CHAPTER 2: METHODOLOGY

The following procedures, unless otherwise stated, were performed at room (ambient) temperature (20-24^{o}C). Analytical grade chemicals were used for all procedures. Unless otherwise stated, all water used was reverse osmosis water further purified using the MilliPore Milli-Q™Water System. All glassware was rinsed with reverse osmosis water before use.

Ethics clearance for these studies had been previously obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Johannesburg, Gauteng, Republic of South Africa): **25/1/92**.

2.1 Patient Data and Sample Storage

Blood samples were collected in ACD Vacutainer™ tubes (Becton, Dickinson & Co., USA) and stored at 4^{o}C. Clinical data were supplied when possible with blood specimens. Blood smears were prepared for analysis.

Staff members of the Red Cell Membrane Unit of Professor Theresa Coetzer, National Health Laboratory Service, separated the buffy coat from the erythrocytes by centrifugation at 2500rpm (1000g) for 15 minutes (Jouan BR3.11 refrigerated to 4^{o}C, Jouan, France). The plasma was discarded whereas the buffy coat was removed and stored at -70^{o}C in a 1.5ml Eppendorf tube (Eppendorf, Germany). The membrane proteins were extracted from the erythrocytes by hypotonic lysis (Coetzer & Palek, 1986) and an aliquot was removed to determine the protein concentration using the Coomassie Plus Protein Assay Reagent Kit (Pierce, USA). The remaining membrane proteins were solubilised (coated in SDS so that the proteins had a net negative charge) by mixing 150µl membranes with 40µl suspension solution (50mM Tris pH8.0, 5mM EDTA, 5% SDS, 25% sucrose), 5µl sucrose loading dye (2.5% sucrose, 0.05% bromophenol blue) and 4µl β-mercaptoethanol (Merck, Germany). The mixture was boiled for one minute and the solubilised protein aliquots were stored at -20^{o}C.

The membrane proteins were analysed by staff of the Red Cell Membrane Unit using sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). A 3.5 to 17 percent non-linear gradient gel (Hoefer SG series gradient maker, Hoefer Scientific Instruments, USA) (Fairbanks *et al*, 1971) and a 12 percent gel (Laemmli, 1970) were used. The relative quantities of each protein present were determined using gel densitometry scanning (GS300 Transmittance/Reflectance Scanning Densitometer with appropriate software, Hoefer Scientific Instruments, USA) The area (according to the gel densitometry scanning analysis) occupied by each protein was compared to the area occupied by band 3. If a protein was deficient, the ratio of that protein to band 3 would decrease. Conversely, if band 3 was deficient, this ratio would increase for all of the membrane proteins. To ensure the reliability of the preparation the patient protein levels were compared to the protein levels of control samples obtained, prepared, separated and analysed at the same time.

Patients for the study were selected according to the type of haemolytic anaemia from which they suffered; this was determined from examination of patient blood smears. Only patients with either HS or HE were chosen. This subset of patients was further limited to 15 HS patients with a band 3 deficiency and nine HE patients with a protein 4.1 deficiency (as indicated by the membrane protein analysis).

2.2 Screening of Erythrocyte Protein 4.1 for Size Variations

2.2.1 Separation of Erythrocyte Membrane Proteins

The Laemmli SDS-PAGE mixture was prepared according to Table 2.1. The 12% resolving (lower) gel was poured into a vertical gel system (The Sturdier Vertical Slab Gel Unit Model SE400, Hoefer Scientific Instruments, USA) and overlaid with water. It was left to set for four to six hours after which the water was poured off. The comb was inserted into the system, the 4% stacking (upper) gel was poured and was left to set for one to two hours.

Table 2.1: Laemmli SDS-PAGE Gel Components

Component	12% Resolving Gel	4% Stacking Gel
30% Acrylamide (w/v)	12.00ml	1.38ml
1% Bisacrylamide (w/v)	3.20ml	1.00ml
4x Resolving Buffer	7.50ml	-
4x Stacking Buffer	-	2.50ml
10% SDS (w/v)	0.16ml	0.02ml
Milli-Q Water	7.00ml	4.90ml
10% APS* (w/v) (fresh)	0.20ml	0.20ml
TEMED**	0.015ml	0.0075ml

**Ammonium persulphate (Promega Corporation, USA)*

***N,N,N',N' Tetramethylethylenediamine (Promega Corporation, USA)*

4x Resolving Gel Buffer (100ml)

- 1.5M Tris 18.17g

Dissolve in 80ml water.

Adjust pH to 8.8 with HCℓ (Saarchem, RSA).

Correct volume to 100ml with water.

4x Stacking Gel Buffer (100ml)

- 0.5M Tris 6.06g

Dissolve in 80ml water.

Adjust pH to 6.8 with dilute HCℓ.

Correct volume to 100ml with water.

The comb was removed and the buffer tanks were filled with Laemmli gel running buffer.

Laemmli Running Buffer (2l)

- 25mM Tris 6.06g
- 191mM Glycine 28.8g
- 0.1% SDS 2.0g

Dissolve in a final volume of 2l water.

45µg aliquots of solubilised protein were loaded in duplicate order onto the gel and were separated for 17 hours at 75V (PS500XT DC Power Supply (500V, 400mA, 20W), Hoefer Scientific Instruments, USA). The gel was removed from the system and one half was used for Western blotting. The duplicate half was stained overnight in Coomassie Blue stain to visualise the membrane protein separation.

Coomassie Blue Stain (1l)

- 0.5g Coomassie Brilliant Blue R-250
- 250ml Iso-propanol
- 100ml acetic acid with sterile water to obtain a final volume of 1l

Dissolve in a final volume of 1l water and stir solution overnight.

Filter through Whatman #1 Filter Paper (Whatman, USA) to remove undissolved dye particles.

The gel was destained overnight in a 10% acetic acid, 10% methanol solution. It was then washed in 10% acetic acid until the background was destained.

2.2.2. Western Blot of Erythrocyte Membrane Proteins

The Hoefer Transblot System (Hoefer Scientific Instruments, USA) was filled with 5l of transblot buffer (pH8.3), which was subsequently left to cool overnight to a temperature of 4^{o}C.

Transblot Buffer (1l)

- 25mM Tris 3.03g
- 192mM Glycine 14.4g
- 20% Methanol 200ml

Dissolve in a final volume of 1l sterile water (do not adjust pH).

The Laemmli gel, filter paper (Whatman #3, Whatman, USA) and nitrocellulose membrane (Hybond-C Super Transfer Membranes [pure nitrocellulose, supported, 0.45 microns], Amersham, UK) were soaked in transblot buffer for five minutes.

The blotting cassette was opened and a cassette sponge was placed into it. A piece of soaked filter paper was placed over the sponge and the gel was laid onto the filter paper. The nitrocellulose membrane was positioned on the gel and covered with a second piece of filter paper. Air bubbles were eliminated by gently rolling a glass rod over the filter paper. A cassette sponge was placed onto the filter paper and the cassette was closed and inserted into the transblot system. The gel faced the cathode while the membrane faced the anode, enabling the movement of the proteins from the gel to the membrane. The protein transfer was performed overnight at 4^{o}C at a constant voltage of 35V and a current ranging from 0.22 to 0.35 amps using a Biorad power pack (Biorad Model 200/2.0 Power Supply, Biorad, USA). The following morning the nitrocellulose membrane was washed in Tris-buffered-saline (TBS, pH7.5) for four minutes at rotor speed three (Hoefer Red Rotor Model #PR70-230V, Hoefer Scientific Instruments, USA) and thereafter left between filter paper to dry. The gel was placed overnight in Coomassie Blue stain to ensure no protein 4.1 remained on the gel (as described in section 2.2.1).

2.2.3 Immunoblot to Detect Erythrocyte Protein 4.1

The nitrocellulose membrane was washed (Hoefer Red Rotor speed two) in 3% bovine serum albumin factor V (BSA) (Roche, Germany) in TBS (pH7.5) for one hour. This prevents non-specific binding of the antibody to the membrane as the membrane is coated with BSA.

TBS Buffer (pH 7.5)

- 0.05M Tris 6.055g
- 0.9% NaCl 9.0g

Dissolve in 800ml water.

Adjust pH to 7.5 with HCl.

Correct volume to 1l with water.

The membrane was incubated (Hoefer Red Rotor speed two) overnight in 25ml rabbit anti-protein 4.1 polyclonal antibody. The antibody was prepared by

Professor Theresa Coetzer at St. Elizabeth's Medical Center, Boston, USA, and was diluted to 1:1000 using 1% BSA in TBS (pH7.5). The membrane was subsequently washed four times (Hoefer Red Rotor speed three) in TBS with 0.05% Tween-20 in order to remove unbound antibody, each wash being five minutes long. The membrane was incubated for one hour (Hoefer Red Rotor speed two) in 25ml goat anti-rabbit antibody conjugated to horseradish peroxidase. This antibody was obtained from Roche (Germany) and was diluted 1:1000 in 1% BSA in TBS. The membrane was rinsed (Hoefer Red Rotor speed three) in TBS (five washes of five minutes each).

Protein 4.1 was visualised using 4-chloro-1-naphthol ($C_{10}H_7ClO$) (Sigma Chemical Company, USA) and hydrogen peroxide (H_2O_2). The 4-chloro-1-naphthol (15mg) was dissolved in 5ml methanol and was mixed with 25ml TBS directly before use. 12.5µl of 30% hydrogen peroxide were added to each 30ml of TBS-methanol-4-chloro-1-naphthol. The membrane was incubated in this solution with gentle agitation until the protein bands appeared, typically within a maximum of five minutes. The membrane was washed in Milli-Q water to remove surplus colour reagent and then dried between filter paper.

2.3 DNA Extraction

DNA was extracted from the buffy coat (stored at -70^{o}C) of the patients selected for the study (section 2.1). The buffy coats were slowly thawed and 100µl were removed before the remaining buffy coat was refrozen. 400µl freshly prepared 0.17M ammonium chloride was added to each 100µl buffy coat in a 1.5ml Eppendorf tube. The solution was mixed by inversion and left at room temperature for 20 minutes before being centrifuged at 14 000rpm (Hägar Designs HM2 centrifuge, Hägar Designs, RSA) for 30 seconds. The supernatant was discarded and the leukocyte pellet was washed in 0.9% sodium chloride. This washing step to remove excess haemoglobin from the leukocyte pellet was typically repeated three times. The pellet was resuspended in 200µl 0.05M sodium

hydroxide and the mixture was placed in boiling water for ten minutes in order to lyse the leukocytes and release the DNA. The sodium hydroxide was neutralised by the addition of 25µl 1.0M Tris-HCl (pH8.0). The DNA was quantitated and stored at –20^{o}C for further studies.

2.4 DNA Quantitation

The DNA sample was diluted one in fifty in a final volume of 500µl. The optical density was recorded in duplicate at both 260nm (A_{260}) and 280nm (A_{280}) using a Beckman DU®-65 Spectrophotometer (Beckman, USA). The DNA concentration was calculated:

$$[DNA]\mu g.\mu l^{-1} = \frac{A_{260} \times 50\mu g.ml^{-1} \times \text{dilution factor}}{1000}$$

Note: The value of 50µg is the standard quantity of DNA present in 1ml of a DNA solution at an A_{260} of 1.00.

The division by 1000 was performed to obtain the DNA concentration in $\mu g.\mu l^{-1}$ instead of $\mu g.ml^{-1}$.

The dilution factor was 50.

The DNA was diluted with Milli-Q water to 0.1µg.µl^{-1}.

The purity of the sample was calculated using the A_{260}/A_{280} ratio. DNA absorbs ultraviolet light at a wavelength of 260nm while protein absorbs ultraviolet light of 280nm. Thus a low A_{260}/A_{280} ratio indicates a sample contaminated with protein. A good A_{260}/A_{280} ratio is 1.8 to 2.0.

2.5 Primer Design and Storage

Primers of 19 to 22 bases in length were designed to amplify exons in band 3 (table 2.2) and protein 4.1 (table 2.3). Excepting the band 3 promoter region, these

primers were usually designed in the introns flanking the exon of interest. The primers were located away from the exon in order to obtain as much exonic sequence as possible when sequencing the DNA. Primers were designed for the band 3 promoter region (within exon 1) and for exons 2, 3, 7, 8, 14 and 20. Primers and polymerase chain reaction (PCR) conditions had been previously optimised by Charlene van Zyl and Natalie Bracher of the Red Cell Membrane Unit for band 3 exons 4, 5, 6, 9, 10, 11, 12, 13, 15, 16, 17, 18 and 19.

Primers were also designed for protein 4.1 exons 4 and 17. Protein 4.1 exons 4 and 17 were chosen for analysis as they are sites of known mutations. Protein 4.1 exons five to 16 and 18 to 21 were left for later studies because of time constraints and exons one to three are non-coding.

The primers were manually examined for 3'-overlaps that could cause the formation of primer dimers, for large regions of complementarity and for palindromes. Primers were procured from Integrated DNA Technologies (USA) or Inqaba Biotec (RSA). The primers were resuspended in sterile 10mM Tris, 1mM EDTA buffer (pH8,0) to a concentration of $1\mu g.\mu l^{-1}$ (Integrated DNA Technologies) or 100µM (Inqaba Biotec) and these stock primers were stored at $-20^{o}C$. Aliquots of the stock primer were diluted with sterile water to a 10µM concentration for PCR. These aliquots were stored at $-20^{o}C$ and were thawed on ice water when required.

Table 2.2: Primer Pairs, Annealing Temperatures and Expected Fragment Sizes for Band 3 Exons

Exon	Sense Primer	Sequence (5'-3')	Antisense Primer	Sequence (5'-3')	Annealing Temperature (^{o}C)	Expected Fragment Size (bp)
1	P180 (20-mer)	CTGAGCAGTTCCCTGTGGAG	P181 (19-mer)	CCACCTGCACCCCCTTGGA	62	680
2/3	P158 (21-mer)	TGTGCCCCACTCAGCCTGTGCC	P159 (21-mer)	AGTGCCCCTCCTGTCCCTGTC	64	399
4	P74 (20-mer)	GGGACGGTCAGGTCAATACT	P75 (20-mer)	AAGGCAGCATGGGAAAGAAC	60	143
5/6	P80 (20-mer)	AAGCCTCACAAGCACAAGCC	P81 (20-mer)	GAGCCATAGTGGAGGAAAGT	58	645
7/8	P160 (20-mer)	ATGTCTACCCTTGATGTCTA	P161 (20-mer)	CAGGCTGAGCCAAGACACGA	54	611
9	P90 (19-mer)	ACCCTGGGCTCCTCGGCCT	P91 (20-mer)	GCAGGTCCCAAGCTTCCCCA	70	275
10	P76 (20-mer)	CTGAGGGGGTTCTGGTTCCT	P77 (20-mer)	TGAGGTGTCTGGGGGTCGGT	65	365

Table 2.2: Primer Pairs, Annealing Temperatures and Expected Fragment Sizes for Band 3 Exons (continued)

11	P82 (19-mer)	TTTCCCCTCACCTCCTCCA	P83 (19-mer)	GTCAGAGGCAAGAGTTAGG	54	292
12/13	P121 (20-mer)	AGTGTCGGAGCTGCTGATCT	P122 (19-mer)	CTTATACACAACCTCCCGT	54	480
14	P162 (21-mer)	GTAGAGTTAGCTGGTGGTATT	P163 (20-mer)	GGAAGCTAAGGGCACTGAGG	54	342
15	P92 (20-mer)	GGGGAGTGACTGGGCACTGA	P93 (21-mer)	ATGAGGACCTGGGGGGTATCA	67	185
16	P96 (19-mer)	CATAACCTCTCTGATTCTC	P95 (20-mer)	GCCAGGGAAAGGTCTCTGCC	60	449
17	P78 (20-mer)	GTGGAGGAGGCAGGGGAGAA	P79 (20-mer)	GGGCAGGAGGATGGTGAAGA	67	343
18/19	P86 (20-mer)	GTGTTTTATTCCCAGCCCCA	P87 (20-mer)	CCCCCTTTACAGGTGAGCAA	64	722
20	P164 (21-mer)	CTGCTGGGGAGATAAAACAGG	P165 (20-mer)	AAGGTGGGGATGTGGAATGG	54	277

Table 2.3: Primer Pairs, Annealing Temperatures and Expected Fragment Sizes for Protein 4.1 Exons 4 and 17

	Exon 4	Exon 17
Sense Primer (5'-3')	P152 (20-mer) GCTCAGGAAGAACTCAGAGA	P156 (21-mer) GATTTAGACAAGAGTCAAGAG
Antisense Primer (5'-3')	P153 (22-mer) TCCACAACACATTCATAAACTG	P157 (20-mer) AGTTCGGAAGGGTGAGTGAG
Annealing Temperature	54^{o}C	54^{o}C
Product Size	209bp	141bp

2.6 Polymerase Chain Reaction Protocols

Initially the Roche PCR Master Kit™ (Roche, Germany) was used for all DNA amplification reactions. Later the Promega PCR Master Mix™ (Promega, USA) was used. The reaction was identical. Reactions were prepared on ice water.

Twenty-five microlitres of two times (2x) PCR Master Kit Master Mix (Roche, Germany) or PCR Master Mix (Promega, USA) were added to each reaction (final volume of 50µl), giving a one times (1x) concentration. The final composition of the Roche PCR Master Kit Master Mix was 2.5U *Taq* Polymerase in Brij®35 0.005% (v/v), 0.2mM each deoxyadenosine 5'-triphosphate (dATP), deoxycytidine 5'-triphosphate (dCTP), deoxyguanosine 5'-triphosphate (dGTP) and deoxythymidine 5'-triphosphate (dTTP), 50mM KCl and 1.5mM $MgCl_2$ in PCR buffer (pH 8.3). The final composition of the Promega Master Mix was 2.5U *Taq* Polymerase, 0.2mM each dATP, dCTP, dGTP and dTTP, and 1.5mM $MgCl_2$ in PCR reaction buffer (pH 8.5).

Up to 2.5µl of each 10µM primer were added to the reaction with a final concentration of 0.1 to 0.5µM. The primers were tested using the Roche PCR Master Kit™ (Roche, Germany), 2µl (200ng) of control DNA known to amplify reliably and 2,5µl of each primer per 50µl PCR reaction. The temperature at which the primers would anneal to the denatured DNA strands was taken to be 2^{o}C below the primer melting temperature. The melting temperature was calculated by multiplying by 4^{o}C for every cytosine or guanine and 2^{o}C for every adenine or thymine. If there was product in addition to the expected fragment, the temperature was raised to one more suitable for the specific binding of the primers. If the product was absent or only present in small amounts the annealing temperature was decreased. If primer dimers were present, the volume of each primer added was dropped to as low as 1µl in a 50µl reaction. Optimal conditions were noted and used for all further amplifications. The optimal annealing temperatures are shown in Tables 2.2 (band 3 primers) and 2.3 (protein 4.1 primers). One to five microlitres of extracted DNA (0.1µg.µl^{-1}) were added to the reaction (concentration of 100ng to 500ng per 50µl reaction). The reaction was made up to a final volume of 50µl with the sterile water provided in the kit.

The DNA was amplified using either the Eppendorf MasterCycler Gradient (Merck, Germany) or the DNA Thermal Cycler (Perkin Elmer Cetus, USA). If the latter was used the reaction mix was overlaid with a drop of mineral oil to avoid evaporation of the sample. This is not necessary with the Eppendorf MasterCycler Gradient machine as the lid is heated. In order to minimise primer dimerisation and non-specific extension of the DNA by *Taq* Polymerase, the machines were heated to 94^{o}C (a modified hot-start) before the samples were transferred from ice water to the machine. Thirty PCR cycles comprising DNA denaturation at 94^{o}C (30 seconds), primer annealing at 54^{o}C to 70^{o}C (30 seconds) and primer extension at 72^{o}C (30 seconds) were performed. The annealing temperature varied according to the primer pair (tables 2.2 and 2.3). On completion of the PCR cycles the samples were incubated for four minutes at 72^{o}C to allow any remaining DNA elongation. PRC product was stored at 4^{o}C.

2.7 Visualisation of PCR Product

The PCR product was visualised using DNA electrophoresis through agarose gels. Agarose (FMC Bioproducts, USA) was dissolved over a Bunsen burner in one times (1x) Tris-acetate-EDTA (TAE) buffer (40mM Tris-acetate, 2mM EDTA). The agarose was measured weight/volume to achieve the required gel percentage.

50x TAE Buffer (1l)

- 2M Tris 242g
- Glacial acetic acid 57.1ml
- 0.5M EDTA 37.2g

Dissolve in 800ml water.
Adjust to pH8.0 with HCl.
Correct volume to 1l with water.
Dilute 1 in 50 with water to prepare 1x working strength TAE buffer.

The agarose solution was cooled slightly and 3µl ethidium bromide ($10\mu g.\mu l^{-1}$) were added to each 50ml of agarose solution. The mixture was poured into a gel-casting chamber and allowed to solidify. The gel was placed into a horizontal electrophoresis system (Horizontal Mini-Gel Kit Model #MGU-200T, C.B.S. Scientific Company, USA) which was filled with one-times (1x) TAE buffer and the casting combs were removed.

Seven to ten microlitres of the reaction product were mixed with 1µl of sucrose loading dye and loaded onto a one percent agarose gel.

6x Loading Dye (Promega, USA)

- 15% Ficoll 400
- 0.03% bromophenol Blue
- 0.03% xylene cyanol FF
- 0.4% orange G
- 10mM Tris-HCl (pH7.5)
- 50mM EDTA

The reaction products were separated at a constant current of 65mA, with an average voltage of 130V (PS500XT DC Power Supply (500V, 400mA, 200W), Hoefer Scientific Instruments, USA) for 30 minutes. The gels were viewed using ultraviolet (UV) light to detect any DNA amplicon. This was realized through the ethidium bromide which binds to double stranded DNA and fluoresces under UV light. The size of the amplicon was approximated through comparison with a concurrently separated 100 base-pair DNA size ladder (Promega, USA). Three microlitres of dilute DNA ladder (stored at 4^{o}C) were loaded. The concentrated DNA ladder (stored at -20^{o}C) was diluted two in five with 25µl sterile water and 5µl loading dye to a final volume of 50µl.

2.8 DNA Precipitation

When DNA amplification was poor, the PCR product was precipitated to concentrate it. The amplicon (in a 500µl PCR tube) was mixed by inversion with 2.5 volumes of ice-cold 100 percent ethanol and incubated at -20^{o}C for 30 minutes. The solution was centrifuged at 14 000rpm (18 500*g*) for 20 minutes in a Sorvall RMC-14 refrigerated microcentrifuge (Sorvall, USA) cooled to 4^{o}C. The supernatant was poured off and the pellet was washed in 100µl 70 percent ethanol and centrifuged at 14 000rpm (Sorvall RMC-14 refrigerated microcentrifuge cooled to 4^{o}C) for ten minutes. The supernatant was removed using a micropipette and the pellet was left to air dry, prior to resuspension in 20µl 1x restriction enzyme buffer.

2.9 Screening for Known DNA Mutations using Restriction Enzyme Analysis

Patients with a band 3 deficiency were screened for the band 3 Pribram, band 3 Cape Town, band 3 Osnabruck I/Lyon, band 3 Tuscaloosa, band 3 Coimbra, band 3 Pribram, band 3 Smichov, band 3 Most and codon 490/codon 760 hotspot mutations using restriction enzyme analysis as shown in Table 2.4. The other mutations were not amenable to restriction digests, either because no restriction enzyme site was changed or because the restriction enzyme was not easily

available. If a number of mutations were present in one exon, SSCP analysis (discussed in section 2.9) was used to screen the exon. Similarly, if no mutation was found using restriction enzyme digests, all of the band 3 exons were screened using SSCP to identify exons in which nucleotides were altered. If a patient was found to have a mutation they were screened for the band 3 Montefiore, Mondego and Genas polymorphisms which are known to modulate the severity of the HS phenotype.

Patients displaying protein 4.1 deficiencies were screened for the protein 4.1 Madrid, Lille and Aravis mutations using similar restriction enzyme analysis (Table 2.5). If no mutation was found using restriction enzyme digests, the protein 4.1 exons for which primers had been designed were screened using SSCP to identify exons in which nucleotides were altered.

If no restriction site for a published DNA mutation was mentioned or the enzyme described was unavailable, the exons of interest were assessed to determine whether the nucleotide changes created or deleted a restriction enzyme recognition site. The DNA sequence was downloaded from the http://www.pubmedcentral.nih.gov website and either the http://darwin.bio.geneseo.edu/~yin/WebGene/RE.html or the http://www.firstmarket.com/cutter/cut.html website was used to search for restriction enzyme sites. If an appropriate and cost-effective enzyme for identification of a mutation was detected (indicated by an asterisk in tables 2.4 and 2.5), the patients were also tested for the mutation.

A restriction enzyme master mix consisting of the restriction enzyme, bovine serum albumin (fraction V, Roche, Germany) and a suitable restriction enzyme buffer diluted in sterile water according to the manufacturer's specifications was prepared with a final volume of 5µl per reaction (Table 2.6). 20µl of amplified DNA were combined with 5µl of the restriction enzyme master mix. The combination was incubated for four hours at the temperature at which the restriction enzyme functioned optimally (Table 2.6). The reaction was stopped by

Table 2.4: Band 3 Mutation Restriction Enzyme Analysis

Mutation	Exon	Amino Acid Change	Nucleotide Change	Enzyme	Restriction Enzyme Site Change	Normal Allele Fragment Sizes (bp)	Mutant Allele Fragment Sizes (bp)
Cape Town	5	E90K	GAG→AAG	***Mbo*II**	Created	269	207, 62
Osnabruck I / Lyon	6	R150X	CGA→TGA	**AvaII**	Deleted	299, 169, 177	299, 346
Tuscaloosa	10	P327R	CCC→CGC	***Aci*I**	Created	57, 46, 262	57, 46, 96, 166
Coimbra*	13	V488M	GTG→ATG	***Hsp92*II**	Created	155, 27, 298	155, 27, 86, 212
Pribram	Intron 12	-	-1g→a	***Taq*I**	Created	153, 327	480
Bicetre	13	R490C	CGC→TGC	***Aci*I**	Deleted	163, 111, 72, 12, 122	163, 183, 12, 122
Pinhal	13	R490H	CGC→TGC	***Aci*I**	Deleted	163, 111, 72, 12, 122	163, 183, 12, 122
Smichov*	15	616:1848delC	ATC→AT	***Hsp92*II**	Deleted	94, 69, 22	163, 22
Most	17	L707P	CTG→CCG	***Aci*I**	Created	142, 201	114, 28, 201
760 Hotspot	17	R760var	CGG→var	***MspA*1I**	Deleted	270, 73	343

** : new restriction enzyme digest*

Table 2.5: Protein 4.1 Mutation Restriction Enzyme Analysis

Mutation	Exon	Amino Acid Change	Nucleotide Change	Enzyme	Restriction Enzyme Site Change	Normal Allele (bp)	Mutant Allele (bp)
Lille*	1	M1T	ATG→ACG	***Hsp92*II**	Deleted	54, 155	209
Madrid*	1	M1R	ATG→AGG	***Hsp92*II**	Deleted	54, 155	209
Aravis*	17	K447del	AAAAAG→AAA	***Xmn*I**	Deleted	64, 77	141

** : new restriction enzyme digest*

Table 2.6: Restriction Enzyme Protocols

Restriction Enzyme	Manufacturer	Restriction Enzyme Buffer	Volume Enzyme Buffer (µl)	Volume BSA (µl)	Volume Restriction Enzyme (µl)	Volume Milli-Q Water (µl)	Digest Temperature	Percentage Agarose Gel
***Mbo*II**	Promega, USA	Buffer B	0.50	0.25	0.33	3.59	37°C	2.0%
***Taq*I**	Promega, USA	Buffer E	0.50	0.25	0.20	4.05	65°C	1.0%
***Aci*I**	Roche, Germany	NE Buffer 3	0.50	0.25	0.25	3.75	37°C	2.5%
***MspA*1I**	Promega, USA	Buffer C	0.50	0.25	0.20	4.05	37°C	1.0%
***Ava*II**	Promega, USA	Buffer C	0.50	0.25	0.20	4.05	37°C	1.5%
***Hsp92*II**	Promega, USA	Buffer K	0.50	0.25	0.30	3.95	37 °C	1.0%
***Xmn*I**	Promega, USA	Buffer B	0.50	0.25	0.30	3.95	37 °C	2.5%
***Eco*81I**	Promega, USA	Buffer M	0.50	0.25	0.20	4.05	37°C	2.0%
***Hae*III**	Promega, USA	Buffer C	0.50	0.25	0.25	3.75	37°C	1.0%

Note: The enzyme mixture was made up as a master mix (5 to 10 times volume)

Each 5µl of the restriction enzyme mixture was added to 20µl PCR product

the addition of 2.5µl blue/orange loading dye (Promega, USA). The restriction enzyme digest products were separated on agarose gels of varying percentages according to the sizes of the restriction products of interest (Tables 2.4 and 2.5). An undigested sample was loaded in conjunction with the digest products as a marker of the original amplicon size. A 100bp DNA size ladder (Promega, USA) (diluted as described in section 2.7) was used to determine the size of the digest products.

2.10 Single Strand Conformation Polymorphism (SSCP) Analysis

2.10.1 Amplification and Preparation of SSCP Samples

Samples for SSCP were amplified using the Roche PCR Core Kit™ (Roche, Germany). Up to 2.5µl of DNA (depending on the optimal quantity determined during the earlier PCRs, section 2.7) was added to 0.25µl Redivue [α-^{32}P]dATP (10mCi/ml, specific activity 3000Ci.mmol^{-1}, Amersham Biosciences, UK), 0.17µl of each deoxynucleotriphosphate (dNTP) (total dNTP volume of 0.68µl), 0.5µl *Taq* Polymerase (Roche, Germany), 1.25µl each primer (10µM) and 2.5µl 10x PCR reaction buffer with $MgCl_2$ in 500µl PCR reaction tubes. The SSCP mixture was made up as a master mix and added to the DNA. Water was added to the vial to a final volume of 25µl. Three unrelated control samples were included in each run. The PCR product was assessed using an agarose gel as described in section 2.7.

PCR products greater than 300bp in size were digested using restriction enzymes, SSCP results being poor on larger fragments. The digests are detailed in Table 2.7. The restriction digest protocols are the same as those described in Table 2.6.

Table 2.7: SSCP Restriction Enzyme Digests

Exons	Initial Product Size (bp)	Restriction Enzyme	Digested Product Sizes (bp)
1	680	*MspA*1I	167, 201, 312
2&3	399	*Hae*III	172, 227
5&6	645	*Ava*II	169, 177, 299
7&8	611	*Ava*II	27, 263, 311
10	365	*Taq*I	115, 250
12&13	480	*Ava*II *Taq*I	158, 254,322 153, 327
14	342	*Ava*II	85, 257
16	449	*Ava*II	147, 302
17	343	*Hae*III	140, 203
18&19	722	*Ava*II & *Mbo*I	59, 118, 264, 281

One to five microlitres of the PCR or restriction enzyme digest product were diluted with SSCP denaturation solution (0.1%SDS, 10mM EDTA pH8.0) to a volume of 10µl. This was mixed 1:1 with stop solution to a final volume of three to five microlitres. The quantity of PCR product used was determined from the intensity of the amplicon bands viewed on the agarose gel and was varied so that similar amounts of DNA were loaded onto the SSCP gel (prepared as described in section 2.10.2). At a later stage this dilution step was eliminated (2.5µl of the PRC product were mixed with 2.5µl of stop solution) in order to increase the intensity of the visual result.

Stop Solution (Promega, USA)

- 10mM NaOH
- 95% formamide
- 0.05% bromophenol blue
- 0.05% xylene cyanol

This mixture was heated for two minutes at 94°C to denature the DNA and up to 5µl were then loaded onto the gel.

Ultimately the DNA amplification process was modified to use either the Roche PCR Master Kit or the Promega Master Mix. The PCR was performed as described in 2.6 but at a volume of 25µl (i.e. half) and 25 microCuries (µCi) (0.25µl) Redivue [α-^{32}P]dATP (10mCi/ml, specific activity 3000Ci.mmol^{-1}, Amersham Biosciences, UK) replaced 0.25µl water. The PCR product was diluted 1:1 with stop solution and loaded directly onto the SSCP gel (4-5µl) after a two minute incubation at 94°C.

2.10.2 Preparation of the SSCP Gel

Table 2.8: SSCP Gel Components for 60ml 0.5x MDE Gel Solution

Component	Quantity
2x MDE* Solution	15.00ml
10x TBE Buffer	3.60ml
Milli-Q Water	40.91ml
10% fresh APS**	480µl
100% TEMED	12µl

**Mutation Detection Enhancement Solution (FMC Bioproducts, USA)*

***Ammonium Persulphate*

10x Tris Borate EDTA (TBE) Buffer (1l)

- 0.9M Tris 54g
- 0.9M Boric Acid 27.5g
- 0.5M EDTA (pH8.0) 2ml

Dissolve in a final volume of 1l sterile water.

Mix 60ml in 1l milli-Q® water to prepare 0.6x TBE buffer (or 100ml in 1l to prepare a 1x TBE buffer for DNA sequencing).

The MDE Solution, TBE buffer, Milli-Q water, APS and TEMED were combined immediately prior to pouring the gel. The mixture was poured between

sequencing gel plates (long plate: 41.9x33.3cm, short plate: 39.4x33.3cm, glass, Gibco-BRL Life Technologies Inc., USA) separated by 0.4mm spacers (Gibco-BRL Life Technologies Inc., USA). A 0.4mm gel casting comb (Gibco-BRL Life Technologies Inc., USA) was inserted with the flat surface touching the gel to create a smooth upper surface and the gel was allowed to set.

The gel was positioned on the Model S2 vertical gel system (Gibco-BRL Life Technologies Inc., USA) and the top and bottom reservoirs were filled with 0.6x TBE buffer shortly before the SSCP samples were loaded. The gel system was attached to a Consort Microcomputer Electrophoresis Power Supply (Consort, USA), the cathode at the top where the samples were loaded.

2.10.3 SSCP Gel Electrophoresis

The samples were separated at room temperature over a period of 17 hours at a constant power of four Watts (4W). The voltage and current were set at maximum values so that they would be non-limiting. The gel was removed on filter paper which was then placed onto an old x-ray film, covered with cling film and placed into a cassette with enhancing screens. A fresh X-ray film (Agfa, Germany) was placed onto the wrapped gel and the cassette was closed. The X-ray film was exposed to the radiation from the SSCP samples overnight at -70^{o}C. The film was developed using X-ray developing machines of the emergency X-ray Unit of Johannesburg General Hospital. The patient samples were compared to unrelated control samples to determine if nucleotide changes were present. These changes are noted by discrete bands at levels differing from the normal control bands.

2.11 DNA Sequencing

2.11.1 DNA Sequencing Reaction

DNA was amplified and viewed on an agarose gel as described in 2.7, only samples uncontaminated by primer dimers or non-specific DNA amplification being used for sequencing reactions. Direct DNA sequencing was performed

using the Sequenase™ Version 2.0 PCR Product Sequencing Kit (Amersham Biosciences, UK).

Seven microlitres of PCR product were mixed with 1µl of Exonuclease I ($10U.\mu l^{-1}$) (Amersham Biosciences, UK) and 1µl of Shrimp Alkaline Phosphatase ($2U.\mu l^{-1}$) (Amersham Biosciences, UK) in a 500µl Eppendorf tube. The former enzyme digests single stranded DNA, removing the primers from the solution, while the latter dephosphorylates free nucleotides. At a later stage 2µl ExoSapIT™ (Amersham Biosciences, UK), a mix incorporating both enzymes, was used instead of the separate enzymes. The mixture was incubated at 37°C for 15 minutes. The enzymes were denatured by incubation at 80°C for a further 15 minutes. This procedure was performed using the Eppendorf MasterCycler Gradient (Merck, Germany) machine.

The treated DNA (9µl) was mixed with 1µl of either the sense or the antisense primer (10µM) and heated in boiling water for three minutes to denature the DNA. The mixture was placed immediately into ice water to prevent reannealing of the amplified DNA strands and to promote annealing of the primer to the single stranded DNA. 2µl Sequenase™ Reaction Buffer, 1µl dithiothreitol (DTT), 2µl Sequenase™ Labelling Mix (diluted one in five with Milli-Q water), 5µCi (0.5µl) Redivue [α-^{32}P]dATP (10mCi/ml, specific activity $3000Ci.mmol^{-1}$, Amersham Biosciences, UK) isotope and two microlitres Sequenase™ DNA Polymerase (diluted one in seven with Sequenase™ Buffer) were added to the ice-cold DNA mixture (final volume 17.5µl). This labelling reaction was incubated at room temperature for two minutes to allow extension of the DNA strands.

A 3.5µl aliquot of the labelling reaction mix was mixed with 2.5µl of each termination mix in 1.5ml Eppendorf tubes. The four termination mixes contained dATP, dCTP, dGTP and dTTP and one of ddATP, ddCTP, ddGTP or ddTTP. The termination mixes were pre-warmed in a 37°C water bath. After addition of the labelled DNA to the termination mixes, the mixture was incubated in a 37°C water bath for a further five minutes. This allowed the Sequenase™ enzyme to

incorporate the dNTPs into the nucleotide sequence, the extension terminating on the incorporation of a dideoxyribonucleotide. The reactions were interrupted by the addition of 4µl of stop solution (Promega, USA) and placed on ice. The samples were heated to 80^{o}C for two minutes before each loading. A loading volume of 2.5µl to 5µl per well was used.

2.11.2 Preparation of the Sequencing Gel

Table 2.9: Sequencing Gel Components for 60ml 8% Acrylamide Gel Solution

Component	***Quantity***
40% Acrylamide	12ml
2% Bisacrylamide	12ml
10x TBE Buffer	6ml
Milli-Q Water	11.2ml
Urea (solid)	24g
10% Fresh APS	480µl
100% TEMED	12µl

The acrylamide, bisacrylamide, TBE Buffer, Milli-Q water and urea of the sequencing gel mixture were mixed according to the quantities indicated in Table 2.9 to give a final volume of 60ml. Once the urea was completely dissolved, the APS and TEMED were added in order to allow polymerisation of the acrylamide molecules. The mixture was immediately poured between sequencing gel plates (as described in section 2.10.2), a comb was inserted with the flat surface touching the gel to create a smooth upper surface and the gel was allowed to set. The top of the gel was covered with 1x TBE buffer and the gel was stored at room temperature overnight.

The gel was positioned on the Model S2 vertical gel system (Gibco-BRL Life Technologies Inc., USA) and the top and bottom reservoirs were filled with 1x TBE buffer. The gel system was attached to a Consort Microcomputer

Electrophoresis Power Supply (Consort, USA), the cathode at the top where the samples were loaded. The gel was preheated to 45^{o}C by applying a constant power of 60 Watts to the gel for one hour before loading.

2.11.3 DNA Sequencing Gel Electrophoresis

The DNA fragments were separated at a constant power of 60 Watts. The voltage and the current were set at maximum values so as to be non-limiting. The samples were separated over a period of two to seven hours. One to three aliquots of each sample were loaded at various times during the separation, the sample being heated at 80^{O}C before each loading. The length of the time of the electrophoresis and the number of loadings depended on the size of the exon being sequenced.

At the completion of the separation, the gel was transferred to old x-ray film and placed into an unscreened cassette. The gel was covered with cling-film to prevent contact with the x-ray film or the cassette. A fresh x-ray film was placed onto the wrapped gel and exposed to the radiation from the sequenced PCR product overnight at –70^{o}C. The film was developed using the X-ray developing machines of the Emergency X-ray Unit of Johannesburg General Hospital. The sequence was read manually and compared to documented band 3 sequences.