Molecular mutations and polymorphisms associated with hereditary haemolytic anaemias in local populations

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A Dissertation Submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, for the Degree of Master of Science.

Johannesburg 1993.

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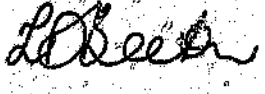
DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg, and has not been submitted for a degree in any other University.



Lesley Dawn Beeton

September 1993.

To Ian and my parents.

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Abstract

Two black South African subjects presenting with Hereditary Elliptocytosis were investigated and the defect defined as SpaI/74, a previously described spectrin variant leading to defective heterodimer self-association and instability of the erythrocyte membrane. Molecular analysis of exon 2 of α spectrin determined that in both probands the change was a point mutation in codon 28, CGT (Arg) to TGT (Cys), previously described in two New Zealand kindred.

It was established in a group of controls and patients, that a change in a TaqI digestion site in the human erythroid β spectrin gene is polymorphic. The frequency of the polymorphism was calculated. There was no statistically significant difference between black and white individuals in this regard.

A normal range of erthrocyte membrane protein content, expressed as a ratio to band 3, was established by densitometric analysis and will be used for comparison and diagnosis of hereditary haemolytic anaemias in local populations.

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Abbreviations

1D	one dimensional
2D	two dimensional
A	absorbance
aa	amino acid
ACD	acid citrate dextrose
APS	ammonium persulphate
ASO	allele-specific oligonucleotide
ASOH	allele-specific oligonucleotide hybridization
b3	erythrocyte membrane protein band 3
bis	N,N'-methylene bisacrylamide
bp	base pair
BSA	bovine serum albumin (fraction V)
Ci	Curie
dATP	deoxy-adenosine 5'-triphosphate
dCTP	deoxy-cytidine 5'-triphosphate
dGTP	deoxy-guanosine 5'-triphosphate
dTTP	deoxy-thymidine 5'-triphosphate
dl	decilitre
DMSO	dimethyl sulphoxide
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid disodium salt
g	relative centrifugal force
g	gram
Hb	haemoglobin
Hct	haematocrit or packed cell volume
HE	hereditary elliptocytosis
HMW	high molecular weight complex
HPP	hereditary pyropoikilocytosis
HS	hereditary spherocytosis
IEF	iso-electric focusing
k	kilo
kb	kilobase
kDa	kilodalton
l	litre
µ	micro

M	molar
mA	milli Ampere
MCV	mean cell volume
ng	nanogram
nm	nanometre
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
retic	reticulocyte
RFLP	restriction fragment length polymorphism
SD	standard deviation
SDS	sodium dodecyl sulphate
Sp	spectrin
SpD	spectrin dimers
SpO	spectrin oligomers
SpT	spectrin tetramers
ss	single-stranded
TAE	Tris/acetate/EDTA buffer
TBE	Tris/borate/EDTA buffer
TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	melting temperature
TPCK	L-1-tosyl-amido-2-phenylethyl chloromethyl ketone
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
U	unit
v	volume
V	Volt
w	weight
W	Watt

1. Introduction

The red cell membrane consists of a lipid bilayer supported by a hexagonal lattice of interacting structural proteins including spectrin, actin and band 4.1 (Liu, Derick and Palek, 1987). Deficiencies and dysfunctions of these protein components lead to instability of the membrane and altered shape, resulting in damage during circulation and premature removal, mainly by the spleen (Hatton and Weatherall, 1991).

Hereditary haemolytic anaemias, Hereditary Elliptocytosis (HE), Hereditary Spherocytosis (HS) and Hereditary Pyropoikilocytosis (HPP), are associated with defective red cell membrane proteins.

In this study, three aspects were investigated.

In Part I, the molecular defect in two local subjects, presenting with clinical signs of HE, was determined. With the availability of the DNA sequences for both α and β spectrin, the polymerase chain reaction (PCR) could be employed to amplify DNA from individuals with HE and HPP, to be followed by DNA sequencing and eventually the elucidation of the molecular defects involved. Known defects resulting in HE were screened for. The defect was defined as SpaI/74, characterized by an increase in *a*

74 kDa polypeptide, leading to defective heterodimer self-association and thus instability of the membrane. The point mutation resulting in the membrane defect was previously described in two unrelated New Zealand kindred (Coetzer *et al.*, 1991). Here, the change (CGT ▸ TGT) is described in two unrelated Black individuals from local populations.

Part II deals with the presence and frequency of a TaqI restriction fragment length polymorphism (RFLP) in the human erythroid β spectrin gene in a group of controls and patients with known α spectrin defects. This study is of importance in linkage analysis.

In Part III, a mean ± standard deviation (SD) for erythrocyte membrane protein content, expressed as a ratio to band 3, was established in local control individuals. This provides a reference in our laboratory and enables comparison and diagnosis of local cases of HE or HS.

1.1. Cell Membranes

Cellular membranes provide compartmentalization and barriers to control movement of solutes and molecules. They are heterogeneous structures (Miller and Ray, 1992).

The lipid bilayer provides the specialized function of acting as a barrier, as well as a host of other functions; for example, transport of specific molecules in or out of the cell, catalysis of membrane-associated reactions and signal transduction, attributed to the integral proteins (Shohet and Bicknese, 1993). Lipid molecules constitute approximately 50 % of the mass of animal cell plasma membranes. They form bilayers spontaneously in the aqueous environment of the cell (In: Alberts *et al.*, 1983). Three types of lipids are found in cell membranes:

i) phospholipid,

ii) cholesterol,

iii) glycolipids.

Cell shape and deformability are defined by a membrane skeleton comprising actin filaments, intermediate filaments and microtubules, as well as a group of skeletal proteins (Pumplin and Bloch, 1993). The membrane skeleton is defined as the insoluble residue which remains after extraction of intact (red) cells or their isolated membranes with the nonionic detergent Triton X-100 (Lux, 1979).

Membrane skeletons have several distinctive features. Firstly, they are based on a protein (for example,

spectrin or fodrin) which offers flexible morphology to the membrane. Secondly, the membrane skeleton creates stability and provides mechanical support for the lipid bilayer and, thirdly, enables attachment of associated proteins (Pumplin and Bloch, 1993).

A theory that is rarely achieved in practice is that which suggests that molecules in the fluid phospholipid membrane bilayer are randomized by thermal motion. Rather, it is proposed by Edidin (1992) that cell membranes consist of lipid and protein domains, whose composition and function vary along the cell membrane surface.

1.2. Erythrocyte membranes

The erythrocyte membrane has provided a model for the study of cellular membranes over the past two decades and has served as a valuable system for studying membrane structure (Luna and Hitt, 1992; Shohet and Bicknese, 1993) by means of biochemistry and electron microscopy. The elucidation of the erythroid membrane structure has lead to proposals of non-erythroid skeleton structure (Pumplin and Bloch, 1993). Non-erythroid isoforms of the major membrane proteins have been identified. The study of the erythrocyte membrane provides insight into specialized membrane domains and how the membrane

skeleton governs basic cell processes, by extrapolation to non-erythroid cells; for example, adhesion and movement (Luna and Hitt, 1992).

Shohet and Bicknese (1993) define the architecture of the erythrocyte membrane, using biophysical approaches, in order to gain insight into the intimate arrangement of membrane components. Biochemistry and electron microscopy have limited resolving power and using the techniques of fluorescence resonance energy transfer, single photon radioluminescence and total internal reflection, Shohet and Bicknese are able to measure distances between molecules in membranes, from a few Angstroms to fractions of a micrometer. In their opinion, subtle rearrangements of architecture may be a mechanism for several haemolytic anaemias.

The normal behaviour of the red cell membrane depends on the highly specialized lipid bilayer composition and associated membrane skeleton proteins (reviewed in Sheetz, 1983; Shohet and Beutler, 1990). Recently, Gascard *et al.*, (1993) proposed the existence of functional phosphoinositide domains in human erythrocyte membranes.

The phospholipids of the erythrocyte membrane are non-randomly, asymmetrically distributed between the inner and outer leaflets of the lipid bilayer. Phosphatidylserine and phosphatidylethanolamine are concentrated in the inner leaflet, whilst phosphatidylcholine and sphingomyelin are concentrated in the outer leaflet (Op den Kamp, 1979). Litman, Hsu and Marchesi (1980) suggested a link between spectrin and specific membrane lipids. The association between phosphatidylethanolamine and phosphatidylserine and spectrin is reviewed in Shohet and Beutler (1990). Spectrin and band 4.1 bind with low affinity to negatively charged phospholipids in the lipid bilayer, such interactions being required for stability of the membrane (Bennet, 1989). The structural organization and interactions of membrane skeletal proteins have a role in regulating the rate of transbilayer movement of lipids across the erythrocyte membrane (Mohandas *et al.*, 1985). That is, they contribute to maintaining phospholipid asymmetry in the membrane lipid bilayer.

Spectrin, a water-soluble, high molecular weight protein, is the key component of the erythrocyte membrane skeleton (Litman, Hsu and Marchesi, 1980; Pumplin and Bloch, 1993), comprising approximately 75 % of its mass (Cohen, 1983). The functional units of spectrin are tetramers

comprised of heterodimers of the α and β subunits (Steck, 1974; Marchesi, 1979).

It has been determined, by means of high resolution negative staining electron microscopy, that the erythrocyte membrane skeleton is arranged in a hexagonal lattice (Liu, Derick and Palek, 1987), of spectrin, actin and band 4.1. (Figure 1). This lattice presents domains important for the function of erythrocytes and erythrocyte membranes and is required for mechanical stability (Edidin, 1992). Junctional complexes at the centre and at six corners of each hexagon are linked to the adjacent complexes by filaments of spectrin tetramers. Spectrin cross-bridges between adjacent junctional complexes are convoluted and relaxed. Liu, Derick and Palek (1987) propose that this contributes to the flexibility of spectrin and is important for deformability of the intact erythrocyte on its 120 day journey through the circulation. When spectrin was extracted from the membrane and incubated at 37°C under hypotonic conditions, there was an increase in dimer content (with a concomitant decrease in tetramer content to 50 %) (Liu and Palek, 1980). Further studies of membrane skeletons enriched with spectrin dimers revealed considerable disruption of the hexagonal lattice (Liu,

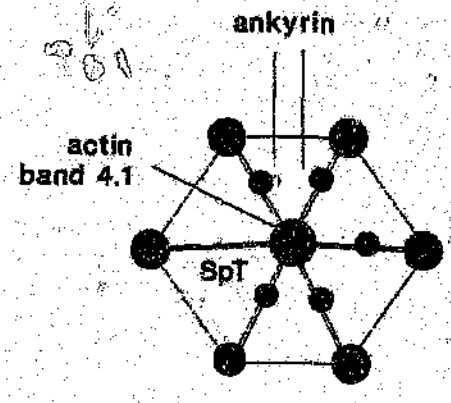


Figure 1. Schematic diagram of hexagonal lattice of red cell membrane.
A spread meshwork representing spectrin tetramers (SpT) and their interaction with actin, band 4.1 and ankyrin.
(Liu, Derick and Palek, 1987).

Derick and Palek, 1987), as determined by electron microscopy.

Linkage of the skeleton to the membrane has been studied. Bennett and Branton (1977) examined the specific association between spectrin and the inner surface of the human erythrocyte membrane by measuring the binding of purified [^{32}P]-spectrin to inside out spectrin-depleted vesicles and to right side out membrane vesicles. Their results suggested that the attachment of spectrin to the cytoplasmic surface of the membrane is due to a selective protein-protein interaction, independent of erythrocyte actin and does not appear to be directly via band 3. However, band 3 could provide a primary anchorage for an intermediate protein, to which spectrin is bound directly. Subsequent work determined that the hexagonal meshwork of spectrin-actin and associated proteins is anchored to the membrane by ankyrin and protein 4.1 (Figure 2). Ankyrin links the anion exchanger (band 3) to the β subunit of spectrin (Bennett and Stenbuck, 1979; Bennett and Stenbuck, 1980; Weaver and Marchesi, 1984; Weaver, Pasternack and Marchesi, 1984). Ankyrin interacts with a number of membrane proteins and is thus capable of linking these proteins to the spectrin-actin skeleton (Davis, Davis and Bennett, 1989).

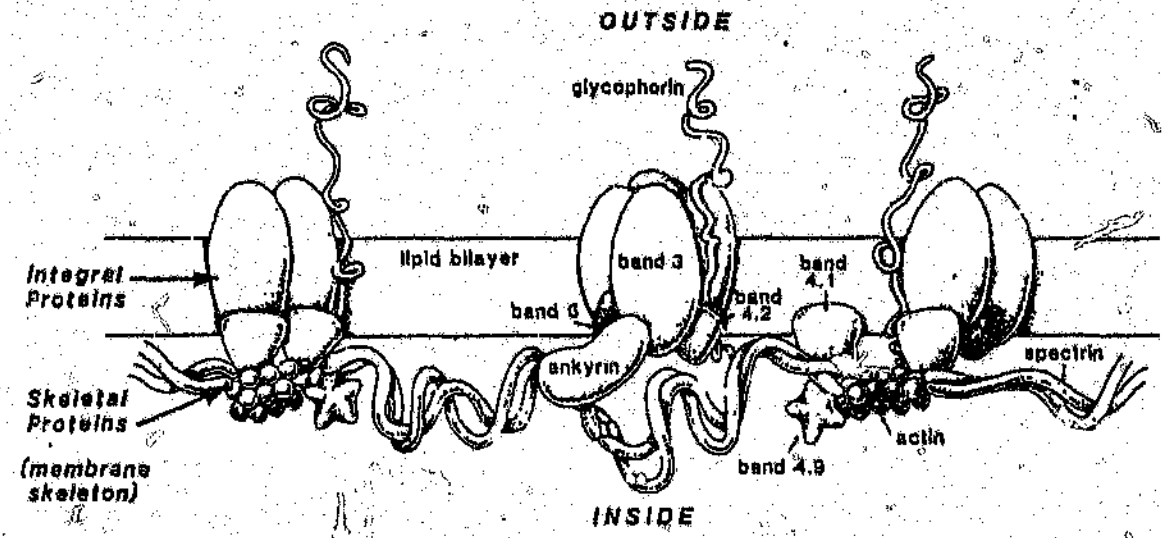


Figure 2. Distribution of proteins in the red cell membrane.

A schematic diagram by Palek (1987) depicting the distribution of proteins in the red cell membrane and their associations.

Litm( ), Hsu and Marchesi (1980) showed that spectrin associates with the inner surface of the red cell membrane by a non-covalent association with a multi-protein complex of band 3, 4.1 and 4.2. Pasternack *et al.*, (1985) showed that band 3 binds protein 4.1 and enables the formation of the spectrin-actin-4.1 complex. Protein 4.1 binds to both α and β spectrin near the ends of the tetramer (Tyler, Reinhardt and Branton, 1980) and also binds band 3 (Pasternack *et al.*, 1985; Jöns and Drenckhahn, 1992) and glycophorin C (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985) (Figure 2). Arginine-rich clusters of the cytoplasmic domain of band 3 serve as a binding site for the membrane-binding domain of protein 4.1. Both binding motifs are highly conserved and it is suggested that they may be associated with the binding of 4.1 to glycophorins (Jöns and Drenckhahn, 1992). Much attention has been focused on the spectrin-spectrin self-association and spectrin-actin-4.1 interactions but it has been suggested that the band 3-ankyrin association may also be important (Low *et al.*, 1991). Band 3-ankyrin linkage of the membrane skeleton to the lipid bilayer is essential for red blood cell stability in the presence of mechanical stress. When the intracellular pH was increased above 8.5, band 3 was gradually released from the membrane, leaving the skeletal interactions intact, but resulting in

instability of the membrane. This lead to the conclusion that vertical linkages between the lipid bilayer and the skeletal proteins are crucial for mechanical integrity (Low *et al.*, 1991).

1.3. Major membrane and membrane-skeletal proteins

All of the major erythrocyte membrane proteins are affected by one or more post-translational modification events; for example, oxidation and proteolysis may be detrimental to protein function, whilst phosphorylation and fatty acid acylation may facilitate function by modifying the properties of a protein (as reviewed by Cohen and Gascard, 1992).

The membrane proteins are resolved according to the methods of Laemmli (1970) and Fairbanks, Steck and Wallach (1971) by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3) (reviewed by Zail, 1986; Shohet and Beutler, 1990).

1.3.1. Spectrin

Spectrin is a heterodimer consisting of an α (Mr = 240 kDa) and a β (Mr = 220 kDa) subunit (Steck, 1974; Marchesi, 1979; Winkelmann and Forget, 1993; Gallagher and Forget, 1993). The molecular weights of the spectrin subunits were established initially by means of

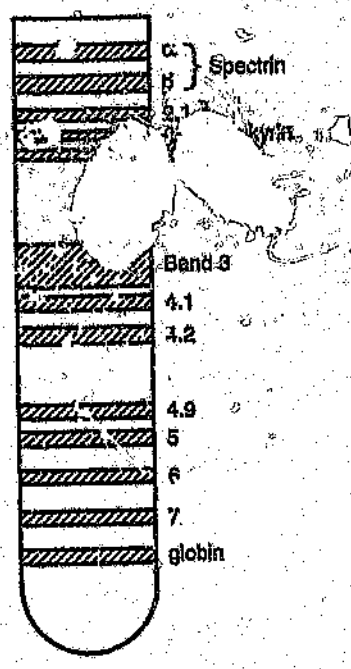


Figure 3. Fairbanks SDS-PAGE of the erythrocyte membrane. A schematic diagram of proteins of the red cell membrane, electrophoresed under reducing conditions using the method of Fairbanks, Steck and Wallach (1971). The proteins are resolved according to size and labelled according to Steck (1974).

SDS-PAGE. Subsequent cloning of the cDNA of the α and β spectrin subunits (Sahr *et al.*, 1990; Prchal *et al.*, 1987; Winkelmann *et al.*, 1988) revealed that the actual molecular weights are slightly higher than those indicated.

Both the α and the β subunits consist of multiple homologous segments, with a periodicity of 106 amino acids. It has been proposed that each homologous segment folds into a bundle of three α helices (Speicher and Marchesi, 1984; Yse *et al.*, 1990) (Figure 4).

The α and β subunits of spectrin associate in heterodimers which in turn are arranged head-to head to form tetramers and oligomers (Morrow and Marchesi, 1981). The subunits combine in an antiparallel fashion to form heterodimers, approximately 100 nm long (Shotton, Burke and Branton, 1979). This (side-to-side) association is a two-step process which occurs with high affinity in seconds (Speicher, Weglarz and DeSilva, 1992). The first step involves contact of complementary nucleation sites on each subunit, located near the actin-binding site of the heterodimer at the tail end of the molecule. In each nucleation site there is an 8-residue insertion in the normal 106-residue repeat unit. Since the repeating 106-residue motif is highly conserved, it is

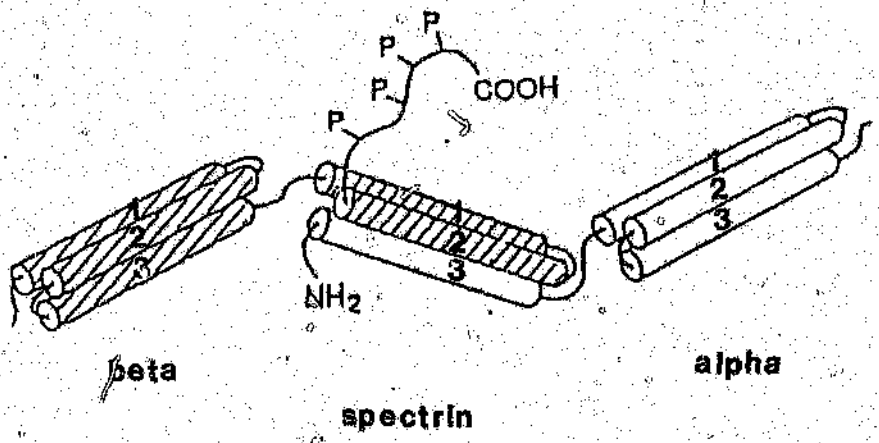


Figure 4. Schematic representation of the proposed α helical structure of spectrin.

Both the α and β chains are composed of multiple triple helical segments connected by short non-helical regions. The figure depicts heterodimer formation between the amino terminus, αI domain, of the α chain and the carboxy terminus, βI domain, of the β chain. The latter contains the phosphorylation sites (p).
(Speicher and Marchesi, 1984; Tse *et al.*, 1990).

thought that the insertion confers conformational properties on the nucleation region and that this may be responsible for the initial association. The remainder of the subunit chains quickly associate along their lengths, to form a twisted, rope-like structure (Speicher, Weglarz and DeSilva, 1992).

Winograd, Hume and Branton (1991) provide experimental evidence that relates the boundaries of the folded, conformational unit to the sequence of repeating motifs. There are non-homologous segments at the amino terminal of α spectrin and at the carboxy terminal of β spectrin. These partial segments are thought to have increased susceptibility to proteolysis which may play a role in defective spectrin heterodimer self-association in mutant spectrin variants (Winograd, Hume and Branton, 1991).

Functional domains have been identified on both subunits, this being indicative of a multifunctional protein (Morrow *et al.*, 1980; Speicher *et al.*, 1980). Similarly, structural domains were identified (Speicher *et al.*, 1982; Yurchenco *et al.*, 1982). The spectrin heterodimer has nine structural domains, resolved after limited tryptic digestion at 4°C (Figure 5). Five domains (αI-V) were defined for the α chain, whilst four domains (βI-IV) were defined for the β chain (Speicher *et al.*, 1982;

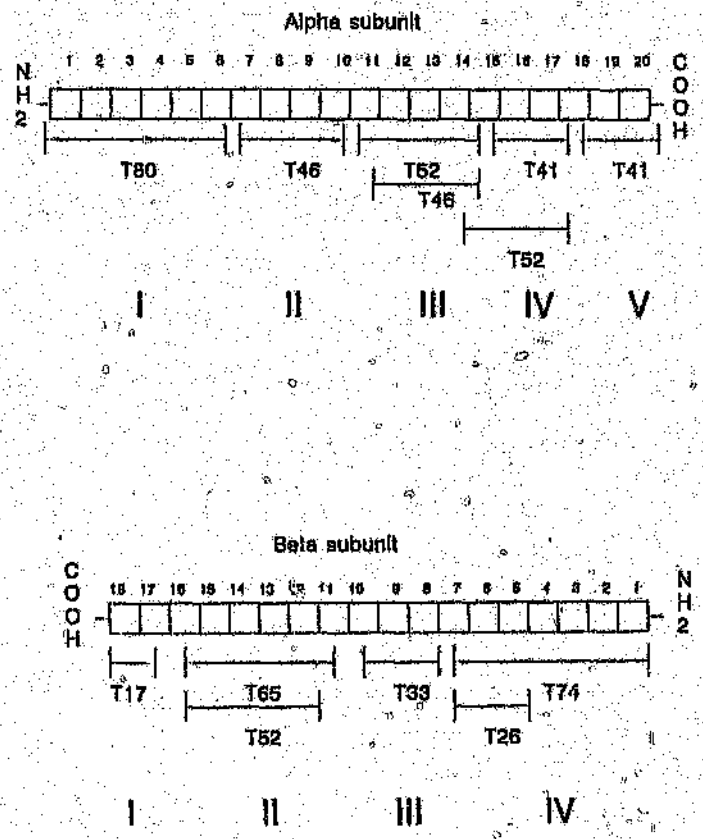


Figure 5. Anatomy of the spectrin molecule.
Schematic representation of the 12 kDa repeat units (106 amino acids each) of the α and β subunits of spectrin. The repeats are numbered from the amino terminus of each subunit, arranged in an antiparallel fashion. The tryptic peptides (T, size given in kDa) and the corresponding domain designation (Roman numerals) are shown.
(Palek, 1987; modified from Speicher and Marchesi, 1984).

Yurchenco *et al.*, 1982).

Spectrin heterodimers associate head-to-head to form tetramers, 200 nm long (DeSilva *et al.*, 1992). Formation of the head-to-head complex produces a triple-stranded conformational unit (Figure 4), differing from the rest of the molecule (Speicher *et al.*, 1993). Higher oligomers can also form by means of insertion of additional dimers at the tetramer site (Morrow and Marchesi, 1981). The tetramer binding site has been determined as a discrete region near the amino terminal of α spectrin (αI domain) and a complementary region near the carboxy terminal of the β subunit (Speicher *et al.*, 1993). Where heterodimer self-association is defective, the tetramer-dimer equilibrium shifts towards spectrin dimers (DeSilva *et al.*, 1992). The majority of spectrin mutations leading to haemolytic anaemias are localized to this region and are known to disrupt tetramer association. Spectrin is eluted from the membrane in the form of tetramers and dimers (Ungewickell and Gratzer, 1978). In solution, the spectrin dimer-tetramer equilibrium depends on temperature and salt concentration. Liu and Palek (1980) demonstrated that spectrin dimers and tetramers are in a reversible equilibrium in the membrane and that this environment favours spectrin tetramers. It was also shown that

conversion of spectrin tetramers to dimers, induced by incubation of membranes in hypotonic conditions at 37 °C, leads to diminished structural stability of the membrane.

In many proteins, including major erythrocyte proteins, phosphorylation or other forms of post-translational modification, may be a mechanism for regulation of protein function or dysfunction in erythroid disorders (Cohen and Gascard, 1992). Spectrin dimers can be phosphorylated, and it has been shown that four phosphorylation sites exist. These are clustered at the extreme carboxy terminal end of β spectrin (Harris and Lux, 1980). Liu and Palek (1980) established that the spectrin tetramer-dimer equilibrium is not influenced by spectrin phosphorylation-dephosphorylation.

The human α spectrin gene is located on chromosome 1q22-1q25 (Huebner *et al.*, 1985). Prchal *et al.*, (1987) characterized cDNA clones for human erythrocyte β spectrin. The β spectrin gene was assigned to chromosome 14 (Prchal *et al.*, 1987; Winkelmann *et al.*, 1988).

In 1990, the full length cDNA sequence of both the α (Sahr *et al.*, 1990) and the β (Winkelmann *et al.*, 1990) human erythroid spectrin genes were published. Sahr *et al.*, (1989) determined the sequence and intron-exon

boundaries encoding the αI domain of human spectrin. Kotula *et al.*, (1991) determined the exon-intron organization of the α spectrin gene, which does not correlate with the 106 aa repeat structure.

Several polymorphisms have been reported involving the αII and αIII domains of α spectrin, as well as the βIII domain of β spectrin (reviewed by Palek and Lambert, 1990; DiPaola *et al.*, 1993).

1.3.2. Ankyrin

Ankyrin (also known as 2.1) is a 210 kDa globular protein, whose function is to anchor spectrin to the cytoplasmic region of band 3 (Figure 2). Protein 2.1 is the progenitor of erythrocyte membrane proteins 2.2, 2.3 and 2.6, reviewed by Steck (1974), by a cascade of proteolytic events (Siegel, Goodman and Branton, 1980) or alternative splicing (reviewed by Bennett, 1992). Isoform-specific alternative splicing of protein 2.2 mRNA removes modulatory domains, resulting in altered binding function of ankyrin (Hall and Bennett, 1987; Bennett, 1992).

Ankyrin can be phosphorylated *in vitro* and this was shown to significantly reduce its affinity for either phosphorylated or unphosphorylated spectrin tetramers but

not spectrin dimers. It is suggested that ankyrin phosphorylation may provide an important mechanism for the regulation of the erythrocyte membrane skeleton (Lu, Soong and Tao, 1985).

Wallin *et al.*, (1984) identified two functional domains of protein 2.1, one associated with binding band 3 and the other involved with binding spectrin. At the same time, Marchesi and his colleagues identified two structural domains, after limited digestion at 0°C with trypsin (Weaver and Marchesi, 1984). One was a basic domain (82 kDa), sensitive to proteolysis and the other was a neutral phosphorylated domain of 55 kDa. The entire cDNA for the ankyrin gene has been cloned (Lambert *et al.*, 1990; Lux, John and Bennett, 1990). This has lead to refinements of the size determination of the ankyrin domains and the assignment of functions. Ankyrin consists of three domains. The N'-terminal (89-95 kDa) membrane-binding domain has a site for band 3 (Davis and Bennett, 1984b; Weaver, Pasternack and Marchesi, 1984; Davis and Bennett, 1990). Lux, John and Bennett (1990) describe a 33 amino acid repeat motif from cDNA information, within the N'-terminal. This motif is conserved in proteins involved with the cell cycle and differentiation. There is an intermediate domain (62 kDa) associated with spectrin binding (Bennett, 1978; Davis and Bennett,

1984a) and a C'-terminal regulatory domain (50-55 kDa) (Weaver, Pasternack and Marchesi, 1984; reviewed by Bennett, 1992; Peters and Lux, 1993).

The ankyrin gene has been assigned to chromosome 8 (Lambert *et al.*, 1990).

1.3.3. Band 3

Band 3 is the major erythrocyte transmembrane protein (Figure 2), comprising 25 % of the total membrane. Band 3 (monomer) has a molecular mass of approximately 90 kDa, but it is found in tetrameric form in the membrane (reviewed by Steck, 1978; Zail, 1986). Band 3 has two domains. The 55 kDa glycosylated transmembrane domain, including the carboxy terminal, is involved in chloride-bicarbonate exchange, whilst the cytoplasmic N'-terminal domain (40 kDa) provides binding sites for ankyrin (Low *et al.*, 1991), protein 4.1 (Jöns and Drenckhahn, 1992; Lombardo, Willardson and Low, 1992), 4.2 and some glycolytic enzymes (reviewed by Cohen and Gascard, 1992). Phosphorylation of band 3 is mainly on serine and threonine residues of the cytoplasmic domain. However, where tyrosine phosphorylation occurs, enzyme binding (glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase and aldolase) is inhibited. It is suggested that phosphorylation of band 3 may regulate the

anion transport function of the protein (reviewed by Cohen and Gascard, 1992).

The full length cDNA sequence of band 3 has been determined (Tanner, Martin and High, 1988). The gene for band 3 has been localized to chromosome 17 (Showe, Ballantine and Huebner, 1987).

1.3.4. Protein 4.1

Protein 4.1 serves a dual function. It increases the affinity of association between spectrin and F-actin and links the skeleton to the membrane, by association with glycophorins (Leto *et al.*, 1986; Shahrokh, Verkman and Shohet, 1991) and the N'-terminus of band 3 (Lombardo, Willardson and Low, 1992). Band 4.1 binds to lipids, namely phosphatidylserine (Cohen *et al.*, 1988). Protein 4.1 is phosphorylated. Several protein 4.1 analogues are found in non-erythroid cells. The gene for band 4.1 has been assigned to chromosome 1 (Conboy *et al.*, 1986).

Protein 4.1 exhibits tissue- and development specific heterogeneity in molecular weight, subcellular localization and primary amino acid sequence (Conboy *et al.*, 1991). Tang *et al.*, (1990); Huang *et al.*, (1993) determined that combinatorial splicing of exons generates at least five isoforms in complex tissue-specific

patterns. One motif is specifically expressed during erythroid maturation and is required for erythroid spectrin-actin binding.

Dramatic changes in morphology and membrane mechanical properties, largely due to the reorganization of the membrane skeletal protein network, occur during erythroid differentiation. It has been shown that protein 4.1 isoforms are expressed and localized differentially during erythropoiesis and that they have diverse functions, which have yet to be established (Chasis *et al.*, 1993). Conboy *et al.*, (1986) cloned the protein 4.1 cDNA in order to characterize the function of protein 4.1 in erythroid and non-erythroid cells.

The major isoform in human erythrocytes is a doublet of approximately 80 kDa. The difference in mobility between the two polypeptides is due to the deamidation of two asparagine residues at positions 478 and 502 in the carboxy-terminal region of protein 4.1 (Inaba *et al.*, 1992). Deamidation at the 502 residue occurs more slowly than at the 478 residue and progresses as the erythrocyte matures. This is responsible for the observed conversion of 4.1b to 4.1a. The ratio of 4.1a to 4.1b increases as red blood cells age, that is, immature cells have a decreased ratio (Mueller *et al.*, 1987). Deamidation is

thus a marker of aging. Inaba *et al.*, (1992) suggest that deamidation may also result in a conformational change of the molecule, which may affect the biochemical characteristics and functions of protein 4.1 during erythrocyte aging.

It is suggested by Conboy *et al.*, (1991) that there are functional domains in protein 4.1 responsible for interaction with other membrane skeletal proteins and that developmentally controlled alternative RNA splicing in the spectrin-actin binding region may regulate the biogenesis of membranes and the mechanics of erythropoiesis. Four functional domains were originally described by Leto and Marchesi (1984). These domains include a 30 kDa N'-terminal membrane-binding domain (Anderson and Lovrien, 1984) with glycophorin and ATP-binding ability (Pasternack *et al.*, 1985), two phosphorylated domains of 16 and 10 kDa and a C'-terminal spectrin-binding domain of 24 and 22 kDa in protein 4.1a and 4.1b, respectively (Tang *et al.*, 1990).

1.3.5. Protein 4.2

The function of band 4.2 (pallidin), 72 kDa, is unknown. Protein 4.2 comprises 5 % by mass of the red cell membrane (Lux and Becker, 1989). Protein 4.2 is located on the inner surface of the membrane. It is associated,

with high affinity, with the cytoplasmic domain of band 3 (Korsgren and Cohen, 1986; Korsgren and Cohen, 1988; Najfeld *et al.*, 1992) and has an interaction with ankyrin in solution (Korsgren and Cohen, 1988).

The full length cDNA has been cloned (Korsgren *et al.*, 1990) and the protein 4.2 gene has been assigned to chromosome 15 (Najfeld *et al.*, 1992).

It has been shown that protein 4.2 is N'-myristolated (Risinger, Dotimas and Cohen, 1992). This is important for membrane binding and may facilitate the association with band 3 or membrane lipids.

1.3.6. Protein 4.9

The function of protein 4.9 (dematin) in the red cell is unknown. Protein 4.9 is a 48 kDa phosphoprotein. It is possibly involved in organizing erythrocyte actin, since it has been shown to cosediment with actin (Siegel and Branton, 1985). It was suggested that protein 4.9 holds short actin filaments together to allow interaction with spectrin tetramers during red cell development.

1.3.7. Actin

Erythrocyte actin is also known as band 5, as seen on SDS-PAGE (Figure 3). It has a molecular mass of 42 kDa

and, in red cells, is mainly of the β actin isotype. Fifteen F-actin monomers are bundled at each junctional complex of the hexagonal skeletal lattice (Atkinson, Morrow and Marchesi, 1982) (Figures 1 and 2).

Actin copurifies with spectrin in a functional relationship involved in the maintenance of red cell shape, Pinder *et al.*, (1978) and is an important component of the spectrin-actin-band 4.1 skeletal complex. Human erythrocyte actin resembles muscle actin. The incorporation of agents influencing actin polymerization in resealed membranes leads to abnormal deformability (Nakashima and Beutler, 1979). That is, actin functions as a membrane structural element to maintain erythrocyte membrane deformability.

1.3.8. Band 6

Band 6 is also known as glyceraldehyde-3-phosphate dehydrogenase.

1.3.9. Band 7

Band 7 is resolved on Fairbanks SDS-PAGE as a 28 kDa protein, whilst on the Laemmli discontinuous buffer SDS-PAGE system three components are determined, that is, 30 kDa, 28 kDa and 26 kDa (Lande, Thiemann and Mentzer, 1982). Band 7 is noted in renal tubules and is believed

to be a member of an ancient membrane channel family (Preston and Agre, 1991), Band 7 may be important in erythrocyte dehydration in sickle disease and in perturbations of water and electrolyte balance in stomatocytic red cells (Lande, Thiemann and Mentzer, 1982).

1.3.10. Glycophorins

Glycophorin A, B, C and D belong to a group of red blood cell transmembrane proteins (reviewed by Cartron and Rahuel, 1992; Chasis and Mohandas, 1992). They are diverse in function.

Glycophorin A (GPA) has a high sialic acid content which provides a negative surface charge on mature erythrocyte membranes (Chasis and Mohandas, 1992). This minimizes cell-cell interactions in the circulation. Glycophorin A carries the MN blood groups, whilst glycophorin B carries the S group (Cartron and Rahuel, 1992).

The cytoplasmic domain of glycophorin C (GPC) interacts with protein 4.1, thus contributing to the regulation of erythrocyte shape, deformability and stability (Anderson and Lovrien, 1984; Anderson and Marchesi, 1985; Reid *et al.*, 1990).

1.4. The erythrocyte

Erythrocytes are heterogeneous with respect to density, size, osmotic fragility, rheology, metabolism and function (Vaysse, Gattegno and Pilardeau, 1992). Fractionation of erythrocytes according to density results in separation according to age. That is, the older the cell, the greater the density (Vaysse, Gattegno and Pilardeau, 1992).

The removal of normal erythrocytes from the circulation, by the spleen, is due to :

i) modification of phospholipids or decreased asymmetry of phospholipids,

ii) modification of carbohydrate residues,

iii) modification of proteins, such as degradation of band 3 by calpain (Kosower, 1993).

Older erythroid cells are marked by the exposure of a senescent cell antigen on band 3. These cells are targeted for destruction by phagocytosis (Kosower, 1993).

Erythrocytes with membrane defects are prematurely removed from the circulation, resulting in haemolytic anaemias.

1.5. Disorders of the red cell membrane

Deficiencies or dysfunctions of red cell membrane skeletal proteins lead to erythrocytes of abnormal shape and with altered deformability. Increased membrane rigidity results in premature removal of erythrocytes from the circulation, resulting in haemolysis.

1.5.1. Hereditary spherocytosis (HS)

The progressive loss of membrane surface area leads to increasingly spheroidal cells, with increased osmotic fragility. This results in increased membrane rigidity and increased susceptibility to destruction in the spleen. Vertical linkages between the lipid bilayer and the membrane skeletal proteins are important for the mechanical integrity of the membrane (Low *et al.*, 1991). This mechanism is important in the pathobiology of HS (Figure 6) (Palek, 1987).

Most individuals with HS have a spectrin deficiency, however, deficiencies or defects of ankyrin, band 3 and 4.2 have also been identified in patients presenting with HS (reviewed by Palek and Sahr, 1992). Subjects with the autosomal dominant form of HS are clinically less severe than those with the apparently recessive form (as reviewed by Lux and Becker, 1989). Complications include mild to moderate anaemia, splenomegaly and jaundice.

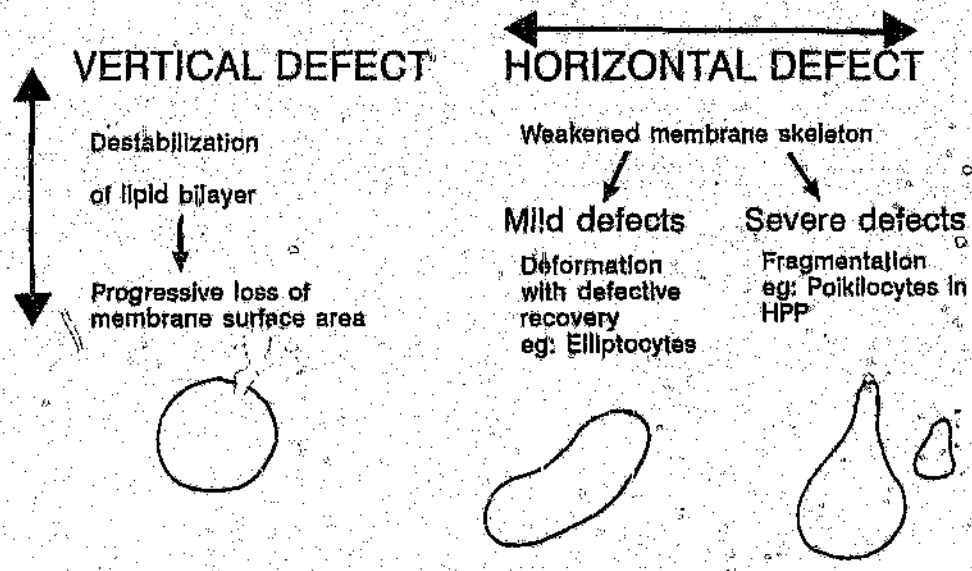


Figure 8. Pathobiology of the membrane lesion in hereditary haemolytic anaemias.

Vertical linkages between the lipid bilayer and the membrane skeletal proteins are crucial for membrane integrity and mechanical stability. Destabilization of the lipid bilayer leads to a loss of membrane material, resulting in increasingly spheroidal cells.

Defective heterodimer self-association results in a weakened skeleton. In mild defects, this leads to a defective recovery from elliptical deformation. More severe defects are associated with destabilization of the membrane, resulting in poikilocytosis. In addition, HPP erythrocytes are partially deficient in spectrin and as a result they lose surface area and form microspherocytes.
(Palek, 1987).

1.5.2. Hereditary elliptocytosis (HE)

This section includes a discussion of Hereditary Pyropoikilocytosis (HPP). In peripheral blood smears from patients with HE the majority of erythrocytes are elliptical (Figure 6). However, this condition is often symptomless, and in some cases the percentage elliptocytes is as low as 10 %, and detected by chance.

HPP is a severe, recessive haemolytic anaemia associated with poikilocytes, microspherocytes and fragmentation on the peripheral blood smear. This is a molecularly heterogeneous group of patients whose red cells are sensitive to thermal stress and who are commonly compound heterozygotes for defective spectrin heterodimer self-association and decreased spectrin content (reviewed by Palek and Sahr, 1992) (Figure 6).

There is a marked heterogeneity in the clinical expression of HE and HPP, ranging from an asymptomatic carrier state to severe transfusion dependent haemolytic anaemia (Coetzer *et al.*, 1990a). Clinical features, when present, include mild haemolysis, periodic jaundice and a degree of splenomegaly (Nurse, Coetzer and Palek, 1992). HE is inherited in an autosomal dominant fashion (Palek and Lux, 1983).

At a molecular level, HE is a heterogeneous group of red cell membrane disorders (reviewed by Lux and Becker, 1989; Palek and Sahr, 1992). Several different membrane defects give rise to elliptical morphology. Several band 4.1 defects have been described (reviewed by Palek, 1987; Palek and Lambert, 1990; Palek and Sahr, 1992). Other defects, involving protein 4.2 or the glycophorins have been identified in HE, but are less common. It has been noted that a decrease in protein 4.2 is seen in several types of inherited haemolytic disorders (Cohen, Dotimas and Korsgren, 1993), associated with increased osmotic fragility and abnormal red cell shape (Najfeld *et al.*, 1992). Spectrin mutations are most common, usually involving the spectrin heterodimer self-association site (reviewed by Palek, 1987). At the protein level, this defect can be detected by analysis of the content of spectrin dimers and tetramers in spectrin extracts. The percentage of spectrin dimers is increased in these patients (see below). All patients with HPP are also partially deficient in spectrin (Coetzer and Palek, 1986).

At the level of protein structure, abnormal spectrin variants are identified by limited tryptic digestion, followed by electrophoretic analysis of the resulting tryptic peptides (Speicher *et al.*, 1980). Under these

conditions, limited tryptic digestion gives rise to a reproducible pattern of peptides, that can be resolved by one (1D) or two dimensional (2D) SDS-PAGE (reviewed by Palek, 1987). In normal individuals, the spectrin heterodimer self-association site, the αI domain, appears as the most prominent spot on 2D SDS-PAGE. The αI domain of α spectrin is an 80 kDa fragment, usually resistant to further limited tryptic digestion (Speicher *et al.*, 1982). In contrast, the 80 kDa spot is markedly decreased in digests of spectrin from patients with HE with defective heterodimer self-association. New spots are visible of lower mass, commonly 74, 46 (Lawler, *et al.*, 1984) or 65 kDa (Lawler *et al.*, 1985). These spectrin variants are designated SpαI/74, SpαI/46 and SpαI/65 respectively. A SpαI/50b variant has also been described (Marchesi *et al.*, 1987). The abnormal peptides originate from the αI domain of α spectrin and can be demonstrated by the use of polyclonal antibodies against the αI domain (Lawler *et al.*, 1984).

At the level of spectrin function, most defects in the red cell membranes of HE subjects are characterized by defective self-association of spectrin dimers to form tetramers. This is studied by determination of the distribution of spectrin dimers, tetramers and oligomers, at 4°C, in crude spectrin extracts of the red cell

membrane (Liu *et al.*, 1981), by means of non-denaturing PAGE. When membranes are extracted at 4°C the relative proportion of spectrin dimers and tetramers in the crude spectrin extract reflects their distribution in the membrane *in situ* (Ungewickell and Gratzer, 1978). Spectrin dimer content is expressed as a percentage of the total spectrin dimer and spectrin tetramer content. All individuals with αI spectrin variants exhibit an increased percentage of spectrin dimers. The percentage spectrin dimers correlates with the clinical severity of the disorder (Coetzer *et al.*, 1987; Coetzer *et al.*, 1990b). Factors influencing this parameter include functional differences between spectrin mutants, the amounts of spectrin mutants present, the stability of the mutant and the possibility of a superimposed genetic defect, involving spectrin synthesis. Hence, HPP subjects, having the highest percentage of spectrin dimers (SpD) as well as a decreased spectrin content, present with the most severe haemolytic anaemia (Coetzer *et al.*, 1987; Coetzer *et al.*, 1990b). In control subjects spectrin exists mainly in the tetrameric form (SpD = 5.0 ± 3.6 %). HE subjects with the SpαI/65 variant have a smaller increase in SpD percentage (SpD = 18.4 ± 4.8 %), when compared with the SpαI/74 or SpαI/46 variants (SpD = 39.4 ± 6.3 %) (Coetzer *et al.*, 1987).

SpaI/74 is the variant that most severely affects individuals, followed by SpaI/46 and finally, SpaI/65, which is the mildest defect (Coetzer *et al.*, 1990a).

Molecular analysis of spectrin variants has revealed several point mutations (reviewed by Palek and Sahr, 1992). The SpaI/46 abnormality is heterogeneous, with three different mutations thus far described (reviewed by Palek and Sahr, 1992). One of these mutations, studied by Gallagher *et al.*, (1992), was identified as a point mutation at position 207 in the αI domain of the α spectrin chain, causing the replacement of a highly conserved leucine residue with a proline. Position 207 is 51 residues away from the amino terminal side of the abnormal tryptic cleavage site. The mutation occurs in helix 2 directly opposite the abnormal cleavage site in helix 3, thus providing support for the proposed triple helical model of spectrin repeats (Figure 4). Further, proline is a helix breaker, thus it was predicted that the disruption of the α-helical structure may result in a defective spectrin chain and hence defective spectrin heterodimer self-association.

The SpaI/65 variant is due to a single mutation involving an insertion of a leucine residue at position 154 (Coetzer *et al.*, 1990b). The SpaI/74 variant is the most

heterogeneous at the molecular level and mutations at five different codons have been noted (reviewed by Palek and Sahr, 1992).

Coetzer *et al.*, (1991) describe a "hot spot" at codon 28 of α spectrin associated with four known point mutations. The arginine residue at codon 28 is changed to histidine, cysteine, serine or leucine. The arginine residue at position 28 seems to be required for functional stability and self-association of spectrin heterodimers. These mutations all give rise to the spectrin variant SpaI/74. The amino acid change alters the conformation of the protein, making a normally protected site at residue 45 or 48 more accessible to tryptic digestion, resulting in increased amounts of the 74 kDa peptide.

Floyd *et al.*, (1991) confirmed that the arginine residue at codon 28 may be mutated to serine or leucine, and further determined that a point mutation at codon 48 changes lysine to arginine, similarly resulting in the SpaI/74 variant. Point mutations at codons 43 and 49 also result in the SpaI/74 variant (Morle *et al.*, 1990).

In conclusion, HE SpaI/74 variants belong to a molecularly heterogeneous group, characterized by an

altered tryptic digest pattern and an increased SpD percentage. The mutation predominantly involves α spectrin, however, more rarely β spectrin may be implicated (Lecomte *et al.*, 1990; reviewed by Palek and Sahr, 1992). In both cases, there is defective heterodimer self-association and hence decreased spectrin tetramer assembly on the membrane. The horizontal interactions of the membrane skeleton are compromised (Palek, 1987). This reduces the stability of the erythrocyte membrane and results in loss of cell shape and the clinical manifestations of HE (Figure 6).

2. Part I: Characterization of a membrane defect in two local cases of Hereditary Elliptocytosis

The molecular characterization of membrane defects requires techniques such as the polymerase chain reaction (PCR) and DNA sequencing, as well as protein biochemistry. Protocols discussed in the literature make use of the availability of the DNA sequences for the α and β subunits of spectrin in the elucidation of molecular defects. These techniques were applied in determining the molecular defect associated with HE in two local probands.

2.1 Subjects

Two unrelated black South African probands presented with HE (Figure 7) (Table I).

The first (TM), a 14 year old male, was previously described by Coetzer and Zail (1981; 1982), by means of protein analysis only, when he presented with HE associated with neonatal poikilocytosis. TM has a haemoglobin (Hb) level of 10.6 g/dl, which is decreased with respect to the normal value due to the anaemia (Table I). The packed cell volume or haematocrit (Hct) is decreased and haemolysis was noted in the peripheral blood sample. Mean cell volume (MCV) is decreased, indicating a microcytic anaemia. Reticulocyte production

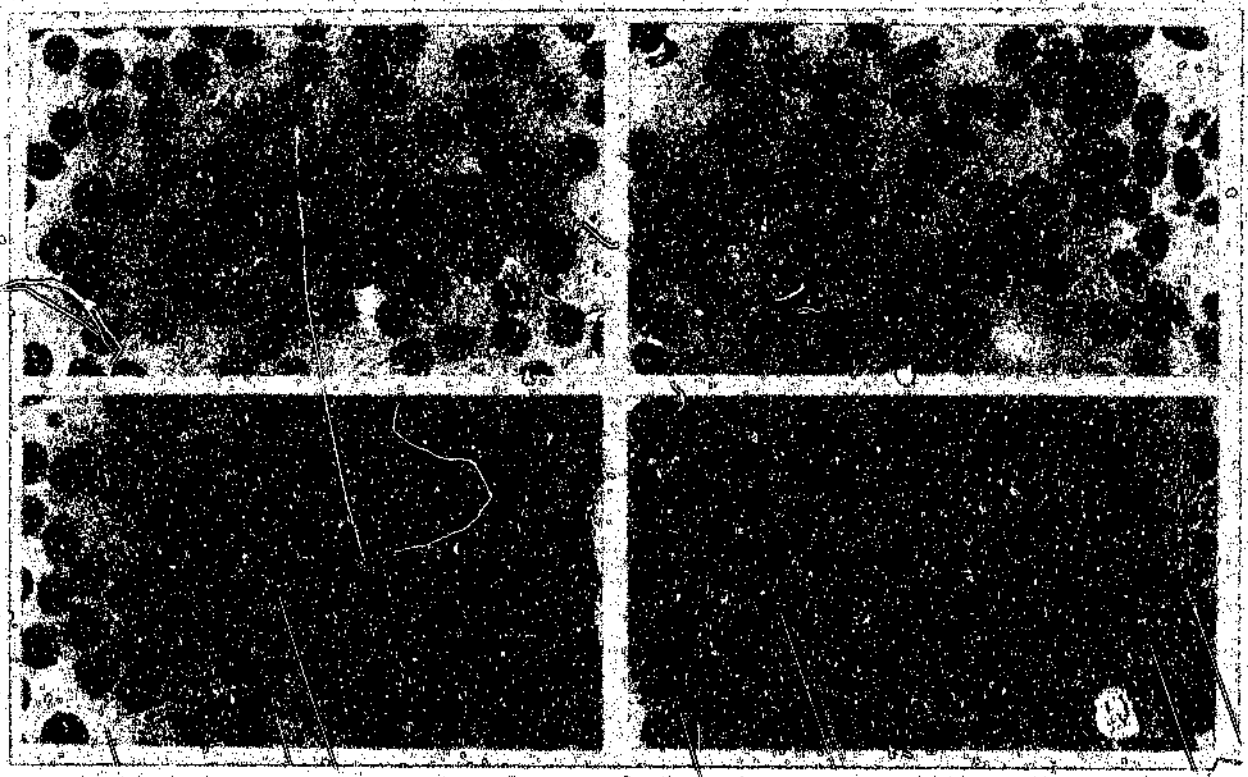


Figure 7. Peripheral blood smears.
Peripheral blood smears, stained with Wright Giemsa stain, showing :
a) Erythrocytes from a normal individual;
b) Erythrocytes from TL, with elliptocytosis;
c) Erythrocytes from TM (mother) who is haematologically normal;
d) Erythrocytes from TM, with elliptocytosis.

Table I. Clinical data.

Subject	Hb	Hct	MCV	Retic
	g/dl	%	fl	%
Normal values (M)	14-18	41-51	82-100	0.5-2.0
(F)	12-16	37-49	82-100	0.5-2.0
TM	10.6	30.1	68.7	8.4
TM (mother)	12.2	35.4	87.2	---
TL	13.5	37.1	85.4	11.0

is increased in order to compensate for the anaemia. There are no siblings and only the mother is available for study. She is haematologically normal (Coetzer and Zail, 1982), however, the haematocrit is on the lower side of normal (Table I).

The second proband (TL) is a 15 year old black male. TL has a low haemoglobin level (Table I) and the haematocrit is decreased due to haemolysis. The MCV is within the normal range, however, reticulocyte production is increased. No siblings are available for study and only the father was investigated. He is haematologically normal.

Neither proband has been splenectomised.

Human ethics clearance (# 25/1/92) was obtained by Dr T Coetzer in order to obtain blood specimens from normal and affected individuals.

2.2. Materials and methods

Chemicals used for protein analysis were reagent grade and all solutions were prepared using distilled, deionised water. Molecular biology grade reagents were used for DNA analysis and solutions were prepared in Milli-Q water.

A list of chemicals and suppliers is provided in the Appendix.

2.2.1. Erythrocyte membrane protein analysis

The methods employed were described previously by Dodge, Mitchell and Hanahan (1963) and Coetzer and Falek (1986). Red cell membrane "ghosts" were prepared by lysis centrifugation. Blood was collected from patients and suitable healthy controls in ACD and centrifuged at 800 *g* at 4°C, for ten minutes. The plasma was aspirated and the buffy coats used for genomic DNA extraction (2.2.3.1.). The erythrocytes were washed three times in approximately ten volumes of cold physiological saline (0.9 % NaCl), to remove any residual white cells. One to two ml red blood cells were lysed by the addition of 40 ml ice-cold hypotonic buffer.

Hypotonic buffer was prepared as follows:

3 mM Na_2HPO_4

0.1 mM EDTA

Adjust pH to 8.0 with NaH_2PO_4.

A 400 mM stock of PMSF was prepared fresh in DMSO and was added to a final concentration of 0.1 mM in the buffer, with vigorous stirring, at room temperature, just before use. The buffer was then cooled to 4°C.

The lysate was centrifuged at 15 000 rpm (JA-17 rotor) in a Beckman J2-21 centrifuge for 15 minutes at 4°C. The supernatant was carefully aspirated. The small, tightly packed "button" of white cells underneath the "ghost" pellet was removed to prevent contamination of the red cell membranes with proteases. The "ghosts" were washed three times in the hypotonic lysis buffer until almost white membranes were obtained and the membranes resuspended in approximately one volume of lysis buffer.

Five aliquots of membranes (80 µl each) were solubilized (2.2.1.2.) and stored at -20°C for SDS-PAGE analysis. The remainder was used immediately for spectrin extraction, after removing aliquots for protein determination.

2.2.1.1. Protein determination

The amount of protein in the membrane sample (µg) was estimated using the method of Bradford (1976). Bradford reagent was prepared as follows:

50 mg Coomassie brilliant blue G-250

25 ml 95 % ethanol

50 ml 85 % phosphoric acid (w/v)

Make up to 500 ml with water and filter through Whatman No.1.

Store at 4°C in the dark.

Membrane aliquots, of 5 µl each, were prepared by adding one to two µl 10 N NaOH to solubilize the membranes and hence to prevent underestimation of protein content, and one ml Bradford reagent. The samples were mixed by gentle vortexing, following which the sample was assayed spectrophotometrically at 595 nm, against a standard curve of dilutions of BSA (Albumin, fraction V), ranging from 1.37 µg/ml to 20.55 µg/ml.

2.2.1.2. Laemmli SDS-PAGE

Membrane protein analysis was carried out by electrophoresis of the proteins in the Laemmli discontinuous buffer system (1970).

The Laemmli resolving gel with a final concentration of 12 % acrylamide was prepared as follows :

12 ml 30 % acrylamide (w/v)
3.2 ml 1 % bis (w/v)
7.5 ml 4x resolving gel buffer (1.5 M Tris pH 8.8)
0.16 ml 10 % SDS (w/v)
7.0 ml water
0.2 ml 10 % APS (w/v) (fresh)
0.015 ml TEMED

Once poured into the gel apparatus (Hoefer 12 cm x 15 cm x 0.15 cm), the gel was overlayered with 300 µl isopropanol and allowed to set for 30 minutes. Once polymerized, the isopropanol was removed.

The 4 % stacking gel was prepared as follows:

1.3 ml 30 % acrylamide (w/v)
1.0 ml 1% bis (w/v)
2.5 ml 4x stacking gel buffer (0.5 M Tris pH 6.8)
0.02 ml 10 % SDS (w/v)
4.9 ml water
0.2 ml 10 % APS (w/v) (fresh)
0.0075 ml TEMED,

and was poured on top of the resolving gel, thus forming

the discontinuous system.

Erythrocyte membranes (2.2.1.) (80 µl) were solubilized in 5x suspension solution (50 mM Tris, 5 mM EDTA, 5 % SDS, 25 % sucrose, pH to 8.0 with HCl) (add one volume suspension solution to four volumes of membranes) and 20 µl 20 % (v/v) β-mercaptoethanol. The samples were boiled for one minute. Aliquots of 45 µg and 50 µg were electrophoresed at 75 V for 18 to 20 hours, in Laemmli running buffer (3.03 g Tris, 14.4 g Glycine, 1 g SDS in 1 litre water).

After electrophoresis, the gels were stained in Coomassie stain (0.5 g Coomassie R-250, 250 ml isopropanol, 100 ml acetic acid in 1 litre and filtered through Whatman No.1), for three hours to overnight, in order to visualise the proteins. Ten percent acetic acid was used to destain gels. Quantitation of membrane proteins, by scanning densitometry at 525 nm using a green filter, was carried out using a Helena Quick Scan Jr TLC densitometer. The area under the peaks was quantitated, and the protein 4.1 content expressed as a ratio to band 3.

2.2.1.3. Fairbanks SDS-PAGE

The non-linear gradient (3.5 - 17 % acrylamide) Fairbanks gel, according to the method of Fairbanks, Steck and Wallach (1971), was employed in order to resolve all the components of the red cell membrane.

The gel mixtures were prepared as follows:

Final [acrylamide]	3.5%	17 %
Component		
40 % acrylamide/1.5 % bis (w/v)	3.0 ml	3.4 ml
10x Tris-Acetate buffer *	3.4 ml	0.8 ml
10 % SDS (w/v)	0.68 ml	0.16 ml
Water	26.9 ml	1.62 ml
25 % glycerol (v/v)	---	2.0 ml
10 % APS (w/v) (fresh)	0.4 ml	0.02 ml
TEMED	0.11 ml	---
0.5 % TEMED (v/v)	---	0.5 ml

* A 10x Tris-Acetate stock buffer was prepared (400 mM Tris, 200 mM Sodium acetate, 20 mM EDTA, pH to 7.4 with glacial acetic acid).

The gel was poured into a Hoefer gel apparatus (12 cm x 15 cm x 015 cm), using a Hoefer gradient maker, as follows:

1. The gradient maker has two reservoirs separated by a screw tap, the front reservoir connected to a peristaltic pump via a second screw tap.
2. Close both taps.
3. Prepare both acrylamide mixtures and add the 3.5 % mixture (34.5 ml) to the rear reservoir.
4. Add the 17 % acrylamide mixture (8.5 ml) to the front reservoir.
5. Stir both gel mixtures at a moderate speed during the preparation and pouring of the gel.
6. Open the interconnecting screw tap and allow the interconnecting chamber to fill with 3.5 % acrylamide mixture. Close the tap.
7. A plunger is lowered into the 17 % acrylamide (front) reservoir until it is about 2 mm from the surface of the liquid, in order to prevent equalisation of the gel mixture volumes when the interconnecting tap is opened.
8. Clamp the tubing at the top of the plunger.
9. Apply the pump at a moderate speed.
10. Open the screw tap between the gradient maker and the pump.

11. Open the interconnecting screw tap, to allow the gel mixtures to mix.

12. The 17 % acrylamide mixture is pumped out first and is overlayered with decreasing concentrations of acrylamide.

Membrane aliquots were solubilized as for Laemmli SDS-PAGE (2.2.1.2.). Aliquots of 20 and 25 µg were electrophoresed at 35 V for 18 to 20 hours, in 1x Tris-Acetate buffer with 0.1 % SDS (w/v). Gels were stained and densitometric analyses carried out as described for Laemmli SDS-PAGE (2.2.1.2.). Membrane protein content was expressed as a ratio to band 3.

2.2.2. Spectrin extraction

Spectrin was extracted as described by Coetzer and Palek (1986), at 4°C. The pellet of white membranes was washed once at 17 000 rpm (in the Beckman JA-17 rotor) at 4°C for 20 minutes, with 30 volumes of ice cold low ionic strength spectrin extraction buffer (0.1 mM Na_2HPO_4, 0.1 mM EDTA, 0.1 mM DTT, pH to 8.0 with NaOH). This buffer was prepared fresh. The supernatant was aspirated and the membrane pellet was resuspended in 0.5 volumes of the remaining buffer. The sample was incubated overnight on ice, in order to extract spectrin from the membranes, and then ultracentrifuged in a Beckman ultracentrifuge at

45 000 rpm in a Ti50 rotor for 30 minutes at 4°C. The spectrin-containing supernatant was carefully removed. One volume of 10x digestion buffer (100 mM $NaPO_4$, 1.5 M NaCl, 50 mM β-Mercaptoethanol, 50 mM EDTA, pH 7.5) was added to nine volumes of the hypotonic extract, making the extract isotonic. Two aliquots of 5 µl each were removed for protein determination (2.2.1.1.).

2.2.2.1. Non-denaturing gel electrophoresis

One set of tube gels (15 tubes of 15 cm x 0.5 cm), poured to a height of 8 cm, was prepared, according to the method of Liu *et al.*, 1981, as follows:

i) 1 % agarose was dissolved by heating in water and cooled down to 42°C.

ii) The following were mixed together as described below:

40 % acrylamide/1.5 % bis (w/v)	3.125 ml
1 % agarose (w/v)	15.0 ml
10 x Tris-Acetate buffer (2.2.1.3.)	5.0 ml
Water	22.0 ml
1.5 % APS (fresh)	2.5 ml
0.5 % TEMED	2.5 ml

The acrylamide, 10x buffer and water were mixed and placed at 42°C. The agarose was prepared and added to the

acrylamide mixture, which was transferred to a stirrer and the APS and TEMED added immediately. The rack of tubes, which were covered at the bottom with Parafilm, was placed in a 37°C water bath and the tubes were filled with the mixture. The gel surfaces were overlayered with 2 to 3 mm of water. The rack was placed in a basin of ice for 3 minutes to set the agarose in the gels and care was taken to keep the tubes vertical. The gels were allowed to polymerize at room temperature for 30 minutes and then stored at 4°C under 1x Tris-Acetate buffer and covered with Parafilm.

Ten and 15 µg aliquots of isotonic spectrin were prepared for electrophoresis by the addition of 1/10 volume of 2 M (70 % w/v) sucrose in 50 % (w/v) bromophenol blue to the sample. The sample was applied to the tube at 4°C and electrophoresed at 20 to 22 V for 18 to 20 hours at 4°C, in 1x Tris-Acetate buffer with 80 µl β-Mercaptoethanol per litre, pre-cooled. After electrophoresis, the gels were extruded from the tubes and stained in Coomassie stain (2.2.1.2.). Densitometric analysis of the samples was performed as described previously (2.2.1.2.) in order to obtain the percentage SpD/(SpD + SpT), according to the method of Liu *et al.*,(1981).

2.2.2.2. Tryptic digest of spectrin extract

One mg/ml trypsin-TPCK was prepared in water and added to 500 µg isotonic spectrin in the ratio of 1:100 (w/w) of trypsin to spectrin. Limited digestion (Lawler *et al.*, (1984) was performed at 4°C on ice for 18 hours and was stopped by the addition of 50 mM PMSF (prepared in DMSO), to a final concentration of 5 mM. The sample was aliquoted into 2x 60 µg for one dimensional (1D) Laemmli SDS-PAGE (2.2.2.3.) and immunoblotting (2.2.2.4.) and 200 µg for freeze-drying, in a 1.5 ml Eppendorf tube, followed by two dimensional (2D) analysis (2.2.2.5.).

2.2.2.3. One dimensional analysis of tryptic digest

The tryptic digest of spectrin was analysed initially by Laemmli SDS-PAGE (10 % final acrylamide), prepared as follows:

<u>10 % Resolving gel</u>

7.5 ml 40 % acrylamide/1.5 % bis
7.5 ml 4x resolving gel buffer (1.5 M Tris pH 8.8)
11.55 ml water
0.15 ml 20 % SDS (w/v)
0.3 ml 0.2 M EDTA (pH 7.4)
1.5 ml 1.5 % APS (w/v) (fresh)
1.5 ml 0.5 % TEMED (v/v)

4 % Stacking gel

0.665	ml	40 % acrylamide/1.5 % bis
2.5	ml	4x stacking gel buffer (0.5 M Tris pH 6.8)
4.62	ml	water
0.05	ml	20 % SDS (w/v)
0.1	ml	0.2 M EDTA (pH 7.4)
1.0	ml	1.5 % APS (w/v) (fresh)
1.0	ml	0.5 % TEMED v/v)

The 60 µg aliquots of sample were electrophoresed at 65 V for 18 to 20 hours in Laemmli running buffer (2.2.1.2.). After electrophoresis, the gels were dismantled and stained with Coomassie stain (2.2.1.2.) or Western blotted onto nitrocellulose for immunodetection (2.2.2.4.).

2.2.2.4. Western blot analysis of tryptic digest

In order to positively identify SpaI variants it was necessary to blot the 1D Laemmli gel and probe with a polyclonal rabbit anti-human primary antibody prepared against the SpaI domain (Coetzer and Palek, 1986). The gel was soaked in transblot buffer (25 mM Tris, 192 mM Glycine, 20 % Methanol) (Towbin, Staehelin and Gordon, 1979) together with nitrocellulose membrane, for blotting. The blot was prepared by semi-dry blotting onto nitrocellulose with a Bio-Rad apparatus at

15 V/150 - 200 mA for 30 minutes at 4°C. The blot was rinsed in TBS (0.05 M Tris, 0.9 % NaCl, pH 7.5) and dried on blotting paper at room temperature. Efficiency of transfer was checked by:

(i) staining the residual gel in Coomassie stain (2.2.1.2.),

(ii) placing the blot in Ponceau S stain, diluted 1 in 10, in water, for two to five minutes. The stain washes off completely with water and does not affect the immunodetection.

For immunodetection the blot was incubated in 3 % BSA in TBS/0.05 % Tween 20, for 1 hour. All subsequent incubations were carried out at room temperature, with shaking. The blot was washed three times for five minutes each in TBS/0.05 % Tween 20. The primary antibody (rabbit anti-80 kDa) (prepared by Dr J. Lawler, Dept of Biomedical Research, St Elizabeth's Hospital, Boston) was diluted 1 in 1000 with 1 % BSA in TBS. This could be stored for several months at -20°C and re-used successfully three times. The blot was incubated for 18 to 20 hours and then washed as before. The secondary antibody (sheep anti-rabbit IgG-peroxidase) was diluted 1 in 1000 with 1 % BSA in TBS for use and could be stored at 4°C for 1 week. After washing, the blot was incubated

in the secondary antibody for 1 hour. The blot was washed twice as before and then the colour development and visualisation of bands was carried out as follows:

30 mg 4-chloro-1-naphthol, dissolved in
10 ml cold methanol
40 ml ice-cold TBS
25 µl 30 % H_2O_2 added just before incubation.

The blot was incubated for approximately 2 minutes, or longer if required, until the bands could be visualised, and then rinsed with water to remove any background staining.

2.2.2.5. Iso-electric focusing (IEF) and 2D SDS- PAGE

For improved resolution of any changes in tryptic digest pattern in a patient compared with a control spectrin extract, IEF analysis, based on a modified method of O'Farrell (1975) and 2D SDS-PAGE (Lawler *et al.*, 1984), were carried out.

The glass tubes (16 cm x 0.3 cm) were washed in chromic acid, rinsed with water and ethanol, dried at 60°C and sealed at their bases with Parafilm. They were placed vertically in a rack and marked to 12 cm. The gel mixture was prepared as follows:

(i) The acrylamide mixture for IEF gels was prepared fresh by mixing 2.838 g acrylamide and 0.162 g bis in 5 ml water and, once dissolved, the volume was made up to 10 ml with water.

(ii) The following were mixed together as indicated:

Component	[Final]
5.5 g urea	9 M
1.33 ml acrylamide mixture	4 %
1.8 ml water	
0.1 ml ampholyte pH 3 - 10	1 %
0.2 ml ampholyte pH 4 - 6	2 %
0.2 ml ampholyte pH 5 - 7	2 %

The mixture was heated at 40°C and, when the urea had dissolved, made up to 10 ml with water. Ten µl 10 % APS (w/v) (fresh) was added and the mixture was degassed for 1 minute, before the addition of 200 µl NP-40 and 7 µl TEMED. The mixture was inverted gently. Ten to twelve gels were poured to the 12 cm mark, using a syringe and a long needle, taking care not to trap air bubbles. They were overlayered with 10 µl 8 M urea (stored at room temperature) and allowed to set for 2 hours, at which time the urea was replaced with 20 µl lysis buffer (9.5 M urea, 2 % NP-40 (w/v), 0.8 % ampholyte pH 5-7 (v/v), 0.8

% ampholyte pH 4-6 (v/v), 0.4 % ampholyte pH 3-10 (v/v), 5 % β-mercaptoethanol (v/v)) and left to set for a further two hours. The gels could be stored at room temperature for not more than a week, before use.

For electrophoresis of freeze-dried samples (2.2.2.2.) the lower chamber of the IEF apparatus was filled with 500 ml anode buffer (0.01 M phosphoric acid) and the tubes were placed in the running tank, removing any air bubbles which may have become trapped underneath. The lysis buffer was removed from the surface of each tube gel and replaced with 20 µl of fresh buffer. This was overlayered with freshly degassed cathode buffer (0.02 M NaOH, degassed for 1 hour) and the upper reservoir filled. The gels were pre-equilibrated as follows:

200 V, 15 minutes
300 V, 30 minutes
400 V, 30 minutes

After the equilibration, the cathode buffer was removed from the tank and from the tops of the gels. Ten µl of overlay solution (9 M urea, 0.4 % ampholyte pH 5-7, 0.4 % ampholyte pH 4-6, 0.2 % ampholyte pH 3-10, stored at -20°C) was syringed onto the remaining lysis buffer which was then removed, taking care not to allow any cathode

buffer to touch the top of the gel. The freeze-dried sample (200 µg) was resuspended in 100 µl lysis buffer to yield 2 mg/ml. The sample was then loaded onto the gel, under the overlay solution. Fresh degassed cathode buffer was syringed over the overlay solution and the upper tank was filled as before. The samples were electrophoresed at 400 V for 16 hours (8 kV.hours) and then for a further 800 V for 1 hour.

After focusing, the tops of the gels were marked with bromophenol blue and then the gels were extruded and equilibrated in O'Farrell buffer with β-mercaptoethanol for 2 hours before loading onto the second dimension SDS-PAGE or snap-freezing for storage at -70°C.

O'Farrell buffer was prepared as follows:
10 % glycerol (w/v)
2.3 % SDS (w/v)
0.0625 M Tris pH 6.8
Make up to 95 ml with water.

For use, 9.5 ml O'Farrell buffer was added to 0.5 ml β-mercaptoethanol.

The second dimension was run on a 10 % SDS-PAGE Laemmli system as described previously (2.2.2.3.), except that

the gel dimensions were 12 cm x 15 cm x 0.3 cm.

The equilibrated IEF gels were placed onto the stacking gel surface, taking care to avoid air bubbles. One % agarose was dissolved by heating in 9.5 ml O'Farrell buffer and 0.5 ml β-mercaptoethanol was added, just prior to pipetting the warm liquid agarose mixture over the IEF gel. One well was formed for loading membranes as size markers. The iso-electrically focused proteins were electrophoresed according to size (kDa) through the second dimension at 75 V for 18 to 20 hours at room temperature, in Laemmli running buffer. The gels were then visualised as described previously using Coomassie stain (2.2.1.2.).

2.2.3. Molecular characterization of membrane defect

Molecular biology methods were employed to further investigate the spectrin variant identified by means of the protein analysis described above. General methods were employed according to Maniatis, Fritsch and Sambrook (1989).

2.2.3.1. Extraction of genomic DNA

Genomic DNA was extracted by the method of Sykes (1983). Blood was collected in ACD tubes, centrifuged for 10 minutes at 800 *g* and buffy coats were removed. Buffy

coats could be frozen at -70°C or used immediately. The samples were decanted into 50 ml conical centrifuge tubes and an equal volume of saline solution (0.2 % Triton X-100 (v/v), 0.9 % NaCl w/v) was added to each tube. This was mixed thoroughly and centrifuged at 800 *g* for 15 minutes at room temperature. The supernatant was discarded and the soft, pink pellet was resuspended in 30 ml saline solution and mixed thoroughly. This was centrifuged as above and the supernatant discarded to yield a whitish pellet. The pellet was dispersed with a large diameter glass rod (3 mm) in a few drops of DNA lysis buffer (7 M urea, 0.3 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.5) and then worked into solution to a final volume of 10 ml. Two ml 10 % SDS were added to each tube, swirled gently and left to stand for 10 minutes at 37 °C. To each tube, 5 ml chloroform and 10 ml Tris-saturated phenol were added, vigorously shaken and centrifuged at 800 *g* for 10 minutes, at room temperature.

Tris-saturated phenol was prepared as follows:

(i) Melt 250 g phenol at 65 °C.

(ii) Add 0.25 g of the anti-oxidant 8-hydroxyquinoline (to a final concentration of 0.1 %).

(iii) Extract the phenol with five x 200 ml 0.1 M Tris pH 8.0, until the pH of the aqueous phase is

greater than 7.6 (as indicated by pH indicator paper).

(iv) Store in a dark bottle at 4°C.

The aqueous phase was removed and extracted again with 5 ml chloroform (800 *g* for five minutes at room temperature). The aqueous phase was transferred to a 50 ml conical flask and 10 ml ice-cold isopropanol (stored at -20°C) was added. The DNA was spooled onto a glass rod, immediately upon addition of the isopropanol and dissolved overnight in 0.25 - 0.5 ml TE (10 mM Tris, 1 mM EDTA, pH 8.0).

2.2.3.2. Quantitation and electrophoresis of extracted DNA

Five µl of DNA was diluted 1 in 200 with 1 ml water and was spectrophotometrically assayed at 260 nm. The concentration (mg/ml) was estimated according to the following formula:

$$\frac{A_{260} \times \text{conversion (50 µg/ml)} \times \text{dilution (200)}}{1000}$$

One A_{260} unit of ds DNA = 50 µg/ml.

The DNA quality was determined by electrophoresis on a 1 % agarose gel prepared in 1x TAE buffer, with 2.5 µl ethidium bromide (10 mg/ml) per 100 ml.

The buffer (50x TAE) was prepared as follows:

121 g Tris

28.55 ml Glacial acetic acid

2.0 ml 0.5 M EDTA pH 8.0

Make up to 500 ml with water, and dilute 1 in 50 with water, to a working strength.

One µg of DNA, to which 0.25 volumes of 1x DNA gel loading dye was added, was loaded into each well and electrophoresed at 100 V for approximately one hour. The DNA was visualised under UV transillumination, at 302 nm.

DNA gel loading dye (10x) was prepared as follows:

50 % glycerol

0.25 % bromophenol blue

0.25 % xylene cyanol

0.1 M EDTA pH 8.0

Dilute 1 in 10 to a working strength with water.

2.2.3.3. Polymerase Chain Reaction (PCR)

a) Amplification of α spectrin DNA

Amplification of specific exons of α spectrin from

genomic DNA was carried out by means of PCR (Saiki *et al.*, 1988) using Taq DNA polymerase. In order to further investigate the SpaI defect, exon 2 (aa 9 - 88) was amplified using oligomer primers complementary to regions of intron flanking the required exon sequence (Sahr *et al.*, 1989; Coetzer *et al.*, 1991).

The upstream primer, a 21mer, was designated P_1 (5' CACATATAAGCGGGGCAACAT 3') and the downstream primer P_2, similarly a 21mer, (5' TAGGGTCTGCTCTGAGGCAAT 3').

Upon amplification under the following conditions a 348 bp fragment was produced.

Reaction mix

Component	*Volume*	*[Final]*
Sterile water	55 µl	
10x reaction buffer*	10 µl	1x
dATP (10 mM)	2 µl	200 µM
dCTP (10 mM)	2 µl	200 µM
dGTP (10 mM)	2 µl	200 µM
dTTP (10 mM)	2 µl	200 µM
P_3 (10 µM)	5 µl	0.5 µM
P_2 (10 µM)	5 µl	0.5 µM
Taq DNA polymerase (5 U/µl)	0.5 µl	2.5 U
$MgCl_2$ (25 mM)	6.5 µl	1.625 mM
Genomic DNA template (0.1 µg/µl)	10 µl	1 µg
Total	100 µl	

*10x reaction buffer (supplied with the enzyme): 500 mM KCl, 100 mM Tris, 1 % Triton X-100, pH 9.0

The reaction was overlayered with 50 to 100 µl mineral oil, to prevent evaporation.

The reaction was amplified for 30 cycles, using a Perkin Elmer Cetus thermal cycler, one cycle consisting of :

94°C, 1 minute, (denaturation),

55°C, 1 minute, (annealing),

72°C, 2 minutes, (extension).

b) Restriction enzyme analysis of PCR product

DNA from normal and affected individuals was screened for known codon 28 mutations using PCR as described, with primers P_2 and P_3. The amplification product was checked by electrophoresis of a 10 µl aliquot on a 1 % agarose gel, against a DNA molecular weight marker (100 bp ladder), in order to determine the correct product size.

If the product was a single, clean band the remaining 90 µl of product was transferred to a new 1.5 ml tube and an equal volume of water added. To this, 50 µl each of Tris-saturated phenol and chloroform were added and mixed thoroughly. The mixture was centrifuged for three minutes in a microfuge. The aqueous phase was removed and 0.1 volumes of 3 M Na Acetate pH 5.2 was added. The DNA was precipitated by adding 2.5 volumes of ice-cold absolute ethanol and placed at -70°C for 15 minutes. Following this the sample was centrifuged in a microfuge for 10 minutes. The pellet was washed for five minutes in a

microfuge in 70 % ethanol and allowed to dry.

If the PCR product was not a single, clean band, the DNA was electrophoresed on a preparative agarose gel and the correct product eluted by means of a spin column (2.2.3.5b.).

The DNA was resuspended in 15 µl water and digested with AhaII at 37°C, for 4 hours, under the following conditions:

DNA	15 µl
BSA (10 mg/ml)	0.2 µl
Water	1.8 µl
10x buffer 2 *	2.0 µl
AhaII	1.0 µl
	20.0 µl

* 10x buffer 2 (supplied with the enzyme):
500 mM NaCl, 10 mM Tris, 10 mM $MgCl_2$, 1 mM DTT,
pH 7.9

The resulting restriction pattern was analysed on a 2 % agarose gel (molecular biology grade), in 1x TAE buffer.

2.2.3.4. Allele-Specific Oligonucleotide Hybridization (ASOH)

a) Preparation of slot blot

Genomic DNA from normal and affected individuals, including positive and negative controls for each of the known codon 28 mutations (Coetzer *et al.*, 1991), were PCR amplified using P_2 and P_3 (2.2.3.3a.).

Ten µl of PCR product was denatured by adding 190 µl denaturing solution (0.4 M NaOH, 25 mM EDTA) and boiled for 2 minutes. The DNA was snap chilled on ice for 15 minutes to maintain ss DNA (Kogan, Doherty and Gitschier, 1987).

Four pieces of Whatman 3M filter paper, as well as a nylon membrane, were cut to fit exactly into the Bio-Rad slot blot apparatus and the unit was sealed tightly.

The filter papers and nylon membrane were pre-wet with water and a vacuum applied until the membrane was just dry. With the vacuum off, the DNA (200 µl) was loaded into the appropriate wells, following which the vacuum was re-applied and the DNA was deposited on the membrane. The sample was washed once with 400 µl 20x SSC (3 M NaCl, 0.3 M NaCitrate), and the vacuum applied until the membrane was dry.

The apparatus was dismantled and the slot blot was rinsed with 2x SSC (diluted with water) and baked at 80°C for 2 hours, in order to fix the DNA to the membrane.

b) Labelling of oligonucleotide probes

The oligonucleotides used were designed to hybridize with the wild type codon 28 (Arg CGT) and each of the four known mutations (Coetzer *et al.*, 1991). The details are as follows:

P_{30} Normal ASO, aa 25 - 31, Tm 68°C (antisense)
5' CACTTCCTGACGCCTCTCCTG 3'

P_{31} Mutant ASO, detects Arg (CGT) to His (CAT), at codon 28, Tm 66°C (sense)
5' CAGGAGAGGCATCAGGAAGTG 3'

P_{32} Mutant ASO, detects Arg (CGT) to Cys (TGT) at codon 28, Tm 66°C (sense)
5' CAGGAGAGGTGTCAGGAAGTG 3'

P_{35} Mutant ASO, detects Arg (CGT) to Ser (AGT) at codon 28, Tm 66°C (sense)
5' CAGGAGAGGAGTCAGGAAGTG 3'

P_{36} Mutant ASO, detects Arg (CGT) to Leu (CTT) at codon 28, Tm 66°C (sense)

5' CAGGAGAGGCTTCAGGAAGTG 3'

Tm was calculated according to the following formula:

$$4°C(G + C) + 2°C(A+T),$$

(Itakura, Rossi and Wallace, 1984).

The end-labelling reaction (Maniatis, Fritsch and Sambrook, 1989) of the oligonucleotide probes was carried out as follows:

Component	µl	[final]
oligonucleotide (0.5 µg/µl)	1	0.5 µg
Water	7	
2x T_4 polynucleotide kinase buffer (140 mM Tris, 20 mM $MgCl_2$, 10 mM DTT, pH 7.6)	15	1x
γ-^{32}P-ATP (6000 Ci/mmol)	5	50 µCi
T_4 polynucleotide kinase (8 U/µl)	2	16 U
	30 µl	

The reaction was incubated at 37°C for 60 minutes and then stopped by adding 1 µl 0.5 M EDTA pH 8.0.

The labelled probes were purified using the Mermaid kit, specific for less than 200 bp of DNA. Three volumes of High Salt Binding solution were added to the terminated reaction mixture (30 µl). Eight µl Glassfog were added and mixed well. The suspension was incubated at room temperature, with vortexing. After 10 minutes, the sample was centrifuged in a microfuge for approximately 15 seconds, to pellet the DNA bound to the Glassfog. The supernatant was discarded and 300 µl Ethanol Wash added to the pellet. This was resuspended by vortexing and was centrifuged briefly to wash the DNA-containing pellet. This step was repeated twice and all traces of ethanol removed before eluting the DNA from the Glassfog. The pellet was resuspended in 50 µl water, incubated at 50°C for five minutes to elute the DNA and then centrifuged for one minute in a microfuge. The elution was repeated giving a final volume of 100 µl. One µl of this was added to 5 ml toluene scintillant and the amount of radio-labelled probe (cpm) determined by liquid scintillation counting, using a β counter (Packard).

c) Hybridization of slot blots

The baked blot was prehybridized for 1 hour at 61°C in approximately 8 ml prehybridization solution, in a sealed hybridization bag.

The following solutions were prepared:

(i) 20x SSPE

3.6 M NaCl

0.2 M Na_2HPO_4

0.02 M EDTA

(ii) 50x Denhardts solution

5 g Ficoll (type 400)

5 g polyvinylpyrrolidine

5 g BSA

Made up to 500 ml with water.

Aliquot and store frozen.

Volume	Component	[final]
12.5 ml	20x SSPE	5x
5.0 ml	50x Denhardts	5x
2.5 ml	10 % SDS	0.5 %
30.0 ml	water	
50.0 ml		

About 4 ml of prehybridization solution was removed and 20 - 50 µl labelled probe added (20 - 50 x 10^6 cpm) to the remaining prehybridization solution in the bag containing the blot. The blot was incubated for 2 to 4 hours at 61°C.

The blot was washed in solutions of increasing stringency, with shaking, as follows:

i) 2x SSPE
   2x 15 minutes, room temperature
ii) 2x SSPE/0.1 % SDS
   2x 4 minutes, 62°C
iii) 1x SSPE/0.1 % SDS
   2x 4 minutes, 65°C.

The blot was then exposed to X-ray film, at -70°C, from 1 hour to overnight, with intensifying screens.

2.2.3.5. Sequence analysis of SpaI defect

a) Preparing the sequencing gel

The sequencing gel was poured the day before it was to be used and allowed to polymerize overnight.

The sequencing gel plates (40 cm x 30 cm x 0.004 cm) were washed three times with Extran and rinsed well with water before wiping with 70 % ethanol. They were assembled, taking care to seal them properly by means of tape and clamps on the sides.

The 8 % gel mixture was prepared as follows:

40 % acrylamide	15.0 ml
2 % bis	15.0 ml
Urea	30.0 g
Water	14.0 ml
10x TBE*	7.5 ml
	75 ml

* 10x TBE: 54 g Tris, 27.5 g Boric acid, 2.0 ml 0.5 M EDTA, pH 8.0. Make up to 500 ml with water.

This was stirred, until the urea had dissolved, and then the catalysts were added (0.6 ml 10 % APS, 15 µl TEMED). The gel was poured using a 25 ml pipette, lifting one corner of the assembled gel plates initially, to guide the flow. The gel was allowed to polymerize overnight at room temperature, covered with cling-wrap.

Buffer for electrophoresis (1x TBE) was prepared by diluting 10x TBE 1 in 10 with water.

For sequencing, the gel was assembled in the BRL model S2 sequencing apparatus and pre-run for 30 minutes, at 60 W, to a surface temperature of ±50°C.

b) Direct sequencing of amplified ds DNA

The PCR products to be sequenced were analysed on 1 % agarose gels. Approximately 100 ng of amplified DNA was electrophoresed on a 1.5 % preparative gel (molecular biology grade), in 1x TAE buffer. The DNA was recovered from the agarose by a spin elution method (Vaux, 1992), replacing glass fibre filter with Whatman No.1 filter paper. The spin column was prepared by cutting the lid off a 1.5 ml Eppendorf tube. The gel slice was placed in a 0.5 ml tube which had been cut in half and perforated five times in the base with a 26 gauge needle. This was placed in a capless 0.5 ml tube, which was perforated once as above and packed with 2 mm x 2 mm Whatman No. 1 filter paper. The entire column was then placed in the capless 1.5 ml tube and centrifuged for five minutes in a microfuge, in order to elute the DNA from the gel. The progress of elution was monitored by visualisation of ethidium bromide in the eluate with UV transillumination. The recovered DNA, in 1x TAE buffer, was transferred to a new 1.5 ml tube and an equal volume of water added. The sample was then phenol/chloroform extracted and ethanol precipitated as before.

The DNA was sequenced following the Sequenase dideoxy chain termination reaction protocol (Sanger, Nicklen and Coulson, 1977).

Firstly, four tubes were labelled G, A, T, C and filled with 2.5 µl of the appropriate dideoxy termination mixture. Each mixture contains 80 µM dGTP, 80 µM dATP, 80 µM dTTP, 80 µM dCTP and 50 mM NaCl. In addition the G mixture contains 8 µM dideoxy-dGTP (ddGTP); the A mix, 8 µM ddATP; the T mix, 8 µM ddTTP and the C mix 8 µM ddCTP.

The template was prepared by resuspending the air-dried, spin-cleaned pellet in 6.5 µl water, 2 µl 5x Sequenase reaction buffer (200 mM Tris, 100 mM $MgCl_2$, 250 mM NaCl, pH 7.5) modified with 10 % formamide (Zhang, Reading and Deisseroth, 1992) and 1.5 µl sequencing primer (0.02 µg/µl), either P_2 or P_3, in a five-fold molar excess of primer to DNA. This suspension was boiled for 10 minutes, to denature the ds DNA and then snap chilled on dry ice for 1 minute. After a 15 - 20 second spin in a microfuge the sample was placed on ice, for annealing of primer to DNA to occur, and was then used immediately.

To the annealed template-primer mixture (10 µl) the following were added in order to incorporate radio-labelled dATP into the DNA template:

- DTT (0.1 M) 1.0 µl
- Labelling nucleotide mix 2.0 µl
  (For use with radio-labelled dATP;
  1.5 µM dGTP, 1.5 µM dTTP, 1.5 µM dCTP;
  diluted 1 + 4 with water)
- α-^{32}PdATP (3000 Ci/mmol) (10 µCi/µl) 0.5 µl
- Sequenase enzyme Version 2.0 2.0 µl
  (diluted 1 + 7 with enzyme dilution
  buffer: 10 mM Tris pH 7.5, 5 mM DTT,
  0.5 mg/ml BSA)

The reaction was mixed thoroughly, by pipette, and incubated at room temperature for two minutes. Each of the tubes (G, A, T, C), prepared initially, was pre-warmed at 37°C and after completion of the labelling reaction, 3.5 µl of it was transferred to each of the G, A, T and C tubes. Each reaction was mixed thoroughly and incubated at 37°C for 5 minutes. The reaction was stopped with the addition of 4 µl stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol) and placed on ice.

The prepared samples were heated in a heating block to 90°C for 4 minutes and 2 µl loaded into each well of a sequencing gel in the order G-A-T-C. The sample was centrifuged briefly and placed on ice. Each loading was run for 1.75 - 2.0 hours at constant power (60 W), with non-limiting parameters (40 mA, 2.4 kV), using a Consort microcomputer electrophoresis power supply. After 3 - 4 loadings the gel was removed from the apparatus and the plates prised apart, leaving the gel on one plate. A used X-ray film was placed on top of the gel and the gel was then lifted off the plate, covered in cling-wrap and autoradiographed with intensifying screens, using Curix film (Agfa). The gel was exposed for 6 -24 hours at -20°C and then developed, using the facilities, by kind permission, of the Johannesburg Hospital X-ray staff.

2.3. Results

2.3.1. Erythrocyte membrane protein analysis

2.3.1.1. Laemmli SDS-PAGE

Aliquots of solubilized membrane samples (45 and 50 µg) were electrophoresed using the Laemmli SDS-PAGE discontinuous buffer system. The gels were Coomassie-stained. The membrane electrophoretic profile of the two probands (TM and TL) is presented in Figure 8. The protein bands are indicated. The profile was normal, as compared with controls, with the exception of band 4.1b in TM and TL, which was increased and hence the ratio of 4.1a/4.1b, in TM and TL, was decreased relative to controls (Table II). This is usually an indication of an increased reticulocyte count. All quantitated values were otherwise within the normal mean ± 2SD, established in our laboratory (Part IV). TM (mother) and TL (father) were both normal (Figure 8, Table II). Protein 4.1 is the principle protein quantitated from the Laemmli gel, as it is best resolved under these conditions, into the 4.1a and 4.1b isoforms. Ankyrin and spectrin content cannot be calculated as ankyrin co-migrates with β spectrin on the 12 % Laemmli SDS-PAGE.

In the figure, only the 45 µg loading is shown, but it should be noted that both 45 and 50 µg loadings were scanned and quantitated.

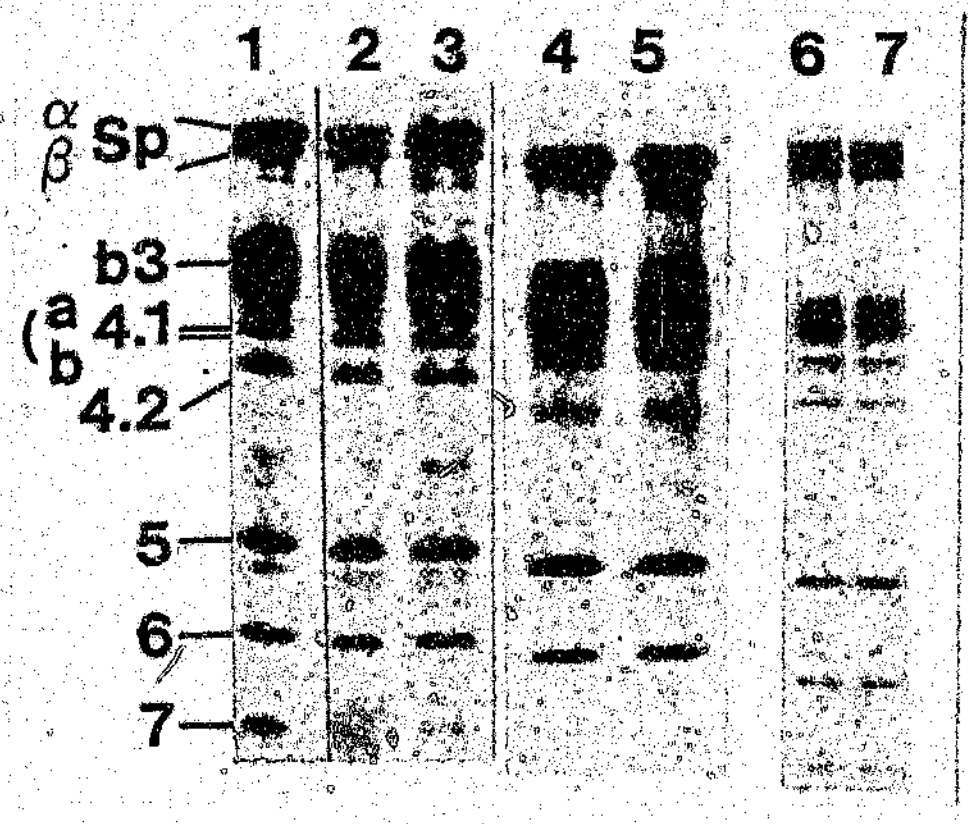


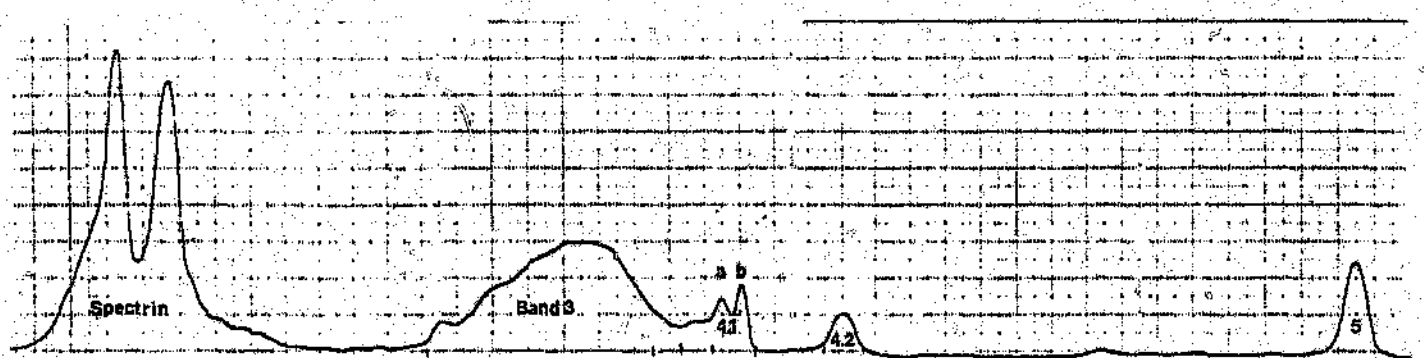
Figure 8. Laemmli SDS-PAGE.
Forty-five µg aliquots of SDS-solubilized membrane extracts were electrophoresed and Coomassie-stained, after which quantitation of densitometric scans of stained gels was carried out (Table II).

1 Control
2 TM
3 TM (mother)
4 Control
5 TL
6 Control
7 TL (father)

Table II. Erythrocyte membrane protein ratios: Laemmli SDS-PAGE.

Subject	4.1a/4.1b	4.1/4.2	4.1+4.2/b3
Control	1.4 ± 0.18	1.5 ± 0.19	0.33 ± 0.08
	n = 49	n = 46	n = 47
TM	0.84	1.48	0.29
TM (mother)	1.36	1.25	0.22
TL	0.87	1.54	0.32
TL (father)	1.54	1.42	0.35

Representative densitometric scan of Laemmli SDS-PAGE (Proband TM)



2.3.1.2. Fairbanks SDS-PAGE

Aliquots of solubilized membrane samples (20 and 25 µg) were electrophoresed using the Fairbanks SDS-PAGE system. The gels were Coomassie-stained and quantitated by scanning densitometry. In the Fairbanks system the membrane protein content is quantitated and expressed as a ratio to the integral protein, band 3. α and β spectrin are resolved and ankyrin (band 2.1) migrates separately to β spectrin. Three of the ankyrin cascade bands (2.1, 2.2 and 2.3) are identified. Band 4.1 is not resolved into the a and b forms. The most anodal protein is globin (Figure 9).

The quantitation of membrane protein content of the probands (TM and TL), as well as TM (mother) and TL (father), showed no differences, relative to controls (Table III), within a mean ± 2SD. Both 20 and 25 µg loadings of membrane protein were scanned and quantitated, however, only the 20 µg loading is shown (Figure 9).

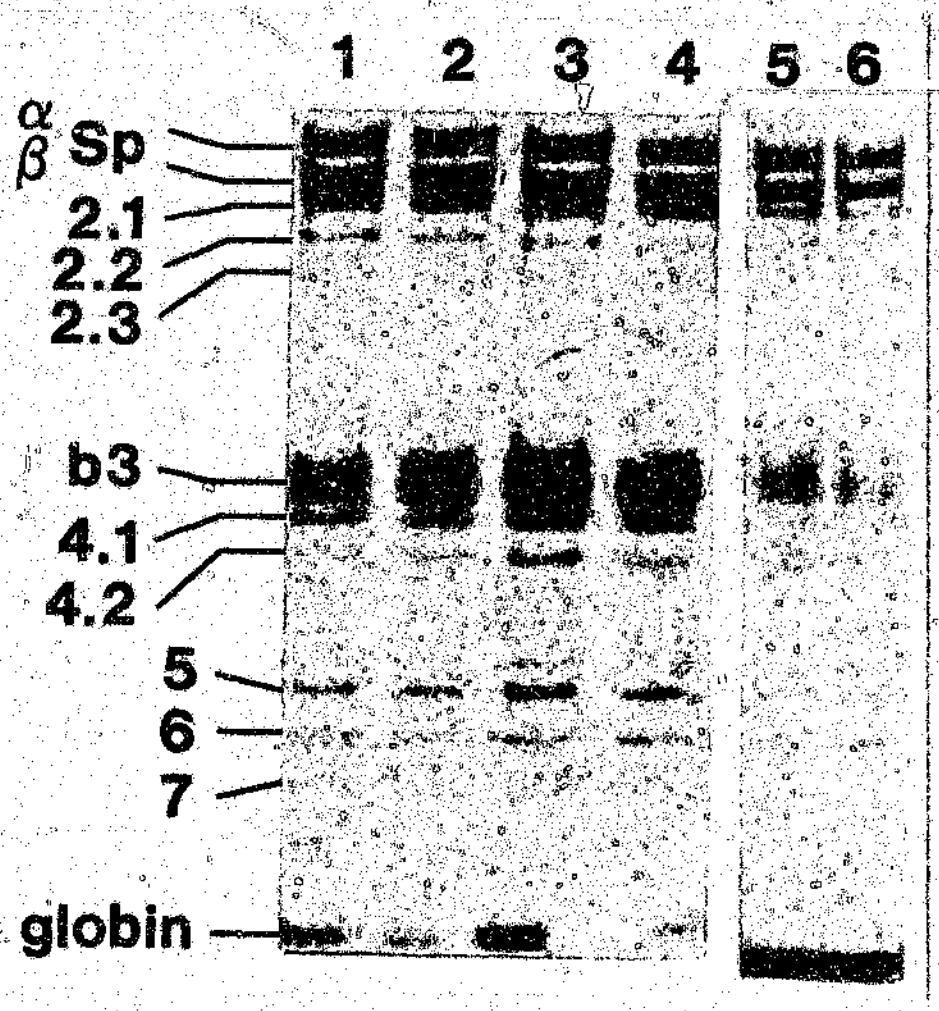


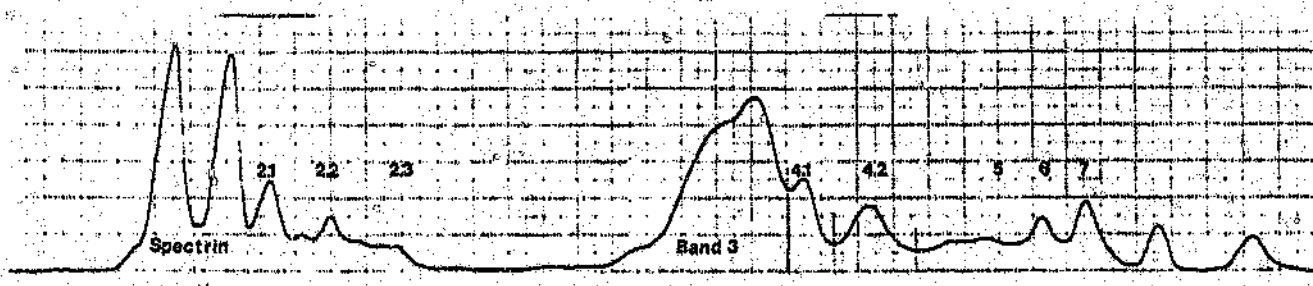
Figure 9. Fairbanks SDS-PAGE.
Twenty µg aliquots of SDS-solubilized membrane extracts were electrophoresed and Coomassie-stained, after which quantitation of densitometric scans of stained gels was carried out (Table III).

1 TL
2 TM
3 TM (mother)
4 Control
5 Control
6 TL (father)

Table III. Erythrocyte membrane protein ratios: Fairbanks SDS-PAGE.

Subject	αSp/βSp	Sp/b3	2.1/b3	4.1/4.2	4.1+4.2/b3
Control	1.11±0.065	1.12±0.11	0.23±0.03	1.00±0.17	0.37±0.056
	n = 40	n = 38	n = 23	n = 37	n = 38
TM	1.11	0.95	0.23	0.99	0.31
TM(mother)	1.09	0.91	0.21	0.90	0.39
TL	1.07	1.08	0.19	1.06	0.41
TL(father)	1.08	1.14	0.20	1.10	0.42

Representative densitometric scan of Fairbanks SDS-PAGE (Proband TM)



2.3.2. Spectrin analysis

2.3.2.1. Non-denaturing PAGE

Ten and 15 µg aliquots of spectrin were subjected to non-denaturing PAGE in order to determine spectrin dimer (SpD) content, expressed as a percentage of total SpD and spectrin tetramer (SpT) in the sample. SpD percentage is an indicator of the presence of a molecular defect giving rise to spectrin variants (Coetzer *et al.*, 1987).

TM and TL have an increased SpD % (Figure 10), 58 % and 57 % (Table IV), respectively, as compared with controls (< 10 %). TM (mother) also has an increased SpD % (Figure 10), 34 % (Table IV), whilst TL (father) has SpD % within the normal range (2.5 % - 11.2 %). The figure shows only the 10 µg aliquot of spectrin, resolved into high molecular weight complex (HMW), spectrin oligomer (SpO), SpT and SpD bands.

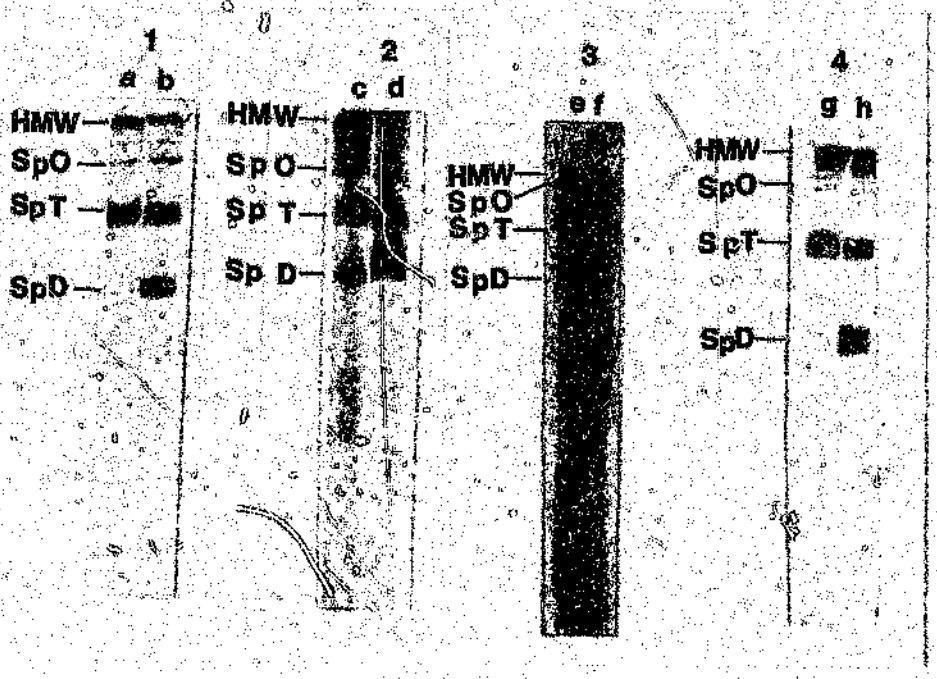


Figure 10. Non-denaturing PAGE.
Ten µg aliquots of spectrin extract were electrophoresed and subsequently Coomassie-stained. SpD content was quantitated from densitometric scans of Coomassie-stained non-denaturing PAGE gels and expressed as a percentage of total SpD and SpT (Table IV). High molecular weight complex (HMW) and spectrin oligomers (SpO) are indicated.

1a Control
b TM (mother)
2c Control
d TM
3e Control
f TL (father)
4g Control
h TL

Table IV. Membrane spectrin dimer (SpD) content (%). SpD content is expressed as a percentage of total SpD and SpT.

Subject	% SpD/(SpD + SpT)
Control	7.33 ± 2.4
Range	[2.5-11.2]
	n = 16
TM	58
TM (mother)	34
TL	57
TL (father)	4

2.3.2.2. One dimensional analysis of tryptic digest of spectrin

Spectrin was extracted from the erythrocyte membranes and subjected to limited tryptic digestion at 4°C. The resulting digest pattern was resolved by Laemmli SDS-PAGE. The pattern was analysed and compared with control digests. Minor differences may be noted between individual control spectrin tryptic digest patterns prepared on different days. It is for this reason that control spectrin is always prepared and electrophoresed at the same time as spectrin from the subjects, to allow for direct compar son.

The probands TM (Figure 11a), TL (Figure 12a) and TM (mother) (Figure 13a) all had an increased band at 74 kDa. A concomitant decrease in the 80 kDa band, relative to the control, was noted. The 80 kDa band represents the normal αI domain of spectrin. It is acceptable to note some 74 kDa product in normal individuals, as a result of limited tryptic cleavage occurring naturally at that point. Western blot analysis of the tryptic digest, using a polyclonal anti-Sp αI domain antibody, confirmed the results and indicated that the increased 74 kDa band observed was of SpαI domain origin in TM (Figure 11b), TL (Figure 12b) and TM (mother) (Figure 13b). This is a previously described

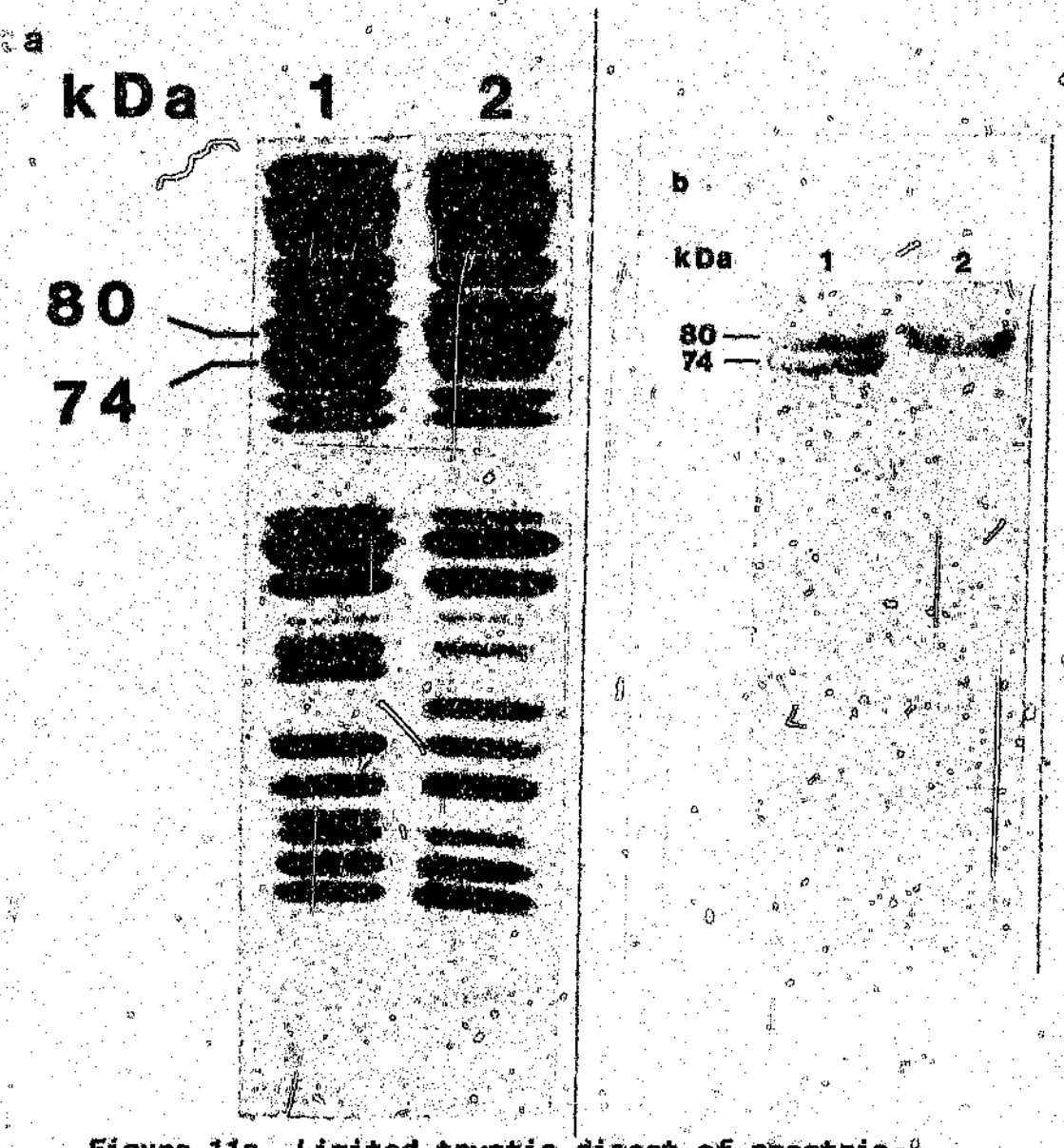


Figure 11a. Limited tryptic digest of spectrin.
Sixty µg digested spectrin was electrophoresed through one dimensional Laemmli SDS-PAGE and Coomassie-stained, in order to visualise an increase in the 74 kDa peptide and a concomitant decrease in the 80 kDa peptide, as compared with a control.

1 TM
2 Control

Figure 11b. Western blot analysis of limited tryptic digest of spectrin.
Digested spectrin was electroblotted onto nitrocellulose and probed using a polyclonal anti-SpaI antibody.

1 TM
2 Control

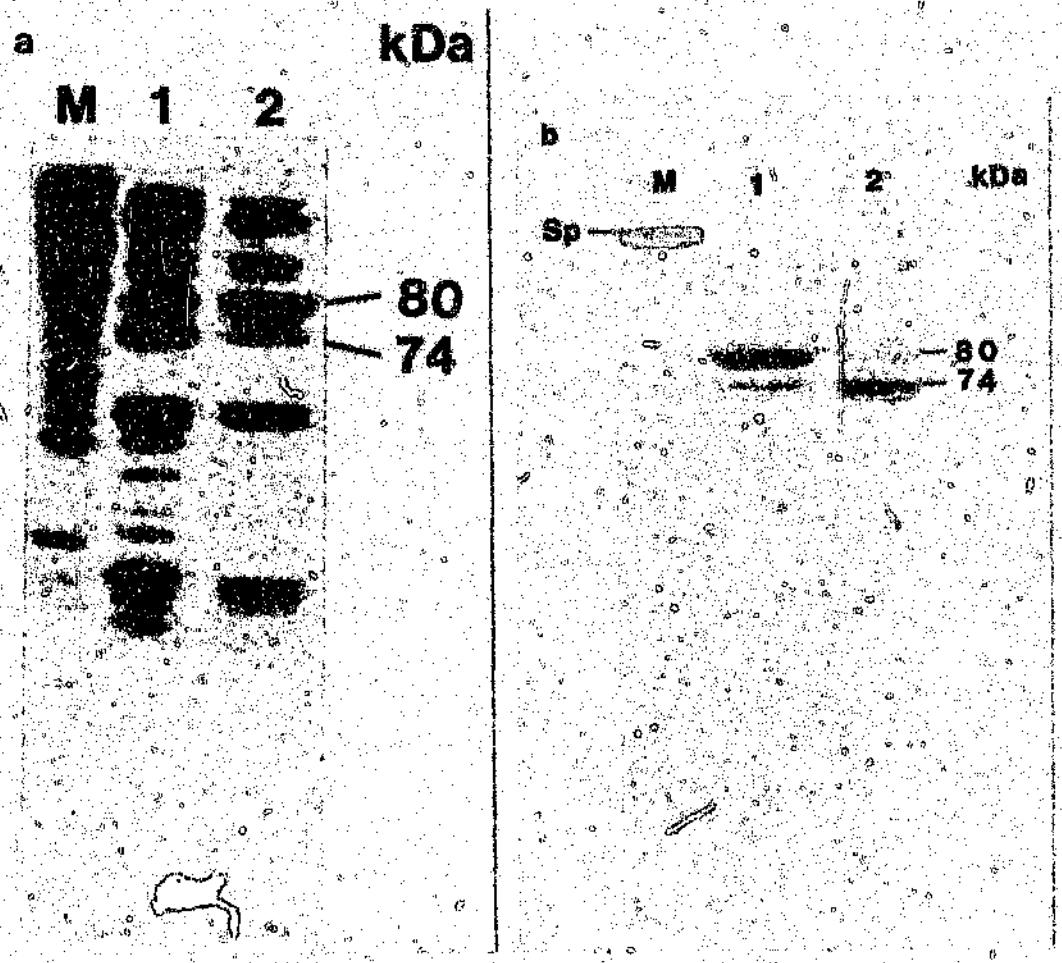


Figure 12a. Limited tryptic digest of spectrin. Sixty µg digested spectrin was electrophoresed through one dimensional Laemmli SDS-PAGE and Coomassie-stained to visualise an increase in the 74 kDa peptide and a concomitant decrease in the αI 80 kDa peptide, as compared with controls.

M Membranes
1 TL
2 Control

Figure 12b. Western blot analysis of limited tryptic digest of spectrin.
Digested spectrin was electroblotted onto nitrocellulose and probed using a polyclonal anti-SpαI antibody.

M Membranes
1 Control
2 TL

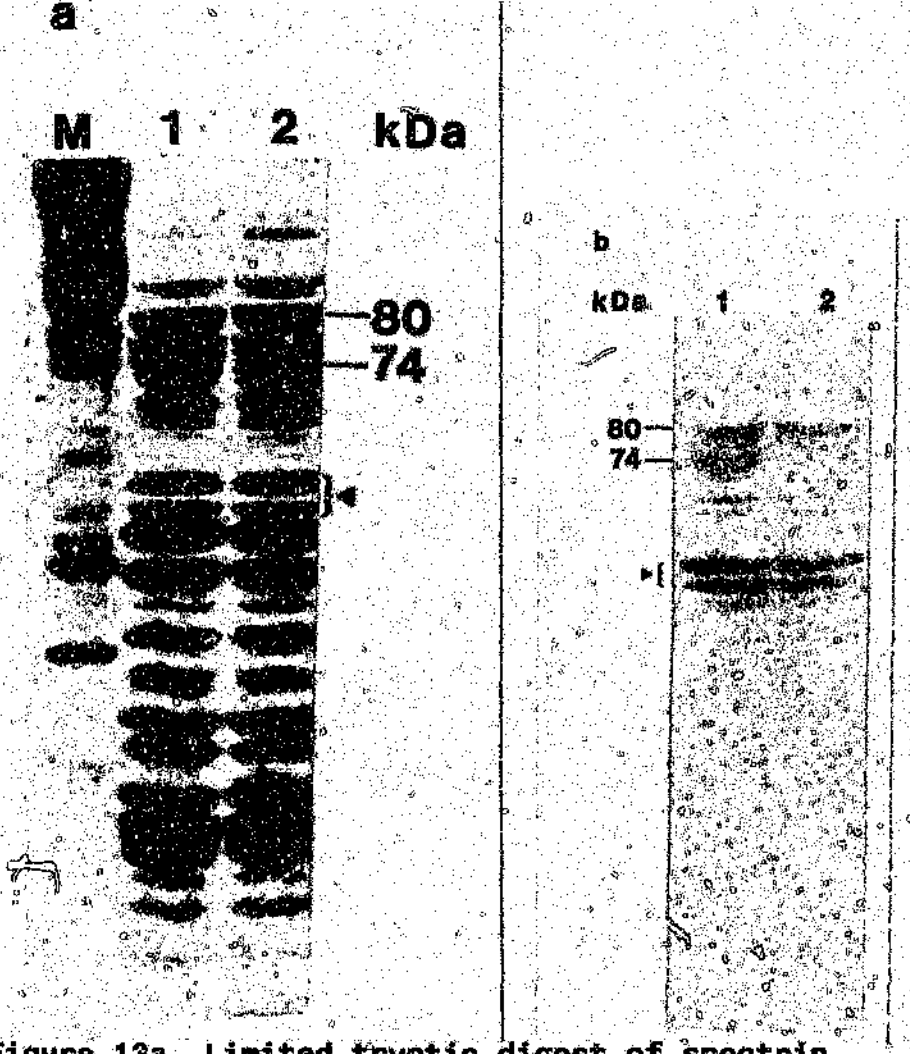


Figure 13a. Limited tryptic digest of spectrin. Sixty µg digested spectrin was electrophoresed through one dimensional Laemmli SDS-PAGE, to visualise the relative amounts of 80 kDa peptide and 74 kDa peptide, as compared with controls. The bands indicated (◂) at approximately 55 kDa are sometimes observed. They have no bearing on the membrane defect.

M Membranes
1 Control
2 TM (mother)

Figure 13b. Western blot analysis of limited tryptic digest of spectrin. Digested spectrin was electroblotted onto nitrocellulose and probed using a polyclonal anti-SpaI antibody.

1 TM (mother)
2 Control
◂ Bands at approximately 55 kDa

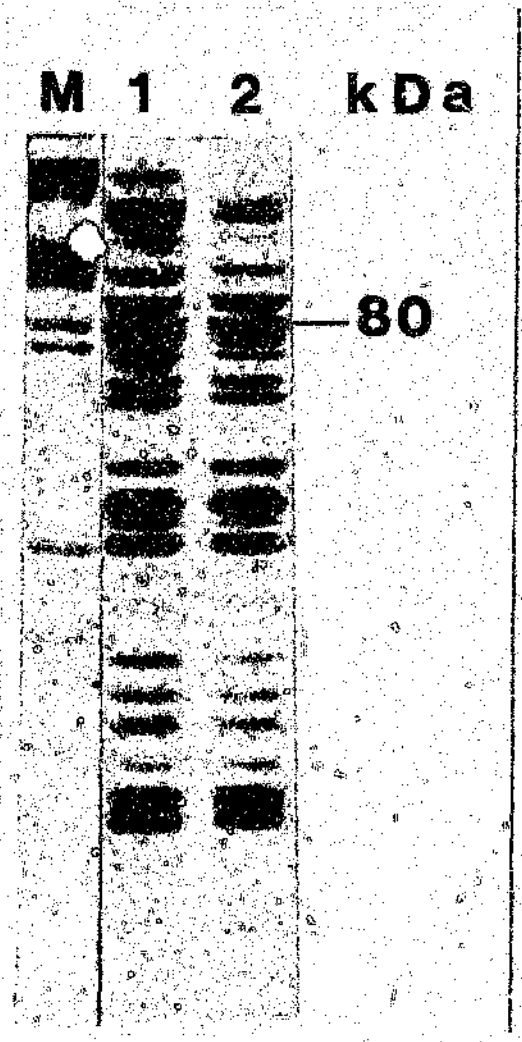


Figure 14. Limited tryptic digestion of spectrin. Sixty µg digested spectrin was electrophoresed through one dimensional Laemmli SDS-PAGE and Coomassie-stained to visualise the 80 kDa peptide. There does not appear to any increased 74 kDa peptide in TL (father), when compared with the control (Western blot not shown).

M Membranes
1 Control
2 TL (father)

variant, namely, SpaI/74. TL (father) was normal with respect to spectrin tryptic digest pattern, with no increased 74 kDa band (Figure 14), when compared to the control. Western blot analysis confirmed the result (not shown).

Two bands at approximately 55 kDa (Figure 13), indicated by ◄, were noted in some tryptic digest patterns. They were however present in the control digest as well and, therefore, do not have any bearing on the membrane defect.

Some variation is noted in the lower molecular weight bands of the limited tryptic digest of spectrin in the proband TM (Figure 11a). This probably represents an αII polymorphism.

2.3.2.3. Two dimensional analysis of tryptic digest of spectrin

In order to further analyze the spectrin variant, the tryptic digests of probands TM and TL were subjected to iso-electric focusing, followed by 2D SDS-PAGE, for improved resolution.

Figure 15b shows proband TM having an increased 74 kDa spot (i), with a decrease in the 80 kDa spot (ii),

relative to the control (Figure 15a). The domains of spectrin, separated initially according to iso-electric point and then according to size, were better resolved under these conditions and no other differences could be detected. The smear in both TM (Figure 15b) and the control (Figure 15a), indicated by ▾, appears to be excess sample loaded onto the IEF gel and which was not focused. It does not affect the pattern or the resolution and the amount of protein which was actually focused was sufficient to visualise all the spots. SDS-solubilized membranes (M) are indicated in order to size the spots.

Trypsin digested spectrin from proband TL was iso-electrically focused and then resolved on 2D SDS-PAGE. Analysis showed an increased 74 kDa spot (i), with a decreased 80 kDa spot (ii) (Figure 16b), relative to the control (Figure 16a).

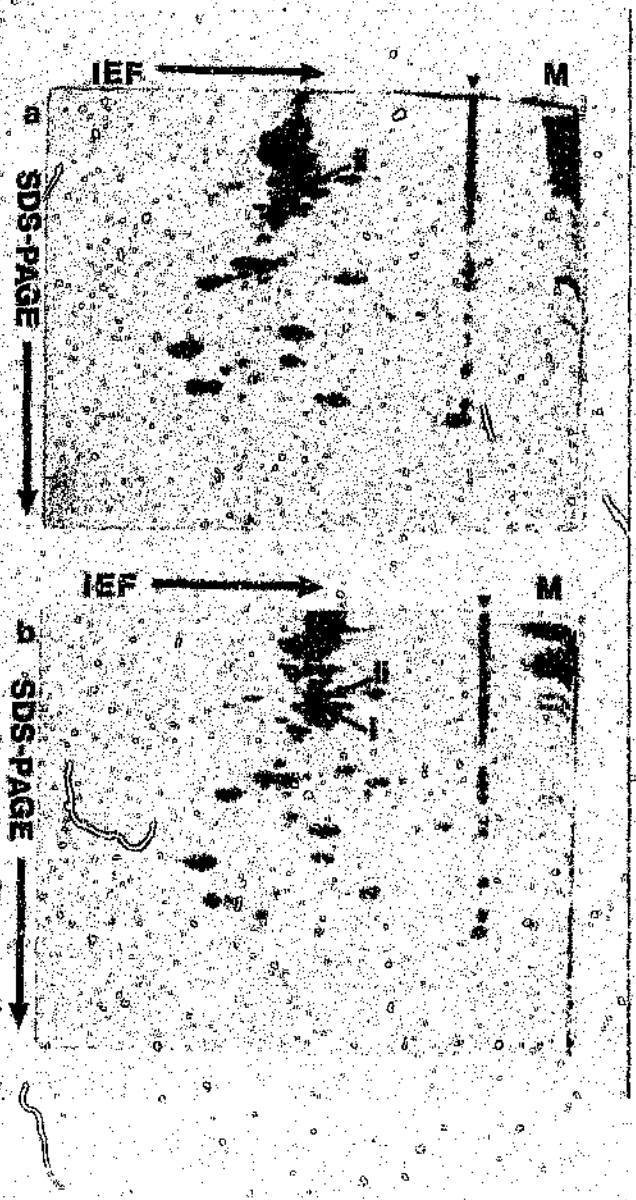


Figure 15. Two dimensional SDS-PAGE.
Following limited tryptic digestion of spectrin, samples (200 µg) were analysed by iso-electric focusing on a gradient of pH 5-7 and, after equilibration, electrophoresed in the second dimension, using 2D SDS-PAGE. In the affected individual (TM), an increased 74 kDa peptide (i →) is observed with a concomitant decrease in the normal 80 kDa peptide (ii →). The smear (◂) is most likely due to excess sample loaded onto the IEF gel which has not been resolved.

a Control
b TM
M Membranes

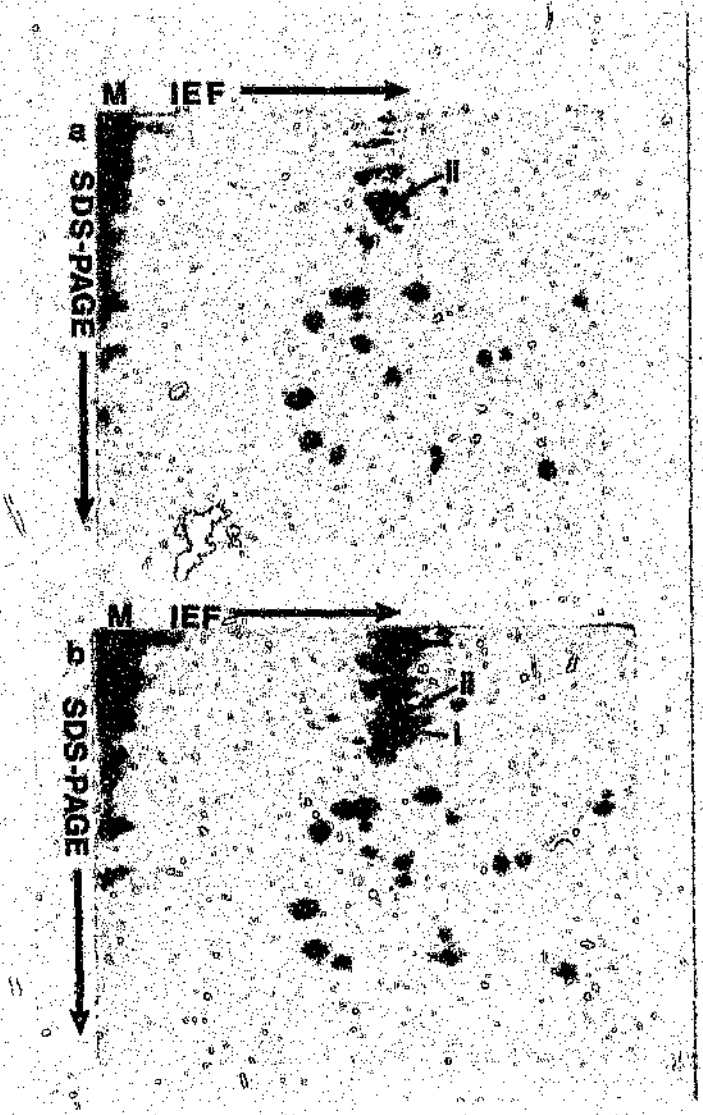


Figure 16. Two dimensional SDS-PAGE.
Following limited tryptic digestion of spectrin, 200 µg aliquots were analysed by isoelectric focusing on a gradient of pH 5-7 and, after equilibration, electrophoresed in the second dimension. In the affected individual (TL), an increased 74 kDa peptide (i →) is observed with a concomitant decrease in the normal 80 kDa peptide (ii →).

a Control
b TL
M Membranes

2.3.3. Molecular characterization of membrane defect

2.3.3.1. Amplification and restriction enzyme analysis of exon 2 of α spectrin

Genomic DNA was extracted from buffy coats from the probands (TM and TL), TM (mother), TL (father) and suitable controls. Aliquots were electrophoresed on an agarose gel to check for the absence of degradation (Figure 17). DNA was PCR amplified using specific primers (Figure 18) to produce a 348 bp fragment, representing exon 2 of α spectrin, the region most commonly associated with SpaI/74. Mutations at codon 28 in exon 2 of α spectrin abolish an AhaII restriction site and hence the PCR amplified and purified products were restricted with AhaII and electrophoresed on 2 % agarose gels.

The probands TM (Figure 19a, lane 4) and TL (Figure 19b, lane 2) as well as TM (mother) (Figure 19a, lane 5) showed three bands after AhaII digestion. The intact 348 bp fragment represents the mutant allele which has lost the AhaII site. The other, normal allele, restricted completely into 222 bp and 126 bp fragments. TL (father) was normal and restricted completely (Figure 19b, lane 3). A positive control previously characterized as being heterozygous for a codon 28 mutation, is included (Figure 19a, lane 3).

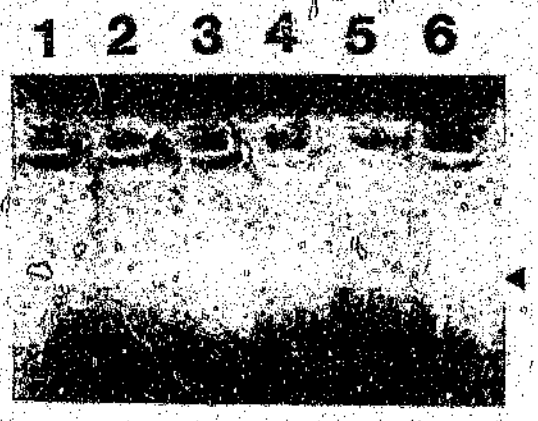


Figure 17. Electrophoresis of extracted genomic DNA. Genomic DNA was extracted and 0.5 ÷ 1.0 µg was electrophoresed on a 1 % agarose gel in 1x TAE, to check the quality.

Lanes 1-6 represent intact, that is, not degraded, DNA. The main band is indicated (◂).

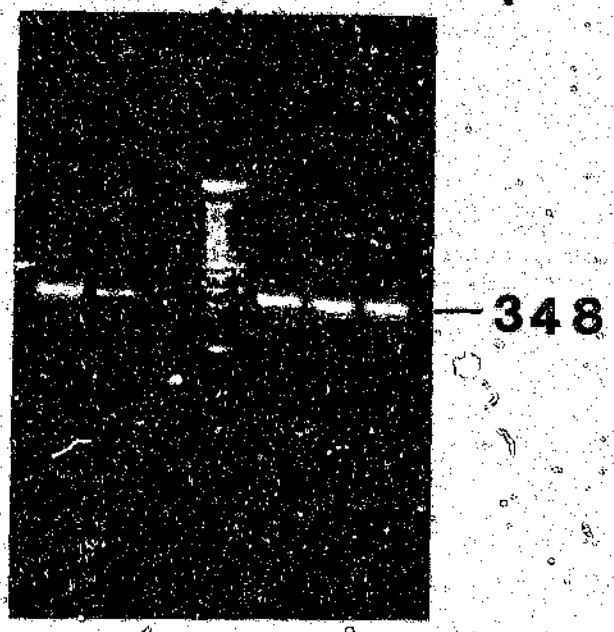


Figure 18. Amplification of exon 2 of α spectrin. Genomic DNA (from the two affected individuals (TM and TL), two controls (normal individuals) and two individuals previously shown to be positive for the SpaI/74 defect at codon 28 was PCR amplified using P_2 and P_3 oligonucleotide primers, to produce a 348 bp fragment, corresponding to exon 2 of α spectrin. The product was electrophoresed on a 1 % agarose gel, with ethidium bromide, in 1X TAE buffer and visualised under UV transillumination.

1 TM
2 TL
3 Control (1)
M 100 bp ladder size marker
4 Control (2)
5 Positive control (1)
6 Positive control (2)

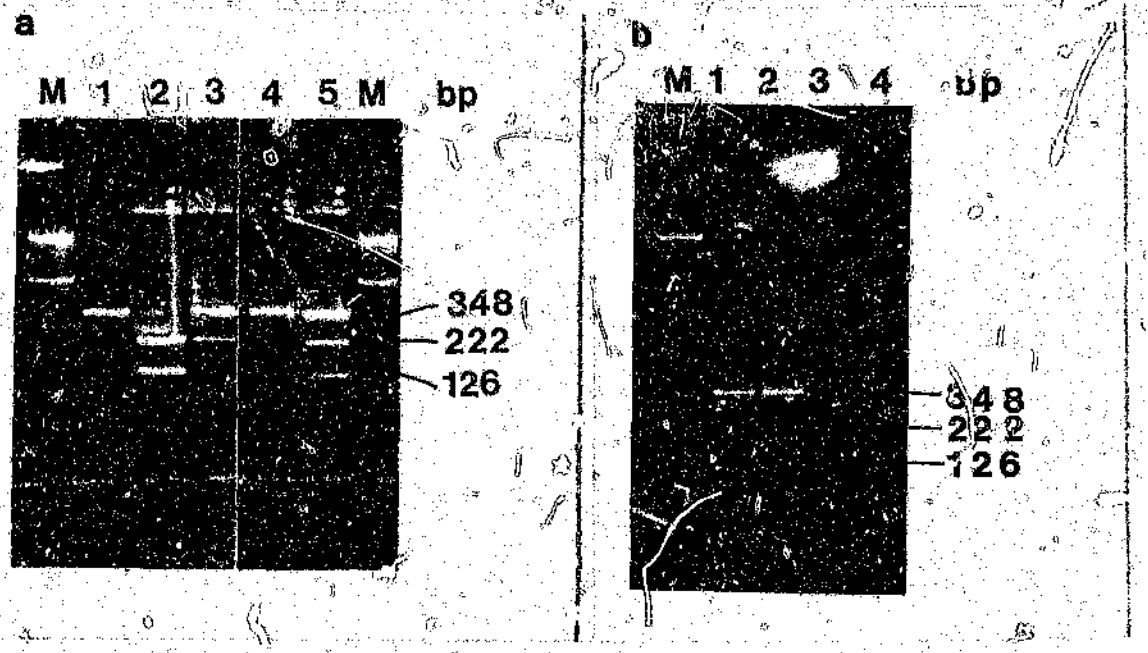


Figure 19. AhaII restriction enzyme analysis.
Genomic DNA was PCR amplified, producing a 348 bp product which was digested with AhaII and electrophoresed on a 2 % agarose gel with ethidium bromide, in 1x TAE buffer. Lane 1 represents the undigested control.

a) The proband (TM), lane 4, and his mother, lane 5, as well as the positive control previously characterized as having a mutation at codon 28, lane 3, are all heterozygous for the mutant allele (348 bp) (which has lost the AhaII site and thus remains intact) and the normal allele, which is restricted into 222 bp and 126 bp fragments by AhaII. The control individual, lane 2, is completely restricted into 222 bp and 126 bp fragments.

b) The proband (TL), lane 2, has both the mutant allele and the normal allele. TL (father), lane 3, and the normal control, lane 4, restrict completely.

M 100 bp ladder size marker

2.3.3.2. Allele-specific oligonucleotide hybridization (ASOH)

AhaII restriction digest results for TM, TL and TM (mother) determined that a mutation exists in codon 28. TL (father) was normal. The four known base changes at codon 28 can be detected by means of allele-specific oligonucleotides. The known mutations are:

i) CGT ▸ CAT (Arg ▸ His)
ii) CGT ▸ TGT (Arg ▸ Cys)
iii) CGT ▸ AGT (Arg ▸ Ser)
iv) CGT ▸ CTT (Arg ▸ Leu)

Genomic DNA was PCR amplified using primers specific for exon 2 of α spectrin (Figure 20). The probands (TM and TL), as well as negative controls, were amplified. Subjects previously described to be positive for each of the known changes were also amplified, as positive controls.

The amplified DNA was slot blotted and hybridized with each of the four oligonucleotide probes. All individuals hybridized with the normal allele-specific oligonucleotide (ASO) (Figure 21(ii)a, lanes 1-7). The

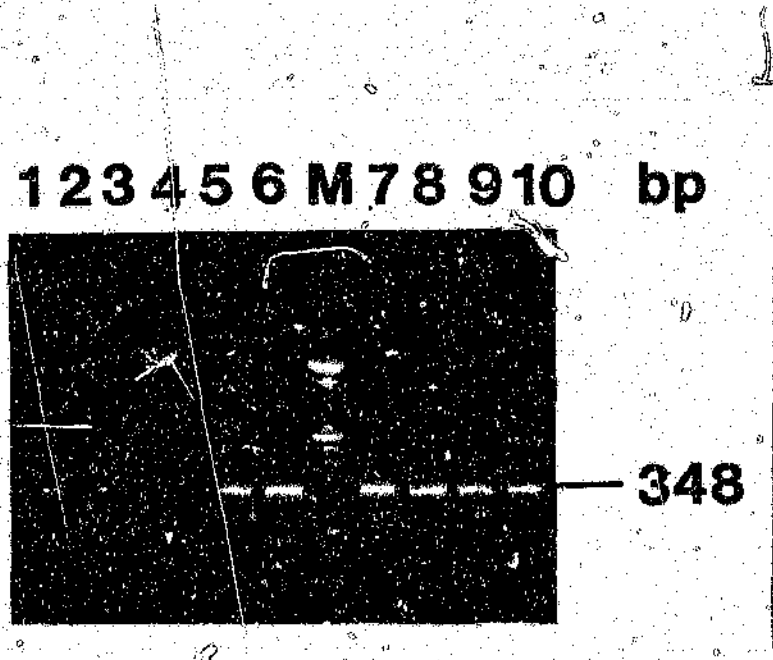


Figure 20. Amplification of genomic DNA for ASOH. Genomic DNA was PCR amplified using primers P_2 and P_3. A 348 bp fragment was produced, representative of exon 2 of α spectrin. Ten µl of each PCR reaction was electrophoresed to check the quality, following which slot blots were prepared. Samples previously characterized as having known point mutations at codon 28 were included as positive controls.

1 TM
2 TM
3 TL
4 TL
5 Control (1)
6 Control (2)
M 100 bp ladder size marker
7 Positive control Arg → Ser
8 Positive control Arg → Leu
9 Positive control Arg → Cys
10 Positive control Arg → His

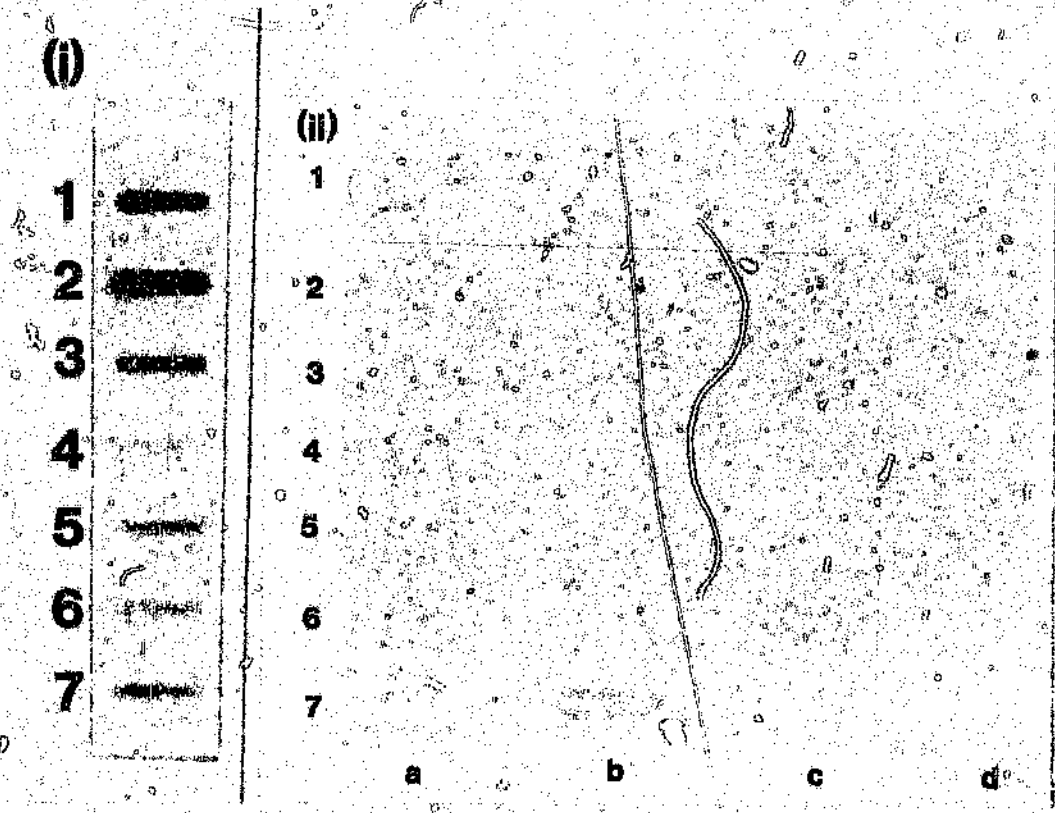


Figure 21. Allele-specific oligonucleotide hybridization. Exon 2 of α spectrin was PCR amplified and slot blots prepared. Oligonucleotide probes to the normal codon 28 sequence as well as four known point mutations at codon 28 were end-labelled and the blots hybridized at 61°C for 2 to 4 hours.

(i) PCR amplified DNA from control individuals (1 to 7) hybridized with the normal ASO probe, to demonstrate a good slot blot.

(ii) 1 Control
2 TM
3 TL
4 Positive control Arg ▸ Ser
5 Positive control Arg ▸ Leu
6 Positive control Arg ▸ Cys
7 Positive control Arg ▸ His

a Normal ASO Arg (CGT)
b Mutant ASO His (CAT)
c Mutant ASO Cys (TGT)
d Mutant ASO Leu (CTT)

positive control (Arg ▸ His) hybridized with the mutant ASO (His) (Figure 21(ii)b, lane 7). Positive control (Arg ▸ Cys) hybridized with the mutant ASO (Cys) (Figure 21(ii)c, lane 6). Positive control (Arg ▸ Leu) hybridized with the mutant ASO (Leu) (Figure 21(ii)d, lane 5). Hybridization with the mutant ASO (Ser) is not shown. TM and TL (Figure 21(ii)c, lanes 2 and 3) hybridized specifically with the mutant ASO (Cys), indicating that this is the mutation in both cases. Since TM and TL hybridize with the normal ASO and the mutant (Cys) ASO they are heterozygous for the mutation at codon 28 in exon 2 of α spectrin.

The halo appearance of the DNA slots (Figure 21(i)) is an artefact which most likely arose during preparation of the slot blot. This appearance was noted on several occasions but did not affect the result or specificity of hybridization. Figure 21(i) represents a slot blot of PCR amplified DNA from control individuals, hybridized with the normal ASO, included to show discrete slot bands.

2.3.3.3. Direct sequencing of ds DNA

PCR amplified DNA of TM and TL was electrophoresed on a preparative agarose gel (Figure 22) and purified for ds direct sequencing of exon 2 of α spectrin. Direct sequencing confirmed the result obtained by ASOH. Both

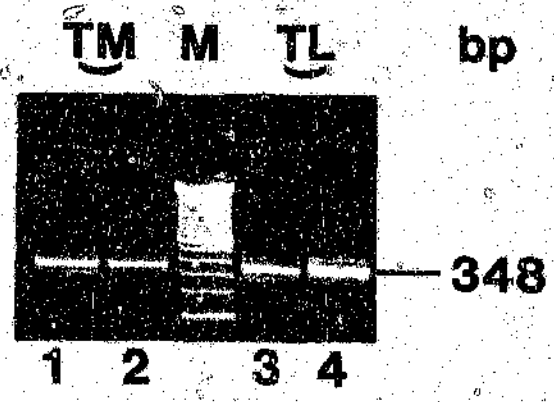


Figure 22. Preparative agarose gel electrophoresis. Genomic DNA was PCR amplified using P_2 and P_3 and approximately 100 ng of product was electrophoresed on a 1.5 % preparative agarose gel to obtain a pure product, without any faint contaminating bands, representing exon 2 of α spectrin. The 348 bp bands were excised and the DNA recovered from the agarose by a spin elution method.

1 TM
2 TM
M 100 bp ladder size marker
3 TL
4 TL

probands were shown to have the codon 28 mutation (Arg ▸ Cys).

Exon 2 of α spectrin was sequenced using P_3, (Figure 23). In this orientation the sense strand was sequenced. Both probands (TL, Figure 23a, TM, Figure 23b) exhibited the same base change, that is, CGT ▸ TGT. This changes the arginine residue to cysteine. This is a known mutation occurring at the "hot spot" codon 28. Direct sequencing enables one to sequence both alleles at once, thus, it could be determined that TM and TL are heterozygous for the mutation, since both the normal "C" and mutant "T" were seen at the same position on the gel.

Further confirmation of the mutation was established by direct sequencing of the antisense strand. Exon 2 of α spectrin of TL and TM was PCR amplified and sequenced with P_2 (Figure 24a and 24b). In this orientation the change reads ACG ▸ ACA.

The entire exon was sequenced and no further changes were discovered in either proband. The intron/exon boundaries were normal, both 5' and 3' to the exon (data not shown).

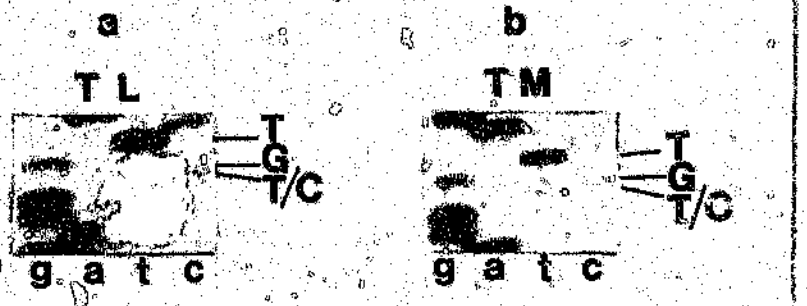


Figure 23.Mutant DNA sequence of a part of the α spectrin gene in TM and TL.

Genomic DNA was PCR amplified and the sense strand sequenced directly using P_3. The point mutation detected in codon 28 of exon 2 of α spectrin changes the normal sequence CGT (Arg) to TGT (Cys). Both probands (TL and TM) were heterozygous for the mutant allele.

a TL
b TM

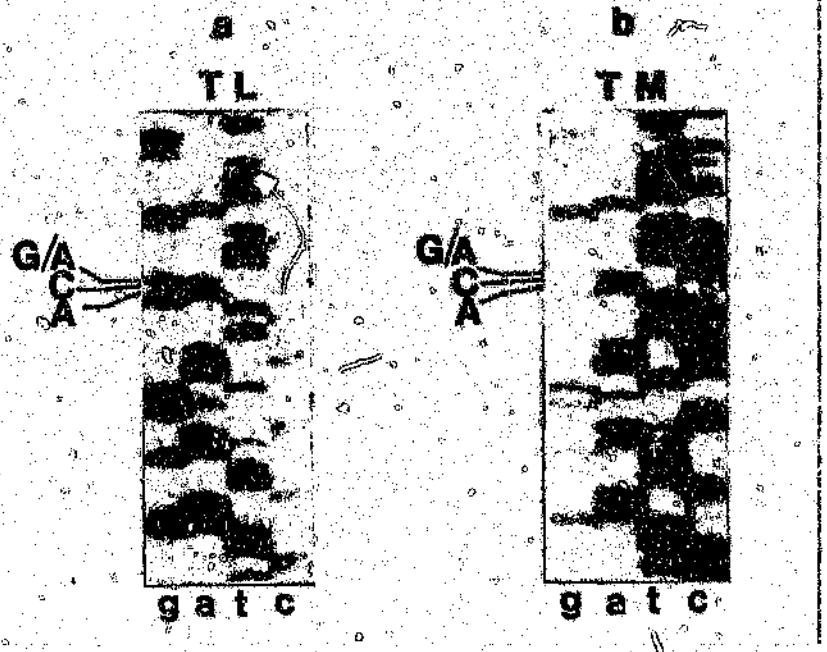


Figure 24. Mutant DNA sequence of a part of the α spectrin gene in TM and TL.

Genomic DNA was PCR amplified and the antisense strand sequenced directly using P_2. A point mutation in codon 28 of exon 2 of α spectrin was detected. This changes the normal sequence CGT (Arg) to TGT (Cys). In this orientation the change reads ACG to ACA. Both probands were heterozygous for the mutation.

a TL
b TM

2.4 Discussion

In both probands, TM and TL, erythrocyte membrane protein content, expressed as a ratio to band 3, was normal. TM (mother) and TL (father) were similarly normal. The ratio of 4.1a:4.1b, as seen on Laemmli SDS-PAGE, was decreased in TM and TL, when compared with controls, as a result of increased reticulocytes to compensate for the anaemia. Immature erythrocytes are characterized by a predominance of protein 4.1b, which decreases as the cell ages (Inaba *et al.*, 1992; Mueller *et al.*, 1987).

Quantitation of SpD content, expressed as a percentage of the total SpD and SpT content, by means of non-denaturing PAGE, is a good indicator of the status of the erythrocyte membrane spectrin. TM and TL both had vastly increased SpD percentages, 58 % and 57 %, respectively, compared with controls (< 10 %). The SpD % of TM (mother) was increased (34 %) but was not as high as TM. TL (father) was normal. These results confirm that TM (mother) is haematologically normal, but is an asymptomatic carrier of the molecular defect (Coetzer *et al.*, 1987).

An increase in the percentage SpD implies that there is defective spectrin αβ heterodimer self-association. Studies have shown that the αβ dimers of spectrin

variants are unable to associate in a head-to-head fashion (reviewed by Palek and Lambert, 1990). That is, the functional units of spectrin, tetramers, cannot be formed and assembled onto the membrane. This disrupts the erythrocyte membrane skeleton and leads to instability of the membrane (Figure 6) (Marchesi *et al.*, 1987; Coetzer *et al.*, 1990b; Tse *et al.*, 1990; Floyd *et al.*, 1991).

SpD studies indicated that the functional unit of spectrin in the probands (TM and TL) is compromised. At the structural level of the protein the defect was further characterized by analysis of the tryptic digest.

Limited tryptic digestion of spectrin at 4°C yields a digest pattern on Laemmli SDS-PAGE. TM, TM (mother) and TL all showed the same pattern. An increased band at 74 kDa was observed, with a concomitant decrease in the 80 kDa band. Western blotting and immunodetection confirmed that the 74 kDa band originates from the 80 kDa band, representing the normal cleavage site of α spectrin. The 74 kDa band represents the product of enhanced cleavage at a normally protected site. This spectrin variant is designated Spαl/74 and has been previously described (Morle *et al.*, 1990; Coetzer *et al.*, 1991; Floyd *et al.*, 1991).

TL (father) was normal, with respect to SpD content and tryptic digest. TL (mother) is unavailable for study, therefore, one must assume that the mother carries the SpaI/74 molecular defect.

Two dimensional analysis of the limited tryptic digest of spectrin provided improved resolution and confirmed the results of the one dimensional analysis.

Several different mutations have been described for SpaI/74 and hence the probands were screened for the presence of these defects. Firstly, PCR amplified genomic DNA was subjected to AhaII restriction digestion. This determines whether or not the molecular defect occurs at codon 28 in exon 2 of α spectrin. Codon 28 appears to be a "hot spot" for mutations (Coetzer *et al.*, 1991). Agarose gel analysis of the restriction digest determined that TL (father) was normal, whereas TM, TM (mother) and TL were heterozygous for a mutation in codon 28 in exon 2 of α spectrin. TM, TM (mother) and TL showed three bands. The 348 bp fragment represents the mutant allele, which has lost the AhaII site due to the point mutation in codon 28, whilst the 222 bp and 126 bp fragments represent the wild type allele.

Secondly, allele-specific oligonucleotide hybridization (ASOH) using probes specific for the four known codon 28 point mutations confirmed the PCR-AhaII restriction digest results and determined that both probands were heterozygous for same change, that is, Arg ▸ Cys (CGT ▸ TGT).

Thirdly, direct sequencing of ds DNA using PCR primers confirmed the results obtained by ASOH analysis.

Both black South African probands therefore carry a "hot spot" mutation at codon 28 and interestingly, this Arg ▸ Cys change has only previously been identified in two unrelated white New Zealand kindred (Coetzer *et al.*, 1991). The authors conclude that the CG dinucleotides at codon 28 underlie a "hot spot" for mutation, most likely due to methylation of the C residue, which then spontaneously deaminates to T, as noted in the probands (CGT ▸ TGT). This mutation is not recurrent. It should be noted that haplotype analysis was not carried out in the local kindred, so it is not known whether this mutation occurs on the same haplotype as the New Zealand kindred.

Arginine is a helix stabilizer and also has a role in stabilizing electrostatic interactions, particularly between subunits of oligomeric proteins (Mrabet *et al.*,

1992). Therefore, where the arginine residue is substituted, as in TM and TL, the α helical structure of the head region of α spectrin is disrupted (Figure 25). The spectrin αβ heterodimers cannot form tetramers and this leads to instability of the erythrocyte membrane and hence the change in cell shape.

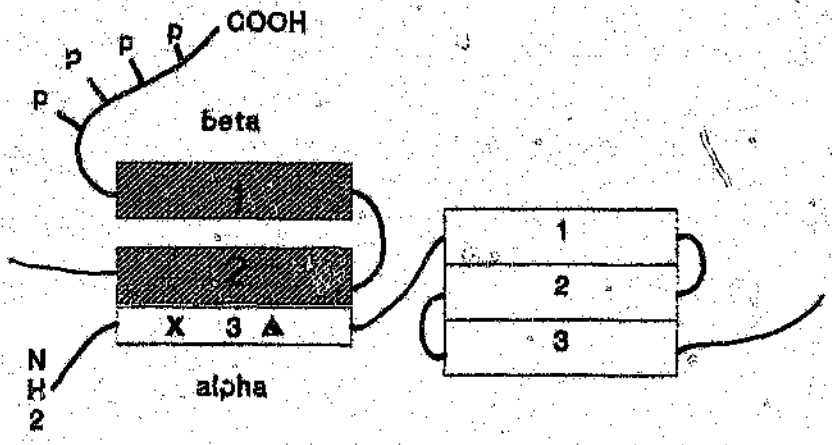


Figure 25.Schematic diagram of the spectrin heterodimer self-association site.

Spectrin αβ heterodimer self-association is weakened in association with SpaI/74 variants. The codon 28 mutation alters the conformation of the head region of α spectrin. The x marks the position of the mutation in codon 28 of helix 3 of exon 2 of α spectrin, which gives rise to enhanced cleavage by trypsin at the normally protected site at position 45 (Arg) or 48 (Lys) (▲) and the observed increased 74 kDa peptide.

2.5 Conclusions

In conclusion, the probands, TM and TL, have been characterized at a molecular level. Both probands carry the SpaI/74 variant and are heterozygous for a "hot spot" point mutation (CGT ▸ TGT) in codon 28 in exon 2 of α spectrin, which results in the incorporation of a cysteine residue instead of an arginine residue into the α spectrin subunit during translation. At the protein level, the amino acid substitution alters the conformation of the head region of spectrin αβ heterodimers such that they cannot form tetramers on the erythrocyte membrane and hence the percentage SpD, relative to the total SpD and SpT, is increased. Enhanced cleavage at a normally protected site gives rise to the increased 74 kDa tryptic peptide observed.

TM (mother), who is haematologically normal, was confirmed to be an asymptomatic carrier for the molecular defect. TL (father) was normal, in all respects.

Thus, the probands, TM and TL, were characterized as having the SpaI/74 variant due to a mutation in codon 28 of α spectrin, leading to the clinical manifestations of HE.

This work is in preparation for publication.

3. Part II: A Taq1 polymorphism in the human erythroid β spectrin gene

3.1 Subjects

The frequency of a Taq1 polymorphism in the human erythroid β spectrin gene was estimated from 84 alleles of unrelated normal individuals and HE patients with known α spectrin defects, both black and white, male and female, from local and North American populations.

Blood was collected in ACD tubes and the buffy coats removed for extraction of genomic DNA (2.2.3.1.).

3.2. Materials and methods

3.2.1. Description of cDNA probe

A 738 bp erythroid β spectrin cDNA probe (supplied by J. Prchal, University of Alabama, Birmingham; Prchal *et al.*, 1987) was hybridized to Southern blots of genomic DNA.

3.2.2. Taq1 digestion of genomic DNA

Ten to 15 µg of genomic DNA (2.2.3.1.), diluted in TE, were digested as follows:

	Volume		[final]
DNA	x	µl	(10-15 µg)
10x buffer *	10	µl	(1x)
100 mM spermidine	4	µl	(2.5 mM)
10 U/µl Taq1	5	µl	(50 U)
Water to make volume to	100	µl	

10x buffer ' (supplied with the enzyme):
100 mM Tris, 50 mM $MgCl_2$, 1 M NaCl, 10 mM β-Mercaptoethanol, pH 8.0.

The reaction mixture was overlayered with mineral oil, to prevent evaporation, and incubated for 16 - 18 hours, at 65°C.

The reaction was stopped with the addition of 2.5 µl 0.5 M EDTA pH 8.0. The mixture below the mineral oil was transferred to a new tube and 10 µl 3 M NaAcetate pH 5.2 added, followed by 250 µl ice-cold absolute ethanol. The digested product was precipitated at -70°C for 30 minutes and then centrifuged in a microfuge for 10 minutes. The pellet was washed once in 70 % ethanol and left to air-dry. The DNA was resuspended in 20 µl TE and 2.5 µl 10x gel loading dye was added (2.2.3.2.).

3.2.3. Preparation of Southern blot

A 0.7 % agarose gel (20 cm x 25 cm) was prepared in 1x TAE buffer, with 2.5 µl ethidium bromide (10 mg/ml) per 100 ml. The samples were loaded into the wells and electrophoresed for 24 - 30 hours at 25 V (20 - 22 mA).

Two lambda DNA molecular weight markers, (EcoRI/HindIII and BstEII digests), were electrophoresed alongside the digested samples.

After electrophoresis, the gel was photographed and incubated in 0.25 M HCl for 10 minutes (Southern, 1975), to depurinate the DNA. After washing in two changes of water, the gel was incubated in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes, following which it was again washed in water and then incubated in neutralising solution (1.5 M NaCl, 0.5 M Tris pH 7.2, 0.001 M EDTA) for 2x 20 minutes.

After washing in water, the gel was capillary blotted onto nylon membrane (Southern, 1975), using 20x SSPE as a blotting buffer, the process taking 12 - 24 hours. The Southern blot was then rinsed in 2x SSPE and baked at 80°C for 2 hours, to fix the DNA, before prehybridization.

3.2.4. Random primed labelling of cDNA probe

The cDNA probe was labelled to high activity using a Random Primed DNA Labelling Kit. Lambda DNA was also labelled in order to be able to size the polymorphic products, according to the DNA molecular weight markers.

The DNA to be labelled was denatured by boiling for 5 minutes, snap-cooled on ice, and the labelling reaction carried out as follows:

i) β Spectrin cDNA probe		ii) Lambda DNA
5 µl (25 ng)	DNA	2 µl (25 ng)
1 µl	dCTP (0.5 mmol/l)	1 µl
1 µl	dGTP (0.5 mmol/l)	1 µl
1 µl	dTTP (0.5 mmol/l)	1 µl
2 µl (1x)	Reaction mix*	2 µl (1x)
5 µl (50 µCi)	α^{32}PdATP (3000 Ci/mmol)	4 µl (40µCi)
1 µl (2 U)	Klenow enzyme	1 µl (2 U)
4 µl	Water	8 µl
20 µl		20 µl

* Reaction mixture:

Hexanucleotide mixture in 10x concentrated reaction buffer, provided by the manufacturer in the kit.

The reaction was incubated at 37°C for 45 minutes and stopped with the addition of 2 µl 0.2 M EDTA pH 8.0. The labelled DNA was purified using a Geneclean kit. Sodium iodide solution (150 µl) was added to each reaction, followed by 5 µl Glassmilk. The mixture was incubated on ice for five minutes, and inverted once or twice during incubation. The DNA-containing Glassmilk was precipitated by centrifugation for 30 seconds and the pellet washed three times in 300 µl NEW (new ethanol wash). The DNA was eluted from the Glassmilk, by means of incubation at 50°C for 3 minutes, into 50 µl TE, followed by centrifugation for 1 minute. The elution step was repeated, to a final volume of 100 µl. Three µl of each

labelled reaction was counted by means of liquid scintillation counting (2.2.3.4b.).

3.2.5. Hybridization of Southern blot

Prehybridization solution was prepared as follows:

		[final]
6.25 ml	20x SSPE (2.2.3.5.c)	5x
2.5 ml	50x Denhardts (2.2.3.5.c)	5x
1.25 ml	10 % SDS	0.5 %
14.0 ml	water	
24.0 ml		

One ml of a 10 mg/ml solution of salmon sperm DNA was denatured by boiling and chilled on ice for 10 minutes, before addition to the prehybridization solution.

The blot was prehybridized at 65°C for 1 hour, in 25 ml prehybridization solution.

The purified, labelled probes were denatured by boiling and were added to the prehybridization solution, together with a further 10 mg salmon sperm DNA. The blot was hybridized overnight at 65°C, in a plastic container.

Following hybridization, the blot was washed three times, with increasing stringency, as follows:

i) 2x SSPE/0.1 % SDS
   15 minutes, room temperature

ii) 0.5x SSPE/0.1 % SDS
   15 minutes, room temperature

iii) 0.1x SSPE/0.1 % SDS
   10 minutes, 50°C

The blot was exposed to X-ray film with intensifying screens for 2 days or longer, at -70°C, and the autoradiograph developed, in order to visualise the bands.

3.3. Results

3.3.1. Southern blot analysis of Taq1 polymorphism

Genomic DNA was digested with Taq1 and electrophoresed on a 0.7 % agarose gel (Figure 26). The gels were Southern blotted onto nylon membrane by capillary action. A cDNA human erythroid β spectrin probe was random prime labelled with α^{32}PdATP and hybridized with the prepared blot.

Figure 27 shows two autoradiographs of Southern blots. The markers (M1, lambda EcoRI/HindIII digest, and M2, lambda BstEII digest) allow size determination of the bands (Figure 28). The BstEII markers were used to provide a standard curve. The log values of the sizes (kb) were plotted against distance moved (cm). The distances moved by the unknown band sizes were read off against the X-axis and the kb value determined by inverse log. In this way the sizes were calculated to be :

(i) Top band 3.5 kb
(ii) Middle band 3.4 kb
(iii) Bottom band 2.7 kb

A 3.4 kb band was constant in all cases (Figure 27). This band was either associated with a 3.5 kb band, lane 21, or a 2.7 kb band, lane 2, or both, lane 1. Resolution was

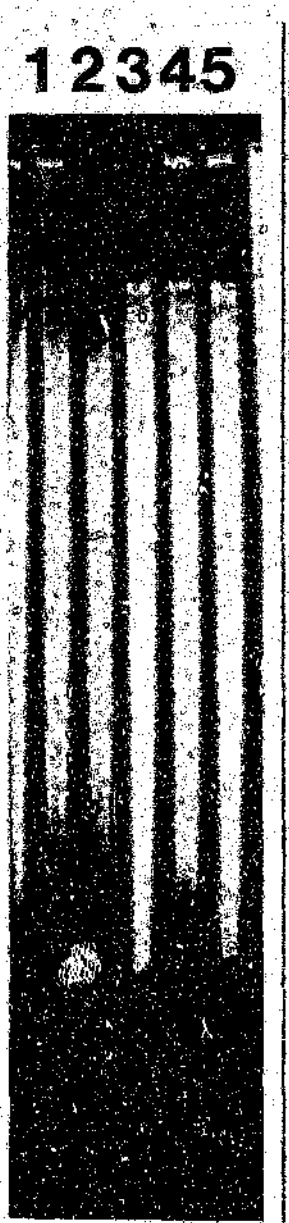


Figure 26.Preparative agarose gel for Southern blotting of genomic DNA.
Genomic DNA, lanes 1 to 5, was digested with TaqI endonuclease and electrophoresed on a 0.7 % agarose gel in 1x TAE buffer, prior to Southern blotting.

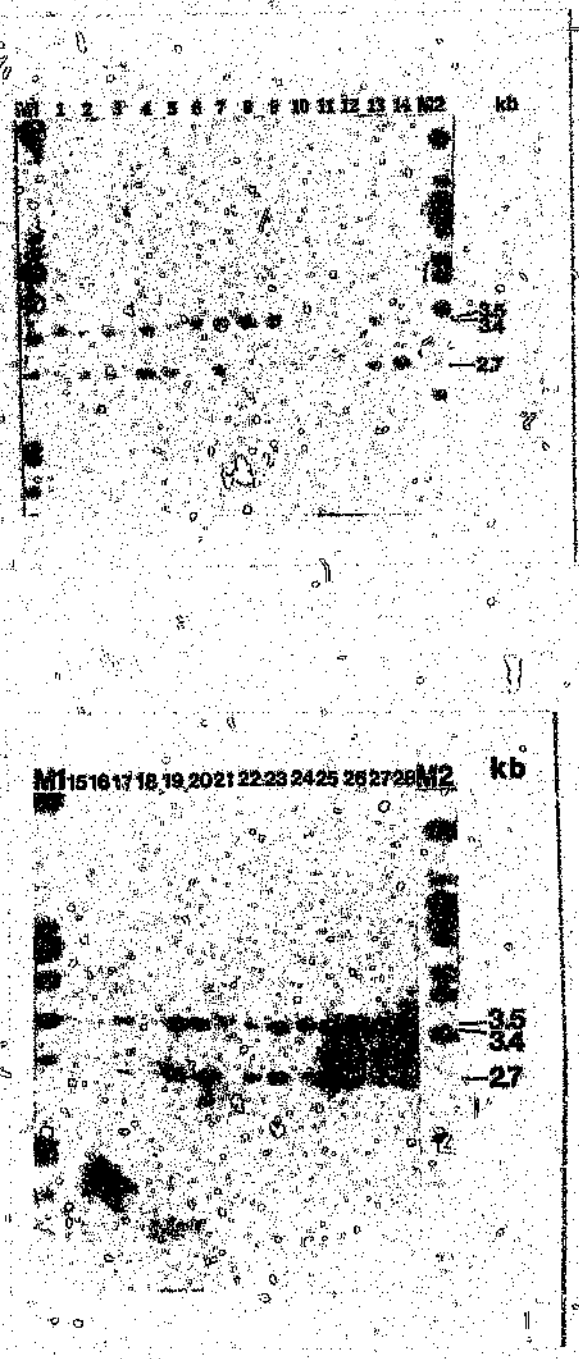


Figure 27.Autoradiograph of Southern blot.
Southern blot of genomic DNA digested with Taq1 (lanes 1 to 28) and hybridized with a cDNA human erythroid β spectrin probe. A constant band at 3.4 kb is seen in all cases associated with either a 3.5 kb band, lane 21, or a 2.7 kb band, lane 2, or both, lane 1.

M1 Lambda EcoRI/HindIII digest
M2 Lambda BstEII digest

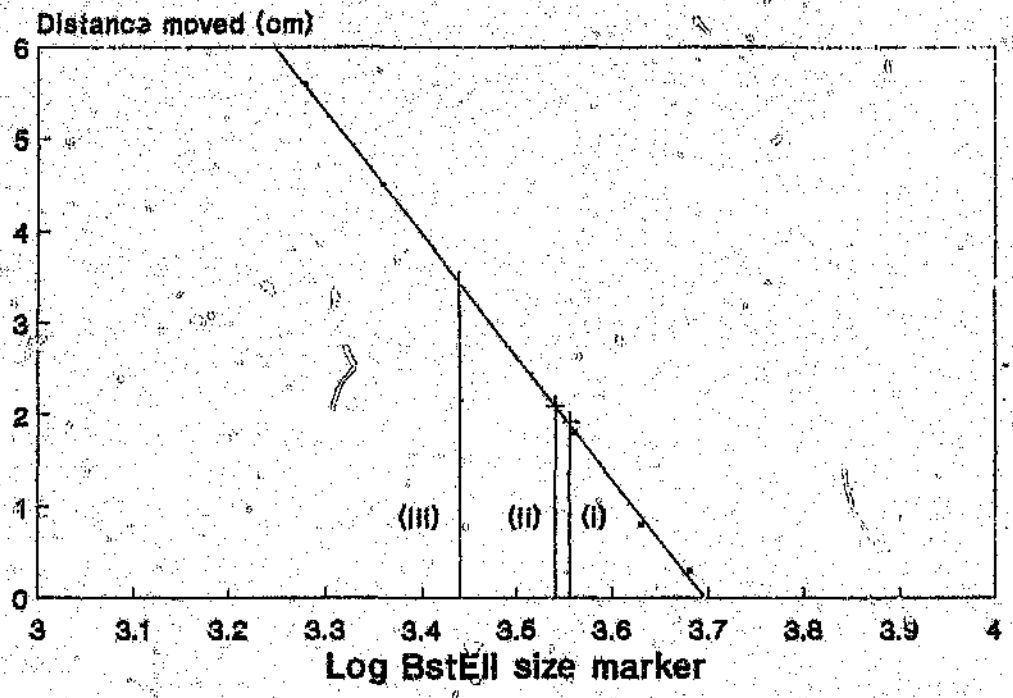


Figure 28. Size determination of polymorphic alleles. The sizes of the three bands observed on the autoradiographs of Southern blots (Figure 27) were determined with reference to a standard curve, produced by plotting log (kb) BstEII digest size marker bands against distance moved (cm).

(i)	Top band	3.5 kb
(ii)	Middle band	3.4 kb
(iii)	Bottom band	2.7 kb

critical since there were only 100 bp separating the top and middle bands. Where results were ambiguous the samples were repeated. The pattern observed represents a two-allele polymorphism depicted schematically in Figure 29. Allele A represents the more common pattern, with two TaqI sites present, giving three fragments of 3.4 kb, 2.7 kb and 0.8 kb. The cDNA probe contains a TaqI restriction site (result not shown) and binds in the region indicated and hence does not detect the 0.8 kb band. Allele a represents the allele without an additional TaqI site, yielding bands of 3.4 kb and 3.5 kb, on digestion with TaqI.

Studies of Mendelian inheritance in three families showed codominant segregation of the polymorphism and confirmed the proposed theory. The heterozygosity was 40 %. Family S represents the pattern of inheritance (Figure 30). The parents are both heterozygous for the polymorphism, having both the A allele and the a allele. All three bands were seen in the parents, that is, 3.5 kb, 3.4 kb and 2.7 kb. The first (i) and third (iii) children are homozygous for the A allele, having only the 3.4 kb and 2.7 kb bands. The second (ii) child was homozygous for the a allele. Only the 3.5 kb and 3.4 kb bands were observed.

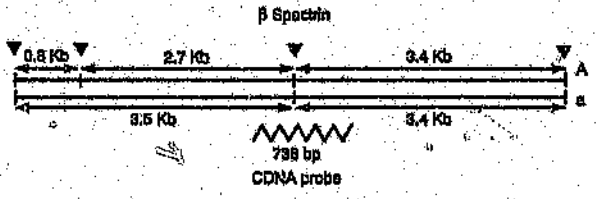


Figure 29. Schematic diagram of Taq1 polymorphism.
A allele, with an extra Taq1 site, producing 3.4 kb, 2.7 kb and 0.8 kb fragments.

a allele producing 3.4 kb and 3.5 kb fragments, on Taq1 digestion.

The cDNA probe binds in the region indicated.

▼ Taq1 sites

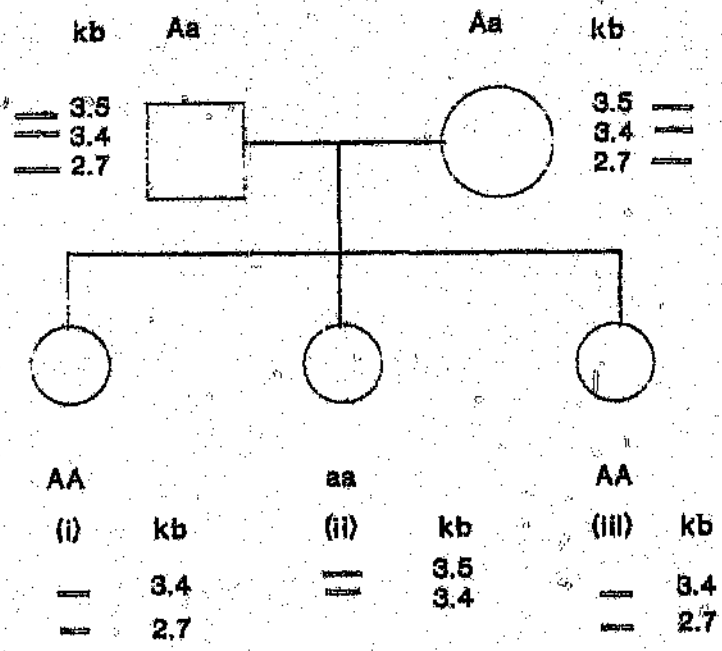


Figure 30.Mendelian inheritance of two-allele polymorphism.
Family S represents the pattern of inheritance of the polymorphism. The parents are both heterozygous for the polymorphism. The offspring are homozygous for either allele. Band sizes were observed, as indicated.

Allelic frequencies were determined by calculating the number of either allele A or allele a, over the total number of alleles studied (Table V). A total of 84 alleles from unrelated individuals were studied. X^2 analysis, where $X^2_{[1]} = 1.43$, $0.5 > p > 0.2$, using the 2x2 contingency table determined that the frequency of the polymorphism in the Black population showed no significant difference to that of the Caucasian population.

Table V. Frequency (*f*) of polymorphism occurrence in Caucasians and Blacks (estimated from 84 alleles of unrelated individuals).

	Caucasian	Black	Caucasian and Black
Allele A			
2.7 + 0.8 kb	*f*=0.787	*f*=0.611	*f*=0.750
Allele a			
3.5 kb	*f*=0.212	*f*=0.389	*f*=0.25
n = alleles	66	18	84

3.4 Discussion

The β spectrin gene is localized on chromosome 14 (Prchal *et al.*, 1987; Winkelmann *et al.*, 1988). The restriction fragment length polymorphism (RFLP) described represents a two-allele polymorphism on the human erythroid β spectrin gene. The polymorphism is characterized by the presence or absence of a Taq1 restriction site and was detected by hybridization of Southern blots with a 738 bp β spectrin cDNA probe. This probe detected three bands, namely a constant band at 3.4 kb associated with either a 3.5 kb band or a 2.7 kb band, or both, as observed on autoradiographs. Studies on three families established that the polymorphism was inherited in a Mendelian fashion. Statistical analysis of the results, by means of the X^2 test, established that there was no significant difference between the frequency of occurrence in Black and Caucasian populations. The populations were assessed independently and combined (Table V). The heterozygosity was 40 %.

Knowledge of this polymorphism and its mode of inheritance may provide information in linkage analysis and enable one to ascribe spectrin defects to one or the other subunit of spectrin. The polymorphism itself is not associated with any disease.

3.5 Conclusions

In conclusion, a two-allele TaqI polymorphism was detected in the human erythroid β spectrin gene. The polymorphism is inherited in a Mendelian fashion and can be used in linkage studies by analysis of the RFLPs. The polymorphism is distributed in both Black and Caucasian populations and there is no significant difference in the frequencies of the polymorphism between the two populations.

A manuscript entitled "A TaqI polymorphism in the human erythroid β spectrin gene" has been submitted for publication.

4. Part III: Densitometric analysis and quantitation of erythrocyte membrane protein ratios in local populations

4.1. Subjects

Blood was collected in ACD from normal individuals from local populations. Erythrocyte membrane protein ratios from black and white individuals, both male and female, were analysed and quantitated.

4.2 Materials and methods

Erythrocyte membranes were prepared by means of lysis centrifugation (2.2.1.) and after protein determination (2.2.1.1.) the membrane proteins were resolved on Laemmli (2.2.1.2.) and Fairbanks (2.2.1.3.) SDS-PAGE. The gels were stained in Coomassie stain. Normal values (mean ± SD) for erythrocyte membrane protein content (expressed as a ratio to band 3) were obtained by scanning densitometry of the stained gels. The gels were scanned at 525 nm on the Helena Quick Scan Jr TLC densitometer and quantitated by calculating the area under the relevant peaks.

In some patients presenting with haemolytic anaemia, it was possible to obtain a reticulocyte count, and this was correlated with the 4.1a/4.1b protein ratio.

In some samples, spectrin was extracted from the

membranes and was subjected to non-denaturing gel electrophoresis (2.2.2.1.). The SpD content expressed as a percentage of SpT and SpD content (mean ± SD) was calculated, in normal individuals, by analysis of scanning densitometry of Coomassie-stained non-denaturing gels.

4.3. Results

4.3.1. Erythrocyte membrane protein analysis

Normal control individuals were analysed with respect to their erythrocyte membrane protein content. Samples were electrophoresed on Laemmli (Figure 31a) (45 and 50 µg) and Fairbanks (Figure 31b) (20 and 25 µg) SDS-PAGE. Only one loading of each is shown in the figure. Laemmli SDS-PAGE was used to quantitate the 4.1a/4.1b ratio and protein 4.1/4.2 ratio. Ankyrin co-migrates with spectrin and hence cannot be quantitated from the Laemmli gel. Fairbanks SDS-PAGE was employed to resolve the remainder of the membrane proteins which were then quantitated and expressed as a ratio to band 3.

The mean ± SD of erythrocyte membrane protein content is shown in Tables VI and VII. The range indicates the highest and the lowest value obtained for control individuals. The values obtained in our laboratory are slightly increased compared with those reported for laboratories in the USA (personal communication, T Coetzer).

4.3.2. Correlation between 4.1a/4.1b and reticulocyte %

Patients presenting with haemolysis usually had a decreased 4.1a/4.1b ratio. Haemolysing subjects are associated with an increased reticulocyte percentage, due

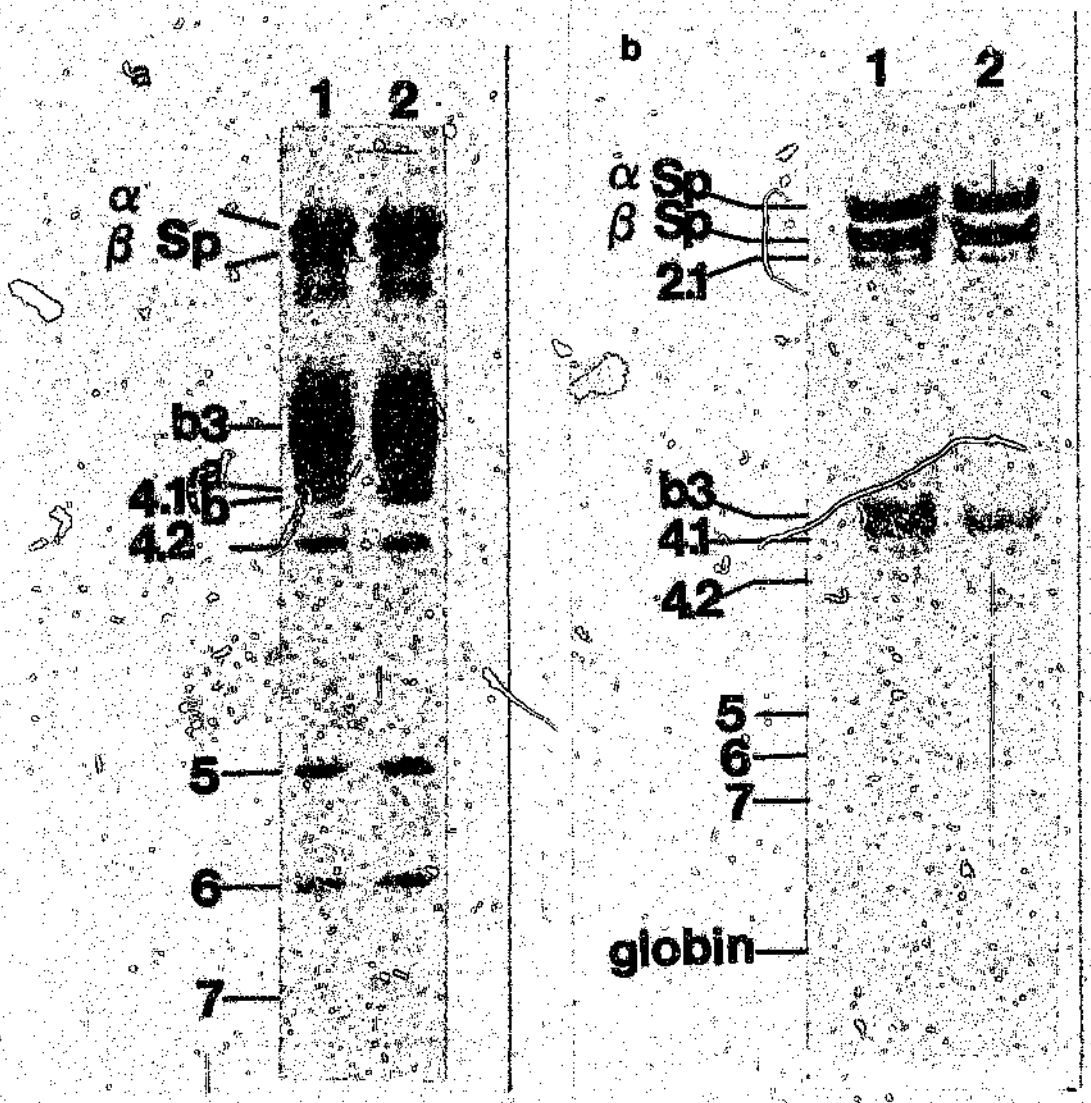


Figure 31. SDS-PAGE of control individuals. Membranes of control individuals were electrophoresed on SDS-PAGE and Coomassie-stained. Quantitation of densitometric scans of stained gels was carried out (Table VI, Table VII).

a) Laemmli SDS-PAGE. Lanes 1 and 2 represent 45 µg aliquots of SDS-solubilized membranes of control individuals.

b) Fairbanks SDS-PAGE. Lanes 1 and 2 represent 25 µg aliquots of SDS-solubilized membrane of control individuals.

Table VI. Erythrocyte membrane protein ratios: Laemmli SDS-PAGE.

	4.1a/4.1b	4.1/4.2	4.1+4.2/b3
	1.4 ± 0.18	1.5 ± 0.19	0.33 ± 0.08
Range	[1.14-1.89]	[1.11-1.88]	[0.2-0.54]
	n = 49	n = 46	n = 47

Table VII. Erythrocyte membrane protein ratios: Fairbanks SDS-PAGE.

αSp/βSp	Sp/b3	2.1/b3	4.1/4.2	4.1+4.2/b3
1.11±0.065	1.12±0.11	0.23±0.03	1.00±0.17	0.37±0.056
Range[1.0-1.27]	[0.89-1.32]	[0.13-0.34]	[0.71-1.45]	[0.27-0.57]
n = 40	n = 35	n = 23	n = 37	n = 38

to compensatory erythrocyte production as a result of anaemia.

A small, preliminary study of patients (n = 10) with an increased reticulocyte count (normal range : 0.5-2.0 %) showed that there was a trend towards an inverse relationship between the 4.1a/4.1b ratio and reticulocyte percentage (Figure 32). That is, as the reticulocyte percentage increases, the 4.1a/4.1b ratio decreases. There was wide scatter due to the small sample number.

4.3.3. Non-denaturing PAGE of spectrin extract

Control individuals (n = 16) were studied with respect to establishing a normal range of SpD content, expressed as a percentage of total SpD and SpT (Figure 33). This parameter allows diagnosis of the variants of HE and HPP subjects and carriers, on the basis of an increase in SpD % over controls (Table VIII). The range indicates the highest and lowest value obtained for control individuals. In this case, the mean ± SD was less than 10 %, as reported previously for normal individuals (Coetzer *et al.*, 1987).

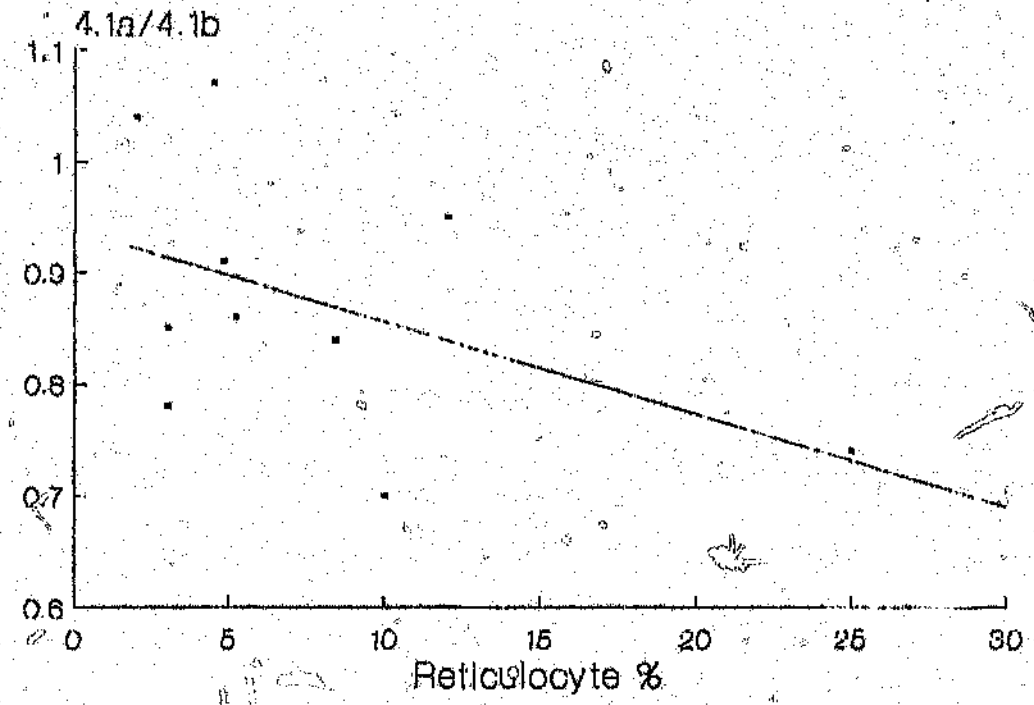


Figure 32. Reticulocyte % versus 4.1a/4.1b ratio. The graph shows an inverse relation between reticulocyte % and 4.1a/4.1b ratio, in individuals with haemolytic anaemia (•) (n = 10), having an increased reticulocyte % (> 2.0 %). In normal individuals the mean ± SD for the 4.1a/4.1b ratio is 1.4 ± 0.18 (Table VI).

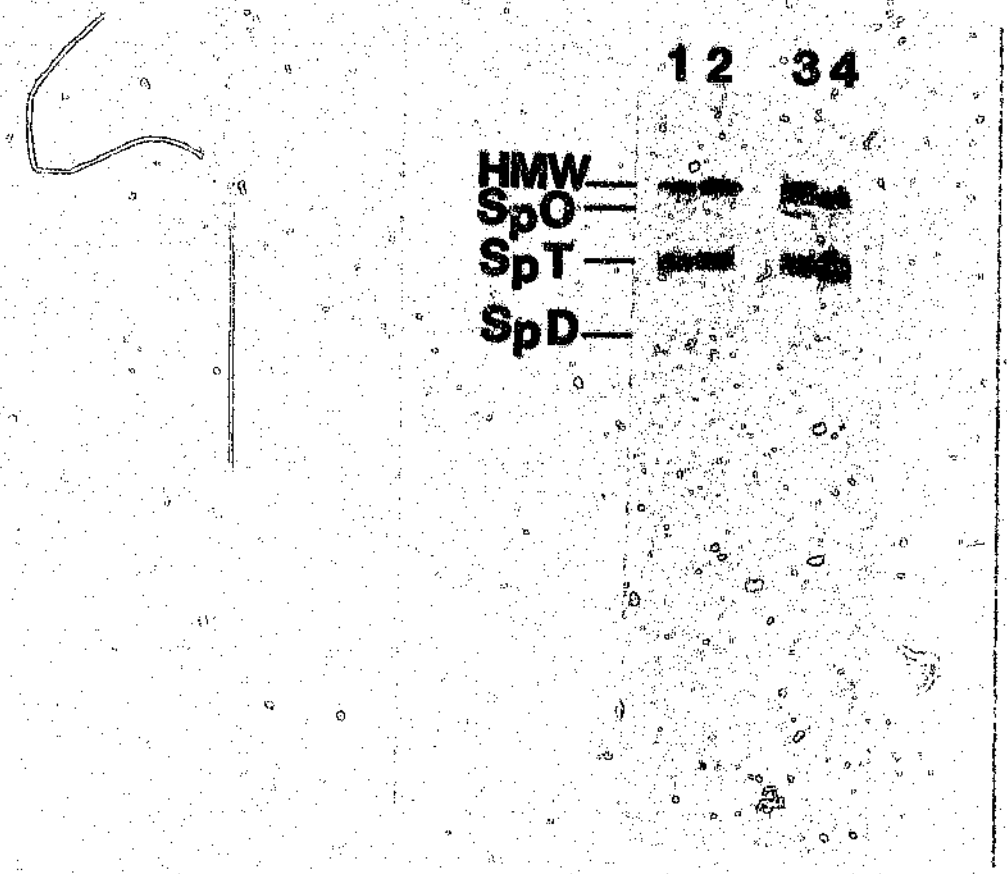


Figure 33. Non-denaturing PAGE
Ten and 15 µg aliquots of spectrin extract from control individuals were electrophoresed and Coomassie-stained, lanes 1-4. SpD content was quantitated from densitometric scans of non-denaturing PAGE gels and was expressed as a percentage of total SpD and SpT (Table VIII). High molecular weight complex (HMW) and spectrin oligomers (SpO) are indicated.

Table VIII. Membrane spectrin dimer (SpD) content (%). SpD content is expressed as a percentage of total SpD and SpT.

% SpD/(SpD + SpT)
7.33 ± 2.4
Range [2.5-11.2]
n = 16

4.4 Discussion

Control individuals were studied with respect to establishing a normal range ± SD of erythrocyte membrane protein content, expressed as a ratio to band 3. In our laboratory the values are only slightly higher than those reported previously (personal communication, T Coetzer). This may be due to differences in gel preparation, chemicals and staining procedures.

Studies of individuals with [illegible] hemolysis (n = 10) show a trend toward [illegible] relationship between reticulocyte percent[illegible] and 4.1a:4.1b ratio. Due to deamidation of asparagine residues in protein 4.1 the amount of the 4.1b form decreases as erythrocytes mature (Inaba *et al.*, 1992). That is, the greater the reticulocyte percentage, the lower the 4.1a:4.1b ratio. The 4.1a:4.1b ratio is thus an indication of the age of the erythrocyte population.

Studies of SpD content, expressed as a percentage of total SpD and Sp1, in normal individuals established a mean ± SD of 7.33 ± 2.8 %, which correlates with published values (Coetzer *et al.*, 1987).

4.5 Conclusions

In conclusion, the normal values ± SD established for use in our laboratory were within the ranges found previously, for both erythrocyte membrane protein content and SpD content. It was observed that a trend existed between increased reticulocyte percentage and decreased 4.1a:4.1b ratio. This ratio is an indicator of the cell population present.

5. Appendix: List of chemicals, equipment and suppliers

Acrylamide	Molecular biology grade, BDH, Poole, England
Agarose:	
LE	SEAKEM, FMC, Rockland, ME, USA
GTG (molecular biology grade)	SEAKEM, FMC, Rockland, USA
Agarose gel systems:	
Hybaid maxi (20 cm x 25 cm)	Hybaid Ltd, Teddington, Middlesex, UK
Single place minigel	OMEG Scientific, Claremont, Cape Town, South Africa
haII	New England Biolabs, Beverly, MA, USA
APS	Stratagene, La Jolla, CA, USA
Ampholytes:	
Biolyte pH 5-7	Bio-Rad, Richmond, CA, USA
Biolyte pH 4-6	Bio-Rad, Richmond, CA, USA
Biolyte pH 3-10	Bio-Rad, Richmond, CA, USA
β counter	United Technologies, Packard, Downers Grove, Ill., USA
bis	BDH, Poole, England
BSA	Fraction V, Boehringer Mannheim, Germany
4-Chloro-1-naphthol	Sigma, St Louis, MO, USA
Coomassie G-250	Bio-Rad, Richmond, CA, USA
Coomassie R-250	BDH, Poole, England
dATP	Boehringer Mannheim, Germany
dCTP	Boehringer Mannheim, Germany
dGTP	Boehringer Mannheim, Germany
dTTP	Boehringer Mannheim, Germany
DNA molecular weight marker	100 bp ladder, BRL, Gaithersburg, MD, USA
DNA thermal cycler	Perkin Elmer Cetus Corporation, Norwalk, CT, USA
DTT	Promega, Madison, WI, USA
Ethidium bromide	Boehringer Mannheim, Germany
Ficoll (type 400)	Pharmacia, Uppsala, Sweden
Filter paper	Whatman International Ltd, Maidstone, England
Formamide	Molecular biology grade, E. Merck, Germany
Geneclean kit	BIO 101, La Jolla, CA, USA
Gradient maker	SG series, Hoefer Scientific Instruments (HSI), San Francisco, CA, USA

Heating block	Hägar designs, Pinelands, South Africa
Hybridization bags	BRL, Gaithersburg, MD, USA
8-Hydroxyquinoline	Sigma, St Louis, MO, USA
J2-21 centrifuge	Beckman, CA, USA
JA-17 rotor	Beckman, CA, USA
β-Mercaptoethanol	E. Merck, Germany
Mermaid kit	BIO 101, La Jolla, CA, USA
Microfuge	Hägar designs, Pinelands, South Africa
Microfuge tubes	Eppendorf, Hamburg, Germany
Mineral oil	Sigma, St Louis, MO, USA
Nunc conical centrifuge tubes	Nunclon, Denmark
Nitrocellulose membrane	Hybond C, Amersham, Buckinghamshire, UK
NP-40	Sigma, St Louis, MO, USA
Nylon membrane	Hybond N, Amersham, Buckinghamshire, UK
Peristaltic pump	Atto Corporation, Tokyo, Japan
τ^{32}PATP	Du Pont NEN, Boston, MA, USA
α^{32}PdATP	Du Pont NEN, Boston, MA, USA
PAGE system	Sturdier SE400, HSI, San Francisco, CA, USA
Phenol	E. Merck, Germany
PMSF	Boehringer Mannheim, Germany
Polyvinylpyrrolidine	Sigma, St Louis, MO, USA
Ponceau S	Sigma, St Louis, MO, USA
Power supply	Microcomputer electrophoresis power supply (E734), Consort, Turnhout, Belgium
Random primed DNA labelling kit	Boehringer Mannheim, Germany
Salmon sperm DNA	Sigma, St Louis, MO, USA
Scintillant	United Technologies, Packard, Groningen, The Netherlands
Semi-dry transfer cell	Transblot SD, Bio-Rad, Richmond, CA, USA
Sequencing apparatus	Model S2, BRL, Gaithersburg, MD, USA
Sequenase kit	United States Biochemical (USB), Cleveland, Ohio, USA
Sheep anti-rabbit IgG-peroxidase	Boehringer Mannheim, Germany
Slot blot apparatus	BIO DOT SF, Bio-Rad, Richmond, Ca., USA

TaqI endonuclease	Boehringer Mannheim, Germany
Taq DNA polymerase	Promega, Madison, WI, USA
TEMED	Bio-Rad, Richmond, CA, USA
Ti50 rotor	Beckman, CA, USA
T4 polynucleotide kinase	Promega, Madison, WI, USA
Trypsin-TPCK treated	Worthington Biochemical, Freehold, NJ, USA
Urea	Bethesda Research Laboratories (BRL), Gaithersburg, MD, USA
UV transilluminator	UVP Inc., San Gabriel, CA, USA
X-ray film	Curix RP-1, Agfa, Germany

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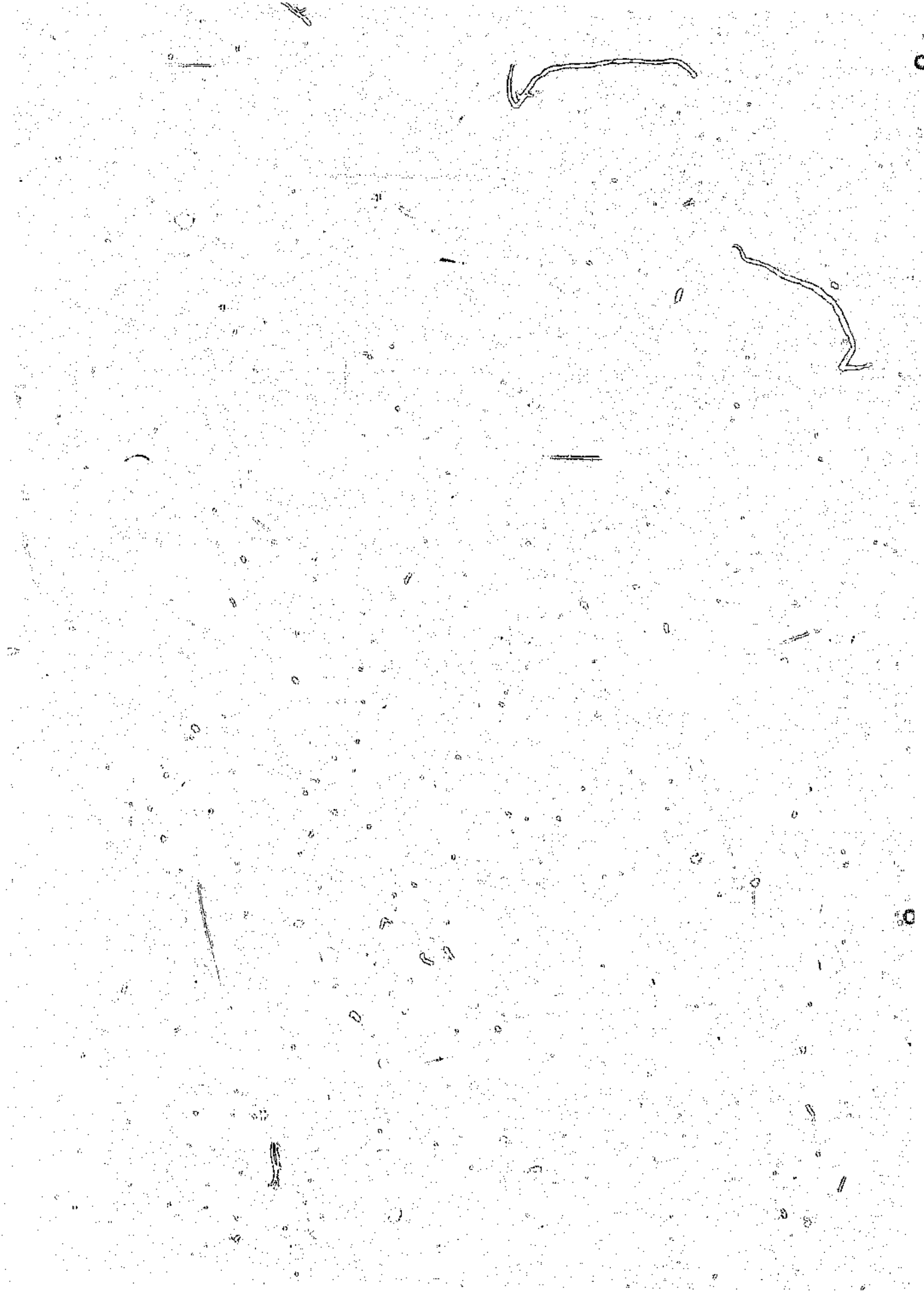

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