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The Role of Erythrocyte Membrane Proteins in Haemolytic Anaemias in South African Populations

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2006-11-16T10:35:04Z
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CHAPTER 3: RESULTS

Fifteen HS patients (Probands/Families A to O) with a band 3 deficiency were examined. The probands, of which five were Black and ten were Caucasian, were from unrelated South African kindred. Samples from family members were available for four of the Caucasian and one of the Black probands. Nine HE patients (Probands/Families 1 to 9) with protein 4.1 deficiencies were identified, two with other family members included for study. Four probands were Caucasian, three were Black African, and two were Indian. These data are detailed in Tables 3.1 (page 82) and 3.2 (page 90) respectively.

Two previously published causative mutations linked to HS were identified within codon 490 of the band 3 gene: band 3 Pinhal and band 3 Bicetre. A novel mutation was isolated in codon 508 but the whether this nucleotide change is the sole cause of HS is uncertain as discussed further in Chapter 4. One patient was found to have the band 3 Memphis I polymorphism. The genetic defects underlying HS in the remaining patients were not identified but a number of known causative mutations were ruled out.

Protein 4.1 mutations causing alterations to protein size (Protein 4.1 Algeria, Protein 4.1 Annecy, an unnamed mutation and Protein 4.1 Hurdle-Mills) were excluded through western blotting, all patients displaying protein 4.1 of normal size. Known point mutations linked to protein 4.1 deficient HE (protein 4.1 Lille, protein 4.1 Madrid and protein 4.1 Aravis) were excluded at the genomic level.

3.1 Band 3 Codon 490 Hotspot Mutations in HS: R490H and R490C

Two different band 3 mutations were identified within codon 490 of the band 3 gene, a known hotspot. These corresponded to the documented band 3 Bicetre (Dhermy *et al*, 1997) and band 3 Pinhal (Lima *et al*, 1999) mutations.

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3.1.1 Clinical Profiles

Proband A is a Black South African female who was 38 years of age at the time at which the blood sample was drawn. She has mild haemolytic anaemia and is neither splenectomised nor transfusion dependent. Her blood smear showed the presence of microcytes, spherocytes, elliptocytes, poikilocytes and pincered cells. Elliptocytes and poikilocytes are not usually associated with HS, but the disorder but was indicated by her band 3 deficiency. Her haematocrit (36.3%, normal=37-49%) was mildly decreased but her reticulocyte count was normal (1.4%, normal<2.0%). Her haemoglobin (11.8g.dl^{-1}, normal=12.0-16.0g.dl^{-1}) and mean cell volume (MCV) (71.8fl, normal=80.0-100.0fl) were decreased. No family members were available for study.

The proband of Family B is a Caucasian male who was seven years of age at the time that the blood sample was drawn. He has severe haemolytic anaemia and is transfusion dependent. His blood smear showed the presence of spherocytes, microspherocytes and pincered cells. A blood sample was also obtained from the mother who is splenectomised, alleviating the more severe symptoms of the disease. Spherocytes and acanthocytes were visible on her blood smear. The splenectomy explains the presence of acanthocytes in her blood smear: the erythrocytes cannot undergo splenic conditioning and the cell membrane remains irregular which manifests as acanthocytosis. The father is not affected but the younger brother, for whom no blood sample was available, shows clinical symptoms of HS. Further haematological data were not available for the proband or his mother.

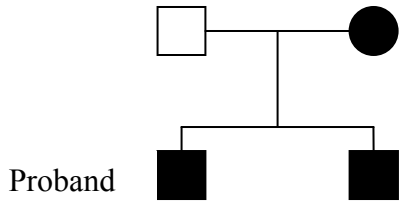

Figure 3.1: HS Pedigree for Family B

White: unaffected; Black: HS

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3.1.2 Erythrocyte Membrane Protein Analysis

SDS-PAGE and protein densitometry analyses were performed by members of the Red Cell Membrane Unit. Fairbanks SDS-PAGE densitometry analysis is used to determine decreases in the quantity of specific proteins by comparing the area occupied by each to the area occupied by band 3. If a protein/band 3 ratio is decreased, the quantity of that protein is decreased, whereas if all of the ratios are increased above normal, band 3 is decreased. Laemmli SDS-PAGE densitometry analysis allows proteins 4.1 a and b to separate and comparison of the area occupied by each isoform can be used to determine the protein 4.1 a/b ratio which can be used as an indicator of reticulocytes, a decreased ratio indicating an increased reticulocyte count. Examples of densitometry scans obtained from SDS-PAGE gels are shown in figures 3.2 (Fairbanks') and 3.3 (Laemmli).

Fairbanks' SDS-PAGE densitometry analysis (scans not shown) showed that proband A had decreased band 3 (76% of normal) and a concomitant decrease in protein 4.2 (83% of normal). Her 4.1a/b ratio was 1.39 which falls within the normal range (1.22-1.88), in concordance with the clinical picture. Her spectrin dimer self-association was normal, ruling out spectrin defects as the cause of her HS.

Proband B (scans not shown) had decreased band 3 (83% of normal) and concomitant protein 4.2 deficiency (87% of normal). This figure is not accurate as the proband is transfusion dependent and hence band 3 from the transfused erythrocytes is included in this figure. His protein 4.1a/b ratio was decreased to 0.94 (normal=1.22-1.88), indicating an increased reticulocyte count as a result of a haematological stress. These results are not accurate because he is transfusion dependent and transfused proteins are examined in addition to his own. His mother (scans not shown) also has severe band 3 deficiency with lower than normal band 3 levels (70% of normal). This is a more accurate reflection of the band 3 deficiency as the cells are not destroyed within the spleen and she is not transfusion dependent as she is splenectomised. Her protein 4.2 levels were 98 percent of normal. She also has a decreased protein 4.1a/b ratio (1.08, normal=1.22-1.88), indicating increased reticulocyte levels.

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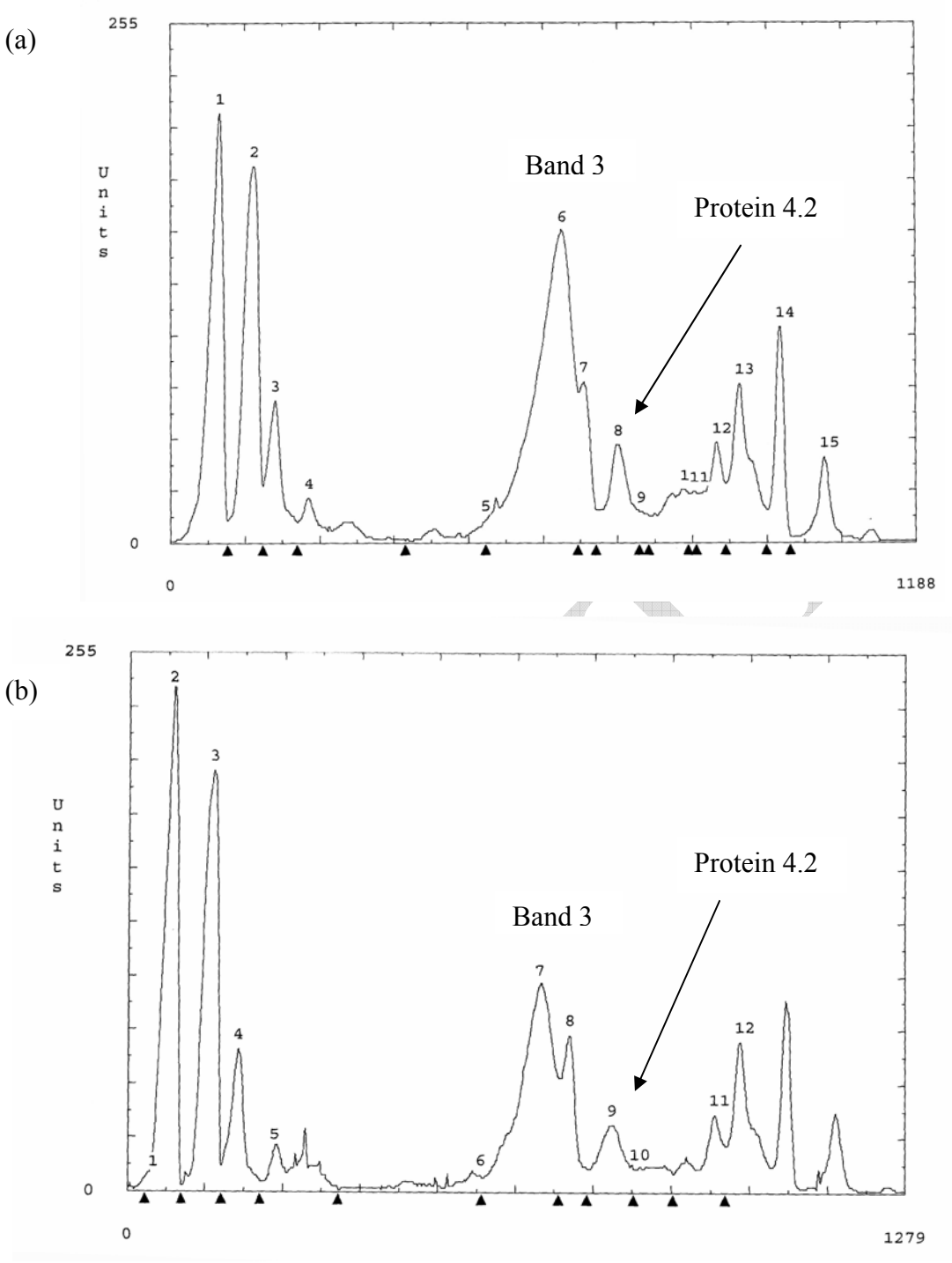

Figure 3.2: Comparison of Fairbanks SDS-PAGE Densitometry Analysis of HS Patient (Father of Proband C) and Associated Control

When compared to the band 3 peak of the control sample (a), the area of the band 3 peak of the patient (b) is significantly lower. The protein 4.2 peak also occupies a decreased area.

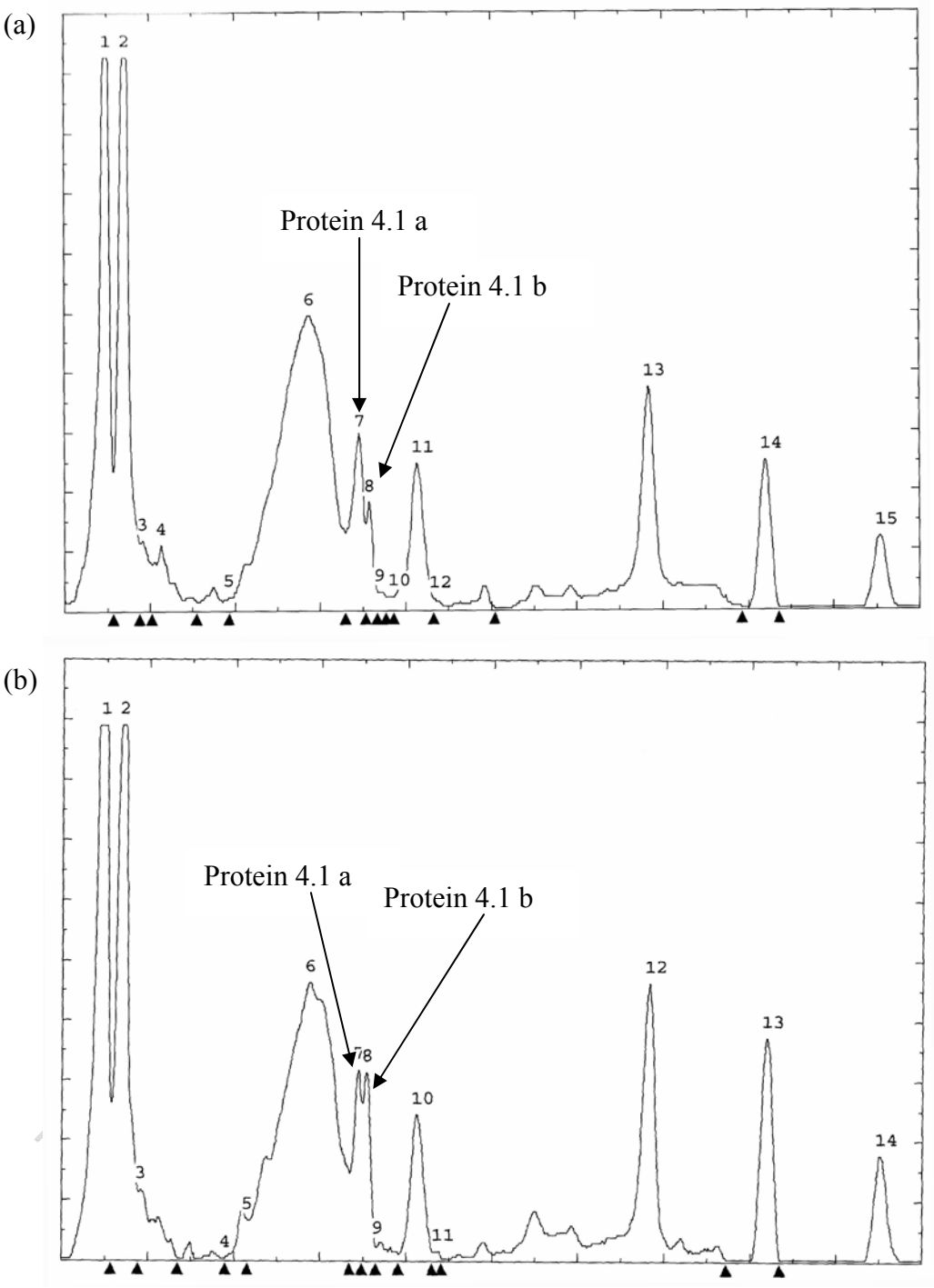

Figure 3.3: Comparison of Laemmli SDS-PAGE Densitometry Analysis of HS Patient (Father of Proband C) and Associated Control

The protein 4.1b peak of the patient (b) occupies a significantly larger area compared to that of the control sample (a), whereas their protein 4.1a peaks occupy a similar area. The protein 4.1 peaks of the patient are of a similar height; those of the control differ.

3.1.3 Band 3 DNA Restriction Enzyme Analysis

Screening of amplified DNA for known hotspots and mutations with restriction enzymes revealed that proband A and the examined members of Family B were negative for the Band 3 Cape Town [E90K], Band 3 Osnabruck/Lyon [R150X], Band 3 Tuscaloosa [P327R], Band 3 Pribram [intron 12, -1g→a] and Band 3 Most [L707P] as well as mutations in the codon 760 hotspot. Each of the three patients were however heterozygous for a change in codon 490 in exon 13 according to the *Aci*I restriction enzyme (C|CGC) digest (see figure 3.4b).

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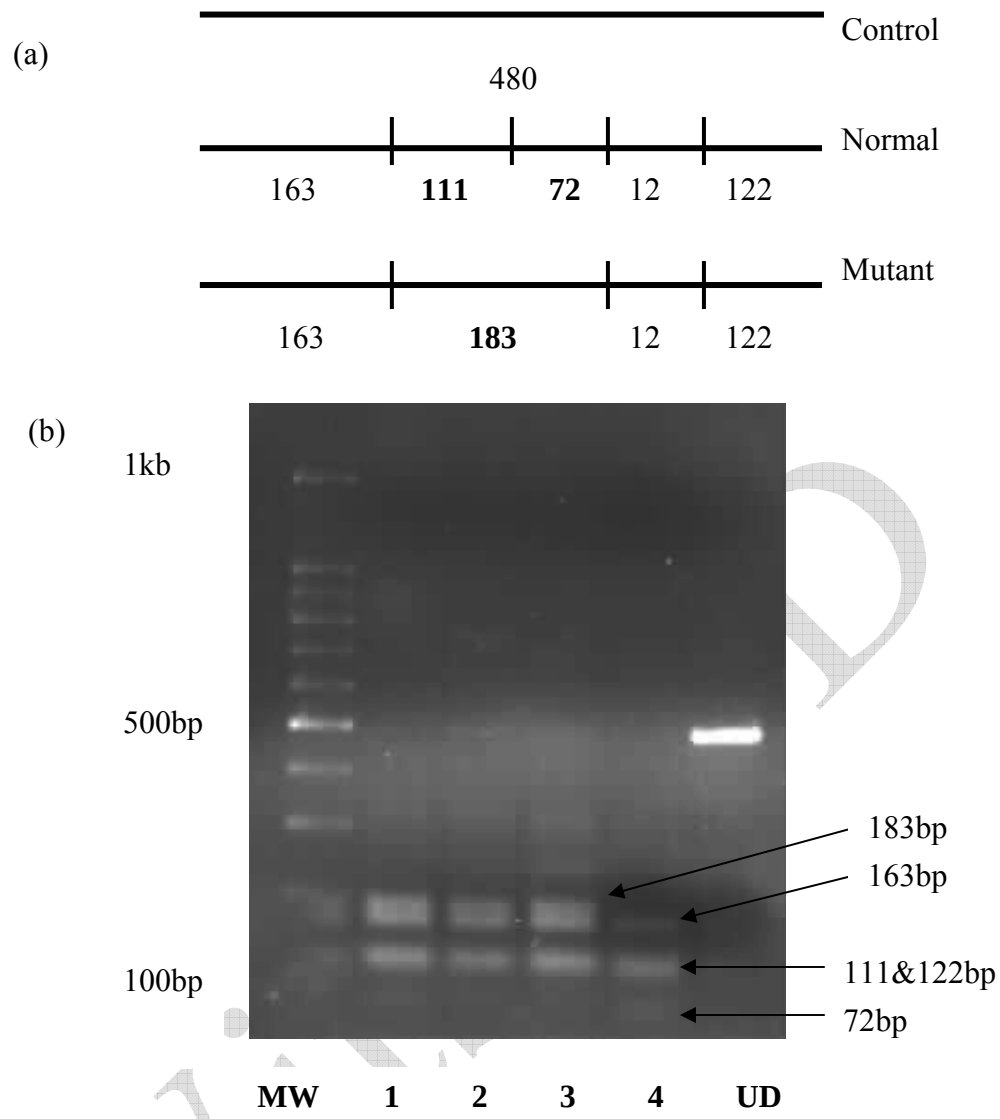

Figure 3.4: *AciI* Restriction Enzyme Analysis of Codon 490

(a) The amplified DNA fragment is 480bp in size and encompasses exons 12 and 13 as well as intron 12. Vertical lines indicate Aci*I digestion sites and the fragment sizes (bp) are shown. The fragment altered by the abolition of an* Aci*I restriction site in codon 490 is indicated in bold font. (b) The digested DNA fragments were separated on a 2.5% agarose gel. The control (lane 4) showed fragments at 163bp, 122 and 111bp (these two bands do not separate) and 72bp. A 12bp band is also created but has moved out of the gel. The* Aci*I recognition site between the 111bp and the 72bp site is lost in heterozygous patients (lanes 1-3), resulting in the presence of a fragment at 183bp. The undigested DNA (UD) is 480bp in size. MW indicates the 100bp DNA ladder.*

3.1.4 Direct DNA Sequencing of Band 3 Exon 13

Direct DNA sequencing of amplified exons 12 and 13 of proband A revealed a heterozygous change in the second nucleotide of codon 490 (see Figure 3.5), an adenine replacing a guanine (CGC→CAC). This nucleotide change results in the replacement of an arginine with a histidine in the mutant band 3 protein. This mutation was described by Lima *et al* (1999) and is known as Band 3 Pinhal.

Proband A is heterozygous for the nucleotide change and hence a doublet is visible on the sequencing gel. Normally only one base is visible as each denatured DNA fragment differs by one base depending on which ddNTP they are terminated. The DNA fragments terminating on the nucleotide change contain different ddNTPs but are otherwise identical, moving through the sequencing gel at the same rate.

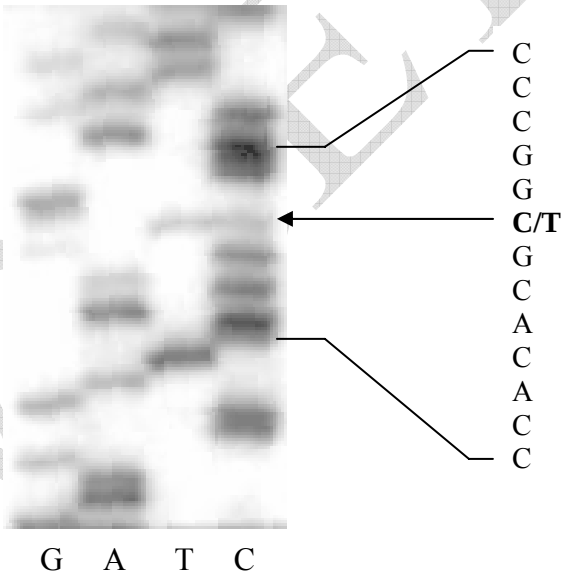

Figure 3.5: DNA Sequence of a Portion of Exon 13 of Proband A Indicating the Pinhal Mutation

The antisense sequence is displayed as the sequence was obtained using the reverse primer. The CGC→CAC mutation thus appears as GCG→GTG. The sequence reads 5'-3' from the bottom of the selected region. The nucleotide change is indicated by an arrow.

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Direct DNA sequencing of amplified exons 12 and 13 of both the proband and the mother of Family B revealed a heterozygous change in the first nucleotide of codon 490 (see Figure 3.6), a thymine replacing a cytosine (CGC→TGC). This change results in the replacement of an arginine with a cysteine in the mutant band 3 protein and is known as Band 3 Bicetre (Dhermy *et al*, 1997).

In this kindred, the normal nucleotide is fainter than the mutated nucleotide but as it is the normal nucleotide for this position it is believed to be present. The mutation was verified as it was also present in the maternal DNA.

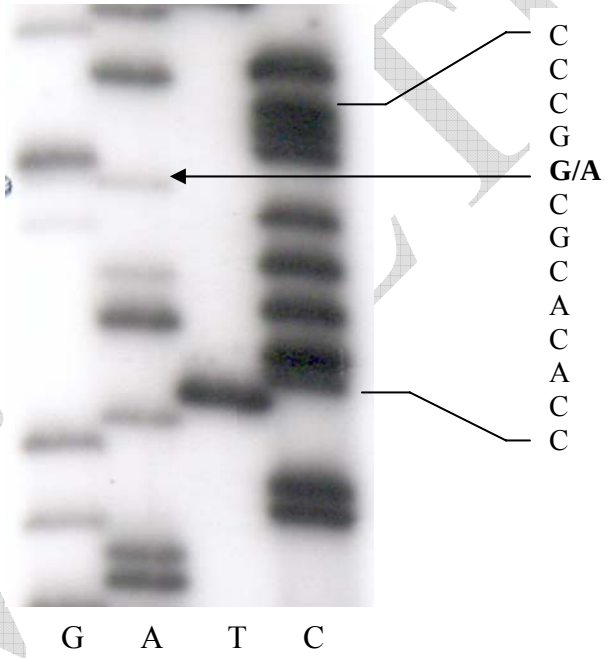

Figure 3.6: DNA Sequence of a Portion of Exon 13 of Proband B Indicating the Bicetre Mutation

The antisense sequence is displayed as the sequence was obtained using the reverse primer. The CGC→TGC mutation thus appears as GCG→ACG. The sequence reads 5'-3' from the bottom of the selected region. The nucleotide change is indicated by an arrow.

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3.2 A Novel Band 3 Mutation: E508K

3.2.1 Clinical Profiles

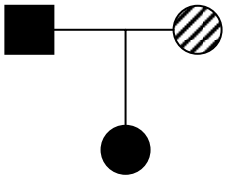

Figure 3.7: HS Pedigree for Kindred C

Solid-HS, Hatched-mild HS

The proband of Family C is a Black African female who presented with clinically severe HS in the neonatal period. Clinical charts indicate that she displayed jaundice, splenomegaly and anaemia and has been transfusion dependent from a young age. Blood was obtained from the proband at age six. Her haematocrit (38.9%, normal child=43.0-55.0%), haemoglobin levels (10.8g.dl^{-1}, normal child=12.0-16.0g.dl^{-1}) and MCV (77.8fl, normal=80.0-100.0fl) were low while her reticulocyte count was elevated (2.7%, normal<2.0%). She displayed extensive microspherocytosis on her blood smear. Her haematological profile would be more dramatically affected if not for blood transfusions every three weeks. She underwent a splenectomy late in the study in order to alleviate her symptoms and transfusion dependency, but no blood samples could be obtained for further examination.

Blood samples and clinical data for both parents were available. The father has mild HS, exhibiting splenomegaly and anaemia. His haematocrit (40.0%, normal=42.0-52.0%) and haemoglobin (14.1g.dl^{-1}, normal=14.3-18.3g.dl^{-1}) were decreased while his reticulocyte count was dramatically increased (8.1%, normal<2.0%). His MCV was normal (87.6fl; normal=79.1-98.9fl) but his peripheral blood smear showed pincered and spherocytic erythrocytes. The mother is clinically asymptomatic, her haemoglobin (13.8g.dl^{-1}, normal=12.0-

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16.0g.dl^{-1}) and MCV (95.4fl, normal=79.1-98.9fl) falling within the expected ranges and her haematocrit (41.0%, normal=42.0-52.0%) being mildly decreased. She displayed some anisocytosis, her reticulocyte count was mildly elevated (2.5%, normal<2.0%) and her spleen was slightly palpable.

3.2.2 Erythrocyte Membrane Protein Analysis

Quantitative red cell membrane protein studies (scans not shown) indicated that proband C had decreased band 3 levels (90% of normal) and decreased protein 4.2 levels (89% of normal). Her protein 4.1a/b ratio of 2.76 (normal=1.22-1.88) was unexpectedly raised for unknown reasons. Due to her transfusion dependency these results could not be taken to be accurate as transfused erythrocyte membrane proteins were also present.

The father of proband C had a decreased band 3 content (65% of normal) and concomitant protein 4.2 deficiency (81% of normal). He also had a decreased protein 4.1a/b ratio of 1.0 (normal=1.22-1.88) confirming the increased reticulocyte count. The mother had decreased band 3 (85% of normal) content but her protein 4.2 levels were 98 percent of normal. This implies that she had an adequate number of protein 4.2 binding sites. Her protein 4.1a/b ratio was normal (1.43, normal=1.22-1.88) although her haematological data did show mildly increased reticulocytes.

3.2.3 Erythrocyte Band 3 DNA Restriction Enzyme Analysis

Screening of amplified DNA for known hotspots and mutations with restriction enzymes revealed that the proband and both parents were negative for the Band 3 Cape Town [E90K], Band 3 Osnabruck/Lyon [R150X], Band 3 Tuscaloosa [P327R], Band 3 Pribram [intron 12, -1g→a] and Band 3 Most [L707P] as well as mutations in the codon 490 and codon 760 hotspots (data not shown). It was however noted that the family members displayed an abnormal exon 12 and 13 *Taq*I digestion pattern. The digestion of exon 13 with *Taq*I showed the homozygous loss of a *Taq*I cleavage site in the proband while both parents were heterozygous for this restriction digest change (see Figure 3.8).

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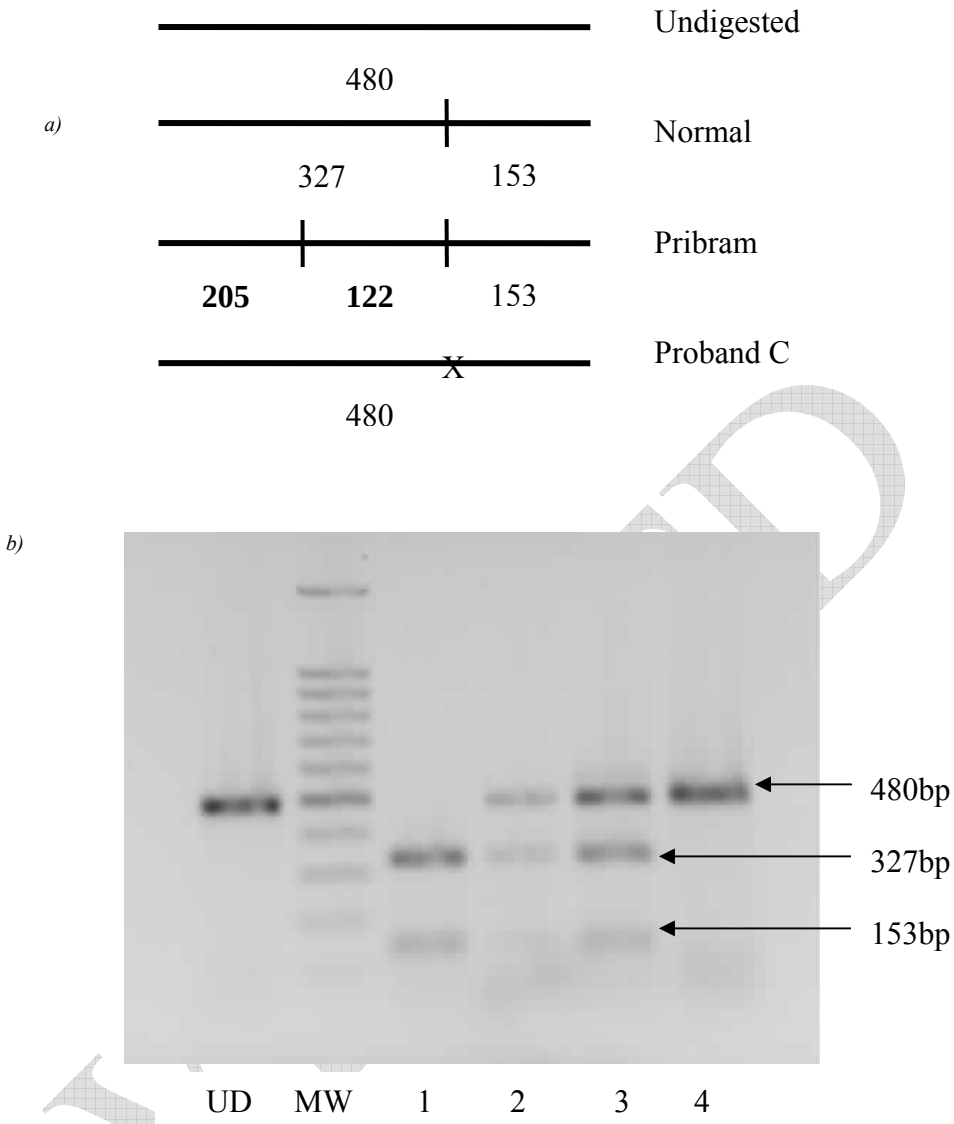

Figure 3.8: *TaqI* Analysis of Band 3 Exons 12 and 13 of Kindred C

(a) Line diagram showing the Taq*I restriction site in exons 12 and 13. The normal* Taq*I restriction site abolished by the novel mutation is marked by an X, while the fragments formed when the Pribram mutation is present are shown in bold. (b)PCR fragments digested with* Taq*I separated on a 1% agarose gel. The control sample (1) shows a complete digest with no fragment at 480bp and digested fragments at 327bp and 153bp. Both parents (2, 3) show a heterozygous pattern with fragments at 480bp, 327bp and 153bp. The proband (4) shows a homozygous mutant pattern, with only the undigested fragment (480bp) visible. The undigested DNA (UD) is 480bp in size. MW indicates the 100bp DNA ladder.*

3.2.4 *TaqI* Analysis of Unrelated Subjects

Randomly selected, unrelated subjects (39 Black and 20 Caucasian) were screened for a similar *Taq*I restriction site loss using identical methods. This analysis was performed to ensure that mutation did not occur frequently within the South African population. If the nucleotide change was common in non-HS subjects, it could not be linked to the disease seen in Family C and would be viewed as a polymorphism. The subjects screened displayed the normal restriction digest patterns, examples of which are shown in Figure 3.9.

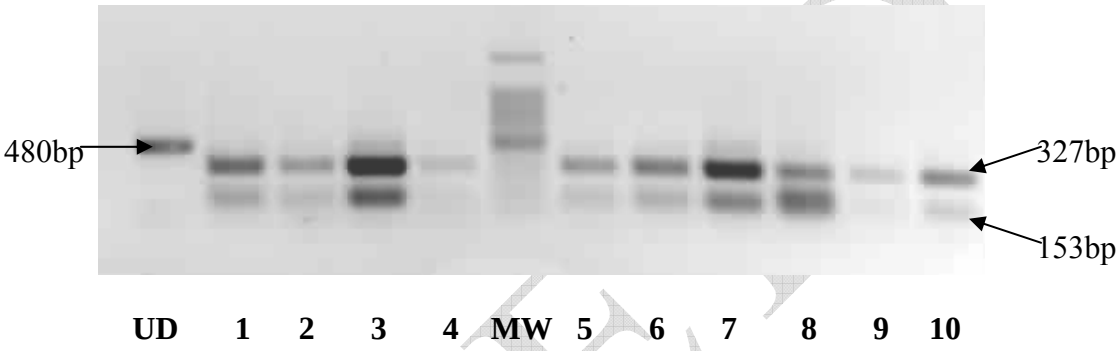

Figure 3.9: *Taq*I Digestion of Band 3 Exons 12 and 13 in Unrelated Subjects

UD is undigested PCR product, lanes one to ten contain random, unrelated non-HS patient samples digested using Taq*I and MW is a 100bp DNA size ladder. DNA fragment sizes are indicated.*

3.2.5 Direct DNA Sequencing of Band 3 Exon 13

Direct DNA sequencing (see Figure 3.10) of exon 13 of the proband, using the reverse primer (P122), revealed a homozygous mutation within the *TaqI* digestion site (TCGA). The guanine residue was replaced by an adenine residue, the resultant DNA fragment reading TCAA. The altered nucleotide is at position one of codon 508 and consequently a lysine (AAG) is encoded instead of a glutamic acid (GAG) in the mutant band 3 protein. The parents are heterozygous for this nucleotide change.

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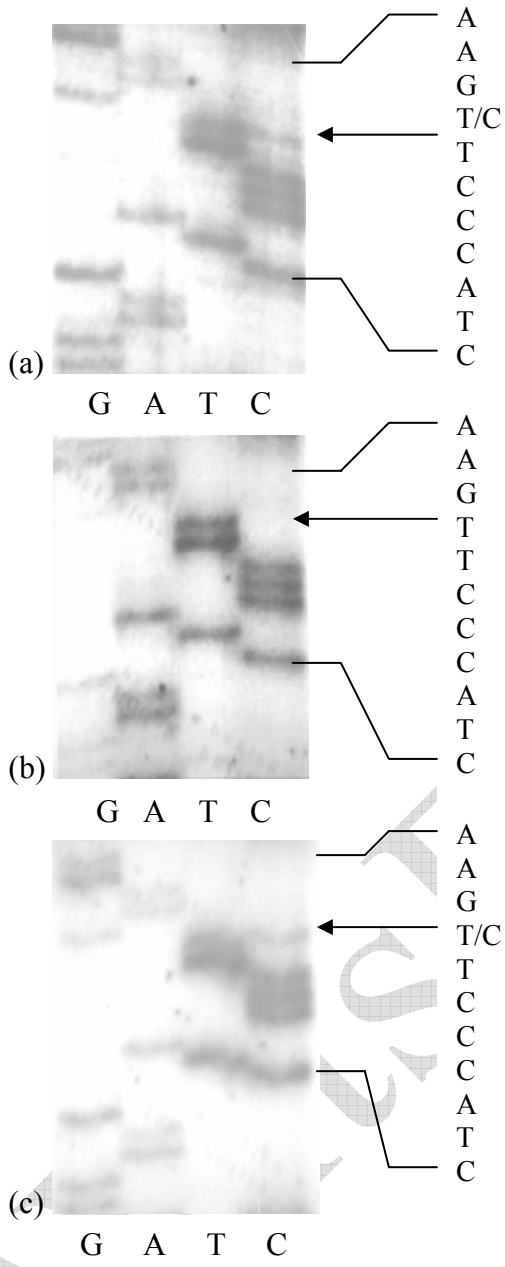

Figure 3.10: DNA Sequence of a Portion of Exon 13 of Family C Indicating a Novel Nucleotide Change

The father (a) and mother (c) of proband C both show heterozygous pattern at nucleotide 508 while the proband (b) shows only the mutant pattern. The antisense sequence is displayed as the sequence was obtained using the reverse primer. The AAG→GAG mutation thus appears as TTC→CTC. The sequence reads 5'-3' from the bottom of the selected region. The nucleotide change is indicated an arrow for each patient.

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3.2.6 Screening for Modulating Factors

A number of techniques were used to determine the presence of any known modulating factors (data not shown). Exon 4 of family C was sequenced to determine if they possessed the Montefiore polymorphism (E40K) but all of the family members showed the normal DNA sequence at this site. Restriction enzyme digests were used to ascertain that the family members possessed neither P147S (the second part of the Mondego polymorphism) nor band 3 Genas (-62G→A), a known promoter polymorphism, both of which have been shown to heighten the severity of band 3 deficiency when found in conjunction with a mutation.

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3.3 Detection of the Memphis Polymorphism

3.3.1Clinical Profile

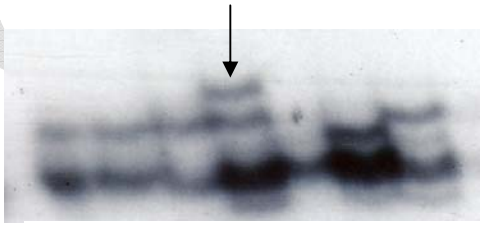
Proband D is a black African male. No other family members were available for study. Little clinical data were available for this patient but his reticulocyte count was normal according to his protein 4.1a/b ratio (1.59, normal=1.22-1.88) and he had spherocytes on his blood smear. According to Fairbanks's SDS-PAGE, his band 3 quantity was slightly decreased (93 percent of normal) and had decreased electrophoretic mobility on a Laemmli SDS-PAGE gel (data not shown).

3.3.2 Band 3 DNA Restriction Enzyme Analysis

Screening of amplified DNA for known hotspots and mutations with restriction enzymes revealed that proband D was negative for the Band 3 Cape Town [E90K], Band 3 Osnabruck/Lyon [R150X], Band 3 Tuscaloosa [P327R], Band 3 Pribram [intron 12, -1g→a] and Band 3 Most [L707P] as well as mutations in the codon 490 and codon 760 hotspots.

3.3.3 SSCP Analysis

SSCP analysis indicated a conformational abnormality in exon 4 of band 3 (Figure 3.11). Exons 2/3, 5/6, 7/8, 9, 10, 11, 12/13 14, 15, 16, 17, 18/19 and 20, as well as the promoter region, showed normal SSCP patterns (data not shown).



P1 P2 P3 P4 C1 C2 C3

Figure 3.11: SSCP Pattern for Band 3 Exon 4

Patients 1 to 3 (P1, P2, P3) show similar patterns to the control samples (C1, C2 and C3). Proband D (P4) has an abnormal pattern, indicated by an arrow.

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3.3.4 Direct DNA Sequencing of Band 3 Exon 4

Sequencing of exon 4 demonstrated that the patient had the band 3 Memphis I polymorphism (Figure 3.12). The point mutation (AAG→GAG) occurs in codon 56. Lysine 56 is replaced by glutamic acid in the band 3 protein with no apparent phenotypic effects. Band 3 Memphis I has been linked to decreased electrophoretic mobility (Jarolim *et al*, 1992) and the diagnosis of this polymorphism fits the decreased electrophoretic mobility of the patient's band 3.

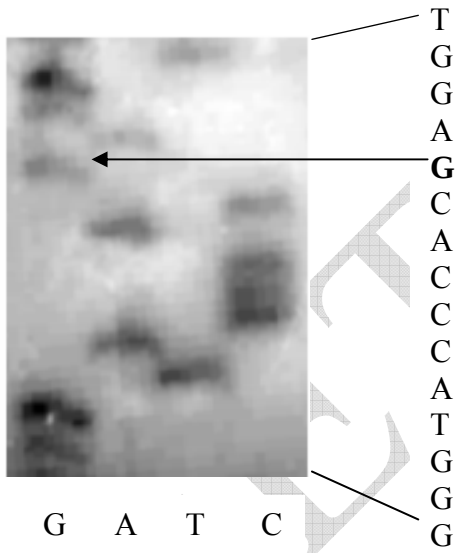

Figure 3.12: Portion of a Direct DNA Sequence of Exon 4 of Proband D Indicating the Band 3 Memphis I Polymorphism

Proband D showed a homozygous pattern at nucleotide 118, the nucleotide change being indicated by an arrow. The sense sequence is displayed as the sequence was obtained using the forward primer. The sequence reads 3'-5' from the bottom of the selected region.

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3.4 Analysis of Band 3-Deficient Hereditary Spherocytosis Patients

Probands A to D were discussed in sections 3.1, 3.2 and 3.3 while the results of probands E to O are amalgamated below. Of the eleven band 3 deficient patients / families discussed below, nine were Caucasian and two were Black. The extent of their band 3 deficiency (as shown in Table 3.1) was calculated from the protein densitometry values obtained from Fairbanks SDS-PAGE. DNA was extracted from the buffy coats of these patients and screened for a variety of known mutations using restriction enzyme digests. In addition to this band 3 exons were screened for possible nucleotide changes using SSCP.

3.4.1 Band 3 DNA Restriction Enzyme Analysis

Screening of amplified DNA for known hotspots and mutations using restriction enzymes revealed that all 11 probands (E to O) were negative for the Band 3 Mondego polymorphism and the Band 3 Cape Town [E90K], Band 3 Osnabruck/Lyon [R150X], Band 3 Tuscaloosa [P327R], Band 3 Pribram [intron 12, -1g→a] and Band 3 Most [L707P] as well as mutations in the codon 490 and codon 760 hotspots (see examples in Figures 3.13 to 3.18).

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Table 3.1: Hereditary Spherocytosis Patients with Band 3 Deficiency

Proband/ Family	Ethnic group	Family Members Examined	Band 3 (%) (Patient/Control)	Degree of Band Deficiency
Proband E	Black African	-	76	Moderate
Proband F	Black African	-	93	Mild
Proband G	Caucasian	-	93	Mild
Family H	Caucasian	Proband Father	85 85	Moderate Moderate
Family I	Caucasian	Proband I1 Proband I2	87 90	Mild Mild
Proband J	Caucasian	-	87	Mild
Proband K	Caucasian	-	92	Mild
Proband L	Caucasian	-	84	Moderate
Proband M	Caucasian	-	88	Mild
Family N	Caucasian	Proband Sister	82 91	Moderate Mild
Proband O	Caucasian	-	93	Mild

The degree of band 3 deficiency was classed:

- *Patient band 3 < 85% → mild deficiency*
- *85%≤ patient band 3 ≥65% → moderate deficiency*
- *Patient band 3 <65% → severe deficiency*

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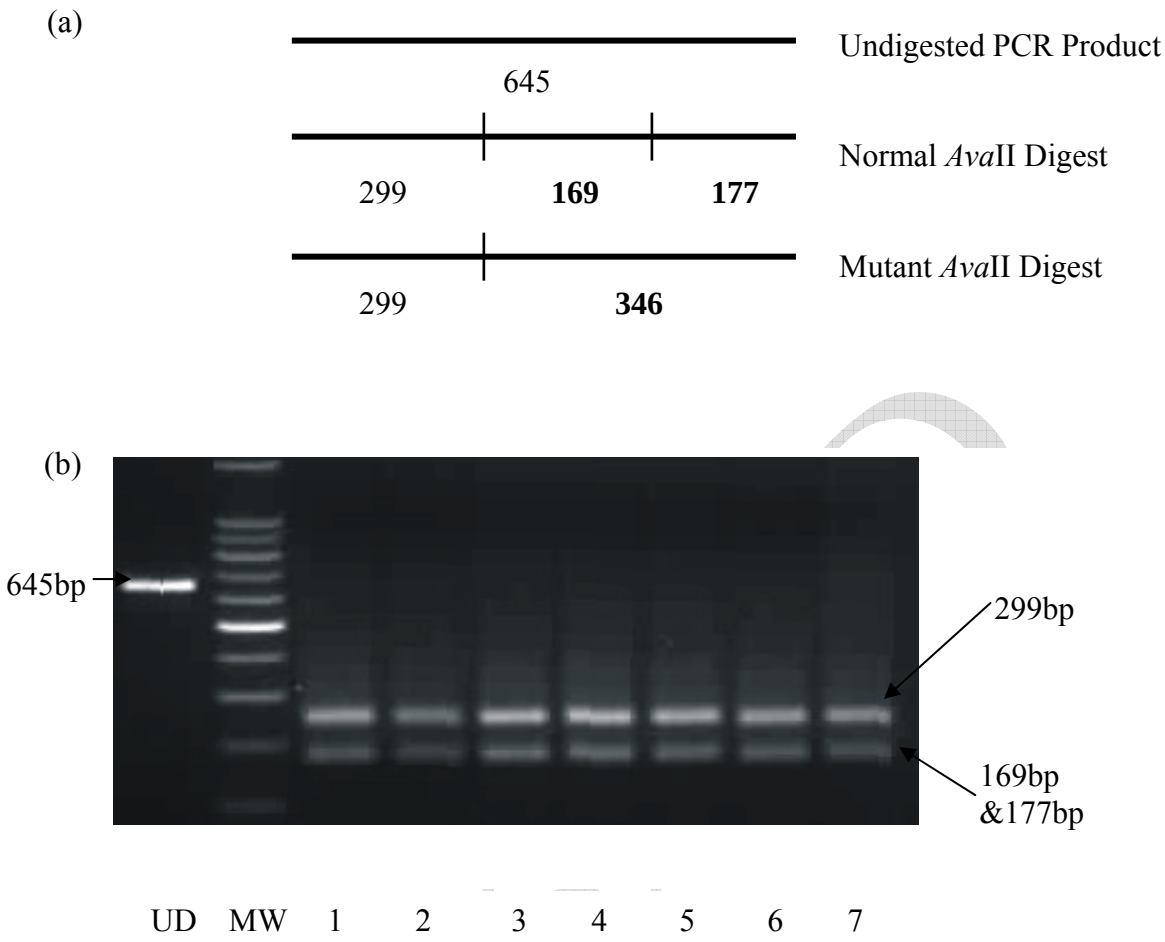

Figure 3.13: *Ava*II Digest for the Band 3 Osnabrück I / Lyon Mutation

(a) Line diagram indicating the expected Ava*II digest fragments. The fragment sizes (in bp) are shown and those altered by the Osnabrück I/Lyon mutation are indicated in bold. The mutation deletes a restriction site and the 645bp fragment is digested into fragments of 299bp and 346bp instead of fragments of 299bp, 169bp and 177bp (the latter two do not separate). (b) Amplified exons 5&6 digested using* Ava*II and separated on a 2% agarose gel. Patients normal at this restriction site are shown in lanes one to seven. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

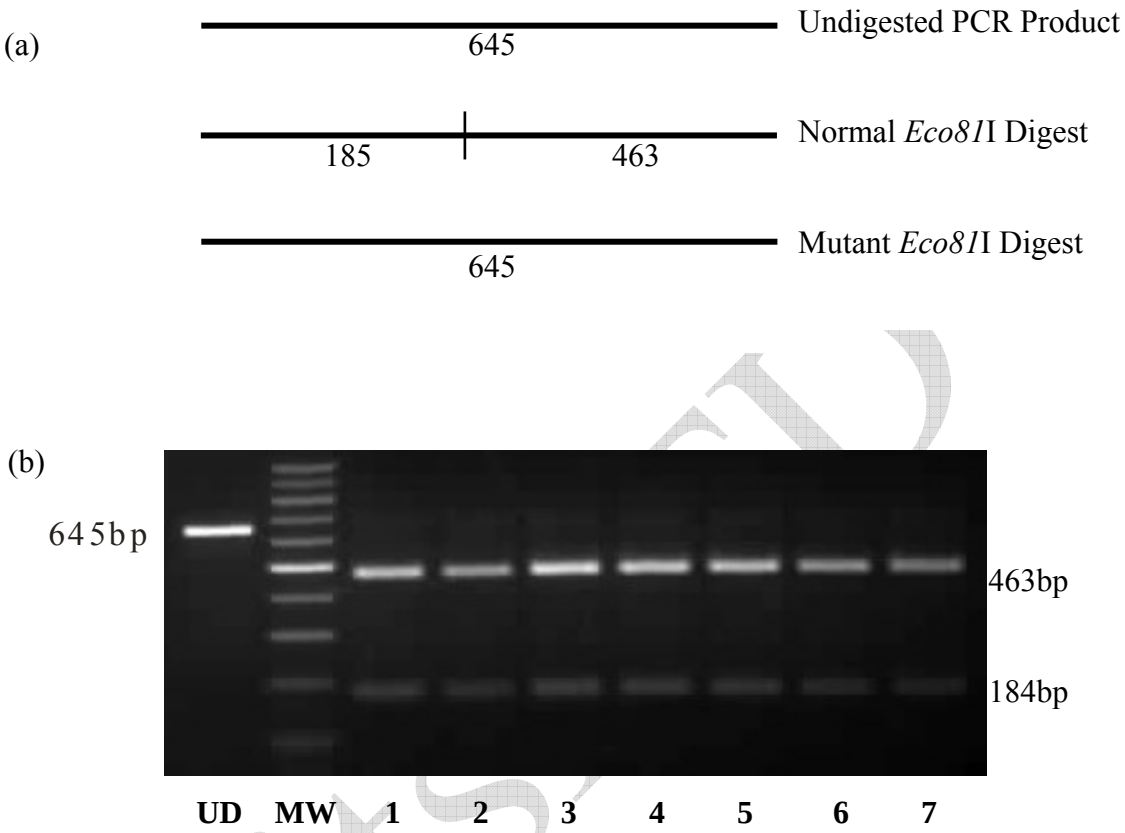

Figure 3.14: *Eco*81I Digest for the Band 3 Mondego Polymorphism

(a) Line diagram indicating the expected Eco*81I digest fragments. The fragment sizes (in bp) are shown and those altered by the Mondego polymorphism are indicated in bold. The nucleotide change deletes a restriction site and the 645bp fragment remains intact instead of being digested into fragments of 463bp and 184bp. (b) Amplified exons 5&6 digested using AvaII and separated on a 1% agarose gel. Patients normal at this restriction site are shown in lanes one to seven. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

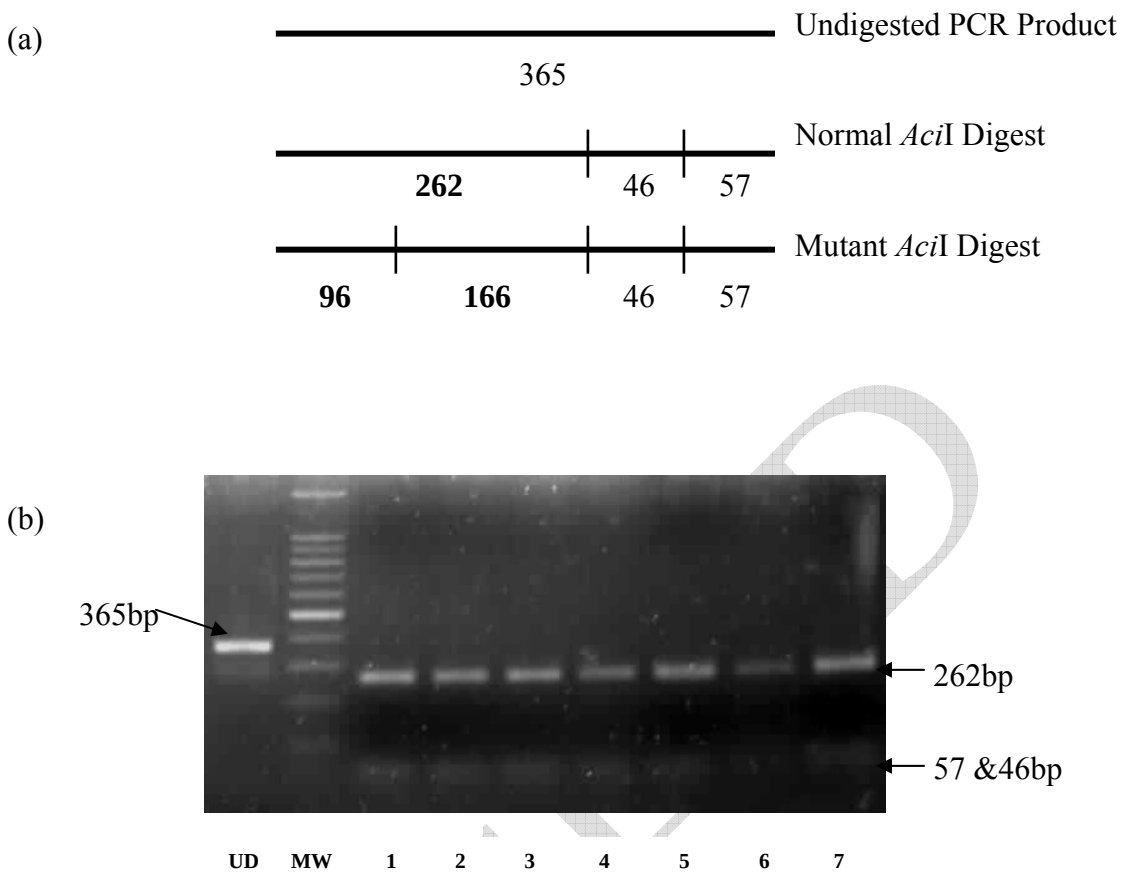

Figure 3.15: *Aci*I Digest for the Band 3 Tuscaloosa Mutation

(a) Line diagram indicating the expected Aci*I digest fragments. The fragment sizes (in bp) are shown and those altered by the Tuscaloosa mutation are indicated in bold. The mutation creates a restriction site and instead of the 365bp fragment being digested into three fragments of 46bp, 57bp and 262bp, the 262bp fragment is further digested into a 96bp and a 166bp fragment. The 46bp and 57bp fragments do not separate. (b) Amplified exon 10 digested using* Aci*I and separated on a 1% agarose gel. Patients normal at this restriction site are shown in lanes one to seven. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

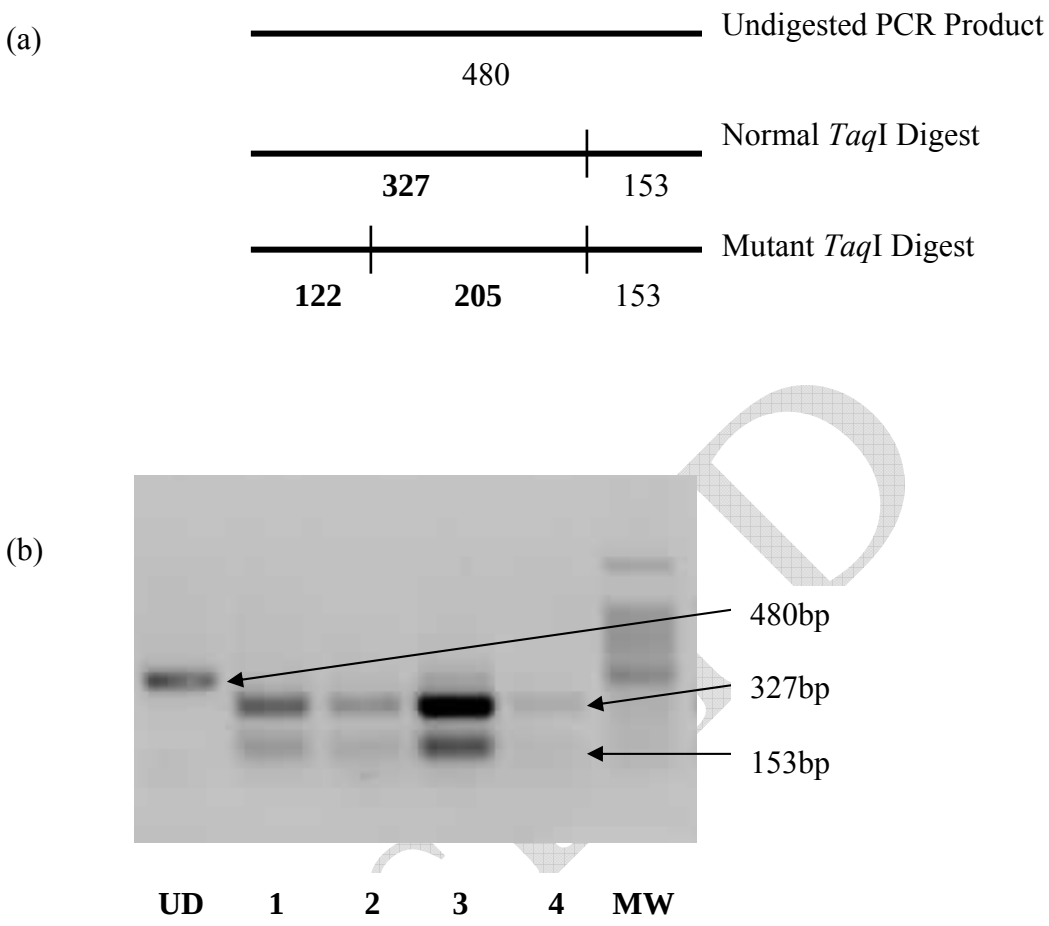

Figure 3.16: *Taq*I Digest for the Band 3 Pribram Mutation

(a) Line diagram indicating the expected Taq*I digest fragments. The fragment sizes (in bp) are shown and those altered by the Pribram mutation are indicated in bold. The mutation creates a restriction site and instead of the 480bp fragment being digested into two fragments of 153bp, 327bp, the 327bp fragment is further digested into a 205bp and a 122bp fragment. (b) Amplified exons 12 and 13 digested using* Taq*I and separated on a 1% agarose gel. Patients normal at this restriction site are shown in lanes one to four. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

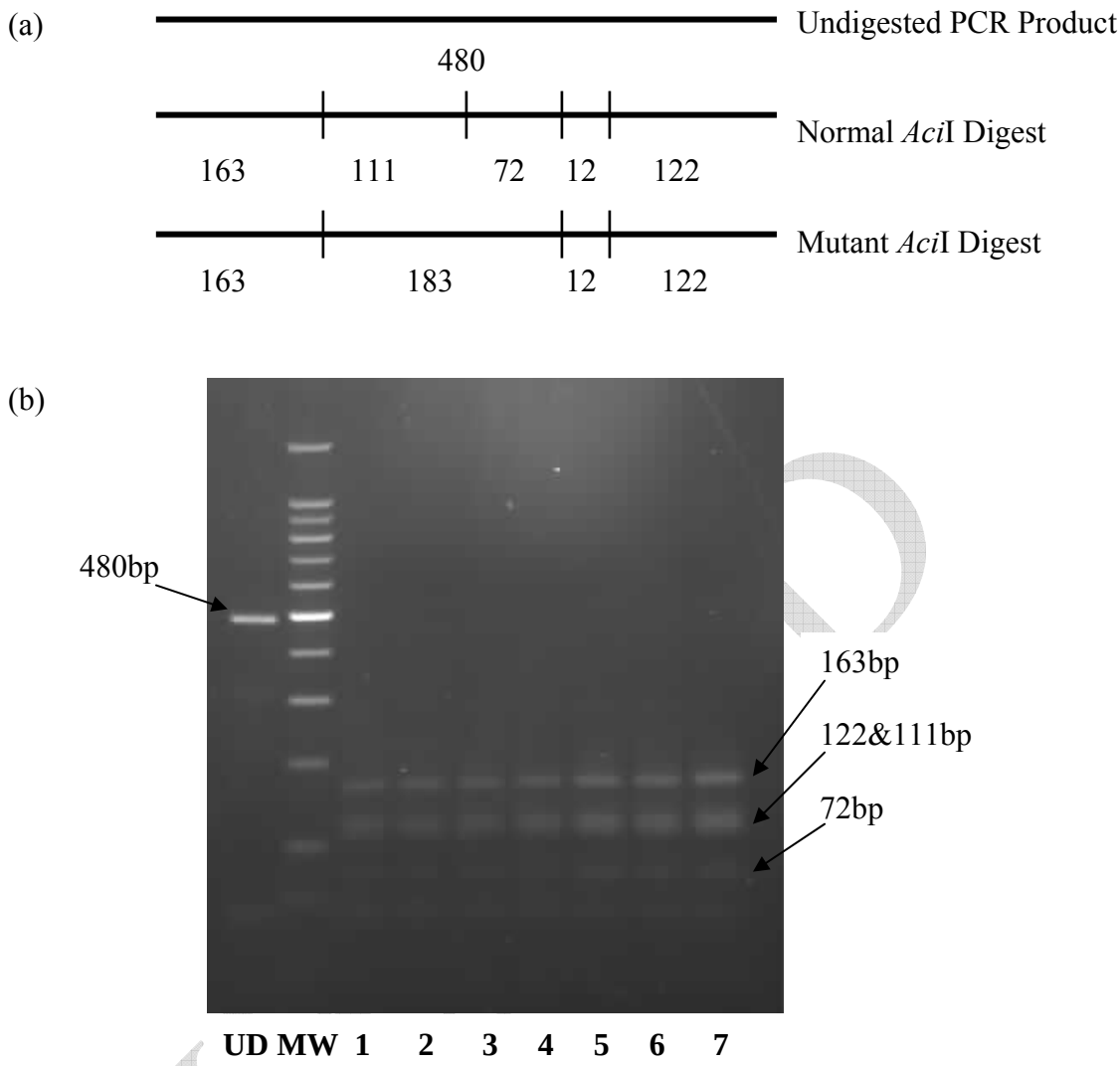

Figure 3.17: *Aci*I Digest for the Band 3 Codon 490 Mutations

(a) Line diagram indicating the expected Aci*I digest fragments. The fragment sizes (in bp) are shown and those altered by the Pribram mutation are indicated in bold. The mutations abolish a restriction site and instead of the 480bp fragment being digested into fragments of 163bp, 122bp, 111bp, 72bp and 12bp; the restriction site between the 111bp and 72bp fragments is lost such that an 183bp fragment is present. (b) Amplified exon 17 digested using* Aci*I and separated on a 2.5% agarose gel. Patients normal at this restriction site are shown in lanes one to seven. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

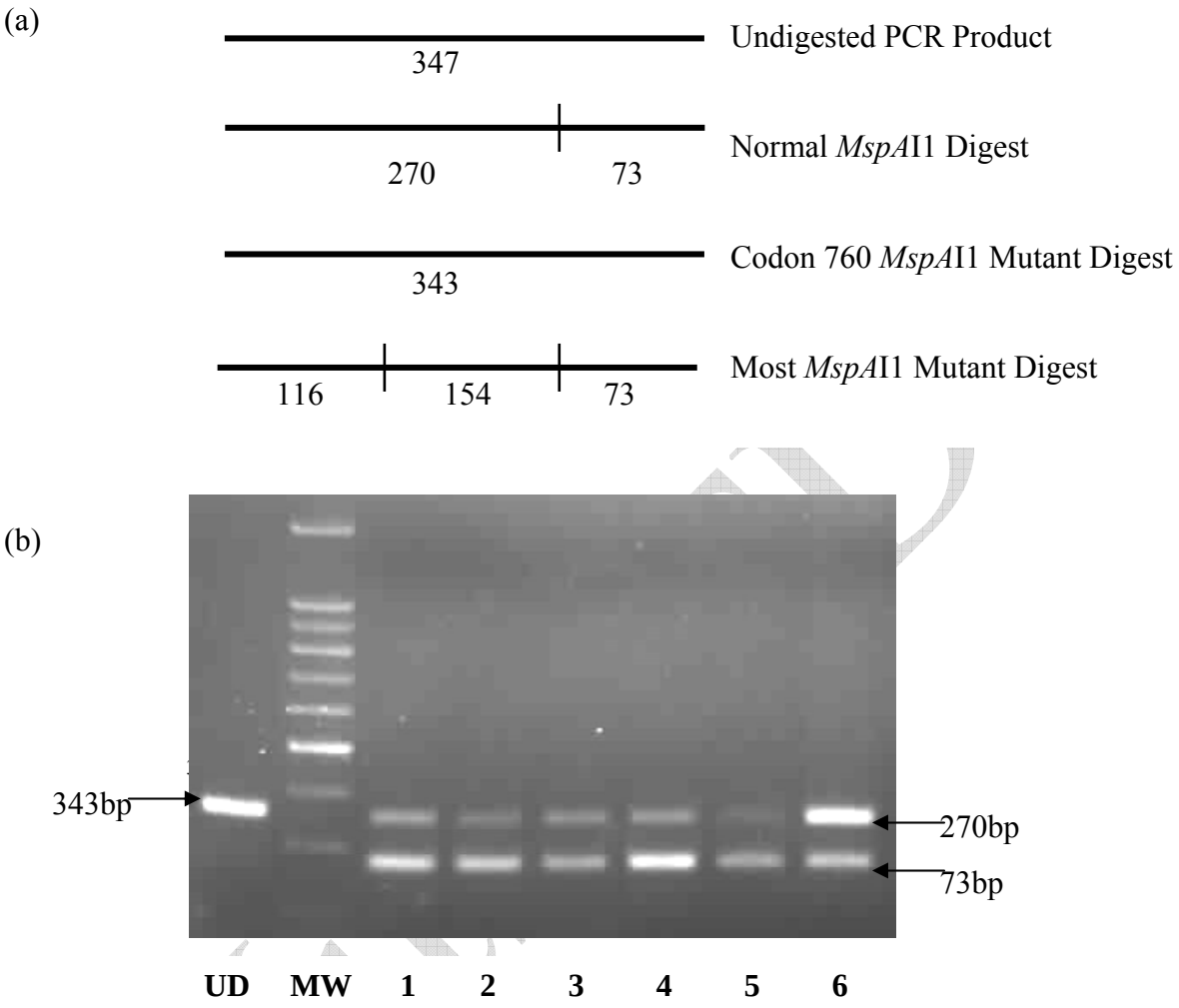

Figure 3.18: *MspA*I1 Digest for Band 3 Codon 760 and Most Mutations

(a) Line diagram indicating the expected MspA*I1 digest fragments. The fragment sizes (in bp) are shown. Band 3 codon 760 mutations abolish a restriction site and instead of the 343bp fragment being digested into a 270bp and a 73bp fragment, it remains intact. The band 3 Most mutation creates a restriction site: in addition to the codon 760 restriction site at 272bp, a second site is created at 116bp and causes the 272bp fragment to be digested. Fragments of 116bp, 154bp and 73bp are hence seen. (b) Amplified exon 17 digested using* MspA*I1 and separated on a 1% agarose gel. Patients normal at this restriction site are shown in lanes one to six. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

3.4.2 SSCP analysis and DNA Sequencing

SSCP analysis was performed on exons with numerous known mutations where restriction digest analysis would be inefficient and also to screen exons for novel abnormalities. PCR products larger than 300bp were successfully digested to fragments smaller than 300bp as described in section 2.10.1.

According to SSCP analysis, only one SSCP change was noted. Proband E displayed a change in exon 9 which was linked to a silent heterozygous polymorphism (GCA→GCC; ala→ala) in codon 250 of band 3 (data not shown). The other patients had no conformational polymorphisms in any of the screened exons (see example in figure 3.19). As SSCP is not infallible, not all mutations will be detected as some nucleotide changes do not alter the conformation of the DNA to a great extent (Saad *et al*, 1993) and some mutations may hence have been overlooked.



Figure 3.19: Example of a Normal SSCP Pattern For Band 3 Exons 12 and 13

The 480bp PCR product was digested into two fragments of 153bp (lower fragment) and 327bp (upper fragment). The SSCP analysis indicated no conformational (i.e. nucleotide) changes in either exon 12 or exon 13 as the patient DNA (J, K, L) samples do not have different patterns to the control samples (1, 2, 3).

3.5 Analysis of Protein 4.1-Deficient Hereditary Elliptocytosis Patients

Of ten patients from nine families with protein 4.1 deficiencies identified, five were Caucasian, three were Black and two were Indian. DNA was extracted from the buffy coats of these patients and screened for three known mutations (Lille, Madrid and Aravis) using restriction enzyme digests. Protein 4.1 was also examined for changes in the size of the protein using western blot protein analysis.

Table 3.2: Hereditary Elliptocytosis Patients with Protein 4.1 Deficiency

Proband/Family	Ethnic group	Family Members	Protein 4.1 (%) (Patient/Control)	Clinical Severity
Family 1	Caucasian	Proband Father Mother	65 67 94	Severe Mild Normal
Family 2	Caucasian	Proband Son	81 99	Severe Normal
Proband 3	Black African	-	77	Severe
Proband 4	Black African	-	76	Mild
Proband 5	Caucasian	-	72	Severe
Proband 6	Indian	-	47	Severe
Proband 7	Indian	-	71	Moderate
Proband 8	Black African	-	68	Moderate
Proband 9	Caucasian	-	91	Moderate

It can be seen that there is a great variation between the protein 4.1 deficiency and the clinical severity. It is possible that other membrane proteins are compensating for the decreased protein 4.1 to different degrees or that polymorphisms in protein 4.1 alter the clinical profile in a manner which has yet to be determined.

WISERD

3.5.1 Screening of Protein 4.1 for Size Variations

The protein 4.1 Annecy, Algeria and unnamed mutations cause a truncated form of the protein while protein 4.1 Hurdle-Mills produces a lengthened protein. The immunoblot was performed as a screen for these truncated or elongated proteins as the electrophoretic mobility of these proteins would be abnormal. The abnormally-sized proteins could move through the acrylamide gel at the same rate as another protein and would not be detected during standard protein analysis, protein 4.1 merely appearing to be absent (homozygous) or decreased (heterozygous) depending on the quantity of normal protein 4.1 in the erythrocyte. The antibody binds specifically to protein 4.1 and would allow for its detection even at an abnormal position. In addition to the known mutations, any amino acid changes altering the size of the protein would be detected using this technique.

Probands one to nine all had protein 4.1 of a normal size according to the immunoblot using rabbit anti-protein 4.1 antibody. A degree of non-specific protein binding was visible in both the patients and the controls (see example in Figure 3.20). Staining of the acrylamide gel with Coomassie Blue Stain after the immunoblot indicated that all of the protein 4.1 and other membrane proteins had been transferred from the acrylamide gel to the nitrocellulose membrane. Only the transfer of spectrin was incomplete. The immunoblots for patients eight and nine were performed by Natalie Bracher.

3.5.2 Protein 4.1 DNA Restriction Enzyme Analysis

Restriction enzyme analysis of known protein 4.1 point mutations Madrid (Exon 4 M1R) Lille (Exon 4 M1T) and Aravis (Exon 17 K447del) showed that none of the patients possessed these mutations (figures 3.21 and 3.22).

WiSETD

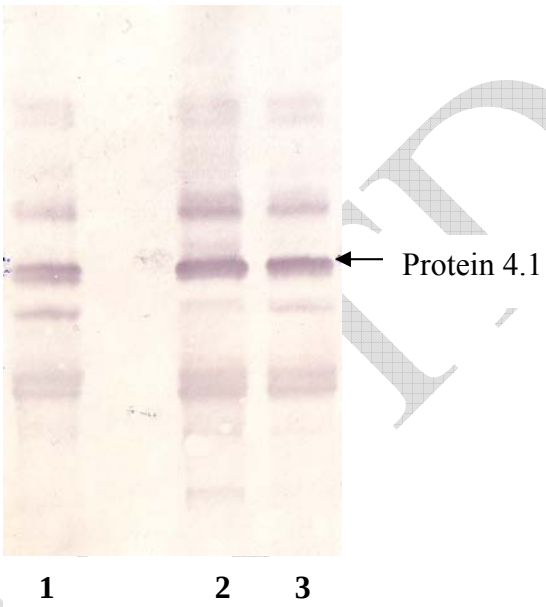

Figure 3.21: Immunoblot Erythrocyte Membrane Proteins from Protein 4.1 Deficient Patients from Family 1

Lane 1 contains a control sample. Lanes 2 and 3 contain patient samples (proband and father respectively) from family 1. The intact protein 4.1 is indicated by the arrow. This band was identified by aligning the immunoblot with red cell membrane proteins on the Coomassie Blue-stained gel (not shown). The other visible bands are believed to be non-specific binding as they are also visible in the control sample.

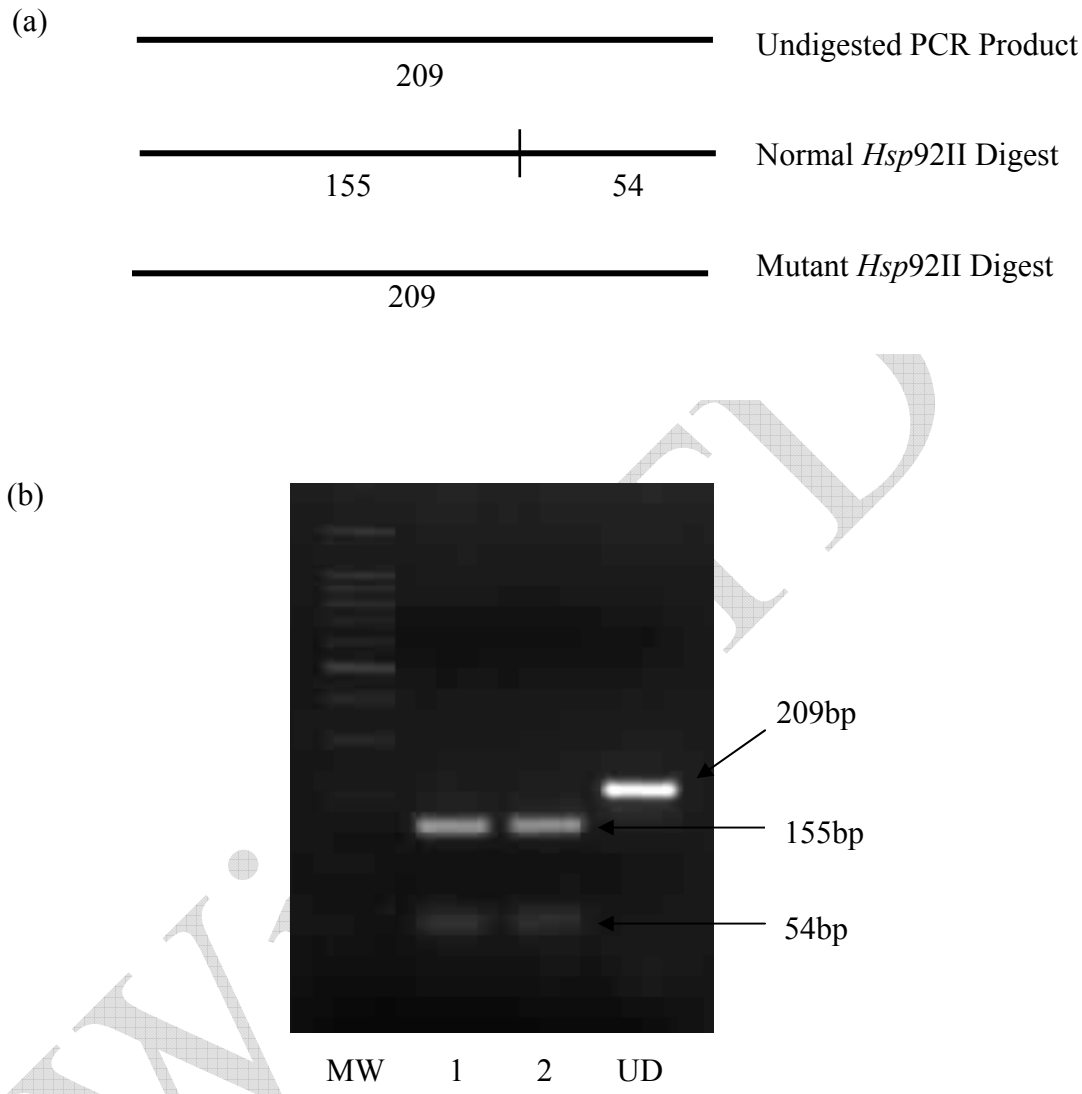

Figure 3.22: *Hsp*92II Digest for the Protein 4.1 Initiation Codon Mutations

(a) Line diagram indicating the expected Hsp*92II digest fragments (bp). The protein 4.1 Lille (M1T: A**T**G→A**C**G) and Madrid (M1R: A**T**G→A**G**G) mutations abolish a restriction site and instead of the 209bp fragment being digested into fragments of 155bp and 54bp, it remains intact. (b) Amplified exon 4 digested using* Hsp*92II and separated on a 2.5% agarose gel. Patients normal at this restriction site are shown in lanes one and two. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*

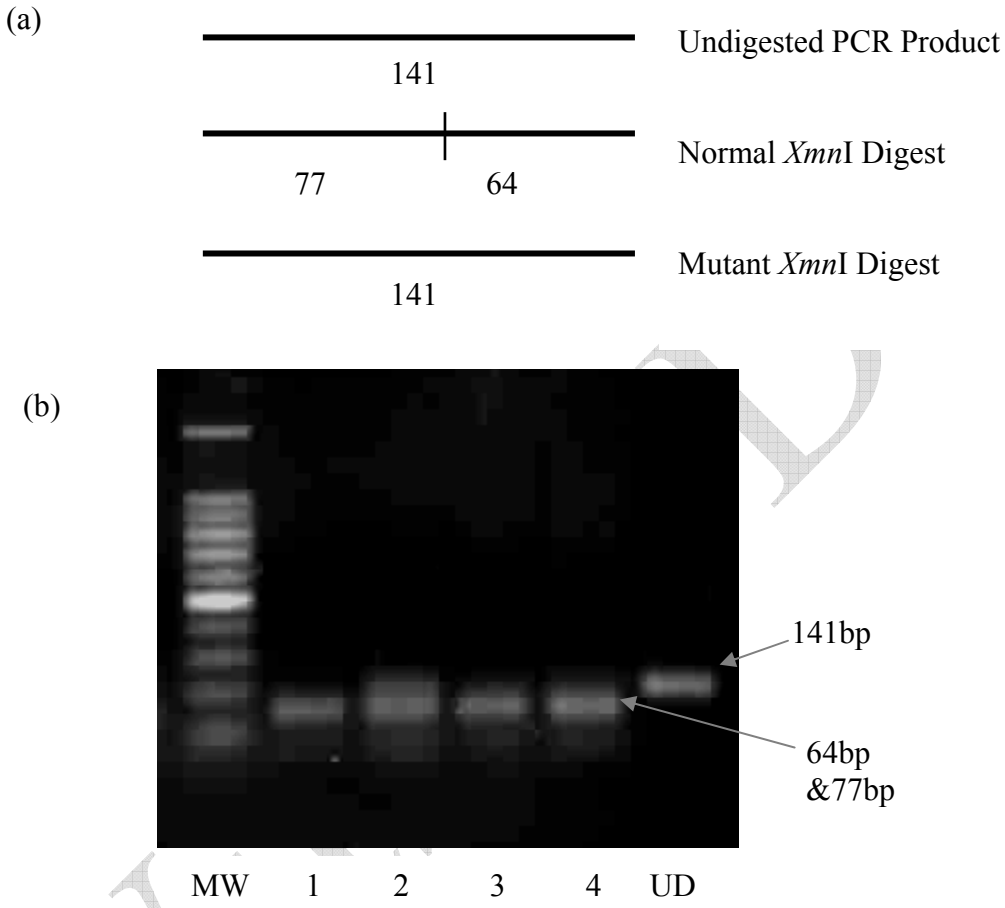

Figure 3.22: *Xmn*I Digest for Protein 4.1 Aravis Mutation

(a) Line diagram indicating the expected Xmn*I digest fragments (bp). The protein Aravis mutation (K447del) abolishes a restriction site and instead of the 141bp fragment being digested into fragments of 64bp and 77bp, it remains intact. (b) Amplified exon 17 digested using* Xmn*I and separated on a 2.5% agarose gel. Patients normal at this restriction site are shown in lanes one to four. UD indicates undigested PCR product and MW indicates the 100bp DNA size ladder.*