THE MOLECULAR BASIS OF ALPHA THALASSAEMIA IN A SOUTH AFRICAN POPULATION

by

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ABSTRACT

The molecular basis of alpha thalassaemia in the so-called 'Cape Coloured' population of the Western Cape was investigated.

Restriction endonuclease digestion, Southern blotting and hybridisation with alpha and zeta globin-specific probes were used to investigate the incidence of the the various alpha thalassaemia determinants and their disorders.

Results indicate that one determinant in this population results from the deletion of a single alpha globin gene on the short arm of chromosome 16. In individuals homozygous or heterozygous for this deletion, digestion with restriction endonuclease Bam H1 shows the presence of a shorter 10,5kb alpha globin-specific fragment as opposed to the 14kb fragment found in normal individuals.

Individuals with both alpha globin genes deleted on the same chromosome i.e. the genotype --/aa, were detected and their alpha thalassaemia determinant characterised by:

1. a family study
2. quantification of the alpha/gamma globin gene ratio, and
3. mapping with the zeta globin probe since the deletion extends into the zeta locus.

The --/ alpha thalassaemia determinant was found to be of Southeast-Asian origin.

A non-deletion form of alpha thalassaemia was also detected in which the alpha globin restriction map appeared to be normal. This condition may have resulted from a point mutation within the alpha globin gene region which affects transcription or RNA processing.

The DNA of infants born with detectable levels of Hb Bart's in their cord blood was investigated in order to estimate the frequency of the single and double gene deletions in this population. The results indicate that infants with Hb Bart's in the 4 - 8% range predominantly have the genotype -a/-a. Using the data obtained the incidence of the heterozygote was calculated according to the Hardy-Weinberg equation. The calculated incidence of the heterozygote (-a/aa) was found to be 16,9%.

ABBREVIATIONS

a	alpha
a^+	$alpha^+$
a^o	$alpha^o$
-a/	minus alpha haplotype
--/	minus minus haplotype
aa/	alpha alpha haplotype
a/b	alpha/beta
Ci	curie ($2,2 \times 10^{12}$ disintergrations / minute)
cm	centimetre
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytidine triphosphate
dGTP	deoxy-guanosine triphosphate
dTTP	deoxy-thymidine triphosphate
fl	femtolitre
g	acceleration due to gravity
hb	haemoglobin
hbH	haemoglobin H
kb	kilobases
MCH	mean cell haemoglobin
MCV	mean cell volume
MED	Mediterranean
mg	milligram
ml	millilitre
pg	picogram
RBC	red blood cells
SEA	Southeast-Asian
tris	tris (hydroxymethyl) amino methane
ug	microgram

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1 INTRODUCTION

The thalassaemias are one of the most common group of monogenic disorders occurring in various populations throughout the world. They are characterised by reduced or absent synthesis of alpha- or beta-like globin chains essential for the formation of normal haemoglobin tetramers (e.g. Hb A: $\alpha_2\beta_2$ and Hb A2: $\alpha_2\delta_2$). The accompanying reduction in haemoglobin synthesis and the imbalance of alpha and beta globin chains result in the characteristic haematological abnormalities of thalassaemia (Weatherall and Clegg, 1981).

Briefly, the disorders are classified into alpha, beta, delta, or delta-beta thalassaemias depending upon which globin chain has been affected. Each of these disorders is heterogeneous and can be further subdivided according to whether, for example, alpha globin chain synthesis is reduced (alpha$^+$ thalassaemia), or absent (alpha^0 thalassaemia) (Weatherall and Clegg, 1982).

This review is confined to the alpha thalassaemias. Beta thalassaemia has recently been reviewed (Weatherall and Clegg , 1981).

1.1 THE ALPHA GLOBIN GENES

In man, the alpha globin genes (alpha 1 and alpha 2), a pseudo-alpha gene and their embryonic zeta globin gene counterparts lie in a cluster on the short arm of chromosome 16 (Kan **et al**, 1975) in the order 5'-zeta 2, pseudo-zeta 1, pseudo-alpha 1, alpha 2, alpha 1 -3' (Lauer **et al**, 1980; Jackson and Williamson, 1980).
Two hypervariable regions (regions in which the distance, in kilobases, between two restriction enzyme recognition sequences varies from one individual to another) exist in this part of the genome. The one hypervariable region lies 3' to the two alpha globin genes and the other lies between the two zeta genes (Higgs, 1981; Orkin, 1978).

The zeta and alpha globin genes are activated at different stages during fetal development. The transcribed mRNA is translated into alpha globin chains, two of which combine with two beta globin chains and an iron-containing pyrrole ring to form a tetrameric haemoglobin molecule. The earliest detectable tetramer is zeta 2-epsilon 2, known as haemoglobin Gower 1. Two other embryonic haemoglobins, alpha 2-epsilon 2 or haemoglobin Gower 2 and zeta 2-gamma 2 or haemoglobin Portland are present in smaller amounts. By eight weeks of gestation the alpha 2-gamma 2 tetramer, haemoglobin F predominates. Prior to birth the haemoglobin F is gradually replaced by haemoglobin A followed shortly afterwards by the appearance of haemoglobin A2. At about six months after birth 97-98% of the haemoglobin in normal infants is haemoglobin A and approximately 2% consists of haemoglobin A2 (Figure 1.1). Some cells do,

however, continue to produce a small amount of haemoglobin F (Old **et al**, 1982).

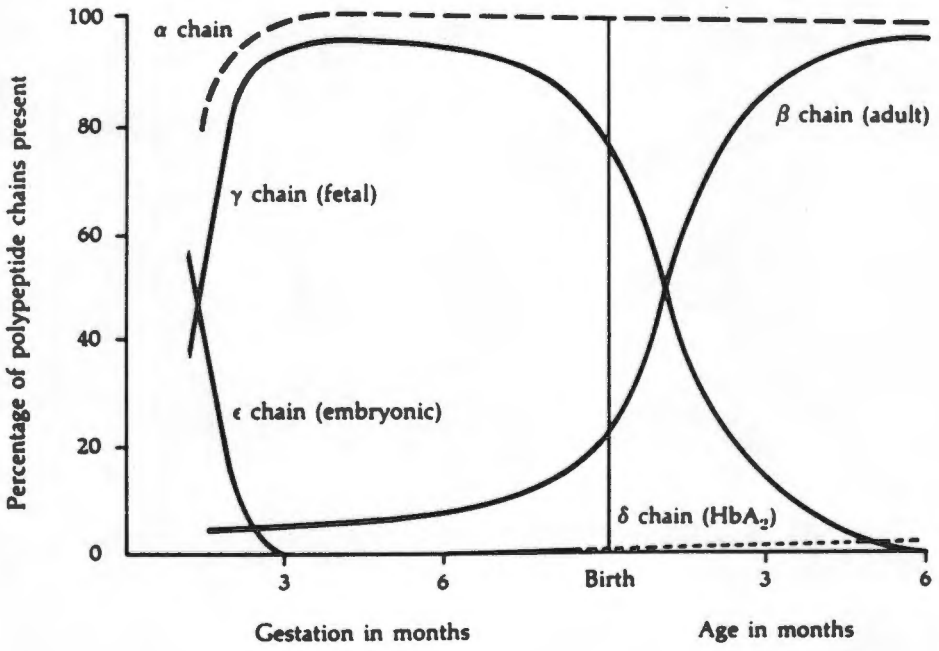

Figure 1.1 The synthesis of haemoglobin chains during development. Haemoglobin F is produced mainly during fetal life. Haemoglobin chains epsilon and zeta coincide and are seen only during the first three months of gestation, after which they are replaced by the gamma and alpha globin chains respectively. The gamma chain is later replaced by the beta and delta chains. (Modified from Weatherall and Clegg, 1981).

The alpha thalassaemias result predominantly from deletions at the alpha globin locus which remove one or both of the alpha globin genes (Kan **et al**, 1975). Non-deletion forms of alpha thalassaemia have also been detected and arise as a result of non-functional alpha globin genes (Kan **et al**, 1977).

1.2 THE CLASSIFICATION OF ALPHA THALASSAEMIA

Early work on alpha thalassaemia in Oriental races suggested that two main genetic determinants of alpha thalassaemia existed - a severe form which was termed alpha thalassaemia 1 and a milder form, alpha thalassaemia 2 . The alpha thalassaemia 1 determinant has been identified as a chromosome from which both alpha globin genes have been deleted, whereas the alpha thalassaemia 2 determinant is a chromosome containing one functional alpha globin gene (Embury **et al**, 1980).

A simpler nomenclature has been proposed by Weatherall and Clegg (Weatherall and Clegg, 1982) and shall be used in this study.

a^+/: alpha thalassaemia determinant associated with decreased alpha globin synthesis.

a^o/: alpha thalassaemia determinant associated with absent alpha globin synthesis.

The interaction of these two determinants may give rise to the following range of disorders:

a^+ heterozygote:

(a^+/a) -three out of the four alpha globin genes are functional.
-this condition is often undetected and is also known as the 'silent carrier'.

a^+ homozygote, a^o trait:

(-a/-a) -two out of the four alpha globin genes
(--/aa) are functional.
-mild hypochromic microcytic anaemia.

a^+/a^o compound heterozygote:

(-a/--)

-only one out of the four alpha globin genes is active.

-hypochromic microcytic anaemia is mild to severe.

-detectable levels of haemoglobin H formed as a result of the aggregation of the excess of beta chains into beta tetramers. In haemoglobin H disease these beta tetramers accumulate in the red blood cell and contribute to shortened red cell survival (Bank **et al**, 1980).

a^o homozygote:

(--/--)

-none of the alpha globin genes are functional.

-causes haemoglobin Bart's hydrops fetalis syndrome.

-high levels of haemoglobin Bart's are detected as a result of the excess gamma tetramers accumulating. (Trace amounts are found in most normal cord bloods possibly as a result of a slight degree of globin chain imbalance during the perinatal period when haemoglobin A replaces haemoglobin F).

1.3 THE MOLECULAR BASIS OF ALPHA THALASSAEMIA

The decreased amount of alpha globin chains in alpha thalassaemia may be caused by, either decreased synthesis of alpha globin chains, or increased degradation of alpha globin chains. By measuring the incorporation of labelled amino acids into the alpha and beta globin chains and calculating the alpha : beta chain synthesis ratio, Weatherall and Clegg (Weatherall and Clegg, 1969) demonstrated that alpha thalassaemia arose as a result of decreased synthesis of alpha globin chains. Recent developments in molecular biology have clarified the molecular basis of the decreased synthesis of alpha globin chains.

In experiments conducted with cell-free systems Benz (Benz **et al**, 1973) demonstrated that there is a deficiency of functional mRNA in haemoglobin H (HbH) disease, because the mRNA isolated from reticulocytes of HbH patients directed the synthesis of a large excess of beta over alpha globin chains. Alpha thalassaemia was shown to result from decreased amounts of alpha globin mRNA relative to beta globin mRNA and thus the disease occurs as a result of decreased transcription or processing of the alpha globin gene (Weatherall **et al**, 1970).

cDNA/DNA liquid hybridization experiments demonstrated that the alpha globin genes are largely or completely deleted in alpha^{o} homozygotes (Hb Barts hydrops fetalis syndrome) (Taylor, 1974). This study was the first demonstration of a gene deletion as the cause of a human genetic disorder. The results of this study were subsequently confirmed (Ramirez **et al**, 1975; Kan **et al**,

1974).

Subsequently, restriction mapping, cloning and sequencing of the alpha globin genes have confirmed that alpha thlassaemia is a heterogeneous disorder caused by deletions which remove both alpha globin genes (alpha^{o} haplotype), or one alpha globin gene (alpha^{+} haplotype) or by non-deletional defects (alpha^{+} haplotype) (Orkin,1978).

1.3.1 ALPHA^{+} THALASSAEMIA

1.3.1.1 DELETIONS

Two different types of gene deletions may give rise to alpha^{+}/ haplotypes (Embury **et al**, 1980).

Firstly, a deletion of 4,2kb involving the 5' alpha globin gene region exists. This is referred to as the leftward deletion or $-a^{4,2}/$.

The second type of deletion giving rise to the alpha^{+}/ haplotype is slightly smaller, i.e. 3,7kb and also involves the 5' alpha globin gene region. As this deletion was shifted to the right of the above example, it became known as the rightward deletion or $-a^{3,7}/$. Both these deletions result from unequal crossing-over at the alpha globin locus during meiosis (Embury **et al**, 1980).

Both these deletions may be detected by restriction endonuclease mapping with restriction enzyme Bam H1 and probed for using a cDNA or genomic alpha globin probe. Normal DNA gives rise to a 14kb Bam H1 alpha globin-specific fragment. However, DNA from a chromosome with the $-a^{4,2}/$ or $-a^{3,7}/$ deletion yields a Bam H1 alpha globin-specific fragment of about 10kb.
(Figure 1.2)

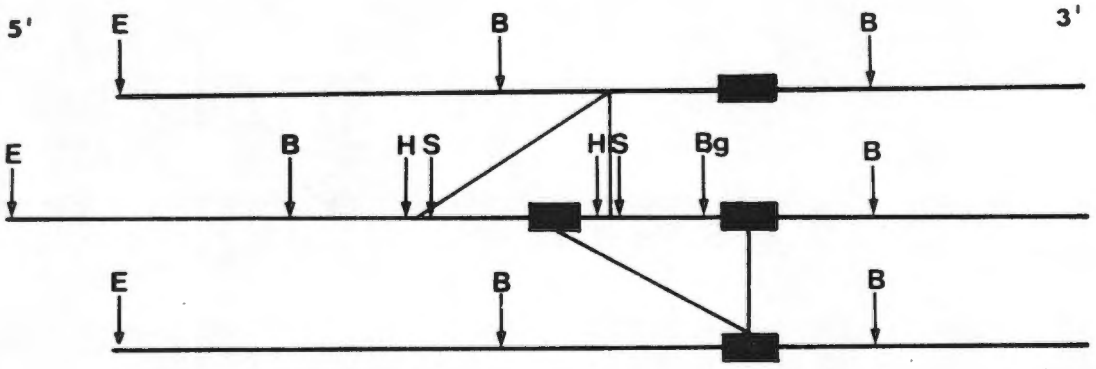

Figure 1.2 The uppermost line represents a chromosome with the Leftward deletion, the middle one represents a normal chromosome and the lower one the Rightward deletion. Restriction endonuclease sites are represented by B (Bam H1), Bg (Bgl 11), E (Eco R1), H (Hpa 1) and S (Sac 1) and the alpha globin genes are represented by the black boxes.

POSSIBLE MECHANISMS FOR HOW THESE DELETIONS AROSE

The deletions causing the heterozygote alpha^{+} thalassaemias with three out four alpha globin genes functional may have arisen from either inter- or intra-chromosomal cross-over events.

A. INTER-CHROMOSOMAL CROSS-OVER DELETIONS.

1. Leftward deletion ($-a^{4,2}/$).

The leftward deletion may arise from a cross-over event occurring between DNA sequences in the shaded area in the following diagram (Figure 1.3).

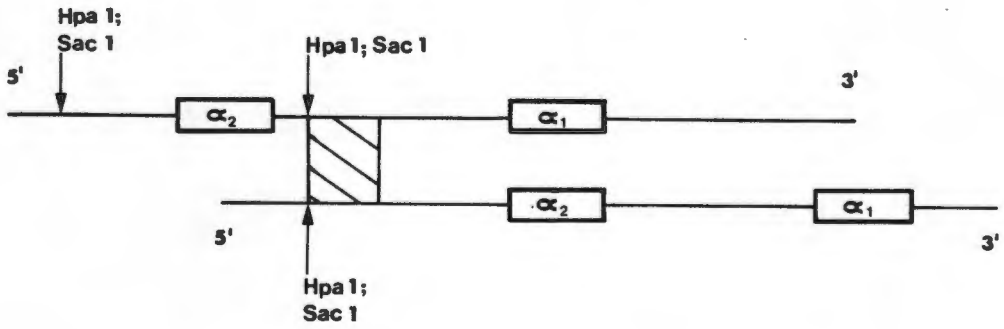

Figure 1.3 Leftward inter-chromosomal cross-over deletion.

The shaded area of the chromosomes contains homologous sequences so that it would be plausible for the two chromosomes 16 to misalign during recombination. This 0,4kb area in which a cross-over would occur is bounded by Hpa 1 and Sac 1 restriction endonuclease sites that

flank the 5' alpha globin locus. With the leftward deletion, i.e. the loss of the 5' alpha globin gene and proximal DNA, the leftmost Hpa 1 and Sac 1 sites would also be lost. This deletion therefore gives rise to a single Hpa 1 or Sac 1 alpha globin-specific restriction fragment. Normal DNA generates alpha globin-specific fragments of 14kb and 4,2kb when digested with Hpa 1 or Sac 1. DNA with the leftward deletion generates only the 14kb fragment.

2. Rightward deletion ($-a^{3,7}/$)

The rightward type of deletion may arise from an inter-chromosomal event occurring between DNA in the shaded area in the diagram below (Figure 1.4).

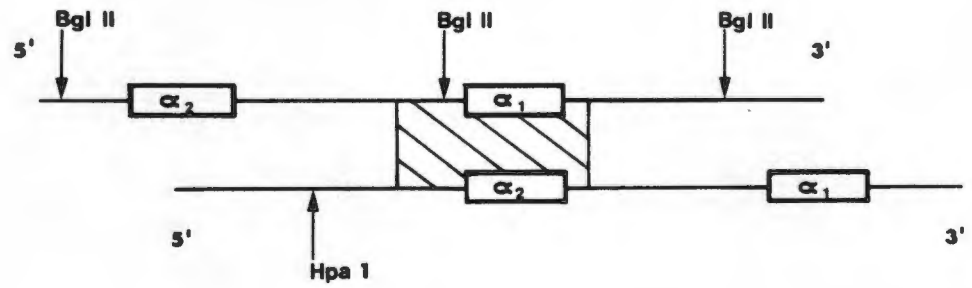

Figure 1.4 Rightward inter-chromosomal cross-over deletion.

This 2,1kb area is flanked by the intergenic Bgl 2 site (top chromosome in Figure 1.4) and by the intergenic Hpa 1 site (bottom chromosome in Figure 1.4). DNA with the rightward deletion generates an alpha globin-specific fragment of approximately 16kb when cut with Bgl 2 since the intergenic Bgl 2 site is deleted. DNA known to contain a normal 3' alpha globin (DNA with the leftward

deletion) generates a 7kb alpha globin-specific fragment because this Bgl 2 site is preserved in the leftward deletion. Restriction endonuclease mapping with Bgl 2 may therefore be utilized to discriminate between the leftward ($-a^{4,2}/$) and rightward ($-a^{3,7}/$) deletions.

B. INTRA-CHROMOSOMAL CROSS-OVER DELETIONS.

1.Leftward deletion.

The leftward deletion would arise from an intra-chromosomal cross-over event occurring between the homologous regions of DNA of a single chromosome as shown in Figure 1.5 below. The shaded area indicates the 0,4kb stretch of homologous DNA.

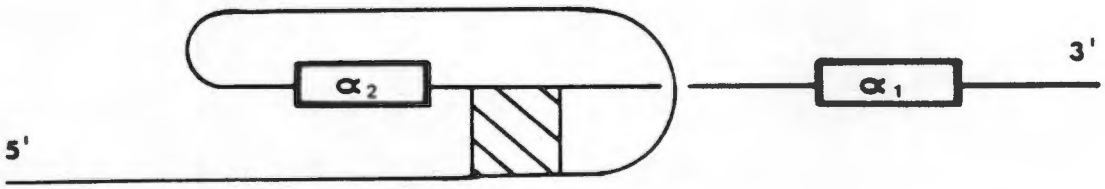

Figure 1.5 Leftward intra-chromosomal cross-over deletion.

2. Rightward deletion.

The rightward deletion may also arise from an intra-chromosomal cross-over event between the homologous DNA regions in the shaded area in Figure 1.6 below.

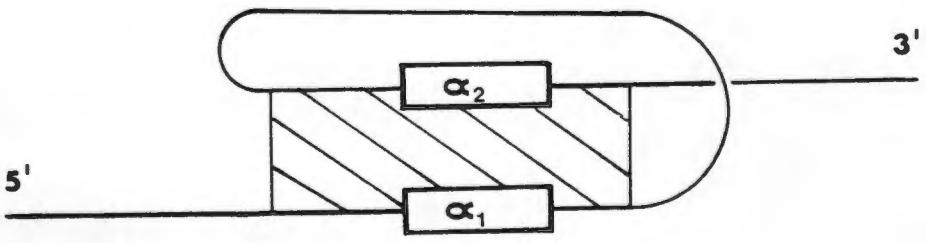

Figure 1.6 Rightward intra-chromosomal cross-over deletion.

One might predict the detection of a reciprocal cross-over product of an inter-chromosomal cross-over event causing the rightward deletion and resulting in a chromosome bearing three alpha globin loci (aaa). This has in fact been reported. Inter-chromosomal crossing-over would thus seem a likely mechanism for the rightward deletion (Goossens **et al**, 1980).

1.3.1.2 NON-DELETIONS

By definition, the non-deletion forms of alpha thalassaemia arise from chromosomes that appear to contain a normal complement of alpha globin sequences, but show decreased alpha globin mRNA production (Hunt **et al**, 1980).

In 1981 Orkin and co-workers described a pentanucleotide deletion within the 5' splice junction of the first intervening sequence in the alpha 2 globin gene of an individual with alpha$^+$ thalassaemia. Following the guanosine of the invariant G-T dinucleotide normally located within such junctions, a deletion of -T-G-A-G-G- was found. A marked deficiency of alpha 2 mRNA was detected in the patient's erythroid cells. They thus concluded that this pentanucleotide deletion within the splice junction was the primary genetic defect in this alpha$^+$ thalassaemic individual. Loss of a functional splice junction has resulted in failure to form stable alpha globin mRNA (Orkin **et al**, 1981).

Another non-deletion form of alpha$^+$ thalassaemia was demonstrated by Goossens (Goossens **et al**, 1982).
A DNA segment from the chromosome containing the alpha 1 and alpha 2 globin loci from a patient with haemoglobin H disease was cloned and sequenced. The alpha 1 globin sequence was found to be identical to that of normal alpha 1 globin DNA sequenced. However, in the alpha 2 globin gene, a difference in the sequence corresponding to amino acid 125 was detected. At this position the normal codon for leucine (-C-T-G-) had been changed to one coding for proline (-C-C-G-). This point mutation

was verified by restriction endonuclease analysis with Eco R2 as it resulted in the loss of an Eco R2 recognition site. The leucine→proline mutation disrupts the H helix (Kleihauer, 1968) of nascent alpha globin chains and thus interfers with alpha 1 : beta 1 dimer formation resulting in an alpha thalassaemia phenotype. The haplotype was thus termed $a^{125\ Leu \rightarrow Pro}a/$.

Recently a human alpha 2 globin gene containing a single point mutation in the poly-A recognition sequence 5' to the poly-A region of the DNA has been shown (Higgs **et al**, 1983). The universal hexanucleotide -A-A-T-A-A-A has been changed to -A-A-T-A-A-G- with the subsequent reduction in alpha globin mRNA synthesis.

1.3.2 ALPHA^0 THALASSAEMIA

Evidence for at least four different determinants of the alpha^0 thalassaemia type exists (Figure 1.7).

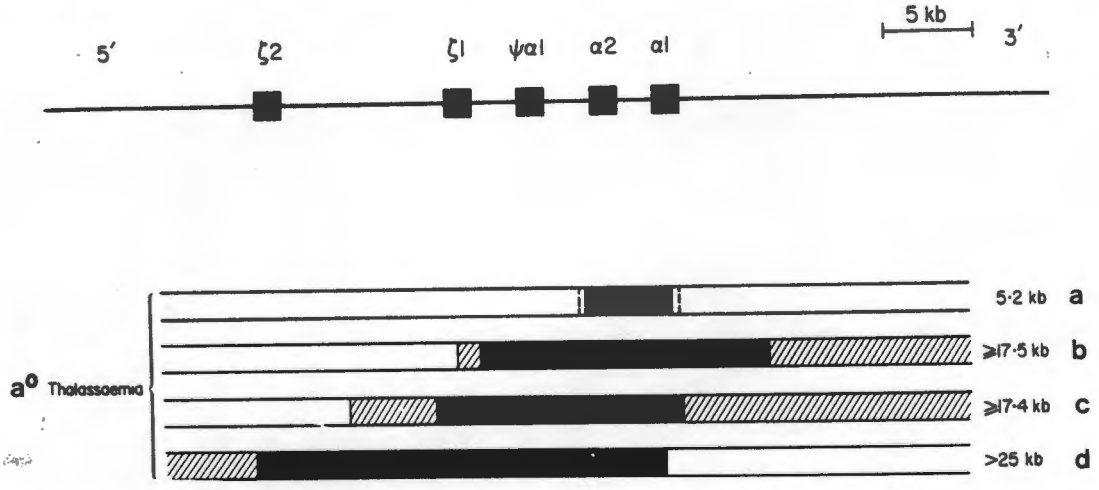

Figure 1.7 The alpha^0 thalassaemia deletions. The areas known to be deleted are shown in black; the shaded areas represent the extremes where the limits of the deletion are not defined (Modified from Weatherall and Clegg, 1981).

A dysfunctional alpha globin gene was reported by Orkin in 1979 (Orkin **et al**, 1979). In 1980 it was shown that this alpha globin gene-specific 2,6kb Eco R1 fragment comprises only the 3' region of the alpha 1 gene from codon 57 onwards (Orkin and Michelson, 1980) This deletion therefore extends for at least 25kb, so that the 5' end of the alpha 1 globin gene, the alpha 2, the pseudo-alpha 1 and the zeta genes are all missing. (Figure 1.7, lane d).

Another example of a dysfunctional alpha globin gene was reported by Pressley (Pressley et al, 1980c). A 5,2kb deletion was found to have removed the alpha 2 globin gene and the 5' region of the alpha 1 globin gene, leaving the 3' region of the alpha 1 gene intact. (Figure 1.7, lane a).

Advances made in determining the overall structure of the zeta-alpha gene cluster and the availibility of probes for the zeta genes have clarified the genetic fine structure of two further alpha^{o} thalassaemia determinants, namely those found in South-east Asia and in the Mediterranean countries. Pressley (Pressley et al, 1980b) demonstrated that these two common alpha^{o} thalassaemia determinants seen in South-east Asia and in the Mediterranean countries have a different molecular basis.

The South-east Asian type of deletion involves the loss of both alpha globin genes and the pseudo-alpha 1 gene and is written as --SEA/ (Figure 1.7, lane b).

The Mediterranean type of deletion involves the loss of both alpha globin genes, the pseudo-alpha 1 gene and the pseudo-zeta 1 gene: hence this deletion is much larger than the former. It is written as --MED/.

It is possible to discriminate between these two alpha^{o} thalassaemia genotypes by restriction endonuclease analysis with Bam H1 and mapping with the zeta globin probe. DNA with the South-east Asian type of deletion yields a 5,9kb and an approximately 20kb zeta globin-specific band, as opposed to bands of 5,9kb and 10,5kb found in normal DNA, since the Bam H1 restriction site between the pseudo-zeta 1 and the alpha globin genes is

removed.

DNA with the Mediterranean type of deletion yields only a 5,9kb band corresponding to the 5' fragment containing the zeta-2 gene in normal DNA. No other zeta-specific bands are seen, indicating that the pseudo-zeta 1 gene and a length of DNA to the right of this region, including the two alpha globin genes, has been deleted

These two deletions giving rise to the alpha^{o} thalassaemia haplotypes have probably arisen by a similar mechanism to that which gives rise to the alpha^{+} thalassaemia haplotypes (Embury **et al**, 1980).

1.4 THE POPULATION GENETICS OF ALPHA THALASSAEMIA

Alpha thalassaemia occurs at a significant frequency in India, South-east Asia, Saudi-Arabia, Africa and the Mediterranean populations (Embury et al, 1979; Lie-Injo et al, 1959; Pembrey, 1975; Nhonoli et al, 1979; Weatherall and Clegg 1981 and Whitelaw et al, 1980). Frequency studies were initially based upon detectable haemoglobin Bart's levels in neonatal cord blood since a decreased number of alpha globin chains results in an excess of gamma globin chains aggregating to form gamma tetramers or haemoglobin Bart's. Haemoglobin Bart's was only detectable in the cord bloods of neonates who subsequently developed alpha thalassaemia. The level of haemoglobin Bart's was related to the severity of the alpha thalassaemia so that mild alpha thalassaemia type 2 had haemoglobin Bart's levels of less than 3% ; alpha thalassaemia type 1 had haemoglobin Bart's levels of between 5 and 10% ; haemoglobin H disease showed haemoglobin Bart's levels of 10 to 25% and the haemoglobin Bart's hydrops fetalis syndrome had almost entirely haemoglobin Bart's.

Haemoglobin Bart's levels were correlated with the number of alpha globin genes deleted by routine quantitation of haemoglobin Bart's levels and restriction endonuclease analysis and, in general, the following pattern was observed:

Alpha thalassaemia genotype	Haemoglobin Bart's
-a/aa	less than 3%
-a/-a	5 to 10%
--/aa	5 to 10%
--/-a	10 to 25%

A considerable number of -a/aa genotypes went undetected as their levels of haemoglobin Bart's were not measurable (Higgs et al, 1982). Thus, alpha globin gene mapping of randomly selected cord bloods is a more reliable method for estimating the frequencies of the -a/aa, -a/-a and --/-a genotypes. However, the frequencies of the different types of alpha thalassaemia determinants vary from one population to another. Mediterranean and Black subjects were found to have mainly the $-a^{3,7}/$ alpha^{+} determinant or rightward deletion, whereas the Chinese subjects investigated demonstrated wide molecular diversity. The $-a^{3,7}/$, $-a^{4,2}/$ and non-deletion forms of alpha thalassaemia were found (Embury et al, 1980).

The two most common alpha^{o} thalassaemia determinants were the --SEA/ and the --MED/ as found in subjects from South-east Asia and the Mediterranean countries respectively. The --/aa genotype was found frequently in Saudi-Arabia, as were non-deletion forms of alpha thalassaemia.

In India and Africa the -a/ determinant predominates and it may be that it has been selected for in these populations. The -a/ determinant occurs frequently in these malarial endemic areas and it has been proposed that the diminished growth of **Plasmodium falciparum** in -a/ erythrocytes may be related to inadequate host cell defence against oxidative stress generated by parasitic growth (Embury et al, 1980). Evolutionary selection of the -a/ determinant would thus endow protection against malaria to the red cells. Similarly, the selective advantage conferred on the individual with the -a/ determinant could result in the high frequency of this

determinant observed in populations living in malarial endemic areas. Interestingly, the frequency of the -a/ determinant is much higher than the frequency of the aaa/ determinant whereas the mechanism for their formation would predict a similar incidence in the absence of differential selection pressure.

2.0 MATERIALS AND METHODS

2.1 Selection of patients.
2.2 Haematological and haemoglobin analysis.
2.3 DNA isolation from whole blood.
2.4 Transformation of lymphoblasts.
2.5 DNA isolation from cultured lymphoblasts.
2.6 Plasmid preparation and purification.
2.7 Restriction endonuclease digestion.
2.8 Agarose gel electrophoresis.
2.9 Southern blotting procedure.
2.10 Nick-translation of the plasmid or insert.
2.11 Hybridization of the ^{32}P-labelled probe to the nitrocellulose filters.
2.12 Autoradiography.
2.13 Densitometric scanning.

2.1 SELECTION OF PATIENTS

Patients with reduced alpha : beta chain synthesis ratios were initially selected for this study in order to analyse the molecular basis for their alpha thalassaemia. Subsequent selection was based on the presence of a microcytic anaemia with normal haemoglobin electrophoresis, haemoglobin A2 and serum iron.

One thousand two hundred and seven cord bloods had been previously screened for Hb Bart's (Provincial Laboratory for Tissue Immunology at the University of Cape Town Medical School, Unpublished results). Forty had been found positive for detectable levels of haemoglobin Bart's. Twenty of these were traced for the purpose of the present study in order to determine the molecular basis and incidence of their deficient alpha globin chain production.

2.2 HAEMATOLOGICAL AND HAEMOGLOBIN ANALYSIS

Blood samples were collected into EDTA tubes. Red cell indices were measured using an electronic cell counter (Coulter ZBI 6), and haematological studies performed using established techniques (Dacie and Lewis, 1975). Haemoglobin was analysed by electrophoresis on cellulose acetate strips and the relative amounts of haemoglobin A2 and H quantified by elution from the strips (Graham and Grunbaum, 1963).

Globin chain synthesis was performed according to the method of Weatherall and Clegg (1969) by the Provincial Laboratory for Tissue Immunology at the University of Cape Town. The alpha : beta chain ratios are expressed as the quotient of the radio-active counts in the alpha and beta peaks.

2.3 DNA ISOLATION FROM WHOLE BLOOD

Total human DNA was isolated from 10 ml of whole blood collected into EDTA-tubes according to the method of Kunkel (Kunkel et al, 1977). The blood was homogenized on ice in 60 ml cold lysis buffer (0,32M sucrose, 5mM $MgCl_2$, 1% triton X-100 and 10mM Tris HCl, pH 7,6). The leukocyte nuclei were pelleted by centrifugation at 2500 g for 20 minutes. The pellets were resuspended in a total volume of 8 ml 75mM NaCl, 25mM EDTA, pH 8,0 with subsequent addition of 0,80 ml 10% SDS. The solutions were vortexed prior to addition of 1 mg of pronase (Sigma). The samples were incubated at 37°C for at least 3 hours. The pronase digestion was stopped by placing the samples on ice. To each, 0,50 ml 5M sodium perchlorate solution was added, followed by extraction with 8ml phenol : chloroform (1:1, saturated with 1mM EDTA, 10mM Tris HCl, pH 7,6). The aqueous and the organic phases were separated by centrifugation at 2500 g for 10 minutes at 10°C. The upper aqueous phase was re-extracted with an equal volume of chloroform : octanol (24:1). The samples were centrifuged at 2500 g for 10 minutes at 10°C to separate the two phases. The aqueous phase was pipetted off and the nucleic acid was precipitated by the addition of two volumes of ice-cold absolute ethanol to the aqueous phase. The nucleic acid was carefully lifted into 1 ml of 1mM EDTA, 10mM Tris HCl pH 7,6 and dissolved overnight.

To the nucleic acid solution 0,10 ml (1/10th volume) 4M NaCl was added followed by 0,05 mg heat-treated RNAse (Sigma). The solution was incubated at 37°C for 1 hour. After digestion, 2 ml sterile distilled water was added

and the solution extracted with an equal volume of chloroform : octanol (24:1) until the interface was clear. The samples were centrifuged at 2500 g for 10 minutes at 10°C to separate the aqueous and organic phases and the DNA precipitated from the aqueous phase by the addition of two volumes of ice-cold absolute ethanol. The precipitate was transfered into a small volume of ice-cold 70% ethanol and washed twice to remove any remaining salt. The DNA pellet was dried under a vacuum and redissolved, by gentle mixing, in 1 ml sterile distilled water at 4°C overnight.

The final DNA concentration was estimated by the determination of the absorbance at 260 nm - i.e. (A_{260}(1cm/1%) = 20). The yield of pure DNA isolated from 10 ml of whole blood ranged from 100 to 300 ug. The DNA samples were stored at -20°C until further use.

2.4 TRANSFORMATION OF LYMPHOCYTES

Five ml whole blood was layered onto 7 ml Ficoll-paque and centrifuged at 2000 g for 10 minutes. After centrifugation four layers were apparent:

1. packed red cell layer,
2. Ficoll-paque layer,
3. band of lymphocytes,
4. plasma layer uppermost.

The lymphocyte layer was removed by inserting a sterile pipette through the plasma layer into the lymphocyte layer and was transfered to a sterile tube. Five ml Hams F-10 medium containing 15% foetal calf serum was added to the lymphocytes. The solution was centrifuged at 2000 g for 5 minutes, the Hams F-10 medium decanted and the pellet resuspended in approximately 2ml (dependent upon the yield of lymphocytes) Epstein-Barr virus transforming medium (van der Westhuyzen **et al**, In Press).

Transformation took 7-21 days during which time the cells were examined regularly and the medium replenished when necessary. Once the transformed cells (lymphoblasts) were visible, fresh medium was added in larger volumes and changed more frequently. The lymphoblasts were harvested when they numbered approximately $1,5 \times 10^8$.

2.5 DNA ISOLATION FROM CULTURED LYMPHOBLASTS

The lymphoblasts were harvested by centrifugation at 2000 g for 10 minutes at room temperature. The pelleted lymphoblasts were washed twice with normal saline and homogenized in 30-60 ml lysis buffer, depending upon the amount of starting material. The protocol for the DNA isolation from whole blood was then followed (Section 2.3).

2.6 PLASMID PREPARATION

E.coli K-12-derived strains carrying plasmids JW101 (Wilson **et al**, 1978) and pBR^{zeta} (Lauer **et al**, 1980) were inoculated from stock cultures into 10 ml L-broth (10 g Difco tryptone, 5 g Difco Bacto yeast extract, 5 g NaCl and 1 g glucose in 1 litre water and autoclaved at 15 psi for 15 minutes). The L-broth contained 20 ug/ml tetracycline (Sigma) or 50 ug/ml ampicillin (Sigma) to ensure growth of only the resistent strains. These starter cultures were incubated overnight at 37°C in a rotary incubator. Ten ml aliquots were used to inoculate four 250 ml L-broth culture flasks containing the appropriate antibiotic. The flasks were incubated at 37°C with constant rotation until the A_{654} of the medium was between 0,60 and 1,00 units (i.e. the cells present were in the logarithmic growth phase).

Chloramphenicol (Sigma), (final concentration = 0,18mg/ml) was then added and the subsequent amplification allowed to proceed overnight with gentle shaking.

The bacterial cells were harvested by centrifugation at 1500 g for 10 minutes at 4°C and the resultant pellets resuspended in 7 ml 25% sucrose, 50mM Tris HCl, pH 8,0. Freshly prepared egg-white lysozyme (1,6 ml of a 25mg/ml solution) was added and the solution gently mixed on ice for 5 minutes. To this, 1,3 ml lytic mix (2% Triton X-100, 6mM EDTA and 50mM Tris HCl, pH 8,0) was added and the mixing continued for a further 20 minutes, on ice. During this period the cells lysed to form a viscous mass. The bacterial cell debris carrying with it the bulk of the bacterial DNA was pelleted by centrifugation at 18000 g for 1 hour at 4°C.

The clear, straw-coloured supernatant was decanted and extracted twice with an equal volume of phenol:chloroform (1:1) followed by two extractions with chloroform : octanol (24:1). To the final clear aqueous phase 1/10 volume of 4M NaCl was added, followed by two volumes of ice-cold absolute ethanol and the nucleic acid precipitated overnight at -20°C. The precipitate was recovered by centrifugation at 2000 g for 10 minutes at 4°C, washed in 70% ethanol and dried under a vacuum. The resultant pellet was redissolved in 3 ml 1mM EDTA, 10MM Tris HCl pH 7,6 at 4°C for at least 1 hour. T1-RNAse (Sigma) was added to a final concentration of 20ug/ml and the solution incubated at 37°C for 30 minutes. The solution was extracted with an equal volume of chloroform : octanol (24:1) and the DNA in the aqueous phase precipitated by addition of 1/10 volume 4M NaCl and two volumes of ice-cold absolute ethanol. The precipitate was recovered by centrifugation at 2000 g for 10 minutes at 10°C, washed twice in a small volume of 70% ethanol and dried under a vacuum. The DNA was then redissolved in 0,25-0,50 ml TE buffer (1mM EDTA, 10mM Tris HCl, pH 8,0).

The plasmid DNA was further purified by chromatography on a Sephadex G-200 column. Twelve fractions of 0,50 ml each were collected into sterile eppendorf tubes. Aliquots of these fractions were electrophoresed on a 0,80% agarose gel to identify the fractions containing the recombinant plasmid. They were pooled and scanned from A_{220} to A_{290} to determine the DNA concentration. The intact plasmid was used for nick-translation or the inserted globin cDNA sequence was released from the plasmid by restriction endonuclease digestion. The latter avoids any spurious hybridisation of the probe to

any plasmid DNA which might have contaminated the patient's DNA.

2.6.1 ISOLATION OF THE GLOBIN SEQUENCES FROM THE RECOMBINANT PLASMIDS

The 1,7kb alpha globin insert (alpha globin cDNA and flanking sequences) may be released from the JW101 plasmid by digestion with restriction endonuclease Mbo 11 (Lauer et al, 1980). The 1,7kb insert was separated from the rest of the plasmid on a preparative agarose gel and electro-eluted into a dialysis bag. The concentration of 1.7kb insert (the entire alpha globin cDNA transcript and some flanking plasmid DNA) was determined by reading A_{260nm}.

Similarly, the zeta globin recombinant plasmid consisted of the vector, pBR322 and a 3,9kb genomic DNA insert. The insert contained the entire zeta globin gene (1,6kb) and 2,3kb of additional flanking DNA. This 2,3kb sequence included a region of repetitive DNA which had to be restricted out to prevent hybridization of the probe to other regions of the genome. The purified recombinant plasmid was therefore digested with restriction endonucleases Eco R1 and Bgl 11 (Malcolm, 1981).

One hundred micrograms of the purified zeta globin plasmid was digested with restriction endonuclease Eco R1 for 4 hours at 37°C. The digestion was terminated by the addition of 1/10th volume 100mM EDTA, pH 8,0 and the plasmid DNA precipitated by the addition of two volumes of ice-cold absolute ethanol. The DNA was redissolved in the appropriate buffer and further digested with Bgl 11. The reaction was terminated by the addition of 1/10 volume 100mM EDTA, pH 8,0 and the entire sample was electrophoresed on an 0,80% agarose gel in conjunction

with a lambda$^{Hind\ III}$ molecular weight marker.
The 6kb band of zeta globin DNA plus pBR322 vector were cut out of the gel and transfered to a dialysis bag containing 0,50 ml electrophoresis buffer. The dialysis bag was placed on the electrophoresis apparatus and perpendicular to the current across it. The DNA was then electrophoresed out of the gel into the buffer surrounding it. The migration of the DNA out of the gel was monitored by placing the dialysis bag on an ultra-violet light box from time to time. When it appeared that all the DNA had migrated out of the gel, the DNA in solution in the surrounding buffer was sucked into a sterile syringe and transfered into a corex tube. The DNA solution was extracted with an equal volume of phenol : chloroform (1:1) and subsequently with an equal volume of chloroform : octanol (24:1). The DNA in solution was precipitated with two volumes of ice-cold absolute ethanol and pelleted by centrifugation at 2500 g for 30 minutes at 10°C. It was washed twice with 70% ethanol, dried under vacuum and redissolved in 0,50 ml sterile water at 4°C overnight. The DNA solution was then scanned from A_{220} to A_{290} and the concentration of the purified zeta globin DNA calculated (Section 2.4). The purified plasmids were subsequently used for nick-translation and hybridisation to digested total human DNA (Sections 2.11 and 2.12).

2.7 RESTRICTION ENDONUCLEASE DIGESTION OF TOTAL HUMAN DNA

Ten micrograms of DNA was digested with 30 units of restriction endonuclease (BRL, Boehringer-Mannheim and New England Nuclear Laboratories) in the presence of the enzyme buffers (composition as recommended by the manufacturers) and nuclease-free BSA (Sigma) at a final concentration of 100ug/ml. The volume in which the DNA was digested was made up to 0,15 ml with sterile distilled water. The digestion was allowed to proceed for a minimum of 6 hours at 37°C and was terminated by the addition of 1/10th volume of 100mM EDTA, pH 8,0. To this, 1/10th volume of 4M NaCl was added and the DNA precipitated by the addition of two volumes of ice-cold absolute ethanol. The precipitated DNA was recovered by centrifugation, washed in 70% ethanol and dried under vacuum. The DNA samples were dissolved in 34 ul sterile distilled water with gentle mixing at 4°C for at least 12 hours prior to electrophoresis.

2.8 AGAROSE GEL ELECTROPHORESIS

To the dissolved DNA, 6 ul orange-G/Ficoll (1% w/v orange-G, 20% Ficoll, 20 mM EDTA) and 4 ul electrophoresis buffer (40mM acetate, 2mM EDTA and 40mM Tris HCl, pH 7,6) was added. A 0,80% agarose gel was prepared by boiling 2 g agarose in 250 ml of the above buffer. Ethidium bromide to a final concentration of 1 ug/ml was added prior to pouring the gel onto a sealed 20 cm by 20 cm glass plate in the gel apparatus. The gel was allowed to set for at least one hour. Prior to loading, the DNA samples were incubated at 37°C for 15 minutes to ensure that the DNA was completely dissolved. The gel apparatus was filled with electrophoresis buffer and the wells flooded. The DNA samples were carefully pipetted into the wells below the surface of the buffer and were run into the gel at 25 volts, 20 milliamps for 10 minutes. The voltage was adjusted to 40 volts and electrophoresis performed for at least 18 hours or until the orange-G front had completely disappeared off the end of the gel.

For optimal resolution of similarly sized bands the gel apparatus was placed at 4°C, the buffer circulated and the DNA electrophoresed for 24 to 30 hours.

Upon completion of the electrophoresis, the gel was viewed on an ultra-violet light box and photographed with a ruler in line with the molecular weight markers. The reciprocal of the mobility of the molecular weight markers was plotted against their known fragment sizes (kilobases). The sizes of the globin DNA fragments were read from the graph after autoradiography.

The DNA was denatured by gentle agitation of the gel in 300 ml 0.50M NaOH, 1,5M NaCl for 3 hours. The gel was rinsed twice in distilled water and neutralized in 0,50M Tris, 20x SSC (3M NaCl, 0,3M Tri-sodium citrate, pH 7,4) for a further 2 hours. The gel was then ready for the Southern blotting procedure (Section 2.9).

2.9 SOUTHERN BLOTTING PROCEDURE

This was performed essentially according to the method of Southern (Southern, 1975).

A platform consisting of four Bijou bottles, placed on their sides, and a 20 cm x 20 cm glass plate was set up in a large developing tray (8 cm deep). A piece of Whatman #1 filter paper, 24 cm x 24 cm, was cut and folded over the glass plate to act as a wick. 20x SSC was poured onto the filter paper to within 2 cm of the plate. Air bubbles between the filter paper and the glass plate were rolled out with a small glass rod. The pre-treated gel (Section 2.8) was slid onto the filter paper on the glass plate, ensuring that no air bubbles were trapped between the gel and the support.

A 20 cm x 20 cm nitrocellulose sheet (Schleicher and Schuell BA 85) was wet by flotation in 100 ml 2x SSC and lowered onto the gel taking care to remove air bubbles. Strips of cling film were placed from the outer edges of the gel to the edges of the tray to ensure diffusion of the 20x SSC through the gel and to prevent evaporation of the 20x SSC during the transfer. Two sheets of Whatman #1 filter paper (20cm x 20cm) were wet by flotation in the 2x SSC and placed on top of the nitrocellulose avoiding air bubbles. A box of tissues was divided in two and placed side by side on top of the filter papers. A 20 cm x 20 cm glass plate, with 4 x 125g weights, was placed on top of the tissues and the whole assembly was placed at 4°C for 30 hours. The level of the 20x SSC was monitored and the saturated tissues replaced with dry ones when necessary.

After DNA transfer had been effected, the wells of the gel were marked on the overlying nitrocellulose, the latter which was then cut into strips compatible with the size of the hybridization box (Section 2.11). The filters were lifted from the gel and rinsed for 10 minutes in 2x SSC. They were blotted dry and the DNA was baked onto the nitrocellulose at 80°C for 2 hours. The filters could be stored indefinitely at 4°C at this stage.

2.10 NICK-TRANSLATION OF THE RECOMBINANT PLASMID OR EXCISED INSERT

In order to prepare a highly radioactive probe, 0,5 ug DNA was was incubated for 90 minutes at 16°C in a solution containing 20uM/l dTTP, dATP, dGTP, 60uCi ^{32}P-dCTP (specific activity = 3000 Ci/mmol), 5mM $MgCl_2$, 10mM 2-mercapto-ethanol, 10ug/ml nuclease-free BSA (Sigma) and 50 mM Tris HCl, pH 7,6 (Rigby **et al**, 1977). Two hundred picograms of DNAse 1 and 2 units of DNA polymerase (BRL Nick-Translation Kit) were added and the final reaction volume made up to 50 ul with sterile distilled water. The reaction was terminated by the addition of 100 ul of a stop-mix solution (10mM EDTA, 0,50% SDS, 10mM Tris HCl, pH 7,6) and placed on ice. The solution was extracted with an equal volume of phenol : chloroform (1:1), the phenol phase re-extracted with TE buffer and the pooled aqueous phases loaded onto a 5 cm x 0,50 cm column of Sephadex G-50 (medium). Twelve fractions (150 ul each) were collected and 1/100 volume aliquots counted for Cerenkov radiation. The fractions of the excluded peak were pooled and the specific activity (in dpm/ug) of the labelled DNA calculated as follows:

Specific activity = 2(sum of the dpm in the pooled fractions) x 100/0,40
= dpm/ug

The radio-labelled DNA was denatured by incubation in a boiling water-bath for 5 minutes, followed by rapid cooling on ice to prevent re-annealing of the DNA strands.

2.11 HYBRIDIZATION OF THE ^{32}P-LABELLED PROBE TO THE NITROCELLULOSE FILTERS

The nitrocellulose filters were wet by flotation in 3x SSC and washed in the following solutions :

1. 3x SSC for 30 minutes at 65°C,
2. 3x SSC, 5x Denhardts* solution for 1 hour at 65°C,
3. 3x SSC, 5x Denhardts solution, 0,10% SDS, 10ug/ml polyadenylic acid and 50ug/ml single-stranded Herring sperm DNA for 1 hour at 65°C in a shaking water-bath.

*Denhardts solution (10x):

2% w/v bovine serum albumin fraction 5,
2% w/v Ficoll (type 400),
2% w/v polyvinylpyrrolidone (type 360).

Each solution was pre-heated to 65°C and the strips of nitrocellulose filters transferred individually with a pair of Millipore forceps.

The filters were hybridized in a hybridization box (designed by A. Jeffries, Leicester University). Ten millilitres of the wash solution #3 were pipetted into the hybridization box, the ^{32}P-labelled single-stranded DNA probe added and the filter strips individually transfered to the hybridization solution ensuring that no air bubbles were trapped between the filters. The hybridization box was tightly sealed and incubated in a shaking water-bath at 65°C for at least 24 hours.

Upon completion of the hybridization, the filters were washed at 65°C to remove unbound and non-specifically bound probe as follows:

1. 3x SSC, 5x Denhardts solution, 0,10% SDS for 2x 30 minutes,
2. 0,10x SSC, 0,10% SDS for 2x 30 minutes (stringent wash).

The amount of radio-activity remaining on the filters was monitored with a Geiger counter, and if significantly above background, the stringent washes were repeated. The filters were blotted dry with Whatman #1 filter paper and further dried at 37°C for 30 minutes. They were re-assembled onto cardboard, covered with cling-film and autoradiographed using Kodak X-omat MA X-ray film and a Dupont intensifying screen. The X-ray film was exposed at -70°C for 1-10 days as required.

2.12 DEVELOPMENT OF THE AUTORADIOGRAPH

The autoradiograph was developed in Kodak X-ray developer for 3 minutes, rinsed in 2% glacial acetic acid for 20 seconds and fixed in Kodak fixative for 6 minutes. The autoradiograph was then washed in running water for 15 minutes and dried.

The distances of the bands from the origin of migration were measured and the sizes of the bands (in kilobases) calculated from the standard curve (Section 2.8).

2.13 DETERMINATION OF THE ALPHA GLOBIN GENE NUMBER BY DENSITOMETRY

The method of Lie-Injo et al (1982) was used to detect the --/ alpha thalassaemia determinant in which both alpha globin genes are deleted.

DNA was digested with restriction enzyme Hpa 1, electrophoresed, blotted onto nitrocellulose, and hybridised with both ^{32}P-labelled alpha globin cDNA and gamma globin cDNA (JW 101 and JW 151, Wilson et al, 1978). The alpha globin genes are located on fragments 14,5kb and 4,2kb in length, whereas the gamma globin genes are on fragments 24kb and 4,8kb in length. The relative quantity of alpha globin genes located on the 14,5kb band was estimated by comparing the density of this alpha globin band divided by the density of the 24kb gamma globin band in the sample, with the ratio obtained from a normal control on the same autoradiogram. Densitometric scanning was done on a Helena Quick Scan Flur-Vis and the relative intensities of the alpha and gamma bands were automatically recorded on a Helena Quick Quant 11.

RESULTS

3.0 INTRODUCTION

3.1 THE MOLECULAR BASIS OF ALPHA THALASSAEMIA

3.2 THE FREQUENCY OF ALPHA THALASSAEMIA

3.0 INTRODUCTION

Haematological analyses have suggested that alpha thalassaemia is prevalent in the local 'Cape Coloured' population.

Original diagnoses were made on the basis of a decreased MCV (mean cell volume) and MCH (mean cell haemoglobin), accompanied by normal haemoglobin electrophoresis and normal haemoglobin A2 and serum iron levels. Some cases were confirmed by alpha : beta chain synthesis ratios.

To investigate the molecular basis of alpha thalassaemia in this population, the confirmed cases were first examined and the study subsequently expanded to include those suggested by the above criteria.

3.1 THE MOLECULAR BASIS OF ALPHA THALASSAEMIA

A total of 25 subjects was examined. DNA from their circulating white blood cells was analysed by restriction endonuclease mapping with Bam H1 and probed with an alpha globin-specific cDNA, JW101 (Wilson **et al.**, 1978). Restriction endonuclease Bam H1 cuts the DNA outside the duplicated alpha globin genes to produce a DNA fragment 14kb in length (Lauer **et al**, 1980). The deletion of one (alpha^{+}) or both (alpha^{o}) alpha globin genes results in the detection of an abnormal 10,5kb Bam H1 alpha globin-specific restriction fragment or no Bam H1 alpha globin-specific fragments, respectively (Figure 3.1).

In 18 subjects, the haematological data could be explained on the basis of their alpha globin restriction patterns alone (Table 1).

TABLE 1. HAEMATOLOGICAL DATA ON SUBJECTS WITH THE -a/ DETERMINANT.

Subject	RBC $x10^{12}/l$	MCV fl	MCH pg	HbA2 %	HbH %	Bam H1 kb	genotype
C1P32	5,30	64	21,2	1.6	n.d.	10,5	-a/-a
C1P42	6,38	62	20,1	1,5	n.d.	10,5	-a/-a
C1P44	5,52	68	21,7	2,9	n.d.	10,5	-a/-a
C1P63	5,69	62	19,5	2,4	n.d.	10,5	-a/-a
C2P11	4,96	75	24,0	2,0	n.d.	14;10,5	aa/-a
C1P37	4,46	71	24,9	2,6	n.d.	14;10,5	aa/-a
C2P32	5,58	70	24,2	2,4	n.d.	14;10,5	aa/-a
C2P35	5,13	74	25,0	2,2	n.d.	10,5	-a/-a
J1P12	6,14	55	18,2	1,5	n.d.	10,5	-a/-a
J1P17	5,49	64	21,3	2,4	n.d.	14;10,5	aa/-a
J1P18	4,70	69	22,1	2,1	n.d.	14;10,5	aa/-a
J1P20	4,91	64	23,0	2,3	n.d.	10,5	-a/-a
J1P26	5,61	66	23,0	2,4	n.d.	10,5	-a/-a
J1P27	5,82	58	20,0	---	---	10,5	-a/-a
J1P28	---	--	---	---	---	14;10,5	aa/-a
J1P43	---	70	24.0	4,0*	---	10,5	-a/-a
J1P47	---	--	---	---	---	14;10,5	aa/-a
J1P63	5,24	69	22,6	2,4	n.d.	10,5	-a/-a

* = co-existent beta thalassaemia

n.d. = not detectable

--- = not done

The subjects with a moderate reduction in MCV and MCH (J1P17, J1P18, J1P28, J1P43 and J1P47) had both the 14kb and 10,5kb Bam H1 alpha globin-specific bands. This indicates that they are heterozygous for the deletion that inactivates one alpha globin gene per chromosome, with the genotype -a/aa and the phenotype alpha thalassaemia 'silent carrier'.

The subjects with a marked reduction in MVC and MCH, and no haemoglobin H detecable in their red blood cells, showed only the 10,5kb Bam H1 alpha globin-specific band. This is indicative of homozygosity for the single gene deletion which inactivates one alpha globin gene per chromosome, with the genotype -a/-a and the phenotype alpha thalassaemia trait.

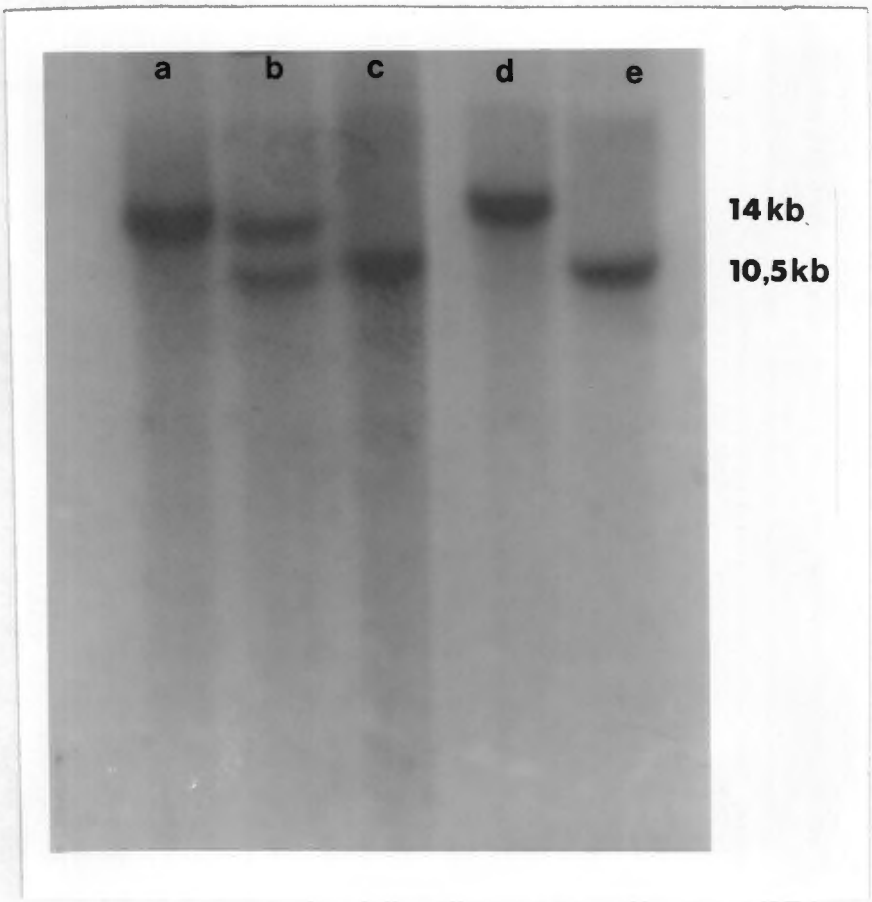

Figure 3.1 Examples of alpha globin-specific Bam H1 restriction endonuclease fragments.
(a) aa/aa, (b) -a/aa, (c) -a/-a, (d) aa/aa, (e) -a/-a.

3.1.1

Both a 'leftward' deletion which removes 4,2kb of the 5' alpha globin gene and the 'rightward' deletion which removes 3,7kb of DNA between the duplicated alpha globin genes have been described (Orkin **et al**, 1979 and Embury **et al**, 1980). To characterize the -a/ deletion encountered in this study further, the DNA of the patients' was digested with restriction endonuclease Bgl 11 which normally produces a 12,5kb alpha globin-specific fragment containing the 5' alpha globin gene and a 7kb alpha globin-specific fragment containing the 3' alpha globin gene. In the 'leftward' deletion, only a 7kb fragment is detected, whereas the 'rightward' deletion removes the intergenic Bgl 11 site and produces an alpha globin-specific fragment 16kb in length (12,5kb + 7,0kb - 3,7kb). Examples of restriction fragment patterns are shown in figure 3.2.

All the subjects studied were found to have the 16kb Bgl 11 alpha globin-specific fragment showing that they had the 'rightward' type of deletion.

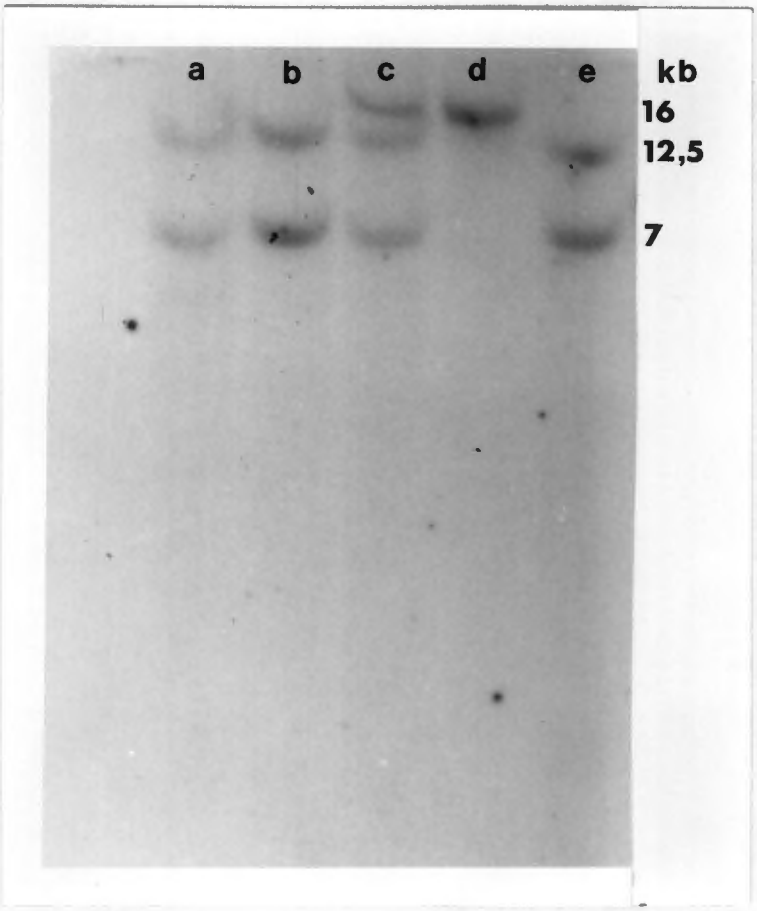

Figure 3.2 Examples of alpha globin-specific Bgl 11 restriction endonuclease fragments.
(a) --/aa, (b) aa/aa, (c) -a/aa, (d) -a/-a, (e) aa/aa.

3.1.2

In some of the cases investigated, the phenotypes could not be explained by their Bam H1 alpha globin-specific fragments alone (Table 2).

TABLE 2. HAEMATOLOGICAL DATA ON SUBJECTS WITH THE --/ AND aa^T/ DETERMINANTS.

subject	RBC $x10^{12}/l$	MCV fl	MCH pg	HbA2 %	HbH %	a/b chains	Bam H1 kb	genotype
C1P35	4,53	57	19,4	0,6	1,1	0,31	10,5	--/-a
C2P14	4,73	57	18,4	0,8	2,0	0,50	10,5	--/-a
C2P8	4,30	63	16,3	1,1	10,2	0,52	10,5	--/-a
C2P12	5,33	63	19,7	2,8	n.d.	0,62	14	aa/aa^T
J1P7	7,02	60	17,0	1,3	3,7	----	10,5	--/-a
C2P10	6,00	65	19,7	2,2	n.d.	----	14	--/aa
C2P30	----	--	----	---	5,4*	----	14	--/aa

aa^T = non-deletion alpha thalassaemia determinant.
* = Hb Barts in the cord blood.
---- = not done

Three subjects (C1P35, C2P14 and C2P8) with detectable levels of haemoglobin H (Table 2) had only the 10,5kb Bam H1 alpha globin-specific fragment. This restriction pattern is consistent with the genotype --/-a or -a/-a. Since the latter genotype would not be expected to result in haemoglobin H disease, a provisional assignment of --/-a was made.

The presence of the --/ determinant in the local population has important clinical implications, and it was therefore sought to establish the molecular basis of haemoglobin H disease in this study more clearly and to confirm that the genotypes in these cases were indeed --/-a, as opposed to the -a/-a genotype which would give rise to the same Bam H1 alpha globin-specific restriction fragment pattern.

3.1.3

A family study was carried out for one of the patients (C2P8, Table 2), in the course of which a fourth case of haemoglobin H disease (J1P7, Table 2) was detected.

Whereas both the propositus (C2P8, figure 3.3) and her brother (J1P7, figure 3.3) had only the 10,5kb Bam H1 alpha globin-specific restriction fragment, their mother (C2P10, figure 3.3) had only the 14kb Bam H1 fragment. She must therefore have donated a chromosome with no alpha globin genes to these two children. Analysis of the cord blood of the child of the propositus (C2P30, figure 3.3) revealed a haemoglobin Barts level of 5,4%, consistent with the inactivation of two out four alpha globin genes, and a diagnosis of alpha thalassaemia trait (Weatherall and Clegg, 1981). DNA extracted from the cord blood (C2P30) showed only the 14kb Bam H1 alpha globin-specific fragment (Table 2) confirming that this child did not inherit any alpha globin sequences from her mother. The red cell indices of the father were within the normal ranges (not shown).

This family study therefore confirms the assignment of the genotype --/aa to the two family members with alpha thalassaemia trait (C2P30 and C2P10) and the genotype --/-a to the two cases of haemoglobin H disease (C2P8 and J1P7, Table 2 and figure 3.3).

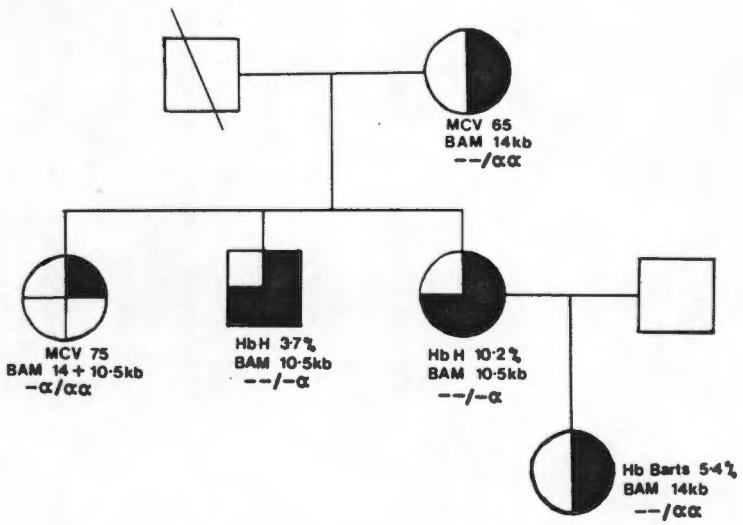

Figure 3.3 The pedigree of the HbH family studied. BamH1 restriction endonuclease fragments (BAM) and the proposed genotypes are indicated. ☐ represents normal individuals, ◳ alpha^{+} thalassaemia carrier (C2P11), ◧ alpha^{o} thalassaemia carriers (C2P10 and C2P30), ■ HbH disease (C2P8 and J1P7) and ⊘ deceased.

3.1.4

One case (C2P12, Table 2) had typical alpha thalassaemia red cell indices, significantly reduced alpha : beta chain synthesis ratios but a normal Bam H1 alpha globin restriction fragment pattern. These findings are compatible with, either an alpha thalassaemia trait with the genotype --/aa, or with a non-deletion type of defect where the alpha globin gene, although structurally intact, has undergone a localised alteration of DNA sequence which affects the expression of the gene.

The method of Lie-Injo (Lie-Injo **et al**, 1982) which measures the relative intensities of the alpha and gamma globin gene fragments was used to distinguish between these two possibilities (see 2.13). Scans of the 24kb gamma and 14,5kb alpha globin-specific Hpa 1 restriction fragments from a normal subject, a --/aa genotype alpha thalassaemia trait (C2P30, Table 2) and from subject C2P12 (Table 2) are shown in figure 3.4.

The density of the alpha globin gene band divided by the density of the gamma globin gene band was 0,661 +/- 0,004 in the normal DNA, 0,332 +/- 0,002 in alpha^{o} thalassaemia trait DNA (50,2% of normal) and 0,657 +/- 0,014 in subject C2P12 (99,4% of normal). These results confirm that subject C2P12 (Table 2, figure 3.4) has a full complement of alpha globin genes and must therefore have inherited a non-deletion type alpha thalassaemia determinant.

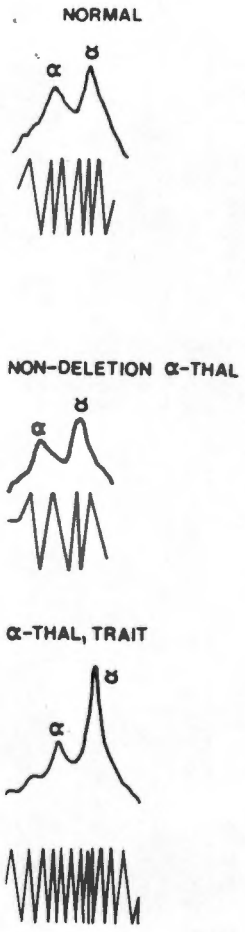

Figure 3.4 Scans of the 24kb gamma globin and 14,5kb alpha globin Hpa 1 restriction endonuclease fragments from a normal subject, an alpha 1 thalassaemia trait (C2P30, Table 1) and from patient C2P12, Table 1.

3.1.5

Restriction mapping with Bam Hl and probing with the zeta globin probe was used to further characterise the --/ determinant.

Four different types of alpha^{0} thalassaemia deletions (Section 1.3.2) involving the zeta globin locus as well as the alpha globin locus have been described, two of which remove both alpha globin genes, namely the --SEA/ and the --MED/ (Weatherall and Clegg, 1981, figure 1.6). DNA with the Southeast-Asian type of deletion yields a 5,9kb and a 20kb Bam Hl zeta globin-specific fragment, whereas DNA with the Mediterranean type of deletion yields only a 5,9kb restriction fragment.

Subjects C1P35 and C2P8 (Table 2) both had the 20kb and 5,9kb Bam Hl zeta globin-specific fragments as well as the normal 10,5kb fragment, confirming the --/ haplotype which is thus of Southeast-Asian origin (figure 3.5).

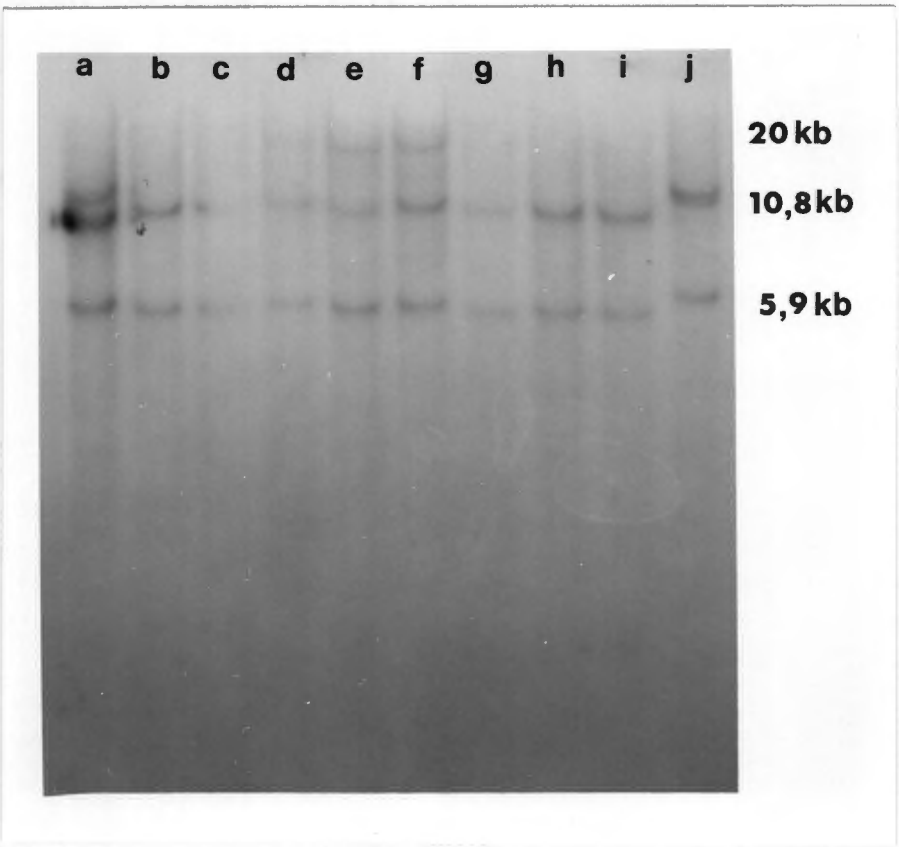

Figure 3.5 Examples of Bam H1 zeta globin-specific restriction endonuclease fragments.
(a - d) normal, (e) --SEA/ (C1P35, Table 2),
(f) --SEA/ (C2P8, Table 2), (g - j) normal.

3.2 THE FREQUENCY OF ALPHA THALASSAEMIA IN THE LOCAL 'CAPE COLOURED' POPULATION.

On account of the considerable number of alpha thalassamia cases which were detected in this population an attempt was made to estimate the frequency of the various alpha thalassaemia determinants. No published frequency studies are available, but a survey of 1207 cord bloods from randomly selected subjects revealed that 40 of these individuals had detectable levels of haemoglobin Bart's (Provincial Laboratory for Tissue Immunology, University of Cape Town, unpublished results), It has been possible to trace 19 out of the 40 subjects for the purpose of the present study. DNA from these subjects was analysed to establish which alpha thalassaemia determinants were present.

Their DNA was mapped with restriction endonucleases Bam H1 and Bgl 11 and probed with the alpha and zeta globin-specific probes. The results are shown, together with their haematological data in Table 3. Their restriction fragment patterns are shown in Figure 3.6.

TABLE 3. HAEMATOLOGICAL DATA ON SUBJECTS WITH DETECTABLE HB BART'S LEVELS.

subject	RBC $x10^{12}/l$	MCV fl	MCH pg	Hb Barts %	Bam H1 kb		genotype
normal	4,4-6,0	82-101	27-34	none	14		aa/aa
J1P52	6,22	65	19	5,9		10,5	-a/-a
J1P53	4,42	73	24	1,1	14	10,5	aa/-a
J1P54	4,78	73	24	2,9	14	10,5	aa/-a
J1P56	5,88	65	20	4,1		10,5	-a/-a
J1P57	4,82	79	24	1,1	14	10,5	aa/-a
J1P58	5,42	61	19	7,1		10,5	-a/-a
J1P59	4,99	68	22	2,5	14		aa^T/aa*
J1P65	5,19	54	15	6,8		10,5	-a/-a
J1P68	4,85	74	25	1,3	14	10,5	aa/-a
J1P69	5,14	74	24	1,0	14	10,5	aa/-a
J1P88	5,09	74	23	1,6	14	10,5	aa/-a
J1P89	5,76	66	20	7,3		10,5	-a/-a
J1P90	4,49	78	26	1,7	14	10,5	aa/-a
J1P91	5,04	72	23	1,7	14	10,5	aa/-a
J1P92	5,27	70	21	5,2	14	10,5	aa/-a
J1P93	5,03	74	24	1,3	14	10,5	aa/-a
J1P94	4,55	83	27	1,5	14	10,5	aa/-a
J1P97	5,18	77	24	1,0	14	10,5	aa/-a
J1P98	5,19	75	24	1,3	14	10,5	aa/-a

* = normal alpha and zeta globin restriction patterns.

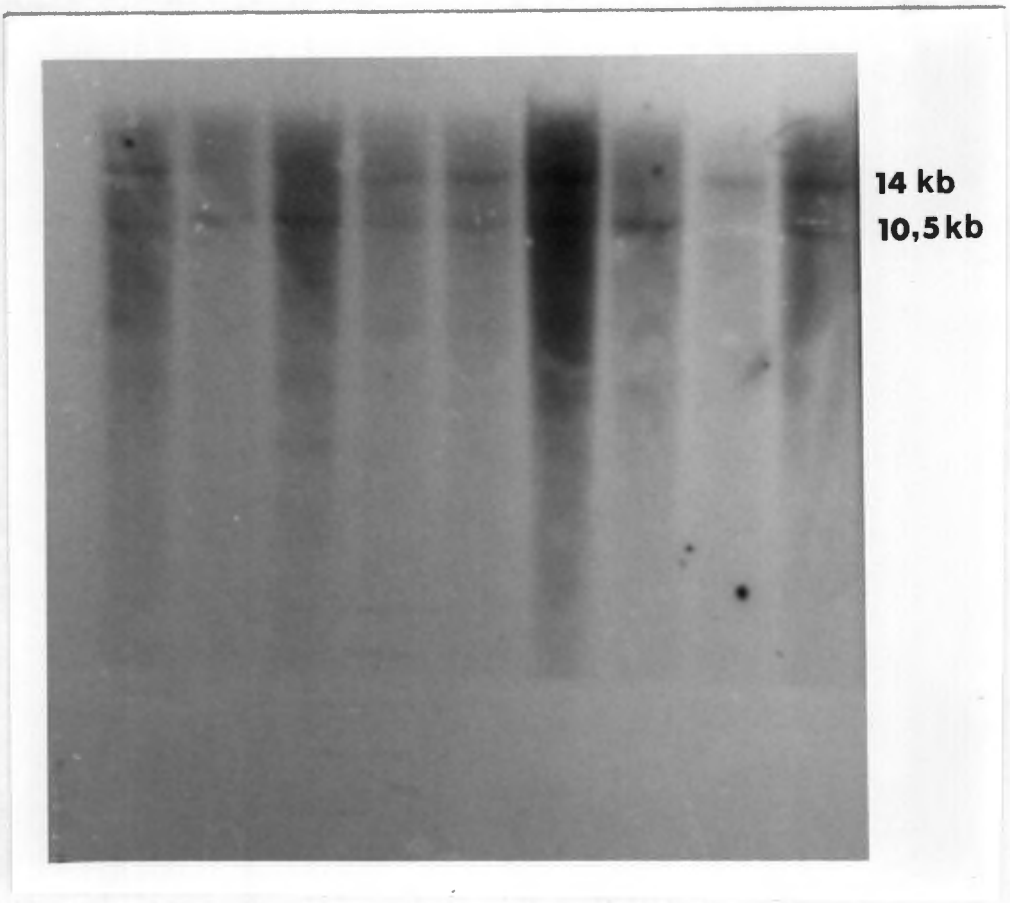

Figure 3.6 Examples of Bam H1 alpha globin-specific restriction fragments.

Using this data it is possible to calculate the frequency of the -a/aa genotype with the Hardy-Weinberg equation as follows:

* $N_{total} = 1207$
** $N_{elevated\ Hb\ Bart's} = 40$
*** $N_{subjects\ analysed} = 19$, and therefore
$N_{effective\ sample\ population} = 19/40 \times 1207$
$= 573,33$

According to the Hardy-Weinberg equation, the genotypes are distributed in the following proportions:

genotype	aa/aa	aa/-a	-a/-a
frequency	p^2	$2pq$	q^2

The observed frequency of the -a/-a genotype is
$5/573,33 = 0,0087$
$= q^2$
therefore $q = 0,0934$
and $p = 1 - q$
$= 0,9066$
therefore $2pq = 0,1693$

The expected number of heterozygotes in this sample is thus $0,1693 \times 573,33 = 97,1$
and the calculated frequency (q) of the -a/ determinant = 0,0934.
For the 'Cape Coloured' population of approximately 2 million, the expected number of heterozygotes is thus approximately $0,1693 \times 2 \times 10^6 = 338600$.

However, the observed number of heterozygotes in this sample is 13, and the observed frequency of the -a/ determinant $23/1146,66 = 0,0200$.

* refers to the number of random neonates initially screened for Hb Bart's.

** The Hb Bart's level was considered to be elevated if Hb Bart's was detectable on cellulose acetate strips followed by quantification in phosphate buffer (Weatherall and Clegg, 1981).

*** Twenty-one subjects out of the forty with elevated Hb Bart's could not be traced.

DISCUSSION

4.1 THE MOLECULAR BASIS OF ALPHA THALASSAEMIA IN THE CAPE COLOURED POPULATION

4.2 THE PREVALENCE OF ALPHA THALASSAEMIA IN THE CAPE COLOURED POPULATION.

4.1 THE MOLECULAR BASIS OF ALPHA THALASSAEMIA IN THE CAPE COLOURED POPULATION

The results of this study indicate that gene deletions are responsible for the majority of cases of alpha thalassaemia detected in the Cape Coloured population investigated.

Table 1 shows that the phenotypes of 18 of the 25 subjects studied could be explained on the basis of the deletion of one or two alpha globin genes. Restriction endonuclease mapping with restriction enzyme Bam H1 and probing with an alpha globin-specific probe provides a useful additional means of diagnosis of alpha thalassaemia in this population. However, the conventional restriction mapping described above will not detect the alpha thalassaemia trait (--/aa) or non-deletion (aa/aa^T) alpha thalassaemia genotypes (Table 2).

The --/ alpha thalassaemia determinant may be detected by analysis of the relative intensities of alpha and gamma restriction fragments compared with a globin internal standard (Lie-Injo **et al**, 1982 and figure 3.3), or by restriction endonuclease mapping with a zeta globin (embryonic alpha globin) probe (Higgs **et al**, 1981).

The predominance of the -a/ haplotype (33/44 alpha thalassaemia determinants) over the --/ haplotype (6/44 alpha thalassaemia determinants) is consistent with the low incidence of haemoglobin H disease and the absence of any reported cases of haemoglobin Bart's hydrops fetalis syndrome in this population. A survey of 1207 fetal cord bloods (Botha, Rees and Du Toit 1975-1978)

did not reveal any haemoglobin Bart's levels in excess of 8% (Section 1.4).

However, the existence of the --/ determinant in this population implies that the haemoglobin Bart's hydrops fetalis syndrome could occur with a low frequency.

The finding that the majority of single gene deletions (-a/) in this population result from the rightward deletion which removes the Bgl II site from between the duplicated alpha globin genes serves to confirm the ubiquity of this deletion (figure 3.2). It has been found in Jamaican and American Blacks (Embury **et al**, 1980 and Higgs **et al**, 1981), Mediterranean and Chinese subjects (Embury **et al** 1980), Malays and Indians (Lie-Injo **et al.** 1982) and Algerians (Whitelaw **et al**, 1980 and Henni **et al**, 1981) and therefore cannot be used to establish the geographical or racial origins of the alpha thalassaemia genes analysed in this study.

Some insight into the origins of the alpha thalassaemia genes found in the Cape Coloured population may be obtained from a study of the ethnic origins of this group (Botha and Pritchard, 1972).
The three main influences were:

1. Southern African peoples, who include the indigenous Khoikhoi and Khoisan, and slaves imported from Madagascar and East Africa during the 17th and 18th century.

2. Asians, who were slaves and political exiles imported by the Dutch East India Company from India and Indonesia.

3. Western Europeans, mainly from the Netherlands.

The incidence of alpha thalassaemia in Western Europeans is known to be low (Weatherall and Clegg, 1981). Although the Khoikhoi have largely been assimilated into the Cape Coloured population, the -a/ determinant has been detected in the San Bushmen (Ramsay, M., Personal Communication).

The finding that 11% of newborn Tanzanians had detectable levels of haemoglobin Bart's (Nhonoli **et al**, 1979) suggests East Africa as one possible source of alpha thalassaemia genes.

The other potential sources are India and Indonesia. In central India the incidence of the -a/ haplotype is very high, whereas haemoglobin H disease and haemoglobin Bart's hydrops fetalis are rare (Brittenham **et al**, 1980). Numerous cases of haemoglobin Bart's hydrops fetalis have however been documented in Indonesia (Wasi **et al**, 1974). The --/ haplotype found in the Cape Coloured population was characterized as being of Southeast Asian origin (Section 3.1.4) because a 20kb Bam H1 zeta globin-specific fragment was detected upon analysis of the DNA of subjects known to have the --/aa genotype (Pressley, 1980). This finding thus confirms Indonesia as the most likely source of the --/ determinant found in the Western Cape.

4.2 THE PREVALENCE OF ALPHA THALASSAEMIA IN THE CAPE COLOURED POPULATION

An attempt was made to estimate the frequency of the alpha thalassaemia determinants because of the large number of cases of alpha thalassaemia which were detected during the course of this study. Using the data from Table 3 (section 3.2), the frequencies of the different determinants were calculated according to the Hardy-Weinberg equation (Emery, 1976).

The frequency of the -a/ determinant (q) was calculated to be 0,0934 and the frequency of the -a/aa genotype (2pq) was calculated to be 0,1693. However, the detected frequency in this sample of the -a/ determinant was 23/1146,66 = 0,0200 and that of the -a/aa genotype (13/573,33) was found to be 0,0226. The fact that only 3,3% of the sample had detectable levels of Hb Bart's implies that Hb Bart's measurement grossly underestimates the frequency of the -a/aa genotype, if the calculated frequency of this genotype is correct.

In studies performed on Jamaican cord blood samples, it was shown that a considerable proportion of the subjects with the -a/aa genotype failed to manifest Hb Bart's (Higgs **et al**, 1982). The prevalence of the -a/aa genotype was thus also underestimated in this population when using the presence of Hb Bart's in fetal cord blood as an indicator of alpha thalassaemia.

The Hardy-Weinberg equation is derived on the assumptions that mating is random and the population homogeneous with respect to the determinants (alleles) considered. The possibility exists that the Hardy-Weinberg equation used to calculate the frequency of the -a/aa heterozygotes may not be valid for the following reasons:

The Cape Coloureds have diverse origins in the indigenous Koikoi, East African, Asian and Western European population groups (Botha and Pritchard, 1972). Social and cultural constraints upon groups of different origin within the Cape Coloured population may have perpetuated the initial heterogeneity to some degree. In addition, the social customs may be operating in such a way as to lead to a significant degree of non-random mating with respect to the alpha thalassaemia genotypes.

On the other hand, a high frequency of the -a/ determinant would not be unexpected in this population, as the founder population groups are known to have high frequencies of this determinant. Although deviations from the Hardy-Weinberg equilibrium in this population may account, in part, for the high (16,9%) heterozygote frequency, the true frequency may not be much less than this.

The results of this Hb Bart's study confirm that the -a/ haplotype is the most common alpha thalassaemia determinant in the Western Cape because most of the subjects with mildly elevated levels (1 - 4% Hb Bart's) of Hb Bart's at birth have the genotype -a/aa.

The levels of Hb Bart's are approximately proportional to the number of alpha globin genes deleted (Section 1.4) and compare favourably with those reported by Lie-Injo (Lie-Injo **et al**, 1982) i.e. subjects with Hb Bart's levels less than 4% had one alpha globin gene deleted and subjects with Hb Bart's leves of between 4 and 8,5% had two alpha globin genes deleted. In most cases the Hb Bart's levels correlated with the number of alpha globin genes deleted.

In conclusion therefore, the results of this study have defined the structural nature of the genetic defects giving rise to alpha thalassaemia in the Cape Coloured population, and have estimated the frequency of the commonest -a/ determinant. The latter also points to the need for a random survey of the Cape Coloured population using alpha globin gene mapping in order to determine the true frequency of the -a/ determinant and the prevalence of alpha thalassaemia in the Western Cape.

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