6: Pressley et al 1980c; 7: Winichagoon et al 1984; 8: Lie-Injo et al 1985; 9: Felice et al 1984; 10: Orkin and Michelson 1980; 11: Nicholls et al 1985; 12: Trent et al 1986a; 13: Higgs et al 1985; 14: Wilkinson et al 1986; 15: Steinberg et al 1986; 16: Weatherall et al 1981; 17: Chang and Kan 1984).

the crossovers occurred (Michelson and Orkin 1983; Higgs et al 1984). The second type of deletion is $-\alpha^{4.2}$, also known as "leftward deletion", consistent with a deletion of 4.2 kb of DNA including the whole of the α2 gene (Embury et al 1979 and 1980). The $-\alpha^{4.2}$ deletion is most likely to have arisen by unequal crossing-over within or near to the X boxes of homology, which are 4.2 kb apart (Figure 1.2). The $-\alpha^{3.7}$ deletion seems to be more common in many populations including Asians in which the $-\alpha^{4.2}$ deletion was originally identified.

A number of individuals have been found in whom three α-globin genes are present on one chromosome. These appear to represent the reciprocal products of the unequal crossover events that produced the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, and are, thus, called $\alpha\alpha\alpha^{\text{anti } 3.7}$ and $\alpha\alpha\alpha^{\text{anti } 4.2}$, respectively (Goossens et al 1980; Higgs et al 1980a; Sancer et al 1980a; Lie-Injo et al 1981; Trent et al 1981). The third α gene is usually functional, (Liebhaber and Kan 1981). However, individuals with a total of five α-globin genes are clinically and hematologically normal.

In several case, the remaining α genes in some $-\alpha^{3.7}$ and $-\alpha^{4.2}$ haplotypes also carry a mutation in the coding sequence. For example, the α-chain variants α^{J} Tongariki (Bowden et al 1982), α^{G} Philadelphia (Sancar et al 1980b; Surrey et al 1980; Morle et al 1984), and α Evanston (Moon-Penn et al 1983; Honig et al 1984) are associated with the $-\alpha^{3.7}$ deletion, and α^{Q} is linked to the $-\alpha^{4.2}$ deletion (Lie-Injo et al 1979; Higgs et al 1980c; Pagnier et al 1982). Presumably, these associations arose either

by mutation in one of the α genes before an unequal crossing-over event, or by mutation in the remaining α gene on the deleted chromosome, or by homologous recombination between chromosomes containing the deletion and variant alleles.

Deletions leading to loss of function of both α genes on a given chromosome (--/) are more difficult to explain and apparently represent illegitimate recombination. A number of different deletions that produce the --/ haplotype have been identified (Figure 1.4). Three distinct deletions have been found to account for the α-thalassemia-1 haplotype in Southeast Asians (Pressley et al 1980c; Winichagoon et al 1984; Felice et al 1984; Chang and Kan 1984; Lie-Injo et al 1985) and three in Mediterraneans (Pressley et al 1980b,c; Orkin and Michelson 1980; Nicholls et al 1985; Trent et al 1986a). Two different deletions have been observed in American blacks (Felice et al 1984; Steinberg et al 1986), and two in north Europeans (Weatherall et al 1981; Higgs et al 1985). One of these deletions, the $--^{BRIT}/$ type, has also recently been detected in an Australian family (Wilkinson et al 1986). A common form of the deletion in Southeast Asians ($--^{SEA-1}/$) involves loss of all α and ψα genes while in four other deletions ($--^{MED}/$, $--^{SEA-2}/$, $--^{BRIT}/$, and $--^{BLACK-1}/$) the lesions also extend to the ψζ. The $-(\alpha)^{20.5}$ deletion involves the 5' portion of the α1 gene and extends to remove the ψζ gene, leaving the ζ gene intact. It is interesting that three haplotypes ($--^{N\ EUR}/$, $--^{SEA-3}/$, and $--^{BLACK-2}/$) have

deletions of the whole α-globin gene cluster. These haplotypes would result in embryonic or fetal wastage in homozygotes rather than a hydrops fetalis phenotype. Whether these three haplotypes have a similar extent of deletion or not is still unknown, since the end points of the deletions have not been determined.

DNA analysis by restriction endonculease mapping has successfully been used to detect various deletions and triplications of the α-globin genes. To distinguish the single (-α/) and triple (ααα/) α-gene alleles from the normal (αα/) haplotype, one may use restriction enzymes that normally cleave outside the two α genes and detect the differences in size of the DNA fragment by hybridization with the α-gene probe. The single α-gene deletion gives a smaller DNA fragment while the triple α-gene allele produces a larger DNA fragment, when compared with the normal. The double α-gene deletion on one chromosome (--/) cannot be identified by use of an α-gene probe. However, it is possible to detect some types of this deletion by observing changes in the restriction fragment containing the ζ or ψζ gene.

Nondeletion forms of α-thalassemia have also been observed, although there are relatively few when compared with the β-thalassemias. They have been described in the Asian (Kan et al 1977; Embury et al 1979; Higgs et al 1981a), Mediterranean (Kan et al 1979; Orkin et al 1979a and 1981a; Higgs et al 1981a; Galanello et al 1983), black (Sancar et al 1980a), and Saudi Arabian (Pressley et al 1980a, Higgs et al 1981a) populations. One such nondeletion allele from an Italian patient, reported by Orkin et al (1981), contains a pentanucleotide deletion that

removes the donor splice site of the IVS-1 of the $\alpha 2$ gene and results in abnormal RNA splicing by activation of a cryptic splice site in the first exon (Felber et al 1982). An unusual form of nondeletion α-thalassemia containing different mutations in both α-genes on the same chromosome has been uncovered in Saudi Arabian individuals (Higgs et al 1983). This haplotype gives rise to Hb H disease in the homozygous state, suggesting that the α-globin production from four α genes only amounts to that from one fully functional α gene (Pressley et al 1980a). Molecular cloning and sequencing has revealed that the $\alpha 1$ gene from such individuals is completely nonproductive, due to a nucleotide deletion at codon 14 causing a frameshift mutation and generation of an in-phase termination codon at position 35. The $\alpha 2$ gene from the same chromosome contains a base change in the polyadenylation signal, altering the normal from AATAAA to AATAAG which results in inefficient production of mature mRNA such that the output from this $\alpha 2$ gene is reduced by about 50%.

In Sardinian patients with Hb H disease caused by a deletion α-thalassemia 1 and a nondeletion α-thalassemia, a mutation in the $\alpha 2$ gene changes the initiation codon ATG to ACG and abolishes the function of this gene. Globin mRNA output from the affected $\alpha 2$ locus is decreased relative to the $\alpha 1$ locus (Pirastu et al 1984a). In an Algerian patient with Hb H disease, a nondeletion α-thalassemia was found to be linked with the $-\alpha^{3.7}$ deletion (Morle et al 1985). The remaining α gene contains a deletion of the two nucleotides at positions -2 and -3 preceeding the ATG codon. This defect causes a reduction of translation efficiency by 30-50% (Morle et al 1986) and results in Hb H

disease in homozygotes. Other nondeletion α-thalassemias appear to result from structural mutations that produce abnormal α-globin chains. Examples of this group are: Hb Constant Spring ($\alpha^{142\ Gln+30\ amino\ acids}$; Milner et al 1971), Hb Suan-Dok ($\alpha^{109\ Leu\rightarrow Arg}$; Sanguansermsri et al 1979), Hb Petah Tikvah ($\alpha^{110\ Ala\rightarrow Asp}$; Honig et al 1981), and Hb Quong Sze ($^{125\ Leu\rightarrow Pro}$; Goossens et al 1982).

Except for the double mutations in Saudi Arabians, Hb Constant Spring in Southeast Asians, and probably the nondeletion α-thalassemia in Sardinians, the other nondeletion α-thalassemias are uncommon. They have usually been observed in individual families with Hb H disease, caused by interaction of an α-thalassemia-1 allele on one chromosome and a nondeletion allele on the other. These nondeletion α-thalassemias are unlikely to be detected in population surveys by the DNA mapping technique.

As mentioned previously, before the DNA mapping technique became available, α-thalassemias were very difficult to detect accurately in adults by conventional hematological and biochemical procedures. Heterozygous α-thalassemia 2 (-α/αα) in particular, was practically impossible to distinguish from the normal condition (αα/αα). Hypochromia and microcytosis, which are associated with homozygous α-thalassemia 2 (-α/-α) and heterozygous α-thalassemia 1 (--/αα), are insufficient criteria for diagnosis of these two genotypes, because these hematological changes can also be observed in other conditions e.g. β-thalassemia and iron deficiency anemia. Although the two later conditions can sometimes be ruled out, one cannot be confident of

such an indirect indicator. The more reliable method of assessing globin chain synthesis is rather tedious and impractical as a screening technique. Examination of Hb Bart's (γ_4) in cord blood (Pootrakul et al 1967; Na-Nakorn and Wasi 1970) has been widely used for the screening of α-thalassemia in populations. However, this technique has been reported to give erroneous results in some cases in differentiating the normal condition and α-thalassemia-2 heterozygotes (Ohene-Frempong et al 1980; Higgs et al 1982). In addition, all the methods mentioned above cannot distinguish the $-\alpha/-\alpha$ genotype from the $--/\alpha\alpha$ one.

By using the DNA mapping technique, one can now accurately detect various deletion forms of α-thalassemia. This technique is so far the most powerful and reliable for the detection of these defects and has been used for population screening. Various genotypes can be distinguished and an accurate frequency of each haplotype can be estimated.

1.4.2 The β-thalassemias

The β-thalassemias are a heterogeneous group of disorders characterized by deficiency of β-globin in erythroid cells (Weatherall and Clegg 1981). The heterozygous state of β-thalassemia is clinically asymptomatic, although such individuals show typical abnormalities of red cell morphology and indices. Individuals with homozygous β-thalassemia, also known as Cooley's anemia or thalassemia major, are usually severely anemic and

require lifelong medical management. Because of the great molecular heterogeneity of the β-thalassemias, many homozygotes for this condition are in reality compound heterozygotes with two different β-gene mutations.

β-Thalassemias are divided into β^+-thalassemia, in which β-chains are present but reduced in amount, and β^0-thalassemia, in which there is virtually no detectable synthesis of β-chains. However, many different mutations of the β gene have been found to cause β^+- and β^0-thalassemias. In contrast to α-thalassemias, β-thalassemias are in general not due to gross deletion of the structural β gene, except in Indians where there is a partial β-gene deletion involving the loss of 619 nucleotides in the 3' portion (Flavell et al 1979; Orkin et al 1979b and 1980; Spritz and Orkin 1982; Kazazian et al 1984a). Point and frameshift mutations due to nucleotide substitutions and nucleotide deletions/insertions, respectively, are the two common causes of β-thalassemias. The reported mutations are listed in Table 1.2 and are divided by the type of alterations they impose upon the β gene responsible for the thalassemic phenotype. Figure 1.5 shows locations of all the mutations in the β gene. The majority of the mutations occur in the 5' half of the gene. Mutations at the gene promoters, in the exons and introns that partially disturb normal RNA splicing, and at the RNA cleavage and polyadenylation signal usually result in β^+-thalassemia, while mutations that produce non-functional mRNA or completely inhibit normal RNA processing give rise to β^0-

Table 1.2 Mutations causing β-thalassemias (modified and extended from Orkin and Kazazian 1984; Kazazian 1985; Antonarakis et al 1985).

Mutation	Type	Ethnic group	Reference
I. Transcriptional mutations (●)			
a. Distal element			
1) -88 C→T (ACATCC)	β^+_+	Am.Black	1
2) -87 C→G (ACACGC)	β^+	Mediter.	2
b. ATA Box			
3) -31 A→G (CGTAAAA)	β^+_+	Japanese	3
4) -29 A→G (CATGAAA)	β^+_+	Am.Black	4
5) -28 A→C (CATACAA)	β^+_+	Kurdish	5
6) -28 A→G (CATAGAA)	β^+	Chinese	6
II. Non-functional mRNA (△)			
a. Nonsense mutations:			
1) codon 15 (G→A)	β^0_0	Indian	7
2) codon 17 (A→T)	β^0_0	Chinese	8
3) codon 39 (C→T)	β^0_0	Mediter.	9,10,11
4) codon 121 (G→T)	β^0	Polish	12
b. Frameshift mutations:			
5) codon 6 (-1,A)	β^0_0	Mediter.	13,14
6) codon 8 (-2,AA)	β^0_0	Turkish	10
7) codon 8/9 (+1,G)	β^0_0	Indian	7
8) codon 16 (-1,C)	β^0_0	Indian	7
9) codon 41/42 (-4,TCTT)	β^0	Indian	7
		Taiwan	15
		Thai	16
10) codon 44 (-1,C)	β^0_0	Kurdish	17
11) codon 71/72 (+1,A)	β^0	Chinese	18
III. Defective RNA processing (◇)			
a. Splice junction changes:			
1) IVS-1 position 1 (GT→AT)	β^0_0	Mediter.	2
2) IVS-1 position 1 (GT→TT)	β^0_0	Indian	7
3) IVS-1 3'-end (-17bp)	β^0_0	Kuwaiti	12
4) IVS-1 3'-end (-25bp)	β^0_0	Indian	19
5) IVS-2 position 1 (GT→AT)	β^0_0	Mediter.	20,21
6) IVS-2 3'-end (AG→GG)	β^0	Am.Black	4

Mutation	Type	Ethnic group	Reference
b. Consensus changes:			
7) IVS-1 position 5 (G→C)	β^+	Indian Chinese	7,22 18
8) IVS-1 position 5 (G→T)	$?_+$	Mediter.	12
9) IVS-1 position 6 (T→C)	β^+	Mediter.	2
c. Internal IVS changes:			
10) IVS-1 position 110 (G→A)	β^+	Mediter.	23,24
11) IVS-1 position 116 (T→G)	?	Mediter.	12
12) IVS-2 position 654 (C→T)	β^0_+	Chinese	18
13) IVS-2 position 705 (T→G)	β_+	Mediter.	25
14) IVS-2 position 745 (C→G)	β^+	Mediter.	2
d. Coding region substitutions affecting processing:			
15) codon 24 (T→A, silent)	β^+_+	Am.Black	26
16) codon 26 (G→A, Hb E)	β_+	Asian	27
17) codon 27 (G→T, Hb Knossos)	β^+	Mediter.	28
IV. RNA cleavage and polyadenylation mutation (O)			
1) AATAAA→AACAAA	β^+	Am. Black	29

References 1: Orkin et al 1984a; 2: Orkin et al 1982a; 3: Takihara et al 1986; 4: Antonarakis et al 1984a; 5: Poncz et al 1982; 6: Orkin et al 1983b; 7: Kazazian et al 1984a; 8: Chang and Kan 1979; 9: Moschonas et al 1982b; 10: Orkin and Goff 1981b; 11: Trecartin et al 1981; 12: Kazazian 1985; 13: Kazazian et al 1983; 14: Chang et al 1983; 15: Kimura et al 1983; 16: Fukumaki et al 1985; 17: Kinniburgh et al 1982; 18: Cheng et al 1984; 19: Orkin et al 1983c; 20: Baird et al 1981; 21: Treisman et al 1982 22: Treisman et al 1983; 23: Spritz et al 1981; 24: Westway and Williamson 1981; 25: Spense et al 1982; 26: Goldsmith et al 1983; 27: Orkin et al 1982b; 28: Orkin et al 1984b; 29: Orkin et al 1985.

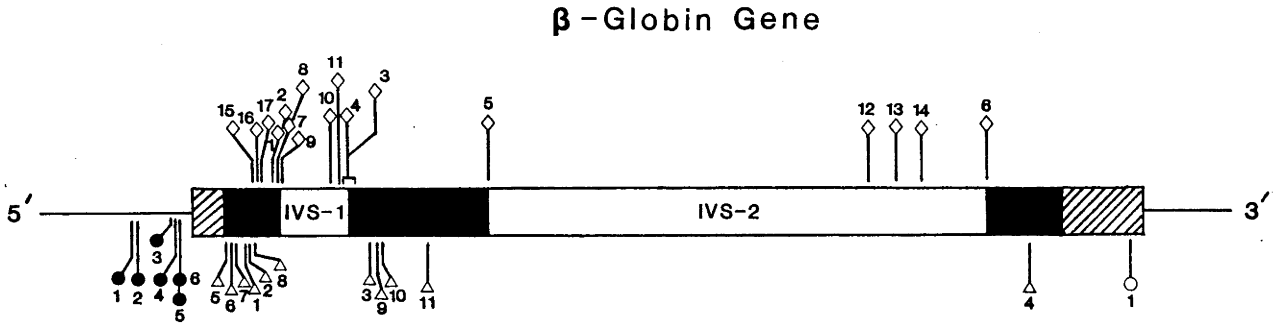


Figure 1.5 Location of the 35 mutations producing β-thalassemias in various ethnic groups. The numbers of each individual mutation correspond to those listed in Table 1.2. The symbols are: (●) Transcriptional mutations, (△) Non-functional mRNA, (◇) Defective RNA processing, (○) RNA cleavage mutation (modified from Kazazian 1985).

thalassemia. Mild β-thalassemia can also be the result of single nucleotide substitutions that produce structural hemoglobin variants. For examples, the β^{E} ($\beta^{26\ Glu \rightarrow Lys}$) and the $\beta^{Knossos}$ ($\beta^{27\ Ala \rightarrow Ser}$) mutations also manifest as β^{+}-thalassemia, because the nucleotide changes responsible for the amino acid substitutions also activate a cryptic doner RNA splicing site resulting in unstable mRNA. However, most of the β-globin variants do not produce β-thalassemia because production of β-globin is normal.

The mechanism of RNA transcription and processing of many cloned β-thalassemia genes has been studied and this topic has recently been reviewed (Orkin and Kazazian 1984; Collins and Weissman 1984; Nienhuis et al 1984).

At least 17 deletions in the β-globin gene cluster are now known, as shown in Figure 1.6. These include β^{o}-thalassemia, HPFH, δβ-thalassemias, γδβ-thalassemias, and abnormal hemoglobins due to hybrid gene (Hb Lepore and Hb Kenya). All of the deletions can be detected by restriction endonuclease analysis. Although many deletions are quite large, none of them has a cytogenetically visible lesion on the short arm of chromosome 11. While nearly all of these deletions are rare, the deletion of the β-globin gene accounts for about 30% of β-thalassemia alleles in Indians (Kazazian et al 1984a).

In some syndromes associated with increased fetal hemoglobin production, no deletions are detected by genomic blotting analysis. As in the deletion forms of HPFH, it appears that the elevated γ-globin expression is in cis to the mutation. A variety

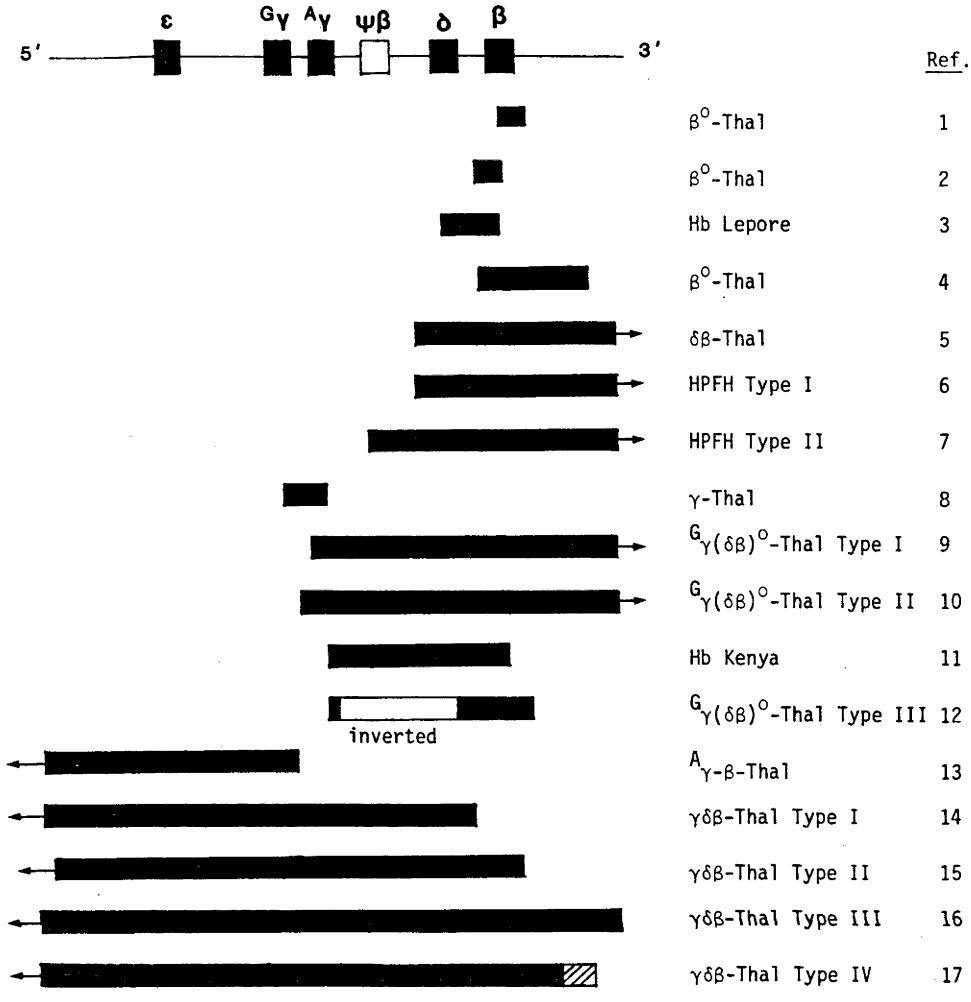


Figure 1.6 Deletions in the β-globin gene cluster. The extent of various deletions within the gene cluster is presented below the map. Filled bars represent areas known to be deleted. Arrows indicate that the endpoints of the deletions have not been determined. Hatched bar indicates that there is uncertainty in the extent of the deletion (redrawn from Kazazian 1985) (references 1: Orkin et al 1979b; 2: Boehm et al 1984; 3: Flavell et al 1978; 4: Gilman et al 1984; 5: Bernards et al 1979; 6: Fritsch et al 1979; 7: Bernards et al 1980; 8: Sakumaran et al 1983; 9: Orkin et al 1979c; 10: Jones et al 1981a; 11: Huisman et al 1972; 12: Jones et al 1981b; 13: Pirastu et al 1984b; 14: Van der Ploeg et al 1980; 15: Orkin et al 1981b; 16: Fearon et al 1983; 17: Pirastu et al 1983).

It should be noted that none of the point mutations upstream of the γ-globin gene has been shown to be directly responsible for the increase in γ-globin chain synthesis.

of these conditions have recently been reviewed (Collins and Weissman 1984; Karlsson and Nienhuis 1985). At least four nondeletion HPFH syndromes have been characterized at the molecular level. They were all found to have point mutations within the promoter region of a γ gene resulting in increased expression (Collins et al 1984 and 1985; Giglioni et al 1984; Gilman and Huisman et al 1984; Gelinas et al 1985).

Initial detection of β-thalassemia is much more simple than α-thalassemia. Individuals with homozygous β-thalassemia present characteristic clinical and hematological manifestations. β-Thalassemia heterozygotes are associated with hematological changes and an increased proportion of Hb A_2 (>3.5%). At the DNA level, restriction endonuclease analysis is unsuitable for screening purposes, since most defects resulting in β-thalassemia are not due to β-gene deletion. It is very useful, however, for characterization of chromosome haplotypes (combinations of polymorphic restriction sites in a region of the chromosome) and frameworks of β-thalassemia genes (Orkin et al 1983a; Orkin and Kazazian 1984). This will provide information regarding how often mutation of the β gene might have occurred in a population, and it is a useful strategy for selecting a specific gene for further study by molecular cloning and sequencing (Orkin et al 1982a). In addition, the analysis of β-gene polymorphisms and detection of the known defective genes by oligonucleotide probes have been used for prenatal diagnosis of fetuses at a very early stage.

1.5 Thalassemia and the malaria hypothesis

The observation that thalassemia was prevalent in Mediterraneans led to the development of the malaria hypothesis. It was suggested by Haldane (1949), that the high frequencies of thalassemia could be due to a selective advantage of heterozygous carriers in malarial infection.

Malaria is a major cause of morbidity and mortality in tropical and subtropical areas. The disease is caused by protozoan parasites of the genus Plasmodium and the severest forms of the illness are associated with the species Plasmodium falciparum. Natural infection in man is transmitted by anopheline mosquitoes. Most of the pathology induced by plasmodial infection is related to the asexual development of the parasite within erythrocytes (Perrin et al 1982). The main clinical manifestations are fever, anemia, and splenomegaly. In endemic areas, malaria is the cause of most febrile illness, and the frequency of splenomegaly in children is used as an index of the prevalence and transmission of malaria (Manson-Bahr and Apted 1982).

Population studies have shown that the prevalence of hemoglobinopathies and other red cell defects correlates geographically with the epidemiology of malaria (for review see Motulsky 1964 and 1975; Livingstone 1971 and 1983). Before the development of techniques to grow the falciparium parasite in vitro, many comparative investigations of the morbidity and mortality resulting from malarial infection were carried out in normal groups and groups who carried several red cell variants. However, thalassemia received less attention than sickle-cell

anemia and G6PD deficiency. The most conclusive evidence indicated that the Hb S heterozygote is at an advantage in the presence of malaria. The technique for continuous culture of the falciparum parasite *in vitro* was developed in 1976 (Trager and Jensen 1976) and has been used extensively to test all the candidate red cell variants. In culture experiments, a selective advantage of the Hb S heterozygote has clearly been confirmed by impairment of parasite growth in the red cells containing Hb AS (Friedman 1978; Pasvol *et al* 1978; see also Luzzatto 1979). Parasite growth is also retarded in erythrocytes with G6PD deficiency (Roth *et al* 1983a,b; Luzzatto *et al* 1983) or Hb CC (Friedman *et al* 1979), while there is contradictory evidence as to whether homozygous Hb E cells do (Nagel *et al* 1981) or do not (Santiyanont and Wilairat 1981) retard growth. However, heterozygous Hb C or Hb E red cells have been shown not to inhibit parasite growth (Friedman *et al* 1979; Nagel *et al* 1981; Santiyanont and Wilairat 1981). Ovalocytic red cells can resist invasion by the parasite (Kidson *et al* 1981) probably because of rigidity of the cell membrane (Mohandas *et al* 1984). Growth of the malaria parasite is markedly retarded in Hb H ($-\alpha/--$) red cells (Ifediba *et al* 1985) but in those of α- and β-thalassemia heterozygotes, parasite growth has been reported as unimpaired (Roth *et al* 1983a, Ifediba *et al* 1985), except in the presence of oxidative stress as imposed by high oxygen tension, menadione, or riboflavin (Friedman 1979). The last study, which tested thalassemic as well as G6PD-deficient erythrocytes, has led to a suggestion that oxidant sensitivity may be the cause of the

protective effect in the red cells of these two conditions.

However, Roth et al (1984) have expressed some doubt concerning the "oxidative stress hypothesis" as the only explanation for growth inhibition of malaria in G6PD-deficient and thalassemic red cells. An alternative hypothesis for β-thalassemia suggests that protection is related to a slightly slower disappearance of Hb F in heterozygous infants, since Hb F by itself exerts an inhibitory effect on parasite growth (Pasvol et al 1977). This situation does not occur in α-thalassemia, thus, cannot be invoked to explain the selective advantage of this condition. Heterozygous α-thalassemia is associated with the presence of Hb Bart's (γ_4) at birth, however this abnormal tetramer disappears in the first three months (Pootrakul et al 1967) and may not participate in protection against malaria.

Although more favourable evidence has been obtained for other red cell variants from the direct culture experiments, the role of malaria selection for thalassemia has not gained adequate support. It is unclear what mechanism the thalassemic red cell possesses to protect against malaria. Although imbalanced globin synthesis and subsequent changes in the red cell are the primary events, some other mechanisms in the body (such as phagocytosis and extraerythrocytic oxidative stress) may be important. These protective mechanisms, which are not yet well understood, may play a major role and may not be observed in culture experiments in vitro. The widespread polymorphic incidence of the thalassemias and their overall correlation with malaria have thus continued to be the only strong evidence in favor of a role for malaria in determining their frequencies.

1.6 Malaria in Papua New Guinea

Malaria is the greatest single public health problem and the most important cause of ill-health in PNG because everywhere below 2,500 meters is potentially malarious. An estimated two-thirds of the population live in areas where the disease is endemic (Ward and Lea 1970). The coast and lowlands (between sea-level and 100 meters) of PNG are the areas of highest endemicity (Figure 1.7) because they provide ideal circumstances for malaria transmission. These include a high environmental temperature and humidity, an adequate rainfall throughout the year, and an abundance of appropriate anopheline breeding-places. The major vectors in the PNG mainland and all the associated archipelagos and islands are three members of the *Anopheles punctulatus* complex (Spencer *et al* 1974) i.e. *A. farauti* Laveran, *A. punctulatus* Donitz, and *A. koliensis* Owen. Three species of *Plasmodium* parasites (*P. vivax*, *P. falciparum*, and *P. malariae*) are known to exist in PNG (van Dijk and Parkinson 1974). *P. vivax* was found to be the predominant species in the earlier surveys but *P. falciparum* has now become the more common species.

In coastal and lowland areas, endemicity of malaria ranges from holo- to mesoendemicity. The holoendemicity in the north coast of PNG was first recorded in 1900 by Robert Koch who noted a parasitemia rate of 80-100% in children under 2 years old but lower rates in the older groups, in his studies at Bogadjim and Bongu villages in what is now Madang Province (Ewers and Jeffrey 1971). He had also postulated the development of immunity to malaria in adults (Ewers and Jeffrey 1971; Ewers 1972). Peters

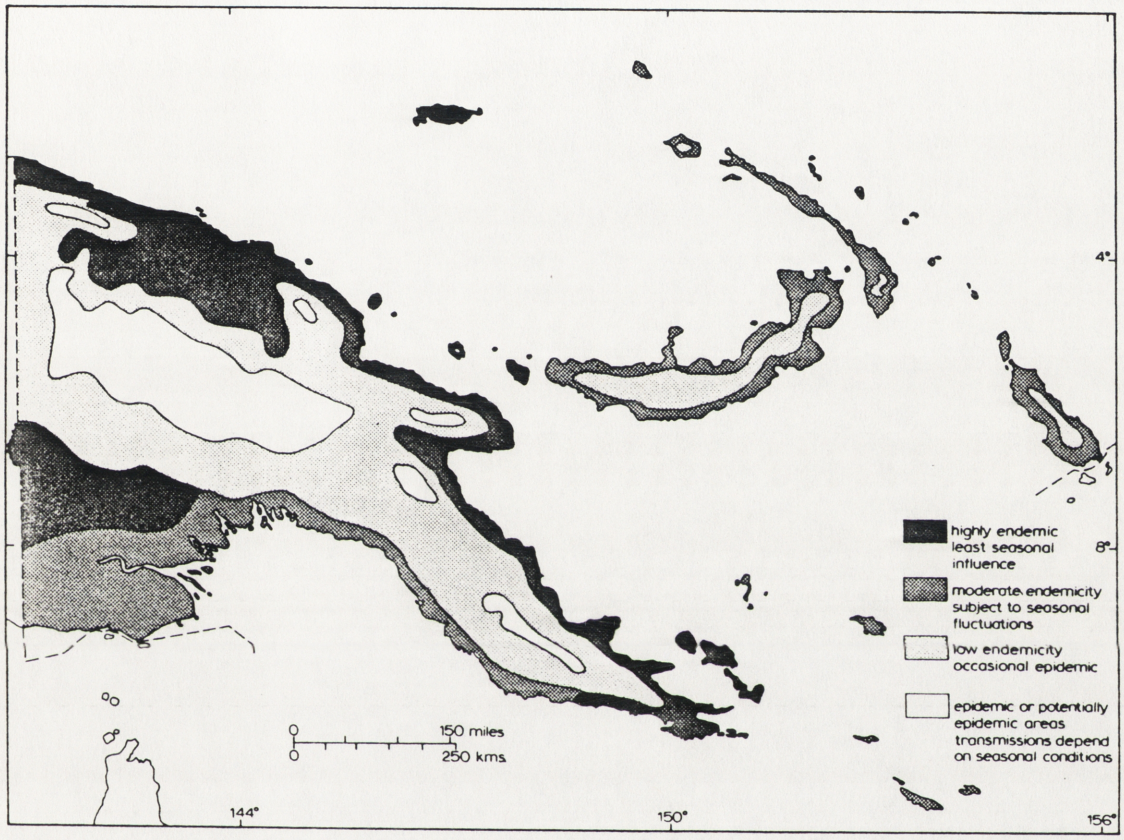


Figure 1.7 Endemicity of malaria in Papua New Guinea (from Ward and Lea 1970).

(1960), from his studies in 1957 in Maprik, East Sepik Province, also concluded that malaria in that area was holoendemic. He found a parasitemia rate of 95% and splenomegaly of 97% in children 2 to 4 years old and also noted that the infant mortality rate near Maprik was over 60%.

Residual spraying with DDT to control malaria was begun in 1957 (Peters 1960; Parkinson 1974) and this showed some effect on the endemicity. A survey by Vines between 1964 and 1965 revealed a parasitemia rate of 52% and splenomegaly of 84% in children 2 to 9 years old on the mainland (Vines 1970). The spraying gradually expanded to cover living areas of approximately 50% of the population in around 1967 and a significant reduction in parasitemia rates after the spraying was demonstrated in later surveys in many areas between 1970 and 1973 (Parkinson 1974). However, the spraying programme has often been unpopular and has been discontinued in some areas (Darlow et al 1981).

At present, the endemicity of malaria in some coastal and lowland areas of PNG is still very high. The exact picture of the endemicity in most of the areas is unknown. The available information does show a high level of stable transmission and hyperendemic malaria. For example, a small survey carried out in 1980 in a village near Madang showed a parasitemia rate in children under 10 years old to be 52% and the spleen rate amongst 2 to 9 year-olds to be 57% (cited in Darlow et al 1981). Another survey in the Ok Tedi region of the Star Mountains, Western Province, revealed a parasitemia rate of 65% and a spleen rate of 88% in children 2 to 9 years old (Cattani et al 1983). In the

latest survey conducted between 1981-1983 by Cattani et al (1986) covering a population of 16,500 in Madang Province, malaria was found to be highly endemic throughout this area. The parasite rate for all Plasmodium species in the one to nine years age group averaged 53.6% in the dry seasons and 56.7% in the wet seasons and the spleen rate in the same age group was about 71%. In addition, the proportion of the population positive for antimalarial antibodies, an indicator of past as well as present infections, as detected by ELISA, was found to increase with age. Greater than 80% of children five years and older and virtually 100% over the age of 14 were ELISA-positive.

In the southern coast of PNG where the climate is rather hot and arid, and influenced by the cool south-eastern trade winds, the degree of malaria endemicity has been lower than in the north. In the highlands of PNG, malaria is absent in many areas. Peters et al (1958) noted that the upper limit of malaria appears to lie between 1800 and 2000 meters, although in some areas such as the Goroka Valley, at 1700 meters, malaria is not endemic. However, in some highland areas there have been unstable hypoendemic patterns with seasonal and periodic epidemics (Black 1954; Spencer et al 1956; Peters et al 1958 and 1960; Sharp 1982). There is evidence that the spread of malaria from the lowlands to the highlands occurred after European contact and socio-economic developments such as road building, airstrip construction, and digging of duck ponds. The highlanders are generally non-immune and susceptible to malarial infection when they move to stay temporarily in lowland areas.

1.7 Aims of this study

It was the aim of this study to examine the incidence and types of thalassemia found in the populations of PNG and to evaluate their distribution in relation to the endemicity of malaria. As described in the previous section, malaria is highly endemic in the lowlands but virtually nonendemic in the highlands. This epidemiological pattern of malaria makes PNG very suitable for a comparative study of the selective effects of malaria on human microevolution. By taking the advantage of this epidemiological pattern and by making use of the recombinant DNA technology, the effect of malarial selection on the defective globin genes was investigated in the populations of PNG.

Initially, populations of lowland and highland PNG were examined with an α-globin gene probe for the frequency and distribution of α-thalassemia 2 caused by a single α-globin gene deletion (-α/). When detected, the -α/ deletion was further analysed for its subtypes and linked polymorphisms. The question of whether α-thalassemia 1 caused by a deletion of both α-globin genes on one chromosome (--/) is present in the populations of PNG was also investigated. The study also covered an examination of the frequency and distribution of β-thalassemia in various groups of PNG and an analysis of polymorphisms associated with β-thalassemic genes and normal β-globin genes. In addition, the problem of whether α-thalassemia 2 might be found in Australian Aborigines and Indonesians who are neighbouring groups of Papua New Guineans but may be under different selection pressures was addressed.

The results of this study are discussed in relation to the importance of malarial selection, migration, random effects, and interaction with other red blood cell characteristics in determining the incidence of thalassemias.

CHAPTER 2

GENERAL METHODS

Chapter 2: General Methods

2.1 Introduction

DNA analysis by restriction endonuclease mapping is the main technique employed to examine α- and β-globin genes in this study. In addition, detection of abnormal hemoglobin by starch-gel electrophoresis (Smithies 1955; Owen et al 1958) and of β-thalassemia by microchromatographic quantitation of Hb A_2 (Huisman et al 1975) were carried out for blood samples obtained in appropriate condition. In this chapter, the procedures used to study DNA by restriction endonuclease mapping are described in detail.

DNA purified from cells or tissues from a person can be cleaved into fragments of defined length by restriction endonucleases. The fragments are then separated according to size by agarose-gel electrophoresis, transferred onto nitrocellulose or nylon membranes (Southern 1975), and incubated with radioactively labelled gene specific probes (Rigby et al 1977). These probes, obtained by molecular cloning techniques, will hybridize specifically with homologous genomic DNA sequences on the membrane. After stringent washing of the membranes and autoradiography, only fragments containing part or all of the gene of interest will be detected. The size of the fragments detectable on the autoradiograph can be estimated by comparison with labelled fragments of known length fractionated on the same gel. This technique enables detection of gross gene deletions and DNA polymorphisms due to nucleotide changes that disrupt or create restriction sites, some of which will also cause a recognisable phenotypic change.

2.2 Sources of plasmid probes

Globin gene specific probes provided by other laboratories had been created by insertion into bacterial plasmids of either complementary DNA (cDNA) synthesized from globin mRNA or genomic DNA fragments containing specific globin genes. Four plasmid probes, JW101, pBRα2, pBRζ, and Pst-β, were used in the study. JW101 and pBRα2 were probes for the α-globin genes. They were supplied by Dr G.B. Forget (Wilson et al 1978) and Dr E. Rubin (Rubin and Kan 1985), respectively. JW101 was initially used but replaced by pBRα2 because the latter gave a better result. pBRζ (Lauer et al 1980) and Pst-β (originally from Dr T. Maniatis of Harvard University, MA, USA) for the ζ- and β-globin genes respectively, were provided through Dr S.L. Thein of MRC Molecular Haematology Unit, John Radcliffe Hospital, Oxford, England. All the probes, except pBRα2, were obtained as purified plasmids. It was therefore necessary to transform bacteria with each plasmid in order to provide a source of continuous supply through the subsequent large scale preparation of the plasmid probe from bacterial cultures.

2.3 Transformation of Escherichia coli with plasmid DNA

The plasmids were introduced into E. coli HB101 by the method routinely performed in our laboratory. All solutions, materials, and glassware used were sterilized and techniques required for C1 level of containment were practiced (Recombinant DNA Monitoring Committee 1985).

(1) A single colony of E. coli HB101 was inoculated into 5 ml of LB (Luria-Bertani) medium and cultured at 37°C overnight with vigorous shaking.

(2) The following morning, 0.25 ml of the overnight culture was added to a flask containing 25 ml of fresh LB medium, to make a 1:100 dilution.

(3) The bacteria were grown in the same conditions for approximately two and a half hours; until an OD_{600} of 0.3-0.6 was reached.

(4) The bacterial culture was chilled on ice for 10 minutes and then transferred to two 15-ml plastic tubes. The bacterial pellet was obtained by spinning in a bench centrifuge at 4°C at top speed for 10 minutes. The supernatant was discarded.

(5) The pellet was resuspended in 10 ml of 10 mM $MgCl_2$. The suspension was left at 4°C for 5 minutes.

(6) The bacteria were again harvested at 4°C and the supernatant was discarded. The pellet was resuspended in 10 ml of 50 mM $CaCl_2$-10 mM Tris-HCl (pH 8.0) and the suspension was again left at 4°C for 10 minutes.

(7) The bacteria were again harvested and the supernatant was discarded. The pellet was resuspended in 1 ml of 50 mM $CaCl_2$ -10 mM Tris-HCl (pH 8.0). This suspension contained competent bacteria.

(8) Forty to 100 ng of plasmid in 100 µl of TE 10-1 (10 mM Tris-HCl,pH 8.0-1 mM EDTA) were added to 0.2 ml of the competent bacteria in a plastic 1.5 ml centrifuge tube, followed by 20 µl each of 0.1 M $CaCl_2$ and 0.1 M $MgCl_2$. The tube was left on ice for 15 minutes.

(9) The bacteria were heat-shocked at 45°C for 3 minutes, and left at room-temperature for 10 minutes.

(10) The bacterial suspension was transferred into a tube containing 10 ml of LB medium and incubated at 37°C for 60 minutes with gentle rocking.

(11) The bacteria were then spun down and resuspended in 0.5 ml of fresh LB medium.

(12) One hundred µl of the suspension were spread on each plate with an appropriate antibiotic (tetracycline for JW101 and Pst-β, and ampicillin for pBRζ) and the plates were incubated at 37°C overnight.

(13) Single colonies of the transformed bacteria were streaked on new plates with the same antibiotic for second selection and isolation.

(14) After incubation at 37°C overnight, the streaked plates were kept as the source of transformed bacteria for the plasmid probe preparation. Stabs of the transformed bacteria were made in vials for longer storage.

2.4 Plasmid probe preparation

A large quantity of plasmid probe was prepared from 1 liter of bacterial culture with the plasmid amplified. The procedure for culturing the bacteria and amplification of the plasmid in rich medium is described by Maniatis *et al* (1982). The method of lysis of bacterial cells by sodium dodecyl sulfate (SDS) originally described by Godson and Vapnek (1973) was followed and modified slightly. Removal of bacterial debris and high-

molecular-weight DNA by centrifugation was performed at 15,000 rpm at 4°C for 30 minutes in a refrigerated superspeed centrifuge (Sorvall RC-5B) instead of in an ultracentrifuge. Plasmid DNA was further purified by centrifugation in cesium chloride gradients as described (Maniatis et al 1982).

2.5 Preparation of human genomic DNA and restriction endonuclease digestion

Genomic DNA was prepared from human leukocytes obtained from 10 to 20 ml of blood exactly as described by Grunebaum et al (1984). This method was relatively simple and up to 50 samples could be processed at once. The DNA prepared by this method was always completely digestible with restriction enzymes.

Restriction enzymes were purchased from various manufacturers. The main sources were Boehringer, Biolabs, and Pharmacia. Digestions were performed under the optimal conditions as specified by the manufacturers. The enzyme buffers were made up at ten times the final concentration and stored at -20°C. Digestions were carried out in a total volume of 50 or 100 µl according to the amount of DNA used.

(1) An aliquot of 5 or 10 µg of DNA was transferred to a 1.5 ml plastic tube.

(2) Distilled water was added to give a final volume of 50 or 100 µl after addition of DNA, buffer and enzyme.

(3) Buffer (5 or 10 µl) and finally enzyme were added (generally 4 Units of enzyme per µg of DNA, except for Bam HI which required 6-10 Units/µg).

(4) The reaction mixture was mixed by brief vortexing, centrifuged for a few seconds and incubated at 37°C (unless specified otherwise by the manufacturers) overnight.

(5) The digestion was terminated by addition of 10 or 20 µl of 2 M NaCl-0.25 M EDTA and 120 or 240 µl of ethanol.

(6) After cooling at -20°C for 1 hour, the DNA pellet was recovered by spinning in an Eppendorf microfuge for 5 minutes. The supernatant was poured off.

(7) The pellet was drained, air-dried for 1-2 hours, and dissolved in 30 µl of TE 20-1 (20 mM Tris-HCl,pH 7.5-1 mM EDTA).

2.6 Agarose-gel electrophoresis

Electrophoresis was usually run on a 0.8% agarose gel in TAE buffer (0.04 M Tris-acetate-0.002M EDTA, pH 8.0). In some cases, a higher percentage agarose gel (1%) was required in order to separate DNA fragments around 1 kb. Agarose gels were cast in a perspex mold with inner dimensions of 155 mm wide X 185 mm long. A comb with 14 or 10 teeth, 10 mm wide and 1 mm thick, could be placed in either dimension of the frame to form the sample wells. A gel volume of 200 ml produced a gel 6 mm thick with sample wells 5 mm deep.

The gel was set on a cooling plate between two electrophoresis tanks containing TAE buffer. Well-dissolved DNA samples, mixed thoroughly with 10 µl of gel-loading buffer (0.25% bromophenol blue - 0.25% xylene cyanol - 25% Ficoll 400 in H_2O)

and heated at 65°C for 10 minutes, were loaded into the sample wells, which contained a small volume of TAE buffer. One well was loaded with standard size markers consisting of 500 ng Hind III digested bacteriophage lambda DNA (Hind III-λDNA, Pharmacia) labelled with ^{32}P nucleotide by nick translation to a total count of about 500 cpm. Paper wicks (Whatman 3MM) were used to connect gel and buffer tanks. Electrophoresis was carried out at 300 V for 30 minutes at constant voltage. After the DNA had entered the gel, the wells were filled with the buffer and the gel was covered with Glad Wrap. The electrophoresis continued at 150 V overnight when the bromophenol blue had usually migrated 10-13 cm. The gel was stained in the buffer containing 2 mg/l ethidium bromide for 30 minutes and photographed by Polaroid camera fitted with a red filter under UV light.

2.7 Southern blotting transfer of DNA fragments

Following electrophoresis, the DNA was partially hydrolyzed by soaking in 0.25 M HCl for 15 minutes prior to alkali denaturation and high-salt neutralization, and then transferred from the gel to the surface of a GeneScreen Plus membrane (NEN Research Products, Du Pont) by the method described by the manufacturer, which was a modification of that originally described by Southern (1975) and known as Southern blotting. GeneScreen Plus membrane, a nylon-based nitrocellulose, is less fragile than the conventional nitrocellulose. After blotting, baking of the membrane at high temperature was not necessary. A membrane carrying DNA fragments could be reused many times.

2.8 Preparation of ^{32}P-labelled probes by nick translation

The sources of DNA used in nick translation were whole plasmids containing the globin-gene sequence, which had been prepared from transformed bacteria (section 2.4). Nick translation kits and [α-^{32}P]dCTP and [α-^{32}P]dATP were purchased from Amersham, UK. Specific activity of both radioactive nucleotides was 3,000 Ci/mmole, concentration 10 mCi/ml. The probes were labelled to a specific activity at least $1x10^8$ dpm/µg DNA (Protocol B or C in the manual supplied with the kit) with one of the radioactive nucleotides. The amount of DNA to be nick translated was calculated according to the requirement for each hybridization. Half a microgram of plasmid probe was enough for at least 6 large membranes (13 cm x 18 cm).

(1) The following components were mixed in a 1.5 ml plastic tube:

a)	Probe	0.5 µg
b)	Nucleotide/buffer (solution 1)	10 µl
c)	[α-^{32}P]dCTP or [α-^{32}P]dATP	5-10 µl
d)	Distilled water (to make up a final volume of	50 µl)
e)	Enzyme solution (solution 2)	5 µl

(2) The mixture was incubated at 15^{o}C for 1 hour. Then, the reaction was stopped by adding 5 µl of 0.5 M EDTA.

(3) The ^{32}P-labelled DNA was separated from the free radioactive nucleotide by passing the mixture through a 5 ml glass-pipette column packed with Ultrogel (AcA 54, LKB), washed with TE 20-5 (20 mM Tris-HCl,pH 7.5-5 mM EDTA).

(4) The column was monitored with a hand-held, end-window Geiger counter and the first peak was collected in a 1.5 ml plastic tube.

(5) The volume of the labelled probe was measured and 5 µl was counted in a scintillation counter. The specific activity of the probe was estimated.

2.9 Hybridization and washing of membranes

A slightly modified version of the method of hybridization described by Nasmyth (1982) was used routinely. Membranes were prehybridized and hybridized in the same solution at 65^{o}C. Two or three large membranes were hybridized in the same bag simultaneously.

(1) Thirty ml of hybridization solution were prepared as follows:

a) A mixture of 13.8 ml of distilled water (=0.46 volumes) and 0.276 ml of 10 mg/ml salmon sperm DNA (=0.0092 volumes) was made up in a 50 ml Corning plastic tube and boiled in a boiling water bath for 10 minutes.

b) The solution was chilled rapidly on ice, and 16.2 ml of Nasmyth's solution (=0.54 volume) were added and mixed well. The Nasmyth's solution contained 1.1 M NaCl, 0.333 M Na_2HPO_4, 0.0111 M EDTA (pH 6.2), 1.85% W/V sodium lauroyl sarcosinate, 18.5% W/V dextran sulfate.

(2) The hybridization solution was added to the bag containing membranes. The volumes used were 20 ml for two and 30 ml for three membranes. All air bubbles were squeezed out. The

bag was sealed and incubated for 2-3 hours in a rocking water bath at 65°C.

(3) The ^{32}P-labelled probe (150-250 ng), which had been boiled for 10 minutes and cooled on ice for 10 minutes, was added to the bag after cutting open one corner.

(4) All air bubbles were carefully squeezed out. The bag was resealed and rubbed all over to distribute the probe evenly.

(5) The bag was reincubated in the rocking water bath at 65°C for at least 24 hours.

(6) The membranes were removed from the bag and washed sequentially in 2x, 1x, 0.5x, and 0.1xSSC plus 0.1% SDS, 30 minutes each at 65°C with adequate agitation (1xSSC=0.15 M sodium chloride-0.015 M sodium citrate). The volume of each washing solution was approximately 250 ml/membrane. (Note: In some cases the hybridization solution was saved for reuse with additional prehybridized membranes.)

(7) After the final washing, the radioactivity on the membranes was checked with a Geiger counter. If the count was much higher than 5 cps, the membranes could be washed longer in the final solution.

(8) The membranes were dried for about 1 hour at room temperature, wrapped in Glad Wrap and mounted onto a used X-ray film with cellotape prior to autoradiography.

2.10 Autoradiography and estimation of DNA-fragment sizes

The mounted membranes were placed with a sheet of Fuji RX X-ray film in a cassette fitted with one or two intensifying screens (Du Pont Lightning Plus). The cassette was kept at -80^{o}C for 1-7 days and the film was subsequently developed in a Kodak X-OMAT M20 film processor. Mobilities of the standard-size markers (the ^{32}P-labelled Hind III-λDNA) were measured. To produce a standard curve, mobilities (in cm) of the markers were plotted against logs of their sizes (in base pairs). The log values of the unknown fragments were read from the standard curve and converted to the lengths in base pairs by taking the anti-log. Estimation of DNA fragments larger than 10 kb by this method was not accurate due to non-linearity of the curve in the large fragment size range.

2.11 Rehybridization of membranes

After hybridization, the probe could be stripped off and the membrane rehybridized with a different probe. This could be done many times and it was found that background tended to be reduced. The membrane was immersed in 100 ml of 0.4 M NaOH and then in the same volume of 1.5 M NaCl-0.5 M Tris-HCl, pH 7.5 for 30 minutes each at room temperature with gentle agitation. The membrane was dried and then hybridized with a new probe without being prehybridized.

2.12 DNA mapping of the α- and β-globin genes

DNA mapping by restriction endonuclease digestion has extensively been used for studies of α- and β-globin gene

clusters, and their common rearrangements and polymorphisms have been well characterized (Embury et al 1979 and 1980; Lauer et al 1980; Phillips et al 1980; Pressley et al 1980a,c; Higgs et al 1981a; Kan et al 1980; Lawn et al 1980; Antonarakis et al 1982a; Orkin et al 1982a; Fearon et al 1983). The information available from these previous reports is beneficial for the selection of restriction enzymes and probes which will provide informative results. In this study, restriction enzymes Bam HI and Bgl II were principally used for analysis of the α-globin gene, and Hgi AI, Ava II, and Bam HI for the β-globin gene. The other enzymes used occasionally were Apa I, Eco RI, Sac I, and Xba I.

2.12.1 Detection of the single α-globin gene deletion and triplicated α-globin genes

The specific restriction sites cleaved by Bam HI in the α-globin gene cluster of chromosomes carrying the normal, single, and triplicated α-globin genes are shown in Figure 2.1. Bam HI generates a DNA fragment of 14 kb containing the ψα, α2 and α1 genes from the normal α-globin gene cluster. Deletion of an α-globin gene results in a smaller fragment of 10.5 kb. In the triplicated condition, the larger fragment of about 18 kb comprising the ψα and three α genes is produced. These three different fragments can be detected by hybridization with the α-globin gene specific probes, JW101 and pBRα2. Only one of the fragments is observed in homozygotes but a combination of two fragments is found in heterozygotes.

The two types of single α-globin gene deletion, the rightward ($-\alpha^{3.7}$/) and leftward ($-\alpha^{4.2}$/) deletions, can be distinguished by digestion of DNA with Bgl II and hybridization with the ζ-globin

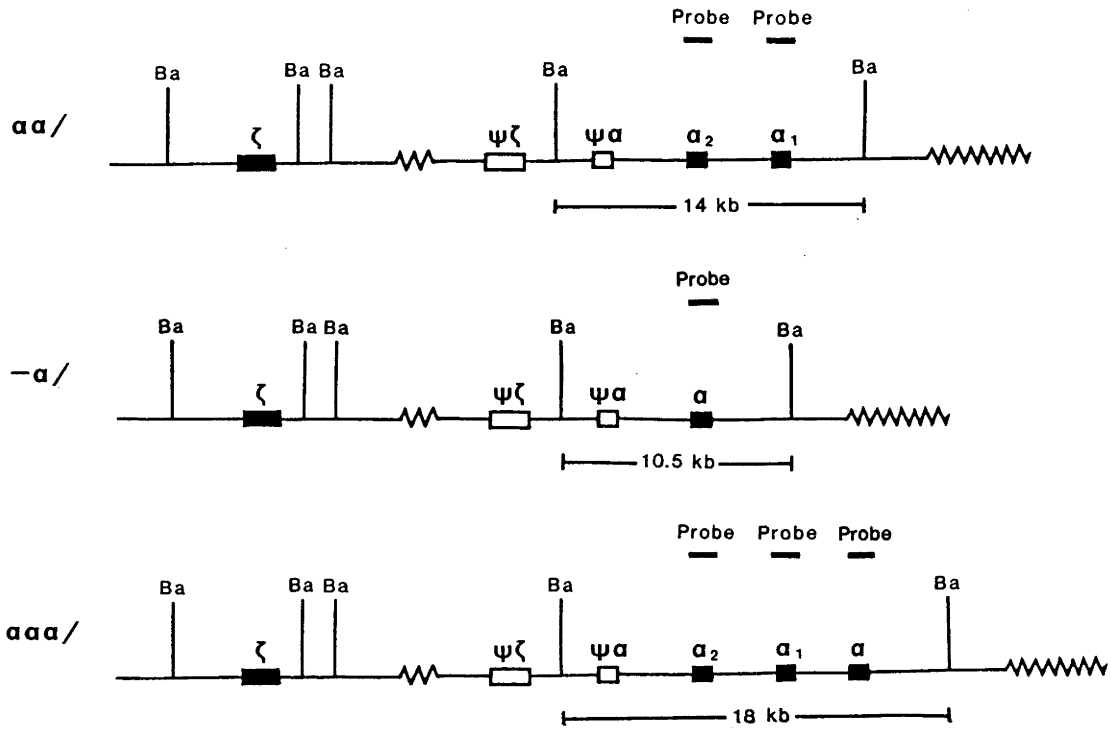


Figure 2.1 The restriction sites for enzyme Bam HI (Ba) in the α-globin gene cluster of chromosomes carrying the normal number of α-globin genes (αα/), the single α-globin gene deletion (-α/), and the triplicated α-globin genes (ααα/); —WW— indicates the regions of hypervariable DNA. The sites of hybridization of the α-globin gene specific probes are indicated. The newly described ψα2 and θ1 genes (see Figure 1.2) are not shown on the maps. These two genes apparently do not hybridize with the probe.

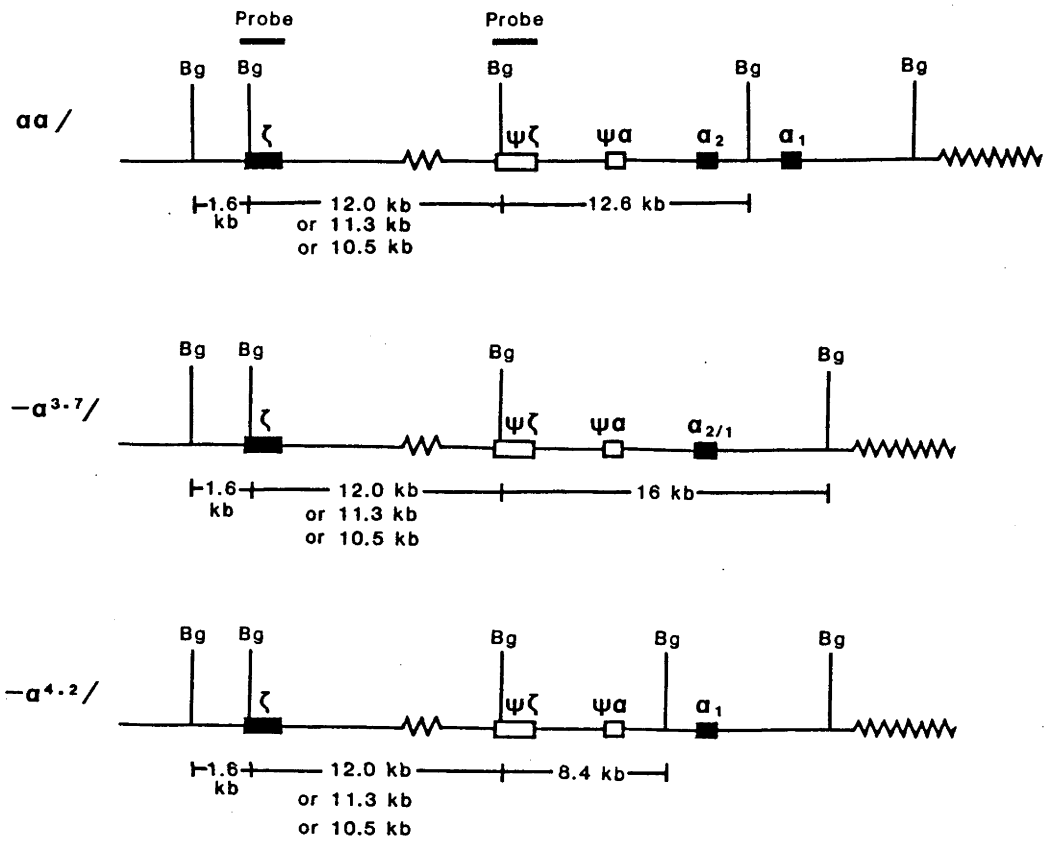


Figure 2.2 The restriction sites for enzyme Bgl II (Bg) in the α-globin gene cluster of chromosomes carrying the rightward ($-\alpha^{3.7}$/) and leftward ($-\alpha^{4.2}$/) deletions. The site of hybridization of the ζ-globin gene specific probe is indicated. The ψα2 and θ1 genes are not shown on the maps (see also the legend of Figure 2.1).

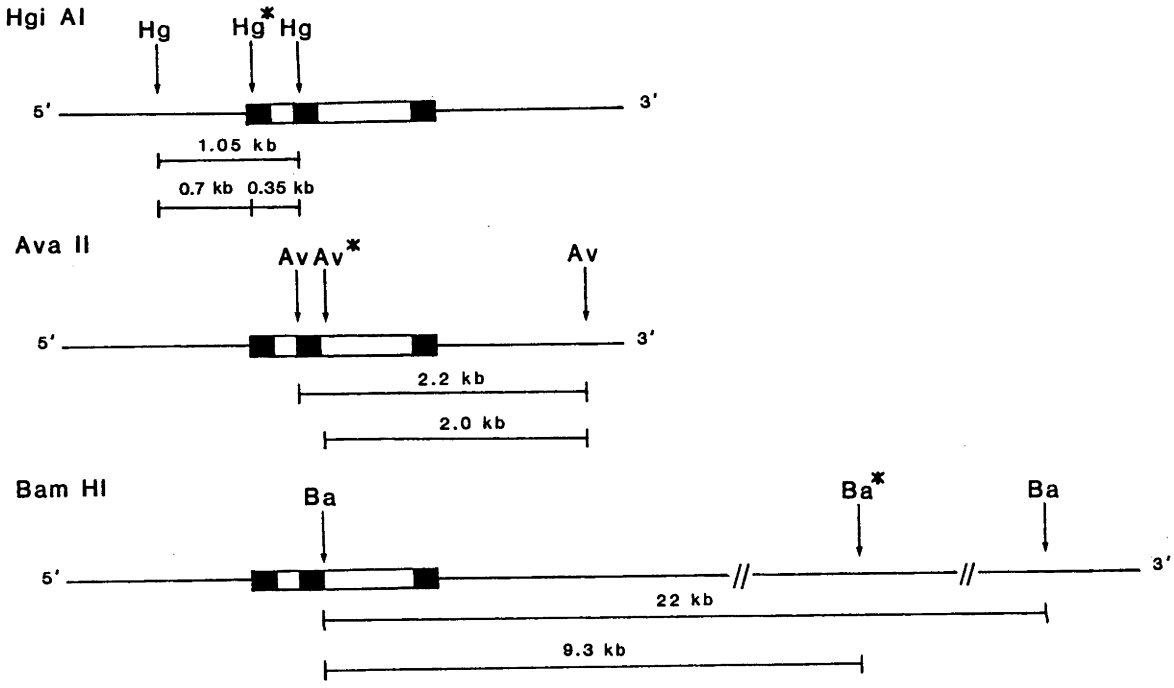


Figure 2.3 The restriction sites for enzymes Hgi AI (Hg), Ava II (Av), and Bam HI (Ba) in the β-globin gene; the asterisks indicate the polymorphic sites. The polymorphic DNA fragment sizes produced by each enzyme are indicated.

Chapter 3: α-Thalassemia in PNG

3.1 Introduction

It is now well established that normal individuals in human populations have duplicated α-globin gene loci (αα/αα). However, before the arrangement of the α-globin loci was completely elucidated, it was thought that there was population heterogeneity in the numbers of the α-globin genes. Melanesians were often cited as an example of a group in which individuals normally had a single α-globin gene locus. The evidence for this hypothesis was obtained from the fact that Hb J Tongariki ($\alpha_2^{115\ Ala\text{-}Asp}\beta_2$) made up 40%-50% of the hemoglobin in Hb J Tongariki heterozygotes (instead of 25%) and that Hb J Tongariki homozygotes lacked Hb A (Abramson et al 1970; Beaven et al 1972). This finding can now be explained by the linkage of an α-globin gene deletion in cis to the α^J-globin gene (Bowden et al 1982). The single α-globin locus in Melanesians is in fact a known defective condition, namely α-thalassemia 2 or α^+-thalassemia, caused by deletion of one of the duplicated α-globin genes (-α/). However, little is known about α-thalassemia in Melanesians, partly because it is difficult to identify α-thalassemia heterozygotes among adults. The first survey of α-thalassemia in PNG, which examined Hb Bart's in cord blood, was conducted by Booth (1981) and showed that 18 out of 50 cord blood samples (36%) collected at the hospitals in Port Moresby and Kerema contained 3-10% of Hb Bart's indicative of α-thalassemia. A recent survey using the same technique with a larger sample size from Madang and also analysis of 30 DNAs of these samples revealed a high occurrence of α-thalassemia in this area of PNG (Oppenheimer et al 1984).

This chapter provides the results of an extensive population survey using DNA mapping to determine the frequency and distribution of α-thalassemia 2 in various regions of PNG where there have been different endemicities of malaria ranging from virtual absence to holoendemicity. The question of whether α-thalassemia 1 or α^{o}-thalassemia (--/) is present in PNG is also examined. Detailed analyses are also carried out for subtypes of the single α-globin gene deletion, a ζ-globin gene deletion and triplicated α-globin genes.

3.2 Populations sampled

Blood samples used for the study were obtained from populations in various parts of PNG. A number of the samples were collected in Port Moresby, Madang, and Goroka during a field-trip to these areas in 1983. The majority of the samples were obtained through collaboration with the folowing workers in PNG. Dr Kuldeep K Bhatia of the PNG Institute of Medical Research, Goroka, provided the samples collected from Karimui, Goroka, Southern Highlands Province, Bundi, and Karkar Island. Dr Graham L Jones formerly of the University of Papua New Guinea, Port Moresby, provided the samples collected from Wanigela. Dr Jacqueline A Cattani of the PNG Institute of Medical Research, Madang, provided the samples collected from villagers in Madang Province. Dr George T Nurse of the Blood Transfusion Service, PNG Red Cross, Boroko, provided samples collected from blood donors who came from many parts of PNG. Altogether, 664 blood samples were tested. Six hundred and two samples were adult blood and 62

were cord blood. Buffy coats were taken from the adult blood samples and whole cord blood cells were separated from plasma. They were frozen and transported on dry-ice by air to Canberra for DNA analysis.

3.3 Results

3.3.1 Survey of the single α-globin gene deletion and triplicated α-globin genes

All the 664 DNA samples were screened for the single α-globin gene deletion and triplicated α-globin genes by Bam HI digestion and hybridization with the α-globin gene specific probe (Chapter 2: section 2.12.1). Figure 3.1 shows an autoradiograph of different α-globin genotypes, resulting from combinations of the normal (αα/), deleted (-α/), and triplicated (ααα/) haplotypes, detected in the populations of PNG by DNA mapping. The prevalence of each genotype in various groups is presented in Table 3.1 and the haplotype frequencies are shown in Table 3.2. Frequencies of the single α-globin gene deletion in various areas and provinces are also illustrated on the PNG map (Figure 3.2). The single α-globin gene deletion was found to have higher frequencies on the northern coast than on the eastern and southern coasts. It reaches 81% in Madang and 68% on Karkar Island. In the East and West Sepik, 11 out of 12 chromosomes tested (frequency 92%) were found to carry the deletion, indicating that this region may be the focus of the highest frequency of this deletion. In contrast to the coastal areas. the deletion was observed in much lower frequencies (0-3%) in several highland regions. In Goroka,

Figure 3.1 Autoradiograph of DNAs from Papua New Guinean individuals digested with Bam HI and hybridized with the α-globin gene probe, showing the different α-globin genotypes.

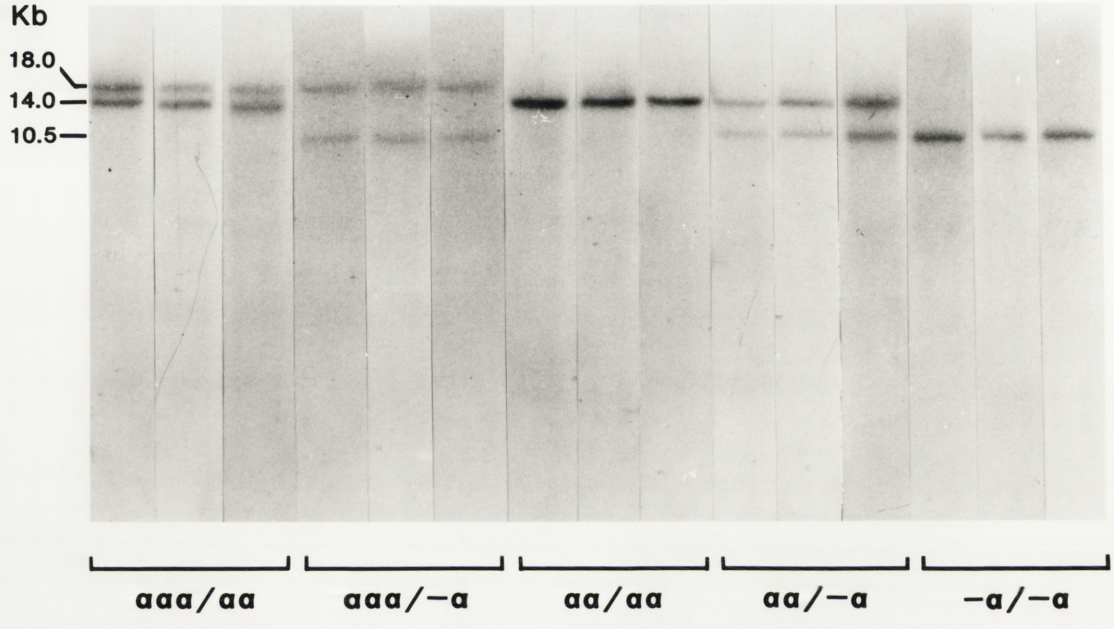

Kb
18.0
14.0
10.5
αα/αα
αα/−α
−α/−α

Table 3.1 α-Globin genotypes detected in various population groups of PNG.

Region	n[a]	αα/αα	-α/αα	-α/-α	αα/ααα	-α/ααα
1. Central Province (P.)						
-Port Moresby	64	42	16	3	2	1
-Wanigela	101	70	14	1	14	2
2. Gulf P.	16	6	6	3	1	0
3. Western P.	11	5	5	1	0	0
4. Milne Bay P.	20	6	8	6	0	0
5. Northern P.	18	3	10	5	0	0
6. Morobe P.	9	5	3	1	0	0
7. Madang P.						
-Madang-1[b]	75	2	24	49	0	0
-Madang-2	19	2	6	10	0	1
-Karkar Is.	28[c]	3	12	13	0	0
-Bundi	16	15	1	0	0	0
8. E. and W. Sepik P.	6	0	1	5	0	0
9. Chimbu P.						
-Karimui	100	65	31	3	1	0
-Chimbu	3	3	0	0	0	0
10. East. Highls. P.						
-Goroka-1[d]	18	17	1	0	0	0
-Goroka-2	62	54	5	2	1	0
11. South. Highls. P.	31	29	2	0	0	0
12. West. Highls. P.	2	2	0	0	0	0
13. Manus P.	4	1	3	0	0	0
14. E. New Britain P.	61	45	14	2	0	0
Total	664	375	162	104	19	4

[a] Numbers of persons tested.

[b] Blood samples from Madang were collected on two occasions; group 1 were obtained from the indigenous inhabitants of 6 villages, and group 2 from blood bank donors. Group 2 is more heterogeneous.

[c] This group includes four Hb J Tongariki heterozygotes: three had the genotype -α/αα and one had the genotype -α/-α.

[d] Group 1 were from adults and group 2 were cord blood samples.

Table 3.2 Frequencies of the normal (αα/), deleted (-α/), and triplicated (ααα/) haplotypes in various population groups of PNG.

Region	Number of chromosomes	Frequency (%)		
		αα/	-α/	ααα/
1. Central P.				
-Port Moresby	128	79.69	17.97	2.34
-Wanigela	202	83.17	8.91	7.92
2. Gulf P.	32	59.38	37.50	3.12
3. Western P.	22	68.18	31.82	0
4. Milne Bay P.	40	50.00	50.00	0
5. Northern P.	36	44.44	55.56	0
6. Morobe P.	18	72.22	27.78	0
7. Madang P.				
-Madang-1	150	18.67	81.33	0
-Madang-2	38	26.32	71.05	2.63
-Karkar Is.	56	32.14	67.86	0
-Bundi	32	96.88	3.12	0
8. East and West Sepik P.	12	8.33	91.67	0
9. Chimbu P.				
-Karimui	200	81.00	18.50	0.50
-Chimbu	6	100.00	0	0
10.East.Highls.P.				
-Goroka-1	36	97.22	2.78	0
-Goroka-2	124	91.94	7.26	0.80
11.South.Highls.P.	62	96.77	3.23	0
12.West.Highls.P.	4	100.00	0	0
13.Manus P.	8	62.50	37.50	0
14.East New Britain P.	122	85.25	14.75	0
Total	1,328			

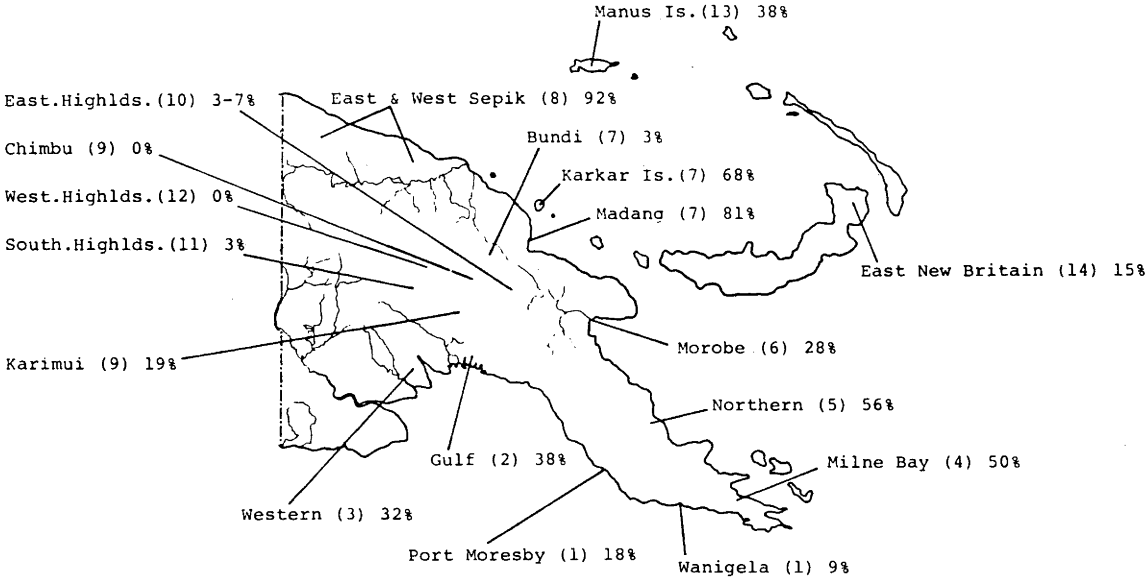


Figure 3.2 The PNG map showing haplotype frequencies (in percent) of the single α-globin gene deletion (-α/) in various areas and provinces. Numbers in parentheses indicate the locations listed in Tables 3.1 and 3.2.

Eastern Highlands, it was found in about 3% of adult blood samples and in 7% of cord blood samples.

The triplicated α-globin gene (ααα/) was detected in one sample each from the Gulf Province, Madang, Karimui, and Goroka. Three out of 64 persons (frequency 2.3%) from Port Moresby and 16 out of 101 (frequency 7.9%) from Wanigela were found to have this haplotype.

3.3.2 Survey of the deletion of both α-globin genes on one chromosome

Deletion of both α-globin genes on one chromosome (--/) results in α-thalassemia 1 or α^{0}-thalassemia, a haplotype that has no production of α-globin chains. The extent of different deletions of the α-globin gene cluster responsible for α-thalassemia 1 has been illustrated in Figure 1.4. The common type of deletion observed in Southeast Asians ($--^{SEA-1}/$) involves absence of the two α genes as well as the ψα genes while the ψζ and ζ genes are present. The deletion also removes the Bam HI site between the ψζ and ψα genes (Pressley et al 1980c: Winichagoon et al 1984), producing a new 17-20 kb fragment of DNA containing the ψζ gene (instead of a normal polymorphic fragment of either 8.9 or 10.0 or 10.7 kb) and a normal 5.5 kb fragment containing the ζ gene (Figure 3.3). The $--^{SEA-1}/$ haplotype can, thus, be detected by Bam HI digestion and hybridization with the ζ-globin gene probe.

To test the technique for detecting this type of the deletion, four DNA samples from Thai individuals who were known to be α-thalassemia-1 heterozygotes (αα/--) or Hb H disease patients (genotypes -α/--, and $\alpha^{CS}\alpha$/--) were used in the control study. DNAs of these four individuals were digested with Bam HI and hybridized with the α- and ζ-globin gene probes (Figure 3.4). The single normal fragment of 14 kb was detected in the individuals with heterozygous α-thalassemia 1 and Hb H disease with Hb Constant Spring after hybridization with the α-globin gene probe. In Hb H disease caused by α-thalassemia 1/α-thalassemia 2, only the 10.5 kb fragment containing a single α gene was detected. When the same membrane was rehybridized with the ζ-globin gene probe, the fragment of about 17 kb, which is specific for the $--^{SEA-1}$/ haplotype, as well as normal fragments were present in all the samples.

More than 600 DNA samples (omitting those from Madang group 2 and Karkar Island) were analysed for the $--^{SEA-1}$/ haplotype or other deletion forms of α-thalassemia 1 that might be observed by variations of the ζ- and ψζ-gene fragments. None of the DNA samples examined showed the 17-20 kb fragment or other abnormal fragments. An example of the autoradiograph of DNA samples from PNG is shown in Figure 3.5. One DNA sample, however, showed a novel fragment of 6.0 kb which hybridized to the ζ-globin gene probe. This particular sample was further analysed and appeared to carry a deletion of the ζ gene (section 3.3.7).

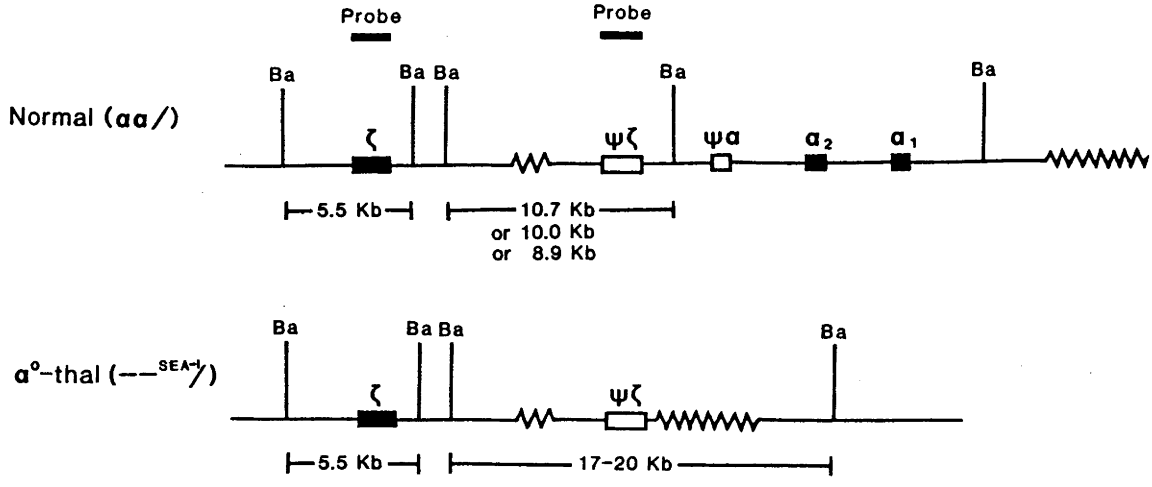
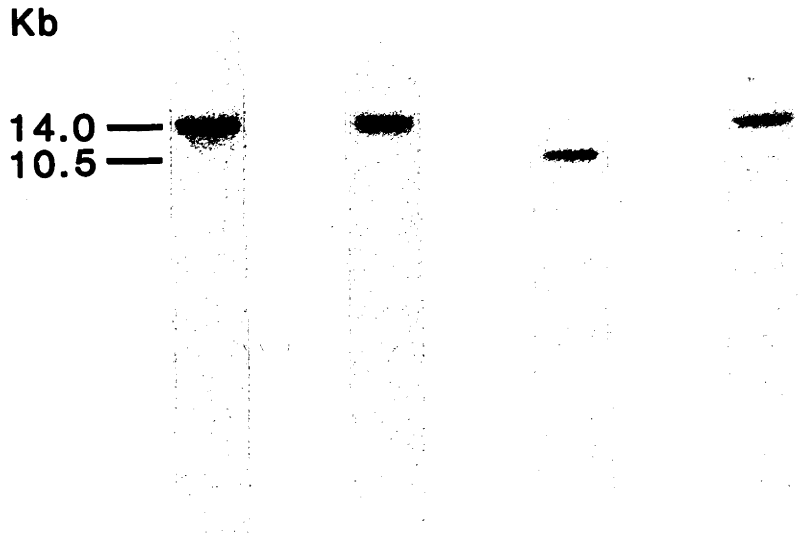


Figure 3.3 Restriction sites of endonuclease Bam HI (Ba) in the α-globin gene cluster of the normal chromosome (αα/) and the chromosome carrying a common type of the deletion of both α-globin genes found in Southeast Asians (−−$^{SEA-1}$/). ∿∿ represents the hypervariable region. The ψα2 and θ1 genes are not shown.

Figure 3.4 Autoradiographs of DNAs from 4 Thai individuals with heterozygous α-thalassemia 1 (αα/--) and Hb H disease (-α/-- and α^{CS}α/--) digested with Bam HI and hybridized with the α-globin gene probe (above). The same membrane was rehybridized with the ζ-globin gene probe (below); the fragment of about 17 kb specific for the -- $^{SEA-1}$/ haplotype was detected.

Bam HI x α-globin-gene probe



Bam HI x ζ-globin-gene probe

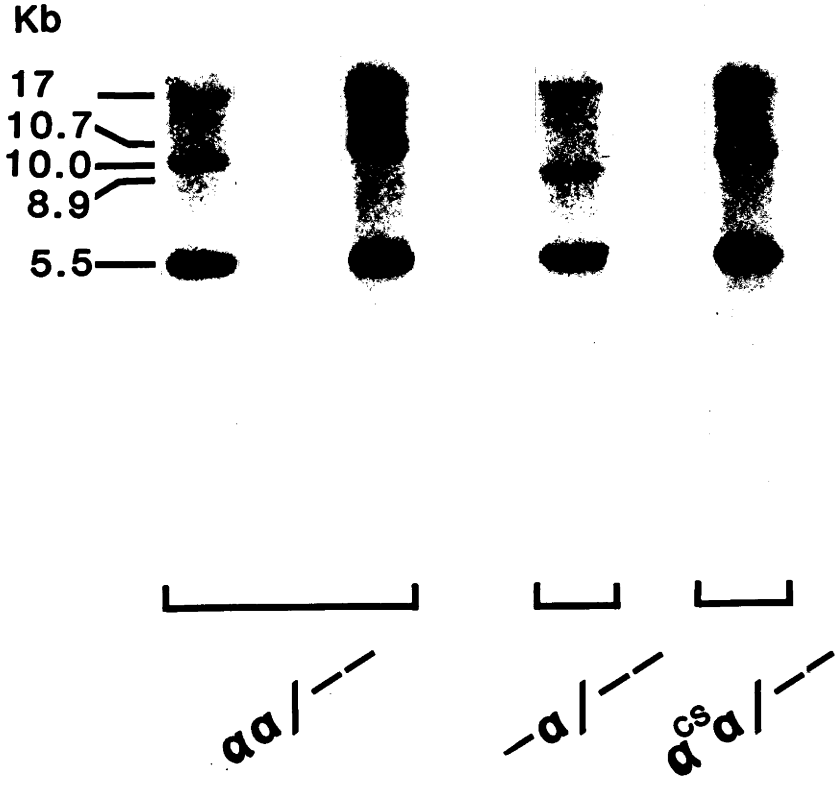
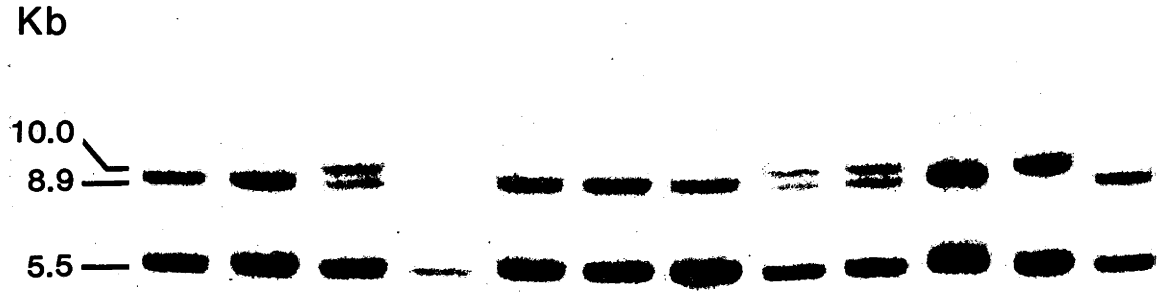


Figure 3.5 Autoradiograph of DNAs from Papua New Guinean individuals digested with Bam HI and hybridized with the ζ-globin gene probe, showing only the normal pattern of DNA fragments. The ζ-gene fragment is 5.5 kb and the ψζ gene fragment is polymorphic, 8.9 or 10.0 kb.

Bam HI x ζ-globin-gene probe



3.3.3 Subtype analysis of the single α-globin gene deletion

The two subtypes of the single α-globin gene deletion, the $-\alpha^{3.7}$ (rightward) and $-\alpha^{4.2}$ (leftward), originally described by Embury et al (1980), were analysed by digesting DNA samples with enzyme Bgl II and hybridizing with the ζ-globin gene probe. The $-\alpha^{3.7}$ deletion can be detected by the presence of a 16 kb fragment and the $-\alpha^{4.2}$ deletion by the presence of an 8.4 kb fragment (Chapter 2: section 2.12.1). Figure 3.6 shows an autoradiograph of the normal genotype, and heterozygotes and homozygotes for the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions observed in the populatios of PNG. Polymorphism of the ζ-globin gene can also be detected in the same autoradiograph. The prevalence of the two subtypes in various groups is presented in Table 3.3. The $-\alpha^{4.2}$ deletion predominates in many groups, reaching 96% of the deletions in Madang and on Karkar Island. In the Gulf, Western, and East New Britain Provinces, and in Karimui the $-\alpha^{4.2}$ deletion is rare and the $-\alpha^{3.7}$ deletion predominant.

3.3.4 Subtype analysis of the $-\alpha^{3.7}$ deletion

The $-\alpha^{3.7}$ deletion can be subdivided into three groups ($-\alpha^{3.7}$I, $-\alpha^{3.7}$II, and $-\alpha^{3.7}$III) based on different points of crossing over in the Z box of homology in the α-globin gene region (Figure 3.7). These subtypes correspond to different restriction

Figure 3.6 Autoradiograph of DNAs from Papua New Guinean individuals with the normal α-globin genotype, heterozygotes and homozygotes for the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions digested with Bgl II and hybridized with the ζ-globin gene probe. The 16.0 and 8.4 kb fragments are specific for the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions, respectively.

Bgl II x ζ-globin-gene probe

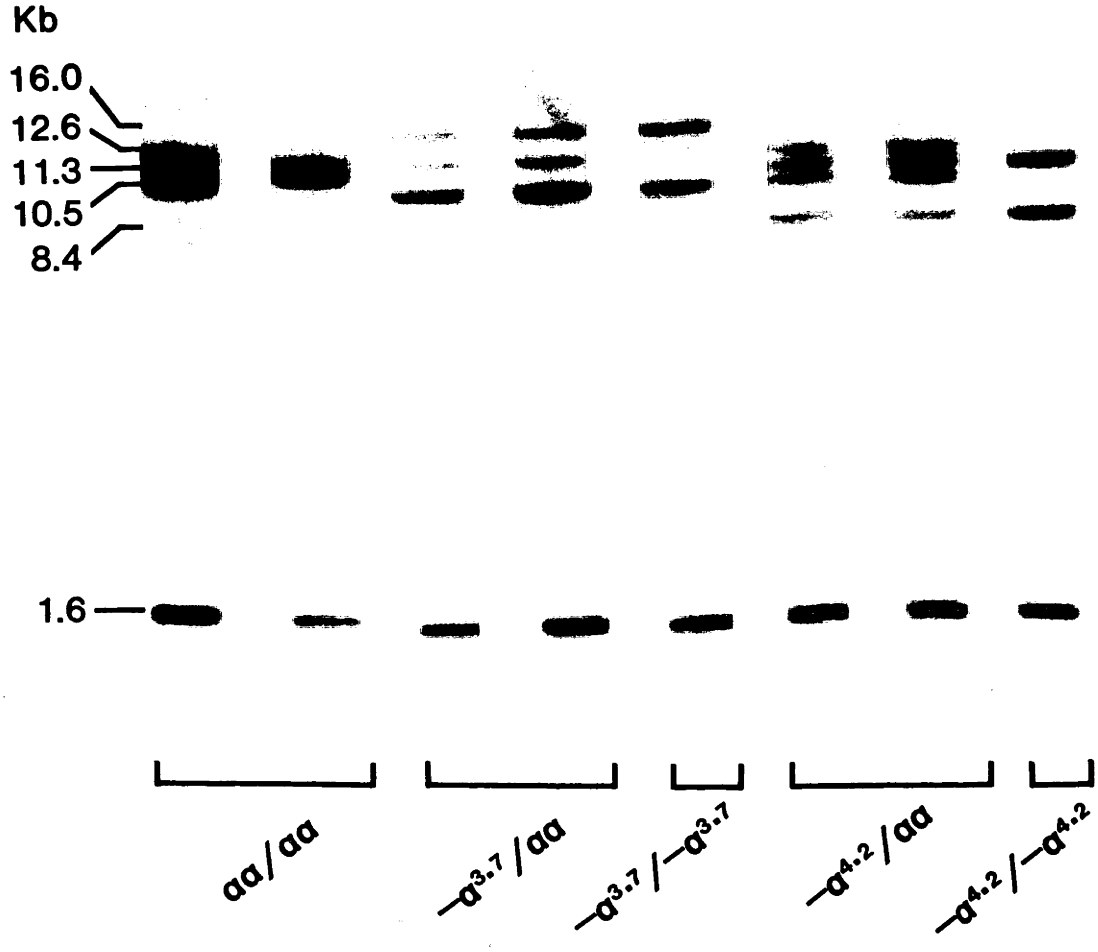


Table 3.3 Prevalences of the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions in various population groups of PNG.

Region	Number of chromosomes	Haplotype			
		αα/	$-\alpha^{3.7}$/	$-\alpha^{4.2}$/	ααα/
1. Central P.					
-Port Moresby	128	102	5	18	3
-Wanigela	202	168	0	18	16
2. Gulf P.	32	19	11	1	1
3. Western P.	22	15	7	0	0
4. Milne Bay P.	40	20	9	11	0
5. Northern P.	36	16	5	15	0
6. Morobe P.	16	12	2	2	0
7. Madang P.					
-Madang-1	150	29	5	116	0
-Madang-2	32	9	3	19	1
-Karkar Is.	56[a]	18	1	24	0
-Bundi	32	31	0	1	0
8. E. and W. Sepik P.	12	1	1	10	0
9. Chimbu P.					
-Karimui	200	162	35	2	1
-Chimbu	6	6	0	0	0
10. East. Highls. P					
-Goroka-1	36	35	1	0	0
-Goroka-2	124	114	1	8	1
11. South. Highls. P.	62	60	0	2	0
12. West. Highls. P.	4	4	0	0	0
13. Manus P.	8	5	0	3	0
14. E. New Britain P.	122[b]	104	12	3	0
Total	1,328	930	98	253	23

[a] Thirteen were not tested for deletion subtypes.

[b] Three were not tested for deletion subtypes.

fragment patterns of the α-globin genes generated using the enzyme Apa I (Michelson and Orkin 1983; Higgs et al 1984). With this enzyme, the normal chromosome (αα/) gives 5 restriction fragments recognizable by the α-globin gene probe. The 6.4 kb fragment, probably containing the ψα gene, is invariant. The others are 2.7 kb (5' α2), 1.9 kb (3' α2), 1.7 kb (5' α1) and 0.89 kb (3' α1). All genotypes contain the 6.4 kb invariant fragment. In addition, the $-\alpha^{3.7}$ deletion arising from crossover I produces only the normal 0.89 kb fragment and a new 2.5 kb fragment. Crossover II produces a single 3.5 kb fragment. Crossover III produces only the normal 2.7 kb fragment and a new 0.7 kb fragment (Figure 3.7). The 0.7 kb fragment in the last subtype does not contain the coding sequence and was unable to be detected by the probe used in the present study. Thus, the $-\alpha^{3.7}$ III subtype was distinguished from the other two by showing the normal pattern of α-globin gene fragments produced by Apa I in DNA samples known to have the $-\alpha^{3.7}$ deletion.

The DNA samples with the $-\alpha^{3.7}$ deletion were further analysed using the enzyme Apa I and the α-globin gene probe. Two subtypes, the $-\alpha^{3.7}$ I and $-\alpha^{3.7}$ III, were detected in the populations of PNG (Figure 3.8 and Table 3.4); 75 out of 84 chromsomes were the $-\alpha^{3.7}$ I subtype and the rest were the $-\alpha^{3.7}$ III subtype. Of 9 chromsomes with the $-\alpha^{3.7}$ III subtype, 8 were found in the East New Britain province on the island of New Britain but only one was detected in the Northern Province (Table 3.4), on the eastern coast of the PNG mainland.

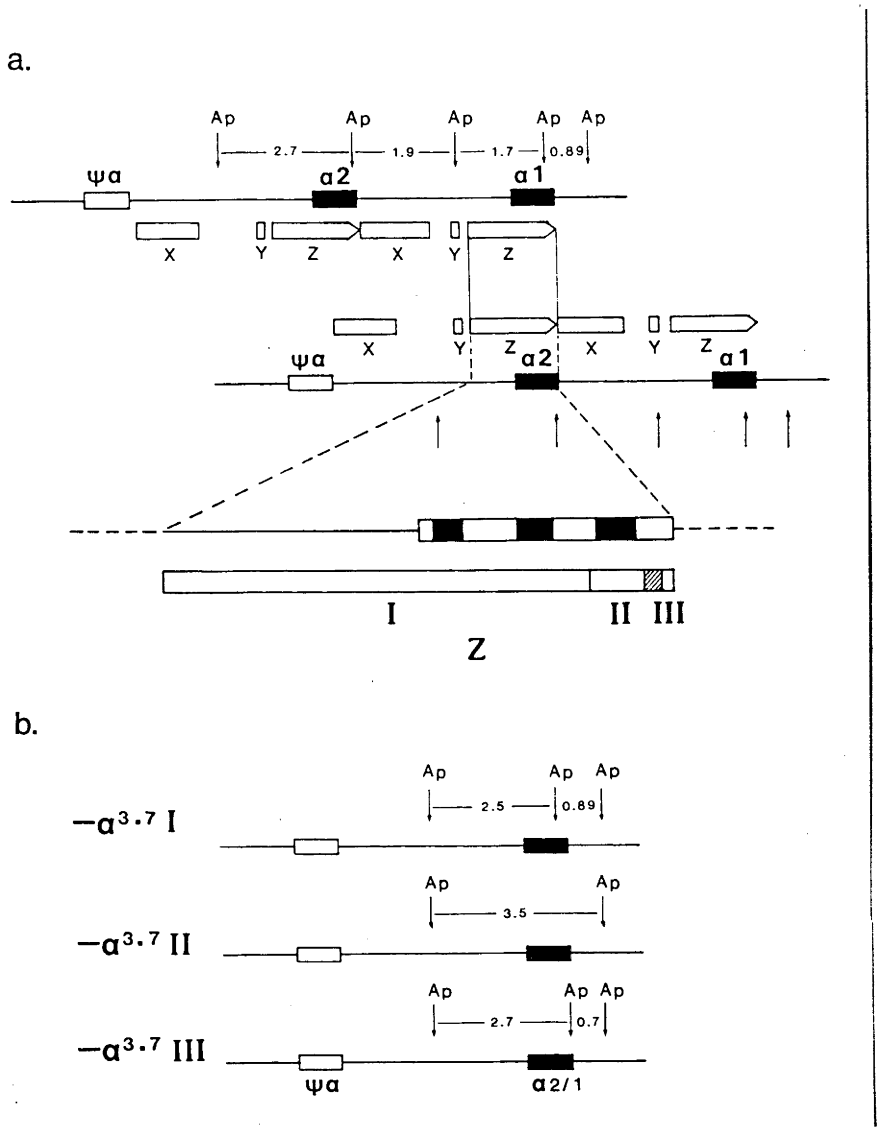


Figure 3.7 a: Mispairing of α-globin genes on two homologous chromosomes during meiosis. X,Y and Z are blocks of highly conserved sequences. Crossing-over in segments I, II and III of the mispaired Z blocks results in the $-\alpha^{3.7}$ deletion subtypes I, II, and III, respectively. Apa I sites (Ap) on both chromosomes and lengths (in kb) of the normal restriction fragments are also indicated.

b: Restriction-fragment lengths of the α-globin gene cleaved by endonuclease Apa I in the three subtypes of the $-\alpha^{3.7}$ deletion.

Figure 3.8 Autoradiograph of DNAs digested with Apa I and hybridized with the α-globin gene probe showing patterns of DNA fragments in the normal individual ($\alpha\alpha/\alpha\alpha$), the $-\alpha^{3.7}$I subtype heterozygotes ($\alpha\alpha/-\alpha^{3.7}$I) and homozygotes ($-\alpha^{3.7}$I/$-\alpha^{3.7}$I), and the $-\alpha^{3.7}$III heterozygotes ($\alpha\alpha/-\alpha^{3.7}$III).

Apa I x α-globin-gene probe

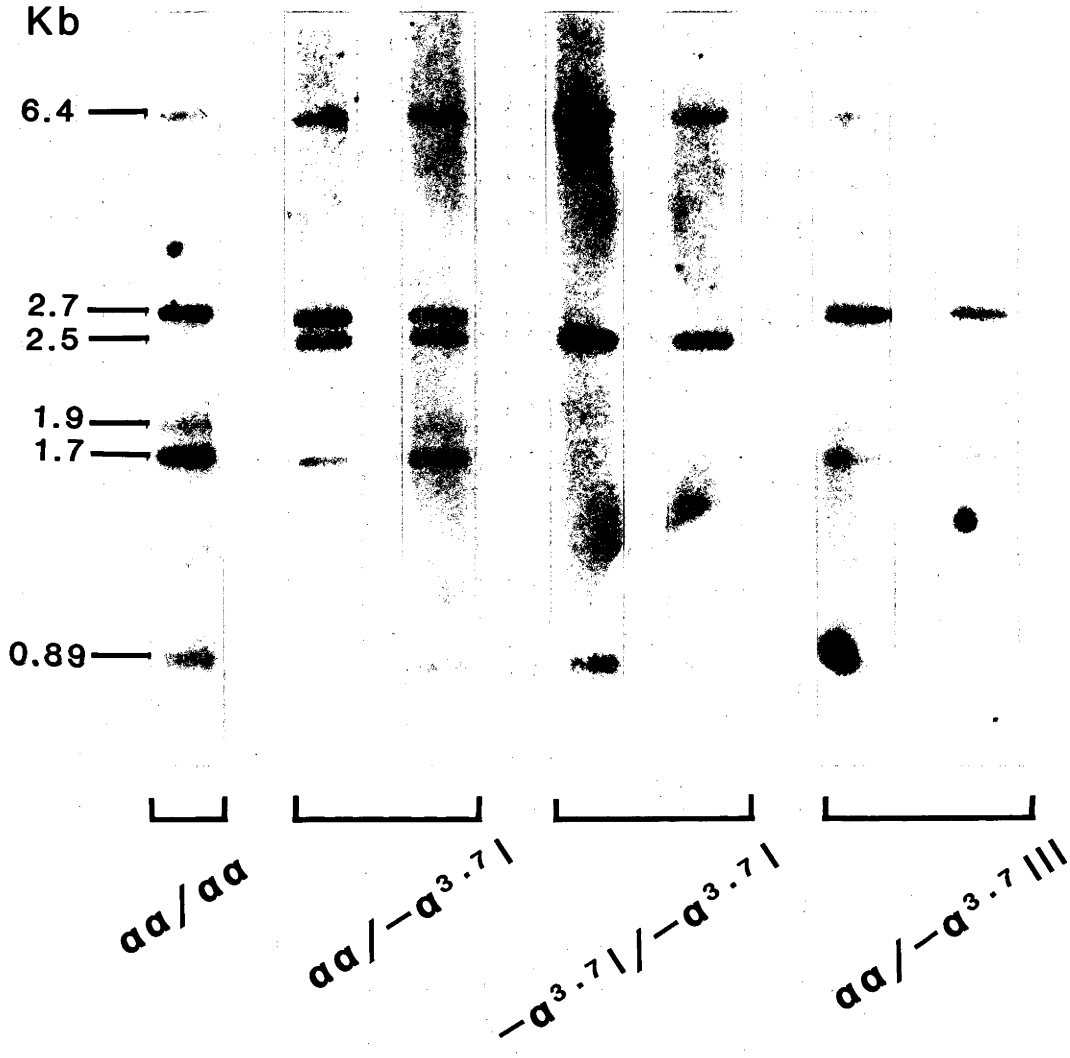


Table 3.4 Subtypes of the $-\alpha^{3.7}$ deletion as detected by Apa I digestion and hybridization with the α-globin specific probe.

Region	Number	Subtype		
		$-\alpha^{3.7}$ I	$-\alpha^{3.7}$ II	$-\alpha^{3.7}$ III
1. Cent.P.(Port Moresby)	3	3	0	0
2. Gulf P.	11	11	0	0
3. Western P.	5	5	0	0
4. Milne Bay P.	9	9	0	0
5. Northern P.	5	4	0	1
6. Madang P.(group 1)	5	5	0	0
7. Chimbu P.(Karimui)	35	35	0	0
8. East. Highlds. P.	1	1	0	0
9. East New Britain P.	10	2	0	8
Total	84	75	0	9

3.3.5 Linked polymorphisms of ζ-globin gene fragments and the single α-globin gene deletion

DNA length polymorphisms due to the hypervariable region between the ζ and ψζ genes tend to be in linkage disequilibrium with variations in the α-globin genes. Digestion with the enzyme Bgl II produces allelic fragments of 10.5, 11.3 or 12.0 kb of the ζ-globin gene. These allelic fragments correspond exactly with the 8.9, 10.0 or 10.7 kb Bam HI fragments of the ψζ gene (section 3.3.2). The polymorphisms detected by using the enzyme Bgl II in 1288 chromosomes with the αα/, $-\alpha^{4.2}$/, $-\alpha^{3.7}$/, and ααα/ haplotypes were analysed. Figure 3.9 shows the Bgl II polymorphism of the ζ-globin gene fragments observed in various α-globin genotypes. Association between polymorphic Bgl II ζ-globin gene fragments and α-globin gene haplotypes is presented in Table 3.6. The 10.5 and 11.3 kb fragments of the ζ-globin gene are common but the 12.0 kb fragment is rare among the populations of PNG. The 11.3 kb fragment, which is more common in the αα/ haplotype than the 10.5 kb fragment, is almost the only fragment associated with the $-\alpha^{4.2}$ deletion. However, both the 10.5 and 11.3 kb fragments are associated with the $-\alpha^{3.7}$ defect. When the subtypes of the $-\alpha^{3.7}$ deletion are considered, the $-\alpha^{3.7}$III subtype seems to associate with the 11.3 kb fragment and both the 10.5 and 11.3 kb fragments are associated with the $-\alpha^{3.7}$I subtype. The 10.5 kb fragment is frequently associated with the ααα/ haplotype.

Figure 3.9 Bgl II polymorphism of the ζ-globin gene fragments observed in various α-globin genotypes in the populations of PNG.

Bgl II x ζ-globin-gene probe

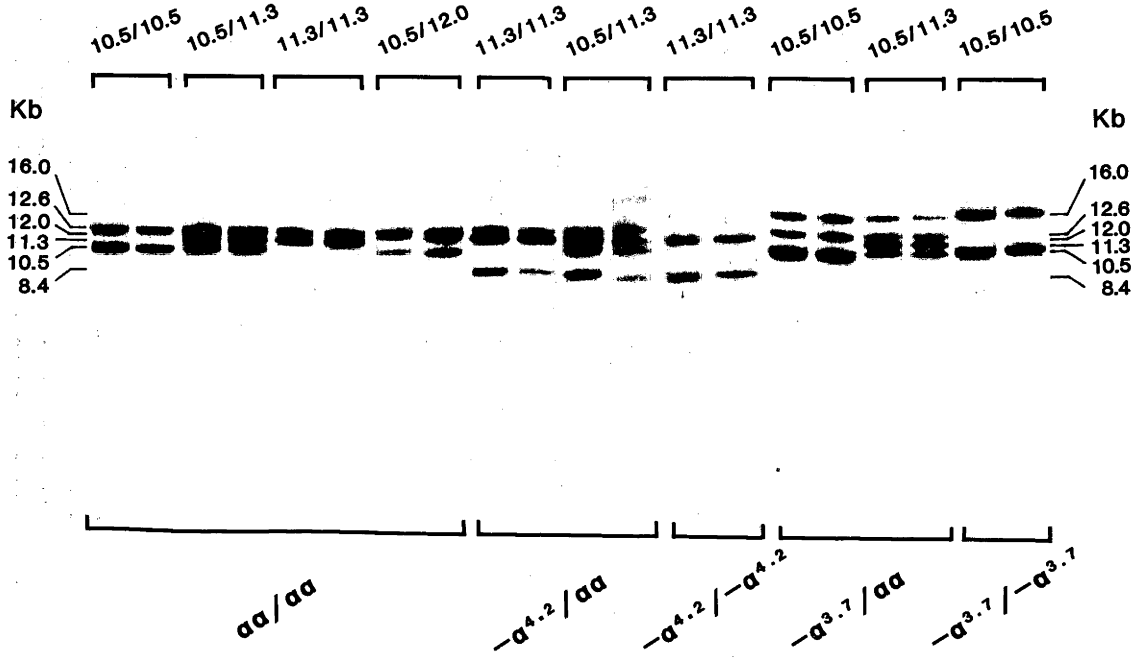


Table 3.6 Association between polymorphic Bgl II ζ-globin gene fragments and α-globin gene haplotypes.

Haplotype	Bgl II ζ-globin-gene fragment			
	10.5 kb	10.5 or 11.3 kb[a]	11.3 kb	12.0 kb etc.
αα/	344	87	472	11[b]
$-\alpha^{4.2}$/	1	47	203	0
$-\alpha^{3.7}$/	35	40	24	1[c]
ααα/	6	16	1	0

[a]This group could not be definitely assigned since both fragments were observed in heterozygotes e.g. αα/$-\alpha^{4.2}$ αα/$-\alpha^{3.7}$ etc.

[b]including : 4 chromosomes with 12.0 kb, 5 chromosomes with either 10.5 or 12.0 kb, and 2 chromosomes with either 11.3 or 12.0 kb.

[c]either 10.5 or 12.0 kb.

3.3.6 Analysis of the triplicated α-globin genes

The triplicated α-globin gene (ααα/), occurring as the reciprocal in the recombination which produces the single α-globin gene deletion, was detected by the presence of an 18 kb Bam HI fragment carrying three α-globin genes (section 3.3.1). To distinguish the $\alpha\alpha\alpha^{anti\ 3.7}$ and $\alpha\alpha\alpha^{anti\ 4.2}$ haplotypes, the membranes containing Bgl II digested DNA were hybridized with the α-globin gene probe. The presence of an extra DNA fragment of 3.7 kb as well as two normal (12.6 and 7.5 kb) fragments specifies the $\alpha\alpha\alpha^{anti\ 3.7}$ while the presence of an extra fragment of 16 kb and only one normal fragment (7.5 kb) specifies the $\alpha\alpha\alpha^{anti\ 4.2}$ (Goossens et al 1980; Higgs et al 1980a; Lie-Injo et al 1981; Trent et al 1981). By this analysis, two DNA samples (one each from Karimui and Goroka), which had both 14 and 18 kb Bam HI fragments of the α-globin genes, showed the extra 3.7 kb Bgl II fragment as well as the two normal Bgl II fragments. Therefore, they were $\alpha\alpha\alpha^{anti\ 3.7}$ heterozygotes ($\alpha\alpha/\alpha\alpha\alpha^{anti\ 3.7}$). Nineteen samples (3 from Port Moresby and 16 from Wanigela), which also had the 14 and 18 kb Bam HI fragments of the α-globin genes, showed an extra 5.7 kb fragment as well as the two normal fragments after Bgl II digestion (Figure 3.10). They were, thus, neither the $\alpha\alpha\alpha^{anti\ 3.7}$ nor $\alpha\alpha\alpha^{anti\ 4.2}$ type but possibly carried a new type of α-globin gene rearrangement i.e. a new triplication on one chromosome. This haplotype is called here "the $\alpha\alpha\alpha^{X}$/". Single and double digestions with several restriction enzymes were performed for DNAs with the $\alpha\alpha/\alpha\alpha\alpha^{X}$ genotype compared with the αα/αα and $\alpha\alpha/\alpha\alpha\alpha^{anti\ 3.7}$ genotypes (Figure 3.10 and Table 3.5). An extra fragment was usually observed, except in double digestion with Bam HI and Eco RI.

Figure 3.10 Autoradiographs of DNAs from: (1) a normal individual ($\alpha\alpha/\alpha\alpha$), (2) an $\alpha\alpha\alpha^{\text{anti 3.7}}$ heterozygote ($\alpha\alpha/\alpha\alpha\alpha^{\text{anti 3.7}}$), and (3) an $\alpha\alpha\alpha^{X}$ heterozygote ($\alpha\alpha/\alpha\alpha\alpha^{X}$) digested with single or combined enzymes and hybridized with the α-globin gene probe.

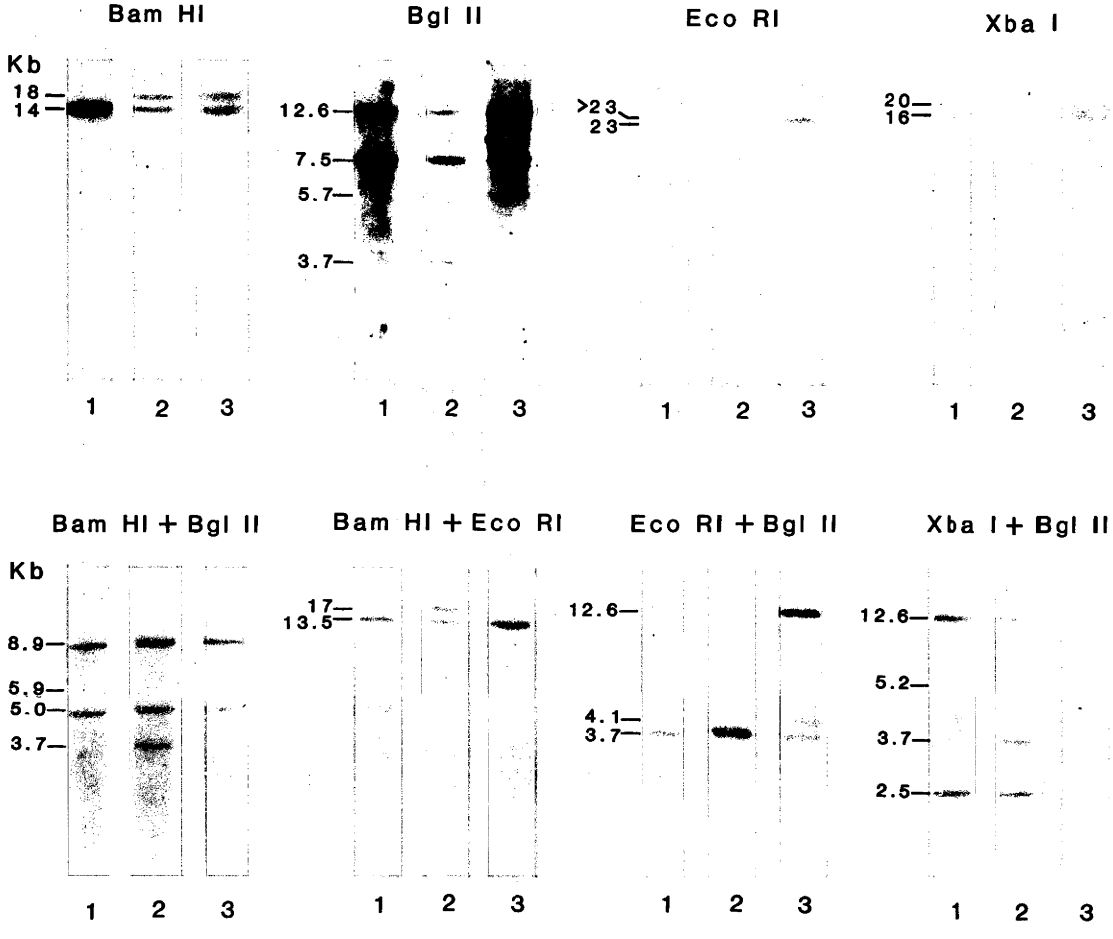

Bam HI
Bgl II
Eco RI
Xba I
Kb
18
14
12.6
7.5
5.7
3.7
>23
23
20
16
1 2 3
Bam HI + Bgl II
Bam HI + Eco RI
Eco RI + Bgl II
Xba I + Bgl II
Kb
8.9
5.9
5.0
3.7
17
13.5
12.6
4.1
3.7
12.6
5.2
3.7
2.5
1 2 3

Table 3.5 Restriction fragments (in kb) of DNAs from individuals with αα/αα, αα/ααα$^{anti\ 3.7}$, and αα/ααα^{X} genotypes digested with single and combined restriction enzymes.

Enzyme	αα/αα (no.1)	αα/ααα$^{anti\ 3.7}$ (no.2)	αα/ααα^{X} (no.3)
Bam HI	14	18	18
		14	14
Bgl II	12.6	12.6	12.6
	7.5	7.5	7.5
		3.7	5.7
Eco RI	23	>23	>23
		23	23
Sac I	14	14	14
	4.3	4.3	5.4
	1.3	3.7	4.3
		1.3	1.3
Xba I	16	20	20
		16	16
Bam HI + Bgl II	8.9	8.9	8.9
	5.0	5.0	5.9
		3.7	5.0
Bam HI + Eco RI	13.5	17	13.5
		13.5	
Eco RI + Bgl II	12.6	12.6	12.6
	3.7	3.7	4.1
			3.7
Xba I + Bgl II	12.6	12.6	12.6
	2.5	3.7	5.2
		2.5	2.5

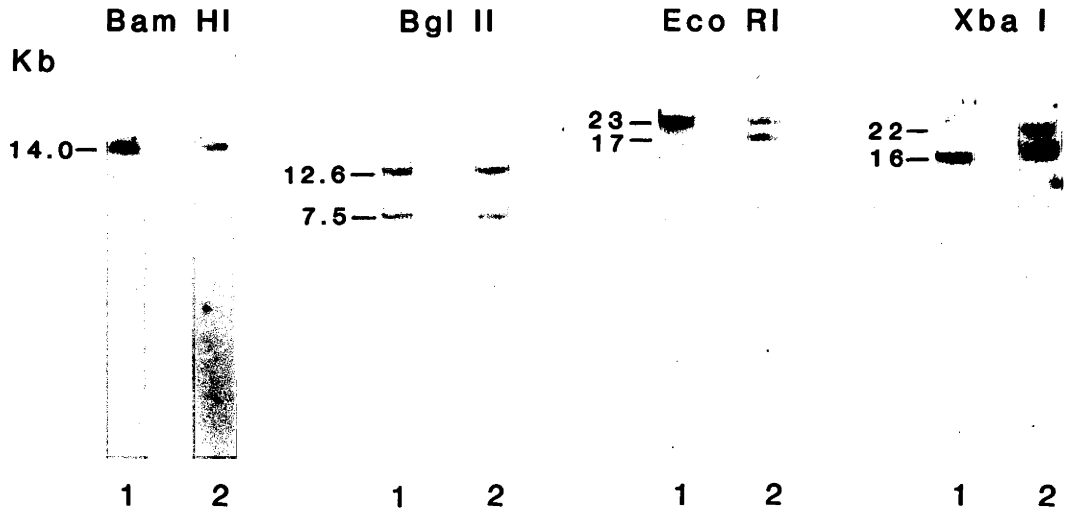
3.3.7 Analysis of the ζ-globin gene deletion

One DNA sample from Port Moresby showed an unusual 6.0 kb fragment with Bam HI when hybridized with the ζ-globin gene probe, although the normal fragment (14 kb) was observed when hybridized with the α-globin gene probe (Figure 3.11). This might be the result of a Bam HI site polymorphism in the ζ-globin gene region on one of the chromosomes or of a deletion in the α-globin gene cluster. Subsequently, it was found that a number of other restriction enzymes also produced an abnormal pattern making a Bam HI polymorphism unlikely and suggesting a large deletion in this cluster. There are two ways in which a deletion might occur and alter the pattern of the ζ genes generated by Bam HI. Firstly, the deletion may have occurred in the 3' region of the cluster, removing all the α-globin genes and changing the size of the Bam HI fragment of the ψζ or ζ gene. Secondly, the deletion may have occurred in the region of the ζ and ψζ genes leaving the α-globin genes intact. To examine these two possibilities, this DNA was further digested with Bgl II, Eco RI, and Xba I and hybridized with both the α- and ζ-globin gene probes.

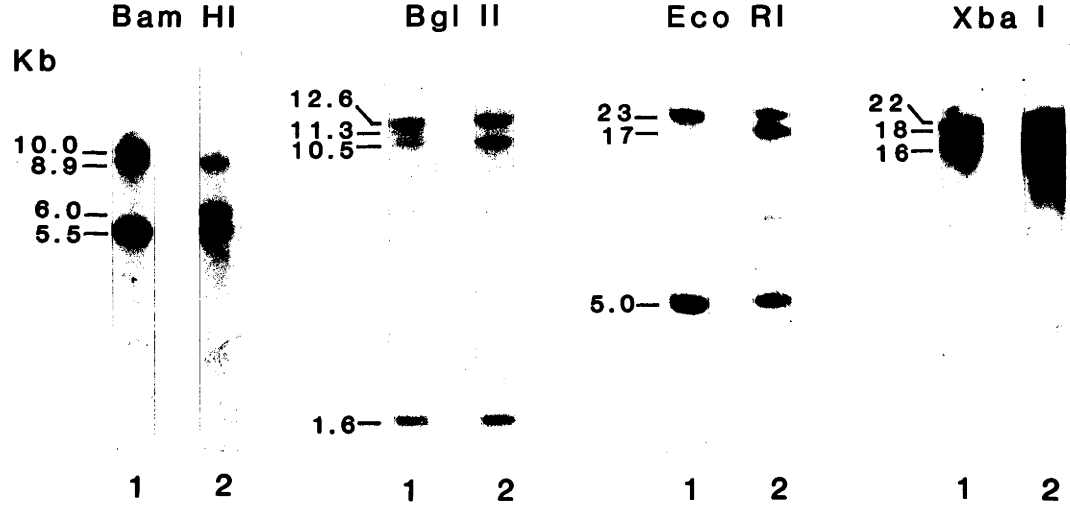
Normal DNA digested with Eco RI and Xba I gives rise to fragments of 23 and 16 kb, respectively, which both contain the ψζ, ψα, α2 and α1 genes when hybridized with the α-globin gene probe (the ψα2 and θ1 genes are not considered). In this unusual DNA sample, extra fragments of about 17 and 22 kb as well as the normal fragments were obtained for each enzyme. The extra fragments hybridized with both the α- and ζ-globin gene probes (Figure 3.11). From these results the site of deletion can be

Figure 3.11 Autoradiographs of a normal DNA sample (1) and the DNA sample with a ζ-globin gene deletion (2) digested with enzymes Bam HI, Bgl II, Eco RI, and Xba I and hybridized with the α- and ζ-globin gene probes.

α-globin-gene probe



ζ-globin-gene probe



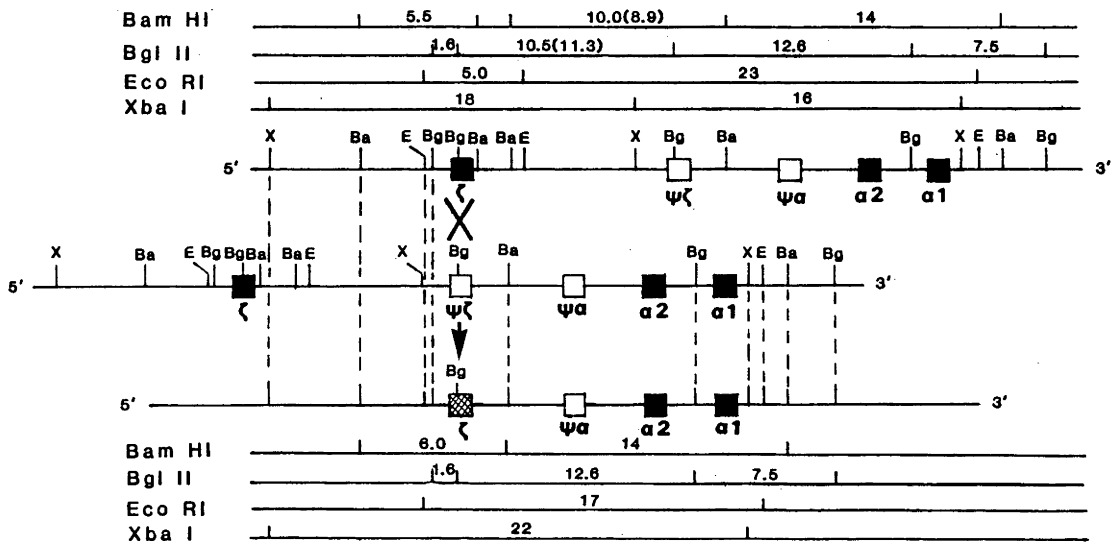


Figure 3.12 Restriction sites of enzymes Bam HI (Ba), Bgl II (Bg), Eco RI (E), and Xba I (X) in the α-globin gene cluster of the normal chromosomes and the chromosome with a ζ-globin gene deletion. The fragment sizes for each enzyme for the normal and deleted chromosomes are shown above and below the gene clusters, respectively. The ψα2 and θ1 genes are not shown.

If as it seems likely the 5' end of the remaining ζ gene is derived from the functional ζ gene, then the remaining gene may be functional, since the nonsense mutation in the nonfunctional ψζ gene is situated at codon 6 (Proudfoot et al 1982).

located 5' to the ψζ gene, removing the Bam HI, Eco RI, and Xba I sites between the ζ and ψζ genes (Figure 3.12). Digestion with Bgl II produced the normal pattern of DNA fragments when hybridized with the α- and ζ-globin gene probes. This would be possible if the deletion was caused by unequal crossing over in the region of the ζ and ψζ genes, in which there is a similar Bgl II site (Figure 3.12).

3.4 Discussion

Before the availability of the DNA mapping technique, it was difficult to estimate accurately the frequency of α-thalassemias in adults, since α-thalassemia-2 heterozygotes (-α/αα) are phenotypically normal and both α-thalassemia-2 homozygotes (-α/-α) and α-thalassemia-1 heterozygotes (--/αα) are asymptomatic. The slightly hypochromic microcytosis in the two latter conditions is an unreliable indicator in populations such as PNG where the incidence of iron deficiency is also high (Kariks and Woodfield 1972). Although screening of α-thalassemias has been more successful using detection of Hb Bart's in cord blood, this technique is unable to reveal genotypes accurately and cannot be used for adults. Using the α-globin gene probe, the presence of a deletion of one or both of the duplicated α-globin genes can be determined. This approach has been employed to estimate the frequency of α-globin gene deletion in the populations of PNG.

The results of screening the populations of PNG by the DNA mapping technique show that α-thalassemia 2, caused by a single

α-globin gene deletion (-α/), is common in coastal and lowland areas but rare in highland regions. It has the highest frequency on the north coast where malaria has been holo- to hyperendemic and lower frequencies in the less malarious areas of the eastern and southern coasts. Thus, the distribution pattern of α-thalassemia 2 is generally very similar to that of the endemicity of malaria (Figures 1.7 and 3.2). This correlation supports the hypothesis that malaria is the selective factor for α-thalassemia 2.

The presence in low frequencies of α-thalassemia 2 in the populations of highland areas where malaria is only sporadically present may be due to genetic exchange occurring with those of the coastal and lowland regions. The great variations in frequency of the -α/ haplotype in the coastal and lowland regions is probably the result not only of different intensities of selection by malaria but also of founder effect, migration and genetic drift, all of which are possible in the small isolated population groups of PNG and which have led to great diversity of the populations as evidenced by linguistic study (Wurm 1983). In addition, the effects of other red blood cell characteristics thought to protect against malaria (Chapter 1: section 1.5) may also have influenced the -α/ haplotype frequency in some localities (discussed in Chapter 6: section 6.4). Although many factors might have affected the -α/ haplotype frequency, malaria seems to be the major one because it is the parasitic disease of red cells that has the strongest impact on morbidity and mortality of every individual living in the lowland areas, and the infectious disease

that determines the distribution and density of the populations in PNG (Riley 1983). Malaria is, at present, highly endemic in the coast and lowlands of PNG (Chapter 1: section 1.6). Thus, this selection pressure is presumably still operating in these regions, although it has somewhat declined following European contact. Since α-thalassemia 2 has no apparent homozygous disadvantage, the mode of selection for α-thalassemia 2 seems to be directional i.e. the polymorphism is not balanced until the frequency of the defect reaches fixation and the defective haplotype (-α/) completely replaces the normal haplotype (αα/) unless the presumed selective pressure is disrupted. Examples of groups in which the -α/ haplotype frequency is close to fixation have been found in the Sepik and Madang areas where the frequencies of this haplotype are more than 80%. In these areas, malaria has long been holoendemic and infant mortality rates due to malarial infection have been extremely high (Peters 1960; Sturt and Stanhope 1968: Townsend 1985).

The more severe form of α-thalassemia, α-thalassemia 1, which is usually caused by dysfunction or deletion of both duplicated α-globin genes on one chromosome (--/), is very rare in the populations of PNG. The common form of this deletion which is found in neighbouring Southeast Asians, the $--^{SEA-1}/$, and other forms of the deletion that could be detected by variations of the ζ or ψζ gene were not observed in the examination of more than 1,200 chromosomes in this study. The 15 reported cases of Hb H disease in PNG indicate that a form of α-thalassemia 1 is present in some southern areas (Ryan et al 1961; Booth 1966; Amato 1977)

but its frequency must be so low that Hb Bart's hydrops fetalis (--/--) has never been observed.

It is not known what the underlying molecular defect of α-thalassemia 1 in PNG is. The defect may be a non-deletion type, partial deletion of the α-globin gene cluster that maintains normal restriction patterns in heterozygotes or a deletion of the whole α-globin gene cluster like that found in American blacks (Felice et al 1984) and Southeast Asians (Winichagoon et al 1984; Chang and Kan 1984; Lie-Injo et al 1985). None of these defects would have been detected in this population study by the DNA mapping technique used. The molecular defect of α-thalassemia 1 in PNG could be characterized by DNA study in those families with Hb H disease.

The rarity of α-thalassemia 1 is perhaps due to the genetic load imposed by segregation of the --/ chromosome which outweighs the possible selective advantage for α-thalassemia 1 heterozygotes (--/αα). Since α-thalassemia 2 is so common in the populations of PNG, α-thalassemia 1 would frequently segregate with α-thalassemia 2 resulting in Hb H disease. In conditions where malaria and other infectious disease are prevalent and health care is poor, persons with Hb H disease would be more susceptible to crisis episodes of acute hemolysis, leading to a reduced life expectancy. Thus, α-thalassemia 1 would decrease in frequency and be eliminated from the gene pool.

Subtype analysis of the single α-globin gene deletion reveals that the $-\alpha^{4.2}$ deletion is generally predominant in most of the regions where the single α-globin gene deletion exists, except in some southern areas (the Gulf and Western Provinces, and Karimui)

and in the New Britain Province where the $-\alpha^{3.7}$ defect is more frequent. Thus, the populations of the north and northeast of PNG represent groups in which the $-\alpha^{4.2}$deletion predominates over the $-\alpha^{3.7}$defect. This may have resulted from founder effect or from the effect of severe malarial selection favouring the $-\alpha^{4.2}$ deletion. The $-\alpha^{4.2}$deletion removes the α2 gene while the $-\alpha^{3.7}$ deletion removes the middle portion between the α1 and α2 genes or the whole α1 gene. Although globin production from the α1 and α2 genes is thought to be equal (Nute 1974; Liebhaber and Kan 1982), mRNA transcription from the α2 gene is greater than the α1 gene (the ratio is 2:1 to 3:1) (Orkin and Goff 1981a; Liebhaber and Kan 1981) and deletion of one or the other gene may have different effects. In fact, Bowden et al (1985) have noted that the $-\alpha^{4.2}$ deletion produces higher Hb Bart's levels in cord bloods than the $-\alpha^{3.7}$ defect indicating a greater imbalance of globin chain production in the $-\alpha^{4.2}$ type. This minute difference of globin-chain imbalance may confer slightly greater protection against malaria and allow the $-\alpha^{4.2}$ deletion to prevail over the $-\alpha^{3.7}$ defect under very strong selection.

Further analysis of the subtypes of the $-\alpha^{3.7}$ deletion shows that the $-\alpha^{3.7}$I subtype is the common form of the $-\alpha^{3.7}$ deletion. It was observed in all groups in which the $-\alpha^{3.7}$ deletion was present (75 out of 84 chromosomes). The $-\alpha^{3.7}$III subtype is very rare in the PNG mainland; one out of 9 chromosomes with the

However, the data does not exclude the possibility of multiple origins for this deletion.

$-\alpha^{3.7}$ III subtype was found in the Northern Province and the rest were detected in the east of New Britain. The $-\alpha^{3.7}$II subtype was not observed in the populations of PNG.

Recently, Hill et al (1985) reported that the $-\alpha^{3.7}$III subtype is unique in Melanesians and Polynesians and not found in other populations from Southeast Asia, India, Saudi Arabia, the Mediterranean or Jamaica, in which α-thalassemia is prevalent. These workers concluded that this observation indicates genetic affinity between the Melanesians and Polynesians, and supports a migration route through Melanesia for the colonizers of Polynesia. However, the results of the present study show that the $-\alpha^{3.7}$III subtype is not as common in the Melanesians of the PNG mainland as in the island Melanesians and Polynesians. This subtype is much more prevalent in the eastern island Melanesians of Vanuatu (formerly called the New Hebrides) and almost the only subtype in Polynesians (Hill et al 1985; Trent et al 1986b). In contrast to previous interpretations (Hill et al 1985), the results of this study suggest that the $-\alpha^{3.7}$III subtype originated in eastern island Melanesia or Polynesia and later diffused west to the offshore islands east of PNG and also to the PNG mainland.

Analysis of DNA length polymorphisms linked to the ζ-globin gene in the individuals carrying the single α-globin gene deletion reveals that a single allele of the polymorphism (the 11.3 kb Bgl II fragment) is almost exclusively associated with the $-\alpha^{4.2}$ deletion. This may suggest that almost all the $-\alpha^{4.2}$ deletions in the populations of PNG have a single origin. One $-\alpha^{4.2}$ haplotype which was associated with the 10.5 kb Bgl II fragment may represent a recently introduced deletion, a new mutation, or a

change of the linked polymorphism by recombination. The $-\alpha^{3.7}$ I subtype is associated with both the 10.5 kb and 11.3 kb Bgl II fragments and the $-\alpha^{3.7}$ III subtype with the 11.3 kb Bgl II fragment. The former may have arisen at least twice on chromosomes carrying the different polymorphisms or there may have been recombination following a single deletion while the latter may have occurred only once. A single origin of the $-\alpha^{3.7}$ III subtype has already been suggested (Hill et al 1985). Thus, there are at least five haplotypes of the single α-globin gene deletion in the population of PNG.

Although the $-\alpha^{4.2}$ deletion is the most common form of the α-globin gene deletion, it is surprising that its reciprocal product, the $\alpha\alpha\alpha^{\text{anti } 4.2}$, has not been observed in the populations of PNG. This result supports the view that the mutation giving rise to the $-\alpha^{4.2}$ deletion (and its reciprocal product) is infrequent, as suggested from the result of analysis of linked polymorphisms. Alternatively, the $\alpha\alpha\alpha^{\text{anti } 4.2}$ haplotype might not have confered any protection against malaria and therefore, may have been eliminated. By contrast, the reciprocal product of the $-\alpha^{3.7}$ deletion, the $\alpha\alpha\alpha^{\text{anti } 3.7}$, was found in these populations. This finding and the result of analysis of linked polymorphisms suggest that the mutation resulting in the $-\alpha^{3.7}$ deletion (and its reciprocal product) might have occurred more frequently than that of the $-\alpha^{4.2}$ deletion in the populations studied. The $\alpha\alpha\alpha^{\text{anti } 3.7}$ may be present in the populations as the result of random genetic drift.

A new type of DNA rearrangement in the α-globin gene region, possibly a new triplication ($\alpha\alpha\alpha^X/$), was detected in the samples from Port Moresby and Wanigela areas. The mechanism of this rearrangement has not been elucidated in this study because of the difficulties in interpreting the results of DNA mapping, which were complicated by fragments of the α-globin genes from the normal chromosome. This problem may be solved by the study of a cloned genomic DNA fragment containing the new triplicated α-globin genes. The $\alpha\alpha\alpha^X/$ haplotype which was found in a high frequency in Wanigela might have originated in this area and later spread to Port Moresby region. This haplotype, like the other triplicated α-globin genes, may not confer protection against malaria. Its frequency in the Wanigela area might be increased because of isolation and inbreeding in this group.

One of more than 600 DNA samples in this study was found to have a deletion in the ζ-globin gene region. The deletion is consistent with an unequal crossing-over between the homologous ζ and ψζ gene regions (Winichagoon et al 1982; Rappaport et al 1984). The haplotype frequency of this deletion is very low (less than 0.08%) in the present study compared with 0.4% observed in combined results of three racial groups (origins not mentioned) in the study by Winichagoon et al (1982). The latter study also reported a surprisingly high prevalence (1.3%) of chromsomes with triplicated ζ genes, which has not been found in the present work. The single ζ-globin gene deletion was observed in an even higher frequency (10%) in American blacks (Rappaport et al 1984). This variation seems to be random and not associated with malaria.

The results of α-globin gene analysis in the populations of PNG presented in this chapter show various rearrangements in the α-globin gene cluster, of which the single α-globin gene deletion (-α/), resulting in α-thalassemia 2, is the most common. In addition, the differences in frequency of the -α/ deletion in these populations of the lowlands and the highlands correlates strongly with the incidence of malaria.

CHAPTER 4

β-THALASSEMIA IN PAPUA NEW GUINEA

Chapter 4: β-Thalassemia in PNG

4.1 Introduction

The presence of β-thalassemia in PNG has been firmly established since the first reports of clinical cases by Ryan in the early 1960s (Ryan 1961a, 1961b and 1962). Later surveys have shown that β-thalassemia is fairly common in some coastal and lowland areas of PNG (Curtain et al 1962; Giles et al 1967; Booth and Garo 1978).

Molecular analysis of β-thalassemia genes in other populations has shown that β-thalassemias are only rarely due to a large DNA deletion in the β-globin gene region (Kazazian 1985). This means that the DNA mapping technique can not be used to study β-thalassemia genes directly unless the mutation involved changes a restriction site (summarized in Antonarakis et al 1985; Kazazian 1985). However, it can be applied to the study of haplotypes (patterns of restriction-site polymorphisms) of normal and abnormal β-globin genes in populations. This kind of study is useful in assessing how often the mutation causing a particular defect has occurred in a population. By haplotype analysis, mutliple mutations of the β^S gene in blacks and the β^E gene in Southeast Asians have been suggested (Antonarakis et al 1982b and 1984b; Wainscoat et al 1983; Kazazian et al 1984c; Pagnier et al 1984). In addition, haplotype analysis combined with the molecular cloning technique have been used to demonstrate the association of haplotypes and specific types of mutations of β-thalassemia in Mediterranean and Asian Indian populations (Orkin et al 1982a; Kazazian et al 1984a and 1984b).

In this chapter, haplotypes of normal and thalassemic β-globin genes of Papua New Guineans were analysed in order to examine whether multiple mutations have occurred in the populations of PNG.

4.2 Samples and methods

Four hundred and fifty blood samples from those collected for the α-thalassemia study were also screened for β-thalassemia trait. In addition blood samples obtained from 4 patients who were β-thalassemia homozygotes and 7 individuals who were parents of the patients or known β-thalassemia heterozygotes, provided by Dr Michael Smiley of Port Moresby General Hospital, were included in the study.

Hb type was checked by starch-gel electrophoresis and Hb A_2 was quantitated by the DEAE-cellulose microchromatographic method (Huisman et al 1975). A level of Hb A_2 less than 3.50% was taken to be normal. In β-thalassemia heterozygotes, the mean value of Hb A_2 was 5.35% (range 4.27-8.11%).

The β-globin gene haplotypes of 41 non β-thalassemic individuals, 17 β-thalassemia heterozygotes, and 4 β-thalassemia homozygotes were analysed by digestion of DNA with restriction enzymes Hgi AI, Ava II, and Bam HI and hybridized with the β-globin gene probe. These three restriction enzymes were chosen because it has been suggested that their polymorphic patterns are correlated with the β-globin gene frameworks obtained from DNA sequencing (Orkin et al 1982a; Antonarakis et al 1982b; Kazazian

et al 1984c). Although β-globin gene haplotypes can be analysed by using more restriction sites located in the 5' portion of the β-globin gene cluster, in the absence of family studies the results would be difficult to interpret due to many possible combinations when individuals heterozygous at several restriction sites are detected. Furthermore, different haplotypes could readily arise from single recombination events in the "hot spot" region between the ψβ- and β-globin genes (Antonarakis et al 1982a, 1982b, and 1984b; Chakravarti et al 1984: Chapter 1: section 1.3.3).

Hgi AI, Ava II and Bam HI have polymorphic sites in exon-1, IVS-2, and at the 3' end of the β-globin gene (Chapter 2: section 2.12.2). Absence of the polymorphic sites (-) produces large DNA fragments (1.05 kb for Hgi AI, 2.2 kb for Ava II, and 22 kb for Bam HI) and presence of the polymorphic site (+) results in small DNA fragments (0.7 kb for Hgi AI, 2.0 kb for Ava II, and 9.3 kb for Bam HI). Eight possible combinations of these 3 polymorphic sites were designated as follows (listed in the order Hgi AI, Ava II, and Bam HI): haplotype a(+++), b(++-), c(--+), d(-++), e(-+-), f(+-+), g(+--), and h(---). To test the technique, DNA from Thai individuals with the normal β-globin genotype or homozygous Hb E, which is a well-characterized condition, were analysed initially (see Appendix).

4.3 Results

4.3.1 Survey of β-thalassemia trait in PNG

Screening for β-thalassemia trait in 450 blood samples from

various regions of PNG showed that 18 individuals were affected. The incidence found in each region is presented in Table 4.1. Six out of 19 samples (31.6%) from the Northern Province had β-thalassemia trait. This incidence is surprisingly high. The finding that β-thalassemia is rare in the highlands of PNG is consistent with the previous reports. The incidence of β-thalassemia in various regions of PNG reported in the literature is summarized in Table 4.2. From the results of this study and the previous surveys (Ryan 1962; Giles et al 1967), it seems that β-thalassemia is prevalent in some localities on the east coast (the Morobe, Northern and Milne Bay Provinces) of PNG.

4.3.2. Clinical and hematological descriptions of β-thalassemia homozygotes

Four patients with homozygous β-thalassemia had clinical and hematological findings typical of the disease (data provided by clinician; see Table 4.3). They were severely anemic and had regularly been transfused. Their mean pre-transfusion Hbs were 4-6 g/dl. In addition, they had splenomegaly (5-10 cm), increased Hbs A_2 and F. Hb type in blood taken before blood transfusion was A_2FA in all the patients. The presence of Hb A in their blood indicates that they carried at least one β^+-thalassemic gene and the genotypes could be either homozygous for β^+-thalassemia or heterozygous for β^+- and β^0-thalassemias. One or both of the parents were investigated. All of them had increased Hb A_2 (range 5.14-7.50%) but not Hb F, compatible with being β-thalassemia heterozygotes.

Table 4.1 Incidence of β-thalassemia in the populations of PNG.

Province	No. examined	No. affected	Percent
1. Central			
-Port Moresby	66	2	3.0
-Wanigela	37	0	0
2. Gulf	16	0	0
3. Western	11	0	0
4. Milne Bay	20	2	10.0
5. Northern	19	6	31.6
6. Morobe	9	0	0
7. Madang			
-Madang-1	18	2	11.1
-Madang-2	75	6[a]	5.5
-Karkar Is.	61	0	0
8. Sepik	6	0	0
9. Manus Is.	3	0	0
10.Chimbu (Karimui)	58	0	0
11.East. Highlds.	18	0	0
12.West. Highlds.	2	0	0
13.South. Highlds.	31	0	0
Total	450	18	4.0

[a]Three were known to be members of the same family, and therefore two were excluded from calculation. If these are included the frequency is 8.0%.

Table 4.2 Reported incidence of β-thalassemia in the populations of PNG.

Province	Locality	No.examined.	No.affected.	%	Ref.
1. Gulf	Kerema	Hosp.patients	4	-	1
	Kerema	22	2	9.1	1
	Kerema and Kairuka	50	3	6.0	2
2. Milne Bay	Oro Bay	26	7[a]	15.4	1
3. Morobe	Markham River	246	7	2.8[b]	3
	Markham River	810	44	5.4[c]	4
4. Madang	Madang and Karkar Is	2000	27	1.4	5
6. Sepik	Sause	354	27	7.6	3
	Abelam	113	2	1.8	3
7. New Britain	Gazelle Peninsula	369	16	4.3	3
8. Chimbu	(unknown)	128	2	1.6	6
9. E.Highlds.	(combined)	238	0	0	3
	Goroka	100	0	0	6
10.W.Highlds.	(combined)	95	2	2.1	3
	(unknown)	94	0	0	6
Total		4645[d]	143	3.1	

[a]Three were related to a patient with β-thalassemia/Hb Lepore disease and not taken into account in the calculation of percentage.

[b]The average of percentages found in three villages (1%,2% and 8%).

[c]The average of percentages found in nine villages, ranging from 0 to 22.8%.

[d]Hospital patients are not included.

(References 1: Ryan 1962; 2: Booth and Garo 1978; 3: Curtain et al 1962; 4: Giles et al 1967; 5: Hornabrook et al 1972; 6: Vaterlaws et al 1981).

Table 4.3 Hematological findings of 4 patients with homozygous β-thalassemia.

Patient no.	Sex	Age (Yr)	Mean pretransfusion Hb (g/dl)	Spleen Size (cm)	Hb type	Hb A_2 (%)	HbF (%)
1	F	4	3.9	5	A_2FA	3.72	13.0
2	M	3	4.9	10	A_2FA	4.79	20.0
3	M	3	4.9	6	A_2FA	4.31	27.3
4	M	3	4.3	5	A_2FA	4.85	38.5

Note: All the data (except Hb type and Hb A_2 levels) were provided by Dr. Michael Smiley of Port Moresby General Hospital.

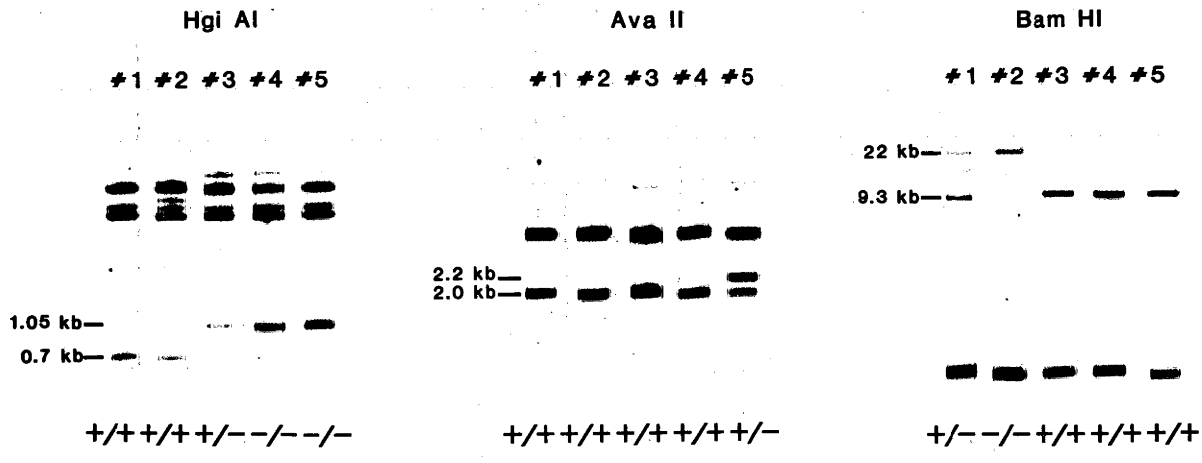
4.3.3 Haplotype analysis of the normal and thalassemic β-globin genes

Haplotypes of the normal β-globin gene were analysed in 41 non β-thalassemic individuals. Figure 4.1 shows an autoradiograph of the common genotypes, which could be assigned unequivocally. The prevalence of each genotype is presented in Table 4.4. Genotype b/d (++-/-++) seems to be most common in this group. The haplotype frequencies are presented in Table 4.5. Haplotypes b(++-) and d(-++) are common in the normal group, accounting for 36.6% and 30.5%, respectively. Haplotypes a (+++) and c(--+) accounted for 17.1% and 14.6%, respectively. Other haplotypes are rare or absent.

In β-thalassemia heterozygotes, genotype a/b (+++/++-) predominates (Table 4.6). Three individuals had genotype a/a (+++/+++) and one had genotype c/c (--+/--+), indicating that β-thalassemic genes are associated with haplotypes a(+++) and c(--+). In 4 β-thalassemia homozygotes, one had genotype a/a and one c/c, and two had genotype a/c (one confirmed by family studies, the other not confirmed). This result verifies the association of β-thalassemic genes with haplotypes a and c.

Figure 4.1 a. Autoradiograph of DNA from 5 non β-thalassemic individuals digested with restriction enzymes Hgi AI, Ava II, and Bam HI and hybridized with the β-globin gene probe.

b. Genotypes derived from combined polymorphic restriction sites of the three enzymes.



Genotypes	#1	:	+++/++−	=	a/b
	#2	:	++−/++−	=	b/b
	#3	:	+++/−++	=	a/d
	#4	:	−++/−++	=	d/d
	#5	:	−−+/−++	=	c/d

Table 4.4 β-Globin genotypes observed in 41 non β-thalassemic individuals.

Genotype	n
a/b	6
b/b	5
a/d	5
d/d	4
c/d	3
c/c	2
a/a	1
a/f	1
b/d*	9
b/c**	5

* This group could be either genotype b/d (++-/-++) or genotype a/e (+++/-+-) but the former is more likely due to the higher frequency of haplotypes b and d (see Table 4.5).

** The most likely genotype of four possible ones [a/h (+++/---), b/c (++-/--+), d/g (+--/-++), and e/f (-+-/+-+) since haplotypes b and c are common while e,f,g, and h are rare or absent.

Table 4.5 Haplotype frequencies of the normal β-globin genes.

Haplotype	n*	Percent
a	14	17.1
b	30	36.6
c	12	14.6
d	25	30.5
e	0	0
f	1	1.2
g	0	0
h	0	0

*Includes 14 assumed to be haplotype b, 5 haplotype c, and 9 haplotype d from equivocal genotypes (see notes to Table 4.4).

Table 4.6 β-Globin genotypes observed in 17 β-thalassemia heterozygotes and 4 β-thalassemia homozygotes.

Genotype	n
(1) β-Thal heterozygotes	
a/b	7
a/a	3
a/c	1
c/c	1
c/d	1
a/d	1
b/c*	1
a/c**	2
(2) β-Thal homozygotes	
a/a	1 (No.1)
c/c	1 (No.2)
a/c***	1 (No.3)
a/c**	1 (No.4)

* The most likely genotype.

** The alternative genotype is d/f.

***This genotype is definite because both parents were also studied.

4.4 Discussion

When compared with α-thalassemia 2, β-thalassemia, which is clinically more important, is less common in the populations of PNG. The correlation of β-thalassemia with malaria endemicity, if it exists, is less clear than that of α-thalassemia 2. High frequencies of β-thalassemia in some places, particularly on the east coast, are likely to be the result of genetic isolation and drift (Giles et al 1967) perhaps in combination with selection by malaria.

If β-thalassemia confers an advantage against malarial infection (Chapter 1: section 1.5), its frequency would be expected to be higher than that found in this population. Without taking random effects into account, there are two factors concerning the frequency of β-thalassemia in populations to be considered. Firstly, the frequency of β-thalassemia is limited by homozygous disadvantage, since β-thalassemia homozygotes have a short life-span and are almost infertile. If such a gene is favoured by selection, its frequency can increase only to a level which ensures that not too many homozygotes are produced. The maximum frequency will be reached when the number of heterozygotes is maximised through advantage of the heterozygotes and disadvantage of the normals and homozygotes under the full operation of selection (a genetic equilibrium). It has been calculated that the heterozygote frequency to maintain a genetic equilibrium of the sickle cell gene is 31.7% (Vogel and Motulsky 1979). For β-thalassemia, it is not known what the maximum heterozygote frequency is. Clearly, the frequency of the β-

thalassemic gene, which generally causes a more severe disease than the sickle cell gene, could never be higher than that of the normal gene. Secondly, increase in the frequency of β-thalassemia may be restricted and the β-thalassemic gene may be eliminated by the presence of other similar but milder defects.

In Southeast Asia where β-thalassemic diseases are well documented, especially cases of β-thalassemia/Hb E disease, the frequency of β-thalassemia is relatively low, varying between 1% and 9% (Wasi 1983). Flatz et al (1965) first pointed out a reciprocal relationship between β-thalassemia and Hb E in Southeast Asia. This is probably due to the fact that combination of the two defects leads to β-thalassemia/Hb E disease which is usually almost as severe as homozygous β-thalassemia, resulting in gene loss. However, Hb E has a higher fitness; Hb E homozygotes have normal life span and fertility whereas β-thalassemia homozygotes have almost zero fertility. In the population in which both defects coexist, Hb E will spread at the expense of β-thalassemia.

In PNG, Hb E is extremely rare, only a single case being observed in more than 10,000 persons tested (Kirk 1980), and none of the other abnormal hemoglobins, except Hb J Tongariki and a few cases of Hb Lepore, are detected. The most common hemoglobinopathy is α-thalassemia 2 (chapter 3). α-Thalassemia 2 may have a similar interaction with β-thalassemia to Hb E, although α- and β- thalassemias are not allelic. Since α-thalassemia 2 is so common, segregation of β-thalassemia in the populations of PNG will often result in a combination of the two

defects. Co-inheritance of α- and β-thalassemias results in less globin-chain imbalance. If the protection against malaria is derived from imbalanced globin chain production, which is the primary abnormality in thalassemic red cells (Chapter 1: section 1.4), then the protective effect is probably reduced when co-inheritance of α-thalassemia 2 and β-thalassemia occurs. Interaction of α-thalassemia 2 with homozygous β-thalassemia may alleviate the severity but occurs less frequently and might not be sufficient to prevent the loss of β-thalassemic genes. Homozygous α-thalassemia 2, similar to homozygous Hb E, is asymptomatic and apparently not disadvantageous. The prevalence of α-thalassemia 2 may, thus, prohibit spreading of β-thalassemia, and the latter may qualify as a "fugitive" allele (Livingston 1983).

The β-thalassemic genes in PNG seem to be of the β^+-thalassemia type. The major molecular defects of β^+-thalassemia are nucleotide substitutions at the promoter regions, or in the exons and introns of the β-globin gene that affect normal RNA processing (Chapter 1: section 1.4.2). The exact molecular defect of β^+-thalassemic genes in PNG awaits study by DNA sequencing. However, DNA analysis has shown that β-thalassemia in PNG is associated with two β-globin gene haplotypes, i.e. haplotypes a (+++) and c (--+). Since these two haplotypes could not be derived from one another by a single recombination event, the mutations producing β-thalassemia genes are most likely to have occurred independently and may have arisen at least twice. By chance, the mutations should have occured on the haplotypes common in the population. However, haplotypes a and c associated with β-thalassemic genes in PNG are less common in the non β-

thalassemic group than haplotypes b and d. This paradox may occur because of fluctuation of the haplotype frequencies in the non β-thalassemic group due to the small sample size or because the mutations originated in sub-population groups in which haplotypes a and c were predominant. Selection which increases the frequency of the β-thalassemia gene, would also increase the frequency of the linked haplotype.

It has been suggested that normal β-globin genes in various populations exist in four forms, namely frameworks 1,2,3 (Asian), and 3 (Caucasian) (Orkin et al 1982a; Antonarakis et al 1982b; Kazazian et al 1984c). These gene frameworks were characterized by DNA sequencing but can be detected in uncloned DNA by DNA mapping using restriction enzymes Hgi AI, Ava II, and Bam HI. Framework 1 is correlated with the polymorphic pattern of +++ (or haplotype a in the present study), framework 2 with ++- (haplotype b), and framework 3 (Asian and Caucasian) with --+ (haplotype c). However, haplotype analysis in non β-thalassemic individuals from PNG and normal individuals from Thailand (see Appendix) revealed the existence of extra polymorphic patterns: haplotypes d (-++) and f (+-+) in Papua New Guineans, and d, e (-+-), and f in Thais. Haplotype d is quite common in the former group (30.5%). The new patterns of polymorphic sites or haplotypes found in these two populations may indicate new β-globin gene frameworks which have never been previously described.

From the results described in this chapter, it can be seen that β-thalassemia in PNG maintains a weaker relationship with malaria than α-thalassemia 2. The prevalence of α-thalassemia 2,

which may interact competitively with β-thalassemia, as well as its homozygous disadvantage, may restrict the spread of β-thalassemia in the malarious areas of PNG. In addition, the haplotype study has shown the association of β-thalassemic genes with two different β-globin gene haplotypes, probably indicating at least two independent mutations.

CHAPTER 5

α-THALASSEMIA IN AUSTRALIAN ABORIGINES

Chapter 5: α-Thalassemia in Australian Aborigines

5.1 Introduction

α-Thalassemia is prevalent in Southeast Asians (Wasi et al 1974, Wasi 1983), lowland and island Melanesians (Booth 1981; Oppenheimer et al 1984; Bowden et al 1985; Chapter 3), and Polynesians (Trent et al 1985 and 1986b). Australian Aborigines are, however, a distinct population group in whom thalassemias and other hemoglobinopathies have never previously been observed (Kirk 1965 and 1981) although they were prehistorically related, and live in close proximity to Southeast Asians and Melanesians. It is of interest to know whether the silent form of α-thalassemia, or α-thalassemia 2, which is undetectable by hematological and biochemical techniques, is present in Aborigines. By using the sensitive and direct technique of DNA mapping, this form of α-thalassemia has now been demonstrated in some Aboriginal groups. This chapter presents the evidence for the occurrence of α-thalassemia 2 in Australian Aborigines as detected by the presence of a single α-globin gene deletion and shows the result of analysis of this defect for its subtypes.

5.2 Populations sampled

DNA was prepared from Aboriginal blood samples collected on various occasions. These samples had been kept in liquid nitrogen for periods of 1 to 15 years. Most of the samples had been used for red cell enzyme studies. Attempts were made to harvest DNA from several hundred blood samples but DNA was obtained from only 134 samples. Good yields of DNA were achieved from the samples stored for the shortest period of time and which had never been

thawed for enzyme studies. The areas where these samples were collected are Kalumburu and Mowanjum (near Derby) in the Kimberly region of Western Australia, Mornington Island in the Gulf of Carpentaria, Cherbourg in Queensland, and Hermannsburg and Yuendumu in the Central Desert. In addition, DNAs prepared from 80 Indonesian blood samples were analysed to compare the subtypes of the α-globin gene deletion with those found in Aborigines. These samples, collected from people who live in Yogyakarta Province on Java Island, were provided by Dr A S M Sofro.

5.3 Results

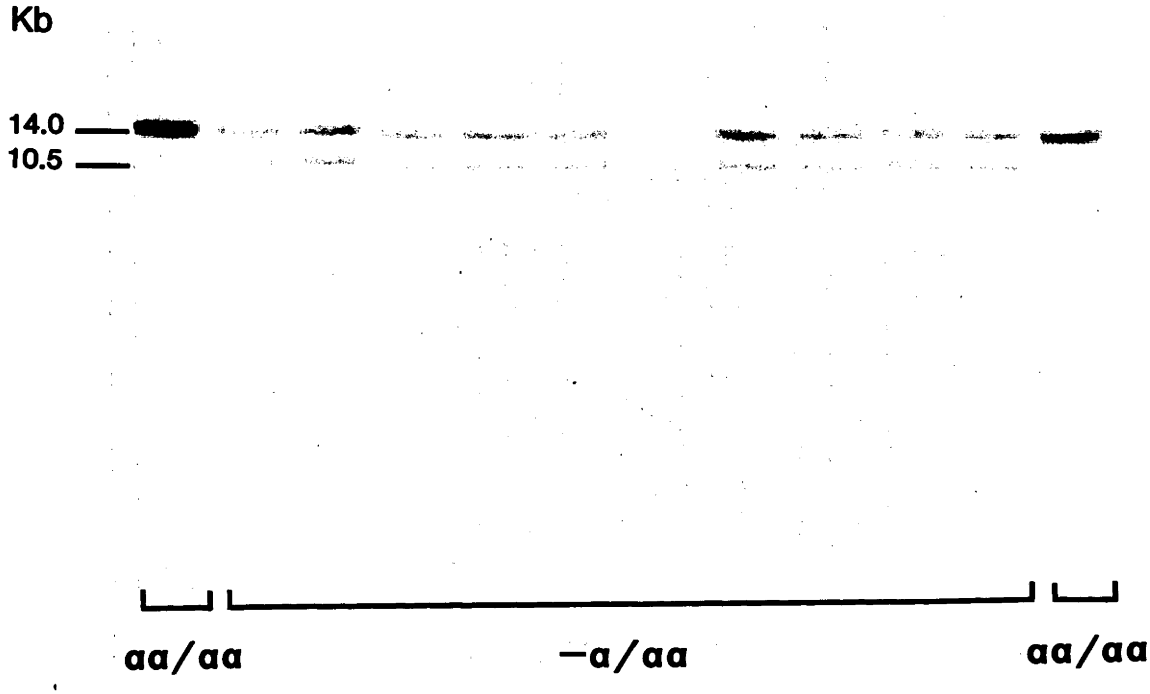
5.3.1 Prevalence of the single α-globin gene deletion

The presence of the deletion form of α-thalassemia 2 (-α/) in Aboriginal DNA samples was demonstrated by Bam HI digestion and hybridization with the α-globin gene probe (Figure 5.1). The -α/ deletion was detected in 17 out of 134 DNA samples; all of them were heterozygotes. Fifteen of these were found in the samples collected from Kalumburu and one sample each from Mowanjum and Mornington Island. The deletion was not found in 6 DNA samples collected from Cherbourg or in 22 samples from the Central desert (Figure 5.2).

The possible existence of the deletion of both α-globin genes on one chromosome (--/) was also examined by rehybridization of the membranes containing Bam HI DNA fragments with the ζ-globin gene probe. None of the samples showed unusual variations of the ζ or ψζ gene fragments.

Figure 5.1 Autoradiograph of DNA from the Aborigines who were normal ($\alpha\alpha/\alpha\alpha$) or α-thalassemia-2 heterozygotes ($-\alpha/\alpha\alpha$) digested with Bam HI and hybridized with the α-globin gene probe.

Bam HI x α-globin-gene probe



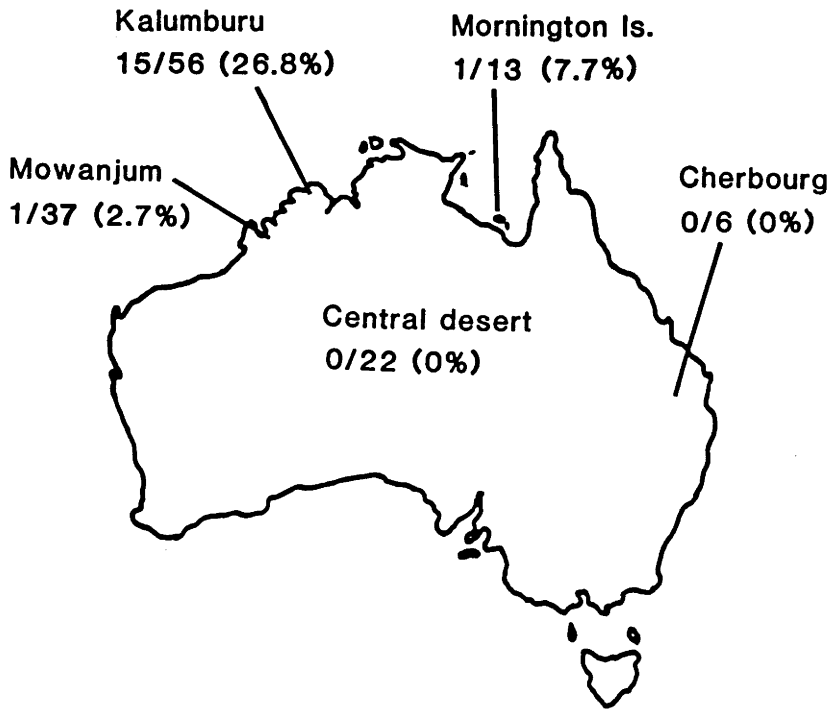


Figure 5.2 Incidence of the single α-globin gene deletion in various groups of Australian Aborigines.

5.3.2 Subtypes of the single α-globin gene deletion and its association with Bgl II fragments of the ζ-globin gene

The Aboriginal DNA samples were further analysed by Bgl II digestion and hybridization with the ζ-globin gene probe. The 16 kb Bgl II fragment of the ψζ gene, specific for the $-\alpha^{3.7}$ deletion, was detected in all the samples with the deletion. Thus, they all carried the $-\alpha^{3.7}$ subtype (Figure 5.3).

Analysis of the polymorphic ζ-globin gene fragment in the Aborigines showed that the 11.3 kb Bgl II fragment was the common allele accounting for about 80% of all chromosomes tested. Nineteen percent carried the 10.5 kb fragment and 1% carried the 12.0 kb fragment. The $-\alpha^{3.7}$ deletion was observed in 15 samples which were 11.3 kb homozygotes and 2 samples which were 10.5 kb and 11.3 kb heterozygotes. Therefore, the $-\alpha^{3.7}$ deletion in this population seems to be always associated with the 11.3 kb Bgl II fragment.

5.3.3 Subtypes of the $-\alpha^{3.7}$ deletion

When the Aboriginal DNA with the $-\alpha^{3.7}$ deletion was digested with the enzyme Apa I and hybridized with the α-globin gene probe, in addition to the normal pattern (Chapter 3: section 3.3.4), 5 samples showed the extra 2.5 kb fragment of the $-\alpha^{3.7}$ I subtype and 9 samples showed the extra 3.5 kb fragment of the $-\alpha^{3.7}$ II subtype (Figure 5.4). One DNA sample was unclassifiable because there were both the 2.5 and 3.5 kb Apa I fragments and the normal

Figure 5.3 Autoradiograph of DNA from the Aborigines with the normal α-globin genotype (αα/αα) and heterozygous $-\alpha^{3.7}$ deletion ($-\alpha^{3.7}$/αα) digested with Bgl II and hybridized with the ζ-globin gene probe.

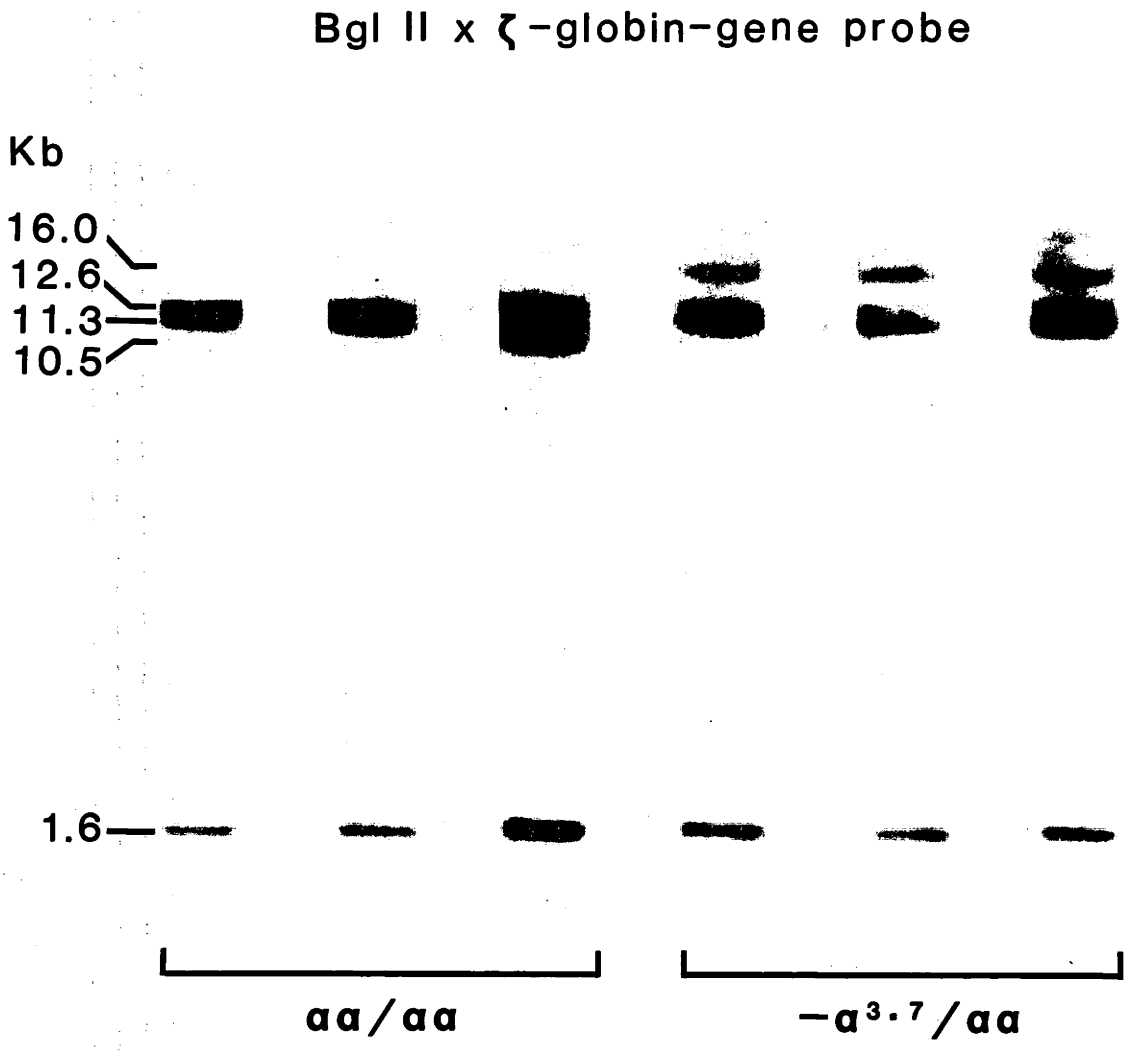
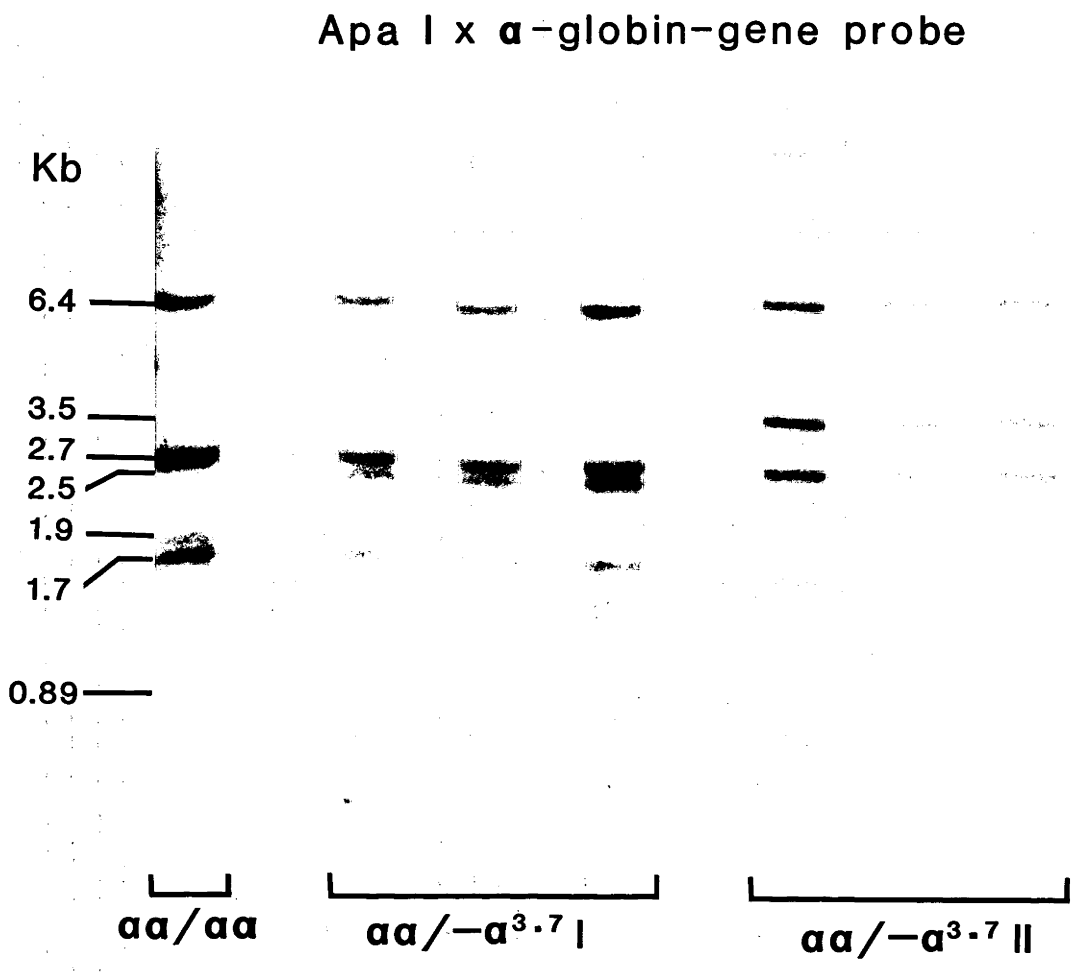

Bgl II x ζ-globin-gene probe
Kb
16.0
12.6
11.3
10.5
1.6
αα/αα
−α3.7/αα

Figure 5.4 Autoradiograph of DNA from the Aborigines with the $-\alpha^{3.7}$ deletion digested with Apa I and hybridized with the α-globin gene probe, showing the $-\alpha^{3.7}$ I and $-\alpha^{3.7}$ II subtypes.


Apa I x α-globin-gene probe
Kb
6.4
3.5
2.7
2.5
1.9
1.7
0.89
αα/αα
αα/−α3.7 I
αα/−α3.7 II

2.7 kb fragment was missing, although this individual was heterozygous for the deletion. He may have either the $-\alpha^{3.7}$I or $-\alpha^{3.7}$II deletion on one chromosome while the other chromosome has two α-globin genes but carries an alteration of the Apa I site.

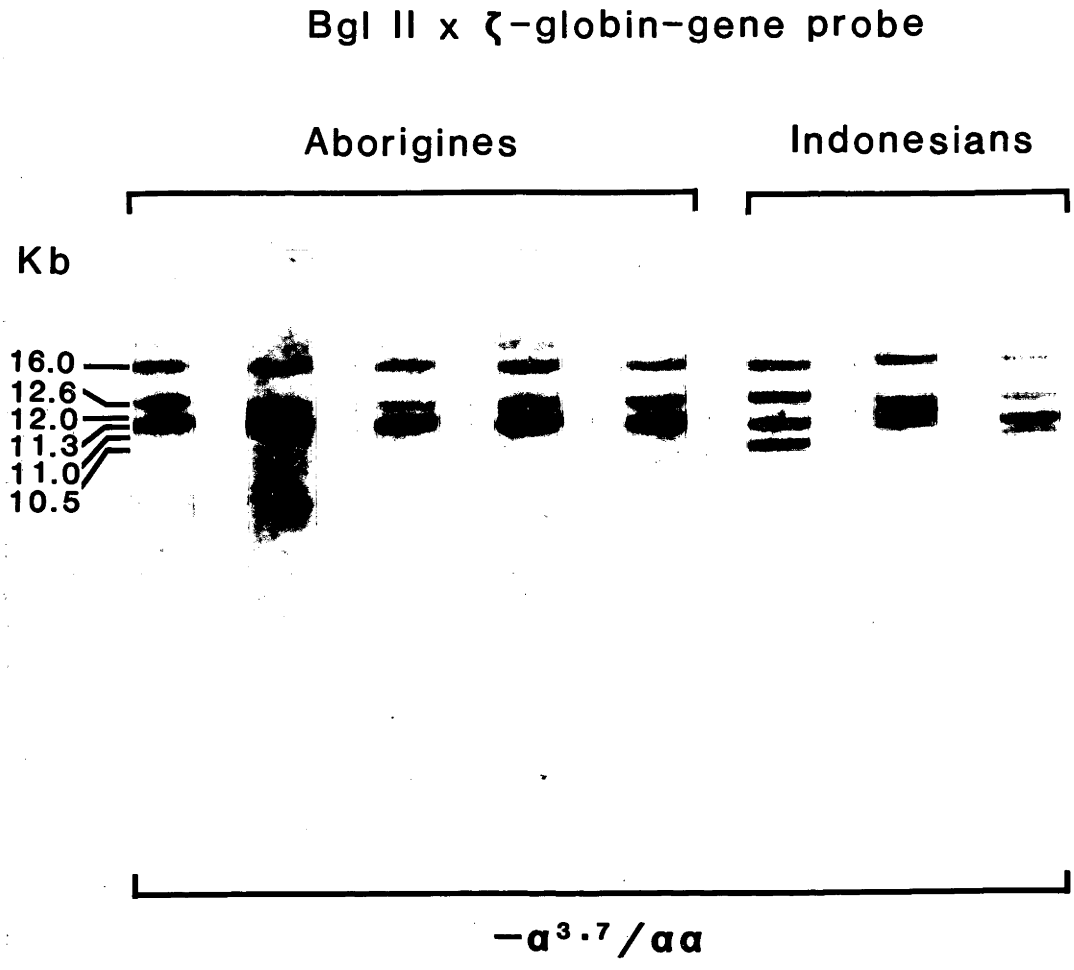
5.3.4 Analysis of the single α-globin gene deletion in Indonesians

To compare the single α-gobin gene deletion of Aborigines and Southeast Asians, 80 blood samples were collected from Yogyakarta, Indonesia, and the DNAs prepared from these samples were similarly analysed. The single α-globin gene deletion was detected in only three samples (haplotype frequency 1.9%). All three were $-\alpha^{3.7}$ I subtype heterozygotes. In these three samples, the 11.3 kb Bgl II fragment of the ζ-globin gene was observed in combination with other Bgl II polymorphic fragments (Figure 5.5). In normal Indonesians the Bgl II ζ-globin gene fragment was very polymorphic; at least five alleles at this locus were detected. The deletion of both α-globin genes on one chromosome, as detected by Bam HI digestion and the ζ-globin gene probe, was not found in these Indonesian samples.

5.4 Discussion

This study has demonstrated for the first time that α-thalassemia 2 is present in Australian Aborigines. It illustrates the power of the DNA mapping technique in revealing the presence of α-thalassemia 2 in a group in which this defect was thought

Figure 5.5 Autoradiograph of DNA from the Aborigines and Indonesians who were heterozygous for the $-\alpha^{3.7}$ deletion digested with Bgl II and hybridized with the ζ-globin gene probe.


Bgl II x ζ-globin-gene probe
Aborigines
Indonesians
Kb
16.0
12.6
12.0
11.3
11.0
10.5
−α3.7/αα

to be absent. All the cases detected here were heterozygotes for the single α-globin gene deletion. Neither homozygotes nor deletion of both α-globin genes on one chromosome were observed. The latter must be very rare or absent in the Aboriginal populations as also indicated by the absence of Hb H disease and Hb Bart's hydrops fetalis.

Two subtypes of the single α-globin gene deletion, the $-\alpha^{3.7}$ I and $-\alpha^{3.7}$ II, were detected. The former has been found to be the common subtype of the $-\alpha^{3.7}$ deletion in several populations including Southeast Asians, Indians, Saudi Arabians, Mediterraneans, Jamaicans (Hill et al 1985), and southern Papua New Guineans (Chapter 3). The $-\alpha^{3.7}$ II deletion which is the more common subtype in the Aborigines has previously been reported in 5 cases of Southeast Asians and 2 cases of Jamaicans (Hill et al 1985) but has not been found in Papua New Guineans (Chapter 3) and other populations. Both subtypes in the Aborigines are linked to the 11.3 kb Bgl II fragment of the ζ-globin gene, the predominant allele in this group.

There are at least three explanations for the presence of the single α-globin gene deletion in the Aborigines: it might be an ancestral gene, it may have been introduced through admixture with other population(s) or it may be a new mutation. Archaeological evidence shows that Aborigines have lived in Australia for at least 50,000 years (White and O'Connell 1982).

Their ancestors are believed to have migrated from Sunda land, the Southeast Asian area which extended at that time close to Sahul land or the Australia-New Guinea land-mass. Australia and New Guinea were later separated when sea levels were rising some 10,000 years ago at the end of the Pleistocene. Morphologically, Aborigines are most closely related to Melanesians, both from New Guinea and other parts of island Melanesia. Furthermore, there are certain genetic markers such as the transferrin allele TF D1 and the group specific component allele GC 1A1 (originally GC Ab) which indicate common genetic ancestry between Aborigines and Melanesians. In contrast, there are some red cell enzyme variants which occur widely in Australia but have never been detected in New Guinea or other parts of Melanesia, indicating a long period of relative isolation (Kirk 1981).

Since one of the deletion haplotypes (the $-\alpha^{3.7}$I subtype linked to the 11.3 kb Bgl II fragment of the ζ-globin gene) found in the Aborigines was also observed in the southern Papua New Guineans (Chapter 3: section 3.3.5), this subtype might be an ancestral genetic defect occurring before their separation, although the chance of co-incident mutations must also be considered. However, the presence of the other haplotype (the $-\alpha^{3.7}$II subtype linked to the 11.3 kb Bgl II fragment of the ζ-globin gene) in the Aborigines without having been detected in Melanesians indicates a different origin of this subtype.

Although new mutations cannot be excluded, the finding that the α-globin gene deletion was present in the northern and northwestern coastal areas but apparently absent in those from the eastern and central areas also suggests that the Aborigines

may have acquired this defect through contact with other population(s). For example, it is well known that the Macassans are a group which have visited these areas (Mulvaney 1975). During the last two centuries or more, these people made journeys regularly each year from the southern Celebes habour (Macassar) to the north and west of Australia by praus and stayed for a period of 6 months in order to collect and process trepang (sea-cucumber). Evidence for their contact with the Aborigines has been found in archaeological materials, culture, arts, and linguistics (Mulvaney 1975).

There might also have been other voyagers from Southeast Asian islands, as suggested by the presence of the GC 1C24 variant in the Tiwi, an Australian Aboriginal population on Bathurst Island in the north of Australia, and in Indonesians on several Lesser Sunda Islands (Kamboh et al 1984). In an attempt to test this hypothesis, a number of samples from Indonesians were collected and analysed. Unfortunately, only three samples in this series were found to carry the single α-globin gene deletion, specifically the $-\alpha^{3.7}$I subtype. No example of the $-\alpha^{3.7}$II was observed. Although the 11.3 kb Bgl II fragment of the ζ-globin gene was present in all the samples, there were also other Bgl II fragments present in every sample (Figure 5.5). Study of Indonesian populations, especially in the Macassans and Lesser Sunda Islanders, will help to elucidate whether the Aborigines have acquired the defect from these people or not.

Since α-thalassemia 2 has been found in many human populations (Higgs and Weatherall 1983), new mutations must be very common. In fact, loss of an α-globin gene by mechanisms

indistinguishable from those occurring *in vivo* has been observed *in vitro*, when a DNA fragment containing the human α-globin gene cluster was cloned in bacteriophage (Lauer *et al* 1980). As both subtypes of the deletion in the Aborigines are associated with the 11.3 kb Bgl II fragment of the ζ-globin gene and since this Bgl II fragment is common in this group (accounting for about 80%), new mutation(s) producing one or both subtypes could also have occurred in the Aboriginal groups.

A high frequency of the α-globin gene deletion in some groups of Aborigines may have been caused by inbreeding. Malaria, which is likely to be the selective factor increasing frequency in some populations (as shown in Chapter 3), has been reported sporadically in Australia. However, its role in maintaining the abnormal gene in Aborigines is unknown, although it has been argued that malaria could never become endemic due to low densities of the aboriginal population (Black 1972).

As a result of the work described in this chapter, α-thalassemia 2 caused by a single α-globin gene deletion has been detected in Australian Aborigines by DNA analysis. However, details of its origin and role in the Aboriginal populations are still obscure and await further studies for clarification.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6: General Discussion

6.1 Introduction

The advent of molecular biology techniques has permitted a great deal of progress in genetic studies. Direct analysis of genes in both the normal and abnormal condition has provided an increased understanding of the structure of genes and their regulation. In addition, in some cases the direct examination of abnormal genes has revealed precisely the type of mutation causing a genetic defect. It is now realized that one genetic defect can result from many different mutations. This has been demonstrated for α- and β-thalassemias as reviewed in Chapter 1. Furthermore, many genetic defects which were previously difficult or impossible to detect can now be revealed. One of these is α-thalassemia. These advances are beneficial not only for clinical use in the diagnosis of various genetic diseases and detection of their carriers but also for genetic studies of populations.

6.2 Defects of globin genes in Papua New Guineans and Australian Aborigines

The DNA mapping technique has been employed in the present investigation for examination of globin genes in the populations of PNG and neighbouring regions in order to characterize their defects. Analysis of the α-globin genes in the populations of PNG (Chapter 3) has revealed that a single α-globin gene deletion ($-\alpha/$), which results in α-thalassemia 2, represents the most common genetically determined red blood cell defect in PNG, and that the distribution of this defect correlates with the endemicity of malaria. This supports the hypothesis that malaria

is the selective factor responsible for increased frequencies of α-thalassemia 2. α-Thalassemia 2 seems to be increasing in frequency because it has no apparent homozygous disadvantage and the presumed selective factor is still in operation. In the Madang and Sepik areas, the defect is at fixation or nearly so.

The -α/ deletion exists in three subytpes ($-\alpha^{4.2}$, $-\alpha^{3.7}$I, and $-\alpha^{3.7}$III) in PNG, and is associated with at least 5 haplotypes, indicating its heterogeneity and multiple origins. The $-\alpha^{4.2}$ deletion is predominant in many areas while the $-\alpha^{3.7}$I defect is more common in some areas in the south. The $-\alpha^{3.7}$III subtype, which is unique in island Melanesia and Polynesia (Hill et al 1985), is present in New Britain but very rare in mainland PNG. This subtype may, thus, have diffused from island Melanesia and Polynesia to island and mainland PNG.

The deletion of both α-globin genes on one chromosome (--/), which results in α-thalassemia 1, has not yet been found in PNG by DNA methods, although α-thalassemia 1 is known to exist from the reported cases of Hb H disease (Ryan et al 1961; Booth 1966; Amato 1977). α-Thalassemia 1 is thus uncommon and this gene is probably fugitive, as previously suggested (Wills and Londo 1981), due to the disadvantages of homozygotes (--/--) and heterozygotes with α-thalassemia 2 (--/-α).

β-Thalassemia has been found in a much lower frequency than α-thalassemia 2 in PNG (Chapter 4), and its patchily increased frequencies in some eastern coastal regions may be due to genetic isolation and drift (Giles et al 1967). The correlation of β-thalassemia with the endemicity of malaria is less pronounced.

The β-thalassemia gene, which causes a more severe disease than α-thalassemia 2, may also be qualified as a fugitive gene when it interacts with α-thalassemia 2 in populations. The common type of this defect in PNG seems to be β^{+}-thalassemia which is not caused by β-globin gene deletion. The β-thalassemic genes in PNG are associated with at least two different β-globin gene haplotypes, suggesting independent mutations which have occurred at least twice.

The prevalence of α-thalassemia in Southeast Asians, Melanesians, and Polynesians raised the interesting question of whether α-thalassemia could be detected by this technique in Australian Aborigines who were thought on clinical grounds to be free of any hemoglobinopathies. This question has been examined (Chapter 5) and the presence of α-thalassemia 2, resulting from the -α/ deletion, in Australian Aborigines has now been demonstrated. Similar to Papua New Guineans, the --/ deletion has not been detected. Two subtypes of the -α/ deletion, $-\alpha^{3.7}$I and $-\alpha^{3.7}$II, have been observed. The $-\alpha^{3.7}$I subtype seems to be similar to that found in southern PNG and may represent a common ancestral gene. The $-\alpha^{3.7}$II subtype has not been detected in Melanesians or Polynesians but has been observed as a rare subtype in Southeast Asians, Mediterraneans, and Jamaicans (Hill et al 1985). However, an attempt to find the latter subtype in Indonesians, who may have originally introduced it into Australian Aboriginal populations, was unsuccessful.

It is clear that there is a correlation between α-thalassemia 2 and malaria, because α-thalassemia 2 is common throughout the "malarial belt" (Higgs and Weatherall 1983), extending from the Mediterranean to the Asia-Pacific area, but infrequent outside this belt. Furthermore, it has a microgeographic distribution in correlation with the endemicity of malaria as observed in PNG.

6.3 Effects of migration, random genetic drift, and selection on α-thalassemia 2 in PNG

Malaria seems to be the most important factor determining the frequency of α-thalassemia 2 in PNG. However, other factors such as migration, and genetic isolation and drift may also have been involved in the distribution of this defect and variation of its frequency.

Extensive linguistic, archaeological, and anthropological studies have provided useful information about past migrations in PNG (Bellwood 1979; Wurm 1983). Several waves of migration occurred from the west. Australoids were the earliest group who entered PNG some 60,000 years ago, and also moved further south to Australia when both regions formed a single land mass. Australoids were displaced in PNG by subsequent waves of Papuan migration. Papuans entered PNG between 15,000 and 4,000 years ago, and some of them also moved to island Melanesia. At the time of the last Papuan migration between 5,000 and 4,000 years ago, Austronesians also moved from island Southeast Asia along the north coast of PNG to Melanesia. These people brought Lapita pottery, pigs and horticulture with them to this area. About 3,000 years ago,

Austronesians migrated to Polynesia, whose present inhabitants are almost certainly their direct descendants.

Between 4,000 and 3,500 years ago, Austronesians who had migrated to the eastern Oceania areas seem to have returned to parts of PNG. They appear to have reached the southwest of New Britain and some coastal areas of the PNG mainland (Wurm 1983). The two main types of Austronesian languages (AN1 and AN2) in PNG may represent languages introduced by these separate waves of Austronesian migration (Cappell 1976).

To what extent have migrations influenced the distribution and frequency of α-thalassemia 2 in PNG? Past migrations have certainly affected the population structure. Papuans, who have occupied most of mainland and island PNG, were the largest group. Later contacts with Austronesians occurred among the coastal groups and islanders, but their admixture with Austronesians seems to be peripheral (Capell 1976; Serjeantson et al 1983). Although linguistically the populations of PNG can be differentiated according to which language stock is spoken (Papuan or Austronesian), there is no evidence for a clear-cut genetic distinction between them, and where genetic distinctions can be made, it is due to marked geographic separation between those groups (Serjeantson et al 1983). The Austronesian admixture among the groups on coastal PNG is estimated to be 15% using the HLA system (Serjeantson et al 1982).

The prevalence of α-thalassemia 2 relates to altitude rather than to linguistic grouping. Oppenheimer et al (1984) have pointed out, from the result of a study of cord blood Hb Bart's,

that the groups with the highest and lowest levels of α-thalassemia are linguistically more closely related than other lowland groups. This has been confirmed from the result of DNA analysis in the present study. For example, the groups with very high frequencies of α-thalassemia 2 from Madang and the Sepik region speak Papuan languages which are in the same language stock as those of groups with very low frequencies from the highland regions. The lowland groups in both language stocks always have high frequencies of α-thalassemia 2, although the frequencies are variable presumably depending on intensities of malarial selection and other factors.

The past migrations seem to have had little effect, if any, on the distribution and frequency of α-thalassemia 2. If Austronesians had interbred with coastal Papuans, which may have occurred at least 3,500 to 5,000 years ago, they would have been subject to selection by malaria, as have the resident Papuans, for about one hundred generations.

More recent migrations, for example, the movements of people between regions within PNG since European contact, may have had more impact on the distribution and frequency of α-thalassemia 2. Following social and economic developments, people have moved from rural areas and villages to the cities, and from highlands to lowlands (and vice versa, although these have been relatively few). Evidence for these recent migrations may be provided by the detection of the -α/ deletion in some highland individuals, especially in the cord blood samples collected from the Goroka Hospital.

PNG has remarkable variation in geography and environment (Allen 1983) which has led to a great diversity of the populations, and has a potential to produce random effects i.e. genetic isolation and drift. The random effects would be significant in a study aiming to compare genetic differentiation among a few small groups. However, the random effects, although they exist, appear to be less profound in the present investigation which observes the overall trends of frequency differences in several groups from the highlands and lowlands.

Selection of α-thalassemia 2 by malaria is thus the most tenable explanation. The frequency of α-thalassemia 2 has a remarkable correlation with malaria endemicity. It has the highest frequencies (68-92%) on the holo- to hyperendemic northern coast and islands, lower (28-56%) on the hyperendemic eastern coast and (9-38%) on the mesoendemic southern coast, and lowest (0-7%) in the nonendemic highland areas. This microgeographic distribution of α-thalassemia 2 in PNG provides strong support for selection of this defect by malaria. It demonstrates, therefore, that in such a small region as PNG where the overall pattern of climate is uniform and where the populations have a common ancestry, the frequencies of a genetic defect, α-thalassemia 2, can vary considerably in correlation with a selective factor, malaria endemicity.

While this chapter was being written, Flint et al (1986) reported the results of their study in PNG as well as island Melanesia. The frequency of α-thalassemia 2 (or α^{+}-thalassemia), but not other unlinked DNA polymorphisms, exhibits an altitude-

dependent correlation with malaria endemicity in PNG and also a latitude-dependent correlation throughout Melanesia. Their results are entirely in agreement with those for α-thalassemia 2 in the present study.

6.4 Interactions between α-thalassemia 2 and other genetic defects in protection against malaria

As might be expected in an area with a high incidence of malaria, other red blood cell abnormalities that have been proposed to confer protection against malaria are also found in PNG. Only one abnormal hemoglobin, Hb J Tongariki($\alpha_2^{115\ Ala \rightarrow Asp}\beta_2$), reaches polymorphic frequency in a limited area (Beaven et al 1972). Since Hb J Tongariki is linked to α-thalassemia 2 and its structural change does not alter α-globin function, selection for Hb J Tongariki should follow that for α-thalassemia 2. However, four other red blood cell abnormalities (α-thalassemia, β-thalassemia, G6PD deficiency, and hereditary ovalocytosis) are widespread in the lowlands of PNG (Chapters 3 and 4; Kidson 1961 a,b; Giles et al 1967; Amato and Booth 1977; Chockkalingam and Board 1980; Chockkalingam 1981: Chockkalingam et al 1982).

G6PD deficiency has been tested and studied extensively. A survey of the published reports and unpublished data available in this department shows that G6PD deficiency has been tested in 5669 males from various parts of PNG (Chockkalingam 1981). Of these 379 (6.7%) were found to be deficient. The frequency ranged from 0% in many highland groups to 53.6% reported by Yoshida et al

(1973) at Butibum in the Markham Valley. More than 5300 people have been screened for ovalocytosis (Amato and Booth 1977; Booth et al 1977; Serjeantson et al 1977; Holt et al 1981). Its incidence in coastal populations of PNG appears to be between 5 and 22.4%. Similar to thalassemias and G6PD deficiency, ovalocytosis is infrequent in the highland populations.

Since these four defects (α-thalassemia, β-thalassemia, G6PD deficiency, and ovalocytosis) result from abnormalities of independent genes, individuals in a population where all are segregating may carry more than one defect. The frequency of each defect favoured by the same or similar selective pressure would be determined by the selective advantage to the population of maintaining more than one favourable defect over the disadvantages of any deleterious effects of the genes singly or in combination with one another. Probable negative relationships between α-thalassemia 2 and α-thalassemia 1 and between α-thalassemia 2 and β-thalassemia in the populations of PNG have already been discussed (Chapter 3: section 3.4 and Chapter 4: section 4.4). α-Thalassemia 2, which is prevalent throughout the lowland areas, seems to have a greater fitness than α-thalassemia 1 and β-thalassemia.

It seems likely that thalassemia (α-thalassemia 2 or β-thalassemia), G6PD deficiency, and ovalocytosis can interact harmlessly with one another or altogether. Both thalassemia and G6PD deficiency are believed to cause intraerythrocytic oxidative stress (Carrell et al 1975) and, especially, G6PD-deficient erythrocytes are known to be susceptible to drug induced

hemolysis. However, it has been found that association of G6PD deficiency and β-thalassemia trait confers a greater resistance to oxidative hemolysis (Siniscalco et al 1961: Carcassi 1974). This may be due to increased activities of two antioxidant enzymes, catalase and glutathione peroxidase, in erythrocytes from G6PD-deficient subjects with β-thalassemia trait (Gerli et al 1982). These subjects have, thus, almost no history of hemolytic crisis. Although an interaction of G6PD deficiency and α-thalassemia has not been examined, a similar effect is presumably present. As α-thalassemia 2 is prevalent in PNG, and also β-thalassemia in some regions, their frequent occurrence with G6PD deficiency would be expected. This may explain why drug induced hemolysis in G6PD-deficient individuals is uncommon in PNG. Amato (1977) noted that "The (G6PD) variants found thus far are associated with severe deficiency of the enzyme; yet from the writer's experience, G6PD deficiency does not appear to be a clinically important or frequent cause of haemolysis. This is all the more surprising in view of the fact that many of the drugs known to cause hemolysis in persons with these variants (e.g. sulphonamides, chloramphenicol, sulphones, aspirin, isoniazid, quinine) are in common use in the hospitals and health centers of PNG."

If the interaction between thalassemia and G6PD deficiency reduces intraerythrocytic oxidative stress and thus resistance to oxidative hemolysis, will it also reduce protection against malarial infection? Although the intraerythrocytic oxidative stress has been proposed as a mechanism for protection against malarial infection (Friedman 1979) and this has been strongly

supported by in vitro culture of the falciparum parasite in G6PD-deficient red cells (Roth et al 1983a, 1983b, and 1986a; Luzzatto et al 1983; Janney et al 1986), it is still uncertain whether oxidative stress is the major or the only protective mechanism (Roth et al 1984), because both α- and β-thalassemia trait red cells, which are believed to accommodate a greater oxidative stress than normal red cells due to excess of free hemoglobin subunits, do not inhibit growth of the falciparum parasite in vitro (Roth et al 1983a; Ifediba et al 1985).

It is thus possible that the major protective mechanisms in thalassemia and G6PD deficiency are different. Thalassemia may require an extraerythrocytic mechanism while G6PD may protect by intraerythrocytic oxidative stress as well as other factors (Roth et al 1984; 1986b). Whatever mechanisms the two defects possess, when both coexist they appear to have additive rather than antagonistic actions. At least it has been found that coexistence of β-thalassemia does not prohibit the protective effect of G6PD-deficient red cells against the growth of falciparum parasites in vitro (Roth et al 1983a).

Less information is available about interactions of thalassemia or G6PD deficiency with hereditary ovalocytosis. Hereditary ovalocytosis by itself is not associated with clinical symptoms or anemia (Lie-Injo et al 1972; Amato and Booth 1977). The oval-shaped red cells result probably from membrane skeletal changes, rendering them resistant to infection by malarial parasites (Kidson et al 1981; Mohandas et al 1984). In the present study, many individuals were known to carry both α-

thalassemia 2 (either heterozygous or homozygous) and ovalocytosis without detrimental effects. In all likelihood, the interaction between G6PD deficiency and ovalocytosis may not cause any clinical symptoms.

Thus, when their geographic distribution and all the possible advantages and disadvantages of these interactions in relation to malaria are considered, it seems likely that thalassemia (more importantly α-thalassemia 2), G6PD deficiency, and hereditary ovalocytosis are cooperative and have supplementary actions in protection against malaria.

6.5 Migrations in PNG and Pacific regions: evidence from DNA analysis

DNA analysis can contribute to the understanding of migrations and contacts among population groups, and the present investigation also provides support for the westward migration of Austronesians to the PNG mainland (section 6.3).

From the study of α-globin gene deletion in populations in the Pacific, Hill *et al* (1985) found that the $-\alpha^{3.7}$III deletion is confined to Melanesia and Polynesia, and this form of the deletion seems to derive from a single origin. They suggested that the $-\alpha^{3.7}$III deletion is a useful population marker, and argued that the presence of this unique mutation in island Melanesians and Polynesians indicates their genetic affinity and supports a migration route through Melanesia for the colonizers of Polynesia.

The present work has also investigated the $-\alpha^{3.7}$III deletion in PNG and found that it is present on the Island of New Britain

but extremely rare on mainland PNG where the other forms of deletion, the $-\alpha^{4.2}$ and $-\alpha^{3.7}$I, are much more common. Therefore, the $-\alpha^{3.7}$III deletion is unlikely to have originated in the mainland PNG but more likely to have been introduced from island Melanesia or Polynesia. Indeed, the $-\alpha^{3.7}$III deletion is very common in island Melanesia (Hill et al 1985; Flint et al 1986) and almost the only subtype in Polynesia (Hill et al 1985; Trent et al 1986b). The $-\alpha^{3.7}$III deletion may, thus, have arisen in island Melanesia or Polynesia and spread westward to island and mainland PNG, consistent with the east-to-west migration of Austronesians as suggested by linguistic studies (section 6.3; Wurm 1983).

Recently, one group have analysed haplotypes associated with the $-\alpha^{3.7}$III deletion compared with normals and have suggested that this defect may have originated in northern Vanuatu (Flint et al 1986). However, the $-\alpha^{3.7}$III deletion appears to have occurred on the haplotype common in both Vanuatu and PNG regions.

If all the data are interpreted in combination with the evidence from other disciplines, it is possible that the $-\alpha^{3.7}$III deletion, which may have arisen in eastern island Melanesia, spread eastward to Polynesia and westward to island and mainland PNG, with both the Austronesian migrations. If this is the case, the $-\alpha^{3.7}$III deletion is most likely to have occurred before Polynesian inhabitation, at least 3,500 years ago. It is also tempting to speculate that Hb J Tongariki, a mutation superimposed on the $-\alpha^{3.7}$III deletion (Higgs et al 1984), may have originated in the Vanuatu region of island Melanesia where the $-\alpha^{3.7}$III

deletion is now very common and this hemoglobin variant can be found (Gajdusek et al 1967). Since Hb J Tongariki is absent in Polynesians (Kirk 1980) although the $-\alpha^{3.7}$ III deletion is present, Hb J Tongariki should have arisen after Austronesians had migrated to Polynesia, less than 3,000 years ago. Hb J Tongariki is now present in New Britain (Abramson et al 1970), on Karkar Island (Beaven et al 1972) and in Madang (Beaven et al 1972) where, in the last two areas, the $-\alpha^{4.2}$ deletion is more common. Therefore, Hb J Tongariki has probably also spread westward from island Melanesia to the islands and mainland of PNG.

6.6 Future research

Although the results of the present work and those reported by others (Oppenheimer et al 1984; Hill et al 1985: Flint et al 1986) have answered several questions concerning hemoglobinopathies in the PNG region, many aspects still need further investigation, some of which have been outlined in Chapters 3, 4, and 5 and require no additional discussion. These include further analysis of the new α-globin gene triplication by DNA cloning and nucleotide sequencing, the study of the molecular defect of α-thalassemia 1 (--/) in the rare families with Hb H disease, and cloning and sequencing of β-thalassemia genes.

There are some population groups which should be examined further. For example, more samples from the Sepik area should be studied, since the -α/ deletion seems to reach the highest frequency, close to fixation, in this region. In addition, the

Fly River region in the Western Province is hyperendemic for malaria and individuals from this region have not yet been studied. Some populations of island PNG (such as New Ireland and Bougainville) have also not been investigated.

It would be interesting to extend the study to other populations outside PNG, about which there is very little or no information. Among these are the people of Irian Jaya who occupy the eastern part of New Guinea, populations on the Southeast Asian islands of Indonesia and the Philippines, and groups in Micronesia to the north and northeast of PNG. Importantly, the groups in Irian Jaya and on the Lesser Sunda Islands of Indonesia have also been exposed to holoendemic malaria, similar to those in PNG. The study of these populations would provide important information about their origin and migration and about the role of hemoglobinopathies in protection from malaria.

Although the presence of α-thalassemia 2 in Australian Aborigines has been established, its extent and frequency in various Aboriginal groups still need to be examined, and further analysis of the α-globin gene cluster in Aborigines in comparison with the results for other groups to the north of Australia will help to clarify the origin of this defect and to understand the relationships of these population groups.

The role of malarial selection for α-thalassemia 2 could also be examined by comparison of the frequencies in various age groups in a highly endemic area or by a longitudinal study following the changes in frequency of a specific group of individuals, begining at the youngest age when the immunity to malaria has not been

developed. These approaches would elucidate the relative morbidity and mortality of the different genotypes (αα/αα, -α/αα, and -α/-α).

The prevalence of several red blood cell genetic defects in PNG provides an opportunity to study various aspects of genetic and physiological interactions between these defects. Hematological and biochemical studies in individuals who carry different genes in combination would increase understanding of the pathophysiological changes and clinical manifestations of these conditions. This could clarify the mechanisms of protection against malaria and assist in development of methods to prevent or treat malaria in genetically more susceptible individuals.

6.7 Final conclusion

The single α-globin deletion (-α/), resulting in α-thalassemia 2, has been shown in this study to be common in PNG and also present in Australian Aborigines. α-Thalassemia 2 is distributed in PNG in correlation with the endemicity of malaria, since it has a range of frequencies from the highest in the intensely malarious lowlands to the lowest in the virtually nonmalarious highlands.

The prevalence of α-thalassemia 2, which has almost no phenotypic effect, in the populations of lowland PNG, seems to limit the spread of other similar but more severe defects such as α-thalassemia 1 (--/) and β-thalassemia. In addition, α-thalassemia 2 seems to have the potential to replace the normal haplotype (αα/) while the genes for the two latter conditions,

which are restricted by homozygous disadvantage, can, at the most, only attain the level of balanced polymorphisms with the normal alleles. This is because an allelic replacement can not occur by an allele that offers only heterozygous advantage. However, it can occur if the allele has an advantage in both the heterozygous and homozygous condition even where the advantage is very slight.

α-Thalassemia 2 appears to be able to coexist with different mild red blood cell genetic defects, G6PD deficiency and hereditary ovalocytosis, which are also widespread in the malarious lowlands of PNG. In some circumstances their interaction seems to have phenotypic advantage. As an example, α-thalassemia 2 may alleviate the deleterious effects of G6PD deficiency by reducing oxidative hemolysis. If as appears highly likely, malaria is indeed the selective factor for all the conditions mentioned above, these findings would suggest that one selective pressure can determine changes in gene frequency at many loci. These changes occur not only because of direct selection for the alleles with higher fitness and against those with lower fitness but also because of competitive and cooperative interactions of these alleles. Some of these changes, thus, occur in concert, the frequency of one allele being dependent on the frequency of different alleles at the other loci.

The additional information provided by the DNA mapping technique has permitted many conclusions about population relationships and selective factors in the PNG region to be substantiated. The application of this technique to other loci and other populations should reveal further interesting interactions in the very near future.

APPENDIX

DNA POLYMORPHISMS OF β^N- AND β^E- GLOBIN GENES IN THAIS

Appendix: DNA polymorphisms of β^N- and β^E-globin genes in Thais

A.1 Introduction

Hb E ($\alpha_2 \beta_2^{26glu \rightarrow lys}$) is the most common hemoglobin variant in Southeast Asia (Flatz 1967; Wasi 1983). The β^E-globin chain is coded by the β^E-globin gene, which is associated with reduced levels of β-globin mRNA, and behaves like a mild β-thalassemia gene (Fairbanks et al 1979 and 1980; Traeger et al 1980; Benz et al 1981). The β^E mutation affects β-globin gene expression by activating an alternative mRNA splice site in exon-1 of the β-globin gene (Orkin et al 1982b). Hb E heterozygotes are asymtomatic and Hb E homozygotes have no obvious clinical manifestations. However, co-inheritance of Hb E and β^+- or β^0-thalassemia genes usually results in a severe β-thalassemia/Hb E disease, which in Thailand and other Southeast Asian countries is more common than homozygous β-thalassemia (Wasi et al 1980 and 1983).

By analysis of restriction fragment length polymorphisms and by direct study of cloned β^E-globin genes, Antonarakis et al (1982b) have suggested that multiple origins of the β^E-globin gene have contributed to its high frequency in Southeast Asia. In this appendix, a study of DNA polymorphisms and haplotypes of normal (β^N-) and β^E-globin genes in Thais is reported. This investigation was made to assess the feasibility of using DNA methodologies to look at the population genetics of β-globin genes. In addition, the information obtained may be useful for prenatal diagnosis of β-thalassemia/Hb E disease in this Thai population.

A.2 Samples and methods

Blood samples of 59 normal Thai individuals (AA) and 50 HbE homozygotes (EE) were provided by Dr Suthat Fucharoen and Professor Prawase Wasi of the Division of Hematology, Department of Medicine, Siriraj Hospital, Bangkok. Clinical examinations, hematologic studies, Hb typing, and quantitation of Hbs A_2, F, and E were performed at the Division of Hematology. The buffy coats taken from 10-20 ml of heparinized blood were sent on dry-ice to Canberra for DNA analysis.

The DNA from all AA and EE individuals was initially screened by using the restriction enzyme Bam HI to detect the polymorphic site at the 3' end of the β-globin gene. Following this, the DNA from 38 AA and 22 EE individuals was analysed by digestion with 2 further restriction enzymes: Hgi AI and Ava II, to detect the polymorphic sites in exon-1 and in IVS-2 of the β-globin gene, respectively. The rest of the samples were not analysed further because they were contaminated with plasmid. Haplotypes resulting from combinations of the presence and absence of the 3 polymorphic sites were designated as in Chapter 4.

A.3 Results

Table A.1 shows the frequencies of the 9.3 kb and 22 kb DNA fragments in DNA from 59 AA and 50 EE individuals digested with the restriction enzyme Bam HI and detected with the β-globin gene probe. About 64% of β^N chromosomes carried the 9.3 kb fragment. In contrast, 80% of β^E chromosomes carried the 22 kb fragment.

Table A.2 shows genotype frequencies in AA and EE individuals obtained by assessing the three polymorphic sites. In the AA group, 27 individuals had unequivocally defined genotypes (homozygous for two or three fragments), 7 individuals could be either one of two possible genotypes (homozygous for one fragment), and 4 individuals were unassignable (triple heterozygotes). Genotypes c/c and a/b were the most common in the AA group. It is likely that all haplotypes (a to h) were present in the AA group but some haplotypes such as g and h must be very rare. The most common haplotypes in the AA group among those assigned unequivocally were haplotypes c (37%), a (24%) and b (20%) (Table A.3). Sixteen EE individuals were b/b homozygotes. The remaining 6 EE individuals were multiple heterozygotes with possible genotypes a/h, b/c, d/g and e/f. In view of the large number of β^E chromosomes carrying haplotype b, the genotype of these individuals is most likely to be b/c. Thus haplotype b accounted for minimum of 73% of β^E chromosomes and probably as many as 86%.

Table A.1 Frequencies of the 9.3 kb and 22 kb Bam HI fragments found in β^N- and β^E-globin genes.

Subject groups	No. of chromosomes	Bam HI fragments	
		9.3 kb	22 kb
AA	118	76 (64.4%)	42 (35.6%)
EE	100	20 (20.0%)	80 (80.0%)

Table A.2 Genotype frequencies obtained by assessing the polymorphic sites for enzymes Hgi AI, Ava II and Bam HI in 38 AA and 22 EE individuals.

Genotypes	n
(a) AA	
c/c	9
a/b	8
a/a	2
d/e	2
c/d	1
b/b	1
b/e	1
a/f	1
c/f	1
f/f	1
a/c* or d/f	3
c/e or d/h	1
b/h or g/e	1
a/e or b/d	1
a/g or b/f	1
Unassignable	4
(b) EE	
b/b	16
b/c**	6

* Underlined genotypes are the more likely ones.

** Most likely genotype. See explanation in text.

Table A.3 Haplotype frequencies (unequivocally assigned) of 54 β^N chromosomes.

Haplotypes	n
a	13 (24%)
b	11 (20%)
c	20 (37%)
d	3
e	3
f	4
g	0
h	0
Unassignable	22

A.4 Discussion

At least 17 restriction site polymorphisms distributed over more than 60 kb of DNA within the human β-globin gene cluster have been described (Collins and Weissman 1984). Analysis of these polymorphic sites has demonstrated that the sites in two regions within the β-globin cluster are non-randomly associated (Antonarakis et al 1985). These two regions are a 34 kb portion including ε-, $^{G}\gamma$-, $^{A}\gamma$-, and ψβ-globin genes (5' to the β-globin gene) and an 18 kb region including the β-globin gene and extending beyond it in the 3' direction. Between these two regions, there is a 9 kb DNA segment containing the δ-globin gene and an apparent "hot spot" for meiotic recombination (Chakravarti et al 1984). Because of this region of increased recombination, the polymorphic restriction sites within and 3' to the β-globin gene are more informative for the analysis of chromosomes bearing normal and abnormal β-globin genes.

Three restriction endonucleases known to have polymorphic sites in the β-globin gene and its 3' flanking region have been used to analyse the β^{N}- and β^{E}-globin genes. Initially, β^{N} and β^{E} chromosomes of Thais were shown to have different frequencies of the polymorphic Bam HI site within this region. This site was present in about 64% of the β^{N} chromosomes but only in 20% of the β^{E} chromosomes. This finding would be beneficial for the identification of β^{E} chromosomes in families with a risk of β-thalassemia/Hb E disease.

It has been suggested that normal β-globin genes in various populations exist in four frameworks which are correlated with

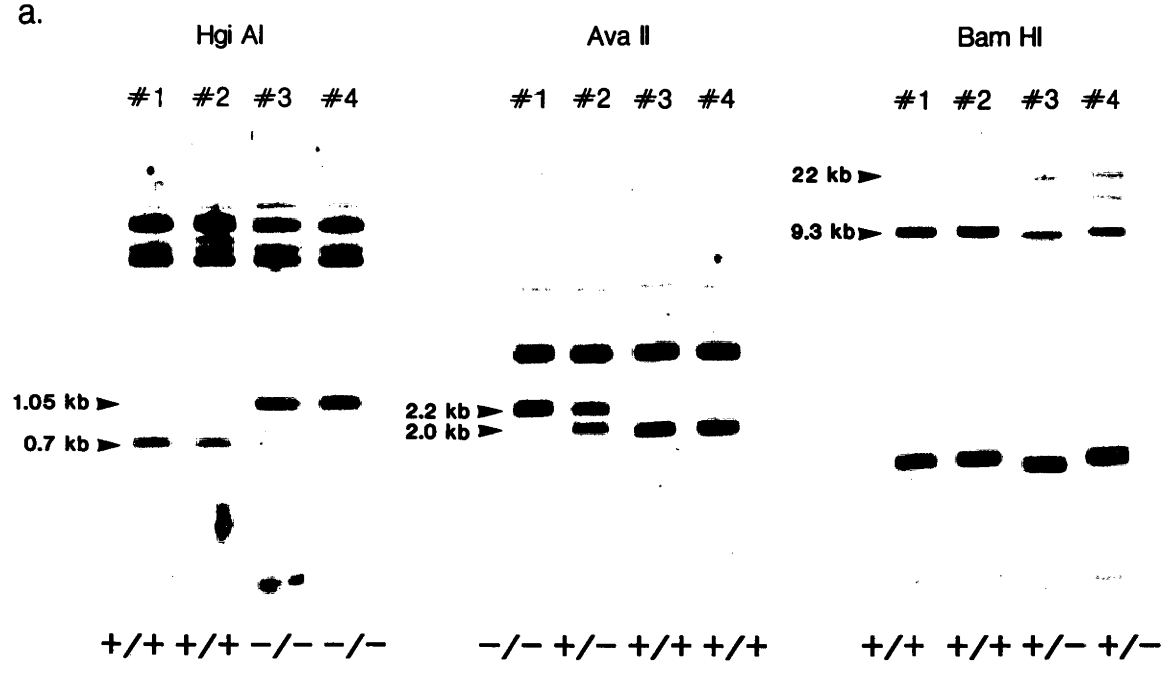
three patterns at the three polymorphic restriction sites of Hgi AI, Ava II and Bam HI (Antonarakis et al 1982b; Orkin et al 1982a; Kazazien et al 1984c). However, the haplotype analysis for normal Thais revealed the possibility of the existence in β^N chromosomes of all the patterns resulting from combinations of the presence and absence of these three polymorphic sites. The common ones were haplotypes c, a, and b, representing 37%, 24% and 20%, respectively, of the β^N chromosomes with unequivocally assigned haplotypes. This analysis also showed the presence of haplotypes d, e, and f. Haplotypes g and h may be present in several genotypes but could not be assigned unequivocally and must be very rare. Examples of the additional patterns are illustrated in Figure A.1. These new patterns of polymorphic sites or haplotypes which are not uncommon in the normal Thais may represent new β-globin gene frameworks which have never been previously described.

In contrast to the β^N chromosomes, the β^E chromsomes were found to be associated with as few as two haplotypes, b and c, with a much greater prevalence of haplotype b (up to 86%). The presence of the β^E-globin gene in two chromosome haplotypes confirmed the work reported by Antonarakis et al (1982b) who studied 20 β^E-globin genes from Cambodians and Laotians and 3 from Thais and suggested the existence of at least two independent origins of the β^E mutation in Southeast Asia. This group also analysed 47 β^N chromosomes from Cambodians and found haplotypes a, b, and c (or frameworks 1,2 and 3) accounting for 18%, 35% and 47% of the β^N chromosomes respectively. It is interesting,

Figure A.1 a. Autoradiographs showing DNA polymorphisms of the β-globin gene in 4 normal (AA) individuals. DNA was digested with Hgi AI, Ava II, and Bam HI and hybridized with the ^{32}P-labeled β-globin probe.

b. Genotypes of these 4 individuals with the newly described patterns of polymorphic sites (or haplotypes).

a.



b.

Genotypes				
#1	:	+−+/+−+	=	f/f
#2	:	+++/+−+	=	a/f
#3	:	−++/−+−	=	d/e
#4	:	−++/−+−	=	d/e

therefore, that haplotypes b and c (the haplotypes associated with β^E chromosomes both in Cambodians and Thais) were apparently higher in frequencies in β^N chromosomes in Cambodians than in Thais. From analysis of the correlation between distribution of Hb E and linguistic groups in Southeast Asia, it has been proposed that Hb E originated in Austroasiatic people, specifically the Mon-Khmer (Flatz 1967; Wasi 1983). The Thais have probably acquired Hb E from the Austroasiatic people who live in close proximity (Wasi 1983). This hypothesis is strengthened by the results of DNA analysis which demonstrated that the β^E mutations occurred on chromosome haplotypes which are common in Khmers or Cambodians (Antonarakis et al 1982b) and that the Thais carry the same β^E chromosome haplotypes as Khmers although the prevalent haplotypes associated with β^E chromosomes are probably less common in β^N chromosomes in Thais (as shown in this study).

Clearly, analysis of β-globin gene haplotypes can provide useful information about the origin of mutations and the relationships of populations. This approach was therefore used to examine the β-thalassemia genes in the PNG populations under consideration in this thesis.

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