MUTATION DETECTION IN X-LINKED DISORDERS: ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY AND PELIZAEUS-MERZBACHER DISEASE.

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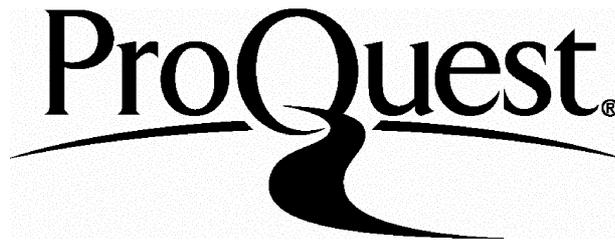
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ABSTRACT

This study was carried out to establish methods of mutation detection in the X-linked disorders: ornithine carbamoyl transferase (OCT) deficiency and Pelizaeus-Merzbacher disease (PMD) and to determine the molecular basis of these disorders. Two main approaches were used: single strand conformation polymorphism (SSCP) analysis and cytosine-guanine dinucleotide (CpG) site analysis.

SSCP screening of the coding regions of the OCT and proteolipid protein genes in 5 families with late onset OCT deficiency and 14 families with PMD, identified sequence variations in 3 and 4 families respectively.

On direct sequencing of the OCT gene 2 novel amino acid substitutions: Ala(176)Thr and Arg(97)Leu were identified and the fourth reported case of Arg(245)Trp. Sequencing of the proteolipid protein gene identified 3 novel changes: Leu(223)Pro, Thr(181)Pro and a G to T change at the -1 position of the 3' acceptor splice site of exon 4. The fourth PMD family which gave a variant SSCP pattern is currently under analysis by a collaborating group.

The second approach was the analysis of 5'CpG3' dinucleotide sites located within the recognition sequences of the restriction enzymes: *Taq*I (TCGA), *Msp*I (CCGG) and *Cfo*I (GCGC), using the principal that mutation abolished digestion.

Genomic DNA or PCR product of exons containing a restriction enzyme site, from patients with classical or late onset OCT deficiency, was digested and the products were analysed by Southern blotting or by separation on high percentage agarose gels respectively. Two point mutations: Arg(109)Gln and Pro(193)Leu, which removed the *Taq*I and *Msp*I sites in exons 5 and 7 respectively, were identified.

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LIST OF ABBREVIATIONS

A	Ampere (s)
A	Adenine
ACRS	Amplification created restriction site
ARMS	Amplification refractory mutation system
ASO	Allele specific oligonucleotide
BME	Beta Mercaptoethanol
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
cm	Centimetre
CNS	Central nervous system
CpG	Cytosine-guanine dinucleotide
CVS	Chorionic villus sample
dATP	2'-Deoxyadenosine 5' triphosphate
dCTP	2'-Deoxycytidine 5' triphosphate
ddATP	2',3'-Dideoxyadenosine 5' triphosphate
ddCTP	2',3'-Dideoxycytidine 5' triphosphate
ddGTP	2' 3'-Dideoxyguanosine 5' triphosphate
ddH_2O	Double distilled water
ddNTP	2' 3'-Dideoxynucleotide 5' triphosphate
ddTTP	2',3'-Dideoxythymidine 5' triphosphate
DGGE	Denaturing gradient gel electrophoresis
dGTP	2'-Deoxyguanosine 5' triphosphate
dITP	2'-Deoxyinosine 5' triphosphate
DM-20	Proteolipid protein isoform
DNA	Deoxyribonucleic acid

dNTP	2'-Deoxynucleotide 5' triphosphate
dpm	Disintegrations per minute
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine 5' triphosphate
EDTA	Ethylenediamine tetraacetic acid
G	Guanine
g	gravitational force
g	gram
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
KAc	Potassium acetate
kb	Kilobase(s)
KCl	Potassium chloride
k_D	Affinity constant
l	Litre
LOD	Logarithm of odds
LS	Labelling solution
M	Molar (moles/litre)
MBP	Myelin Basic Protein
5-mC	5-methyl cytosine
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
MOPS	3-N-morpholino-propanesulphonic acid
mRNA	Messenger ribonucleic acid
MRI	Magnetic Resonance Imaging
NaCl	Sodium chloride
NaOH	Sodium hydroxide

ng	Nanogram
nM	nanomolar
nm	Nanometre
OCT	Ornithine carbamoyl transferase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PLP	Proteolipid protein
pM	Picomolar
PMD	Pelizaeus-Merzbacher disease
PNS	Peripheral nervous system
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
SSCP	Single strand conformation polymorphism
T	Thymine
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMAC	Tetramethyl ammonium chloride
TEMED	(N,N,N',N'-)tetramethylethylenediamine
Tm	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V	Volts
W	Watts
μM	Micromolar
μg	Microgram
μl	Microlitre

1.0.0   <u>INTRODUCTION</u>

1.1.0   **X-LINKED DISORDERS**

1.1.1   **X-LINKED INHERITANCE**

In addition to 22 pairs of homologous autosomes, an individual has 2 nonhomologous sex chromosomes, the X and Y. A female has 2 X-chromosomes, one inherited from each parent, while a male has an X-chromosome inherited from his mother and a Y chromosome inherited from his father. Disorders resulting from defective genes on these chromosomes are termed sex-linked and are characterised by specific patterns of inheritance.

The majority of sex-linked disorders are X-linked and these can be inherited in either a recessive or a dominant manner. X-linked recessive disorders are typified by a predominance of affected males and clinically unaffected carrier females who transmit the disorder to the next generation. This is explained by considering that a male has only one X-chromosome and if this is defective he will be affected, while a female with one defective chromosome, has a second functional chromosome, and will only be a carrier for a recessive disorder.

A female carrier of an X-linked recessive disorder has an equal chance of passing on either of her 2 X-chromosomes thus, on average, half her daughters will be carriers and half her sons affected. If an affected male reproduces (Fig. 1a), he will pass on his defective X-chromosome to all of his daughters who will be carriers, and through them to his grandsons. His sons will inherit his Y chromosome and will be unaffected. In many X-linked disorders males do not reproduce and transmission is solely by carrier females. This results in a characteristic 'knights' move' pedigree pattern of affected males (Fig. 1b).

X-linked inheritance in females is complicated by the inactivation of one X-chromosome (Lyonisation) in each somatic cell to ensure that the amounts of X-linked gene products
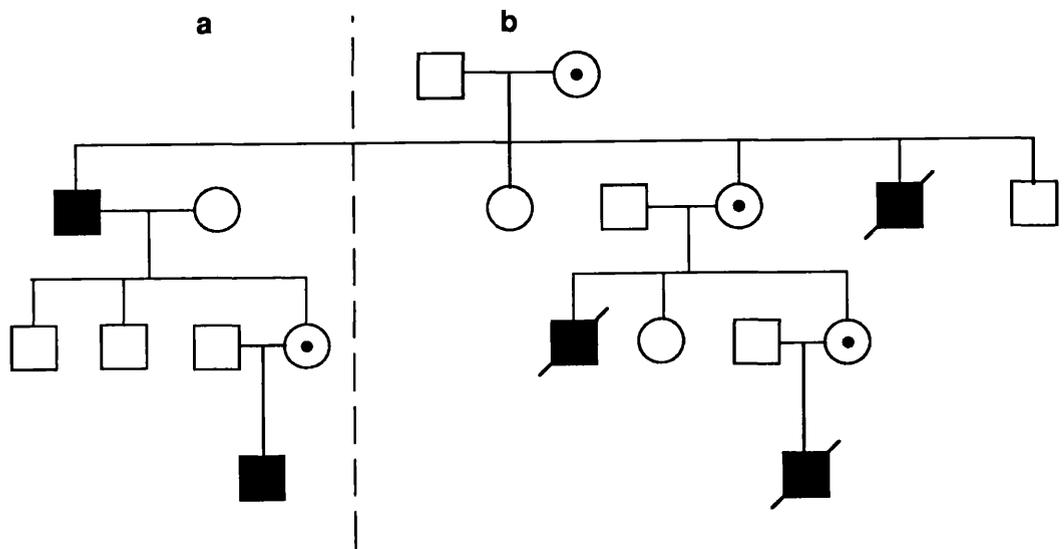
FIGURE 1 a. A typical pedigree of an X-linked recessive disorder in which males reproduce showing transmission from an affected male, via a carrier daughter, to a grandson.

b. A typical pedigree of an X-linked recessive lethal disorder showing the characteristic 'knights' move ' pattern of affected males.

In each case squares and circles represent males and females and completely shaded and dotted shapes indicate affected individuals and carriers respectively.

are equivalent in both sexes (reviewed by Conner and Ferguson-Smith. 1987). X-inactivation occurs early in development and the choice between maternal and paternal chromosomes is random for a given cell, but once established, is identical for all daughter cells. As a result, a female is effectively a mosaic, with a percentage of cells having an active paternal chromosome and the maternal X active in the remainder, and, as the process is random, in a minority of females one or other X-chromosome may be predominantly active. In an X-linked recessive disorder this may result in a manifesting carrier female if the mutant chromosome is the one which is primarily active.

Additionally, manifesting carriers may arise as a result of: X-autosome translocation in which the unaffected X-chromosome is preferentially inactivated to prevent partial monosomy for the autosome involved; a second mutation on the other X-chromosome; monosomy (Turner syndrome) for the short arm of the X-chromosome in which the intact chromosome with the mutation cannot be inactivated; nonrandom X-inactivation during the monozygotic twinning process, and potentially, as a result of uniparental disomy (Conner and Ferguson-Smith. 1987, Nance. 1990, Richards et al. 1990, Spence et al. 1988).

1.1.2   THE MUTATION RATE IN X-LINKED DISORDERS

In contrast to autosomal recessive disorders, X-linked recessive disorders in which affected males do not reproduce, are maintained in the population by new mutations.
Mutations can occur during both oogenesis and spermatogenesis and result in affected males and carrier females in the former case and, as a male cannot pass on his X-chromosome to his sons, in carrier females in the latter. Thus, in isolated sporadic males with noncarrier mothers, the mutation is of maternal origin.

Haldane showed (reviewed by Weatherall. 1991) that the proportion of new mutations among males with X-linked disorders is given by the equation:

$$(1-f)\mu \; / \; 2\mu + v$$

where: f = the effective fertility of males

v = the mutation rate in male gametes per generation

μ = the mutation rate in female gametes per generation

For a lethal disorder in which males fail to reproduce (f = 0) the equation becomes:

$$\mu\ /\ 2\mu + v$$

If no mutations occur in spermatogenesis (v = 0), 50% of affected males will have a carrier mother and 50% will have a new mutation. Similarly, if no mutations occur in oogenesis (μ = 0), no males will have a new mutation. If the mutation rate in both sexes is equal, a 1/3 of males will have new mutations, 2/3 will inherit the mutant gene from a carrier mother.

In practise the relative proportions are gene dependent and for several X-linked disorders, including haemophilia B (Montandon et al. 1992), a higher mutation rate for spermatogenesis than oogenesis has been reported.

A similar mutation rate has been reported by Stephens et al. (1992) in the autosomal disorder neurofibromatosis type I which led them to postulate a role for genomic imprinting that either enhanced mutation of the paternal allele or conferred protection on the maternal allele. For example, the relative hypermethylation of the vertebrate sperm genome compared to that of the ovum (Monk et al. 1987) may result in spontaneous deamination of 5-methyl cytosine in the paternal genome.

The mutation rate for a particular locus varies with gene size, structural and sequence dependent susceptibility to mutation, for example, the presence of repeat elements and the degree of 5-methylation of cytosine residues, and reflects the proportion of a gene at which a change will result in disease and in some disorders a paternal age of onset effect.

1.2.0   <u>DNA ANALYSIS FOR GENETIC</u>
<u>COUNSELLING</u>

At the molecular level, carrier detection and prenatal diagnosis are provided by tracking the mutant gene through a family (indirect analysis) or by identification of the causative mutation (direct mutation analysis).

1.2.1   <u>INDIRECT ANALYSIS</u>

Polymorphic DNA fragments are used as genetic markers to track a disorder through a family. Two main types are used: restriction fragment length polymorphisms (RFLPs) and microsatellite repeats (reviewed by Weatherall. 1991, Davies and Read. 1990, Weissenbach et al. 1992).

In the human genome, on average, 1 in every 150 bases is polymorphic and about 1 in 6 of these abolishes or creates a restriction site causing an RFLP. Analysis involves the digestion of genomic DNA with an appropriate restriction enzyme, separation of the digestion products by agarose gel electrophoresis, Southern blotting and hybridisation with a radiolabelled probe. This can be either a random genomic fragment linked to the disorder of interest (intergenic) or, ideally, a cDNA or partial gene probe (intragenic).

Alternatively the region may be amplified by the PCR, the product digested, separated by agarose or polyacrylamide gel electrophoresis and visualised directly by ethidium bromide staining and ultraviolet illumination.

Recently highly polymorphic markers for linkage have been developed based on runs of CA dinucleotides. These microsatellites, or CA repeats, are spaced frequently and apparently fairly evenly throughout the genome and are detectable by PCR methods. An entire genome map, developed by Genethon, has been published and the number is ever increasing (Weissenbach et al. 1992).

In each case the approach is to find a marker for which the female at risk is heterozygous, having different alleles of an RFLP or differing numbers of a given repeat element on each chromosome. Family studies are then conducted to establish with which form of the marker the disease is segregating, that is the phase. Once this is established, carrier detection and prenatal diagnosis may be possible.

1.2.2   <u>DIRECT ANALYSIS</u>

DNA sequencing is the most desirable course of action as it enables the direct identification of mutations. However, sequencing is labour intensive and for screening a large number of samples or for searching long stretches of DNA it is often more productive to use a prescreening technique to localize the approximate position of a mutation before sequencing. Several such screening methods have been reported, the most widely used being: single strand conformation polymorphism analysis (Orita et al. 1989a, 1989b), denaturing gradient gel electrophoresis (Fischer and Lerman. 1983, Myers et al. 1985a, 1988), chemical mismatch analysis (Cotton et al. 1988), heteroduplex analysis (White et al. 1992) and the analysis of cytosine-guanine (CpG) containing restriction sites (Grompe et al. 1991).

1.2.2.1   <u>SINGLE STRAND CONFORMATION POLYMORPHISM</u>
<u>ANALYSIS</u>

Single strand conformation polymorphism (SSCP) analysis was first described by Orita et al. (1989a, 1989b) and is based on the principle that single stranded nucleic acid under nondenaturing conditions adopts a specific conformation which is determined by the intramolecular interactions within the strand and hence by the sequence. On denaturing polyacrylamide gel electrophoresis nucleic acid will migrate according to size, but under nondenaturing conditions the mobility is also affected by the conformation of the molecule. Thus if the sequence is altered, for example by a mutation, the conformation may change and result in an altered mobility on electrophoresis.

Considering an X-linked recessive disorder (Fig. 2). A noncarrier female has 2 identical alleles. On denaturation these give 2 identical sense and 2 identical antisense strands, which form 2 possible conformations and produce 2 bands on electrophoresis. A carrier female has both mutant and wild type alleles. On denaturation these result in 2 nonidentical sense and 2 nonidentical antisense strands, which form 4 different conformations and produce 4 bands on electrophoresis. Of these bands, 2 are equivalent to the wild type pattern, while 2 are different.

A male has only 1 X-chromosome, with only a single copy of each X-linked allele. This results in 2 possible conformations and two bands on electrophoresis, where an unaffected male with the wild type allele has the wild type pattern on electrophoresis and an affected male with the mutant allele has the mutant pattern on electrophoresis.

In reality a single strand may adopt several conformations under a given set of conditions (Hayashi. 1991) and hence may produce several bands on SSCP analysis. Variations in these cases are detected as alterations in the overall pattern.

Practically, genomic or cDNA is amplified by the PCR and simultaneously radiolabelled by the inclusion of a radiolabelled deoxynucleotide triphosphate or by the use of an end labelled primer. The labelled DNA fragments are digested to an optimal size of about 200bp, denatured and analysed by nondenaturing PAGE. As conformation is also partially determined by local environmental conditions, for example ionic strength, temperature and the presence of solvents, analysis may be carried out simultaneously under 2 or more sets of conditions to increase the probability that a change, if present, is detected.

The technique has been used for mutation detection in a wide range of disorders including cystic fibrosis (Plieth et al. 1992), neurofibromatosis type 1 (Cawthorn et al. 1990), phenylketonuria (Labrune et al. 1991), the B1 variant of Tay-Sachs disease (Ainsworth et al. 1991), haemophilia A (Effrosini et al. 1992) and B (Fraser et al. 1992), ornithine carbamoyl transferase deficiency (Tuchman et al. 1992) and Pelizaeus-Merzbacher disease (Doll et al. 1992).
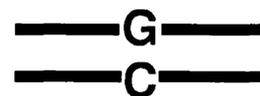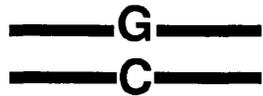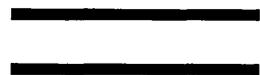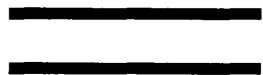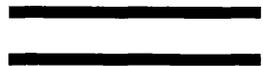
FIGURE 2: The characteristic pattern seen on SSCP analysis of an X-linked recessive disorder.

1.2.2.2   DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) involves the electrophoresis of double stranded DNA through an acrylamide gel containing a linear gradient of DNA denaturants such as formamide, urea or temperature (Fischer and Lerman. 1983, Myers et al. 1985a, 1988).

The technique is based on the fact that a DNA fragment is composed of several regions called melting domains, each with a specific Tm, and as the temperature or denaturant concentration is raised each domain melts cooperatively in a characteristic pattern. The Tm for a region depends on the hydrogen bonding and stacking interactions within that region and hence on the DNA sequence. If this is altered, the interactions will be affected, and the Tm of the region may change.

DGGE is carried out at a constant temperature just below the lowest Tm value. On electrophoresis DNA migrates at a rate determined by its molecular weight until it reaches a point at which the denaturant concentration and temperature are equal to the Tm of the lowest domain. At this point the region melts and the disordered structure is retarded by the gel matrix. As two fragments differing by a base change have different Tms, they will melt at different positions, and can be separated.

Genomic, cDNA and PCR fragments of between 100-1000bp can be analysed and the band patterns are visualised by ethidium bromide staining and UV illumination. Alternatively, radiolabelled PCR products or heteroduplexes formed with labelled wild type DNA may be used to detect differences in structure.

This technique has been used extensively in the analysis of a range of genes including the factor IX gene in haemophilia B (Attree et al. 1989), the beta-globin gene in thalassaemia (Cai and Kan. 1990) and the adenomatous polyposis coli gene in familial adenomatous polyposis (Fodde et al. 1992).

1.2.2.3   CHEMICAL MISMATCH ANALYSIS

This method (Cotton et al. 1988) is based on the chemical cleavage of mismatches in wild type (radiolabelled) and mutant DNA heteroduplexes. The heteroduplexes are incubated with osmium tetroxide, which recognises mismatches involving cytosine or thymine, or with hydroxylamine, which recognises mismatches involving cytosine. After modification, the samples are incubated with piperidine which cleaves the DNA at the modified base. The samples are separated by denaturing PAGE and are visualised by autoradiography.

The separate analysis of each strand potentially enables all mismatches to be identified and this technique has been used, for example, to analyse the ornithine carbamoyl transferase gene (Grompe et al. 1989) and the beta-hexoseaminidase (Hex A) gene (Akli et al. 1991) in Tay-Sachs disease

1.2.2.4   CYTOSINE-GUANINE DINUCLEOTIDE SITE ANALYSIS

Mutations at 5'CpG 3' dinucleotide sites have been reported to make up some 40% of point mutations in a range of disorders (Koeberl et al. 1990, Cooper and Krawczak. 1990). These include the factor VIII gene in haemophilia A (Youssoufian et al. 1988), the phenylalanine hydroxylase gene in phenylketonuria (Abadie et al. 1989) and the Hex A gene (Paw et al. 1991).

The high mutation rate is explained by the spontaneous deamination of cytosine in nucleic acid to form uracil or, in the case of 5-methylcytosine, thymine (Coulondre et al. 1978, Wang et al. 1982). Uracil is foreign to DNA and is readily excised by uracil glycosylase (Lindahl et al. 1982) and replaced by cytosine but thymine, as a normal base, is not removed. The repair system detects only a guanine-thymine mismatch, which in approximately half of the cases is corrected to adenine-thymine, resulting in the generation of an irreversible point mutation.

As the DNA sequence is symmetrical, deamination can occur in either strand, and results

in a cytosine (C) to thymine (T) or a guanine (G) to adenine (A) change depending on whether the mutation originated in the sense or antisense strand respectively. Mutations at these sites typically show a high level of independent recurrence.

5-Methylcytosine (5-mC) is the most common DNA modification in eukaryotic genomes and is believed to be involved in processes such as genomic imprinting and X-inactivation. In vertebrate genomes some 90% of 5-mC occurs within the dinucleotide 5' CpG 3' (Grippo et al. 1968) and consequently these sites represent an abundant class of potential mutation hotspots in genes which are methylated in the germ line .

A proportion of 5'CpG 3' dinucleotides fall within the recognition site of a restriction enzyme, for example: *Taq*I (TCGA), *Msp*I (CCGG) or *Cfo*I (GCGC). Mutations at these sites alter the recognition sites of the enzymes, abolishing digestion, and can readily be detected by the digestion and analysis of genomic DNA or PCR product by Southern blotting or on high percentage agarose gels respectively.

1.2.2.5   **ADDITIONAL TECHNIQUES**

Additional techniques of mutation screening include heteroduplex gel analysis (White et al. 1992), RNase A cleavage (Myers et al. 1985b, 1988) and exon scanning (Kaufman et al. 1990).

Heteroduplex gel analysis is based on the fact that on nondenaturing PAGE, heteroduplexes of wild type and mutant DNA migrate at a slower rate than their corresponding homoduplexes. In this method genomic or cDNA is amplified and simultaneously labelled by the PCR, the products are denatured, allowed to anneal with wild type DNA and are analysed by PAGE.

RNase A cleavage involves the digestion of mismatches in a duplex formed between probe RNA and target DNA, followed by analysis of the resulting digestion products by denaturing PAGE, where the presence of a cleaved fragment indicates a mutation.

Fragments of 100-1000bp can be analysed.

A similar technique, exon scanning, involves the formation of heteroduplexes between an RNA probe and the exons of the target gene. The introns, which constitute most of the gene, loop out as single stranded DNA. The heteroduplex is digested with RNase A and analysed by denaturing PAGE.

1.2.2.6   **DIRECT SEQUENCING OF PCR PRODUCT**

Direct sequencing of PCR product requires a pure, single stranded DNA template. This can be generated by a variety of techniques including asymmetric PCR, M13 cloning and, most recently, magnetic bead technology (Fig. 3).

This technique results in the production of pure, single stranded DNA template starting from impure, double stranded PCR product. It is based on the use of magnetic beads (Dyna M-280 Streptavidin beads, Dynal), small, superparamagnetic, polystyrene particles which are coated with streptavidin and can be isolated from solution by virtue of their magnetic properties.

A PCR reaction is carried out using two primers, one of which has a biotin group attached to its 5'terminus. The proteins avidin and biotin have a very high affinity ($K_D=10^{-15}$M) for each other and, on mixing, the biotinylated PCR product binds strongly to the avidin coated Dyna-beads.

The Dyna-beads and the bound PCR product are isolated from the other PCR reagents by placing the reaction tube in a magnetic particle concentrator. The beads are attracted to the magnet within the particle concentrator and they adhere to the side of the tube closest to the magnetic force. This enables the supernatant containing the unincorporated nucleotides, unbiotinylated primer, genomic DNA and other PCR reagents to be pipetted off.

B →

PCR using a 5'
biotinylated (B) primer

B

Mix with avidin (A)
coated magnetic beads

A\C/A
A

The biotinylated PCR
product binds strongly to
the avidin coated beads

AB=
AB=

Pipette off
supernatant
containing
PCR reagents

Place in a magnetic
particle concentrator

Beads and bound double
stranded PCR product
isolated and resuspended

AB =
AB=
Magnet

Pipette off
supernatant
containing
unbiotinylated
strand

Denature with NaOH
and replace in
particle concentrator

Single stranded PCR
product isolated, washed
and resuspended

AB —   —
AB—   —

Single stranded PCR product

SEQUENCE

FIGURE 3: Preparation of single stranded DNA template for sequencing by
magnetic (Dyna) bead technology

Once the magnet is removed, the beads are released and can be resuspended. Single stranded DNA is produced by denaturation of the double stranded PCR product using sodium hydroxide solution. The supernatant containing the unbiotinylated strand is pipetted off after placing the reaction tube in the magnetic particle concentrator. The single stranded product is neutralised, washed and resuspended in a small volume before sequencing directly. The unbiotinylated strand may be sequenced after neutralisation and precipitation.

1.2.3   IDENTIFICATION OF SPECIFIC MUTATIONS

Once the causative mutation has been identified, carrier detection and prenatal diagnosis are provided using the identifying technique or by sequencing. Alternatively, specific base changes can be detected using a technique such as allele specific oligonucleotide (ASO) analysis, amplification created restriction site (ACRS) analysis or the amplification refractory mutation system (ARMS).

1.2.3.1   ALLELE SPECIFIC OLIGONUCLEOTIDE ANALYSIS

Point mutations can be detected using short complementary oligonucleotides (19-21bp) in a technique known as allele specific oligonucleotide (ASO) analysis. Oligonucleotides long enough to hybridise to a specific sequence, but short enough to allow a mismatch to affect hybridisation, are used. The DNA to be analysed is amplified by the PCR, dot blotted in duplicate onto nylon membranes, and hybridised with either radiolabelled mutant or wild type oligonucleotide probe.

The probe binds to the target DNA with a characteristic, sequence dependent Tm. This is reduced in the case of a mismatch and thus, by washing at a temperature equal to the wild type Tm, a mismatched probe can be selectively removed. On analysis with mutant and normal probes, only those samples with mutant and wild type sequence respectively, will show hybridisation. Heterozygotes will show hybridisation with both probes.

This technique has been widely used in mutation screening, for example, in cystic fibrosis (Serre et al. 1991) and phenylketonuria (Tyfield et al. 1991).

1.2.3.2   AMPLIFICATION CREATED RESTRICTION SITE ANALYSIS

The ACRS technique is based on the principle that mismatches at positions other than the terminal 3' base of a primer do not significantly reduce the efficiency of PCR amplification. Thus, the modification of a primer sequence in the vicinity of an otherwise undetectable sequence change can result in the creation of a restriction site in the final PCR product in the presence (absence) of this change. After amplification the product is digested and analysed by agarose or polyacrylamide gel electrophoresis. Individuals with the wild type or mutant allele can be identified by the presence or absence of digestion.

This technique has been used, for example, to detect an exon 2 base change in the ornithine carbamoyl transferase gene, by the creation of a DraI site (Petty et al. 1991).

1.2.3.3   AMPLIFICATION REFRACTORY MUTATION SYSTEM

The ARMS technique (Newton et al. 1989) is based on the principle that the introduction of a 3'-mismatched base into an oligonucleotide primer will reduce the efficiency of this primer in the PCR reaction. Two same sense primers are used: one refractory to PCR on the mutant DNA, the other refractory to PCR on wild type DNA, and a common antisense primer. For each DNA sample analysed, 2 reactions are carried out, one each with either the mutant or the wild type sense primer.

An individual homozygous for the mutant or the wild type allele shows amplification with only the mutant or wild type primer respectively, while a heterozygote shows amplification with both primers. A set of unrelated control primers is included in each reaction to ensure that the failure of amplification is not due to experimental conditions. This technique has been applied in a range of disorders including cystic fibrosis (Newton et al. 1991).

1.3.0   ORNITHINE CARBAMOYL TRANSFERASE
DEFICIENCY

1.3.1   CLASSIFICATION AND CLINICAL FEATURES

Ornithine carbamoyl transferase deficiency (EC 2.1.3.3, McKusick No.311250) is the most common of the inherited disorders of the urea cycle. It arises from the reduced activity or absence of the mitochondrial matrix enzyme ornithine carbamoyl transferase which catalyses the second step of the cycle, the condensation of ornithine and carbamoyl phosphate to form citrulline (Brusilow and Horwich. 1989).

The urea cycle (Fig. 4) consists of a series of five biochemical reactions which prevent the build up of toxic nitrogenous compounds by removing surplus nitrogen as urea and which form part of the pathway for the de-novo synthesis of arginine. No alternative pathway for urea synthesis exists and thus a block at any stage results in severe clinical manifestations and, if complete, death from ammonia intoxication. Each reaction of the cycle is characterised by a specific disorder arising from a defect in the enzyme catalysing that stage. The five disorders are deficiencies of carbamoyl phosphate synthetase, arginase, argininosuccinate synthetase and argininosuccinase which are all inherited in an autosomal recessive manner and also the X-linked recessive ornithine carbamoyl transferase (OCT) deficiency (Brusilow and Horwich. 1989).

OCT deficiency is classified into classical and late onset forms depending on the age of presentation. In addition, a proportion of heterozygous females is symptomatic.

1.3.1.1   CLASSICAL ORNITHINE CARBAMOYL TRANSFERASE
DEFICIENCY

Males with classical OCT deficiency present in the neonatal period with vomiting, lethargy, convulsions and hyperammonaemia, after a very brief, initial period of good

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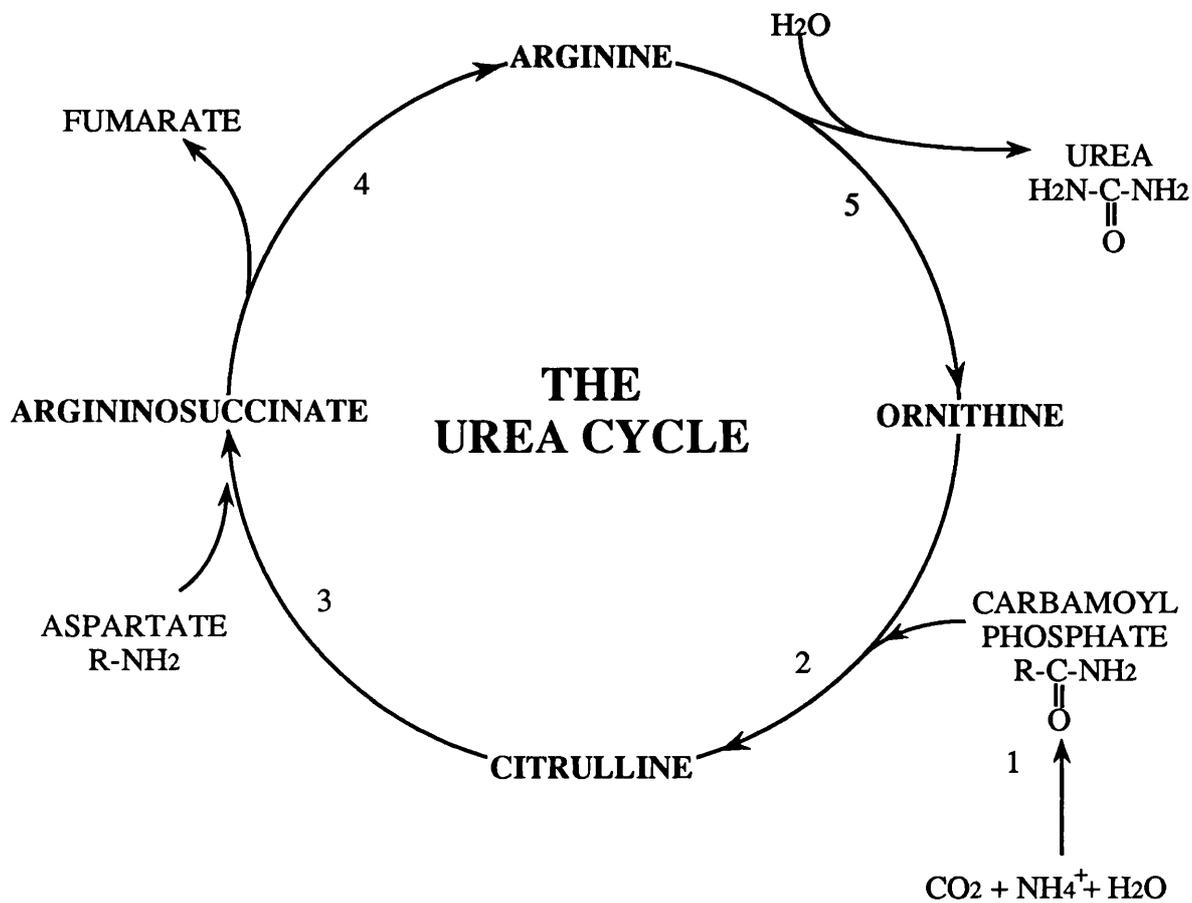
Figure 4: The urea cycle of terrestrial vertebrates.

Reactions 1 – 5 are catalysed by the enzymes: carbamoyl phosphate synthetase, ornithine carbamoyl transferase, argininosuccinate synthetase, argininosuccinase and arginase respectively.

health and, unless treated intensively, die in the first few months of life during hyperammonaemic coma (Brusilow and Horwich. 1989, Matsuda et al. 1991). The small proportion of males which survives shows cumulative neurological impairment with each hyperammonaemic episode (Matsuda et al. 1991).

Residual OCT enzyme activities in this group have been reported to range from 0-9%, with a mean of 3.7% (Matsuda et al. 1991).

1.3.1.2   <u>LATE ONSET ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY</u>

Although the majority of cases presents in the neonatal period, a proportion, some 10% (Finkelstein et al. 1990a), does so in infancy, during childhood or in adult life, with a wide range of phenotypes (Drogari and Leonard. 1988, Finkelstein et al. 1990a). These include vomiting, seizures, lethargy, irritability, coma, failure to thrive, developmental delay, episodic encephalopathy, visual disturbances, confusion, nausea and behavioral problems.

Drogari and Leonard (1988) identified 28 cases of late onset deficiency from the literature and reported a further 6 cases. Since then a further 58 late onset cases have been identified (Maddalena et al. 1988a, Largilliere and Farriaux. 1988, Finkelstein et al. 1990a, 1990b, Mizoguchi et al. 1990, Legius et al. 1990, Matsuda et al. 1991, Tuchman et al. 1991, Feldmann et al. 1992).

In addition, asymptomatic males, as diagnosed by pedigree or direct mutation analysis, have been reported by Finkelstein et al. (1990a) in a large family with 7 affected and 3 asymptomatic males and in 2 families with an Arg(245)Trp change by Hata et al. (1991). In the former case, the disorder was transmitted via the carrier daughters of the asymptomatic males to affected grandsons and carrier granddaughters. Significant residual enzyme activities of 26, 19 and 16% were detected in these families.

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The factors precipitating hyperammonaemia include infection (Maddalena et al. 1988a) and an excessive protein intake (Hata et al. 1991, Legius et al. 1990) but are unpredictable and, in many cases, a precipitating factor cannot be identified.

1.3.1.3 SYMPTOMATIC AND ASYMPTOMATIC CARRIER FEMALES

Heterozygous females show very variable manifestations. These range from virtually asymptomatic with possible protein avoidance to, in 15-20% of cases, severe protein intolerance, vomiting, lethargy, extreme irritability, ataxia, developmental delay, delayed physical growth, seizures, recurrent hyperammonaemia and, in a proportion of cases, death during hyperammonaemic coma (Batshaw et al. 1986, Rowe et al. 1986, Bonaiti-Pellie et al. 1990). Enzyme activities of 2-55% have been reported in symptomatic females (Rowe et al. 1986).

The wide range of symptomatic variation and enzyme activity is explained by the randomness of X-inactivation. This has been demonstrated by the presence of patches of activity on *in situ* hybridisation of liver tissue from a female heterozygous for OCT deficiency (Ricciuti et al. 1976) and in the liver of the sparse fur^{ash} mouse (Mrozek et al. 1991). The proportion of active wild type to mutant allele is predicted to be a major factor in the determination of disease severity. In the majority of carriers, the proportions will be roughly equal and manifestations are expected to be limited and detectable only under severe metabolic stress but, in a small number, the mutant allele will be predominantly active resulting in severe OCT deficiency.

1.3.1.4 THERAPY

Treatment of OCT deficiency (Drogari and Leonard. 1988, Brusilow and Horwich. 1989) is limited and is based on the restriction of dietary protein intake, supplements of arginine and citrulline and the activation of alternative pathways of nitrogen elimination using sodium benzoate and sodium phenylacetate. These are conjugated with glycine and glutamine in the liver to form hippurate and phenylacetylglutamine which are rapidly

excreted in urine thereby removing excess nitrogen.

In a limited number of cases (reviewed by Todo et al. 1992) liver transplants have been carried out with a success rate of about 60%.

1.3.2   <u>THE ORNITHINE CARBAMOYL TRANSFERASE GENE</u>

The OCT gene has been mapped using somatic cell hybrids and *in situ* hybridisation to the short arm of the X-chromosome, at Xp21.1 (Lindgren. 1984). Human OCT cDNA and genomic clones have been isolated, cloned and sequenced and the structure of the gene has been determined (Horwich et al. 1984, Hata et al. 1986, 1988a, 1988b). The gene is expressed in the hepatic cells of the liver and, to a lesser extent, in the jejunal mucosa, from nuclear DNA.

The 73kb gene is divided into 10 exons (Fig. 5) separated by 9 introns of variable length, which range from 80bp to 21.7kb. The gene is translated to give a mRNA of approximately 1700bp, some 2% of the total gene. The mRNA is translated to give a 354 amino acid protein which is imported into the mitochondrial matrix via a 32 amino acid N-terminal leader sequence, encoded by the first and part of the second exon. After import, the leader sequence is proteolytically removed and the 322 amino acid chains are assembled to form the homotrimeric active enzyme (Horwich et al. 1984).

The gene contains several sequence motifs which are conserved across species, being present in yeast, *E.coli* and human OCT enzyme, as well as in the aspartate transcarbamylases. This suggests conservation through evolution and hence a role in enzyme function.

A comparison of the sequences of human, *E.coli* and yeast OCT cDNA (Horwich et al. 1984) revealed a region of near identity in exon 3, corresponding to amino acids 50-63 of the mature protein. This region contains the highly conserved sequence motif Ser-Thr-Arg-Thr-Arg and represents the putative binding site for carbamoyl phosphate. A

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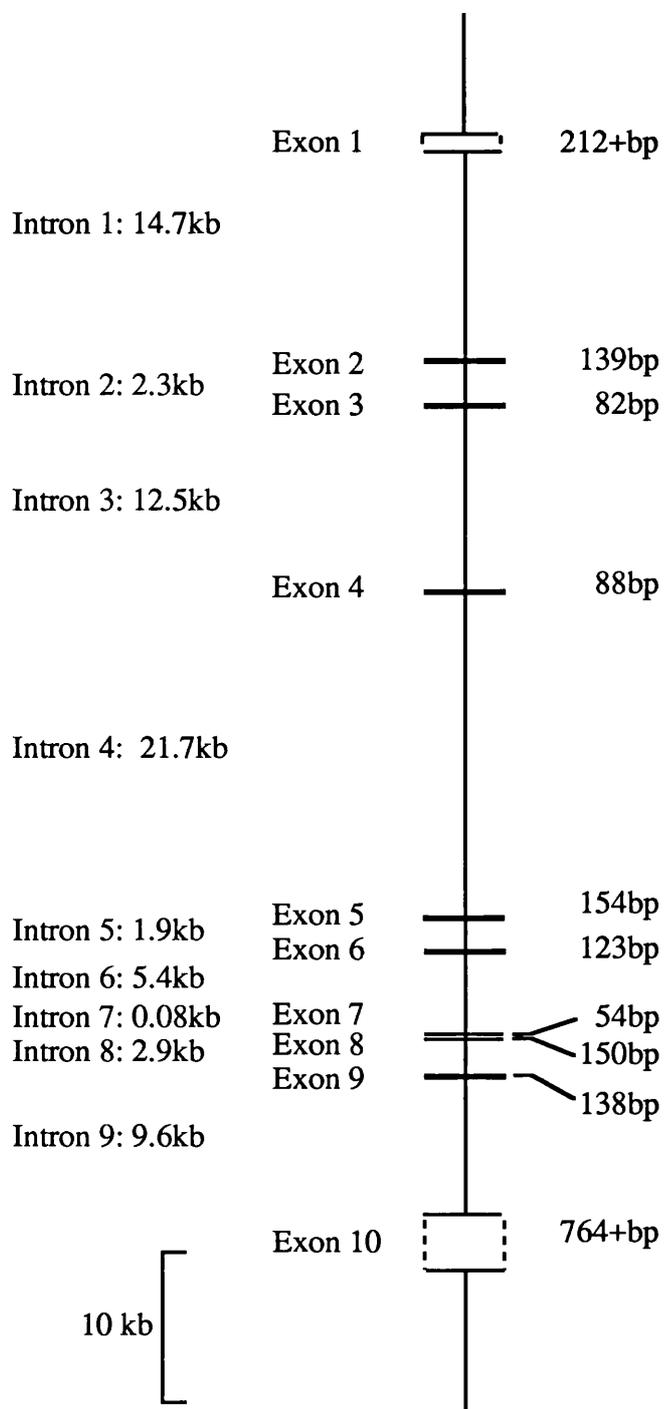
FIGURE 5: The structural organisation of the 73 kb ornithine carbamoyl transferase gene. The structure of the gene is represented schematically by a line, with the exons as bars. Solid bars represent coding sequences, and open bars represent 5' and 3' untranslated sequences. (Figure based on the data of Hata et al.1988.)

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comparison of the sequences of OCT and E.coli aspartate transcarbamylase (Horwich et al. 1984) identified, in addition to residues 50-63, a region of near identity at amino acids 268-273 in exon 9. This region contains the sequence motif Phe-Leu-His-Cys-Leu-Pro and represents the putative binding site for ornithine (Kraus et al. 1985). Regions of homology were also identified at residues 96-103 (exons 4-5) and 141-154 (exons 5-6), though these are of unknown aetiology.

1.3.3 THE MUTATION RATE AT THE ORNITHINE CARBAMOYL TRANSFERASE LOCUS

From the calculations of Haldane (reviewed by Weatherall. 1991), 1/3 of affected males are expected to have a new mutation, 2/3 to have inherited a mutant allele from a carrier mother, assuming that the mutation rate in both sexes is equal.

A study by Hauser et al. (1990) found that on biochemical analysis, 73% and 33% of the mothers of affected males and females respectively, were carriers. On the assumption that the test was 100% accurate, they concluded that 27% and 67% of males and females respectively had new mutations. Conversely, Bonaiti-Pellie et al. (1990) estimated that whilst 57% of affected females had a new mutation, virtually no mutations occurred in oogenesis. They quoted the upper limit of the confidence interval (0-16%) from their analysis, as the maximum probability that an affected male had a new mutation.

1.3.4 MUTATIONS AT THE ORNITHINE CARBAMOYL TRANSFERASE LOCUS

Ornithine carbamoyl transferase deficiency is caused by mutations at the OCT locus. As predicted for an X-linked, lethal disorder, the mutation in each family arises independently and, with the exception of CpG dinucleotide changes, is unique. To date four main types of mutation have been reported to result in OCT deficiency: large deletions, point mutations associated with CpG dinucleotides, splicing mutations and lastly other small changes or point mutations.

1.3.4.1   TOTAL OR PARTIAL GENE DELETIONS

Total or partial gene deletions have been detected in approximately 10-20% of cases by Southern blot analysis, failure of PCR amplification or the nontransmission of a maternal allele on linkage analysis.

Grompe et al. (1991) and Suess et al. (1992) each reported 1 partial and 2 total gene deletions in 3 of 18 (17%) and 3 of 13 (23%) families analysed respectively. In the latter case the partial deletion encompassed exons 7 and 8, in the former case exon 9. Liechti-Gallati et al. (1991) reported 3 deletions in 22 (14%) families, while Rozen et al. (1985) found one partial deletion in 15 families (7%) associated with the loss of the 17.5kb *Msp*I band on Southern blot analysis. This corresponded to exon 10 and the 3' untranslated region.

Two males with a late onset deficiency have also been shown to exhibit a somatic mosaicism for partial gene deletions of exons 7-9 (Legius et al. 1990) and exons 5-8 (Maddalena et al. 1988a) respectively.

1.3.4.2   CHANGES AT CYTOSINE-GUANINE DINUCLEOTIDE SITES

A major class of point mutations is those occurring at the mutation prone 5'CpG 3' dinucleotide sites. The OCT gene coding region contains 18 5'CpG 3' dinucleotides which represent 36 potential mutations and of these, 26 result in amino acid changes - 24 of which are nonconservative. Mutations at these positions recur independently and have been reported in 19 cases, at 8 sites (Table 1).

A proportion of these dinucleotides occur within the restriction sites for *Taq*I, *Msp*I or *Cfo*I enzymes. The OCT gene coding region contains 4 *Taq*I sites in exons 1, 3, 5 and 9. Changes at the 5'CpG 3' dinucleotides within the sites in exons 3, 5 and 9 are detected on Southern blot analysis as an alteration of the expected band pattern (Fig. 6). Mutations at the *Taq*I site in exon 3 are identified by the presence of a new 3.5kb band. Mutations

TABLE 1 - A survey of cytosine-guanine dinucleotide sites in the ornithine carbamoyl transferase gene and mutations reported therein.

EXON	CODON	AMINO ACID	POSSIBLE AMINO ACID CHANGES	MUTATIONS REPORTED	PHENOTYPE	RESTRICTION SITE ALTERATION
1	23	-9	Arg (CGA) -> Ter (TGA) Gln (CAA)	C -> T Grompe et al. 1991	Classical	TaqI
1	26	-6	Arg (CGG) -> Trp (TGG) Gln (CAG)	G -> A Grompe et al. 1989	Classical	
				G -> T polymorphism; Trp present in normal mouse gene. Veres et al. 1987	Wild type	
2	40	8	Arg (CGT) -> His (CAT) Cys (TGT)			HaeIII for C
2	49/50	17/18	Thr/Gly (ACC GGA) -> Arg (AGA)	G -> T, Gly (GGA) -> Ter (TGA) Feldmann et al. 1992	Symptomatic female	MspI
3	92	60	Arg (CGA) -> Ter (TGA) Gln (CAA)	G -> A Grompe et al. 1991 C -> T Liechti-Gallati et al. 1991 C -> T Grompe et al. 1991	Classical Classical Classical	TaqI
4	125	93	Thr (ACG) -> Met (ATG)			
4	127	95	Thr (ACG) -> Met (ATG)			
4	129	97	Arg (CGT) -> His (CAT) Cys (TGT)	G -> T Arg (CGT) -> Leu (CTT) Strautnieks et al. This report	Late onset	MspI

EXON	CODON	AMINO ACID	POSSIBLE AMINO ACID CHANGES	MUTATIONS REPORTED	PHENOTYPE	RESTRICTION SITE ALTERATION
5	141	109	Arg (CGA) -> Ter (TGA) Gln (CAA)	C -> T Maddalena et al. 1988b C -> T Hata et al. 1989 C -> T Grompe et al. 1991 G -> A Suess et al. 1992 G -> A x 2, Maddalena et al. 1988b C -> T x 2 Matsuda et al. 1991 G -> A Strautnieks et al. This report	Symptomatic female Symptomatic female Classical Classical Classical Classical Symptomatic female	*Taq*I
5	178	146	Thr (ACG) -> Met (ATG)			
6	194/5	162/3	Ile/Gly (ATC GGG) -> Arg (AGG)			
6	207/8	175/6	Ser/Ala (AGC GCA) -> Thr (ACA)	G -> A Strautnieks et al. This report	Late onset	*Cfo*I
6	209	177	Ala (GCG) -> Val (GTG)			
6	211/212	179/180	Phe/Gly (TTC GGA) -> Arg (AGA)			
7	225	193	Pro (CCG) -> Leu (CTG)	C - >T Hentzen et al. 1991 C - >T Strautnieks et al. This report	Classical Classical	*Msp*I

EXON	CODON	AMINO ACID	POSSIBLE AMINO ACID CHANGES	MUTATIONS REPORTED	PHENOTYPE	RESTRICTION SITE ALTERATION
8	254	222	Ala (G<u>C</u>G) -> Val (GTG)			*Cfo*I
8	277	245	Arg (<u>C</u>GG) -> Gln (CAG) Trp (TGG)	C -> T x 2 Hata et al. 1991 C -> T Finkelstein et al. 1990b C -> T Strautnieks et al. This report	Late onset Late onset Late onset	*Fnu*4HI for C
9	320	288	Arg (<u>C</u>GA) -> Ter (TGA) Gln (CAA)	G -> T Arg (CGA) -> Leu (CTA) Grompe et al. 1991/Spence et al. 1989	Classical	*Taq*I

BAND (kb)   NORMAL   EXON 3   EXON 5   EXON 9   CORRESPONDING EXON

BAND (kb)	NORMAL	EXON 3	EXON 5	EXON 9	CORRESPONDING EXON
4.6 —	▬	▬	▬	▬	— 2/4
4.5 —				▬ *	
3.7 —	▬	▬	▬	▬	— 10
3.6 —	– – –	– – –	– – –	– – –	— 3
3.5 —		▬ *			
3.4 —			▬ *		
2.6 —	▬	▬	▬	*	— 9
2.0 —	– – –	– – –	– – –	– – –	— 1 + 9
1.8 —	▬	▬	*	▬	— 5
1.7 —	– – –	– – –	*	– – –	— 5
1.5 —	▬	▬	▬	▬	— 6/7/8
0.9 —	▬	▬	▬	▬	— 1
0.8 —	▬	▬	▬	▬	— 2/4
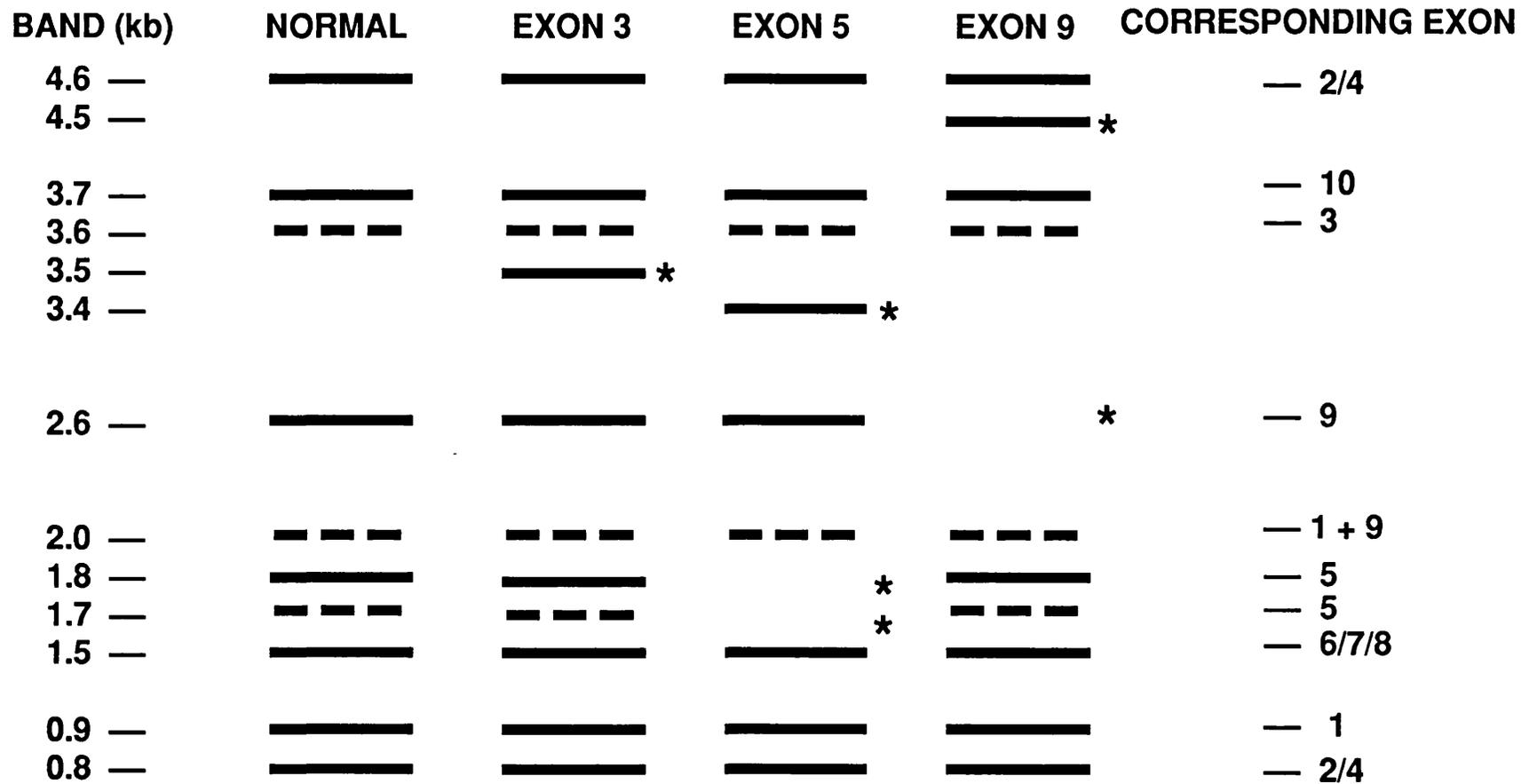
FIGURE 6: The characteristic band patterns obtained on Southern blot analysis of DNA containing mutations within the *Taq*I sites of exons 3, 5 and 9. Abnormal or absent bands are marked by an asterisk. Faint bands are shown by a dashed line. The exons corresponding to each band, as mapped by Grompe et al. (1991), are indicated on the right of the diagram. The bands representing exons 2, 4 and 6–8 could only be assigned to either the 3' or 5' half of the gene and thus the bands marked 2/4 and 6/7/8 may represent either exon 2 or 4 and 6, 7 or 8 respectively.

at the exon 5 *Taq*I site are identified by the presence of a new 3.4kb band, together with the loss of 2 bands of 1.7 and 1.8kb. The loss of the exon 9 *Taq*I site is characterised by the replacement of the constant 2.6kb band with a novel 4.5kb band.

The loss of the exon 1 *Taq*I site results in the creation of a new 2.6kb band and the loss of 1.9 and 0.7kb bands. But, as this 2.6kb band cosegregates with the constant 2.6kb band and the lower bands are faint and readily lost from the gel during electrophoresis, a mutation at this site cannot be detected by Southern blot analysis. Exon 1 changes are detected by *Taq*I digestion of the exon 1 PCR product, followed by analysis on a high percentage agarose gel.

The OCT gene contains three *Msp*I sites, in exons 2, 4 and 7, and 2 *Cfo*I sites in exons 6 and 8. These can similarly be analysed by a combination of Southern blot analysis and digestion of PCR products. In addition, certain 5'CpG 3' sites can be partially analysed by digestion with other enzymes, in cases where the enzyme recognition site partially encompasses a 5'CpG 3' site. Thus, in the 5' untranslated region immediately adjacent to the start codon, the C of the CpG site can be analysed using *Hinf*I.

1.3.4.3   SPLICE SITE AND CODING SEQUENCE CHANGES

The second class of point mutations in OCT deficiency are splice site changes (Table 2) which have been identified in 5 cases and appear to represent, as suggested by Carstens et al. (1991), a common mechanism in the pathogenesis of this disorder. The remaining changes reported in OCT deficiency are predominantly random coding changes (Table 3).

1.3.4.4   MUTATIONS RESULTING IN LATE ONSET ORNITHINE
CARBAMOYL TRANSFERASE DEFICIENCY

Of the molecular defects identified, 6 are associated with a late onset phenotype. These are in exon 3: the amino acid changes Arg(62)Thr and Gly(47)Glu (Tuchman et al. 1992,

TABLE 2 -   Splice site changes reported in ornithine carbamoyl transferase deficiency

LOCATION	SPLICE SITE	POSITION	CHANGE	EFFECT	REFERENCE	PHENO-TYPE
Intron 1	5' donor	-1	G -> A	Reduced levels of mRNA	Grompe et al. 1989	Classical
Intron 2	3' acceptor	-1	G -> A	Not investigated	Tuchman et al. 1992	Classical
Intron 4	3' acceptor	-2	A -> T	Utilization of a cryptic site in exon 5, 12bp deletion of exon 5	Carstens et al. 1991	Classical
Intron 4	5' donor	-1	G -> A	2 mRNA products at 5% of normal levels, one with a missense mutation, the other elongated due to the activation of a cryptic splice site in intron 4	Hodges & Rosenberg. 1986	Sparse fur[ash] mouse mutant
Intron 4	5' donor	-1	G -> T	Not investigated	Strautnieks et al. This report	Late onset
Intron 7	5' donor	+2	T -> C	Skipping of exon 7	Carstens et al. 1991	Classical
Intron 7	5' donor	+3	A -> G	Skipping of exon 7	Carstens et al. 1991	Classical

TABLE 3 - Point mutations reported in ornithine carbamoyl transferase deficiency, excluding cytosine-guanine dinucleotide site changes and splice site changes

EXON	CODON	AMINO ACID	CHANGE	EFFECT	REFERENCE	PHENOTYPE
2	45	13	Leu (CTA) -> Pro (CCA)	Missense mutation	Grompe et al. 1989	Classical
3	87	55	Glu (GAG) -> Lys (AAG)	Missense mutation	Feldmann et al. 1992	Late onset
3	79	47	Gly (GGG) -> Glu (GAG)	Missense mutation	Tuchman et al. 1992	Classical
3	94	62	Arg (AGA) -> Thr (ACA)	Missense mutation	Tuchman et al. 1992	Late onset
5	135	103	Deletion of G at Nu 403	Frameshift mutation	Tuchman et al. 1992	Classical
5	154	122	Glu (GAA) -> Ter (TAA)	Premature termination	Grompe et al. 1989	Classical
5	162	130	Gly (GGG) -> Arg (AGG)	Missense mutation	Feldmann et al. 1992	Classical
6	216	184	Gln (CAG) -> Glu (GAG)	Missense mutation	Grompe et al. 1989	Classical
9	304	272	Leu (TTG) -> Phe (TTT)	Missense mutation	Tuchman et al. 1992	Late onset
10	345	313	Tyr (TAC) -> Asp (GAC)	Missense mutation	Tuchman et al. 1992	Symptomatic female

Feldmann et al. 1992), the former in the putative exon 3 carbamyl phosphate active site. The remainder are in exon 9, a Leu(272)Phe change at the ornithine binding site (Tuchman et al. 1992), and in exon 8, an independently recurring Arg(245)Trp change (Finkelstein et al. 1990b, Hata et al. 1991).

In addition, somatic mosaicism for intragenic deletions of exons 7-9 and 5-8 has been reported in 2 cases (Legius et al. 1990, Maddalena et al. 1988a).

1.3.5   **DIAGNOSIS, CARRIER DETECTION AND PRENATAL DIAGNOSIS**

Biochemically OCT deficiency is characterised by low plasma levels of citrulline, arginine and urea, increased levels of carbamoyl phosphate and glutamine, and orotic aciduria during hyperammonaemic episodes. Determination of this profile enables the elimination of other urea cycle defects and suggests OCT deficiency.

Early diagnosis of affected individuals is essential to ensure survival, optimum clinical management, and development. Diagnosis is based on the clinical and biochemical profiles, but can be complicated by the often reduced severity of the disorder. At its extreme (Hata et al. 1991, Finkelstein et al. 1990a), asymptomatic males have been reported, and diagnosis in such cases may require confirmation through invasive liver biopsy and enzyme analysis. Identification of such males is especially important as any daughters will be obligate carriers for a potentially lethal disorder.

Carrier detection and prenatal diagnosis are routinely provided through a combination of family history and restriction fragment length polymorphism (RFLP) and biochemical analysis.

In a family showing X-linked inheritance of OCT deficiency, obligate carriers are identified as women having, for example, an affected sib and child, an affected child and

TABLE 4 - Intragenic polymorphisms at the ornithine carbamoyl transferase locus

RESTRICTION ENZYME	ALLELES (kb)	METHOD OF DETECTION	INFORMATIVITY (%)	REFERENCE
MspI	6.2/6.6	Southern blot	\ 69%	Rozen et al. 1985 Fox et al. 1986
MspI	4.4/5.1	Southern blot	/	
BamHI	5.2/18	Southern blot	29%	"
TaqI	3.6/3.7	Southern blot	11%	"
DraI	0.100 + 0.016/0.116	Analysis of exon 2 PCR product	44%	Petty et al. 1991

grandchild, or an affected child and nephew or niece. The presence of more than one affected child in a sibship suggests carrier status, but is not absolute, as the possibility of germline mosaicism remains (Hall. 1988).

Five intragenic polymorphisms have been reported in the OCT gene. These are detected by Southern blot analysis using OCT cDNA, or by the digestion and analysis of PCR product (Table 4). Restriction enzyme digestion identifies, in addition, gross deletions, rearrangements and changes at 5'CpG 3' dinucleotides within *Taq*I and *Msp*I sites which are estimated to be the causative change in approximately 30-40% (1.3.4.2) of OCT deficiency cases.

Two main biochemical tests are used to the determine the carrier status of asymptomatic females; the alanine protein loading test (Hokanson et al. 1978) and the allopurinol test (Hauser et al. 1990). These have been reported to detect some 92-96% of obligate carrier women (Pelet at al. 1990, Becroft et al. 1984, Hauser et al. 1990), with no false positives reported. Thus a positive test result strongly suggests carrier status, but a negative test, while significantly reducing the risk, does not eliminate it.

Prenatal diagnosis is carried out by RFLP analysis in cases where the mother is informative for an RFLP and, ideally, her carrier status has been confirmed by family history or biochemical testing. In situations where this is not possible, fetal sexing of a CVS and selective termination of all male fetuses has been used to prevent the birth of affected males with the disorder.

1.4.0   PELIZAEUS-MERZBACHER DISEASE

Pelizaeus-Merzbacher disease (PMD) is a rare dysmyelination disorder characterised by a lack of myelin and a loss of myelin producing cells in the central nervous system (CNS). The peripheral nervous system (PNS) is unaffected.

1.4.1   **MYELIN**

Myelin is a highly specialised membrane specific to the nervous system which forms the insulatory covering, or sheath, of nerve fibres. The sheath is derived from the plasma membrane of glial cells called oligodendrocytes in the CNS and Schwann cells in the PNS. Flattened cytoplasmic projections from these cells wrap each axon in a spiral fashion creating regions of compact multilammelar sheath, interrupted periodically, by unmyelinated nodes of Ranvier (Morel. 1984).

This insulatory covering increases the rate at which nerve impulses are conducted by preventing current flow across the majority of the nerve fibre. This enables action potentials to leap from node to node in a process known as saltatory conduction and, as the local current flow is limited, voltage gradients are less readily dispersed and subsequent action potentials are generated more rapidly than in unmyelinated fibres.

The myelin membrane is composed predominantly of lipids (approximately 75%) arranged in a bimolecular sheet and contains 2 main classes of proteins. The first class consists of the highly hydrophilic myelin basic proteins (MBPs) which adhere to the surfaces of the membrane. The second class is made up of highly hydrophobic, integral membrane proteins. These are represented by the proteolipid proteins (PLPs): PLP and DM-20 in the CNS, and Po glycoprotein and peripheral myelin protein-22 (PMP-22) in the PNS.

Other components include carbohydrates, glycolipids (the most common being galactocerebroside and myelin associated glycoprotein) and an assortment of enzymes including 2'3'-cyclinucleotide 3' phosphohydrolase (Morel. 1984).

1.4.2   **CLASSIFICATION, INHERITANCE AND CLINICAL**
                              FEATURES

The disorder was first reported by Pelizaeus in 1885 and subsequently by Merzbacher in 1910 and, whilst it has been reported to be both clinically and genetically heterogeneous,

2 main forms: classical and connatal PMD are recognised (Scheffer et al. 1991). These are distinguished predominantly by the rate of clinical progression, the age of death and the degree of CNS hypomyelination.

Both forms show onset by 6 months, with symptoms being present potentially at birth, but the connatal form progresses rapidly resulting in death in the first decade. The classical form has a more protracted course, with affected individuals surviving to the second or third decade.

PMD presents with transient laryngeal stridor, nystagmus, muscular hypotonia with poor head control and developmental delay. Surviving children develop slowly progressive psychomotor retardation and spastic quadriplegia, and are subject to involuntary movements and seizures. Affected individuals have difficulty in swallowing and talking and may have no bladder or bowel control (Scheffer et al. 1991). Death is often due to infection (pneumonia) or asphyxiation and treatment of PMD is palliative only and may be withheld as no chance of improvement exists.

Both forms are inherited in an X-linked recessive manner and, in addition, an autosomal recessive connatal form has been suggested to explain the occurrence of occasional, severely affected females (Begleiter and Harris. 1989, Cassidy et al. 1987, Pratt et al. 1992). The recent identification of mutations at a common locus in both forms (Section 1.4.4.3) indicates that they may represent extremes of the same disorder. The presence of affected females in some connatal pedigrees may reflect dominant expression with certain genotypes, unfavourable lyonisation, or defects at an additional locus in these families resulting in the same clinical phenotype.

Carrier women of classical PMD are generally asymptomatic, though Raskind et al. (1991) noted mild hyperreflexia and subtle eye movement abnormalities in a carrier woman and early onset dementia in her carrier mother. They suggested that these symptoms may represent clinical expression in females.

1.4.3   THE PATHOLOGY OF PELIZAEUS-MERZBACHER DISEASE

PMD has been shown, by the absence of myelin breakdown products in affected CNS tissue, to result from a failure of myelin synthesis (dysmyelination) as opposed to the breakdown of pre-existing myelin (demyelination).

Typically, PMD is characterised by a lack of mature oligodendrocytes in the CNS, associated with severe (classical) or total (connatal) hypomyelination (Koeppen et al. 1987, Iwaki et al. 1993) and the absence of PLP and DM-20 proteins. In addition a reduction of the other myelin components specifically MBP, is noted, as well as abnormalities of myelin specific lipids and astrocytes.

Occasional cases with atypical characteristics have been reported including a case with a lack of myelin specific lipids, specifically sphingomyelin, cerebrosides and sulphatides, but normal levels of PLP (Iyoda et al.1989). Hudson et al. (1989a) have reported an X-linked case with normal levels of oligodendrocytes and a high level of preserved myelin. Both cases were diagnosed as classical PMD, though in the former the severity and symptoms were atypical, whilst no change was found on sequencing of the PMD candidate gene in the latter.

1.4.4   THE CANDIDATE GENE: PROTEOLIPID PROTEIN

The candidate gene in Pelizaeus-Merzbacher disease is proteolipid protein. It was selected for two reasons. Firstly it was the only major myelin component which mapped to the human X-chromosome, at Xq22 (Willard and Riordan 1985, Mattei et al. 1986). Secondly the dysmyelinating mouse model jimpy, which is pathologically and phenotypically similar to PMD, was shown to have a mutation at the PLP locus which resulted in aberrantly spliced mRNA transcripts (Nave et al. 1987).

1.4.4.1   THE PROTEOLIPID PROTEIN GENE AND PROTEIN

The proteolipid protein gene was mapped to the X-chromosome, at Xq13-q22, by Willard and Riordan (1985) using a bovine cDNA probe on Southern blot analysis of somatic cell hybrid DNA. It was subsequently localised to Xq21.2-q22 by Willard et al. (1987) and Yang et al. (1987), using somatic cell hybrids and deletion mapping respectively. The most specific localisation is to Xq22 by Mattei et al. (1986) using *in situ* hybridisation.

The 17kb gene encodes a 276 amino acid protein which has been cloned and sequenced (Fig. 7) (Stoffel et al. 1984, Diehl et al. 1986) and consists of 7 exons of varying size separated by 6 introns (Fig. 8), (Diehl et al. 1986, Hudson et al. 1989a).

A second isoform is produced by the alternative splicing of the primary PLP transcript. This 241 amino acid DM-20 isoform lacks an internal 35 amino acids (116-150) and is produced by the utilization of an alternative 5' donor splice site 105 nucleotides within exon 3 (Nave et al. 1987).

Both proteins are extremely hydrophobic and consist of 5 strongly hydrophobic sequences of 26, 30, 39, 12 and 36 residues respectively, which are linked by highly charged hydrophilic sequences. For exons 2-5, each hydrophobic domain together with the adjacent hydrophilic region, corresponds to an exon (Diehl et al. 1986).

Exons 6 and 7 of the gene contain the C-terminal hydrophobic domain with exon 7 containing, in addition, the C-terminal hydrophilic sequence and the 3' untranslated region. Exon 1 contains only the N-terminal methionine codon and the first base of the first codon of the mature protein. The remainder consists of 5' untranslated sequence.

Several models for PLP topology (Laursen et al. 1984, Stoffel et al. 1984, Hudson et al. 1989b, Popot et al. 1991) within the myelin membrane have been proposed. In all models, regions of the hydrophobic domains are proposed to form alpha helices within the lipid bilayer and the charged hydrophilic segments to remain external to the membrane and

I AAGAAAATGA AACAATTGGC AGTGAAAGGC AGAAAGAGAA GATGGAGCCC TTAGACAAGG GAGTATCCCT GAGTAGGTGG GGAAAAGGGG AGGAGAAGGG GAGGAGGAGA GGAGGAGGAA   -205

AGCAGGCCTG TCCCTTTAAG GGGGTTGGCT GTCAATCAGA AAGCCCTTTT CATTGCAGGA GAAGAGGACA AAGATACTCA GAGAGAAAAA GTAAAAGACC GAAGAAGGAG GCTGGAGAGA   -85
　　　　　　　　　　　　　①　　②

③　　Met G(ly)　　　　　　　　1
CCAGGATCCT TCCAGCTGAA CAAAGTCAGC CACAAAGCAG ACTAGCCAGC CGGCTACAAT TGCAGTCAGA GTCCCAAAGA C ATG G gtaagtttcaaaaactttag...   +1

II   (G)ly Leu Leu Glu Cys Cys Ala Arg Cys Leu Val Gly Ala [Pro] Phe Ala Ser Leu Val Ala Thr Gly Leu Cys Phe Phe Gly Val Ala   29
...ttcccctttcttcttccccag GC TTG TTA GAG TGC TGT GCA AGA TGT CTG GTA GGG GCC CCC TTT GCT TCC CTG GTG GCC ACT GGA TTG TGT TTC TTT GGG GTG GCA   +87

Leu Phe Cys Gly Cys Gly [His] Glu Ala Leu Thr Gly Thr Glu Lys Leu Ile Glu Thr Tyr Phe Ser Lys Asn Tyr Gln Asp Tyr Glu Tyr Leu Ile Asn Va(l)   63
CTG TTC TGT GGC TGT GGA CAT GAA GCC CTC ACT GGC ACA GAA AAG CTA ATT GAG ACC TAT TTC TCC AAA AAC TAC CAA GAC TAT GAG TAT CTC ATC AAT GT gtaa   +188
gtacctgccctcccac...

III   (Va)l Ile His Ala Phe Gln Tyr Val Ile Tyr [Gly][Thr] Ala Ser Phe Phe Phe Leu Tyr Gly Ala Leu Leu Leu Ala Glu Gly Phe Tyr   81
...ttgtctacctgttaatgcag G ATC CAT GCC TTC CAG TAT GTC ATC TAT GGA ACT GCC TCT TTC TTC TTC CTT TAT GGG GCC CTC CTG CTG GCT GAG GGC TTC TAC   +243

Thr Thr Gly Ala Val Arg Gln Ile Phe Gly Asp Tyr Lys Thr Thr Ile Cys Gly Lys Gly Leu Ser Ala Thr Val Thr Gly Gly Gln Lys Gly Arg Gly Ser Arg   116
ACC ACC GGC GCA GTC AGG CAG ATC TTT GGC GAC TAC AAG ACC ACC ATC TGC GGC AAG GGC CTG AGC GCA ACG GTA ACA GGG GGC CAG AAG GGG AGG GGT TCC AGA   +348

Gly Gln His Gln Ala His Ser Leu Glu Arg Val Cys His Cys Leu Gly Lys Trp Leu Gly His Pro Asp Lys   150
GGC CAA CAT CAA GCT CAT TCT TTG GAG CGG GTG TGT CAT TGT TTG GGA AAA TGG CTA GGA CAT CCC GAC AAG gtgatcatcctcaggatttt...   +450

IV   [Phe Val Gly Ile [Thr] Tyr Ala Leu Thr Val Val [Trp] Leu Leu Val Phe Ala Cys Ser Ala Val Pro Val Tyr Ile Tyr Phe Asn Thr   179
...acccatgtcaatcattttag TTT GTG GGC ATC ACC TAT GCC CTG ACC GTT GTG TGG CTC CTG GTG TTT GCC TGC TCT GCT GTG CCT GTG TAC ATT TAC TTC AAC ACC   +537

Trp [Thr] Thr Cys Gln Ser [Ile] Ala Phe Pro Ser Lys Thr Ser Ala [Ser Ile] Gly Ser Leu Cys Ala [Asp] Ala Arg Met Tyr G(ly)   207
TGG ACC ACC TGC CAG TCT ATT GCC TTC CCC AGC AAG ACC TCT GCC AGT ATA GGC AGT CTC TGT GCT GAT GCC AGA ATG TAT G   gtgagttagggtacgggtgc...   +619

V   [(G)ly Val Leu Pro Trp Asn Ala Phe [Pro] Gly Lys [Val] Cys [Gly] Ser Asn [Leu] Leu Ser Ile Cys Lys Thr Ala Glu   231
...gcttttgtgtcttacttag GT GTT CTC CCA TGG AAT GCT TTC CCT GGC AAG GTT TGT GGC TCC AAC CTT CTG TCC ATC TGC AAA ACA GCT GAG gtgagtgggttattt   +693
gggtt...

VI   Phe Gln Met Thr Phe His Leu Phe Ile Ala [Ala] Phe Val Gly Ala Ala Ala Thr Leu Val Ser Leu   253
...ctcttttcattttcctgcag TTC CAA ATG ACC TTC CAC CTG TTT ATT GCT GCA TTT GTG GGG GCT GCA GCT ACA CTG GTT TCC CTG gtgagttgactttgaatgat...   +759

VII   Leu Thr Phe Met Ile Ala Ala Thr Tyr Asn Phe Ala Val Leu Lys Leu Met Gly Arg Gly Thr Lys Phe Stop   276
...ctctcttctgttccctacag CTC ACC TTC ATG ATT GCT GCC ACT TAC AAC TTT GCC GTC CTT AAA CTC ATG GGC CGA GGC ACC AAG TTC TGA TCCCCCGTAG   +841

AAATCCCCCT TTCTCTAATA GCGAGGCTCT AACCACACAG CCTACAATGC TGCGTCTCCC ATCTTAACTC TTTGCCTTTG CCACCAACTG GCCCTCTTCT TACTTGATGA GTGTAACAAG...   +961

FIGURE 7: The nucleotide sequence of the proteolipid protein (PLP) gene (Diehl et al. 1986). Nucleotide 1 is the G of glycine, the first amino acid of mature PLP. Underlined coding sequences represent the hydrophobic domains of the protein. In the 5' untranslated region the proposed CAAT (1), TATA (2) and possible cap site (3) are underlined. The circles indicate amino acid residues at which mutations have been identified in PMD cases or animal models.
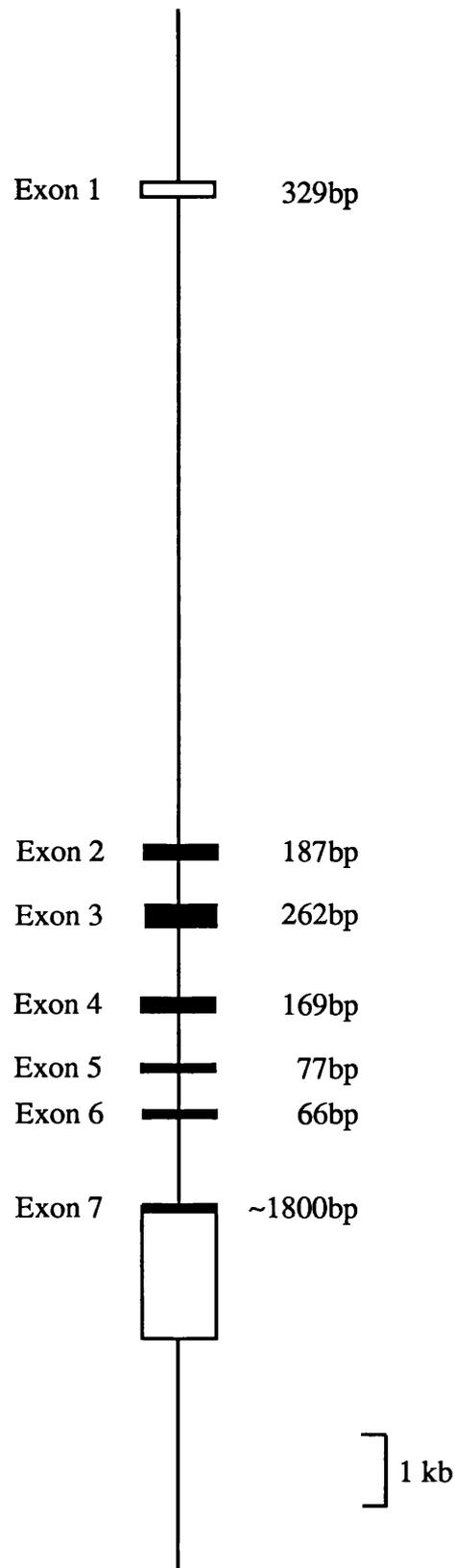
FIGURE 8: The structural organisation of the 17 kb proteolipid protein gene. The structure of the gene is represented schematically by a line, with the exons as bars. Solid bars represent coding sequences, and open bars represent 5' and 3' untranslated sequences. (Figure based on the data of Hudson et al.1989a.)

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form, in part, beta sheets.

1.4.4.2   <u>THE FUNCTIONS OF THE PROTEOLIPID PROTEINS</u>

In the CNS, the proteolipids comprise some 50% of the total myelin protein and are incorporated into the myelin sheath as integral membrane proteins involved in the formation and maintenance of the myelin sheath. Their precise function is unclear but they are likely to promote adhesion and compaction of the successive turns on the extracelluar surfaces of the myelin lamellae, possibly via the homophilic interactions between between PLP molecules of opposed extracelluar faces (Hudson et al. 1989a). In the PNS, proteolipid protein is not incorporated into myelin (Puckett et al. 1987) and PMP-22 and the glycoprotein Po are substituted.

A second role for the PLPs in glial cell differentiation has been suggested by Nadon et al. (1990). This is supported by the recent identification of a mutation at the PLP locus of the rumpshaker mouse mutant (Schneider et al. 1992) which allows the normal maturation of oligodendrocytes. This mutant is characterised by a lack of PLP, but relatively normal levels of DM-20 and MBP, suggesting that DM-20 is the critical factor required for glial cell differentiation.

This is further supported by evidence from other dysmyelinating mutants, for example the shiverer mouse, in which a deletion in the myelin basic protein gene does not interfere with oligodendrocyte differentiation (Newman et al. 1987).

Studies have shown that DM-20 is produced early in development, before the appearance of oligodendrocytes, by a range of nervous tissue cells including nonglial types (Ikenaka et al. 1992). As development progresses the ratio of DM-20 to PLP expression decreases with PLP forming the main isoform and occurring at a 2-10 fold higher level (Nave et al. 1987, Schneider et al. 1992) in the adult CNS. In the PNS, DM-20 remains the predominant isoform (Pham-Dinh et al. 1991b).

The production of PLP correlates with the presence of myelinating cells and, unlike DM-20, this isoform is expressed almost exclusively in these cells (Ikenaka et al. 1992).

Thus the proteolipid proteins are predicted to have a dual role in CNS myelin; acting both as a structural component and as a signal for oligodendrocyte differentiation.

1.4.4.3 MUTATIONS AT THE PROTEOLIPID PROTEIN LOCUS

Mutations at the PLP locus result in defective myelination in several species and are proposed to result in a position dependent loss of function, affecting either the structural or the regulatory role of PLP, or both (Schneider et al. 1992).

Point mutations resulting in amino acid changes have been previously reported in 8 cases of PMD and of these 6 (75%) occurred in exons 4 or 5. In addition a complete gene deletion and an insertion/deletion event has been reported (Table 5).

However, no changes in the PLP gene have been identified in an additional 26 cases -14 of which were reported to be consistent with X-linked inheritance. Hudson et al. (1989a), Pham-Dinh et al. (1991a), Pratt et al. (1991) and Doll et al. (1992) found no sequence changes on analysis of the coding region and splice site junctions in 1 case of apparently X-linked PMD, 13 cases consistent with X-linked inheritance and in a further 12 cases of PMD of unknown mode of inheritance.

If a defect of the PLP gene is responsible for PMD in these families, noncoding regions must be involved as reported, for example, in haemophilia A (Naylor et al. 1992). Genetic linkage analysis in those families not showing a PLP mutation is required to establish the locus involved.

Linkage analysis has been carried out in 2 large classical X-linked families by Johnson et al. (1991) and Maenpaa et al. (1990) but, whilst the results were consistent with the involvement of the PLP gene, a significant LOD score was not obtained. Similarly,

TABLE 5 - Mutations of the proteolipid protein gene reported in Pelizaeus-Merzbacher disease

LOCATION	AMINO ACID	MUTATION	FAMILY HISTORY	PHENOTYPE	REFERENCE
-	-	Complete gene deletion	X-linked	Classical	Raskind et al. 1991
Exon 2	14	Pro (CCC) -> Leu (CTC)	X-linked	Connatal	Trofatter et al. 1989
Exon 3	73	Gly (GGA) -> Arg (CGA)	2 affected brothers	Leucodystrophy	Doll et al. 1992
Intron 3	-	G -> T at -1 position of 3' acceptor splice site of exon 4	X-linked	Classical	Strautnieks et al. This report
Exon 4	155	Thr (ACC) -> Ile (ATC)	X-linked	Classical	Pratt et al. 1991
Exon 4	162	Trp (TGG) -> Arg (CGG)	X-linked	Classical	Hudson et al. 1989a
Exon 4	181	Thr (ACC) -> Pro (CCT)	X-linked	Classical	Strautnieks et al. This report
Exon 4	195/6	Frameshift as a result of an insertion/deletion	X-linked	Classical	Pham-Dinh et al. 1993
Exon 4	202	Asp (GAT) -> His (CAT)	3 affected brothers	Undetermined	Doll et al. 1992
Exon 5	215	Pro (CCT) -> Ser (TCT)	2 affected brothers	Classical	Gencic et al. 1989
Exon 5	218	Val (GTT) -> Phe (TTT)	X-linked	Classical	Pham-Dinh et al. 1991a
Exon 5	220	Gly (GGC) -> Cys (TGC)	2 affected brothers	Connatal	Iwaki et al. 1993
Exon 5	223	Leu (CTT) -> Pro (CCT)	2 affected brothers	Classical	Strautnieks et al. This report

Trofatter et al. (1989) analysed a large X-linked family with connatal PMD using the presence or absence of an exon 2 point mutation and disease status. Tight linkage of the mutation with PMD was noted; LOD score = 4.62 at theta= 0, with no recombinations. To date no recombinations between PMD and the PLP gene have been reported.

Molecular defects in the PLP gene have also been characterised in a number of X-linked dysmyelinating disorders of the CNS in animals (Table 6). All but the rumpshaker mouse (rsh) are characterised by a similar neurological phenotype; that is dysmyelination and a lack of mature oligodendrocytes. Rumpshaker conversely, though myelin deficient, has a normal life span and a normal complement of mature oligodendrocytes. Proteolipid protein is also present, though predominantly as the DM-20 isoform.

1.4.5   **DIAGNOSIS, CARRIER DETECTION AND PRENATAL DIAGNOSIS**

Diagnosis of affected males with PMD is based on the combined analysis of the pathological and clinical profiles. It is complicated by the fact that both the clinical symptoms and the MRI evidence of a dysmyelinating disease are common to a range of disorders. Before the discovery of the rumpshaker mouse mutant, the only definitive diagnostic feature was a lack of mature oligodendrocytes, the analysis of which required an invasive and dangerous brain biopsy. With the discovery of rumpshaker mouse, the pathological and clinical phenotypes will require redefinition.

Due to the severity of PMD symptoms and the inability to treat this disease, reliable carrier detection and prenatal diagnosis are in great demand. The important question in families with a sporadic affected individual, but no other family history of PMD, is the carrier status of the mother and hence the risk to future pregnancies.

Carrier detection is extremely difficult as females are generally asymptomatic and no biochemical tests exist. Boltshauser et al. (1988) used magnetic imaging to study a 5 generation family with X-linked classical PMD and the subsequent identification of the

TABLE 6 -   Mutations of the proteolipid protein gene reported in animal models of Pelizaeus-Merzbacher disease

LOCATION	AMINO ACID	MUTATION	MUTANT	REFERENCE
Exon 2	36	His (CAT) -> Pro (CCT)	Shaking pup dog	Nadon et al. 1990
Exon 3	74	Thr (ACT) -> Pro (CCT)	Myelin deficient rat	Boison et al. 1989
Exon 4	186	Ile (ATT) -> Thr (ACT)	Rumpshaker mouse	Schneider et al. 1992
Intron 4	-2 position of 3' acceptor splice site	AG -> GG Results in skipping of exon 5 and a frameshift mutation	Jimpy mouse	Nave et al. 1987
Exon 6	242	Ala (GCG) -> Val (GTG)	Jimpy [msd] mouse	Gencic and Hudson. 1990

causative mutation in this family (Pratt et al. 1991) enabled an evaluation of the accuracy of this technique. There were found to be 5 concordances and 1 discrepancy - the latter in a 23 year old girl who exhibited an 'unequivocal abnormal MRI' and was incorrectly diagnosed as a carrier. As females of this age will constitute the majority of cases presenting for carrier detection this test has limited application.

Limited carrier detection has been carried out in families with X-linked PMD using RFLP analysis. This method is based on the assumption that the disorder is caused by a mutation at the PLP locus (Maenpaa et al. 1990, Bridge et al. 1991) and has been complicated by the failure to find mutations in approximately 75% of PMD cases at the PLP locus.

Further the PLP gene is very highly conserved and to date only 3 polymorphisms have been reported. Two *Msp*I polymorphisms of 4 / 4.5kb and 2.6 / 2.7kb are detected on Southern blot analysis and have allele frequencies of 0.92 / 0.08 (Wu et al. 1987) and 0.35 / 0.65 (Raskind et al. 1992) respectively. A polymorphism which creates an *Aha*II site in exon 4 has been reported to occur with allele frequencies of 0.74 / 0.26 (Trofatter et al. 1991).

2.0.0 MATERIALS AND METHODS

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2.1.0   **STOCK SOLUTIONS**

2.1.1.   **QIAGEN PLASMID MINI PREPARATION SOLUTIONS:**

Buffer P1 (pH 8.0)

50mM   Tris-HCl
10mM   EDTA
100μl   RNase A   (100μg/ml)

Buffer P2

200mM NaOH
1% SDS

Buffer P3

2.55mM KAc (pH 4.8)

Buffer QBT   (pH 7.0)

750mM NaCl
50mM   MOPS
15%   Ethanol
0.15%   Triton X-100

Buffer QC   (pH 7.0)

1M   NaCl
50mM MOPS
15%   Ethanol

Buffer QF   (pH 8.2)

1.25M NaCl
50mM MOPS
15%   Ethanol

2.1.2 **RANDOM HEXANUCLEOTIDE AND END LABELLING SOLUTIONS**

TM solution (pH8.0)

250mM Tris - HCl
25mM $MgCl_2$
50mM BME

DTM solution

100μM dATP, dGTP, dTTP in TM

OL (pH 7.5)

250μl of random hexanucleotides (90 units/ml)
1mM EDTA
1mM Tris - HCl

Labelling solution (LS) (pH 6.6)

25μl 1M HEPES
25μl DTM
7μl   OL

10 x Kinase reaction buffer (pH 8.0)

670mM Tris - HCl
100mM $MgCl_2$
100mM DTT

2.1.3 **SOUTHERN BLOT AND DOT BLOT ANALYSIS SOLUTIONS**

20 x Standard Saline Citrate (SSC)

3M NaCl
0.3M Sodium Citrate
pH adjusted to 7.0 with Sodium hydroxide

10 x Tris Acetate Buffer (E-Buffer/TAE)

0.4M Tris Acetate
10mM EDTA
pH adjusted to 7.7 with glacial acetic acid

Denaturing solution

1.5M NaCl
0.5M NaOH

Neutralising solution (pH 7.2)

1.5M   NaCl
0.5M   Tris-HCl
1mM   EDTA

100 x Denhardts solution

2%   BSA
2%   Ficoll
2%   Polyvinylpyrollidone

Hybridisation/Prehybridisation mix

10 x Denhardts solution
Boiled, sonicated salmon sperm DNA (50μg/ml)
4 x SSC
1% SDS

2.1.4   **SEQUENCING SOLUTIONS**

TES   (pH 8.0)

100mM NaCl
10mM Tris-HCl
1mM   EDTA

<u>5 x Sequenase reaction buffer</u> (pH 7.5)

200mM Tris-HCl
100mM MgCl$_2$
250mM NaCl

<u>5 x Labelling mix (dGTP)</u>

7.5μM dGTP
7.5μM dCTP
7.5μM dTTP

<u>5 x Labelling mix (dITP)</u>

 15μM dITP
7.5μM dCTP
7.5μM dTTP

<u>Enzyme dilution buffer</u> (pH 7.5)

10mM Tris-HCl
5mM   DTT
0.5mg/ml BSA

<u>ddG/T/C/ATP Termination mix (dGTP)</u>

80μM dGTP
80μM dTTP
80μM dCTP
80μM dATP
8μM ddG/T/C/ATP
50mM NaCl

<u>ddGTP Termination mix (dITP)</u>

160μM dITP
80μM dTTP
80μM dCTP
80μM dATP
8μM   ddGTP
50mM NaCl

ddT/C/ATP Termination mix (dITP)

80μM dGTP
80μM dTTP
80μM dCTP
80μM dATP
8μM ddT/C/ATP
50mM NaCl

Stop solution

95%   Formamide
20mM   EDTA
0.05% Bromophenol Blue
0.05% Xylene Cyanol FF

2.1.5   **BACTERIAL CULTURE SOLUTIONS**

L-Broth

5g   Tryptone
5g   NaCl
2.5g Yeast Extract

Agar

500ml L-Broth
4.5g Agar

2.1.6   **GENERAL SOLUTIONS**

5 x Tris-borate-EDTA (TBE) (pH 8.0)

0.45M Tris-HCl
0.01M EDTA (pH 8.0)
0.45M Boric acid

<u>10 x TEN (STE)</u> (pH 8·0)

0.1M   NaCl
10mM   Tris-HCl
1mM   EDTA

<u>Tris-EDTA (TE)</u>

10mM   Tris-HCl
1mM   EDTA
pH adjusted to 8.8 with NaOH

<u>10 x PCR reaction buffer (Promega)</u> (pH 9·0)

500mM   KCl
100mM   Tris-HCl
15mM $MgCl_2$
0.1%   Gelatin
1%   Triton X-100

<u>Loading buffer</u>

0.2%   Bromophenol blue
0.1M   EDTA
50%   Glycerol

2.2.0   METHODS

2.2.1   **EXTRACTION AND PRECIPITATION OF DNA**

2.2.1.1   **EXTRACTION OF HUMAN DNA FROM BLOOD**

DNA was obtained from the nuclei of peripheral leucocytes using the guanidinium hydrochloride method of extraction (Jeanpierre. 1987). All stages were carried out in a safety hood unless stated.

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Blood samples were collected and stored in 10ml EDTA-coated tubes at -70°C. To extract DNA, the tubes were inverted into 50ml tubes, thawed quickly to avoid a temperature rise above 4°C and the volumes made up to 50ml with ice cold ddH_2O. The samples were centrifuged for 20 minutes at 2.5-3000rpm and 4°C in an IEC CENTRA-7R refrigerated centrifuge and the supernatants were decanted off. The water lysed the red cell component which remained in solution on centrifugation and was separated from the leucocyte pellet.

The nonnuclear membranes in the pellet were disrupted by adding 25ml of ice cold 0.1% Nonidet P40 and vortexing until the pellets were completely resuspended. On centrifugation, as previously, the membranes remained in solution and were separated from the nuclear pellet.

The nuclear membranes were disrupted by adding 7ml of 6M guanidinium hydrochloride and 0.5ml of 7.5M ammonium acetate (pH 7.5) to the samples and vortexing until the pellets were totally resuspended. 0.5ml of 20% sodium sarkosyl and 150μl of fresh proteinase K (10mg/ml) were added and the samples were mixed gently by inversion and incubated at 60°C for 90 minutes. All further steps were carried out outside the safety hood.

The DNA was precipitated by adding 2 volumes (17ml) of absolute ethanol and was spooled out gently with a blunt ended glass pasteur pipette. It was transferred to a bijoux containing one ml of TE and redissolved by placing on a rotary mixer for 16-24 hours at 4°C. The DNA was purified by reprecipitating with 1/10 volume (100μl) of 3M sodium acetate (pH 5.2) and 2 volumes (2ml) of ice cold absolute ethanol. It was spooled out, air dried to evaporate any residual alcohol, redissolved in 1ml of TE and stored at -20°C.

If smaller blood volumes or clotted samples were used, all volumes were halved, and, in cases of minimal precipitation, samples were placed at -20°C for 16 hours before centrifuging as previously.

2.2.1.2   **ETHANOL PRECIPITATION OF DNA**

DNA (genomic, PCR products, oligonucleotide primers and probes) was isolated, purified and concentrated by ethanol precipitation in the presence of ammonium or sodium acetate.

DNA was precipitated by adding a 1/2 or a 1/10 volume of 8M ammonium acetate (pH 7.5) or 3M sodium acetate (pH 5.2) respectively, mixing, adding 2 1/2 volumes of ice cold ethanol and leaving at -70°C for 1/2-16 hours.

The precipitate was collected by centrifuging for 30 minutes at 10,000rpm and 4°C in a MSE Microcentaur bench centrifuge and pipetting off the supernatant. Coprecipitated salt was removed by resuspending the DNA in 70% ethanol (50-100µl), recentrifuging, pipetting off the supernatant and air or vacuum drying the DNA before resuspending in water or TE and storing at -20°C.

Ammonium acetate was used preferentially in cases where the reduction of nucleotide coprecipitation (PCR product purification) was a factor, sodium acetate for probes to be end labelled by T4 polynucleotide kinase which is inhibited by ammonium ions.

To recover DNA present at low concentrations, precipitation was effected in a reduced volume, by substituting 2 1/2 volumes of ethanol with one volume of isopropanol.

2.2.1.3   **PHENOL CHLOROFORM EXTRACTION**

Phenol chloroform extraction was used to isolate DNA probes from agarose gel slices and to purify genomic DNA and PCR products.

The phenol was initially equilibrated to a pH greater than 7.8, to prevent the loss of DNA into the organic phase. An equal volume of 0.5M Tris-HCl (pH 8.0) was added and the solution was placed on a magnetic stirrer for 15 minutes at room temperature, before being allowed to settle. The aqueous layer was removed, the pH of the phenol was

determined using pH paper and the process was repeated until a pH of 7.8 or greater was obtained. The pre-equilibrated phenol was stored in a light tight bottle at 4°C.

To purify DNA, an equal volume of pre-equilibrated phenol was added to the DNA in a polypropylene tube. The sample was vortexed (DNA < 10 kb) to form an emulsion and centrifuged at 10,000rpm for 5 minutes at room temperature. The DNA partitioned into the aqueous phase (the upper layer) which was removed to a clean tube, while the protein partitioned into the organic phase and was removed.

To extract DNA from a gel slice, TE (1.5ml/g agarose) was added and the slice was incubated at 65°C until melted (5-10 minutes). The melted gel solution was mixed with an equal volume of phenol and extracted as previously. The gel collected at the interface between the 2 phases and was left behind on removal of the aqueous phase. Phenol extraction was repeated until no further gel deposits were seen at the interface. Traces of phenol in the aqueous solution were removed by extracting twice with chloroform, as for phenol extraction, and isolating the DNA by ethanol or isopropanol precipitation (2.2.1.2). All extractions were carried out in a fume hood.

2.2.1.4   **DETERMINATION OF DNA CONCENTRATION**

DNA concentrations were determined by measuring the optical density (OD) at 260nm using a PU 8620 UV/VIS/NIR spectrophotometer (Phillips). One OD unit is equivalent to 50 and 40ng/μl of double stranded and single stranded DNA respectively, based on the assumption that DNA contains approximately equal amounts of purine and pyrimidine bases.

The concentration of olignucleotides, between 11 and 25bp in length, was determined using a calculation based on the sum of the extinction coefficients of the nucleotides (X) and the absorbence at 260nm. These were 15200, 7050, 8400 and 12010 for dATP, dCTP, dTTP and dGTP respectively, where $1/X \times OD_{260}$ = concentration (moles/litre).

DNA purity was estimated by determining the ratio of the absorbences at 260 and 280nm, where a ratio of between 1.8 and 2 indicated a pure preparation.

2.2.2   THE POLYMERASE CHAIN REACTION (PCR)

2.2.2.1   OLIGONUCLEOTIDE PRIMERS

Primers were synthesised on an Applied Biosystems 381A DNA synthesiser.
5' Biotinylation was carried out during oligonucleotide syntheses using DMT-biotin-C-6-PA (CRB), according to the manufacturer's instructions.

Primer sequences (20-26bp) were manually selected from the available sequence data on the basis of: a random base distribution with no polypyrimidine or purine tracts, an equal proportion of GC to AT bases and a lack of complementarity between the two primers. In addition sequences with comparable (+/- 5°C) and, where possible, high (55°C+) Tms were selected. In several cases, for the OCT gene, primer design was limited by the scarcity of available sequence data. This necessitated the use of poorly matched primers with low Tm values or primers which failed to amplify complete exons and splice site junctions.

2.2.2.2   PRIMER SEQUENCES AND REACTION CONDITIONS FOR THE AMPLIFICATION OF THE ORNITHINE CARBAMOYL TRANSFERASE GENE

Primer sequences for exons 5 and 7+8, 5' untranslated sequence and the intronic sequences immediately flanking each exon of the OCT gene were obtained from the published literature (Hata et al. 1986, 1988a,1988b, 1989, Finkelstein et al. 1990b).

Additional primer sequences for exons 1, 3, 5, 9 and 10 and the intronic sequences surrounding exons 1, 3, 4 and 10 were provided by Drs Grompe and Caskey (Baylor College of Medicine, Houston) and Professor Matsuda (Kumamoto University Medical

School, Kumamoto) respectively.

Primer sequences are given in Table 7. Due to the limited size of intron 7 (80bp), exons 7 and 8 were conveniently amplified together. In the case of exons 7+8, primers were synthesised both with, and without, 5' biotin groups.

2.2.2.3 PRIMER SEQUENCES AND REACTION CONDITIONS FOR THE AMPLIFICATION OF THE EXONS OF THE PROTEOLIPID PROTEIN GENE

Primer sequences (Table 8) and a complete set of oligonucleotide primers, for the amplification of the seven exons of the proteolipid protein gene, were provided by Dr L.Hudson (Gencic et al. 1989). Subsequent syntheses were based on this data.

2.2.2.4 THE POLYMERASE CHAIN REACTION

Individual exons were amplified using the polymerase chain reaction (PCR). Reactions were carried out in a 100μl volume using 200ng of genomic DNA, 50picomoles of each primer, 0.2mM each of dATP, dGTP, dCTP and dTTP, 1 x reaction buffer (Promega), 10^{-5}M TEMAC and 1.5-2.5 units of *Taq* polymerase (Promega) enzyme. Reactions were overlaid with 50μl of mineral oil to prevent evaporation and amplified for 30 cycles on Biometra TRIO-Thermoblock or PHC-2 water cooled PCR machines.

Each cycle consisted of three stages: a denaturing step (94°C, 1 minute) to denature the double stranded template, an annealing step (primer dependent temperature, 30 seconds) to enable the primers to bind to the complementary DNA strands and an extension step (72°C, 1 minute) during which they were extended by the 5' to 3' activity of *Taq* polymerase.

The reactions were denatured at 94°C for 5-10 minutes, cooled to the annealing temperature, the enzyme was added in 10μl of water and cycling was commenced at the

Exon	Position	Name	Sequence	Size (bp)	Annealing conditions (°C)
1	Intron 1	A	5' CCT AAA TCA AAC CCA AGT CTC TGA CC	218	56
	5' untranslated	B	5' CTC ACT GCA ACT GAA CAC ATT TCT T		
2	Intron 1/Exon 2	C	5' TTT TAA ATC TCT TTT TAC AGG TG	135	48
	Exon 2	D	5' TTT CAG ATC TGC TGA TAG CCA TAG C		
3	Exon 3/Intron3	E	5' GTG TGA ATT TGG CAG TGG ACT TAC C	283	60
	Intron 2	F	5' GAT CCC CGG GTA CCG AGC TCG AAT T		
4	Intron 3/Exon 4	G	5' TTA ATT GTA GGC TTA GCA CTT	119	48
	Intron 4	H	5' (B)-GAG AAG AAA ATA TTT ACA AAC		
5	Exon 5/Intron 5	I	5' CCT GGA GCG TGA GGT AAT CAG CCA G	156	56
	Exon 5/Intron 4	J	5' GTG TAT TGT CTA GCA TGG CAG ATG C		
6	Intron 5	K	5' CAT CTC CTT CAT CCC GTG CCT TTT AG	171	56
	Intron 6/Exon 6	L	5' (B) - TCA AGG CAA AAA GTT TCC CTA CCT		
7&8	Intron 6/Exon 7	M	5' (B) - TCC TTC CTC CTT TAG GGT TAT GAG	322	56
	Intron 8/Exon 8	N	5' (B) - CCT GAG AGA GCA TCA ATT TGT AC	} 166	
8	Exon 8	O	5' (B) - CCA AGC TGT TGC TGA CAA ATG ATC		56
9	Intron 9	P	5' CCA TTC CTT GTT TCT TGC TT	182	50
	Intron 8	Q	5' GGT CTT ATC CCC ATC TCT TT		
10	Intron 9/Exon 10	R	5' GAG AAG AAA ATA TTT ACA AAC	160	48
	3' untranslated	S	5' GAT TCT CTT CTC TTT TCC CCA TAA AC		

TABLE 7: PCR amplification primers for the exons of the OCT gene. Biotinylated primers are indicated by a "B".

Exon	Position	Sequence	Size (bp)
1	Intronic 3'................................	5' CCC GAA CCC CAA AGT TGG AA 3'	511
	Intronic 5'................................	5' AAG TTT CCA GGA CAT CTT CA 3'	
2	Intronic 3'................................	5' TGC TAG TTA CTA AGG TAC TG 3'	362
	Intronic 5'................................	5' TGA GCA CAG GGC CTG GCA GA 3'	
3	Intronic 3'................................	5' ACG AGG CCA CAG ACT CGC GC 3'	412
	Intronic 5'................................	5 ACC TCA CTT ATG TCG GGA AA 3'	
4	Intronic 3'................................	5 ACC ACC CTC CTT ACA CTA AG 3'	335
	Intronic 5'................................	5' ACT CCA GGA TCT CCC AGT TT 3'	
	Intronic 3'................................	5' CTT CCA TAG TGG GTA GGA GA 3'	Nested sequencing
	Intronic 5'................................	5' (B) - GAT CTG CAG GCT GAT GCT GA 3'	primers
5	Intronic 3'................................	5' ATT GAA GGC CAT GGG TGT AA 3'	236
	Intronic 5'................................	5' AGA GAT GGA AGA AGG GCT CT 3'	
	Intronic 3'................................	5' TGG TAT TAG CTA CTC CCT TG 3'	Nested sequencing
	Intronic 5	5' (B) - CCC CGT AAC TCC ATA AAG CT 3'	primers
6	Intronic 3'................................	5' TTG CCT TTC AGA ATA GCT GT 3'	239
	Intronic 5'................................	5' AAA GAT ATC AAC ACA TTC AG 3'	
7	Intronic 3'................................	5' GGT GGC AAA GGC AAA GAG TT 3'	261
	Intronic 5'................................	5' TTT TAT TTC TAC CCT TCC TC 3'	

TABLE 8:   PCR amplification primers for the exons of the PLP gene. Biotinylated primers are indicated by a "B".

extension step. After completion, a final step of 10 minutes at 72°C was carried out to ensure complete extension.

For each set of reactions, a control reaction lacking DNA was included to monitor the exposure of the reagents to contaminant DNA. All reactions and reagent preparations were carried out under sterile conditions.

The PCR products were checked for specificity by electrophoresis of a 5-10μl sample on a 1.5-2% agarose gel against a lambda kb ladder (Gibco BRL) molecular weight marker. The gel contained 1-2μl of ethidium bromide (0.5mg/ml) and was visualised on a UV transilluminator (UVP). Mini gels were prepared as described in section 2.2.5.1, except that preparation and electrophoresis was in 1 x TBE on mini (8 x 10cm, BRL) or midi (12.5 x 15cm, NBL) gel systems at 50-100V for 1/2-2 hours.

In cases of nonspecificity the reaction conditions were modified (annealing temperature increased, annealing and extension times decreased), the enzyme and primer concentrations were reduced, a magnesium titration (0.5-5mM) was carried out, or the number of cycles were reduced.

Consistently nonspecific PCR products were reamplified using nested primers or after the separation of the nonspecific PCR product (10μl) on a 3-4% percentage 1:1 agarose: Nusieve-GTG-agarose gel. After electrophoresis the gel was placed on a UV transilluminator and the required band was excised by comparison with a lambda kb ladder molecular weight marker. Sterile ddH$_2$0 (1.5ml/mg of gel) was added and the sample was freeze thawed 3-4 times to diffuse the DNA out of the gel. The PCR was repeated using 2-5μl of the solution as a template and reducing the cycle number to 20.

All equipment was prewashed or sterile to reduce the chance of contamination during band excision.

2.2.3 RESTRICTION ENZYME ANALYSIS OF POLYMERASE CHAIN REACTION PRODUCT

Twenty microlitres of amplified PCR product was digested with the appropriate restriction enzyme in a volume of 30μl, according to the manufacturer's (NBL, NEB) instructions. *Fnu*4HI, *Cfo*I, *Msp*I, *Hinf*I, *Bsa*HI, *Eco*RI and *Rsa*I digests were incubated at 37°C for 4 hours, *Taq*I digests at 65°C for 1 hour, with 2-20 unit enzyme. For 4 hour digests, an additional 1-10 units of enzyme was added after 2 hours.

For analysis, the products were mixed with 5-10μl of loading buffer or stop solution (U.S.B.) and electrophoresed on 3-4% Nusieve-GTG-agarose gels as described previously (Section 2.2.2.4), for 1-5 hours at 50V and 4°C. The digests were visualised by UV transillumination and ethidium bromide staining.

2.2.4 PREPARATION OF PROBES FOR SOUTHERN BLOT ANALYSIS

The 2kb, PLP cDNA (pRL1) probe was obtained as a glycerol stock, the OCT cDNA as an aliquot of PUC18 plasmid DNA containing the 1.5kb probe insert.

To produce sufficient quantities of the OCT cDNA probe for labelling and to set up a glycerol stock, the plasmid DNA was transfected into competent *E.coli* cells. Transformants were isolated by virtue of the antibiotic resistance (ampicillin) conferred on the bacteria by the plasmid DNA. On plating out on to agar plates containing ampicillin, only those cells which had taken up plasmid DNA formed colonies. These transformed colonies were cultured to amplify the probe insert, the plasmid DNA was isolated and the inserted probe was extracted.

2.2.4.1   TRANSFORMATION OF COMPETENT *E.COLI* CELLS WITH PLASMID CONTAINING PROBE DNA

The plasmid DNA was transfected into competent *E.coli* cells using the calcium chloride method. The bacteria were treated with ice cold $CaCl_2$ and briefly heated to induce a transient state of competence, during which they were incubated with, and internalised, plasmid DNA.

Competent *E. coli* (HB101) cells were prepared from a glycerol stock. 10μl of stock was added to 10ml of sterile L-Broth in a universal tube and the cells were cultured for 16 hours in a 37°C orbital incubator (Gallenkemp). This, and all subsequent stages, until the isolation of the plasmid DNA, were carried out in a safety hood using aseptic technique.

100ml of sterile L-Broth was placed in a 500ml flask, inoculated with 1ml of the 16 hour culture and placed in the 37°C orbital incubator for 2-4 hours until the cell density was approximately 5×10^7/ml. The density was determined by measuring the OD of 1ml aliquots at 30 minute intervals spectrophotometrically. For the *E.coli* HB101 strain an OD_{550} of 0.5 represented the required cell density.

The culture medium was chilled on ice for 10 minutes to stop growth and the cells were collected by centrifugation for 5 minutes at 4,000rpm and 4°C, in an IEC CENTRA-7R refrigerated centrifuge. The supernatant was decanted off into 10% Chloros and the pellet was resuspended in 50ml of an ice cold solution of 50mM $CaCl_2$ and 10mM Tris-HCl (pH 8.0). The cell suspension was chilled on ice for a further 15 minutes and centrifuged, as previously. The supernatant was removed, the pellet was resuspended in 15ml of an ice cold solution of 50mM $CaCl_2$ and 10mM Tris-HCl, and 0.2ml aliquots were dispensed into prechilled tubes. These were stored at 4°C for 12-24 hours.

The plasmid DNA was diluted with TE, to a concentration of 40ng/100μl, and 100μl aliquots were added to duplicate competent cell suspensions. These were chilled on ice for 30 minutes. A control reaction containing only TE was also set up. The reactions were

heat shocked at 42°C for 2 minutes, to induce the bacteria to take up the plasmid, before adding 1ml of L-Broth and incubating at 37°C for 30 minutes. This allowed cell recovery and the expression of the ampicillin resistance.

The cells were plated out on to ampicillin (50μg/ml) infused agar in sterilin-petri dishes (90mm). These were dried at room temperature and incubated, inverted, at 37°C for 16-24 hours. As ampicillin resistant cells secreted beta-lactamase which inactivates ampicillin, the cells were plated at a range of densities (20, 40 and 60μl/plate) to ensure at least one sparsely colonised plate and hence true transformants.

The plasmid DNA was cultured by placing a single colony from each duplicate plate into 10ml of L-Broth containing 50μg/ml of ampicillin and incubating in an orbital incubator at 37°C for 16 hours. The plates were stored at 4°C.

2.2.4.2   **GLYCEROL STOCK PREPARATION**

Bacterial cultures can be stored indefinitely at -70°C in a solution of 5% glycerol. To prepare a glycerol stock, 3.4ml of culture solution was vortexed with 600μl of sterile glycerol, before separating into two cryovials and storing at -70°C. To revive the culture 5μl aliquots were pipetted of the surface of the frozen sample and the cryovial was replaced immediately to storage.

2.2.4.3   **EXTRACTION AND PURIFICATION OF PLASMID DNA**

A Qiagen minipreparation kit was used according to the manufacturer's protocol (Diagen). Plasmid DNA was extracted from bacterial cells in three steps: bacterial cells were lysed and the plasmid DNA was adsorbed to an ion exchange column, the column was washed to remove cell debris, and the plasmid DNA was eluted.

50ml of sterile L-Broth containing the appropriate antibiotic (50μg/ml) was inoculated with a 5μl aliquot of glycerol stock and incubated for 16 hours in a 37°C orbital incubator.

The culture was chilled on ice for 10 minutes and the cells were collected by centrifuging at 4,000rpm and 4°C for 20 minutes in an IEC CENTRA-7R refrigerated centrifuge.

The bacterial cell walls were lysed by resuspending the pellet in 300μl of buffer P1 containing RNase A (100μg/ml), adding 300μl of buffer P2, mixing by inversion and incubating at room temperature for 5 minutes. The bacterial DNA was precipitated out and removed by adding 300μl of buffer P3, mixing thoroughly by inversion and centrifuging as previously. The bacterial DNA was pelleted out while the plasmid DNA remained in solution.

A Qiagen column was equilibrated with 1ml of buffer QB and the supernatant was added to the column. The negatively charged DNA adsorbed to the column and cell debris was removed by washing with buffer QC (2 x 1ml). The DNA was eluted by adding 0.8ml of buffer QF to the column and was reprecipitated using 0.64ml of room temperature isopropanol and centrifuging at 10,000rpm for 15 minutes in a MSE Microcentaur bench centrifuge. The DNA was washed with 70% ethanol, recentrifuged for 5 minutes, air dried and resuspended in 0.5ml TE

2.2.4.4   ISOLATION OF DNA PROBE FROM PLASMID DNA

The probe insert was cut out of the plasmid vector by restriction enzyme digestion with the appropriate enzyme, in the case of OCT and PLP cDNA: *EcoR*I, in a 40μl volume as described previously (2.2.3), where the PCR product was replaced by plasmid DNA. Separation was effected by electrophoresis in a 0.8% agarose mini gel at 50V and 4°C, until the plasmid and insert bands were clearly separated (approximately 3 hours). To enable the insert to be identified, undigested plasmid DNA and a molecular weight marker (*Bst*EII digested lambda DNA) were electrophoresed alongside the digest. The gel was examined on the UV transilluminator and the insert band was identified, excised and isolated by phenol chloroform extraction (2.2.1.3). The probe was diluted in TE to a give a concentration of 10ng/μl.

2.2.5   SOUTHERN BLOT ANALYSIS

2.2.5.1   RESTRICTION ENZYME DIGESTION AND AGAROSE GEL ELECTROPHORESIS

Genomic DNA (20μl, 5-20μg) was digested with restriction enzyme, according to the manufacturer's instructions (N.B.L.), in a total reaction volume of 40μl. The digests were incubated at 65°C for 1 hour (*Taq*I) or 37°C for 4 hours (*Msp*I, *Pst*I) with 20 units of enzyme. In the case of 4 hour digestions, an additional 10 units of enzyme were added after 2 hours.

In cases of consistent nondigestion, the DNA was reprecipitated with 1ml of absolute ethanol and redissolved for 16 hours at 4°C on a rotary mixer.

The digests were separated on a 0.8% agarose gel in 1 x E-buffer. 3.2 g of agarose and 20μl (0.5mg/ml) of ethidium bromide were added to 400ml of 1 x E-buffer in a conical flask and melted in a microwave. The ends of a 20 x 25cm gel tray (BRL) were sealed with tape, the cooled gel solution (60°C) was poured in and a 20 well comb was inserted. The gel was allowed to set for 1-2 hours at room temperature.

The samples were mixed with 10μl of loading buffer, loaded on to the gel and electrophoresed at 90-110V for 16-35 hours, depending on the sizes of the polymorphic alleles to be separated, against *Bst*EII and *Hind*III digested lambda DNA as molecular weight markers. The gel was visualised on a UV transilluminator and photographed (Mitsubishi videocopy processor) against a ruler to position the marker bands.

2.2.5.2   TRANSFER OF DNA TO HYBOND-N/N+ NYLON MEMBRANE

The gel was placed in a photographic tray on a shaking platform and washed in denaturing and neutralising solutions for 1 hour and 30 minutes respectively. For gels to be blotted onto Hybond-N+, the neutralisation step was omitted.

The gel was positioned on a sponge covered in 3MM Whatman paper, where the edges of the paper overlapped into a tray of 20 x SSC. A piece of Hybond-N/N+ membrane and 2 pieces of filter paper were cut to size and presoaked in 2 x SSC. The Hybond-N/N+ membrane was placed on the gel and covered with the filter paper. Any air bubbles were removed by gently rolling the surface of the gel with a 10ml plastic pipette. The edges of the tray were covered with cling film to prevent evaporation and paper towels, to a depth of about 5cm, were placed on the gel to draw the 20 x SSC and hence the DNA onto the filter.

After 16 hours, the filter was removed from the gel and rinsed in 2 x SSC to remove salt and gel debris. Hybond-N+ filters were fixed for 10 minutes in 0.4M NaOH and neutralised by rinsing in 2 x SSC, until the pH of the SSC solution was 7.0. Hybond-N filters were blotted to remove excess moisture and fixed by baking at 80°C for 2 hours.

2.2.5.3 **RANDOM HEXANUCLEOTIDE LABELLING OF DNA PROBES**

This method involved the annealing of random oligonucleotide sequences to denatured probe DNA, followed by extension across the intravening sequences using the 5' to 3' polymerase activity of the large (Klenow) fragment of *E.coli* DNA polymerase I. Alpha-^{32}PdCTP was included in the extension mix and was incorporated in a template directed manner to produce two complementary labelled strands.

Plasmid or insert DNA was diluted in TE to a concentration of 10ng/μl and 50ng of probe were labelled per reaction. The probe (5μl) was placed in a 1.5ml tube, with the lid pierced to avoid a build up of pressure, and was denatured for 2 minutes in a boiling water bath and placed on ice. To this was added, on ice: 11.4μl of LS, 1μl BSA (10mg/ml), 3-5μl (depending on the activity date) of alpha-^{32}PdCTP (3000Ci/mmol), water to a final volume of 25μl and 2.5 units of Klenow. The reaction was mixed and left at room temperature for 2.5-4 hours.

2.2.5.4 REMOVAL OF UNINCORPORATED NUCLEOTIDES BY COLUMN CHROMATOGRAPHY

After labelling, unincorporated nucleotides were removed by gel filtration chromatography though a column of Sephadex G-50. This reduced the level of nonspecific binding to filters. Two peaks of radioactivity were observed: large DNA probes (first peak) were excluded from the matrix and were eluted ahead of the unincorporated nucleotides (second peak) which were retarded by the beads.

Medium grain Sephadex G-50 beads were preswollen in 3 x SSC for 1 hour at 60°C. The tip of a small pasteur pipette was plugged with a small amount of nylon wool and filled with Sephadex solution. This was allowed to settle, before topping up with more solution, until the pipette was 3/4 full.

A series of 1.5ml tubes were labelled and placed in a rack and the column was positioned above tube 1. The probe was diluted with 75µl of 3 x SSC, added to the column and the drips were collected in the tube below. When all the liquid had been collected, the rack was moved so that next tube was under the column. 100µl aliquots of 3 x SSC were added and the drips were collected in the series of tubes until the first peak of counts had been collected. The 4 tubes with the highest count were pooled and the amount of labelled DNA (dpm/µl) was determined using a Bioscan QC2000 Bench top beta counter.

Alternatively, separation was carried out by centrifugation through Sephadex spun columns in a Denley BS 400 centrifuge. To prepare the column, a plastic 1ml syringe was plugged with nylon wool, filled with Sephadex G-50 and equilibrated with 1 x TEN buffer. The column was placed in a disposable centrifuge tube, 0.1ml of Sephadex was added and it was centrifuged at 1600g for 4 minutes at room temperature. This was repeated until the column was 9/10 full, after which it was washed three times by adding 0.1ml of 1 x TEN and centrifuging as previously.

The probe was diluted with 75µl of 1 x TEN and applied to the column, which was

placed in a decapped 1.5ml tube. On centrifugation, as previously, the probe collected in the tube while the unincorporated nucleotides remained in the column. The amount of labelled DNA (dpm/µl) was determined and the probe was stored at -20°C.

2.2.5.5 PRE-ANNEALING OF LABELLED PROBE

Probes rich in repetitive sequences were pre-annealed with a solution of 1.2 x SSC, 1.2 x TE and 1.4 x sonicated human DNA (10mg/ml), after labelling. The solution and probe mixture was denatured for 5 minutes in a boiling water bath before incubating at 65°C for 30 minutes.

2.2.5.6 HYBRIDISATION OF LABELLED PROBE TO FILTERS

The hybridisation step was divided into three steps: prehybridisation, hybridisation and filter washing.

Prehybridisation and hybridisation were carried out at 65°C for 16 hours in bags in a shaking water bath or in glass hybridisation bottles (Hybaid) in a rotary hybridisation oven (Hybaid).

In the former method a piece of plastic tubing, a little larger than the filter, was prepared, the filter was soaked thoroughly in 2 x SSC, placed in the tubing and the edges sealed with a heat sealer. A corner was cut, hybridisation mix (5-10ml/filter) was pipetted in and any air bubbles, which inhibited hybridisation, were removed by rolling a 10ml plastic pipette across the bag and resealing.

In the second method, the presoaked filter was rolled up and placed in a hybridisation bottle with sufficient hybridisation mix to just cover the filter.

With both methods, up to 4 filters could be hybridised simultaneously by using nylon mesh membrane (Hybaid) to separate the filters and adjusting the hybridisation mix

accordingly.

In the prehybridisation step, nonspecific sites in the DNA to which the probe would otherwise bind, were saturated by incubation with a range of components including sonicated salmon sperm DNA, Ficoll BSA and polyvinyl pyrollidone. This reduced the level of nonspecific background hybridisation.

After prehybridisation, the bulk of the solution was removed, leaving just sufficient to cover each filter. The radiolabelled probe was denatured in a boiling water bath for 5 minutes and added to the filters, to give a final concentration of 10^6 dpm/ml.

2.2.5.7   **WASHING OF LABELLED FILTERS**

After hybridisation, the filters were washed in a range of solutions of increasing stringency (increasing temperature, decreasing salt solution).

Initially, the unhybridised probe was removed by 3, 10 minute washes in 2 x SSC, 0.1% SDS, at room temperature. The filters were monitored using a beta emission counter and washed at 65°C in 3 x SSC, 0.1% SDS for 10-30 minutes. After remonitoring, the filters were washed in solutions of decreasing salt concentration (to 0.1 x SSC) until the background signal was minimal.

The filters were blotted, sealed in clingfilm and exposed to X-ray (Kodak-X-OMAT-AR) film, backed by intensifying screens, at -70°C for 16 hours. After developing the filters were rewashed, in cases of high nonspecific background, and reexposed, as required, for up to 3 weeks.

For storage, the filters were stripped of probe by washing with boiling 10mM Tris-HCl for an hour at room temperature. They were monitored, blotted, air dried and stored between tissue paper at room temperature.

2.2.6   DOT BLOT AND ALLELE SPECIFIC
OLIGONUCLEOTIDE ANALYSIS

2.2.6.1   TRANSFER OF POLYMERASE CHAIN REACTION PRODUCT
TO HYBOND-N/N+ NYLON MEMBRANE

In this procedure multiple PCR samples were spotted, in dots of uniform diameter, on to a Hybond-N/N+ membrane using a Minifold Tm filtration manifold (Schleicher and Schuell). The manifold consisted of a block containing a series of ordered slots into which the samples were applied, and fitted into a suction platform. This contained a sheet of Hybond-N/N+ membrane on to which the samples were drawn as suction was applied via a vacuum pump.

Pieces of Hybond-N/N+ nylon membrane and 3MM Whatman paper were cut to the size of the manifold (10 x 13cm) and soaked in 2 x SSC. The paper was placed on the base of the manifold, the filter on top of the paper and the top of the manifold was clamped down and suction applied. A slow flow rate was required for efficient DNA binding and the suction was adjusted so that the buffer took 5-10 minutes to aspirate through the filter.

PCR products (40-60µl) were denatured by adding 0.54ml of denaturing solution and standing at room temperature for 15 minutes. Samples which were to be blotted on to Hybond-N membrane were neutralised by placing on ice and adding 0.5ml of cold neutralising solution. The pH was checked using pH paper, 5µl of loading buffer was added to visualise the products and the samples (100µl) were applied to the wells in duplicate. After filtration was complete, the wells were washed with 0.5ml of 20 x SSC, the apparatus was dismantled and the filter was air dried for an hour before fixing as described previously (2.2.5.2).

2.2.6.2 END LABELLING OF OLIGONUCLEOTIDE PROBES WITH T4 POLYNUCLEOTIDE KINASE

Oligonucleotide probes were 5' end labelled with gamma-^{32}PdATP, using T4 polynucleotide kinase, which transfers the gamma phosphate group of ATP to the 5'OH group of the probe.

30 picomoles of probe were labelled per reaction and to this was added sequentially: 2μl of 10 x kinase buffer, ddH$_2$0 to a final volume of 20μl, 4μl of gamma-^{32}PdATP and 5 units of kinase. The reaction was incubated for 45 minutes in a 37°C water bath, before removing the unincorporated nucleotides by glass column chromatography as described previously (2.2.5.4).

The efficiency of end labelling was determined by scintillation counting. One microlitre aliquots were spotted onto 4, DE-81 ion exchange filters and allowed to dry for 20 minutes. The negatively charged probe bound to the filter, whilst unincorporated nucleotides bound poorly and were removed in a series of washes. Two of the filters were washed before scintillation counting, to determine the signal due to labelled probe, while two were measured directly, to show the total signal of both incorporated and unincorporated label.

The filters were washed for 3 x 5 minutes in 5% NaPPi, 1 x 5 minutes in ddH$_2$0 and finally dehydrated by rinsing for one minute in absolute ethanol before drying. They were placed in scintillation tubes, 2.5ml of scintillant was added and the radioactive count was determined against an internal scintillant only control. The labelling efficiency was determined by comparing the counts from the washed and the unwashed filters.

2.2.6.3 HYBRIDISATION OF LABELLED PROBE TO FILTERS AND FILTER WASHING

OCT exon 5 PCR product from family PD was screened for the 2 possible mutant changes

at amino acid 109 of the mature protein. Three identical blots were prepared. One was hybridised with the wild type oligonucleotide probe, and the other two, with the two possible mutant sequences. The probe sequences were as follows:

Wild type   5' GTA TTG GCT **CGA** GTG TAT A 3'

Arg->Gln mutant   5' GTA TTG GCT CAA GTG TAT A 3'

Arg->Stop mutant   5' GTA TTG GCT TGA GTG TAT A 3'

Hybridisation was carried out as described previously (2.2.5.6), at 47°C. The blots were washed for 3 x 10 minutes in 3 x SSC, 0.1% SDS at room temperature and once at an allele specific temperature in 3 x SSC, 0.1% SDS for 30 minutes, before exposing to X-ray film for 2-16 hours.

The critical temperature for each probe was determined by washing each filter in a series of solutions of increasing temperature (1-2°C), from a base temperature of 48°C, based on the data of Hata et al. (1989). The allele specific temperatures were 53°C (Arg-> Gln mutant probe), 55°C (Arg->Stop mutant probe) or 57°C (wild type probe).

2.2.7   **ACRYLAMIDE GEL ELECTROPHORESIS**

Denaturing polyacrylamide gels were used for the analysis of sequenced PCR products, nondenaturing polyacrylamide gels for single strand conformation polymorphism analysis. Gel preparation and electrophoresis were carried out using BRL equipment and on a BRL model 2 sequencing apparatus.

2.2.7.1   **ACRYLAMIDE GEL PREPARATION**

A large (42 x 33cm) and a small (39 x 33cm) glass plate were washed with detergent, rinsed with tap water and ddH$_2$O, and wiped with 70% industrial methylated spirits. The

smaller of the two plates was placed in a fume hood and the prewashed surface coated with a siliconising fluid (Repelcote, Sigmacote). The silicon coating prevented the gel from adhering to the small plate and ensured that on plate separation, the gel remained bound to only one, the large, plate.

The gel mould was formed by placing the large plate on a flat surface, arranging three 0.4mm spacers along the sides and base and placing the smaller plate (siliconised face inwards) on top. After ensuring that the sides and bases of both plates were aligned, the edges were clamped (at least 3 per side) with 5cm length bulldog binder clips, and a water tight buffer cavity was formed by placing foam pads on the spacers at the point where the smaller plate ended.

Two gel solutions were used: ACCUGEL 40™ and PROTOGEL™, which differed in the ratio (19:1, 37.5:1) of acrylamide to bisacrylamide solution and hence the potential for crosslinking in the gel. Sequencing gels, in which separation depended on size alone, were prepared with the more crosslinked ACCUGEL 40™, while SSCP gels, in which separation depended also on conformation, were prepared with the less crosslinked PROTOGEL™, to enable slight differences in shape to be more readily detected.

A 6% gel solution (200ml) was made up in filtered 1 x TBE and fresh ammonium persulphate (0.5%) was added to promote free radical formation and hence polymerisation. Denaturing gels contained in addition 8.3M urea, which was dissolved by heating the gel solution in a 65°C water bath followed by cooling at room temperature, while 20°C SSCP gels contained 5-10% glycerol.

Prior to gel pouring, the plates were wedged to create a 45°C incline. This reduced the hydrostatic pressure at the base of the mould and prevented leakage. The base was presealed with 2-3ml of gel solution to which 20µl of the polymerisation catalyst TEMED had been added. A syringe (5ml) was filled with gel solution, placed at right angles to the space between the two plates and the gel was poured down one side.

Once the base had set (2-5 minutes), 25µl of TEMED was added to 50ml of gel solution and a 50ml syringe (without plunger) was positioned on one side of the gel mould and filled with gel solution. A steady and continuous flow of solution was maintained until the mould was full and any air bubbles were dispelled by gently raising the plates until vertical and tapping below the bubble. A shark's tooth comb (sequencing gels), or a 32 well comb or a shark's tooth comb with each second tooth removed (SSCP gels), was inserted into the top of the gel and clamped with bulldog clips.

The gel was left to polymerise for two hours at room temperature before electrophoresis or storage for 2-24 hours at room temperature. For storage the top of the gel was covered with paper towels soaked in 1 x TBE and sealed with clingfilm.

2.2.7.2 ACRYLAMIDE GEL ELECTROPHORESIS

The base of the BRL sequencing apparatus was filled with 1 x TBE (500ml). The clips and the spacer at the base of the gel were carefully removed, the plates were attached to the apparatus and the upper reservoir was filled. The comb was removed and the wells were washed to remove any unpolymerised acrylamide, using a hypodermic needle and a 5ml syringe. Air bubbles at the base of the gel plates were similarly removed. The sequencing apparatus was attached to a Consort E734 power pack and the gel was prerun for 1 hour at 55-60W (sequencing gel) or for 1/2 hour at 460-700V (SSCP gel). This equilibrated the gel with the buffer and, in the case of sequencing gels, preheated the gel to the optimum running temperature of 55-60°C.

After prerunning, the wells were rewashed, the shark's tooth comb was replaced (if appropriate), the samples were loaded and electrophoresis was carried out.

2.2.7.3 GEL FIXING, DRYING AND AUTORADIOGRAPHY

After electrophoresis, the gel plates were removed from the apparatus, placed on a flat surface and the smaller plate was eased away from the larger one. The gel was blotted

onto 3MM Whatman paper, covered in cling film and dried under vacuum on a ATTO gel drying processor (AE3700) at 80°C for 1/2-2 hours. The cling film was removed and the gel was exposed to X-ray (Kodak X-OMAT-AR) film for 3-20 hours at -70°C.

Before drying, sequencing gels were presoaked for 5-15 minutes in a solution of 10% methanol and 10% acetic acid, to fix the gels and to wash away excess urea.

2.2.8   **SINGLE STRAND CONFORMATION**
            POLYMORPHISM (SSCP) ANALYSIS

2.2.8.1   **AMPLIFICATION AND LABELLING OF INDIVIDUAL**
            EXONS USING THE POLYMERASE CHAIN REACTION

Individual exons were amplified using the polymerase chain reaction. The reactions were carried out in a 50μl volume using 100ng of genomic DNA, 20-25 picomoles of each primer, 0.02mM dCTP, 0.2mM each of dATP, dTTP, dGTP, 0.1μl of alpha-^{32}PdCTP (3000Ci/mmol), 1 x reaction buffer (Promega) and 10^{-5}M TEMAC. Reactions were overlaid with approximately 25μl of mineral oil and amplified as described previously (Section 2.2.2.4), for 25 cycles. The PCR products were analysed by running a 5-10μl sample on a 1.5-2% agarose gel (2.2.2.4), followed by ethidium bromide staining and UV illumination. Only specific, single band products were suitable for analysis.

In addition, for the analysis of the PLP gene, PCR products of 300bp or larger were digested with an appropriate restriction enzyme, as described previously (Section 2.2.3), to bring the fragment size closer to the optimal 200bp or less (Hayashi. 1991).

Exon 1 and 3 PCR products were digested with *Msp*I. The 512bp exon 1 product was digested to fragments of approximately 400 and 112bp, while the 412bp exon 3 product was cut between codons 83 and 84, to fragments of approximately 166 and 246bp. The 335bp exon 4 product was digested by *Rsa*I into three fragments: a central 111bp section produced by digestion at codon 174 and 12bp into intron 4, and two fragments of

approximately 143 and 81bp.

2.2.8.2 NONDENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

For SSCP analysis, 4µl of each PCR product was mixed with an equal volume of stop solution (U.S.B.) and denatured at 94°C for 5-10 minutes on a Techne PHC-2 PCR machine. The samples were placed on ice or in a dry ice/ethanol bath for several minutes, centrifuged briefly at 10,000rpm to spin down condensation, and replaced on ice to prevent reannealing. 1/2-3µl of each sample was loaded onto a 6%, 37.5:1 acrylamide to N,N'-methylenebisacrylamide, nondenaturing polyacrylamide gel on a BRL model 2 Sequencing System.

Electrophoresis was carried out under two conditions: 4°C and 20°C (room temperature), to increase the probability that a change, if present, would be detected. Running conditions were 340-700V for 6-18 hours. Gels were transferred onto 3MM Whatman paper, dried under vacuum at 80°C and exposed to X-ray (Kodak-X-OMAT-AR) film for 3 hours to 1 week at -70°C in the absence of intensifying screens.

2.2.9 DIRECT SEQUENCING OF POLYMERASE CHAIN REACTION PRODUCT USING MAGNETIC BEAD TECHNOLOGY

2.2.9.1 PREPARATION OF BIOTINYLATED POLYMERASE CHAIN REACTION PRODUCT

A PCR reaction (2.2.2.4) was carried out in a 100µl volume for 30 cycles using 5-20picomoles of each primer, one of which was biotinylated at the 5' terminus. The PCR product was analysed by running a 7µl sample on a 1.5-2% agarose minigel (2.2.2.4). A single, specific band of good intensity was required for sequencing.

2.2.9.2   MAGNETIC (DYNA) BEAD PREPARATION

The required volume of beads (Dyna-M-280 Streptavidin beads, Dynal, 30μl per reaction) was prewashed with 2 x 100μl of TES to remove preservatives. A 1.5ml tube containing the beads was placed in the magnetic separation device (magnet, Promega) for 30 seconds and the supernatant was removed. The tube was removed from the magnet, the beads were resuspended in TES by gentle repeated flushing with a pipette, and were replaced in the magnet. After 30 seconds the supernatant was removed and the beads were resuspended in TES.

2.2.9.3   PREPARATION OF SINGLE STRANDED TEMPLATE

To produce single stranded DNA, 50-80μl of PCR product (avoiding oil) and 30μl of prewashed M-280 streptavidin Dyna-beads were mixed and incubated for 5 minutes at room temperature. This enabled the DNA to adsorb to the beads and the reaction was tapped periodically to prevent settling. The reaction tube was placed in the magnet and left for 30 seconds. The beads with bound double stranded PCR product collected to one side, while the supernatant containing the unincorporated nucleotides, the unbiotinylated primer, genomic DNA and other PCR reagents was removed.

To denature the double stranded product, the reaction was removed from the magnet, incubated with 100μl of 0.1M NaOH at room temperature for 5 minutes and replaced in the magnet. After 30 seconds the supernatant containing the unbiotinylated PCR strand was removed. The single stranded product was washed with 2 x 100μl of TES and 2 x 100μl of ddH$_2$O, resuspended in 7μl of ddH$_2$0 and stored at -20°C.

2.2.9.4   THE SEQUENCING REACTION

The sequencing reaction was carried out using a U.S.B. Sequenase kit, according to the manufacturer's protocol. The reaction was divided into three stages: annealing, labelling and chain termination.

In the first step, unbiotinylated sequencing primer was annealed to the single stranded PCR template. Two microlitres of 5 x reaction buffer and 1µl of primer (2-5picomoles) were added to 7µl of DNA template and incubated in a 65°C water bath for 2 minutes. The reaction was cooled slowly to 30°C by placing in a shallow beaker of water from the 65°C water bath and standing at room temperature for 30-45 minutes. Once annealed, the template was placed on ice and sequenced within 2 hours.

In the labelling step, the primer was extended using limiting concentrations of dNTPs, including radioactively labelled dATP. This resulted in a range of labelled extension products from a few, to several hundred nucleotides, in length.

One microlitre of 0.1M DTT, 2µl of diluted labelling mix, 0.5µl of alpha-^{35}SdATP (1000Ci/mmol) and 1.63 units of Sequenase version 2.0 enzyme were added to the annealed template-primer and the reaction was incubated for 1.5-3 minutes at room temperature or in a 20°C water bath.

The range of sequence data that could be obtained from a reaction was determined by the concentration of the labelling mix, the incubation time and the reaction temperature. To obtain sequence within 30 nucleotides of the sequencing primer: the labelling mix was diluted 15 to 20 fold, the labelling time was 1.5 minutes and the extension temperature was below 15-20°C.

In the termination step, the concentration of the dNTPs was increased and a ddNTP was added. Rapid extension of the labelled DNA occurred, ceasing for each strand, with the incorporation of a ddNTP. 2.5µl of each termination mix (ddATP, ddGTP, ddTTP, ddCTP) was aliquoted into separate tubes and prewarmed at 37°C for 5 minutes before adding 3.5µl of the labelling reaction and incubating at 37°C for 3 minutes. The reactions were stopped by adding 4µl of stop solution and stored at -20°C for up to 1 week.

Secondary structure, which resulted in premature termination and compressed regions on electrophoresis, was eliminated by terminating at 45°C and substituting the nucleotide

analog dITP for dGTP. dITP formed weaker secondary interactions than dGTP, but, as the quality of the resulting sequence was reduced, the reactions were run in conjunction with dGTP.

2.2.9.5   DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The labelled strands were separated from the beads by denaturing the reactions at 65°C for 5 minutes, placing them in the magnet and removing the supernatants. These were heated to 85°C for 2 minutes, $2\mu l$ was loaded onto a 6% denaturing gel (2.2.7.2) and electrophoresis was carried out at 55-60W for 1-3 hours.

Alternatively, the sequencing products were denatured at 65°C and loaded without prior separation from the beads. These remained in the wells and did not interfere electrophoresis.

Approximately 300-400bp of sequence was obtained from a gel. Sequence close to the primer (0-200bp) was obtained after 1-1.5 hours, while 2-3 hours was required to separate more distant (200-400bp) bands. In cases where all the sequence was required, identical samples were loaded, at 1.5 hour intervals onto one gel.

After electrophoresis the gel was fixed, dried and autoradiographed as described previously (2.2.7.3).

2.3.0   DNA PROBES AND RESTRICTION
FRAGMENT LENGTH POLYMORPHISMS (RFLPs)

2.3.1   THE ORNITHINE CARBAMOYL TRANSFERASE GENE

Two *Msp*I polymorphisms (Fig.9) with 6.6 and 6.2kb (upper), and 5.1 and 4.4kb (lower) alleles were detected using a 1.5kb OCT cDNA probe covering exons 1-10 of the OCT gene. The probe was kindly provided by Dr S Bhattacharya.

EXON   BAND SIZE (kb)

10 -   - 17.5

5 -   ← 6.6
5 -   ← 6.2
9 -   - 5.4
1 -   ← 5.1
1 -   ← 4.4

2/4 -   - 3.5

3 -   - 2.4

6/7/8 -   - 1.9
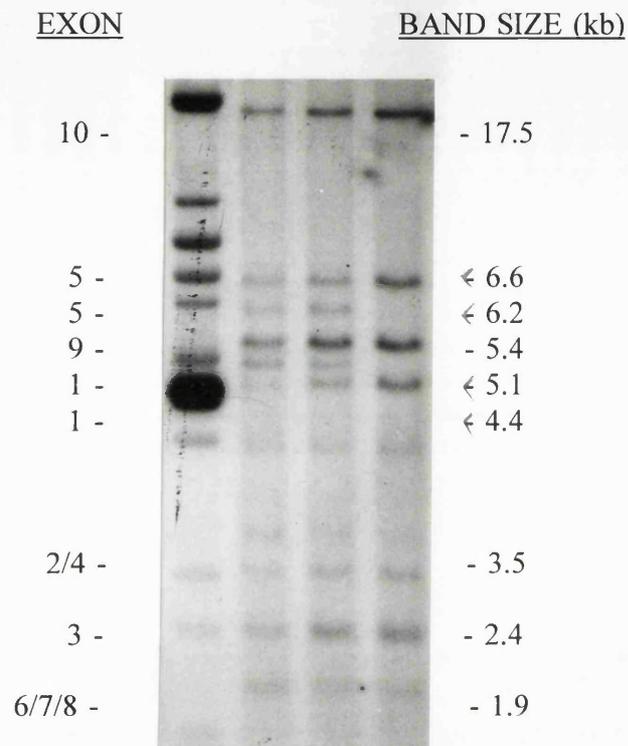
FIGURE 9 Southern blot analysis of *Msp*I digested DNA showing the characteristic pattern on hybridisation with OCT cDNA. The arrows indicate the polymorphic 4.4/5.1 and 6.2/6.6kb bands. Track 1 contains *Bst*EII digested lambda DNA as a molecular weight marker. The final post hybridisation wash was at 65°C for 30 minutes in a solution of 0.5 x SSC and 0.1% SDS.

The exons corresponding to each band, as mapped by Grompe et al. (1991), are indicated on the left. The bands representing exons 2,4 and 6-8 could only be assigned to either the 3' or 5' half of the gene and thus the bands marked 2/4 and 6/7/8 may represent either exon 2 or 4, and 6, 7 or 8 respectively.
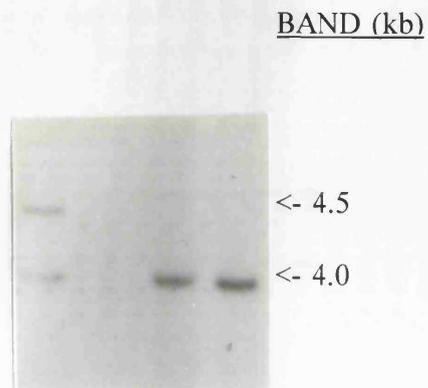
FIGURE 10 The intragenic PLP 4.0/4.5kb *Msp*I polymorphism. The arrows indicate the polymorphic bands detected on Southern blot analysis of genomic DNA by probe pRL1, a 2kb PLP cDNA.

BAND (bp)

<- 227
<- 183

<- 44
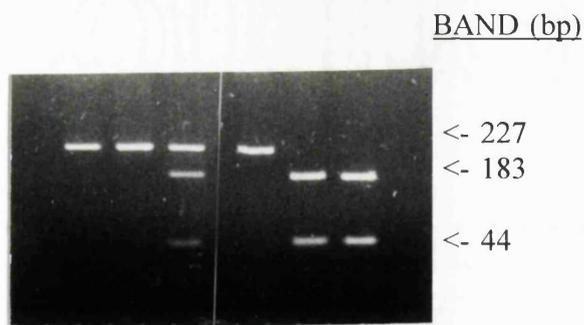
FIGURE 11 The characteristic pattern obtained on *Aha*II digestion of PCR product of exon 4 of the PLP gene. In the presence of the cutting site the 227bp product is digested to fragments of 183 and 44bp. Digestion fragments are separated on a 4% Nusieve-GTG-agarose gel and visualised by ethidium bromide staining.

TABLE 9- Details of probes and Polymorphisms in the Xq21.3-q22 region

CHROMOSOMAL LOCATION	SYMBOL	PROBE	ENZYME	CONSTANT BANDS (kb)	POLYMORPHISM (kb)	FREQUENCY	PROBABLE FREQUENCY OF RECOMBINATION WITH PLP	FINAL WASH CONDITIONS
Xq21.3	DXS3	p19-2	*TaqI* *MspI*	- -	3.0+2.0/5.0 4.4/12.0	0.62/0.38 0.75/0.25	) 8%)	) 0.5 x SSC)
Xq21.33 - q22	DXS178	p212-9	*TaqI*	7.0	1.8/3.2	0.30/0.70	2%	0.2 x SSC
Xq21.33 - q22	DXS101	CX52.5	*MspI*	11.0, 6.0, 2.5,2.0,1.9	7.5/7.7	0.65/0.35	2%	3-5 x SSC
Xq22	PLP	pRL1	*MspI*	1.8	4.0/4.5	0.92/0.08	Zero	0.5 x SSC
Xq22	PLP	Exon 4 PCR Product	*AhaII*	-	0.044 + 0.183/0.277	0.26/0.74	Zero	-
Xq22	DXS94	pXG-12	*PstI*	-	6.5/7.2	0.52/0.48	2%	0.2 x SSC
Xq22	DXS17	S21	*TaqI*	4.0, 1.0	2.0/2.2	0.35/0.65	4%	0.2 x SSC

2.3.2   <u>THE PROTEOLIPID PROTEIN GENE</u>

Two intragenic polymorphisms within the PLP gene were analysed. A 4.0/4.5kb *Msp*I polymorphism was detected by hybridisation of pRL1, a 2kb PLP cDNA (Wu et al. 1987), to Southern blots of *Msp*I digestion products (Fig.10). The second polymorphism created an *Aha*II site within exon 4 (Trofatter et al. 1991) and was analysed by digestion of exon 4 PCR product, followed by the separation of the resulting fragments on a high percentage agarose gel (Fig.11).

Intergenic *Msp*I, *Taq*I and *Pst*I polymorphisms from the Xq21.3-q22 region were detected with probes S21, p19.2, CX52.5, pXG-12 and p212-9. Details of all probes and polymorphisms analysed are given in Table 9. The intragenic pRL1 probe was kindly provided by Dr C. Kinnon.

2.4.0   **MATERIALS**

All chemicals, with the exception of those listed below, were of AnalaR grade and were obtained from British Drug Houses, UK or were supplied by Sigma.

Agarose was supplied by the Palliard Chemical Company, U.K.

Accugel and Protogel were supplied by National diagnostics, U.K.

Bacto-Agar was supplied by Difco Laboratories, U.S.A.

^{32}P-dCTP (3000Ci/mmol) was supplied by ICN Biomedicals Ltd, U.K. and Amersham International plc.

Alpha-^{35}S-dATP (1000Ci/mmol), ^{32}P-dATP (3000Ci/mmol), the Qiagen mini-preparation kit and Hybond-N/N+ were supplied by Amersham International plc.

Dyna-M-280 Streptavidin beads were supplied by Dynal U.K.

dNTPs, hexadeoxyribonucleotides, Ficoll 400 and G-50 Medium grade Sephadex were obtained from Pharmacia.

The Sequenase version 2 sequencing kit and stop solution were obtained from United States Biochemical.

Nusieve-GTG-agarose was supplied by Flowgen.

Taq Polymerase and reaction buffer were supplied by Promega, U.K.

Kodak XAR-5 X-ray film, was supplied by the Eastman Kodak Co. U.S.A.

All restriction enzymes and their respective buffers were obtained from Northumbria Biologicals Limited, with the exception of *Fnu*4HI, *Cfo*I, and *Bsa*HI which were obtained from New England Biolabs.

Water saturated distilled phenol was obtained from Rathburn Chemicals Ltd. U.K.

3.0.0 RESULTS: ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY

3.1.0 CASE STUDIES: PEDIGREES AND CLINICAL DETAILS

3.1.1 CLASSICAL ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY

FAMILY PD

The pedigree of family PD is shown in Figure 12A. The female proband (II-3) is 3.5 years of age and presented at the age of 21 months with developmental delay (around the 9 to 10 month level) and poor growth (-3.62SD). Increased urine glutamine and orotic acid confirmed OCT deficiency. On a low protein diet, sodium benzoate and arginine, she has done well and her growth has caught up somewhat (now being only - 1.96SD). At 3.5 years of age her development is now borderline normal. There was no previous family history and protein load tests in the mother (I-2) and sister (II-I) proved negative.

FAMILY TM

The pedigree of family TM is shown in Figure 12B. The male proband (II-2) and his elder brother (II-1) were diagnosed as suffering from classical OCT deficiency. There was no previous family history.

FAMILY HM

The pedigree of family HM is shown in Figure 12C. The male proband (III-1) was diagnosed as suffering from classical OCT deficiency. There was no previous family history and protein load tests were found to be abnormal in the mother (II-6) and sister (III-2) of the proband (III-1), but not the grandmother (I-2) or aunt (II-2).

I

II

A (Family PD)

I

II

B (Family TM)

I

II

III

C (Family HM)
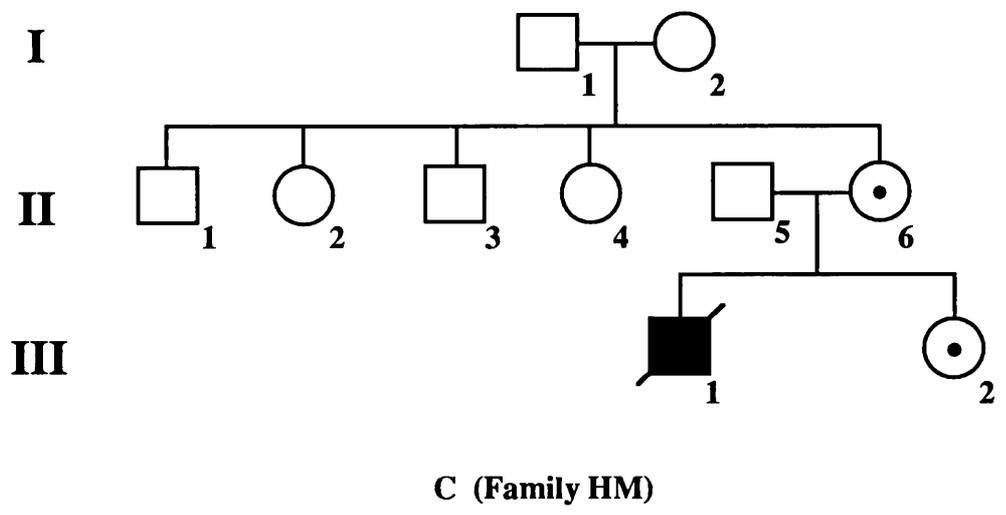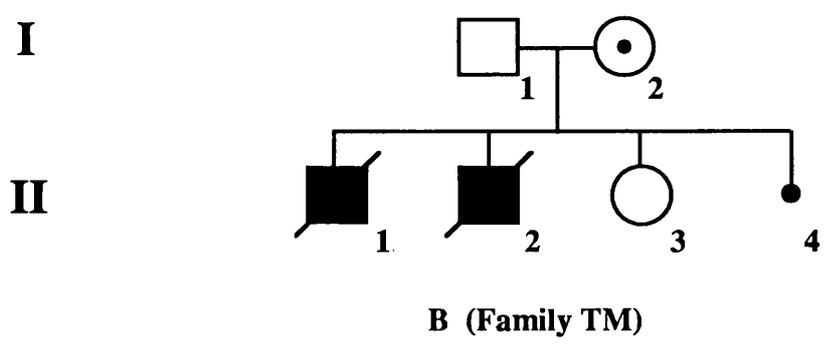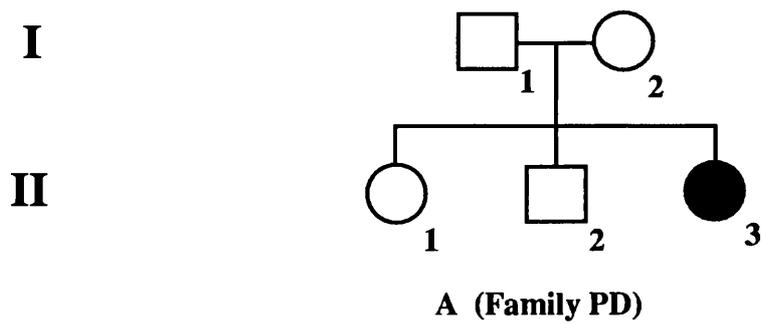
<u>FIGURE 12:</u> Pedigrees of families with classical OCT deficiency in which sequence variations were identified. Females are indicated by circles, males by squares, where a dotted circle represents a carrier female and a shaded shape an affected individual.

3.1.2   **LATE ONSET ORNITHINE CARBAMOYL**
              TRANSFERASE DEFICIENCY

FAMILY CFA

The pedigree of family CFA is shown in Figure 13A. The affected male (II-1) has been previously described by Drogari and Leonard (1988) as case 1. He was first shown to have elevated levels of plasma ammonia at 12 years of age, following a hospital admission for 'confusion'. At 14 years of age he was admitted to hospital, deeply unconscious, following a high protein meal the previous night. His plasma ammonia concentration was 292μM. He has since been treated with a low protein diet, arginine supplements and sodium benzoate and has subsequently qualified as a doctor. There was no previous family history and protein load tests were found to be abnormal in his mother (I-2) and sister (II-2).

FAMILY FAR

The pedigree of family FAR is shown in figure 13B. The affected males (III-2, III-4) have previously been described as cases 2 and 3 by Drogari and Leonard (1988). The oldest son (III-1) in the family was well until 13 months of age, when he started to vomit and became drowsy. He died at 16 months of age after a short encephalopathic illness, called at the time 'cerebral degeneration', and, in view of the subsequent family history, now believed to be OCT deficiency. The second child (III-2) in the family was diagnosed as having OCT deficiency at the age of 17 months after an episode of vomiting during which he became drowsy and floppy. The plasma ammonia concentration was 330μM, with abnormal liver function and raised urine orotic acid concentration. The residual ornithine carbamoyl transferase activity was 1-5%, confirming a diagnosis of OCT deficiency. He is now at 17 years of age progressing well on a low protein diet. III-4 was investigated at the age of one month and is developing normally at 12 years of age, with only one reported incidence of unconsciousness at the age of 13 months.
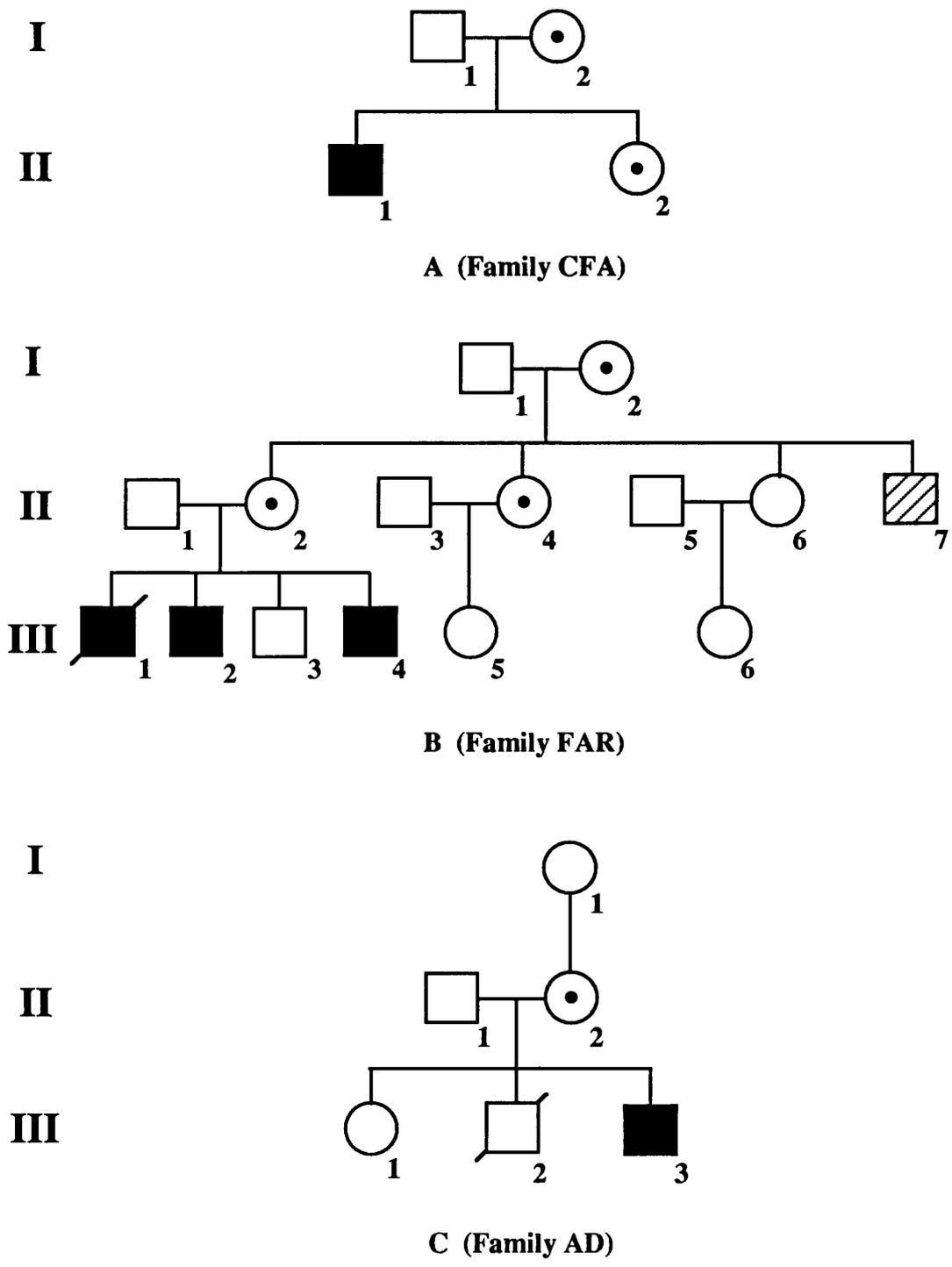
FIGURE 13: Pedigrees of families with late onset OCT deficiency in which sequence variations were identified. Females are indicated by circles, males by squares, where a dotted circle represents a carrier female and a shaded shape an affected individual. A partially shaded shape indicates an asymptomatic individual.

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The clinically undetected male (II-7) had episodes of screaming and vomiting between the ages of 5 months and 3 years, but has since remained well.

Although no previous family history of OCT deficiency had been noted in this family, the sisters (II-4, II-6) and mother (I-2) of II-2 retained a significant risk of carrier status. Protein load testing of the three daughters of I-2 identified two (II-4, II-2) as carriers and excluded the third (II-6).

FAMILY AD

The pedigree of family AD is shown in figure 13C. The affected boy (III-3) was well until 8 months of age when he became irritable, feverish and fed poorly. Increasing drowsiness with abdominal distension and constipation led to an initial diagnosis of viral encephalopathy. Subsequent investigations to eliminate a possible metabolic abnormality identified hyperammonaemia and raised levels of transaminases, methionine, alanine and glutamine. A percutaneous liver biopsy showed that the residual OCT activity was 5.5-20% and a diagnosis of OCT deficiency was made. Currently at the age of 6 years he is developmentally retarded, having little vocabulary and limited social skills, and has moderately severe spastic paraparesis. There was no previous family history and the protein load test identified his mother (II-2) as a carrier.

3.2.0   SOUTHERN BLOT ANALYSIS

3.2.1   DELETION SCREEN

Twenty nine families with classical or late onset OCT deficiency were screened for partial or total deletions of the OCT gene. Genomic DNA from 7 males and 22 symptomatic or carrier females was digested with *Taq*I. DNA from 7 males and 13 symptomatic or carrier females was digested with *Msp*I. In each case the digestion products were analysed by Southern blotting using OCT cDNA as a probe.

An unusual band pattern was identified in one case, in family TM (Fig. 12B). The creation of a novel 3.1kb band and the partial loss of the 4.6kb band was detected on *Taq*I analysis in a boy (II-2) with classical OCT deficiency and his carrier mother (I-2) (Fig. 14). The change may represent a deletion, rearrangement or the creation of a new *Taq*I site within the 4.6kb fragment. No change was detected in the characteristic *Msp*I pattern on analysis of DNA from the affected boy (II-2) in this family.

No additional deletions or rearrangements were seen in the 29 and 20 families investigated with *Taq*I and *Msp*I respectively.

3.2.2 DETECTION OF MUTATIONS AT CYTOSINE-GUANINE DINUCLEOTIDES WITHIN *TAQI* AND *MSPI* RESTRICTION ENZYME SITES

Twenty nine families with classical or late onset OCT deficiency were screened for changes at the 5'CpG 3' dinucleotide sites within the 4 *Taq*I sites located in the OCT coding region (Fig. 6).

Southern blot analysis of *Taq*I digested DNA using OCT cDNA as a probe, identified an altered band pattern in one family, in a symptomatic female. The proband (II-3) in family PD (Fig. 12A) showed the characteristic pattern of the loss of the exon 5 *Taq*I site, that is the gain of a 3.4kb band and loss, here partial as predicted for a heterozygote, of two bands of 1.8 and 1.7kb (Fig. 15). Her mother (I-2) and sister (II-1) had only the wild type pattern and hence were not carriers.

Twenty families were screened for changes at *Msp*I sites. Genomic DNA from 7 males and 13 symptomatic or carrier females was digested with *Msp*I and probed with OCT cDNA. No altered band patterns were seen.

BAND
SIZE
(kb)

4.6 ->

3.7 -
3.4 -
3.1 ->
2.6 -

2.0 -
1.8 -
1.5 -

1   2   3   4   5
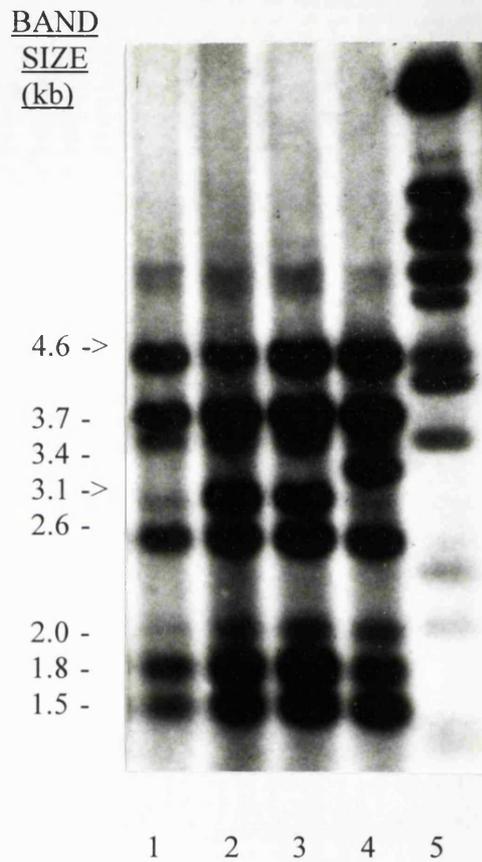
FIGURE 14 Southern blot analysis of *Taq*I digested DNA from the carrier mother (I-2) (Track 3) and the affected male (II-2) (Track 2) in family TM (Fig. 12B). The arrows indicate the novel 3.1kb band and the 4.6kb band which is partially lost. Track 5 contains *Bst*EII digested lambda DNA as a molecular weight marker, track 1; control DNA. Track 4 contains, for comparison, DNA from the proband (II-3) in family PD, in whom a mutation at the *Taq*I site in exon 5 results in the creation of a new 3.4kb band.

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BAND SIZE
kb

4.6 -

3.7 -

3.4 ->

2.6 -

2.0 -

1.8 ->
1.7 ->

1.5 -

1   2   3   4   5   6   7
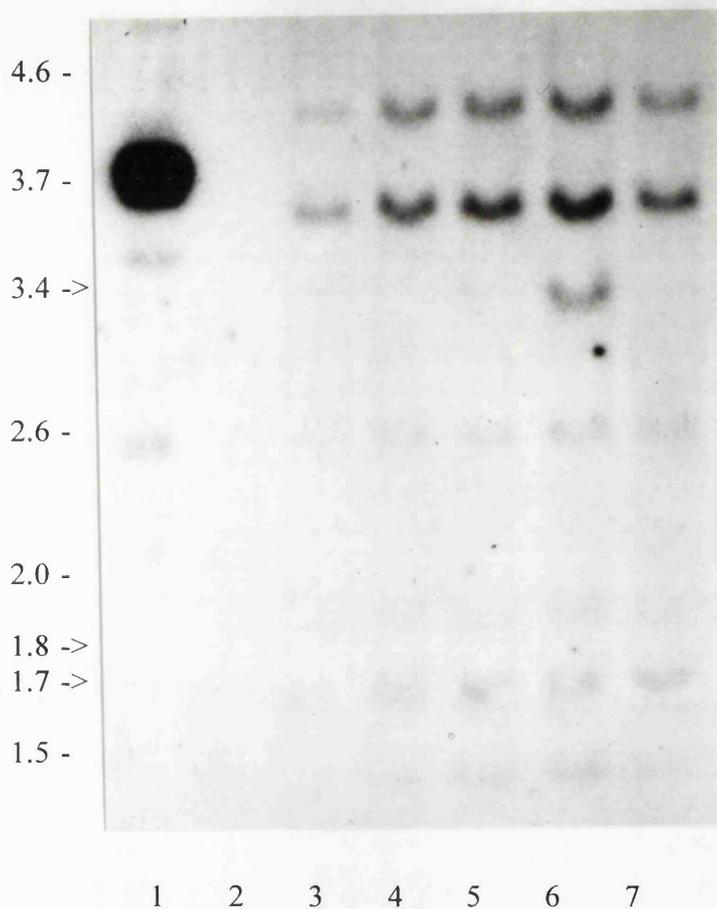
FIGURE 15 Southern blot analysis of *Taq*I digested DNA from family PD (Fig. 12A) showing, in the symptomatic female (II-3), the characteristic pattern of a mutation in the exon 5 *Taq*I site. The arrows indicate the novel 3.4kb band and the 1.8 and 1.7kb bands which are partially lost. Track 1 contains *Bst*EII digested lambda DNA as a molecular weight marker, tracks 3-7: DNA from II-2 (brother), II-1 (sister), I-1 (father), II-3 (proband) and I-2 (mother) respectively. Track 2 is blank.

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The Southern blot result in family PD (Fig. 12A) was verified by *Taq*I digestion of exon 5 PCR products (Fig. 16) and separation of the products on a high percentage agarose gel. In all family members, excepting the proband (II-3), the 156bp product was completely digested to 121 and 35bp fragments. II-3 showed partial digestion having a 121bp product from her unaffected X-chromosome and an undigested 156bp fragment from the defective X-chromosome. The 35bp band was lost from the gel during electrophoresis.

3.2.3.2   ALLELE SPECIFIC OLIGONUCLEOTIDE ANALYSIS

Exon 5 PCR products from family PD were tested for the 2 probable mutations resulting from a deamination at the 5'CpG 3' site within the *Taq*I restriction site. These were either a C to T change, or a G to A change. Radiolabelled oligonucleotides corresponding to the sense strand of the normal sequence and to the sense strands of the 2 potential mutant alleles were hybridised to triplicate dot blotted filters. The proband (II-3) showed clear hybridisation to the normal oligonucleotide, but with reduced intensity as expected for a heterozygote, and strong hybridisation to the oligonucleotide corresponding to the arginine to glutamine change (Fig. 17). Her mother (I-2) showed only hybridisation to the wild type sequence indicating that II-3 represented a new mutation.

3.3.0   PCR BASED TESTING FOR MUTATIONS AT CYTOSINE-GUANINE DINUCLEOTIDE SITES

3.3.1   RESTRICTION ENZYME ANALYSIS

The restriction sites analysed and their corresponding exons are given in Table 10. DNA from affected individuals or known carriers from 29 families was screened for
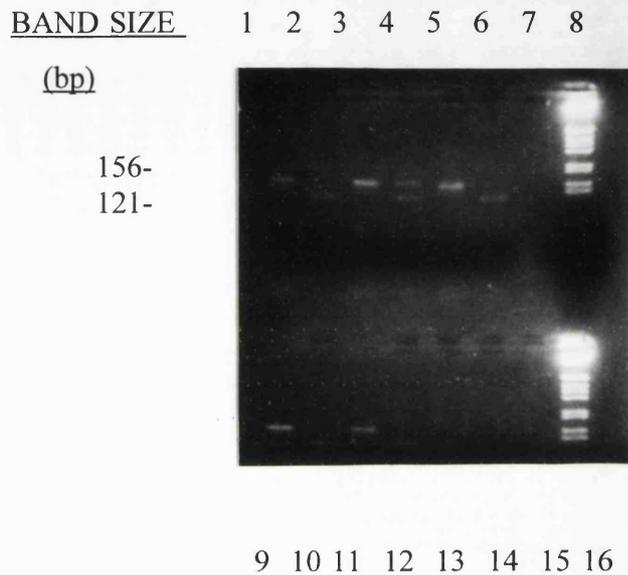
FIGURE 16 Analysis of *Taq*I digested exon 5 PCR products from family PD (Fig. 12A) on a 4% Nusieve-GTG-agarose gel. All family members, excepting the proband (II-3), showed complete digestion of the 156bp product to 121 and 35bp fragments, while she showed partial digestion, having both the 156 and 121bp fragments. The 35bp band was lost from the gel during electrophoresis. Tracks 1-2, 3-4, 5-6, 9-10 and 11-12 contain DNA from I-1 (father), II-3 (proband), II-1 (sister), II-2 (brother) and I-2 (mother) respectively, where each pair of tracks shows the amplified product undigested on the left and digested with *Taq*I on the right. Tracks 7 and 13-15 are blank, 8 and 16 contain lambda kb molecular weight marker.

MOTHER (I-2)

PROBAND (II-3)

SISTER (II-1)

BROTHER (II-2)

FATHER (I-1)

A   B
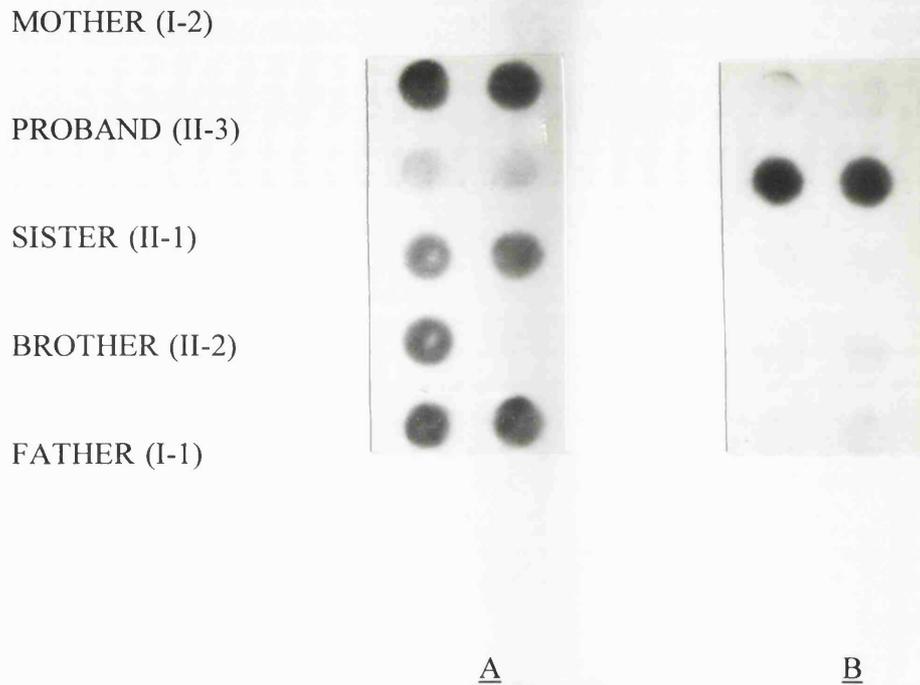
FIGURE 17 Detection of the G to A point mutation which results in the Arg(109)Gln change in family PD (Fig. 12A), using allele specific oligonucleotide hybridisation. (A) Exon 5 PCR products from family members hybridised with the wild type oligonucleotide. Duplicate samples were applied in each case, except for the brother (II-2). (B) Exon 5 PCR products from family members hybridised with the oligonucleotide detecting the G to A change.

Washes were at 57°C and 53°C for wild type and mutant (Arg->Gln) probes respectively and the symptomatic female showed hybridisation with both the wild type and the Arg->Gln oligonucleotides.

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EXON	ENZYME	SIZE OF DIGESTION PRODUCTS (BP)
1	*Hinf*I (GANTC)	218 $\longrightarrow$ 79 + 139
1	*Taq*I (TCGA)	218 $\longrightarrow$ 173 + 45
4	*Msp*I (CCGG)	119 $\longrightarrow$ 25 + 94
6	*Cfo*I (GCGC)	171 $\longrightarrow$ 64 + 107
7	*Msp*I (CCGG)	322 $\longrightarrow$ 26 + 296
8	*Cfo*I (GCGC)	322 $\longrightarrow$ 106 + 216

TABLE 10: 5' CpG 3' dinucleotide containing restriction sites analysed. The table shows the location of each site and the expected sizes of the digestion products on digestion of PCR amplified product from each exon. In the case of exon 7 and 8 CpG sites, exon 7 + 8 PCR product was analysed

mutations at the exon 1 *Taq*I site. Exon 1 was amplified by the PCR and the products were digested with *Taq*I and separated on high percentage agarose gels (Fig. 18). In each case the 218bp product was digested to a band of 173bp, showing that there were no mutations at the *Taq*I site in this exon. The other 45bp fragment was lost from the gel during electrophoresis.

The subsequent assignment of the individual OCT exons to bands on a *Msp*I blot (Grompe et al. 1991) mapped exons 2, 4 and 7 to bands which, in many blots, were of variable intensity (Section 2.3.1, Fig. 9). Also, a mutation was only detectable in a heterozygous female if it resulted in an altered band pattern. In the majority of families investigated the proband was a symptomatic female or insufficient DNA was available from affected males necessitating the analysis of carrier females.

To overcome these difficulties, the *Msp*I sites in exons 4 and 7 were screened by digesting PCR product from these exons and separating the resulting fragments on high percentage agarose gels.

Analysis of the exon 7 *Msp*I site in 33 families with late onset or classical OCT deficiency, comprising 54 X-chromosomes, identified one family with classical OCT deficiency in which digestion of the 322bp exon 7-8 PCR product was abolished. In family HM (Fig. 12C), the affected male (III-1) showed no digestion, while the carrier mother (II-6) and sister (III-2) showed partial digestion and the unaffected father (II-5) showed complete digestion of the 322bp PCR product to fragments of 26 and 296bp (Fig. 19).

Hentzen et al. (1991) have shown that this change replaces a 5.4kb constant band by a novel 5.8kb band on Southern blot analysis of *Msp*I digested DNA. Southern blot analysis of DNA from the carrier mother (II-6) showed the presence of both the wild type 5.4kb band and the mutant 5.8kb band (Fig. 20), as predicted.

Analysis of the exon 4 *Msp*I site in 29 families with late onset or classical OCT

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FIGURE 18 The characteristic pattern obtained on analysis of *Taq*I digested exon 1 PCR product on a 4% Nusieve-GTG-agarose gel. The 218bp product was digested to 173 and 45bp fragments when the *Taq*I site was present. The 45bp band was lost from the gel during electrophoresis. Tracks 1, 3, and 5 contain undigested product, tracks 2, 4 and 6, *Taq*I digested product, track 7 is blank, track 8 contains lambda kb molecular weight marker.

BAND
SIZE
(bp)

322-
296-

Control

Undigested

1   2   3   4   5
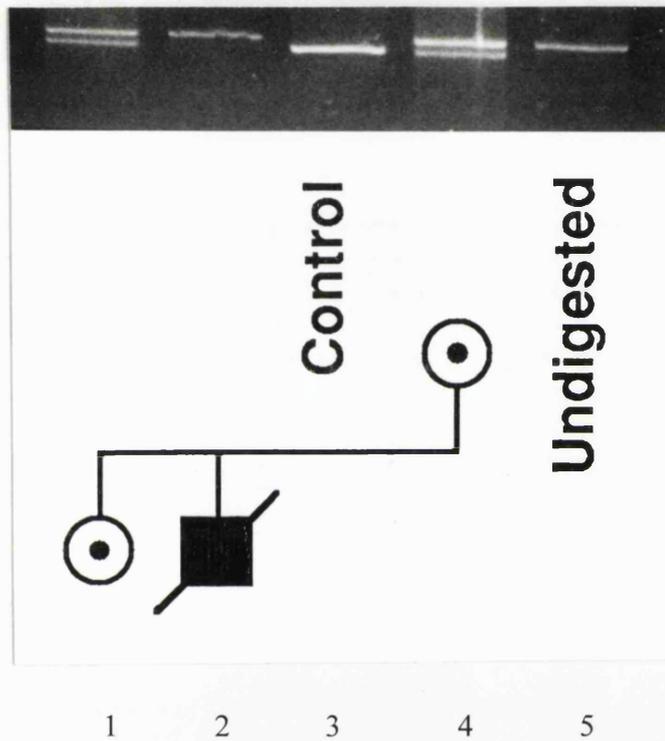
FIGURE 19 Analysis of *Msp*I digested exon 7-8 PCR products from family HM (Fig. 12C) on a 4% Nusieve-GTG-agarose gel. The 322bp wild type DNA was digested to 26 and 296bp fragments, while mutant DNA showed no digestion. Tracks 1-5: carrier sister (III-2), affected male (III-1), wild type control, carrier mother (II-6) and undigested DNA. Symbols are as for Figure 1.

BAND SIZE

bp

6.6 -

6.2 -

5.8 ->

5.4 ->
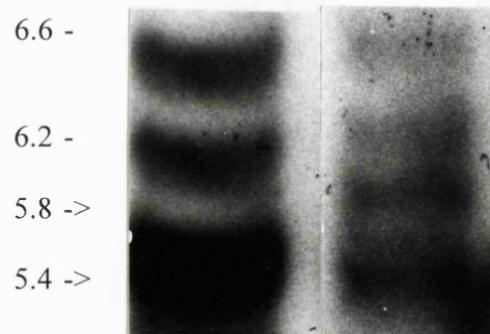
FIGURE 20   Southern blot analysis of *Msp*I digested DNA from family HM (Fig. 12C) showing the characteristic pattern of a mutation within the *Msp*I site in exon 7. The arrow indicates the novel 5.8kb band which the carrier mother (II-6), as a heterozygote, has in addition to the wild type 5.4kb band. Track 1: control DNA, track 2; II-6.

deficiency, identified a change in one family with late onset OCT deficiency. In family AD (Fig. 13C) the affected boy (III-3) showed no digestion of the 119bp PCR product, while his carrier mother (II-2) showed partial digestion, having both the mutant (119bp) and the wild type (25 + 94bp) fragments (Fig. 21).

This change was not seen on digestion of exon 4 PCR products from the remaining 4 late onset males or from carrier females and affected individuals with classical OCT, deficiency making up 51 X -chromosomes.

Analysis of the *Cfo*I sites in exons 6 and 8 in 33 families identified a change in exon 6 in a family with late onset OCT deficiency. In family CFA (Fig. 13A) the affected male (II-1) showed no digestion, while his carrier mother (I-2) and sister (II-2) showed partial digestion (Fig. 22) and his unaffected father (I-1) complete digestion of the 171bp exon 6 PCR product to 64 and 107bp fragments.

This change was not seen on digestion of PCR products from the 4 remaining late onset males or in an additional 50 X-chromosomes from carrier females and affected individuals with classical OCT deficiency.

No changes were identified on *Cfo*I restriction enzyme analysis of exon 8 or on *Hinf*I restriction enzyme analysis of exon 1 (Fig.23).

3.3.2 SEQUENCE ANALYSIS IN FAMILIES SHOWING UNUSUAL DIGESTION PATTERNS

Sequence analysis of exon 7 and 8 PCR product from the carrier mother (II-6) of family HM (Fig. 12C) identified a C to T change at nucleotide 674 of codon 225. This resulted in the replacement of a proline (CCG) by a leucine (CTG) at amino acid 193 of the mature OCT protein. The carrier mother (II-6) had both the C and T bands, while the wild type control had only the C band (Fig. 24).

BAND
SIZE
(bp)
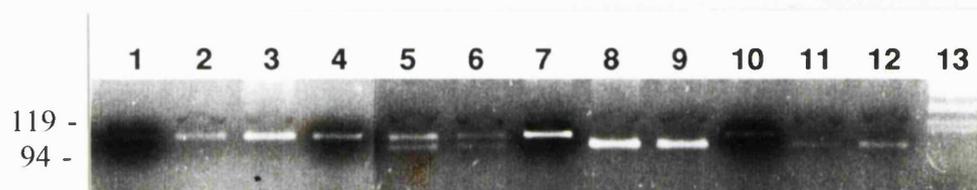
FIGURE 21 Analysis of *Msp*I digested exon 4 PCR product from family AD (Fig. 13C) on a 4% Nusieve-GTG-agarose gel. The 119bp wild type DNA was digested to 25 and 94bp fragments, while mutant DNA showed no digestion. Tracks 1-3 represent the affected boy (III-3) who showed no digestion, tracks 4-6 the carrier mother (II-2) who showed partial digestion, tracks 7-9 and 10-12, the father (II-1) and noncarrier maternal grandmother (I-1) who showed complete digestion. Track 13 contains lambda kb ladder molecular weight marker. In each triplet, the first track contains undigested, the second and third digested product. Symbols are as for Figure 1.
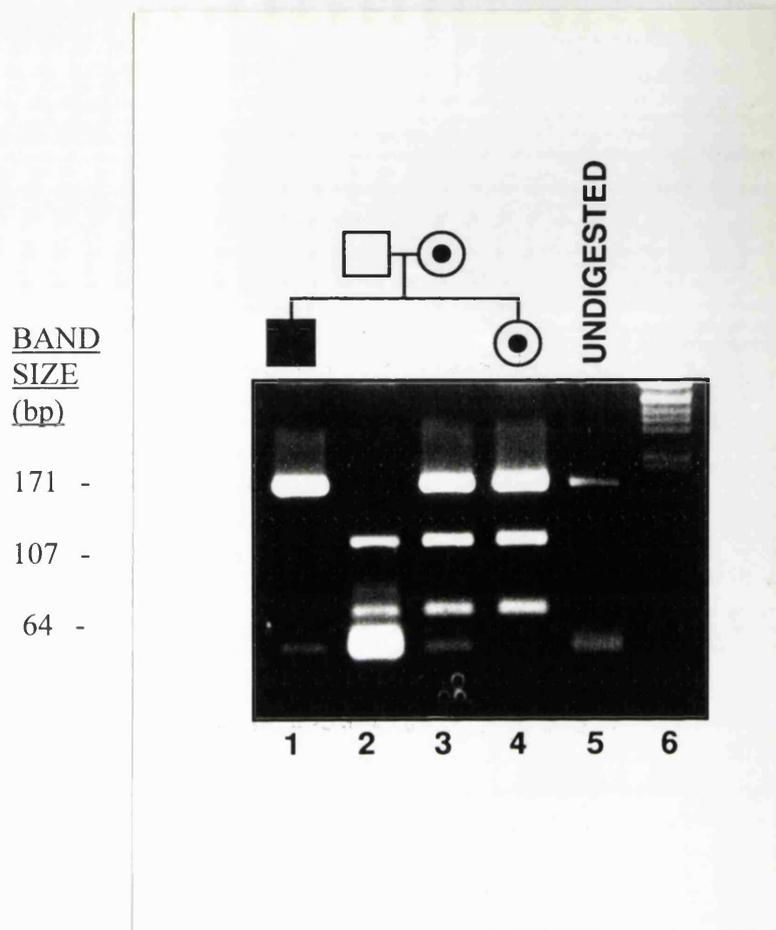
FIGURE 22 Analysis of *Cfo*I digested exon 6 PCR product from family CFA (Fig. 13A) on a 4% Nusieve-GTG-agarose gel. The 171bp PCR product was completely digested to 64 and 107bp fragments in the unaffected father (I-1) (track 2), while the proband (II-1) (track 1) showed no digestion and the carrier mother (I-2) and sister (II-2) (tracks 3 and 4) showed partial digestion. Track 5 contains undigested product and track 6 lambda kb ladder molecular weight marker. The lowest band represents unincorporated primer. Symbols are as for Figure 1.

BAND SIZE

bp

218 ->

139 ->

79 ->
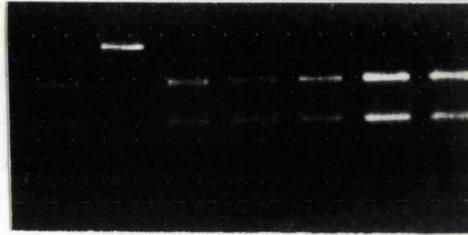
A

BAND SIZE

bp

322 ->

216 ->

106 ->
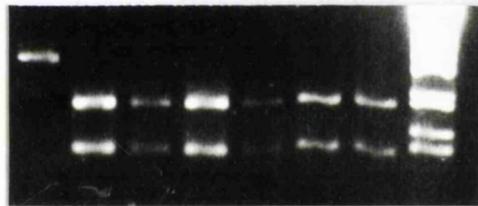
B

FIGURE 23 The characteristic patterns obtained on *Hinf*I and *Cfo*I digestion of PCR product of exons 1 (A) and 7+8 (B) of the OCT gene respectively. In the presence of an intact restriction site, the 218bp and 322bp products were digested to fragments of 79 and 139, and 106 and 216bp respectively. Digestion fragments were separated on 4% Nusieve-GTG-agarose gels and visualised by ethidium bromide staining.

CARRIER FEMALE (II-6)

```
                5'
            /   G
    GLU         A          C     A     T     G
            \   G
            /   C
  PRO/LEU       C/T   ->
            \   G
            /   G
    ASP         A
            \   T
            /   G
    ALA         C
            \   T
                3'
```

WILD TYPE CONTROL

```
                5'
            /   G
    GLU         A          C     T     A     G
            \   G
            /   C
    PRO         C     ->
            \   G
            /   G
    ASP         A
            \   T
            /   G
    ALA         C
            \   T
                3'
```
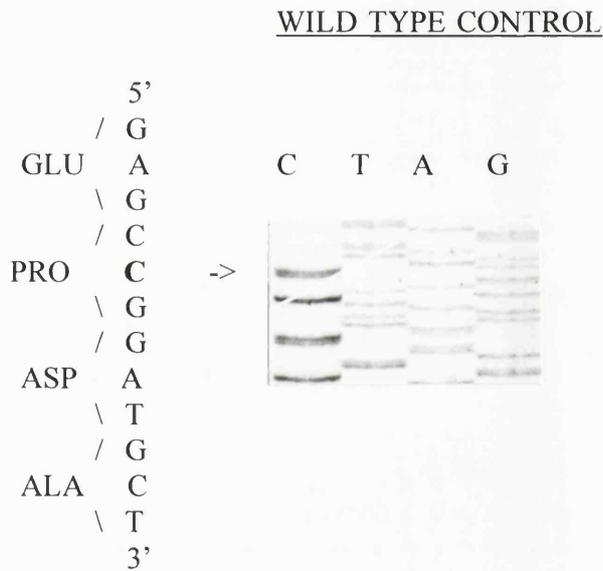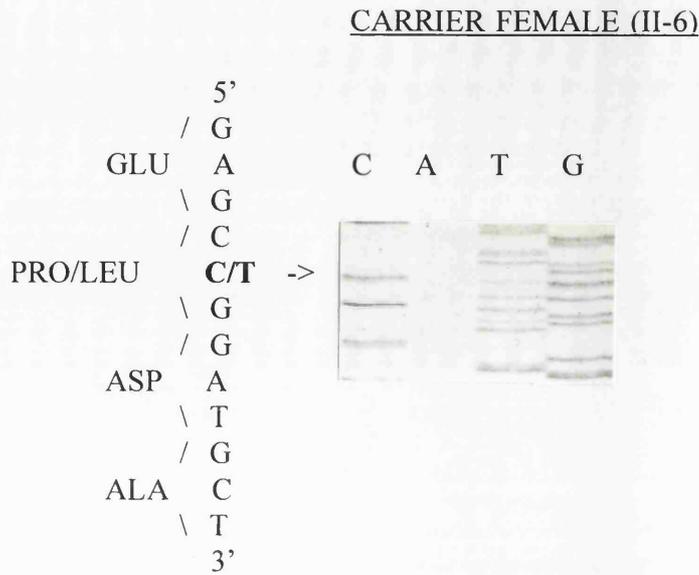
FIGURE 24 DNA sequence of exon 7 and 8 PCR product from the carrier mother (II-6) in family HM (Fig. 12C). The arrow indicates the C to T change at nucleotide 674, which resulted in the substitution of a proline by a leucine at amino acid 193 of the mature OCT protein. The carrier woman had both C and T bands, while the wild type control had only the C band. Band A in the carrier woman was too faint to detect. In each cases the CC and GG doublets were poorly resolved.

In family AD (Fig. 13C), sequence analysis of exon 4 identified a G to T change at the last base of exon 4 in the affected boy (III-3). This change, at nucleotide 386 of codon 129, was predicted to cause an arginine (C$\underline{G}$T) to leucine (C$\underline{T}$T) change at amino acid 97 of the mature protein (Fig. 25) and to alter the sequence of the 5' donor donor splice site of exon 4 (Fig. 26).

Sequencing of exon 6 PCR product in family CFA (Fig. 13A), identified a G to A change at the first nucleotide (622) of codon 208 in the affected boy (II-1) and his mother (I-2) and sister (II-2) who were heterozygous for the change. This was predicted to cause an alanine ($\underline{G}$CA) to threonine ($\underline{A}$CA) change at amino acid 176 of the mature OCT protein (Fig. 27).

3.4.0   LATE ONSET ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY

3.4.1   SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS

SSCP analysis of exons 1, 2, 4, 5, 6, 7+8, 9 and 10 (Table 11) of the OCT gene, in 5 families with late onset OCT deficiency, identified sequence variations in 3 families: AD, CFA and FAR, in exons 4, 6, and 8 respectively. Additional changes were not seen in these families, or in the remaining two late onset families.

In each case, analysis was carried out at both 4°C and 20°C to maximize the probability that a change, if present, was detected.

In family AD (Fig. 13C), SSCP analysis of exon 4 PCR product (Fig. 28) identified a variant pattern (bands 2, 4, 5, 6 and 7) in the affected male (III-3). The unaffected control had only the wild type pattern (bands 1, 3 and 5). This change was detected on analysis at 4°C, but not 20°C, and the mutant pattern was not seen on analysis of the remaining 4 late onset families.

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AFFECTED MALE (III-3)

```
          5'            T     G     A     C
       /  G
ALA       C
       \  C
LEU  /  C
       \  T      ->
          g
          t
          t
          t
          g
          3'
```

UNAFFECTED FATHER (II-1)

```
          5'            T     G     A     C
       /  G
ALA       C
       \  C
ARG  /  C
       \  G      ->
          g
          t
          t
          t
          g
          3'
```
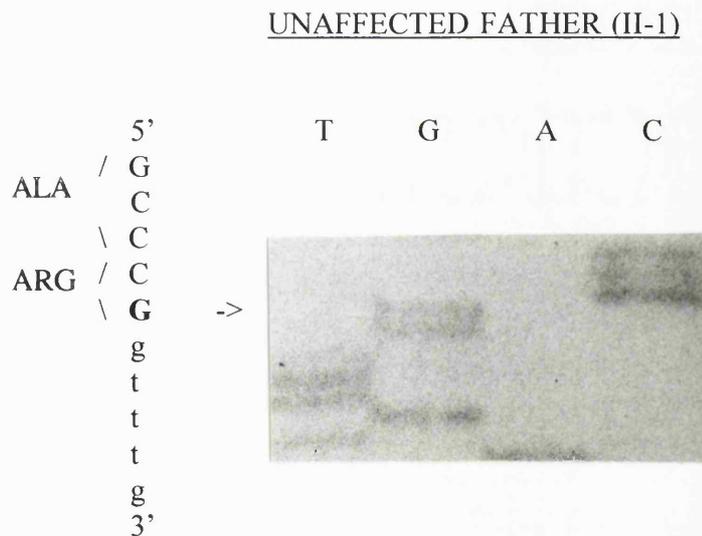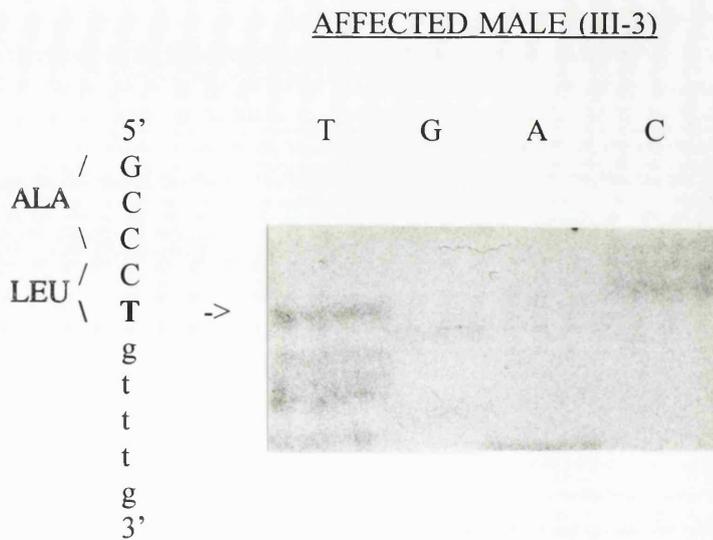
FIGURE 25   DNA sequence of exon 4 PCR product from the proband (III-3) and his unaffected father (II-1) in family AD (Fig. 13C). The arrow indicates the G to T change at the -1 position of the 5' donor splice site. This was predicted to cause an Arg to Leu change at amino acid 97 of the mature protein. The affected boy (III-3) had only the mutant T band, while the unaffected father (II-1) had only the wild type G band. The exonic bases are in capitals, intronic bases in lower case lettering.

```
        -INTRON 4-                    -INTRON 4-

5' G C C C G g t t t g  3'  ->  5' G C C C T g t t t g 3'


      --EXON 4--                    --EXON 4--
```

FIGURE 26 A schematic representation of the exon 4 / intron 4 boundary of the OCT
gene, showing the G to T change at the last base of exon 4 in family AD. This
occurred at the -1 position of the 5' donor splice site. The exonic bases are in capitals,
the intronic bases in lower case lettering.
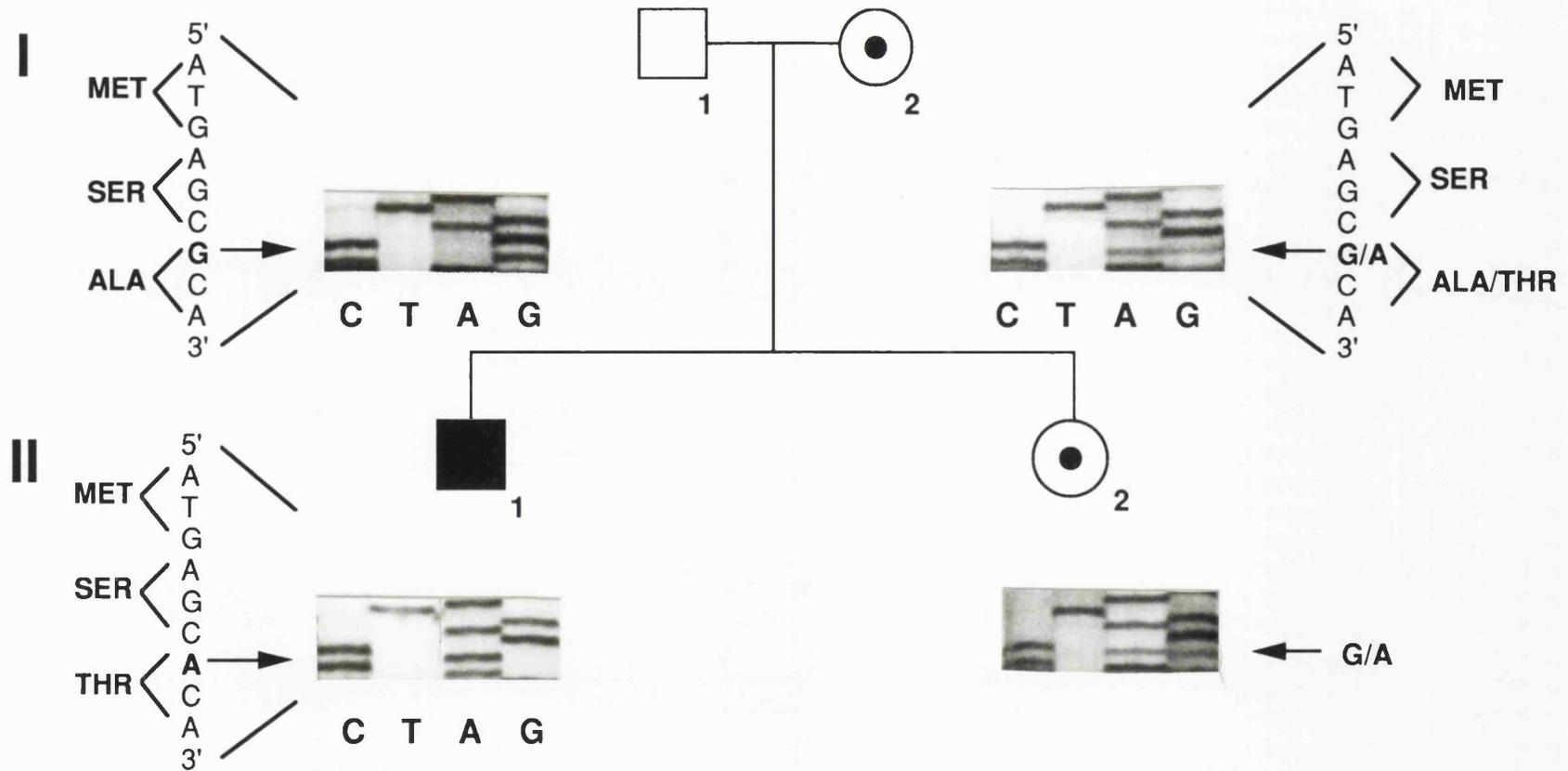
FIGURE 27: DNA sequence of exon 6 PCR product from family CFA (Fig. 13A). The arrow indicates nucleotide 622. At this position, a G to A change results in the substitution of an alanine by a threonine, at amino acid 176 of the mature protein. The affected boy (II-1) had only the mutant A band, his carrier mother and sister (I-2, II-2) had both A and G bands and the unaffected father, the normal G band. Symbols are as for figure 1.

EXON	1		2		4		5		6		7+8		9		10	
CONDI-TIONS	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C
FAMILY AD	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
CFA	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
FAR	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
AA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
HB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 11: The results of SSCP analysis of exons 1,2,4,5,6,7+8,9,10 of the ornithine carbamoyl transferase gene in 5 families with late onset OCT deficiency.

136
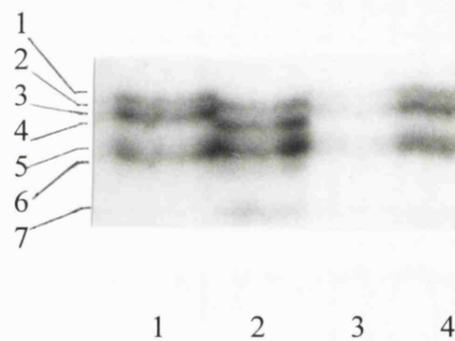
BAND

FIGURE 28 SSCP analysis of the 119bp exon 4 PCR product from the proband (III-3) in family AD (Fig. 13C) on a 6% nondenaturing polyacrylamide gel at 4°C for 16 hours. Tracks 1, 3 and 4 show the wild type pattern (Bands 1, 3 and 5), while track 2 contains DNA from III-3 and shows the variant pattern (Bands 2, 4, 5, 6 and 7).

SSCP analysis of exon 6 PCR product in family CFA (Fig. 13A) identified a variant pattern (bands 2 and 3) in the affected male (II-1) and his mother (I-2) and sister (II-2), who as heterozygotes had both the wild type (1 and 3) and variant (2 and 3) band patterns (Fig. 29). This change was detected on SSCP analysis at both 4°C and 20°C and was not seen on analysis of the remaining 4 late onset families.

SSCP analysis of exon 7 and 8 PCR product identified a variant band pattern in family FAR (Fig. 13B) which segregated with disease status. Bands 2, 3 and 5 represent the mutant pattern which the 2 affected males (III-2, III-4) had inherited from their obligate carrier mother (II-2). She, as a heterozygote, had both the wild type (1, 2, 4 and 6) and the variant (2, 3 and 5) band patterns, while the unaffected brother (III-3) and father (II-1) had only the wild type band pattern (Fig. 30). This change was detected on analysis at 4°C and was not observed on analysis of the remaining 4 late onset families.

3.4.2   SEQUENCE ANALYSIS OF FAMILIES SHOWING A VARIANT PATTERN ON SSCP ANALYSIS

The basis of the SSCP variant in family FAR (Fig. 13B) was investigated by DNA sequencing. Exons 7 and 8 were amplified and the product biotinylated at the 5' end of exon 7 using primers M and N. On sequencing, using a primer (N) within intron 8, no changes were identified within exon 7 and intron 7 in this family. As the sequencing primer (N) was immediately adjacent to the 3' end of exon 8 only a limited amount of exon 8 sequence was obtained. The subsequent sequencing of exon 8 PCR product biotinylated at the 3' end (primer N), using a sequencing primer (O) within exon 8, enabled the mutation in this family, a C to T change at nucleotide 829, codon 277, to be identified.

The change was predicted to result in an arginine to tryptophan substitution at amino acid 245 of the mature OCT protein (Fig. 31). It was found in both clinically affected males (III-2, III-4), who had only the mutant T sequence, and the carrier mother
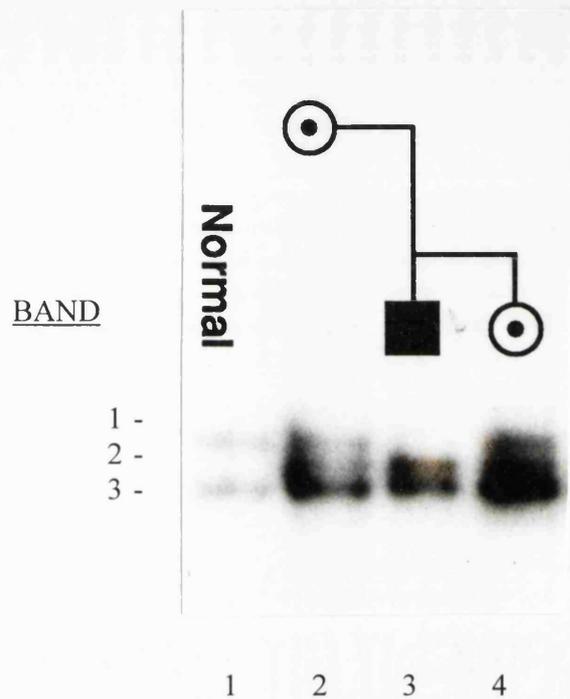
FIGURE 29 SSCP analysis of exon 6 PCR product from family CFA (Fig. 13A) on a 6% nondenaturing polyacrylamide gel at 4°C for 16 hours. Track 1 contains wild type DNA, tracks 2-4, PCR products from I-2 (carrier mother), II-1 (proband) and II-2 (carrier sister) respectively. Bands 1 and 3, and 2 and 3 represent the wild type pattern and the mutant pattern respectively. Symbols are as for figure 1.
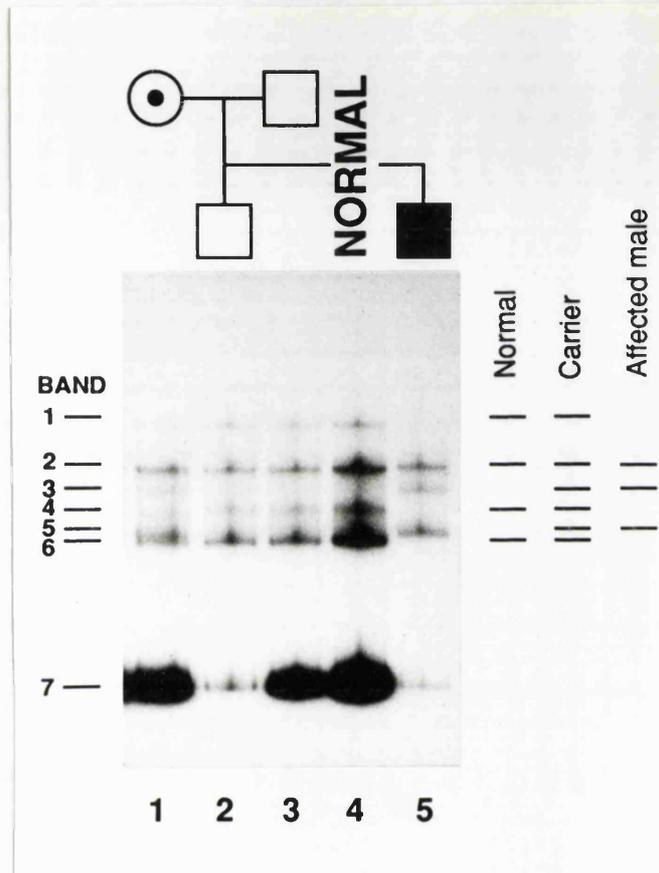
FIGURE 30 SSCP analysis of the 322bp exon 7 and 8 PCR product from family FAR (Fig. 13B) on a 6% nondenaturing polyacrylamide gel at 4°C for 16 hours. Tracks 1-5 contain DNA from II-2 (carrier mother), III-3 (unaffected male), II-1 (father), wild type DNA and III-4 (affected male). The affected male (III-4) had only the mutant pattern (Bands 2,3,5), while the unaffected father (II-1) and the unaffected male (III-3) had only the wild type pattern (Bands 1,2,4 and 6). The carrier mother (II-2), as a heterozygote, had both the wild type and the mutant pattern. Band 7 represents double stranded PCR product. Symbols are as for Figure 1.

140
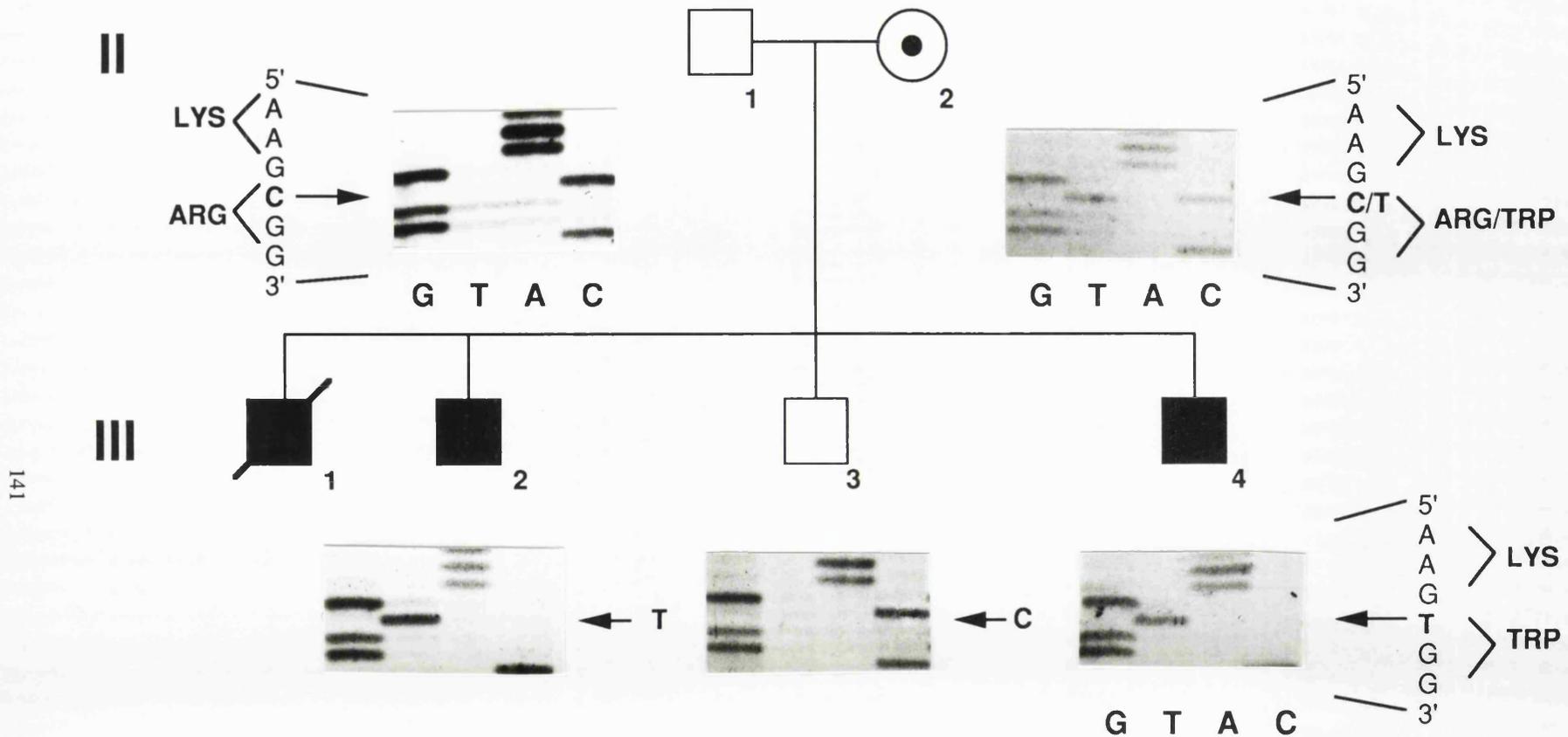
FIGURE 31: DNA sequence of exon 8 PCR product from family FAR (Fig 13B). The arrow indicates nucleotide 829. At this position a C to T change is predicted to result in the replacement of an arginine by a tryptophan at amino acid 245 of the mature protein. The affected males (III-2, III-4) had only the mutant T band, the unaffected male (III-3) and the father (II-1) had only the wild type C band, while the carrier mother (II-2) had both the C and T bands. Symbols are as for Figure 1.

(II-2), who had both C and T bands. The father (II-1) and the unaffected male (III-3) had only the wild type C band.

Sequence variations in families AD and CFA, in exons 4 and 6, were simultaneously identified by the analysis of 5'CpG 3' dinucleotide sites by *Msp*I and *Cfo*I restriction enzyme digestion respectively. The results of sequence analyses in these families are given in Section 3.3.2, Figures 25, 26 and 27.

3.4.3 AN EXTENDED FAMILY STUDY IN A CASE OF LATE ONSET DEFICIENCY

Following the identification of the Arg(245)Trp change in family FAR (Fig. 13B) the extended family were screened for this change.

At this stage the carrier status of II-2, I-2, II-4 and II-6 was uncertain. The disorder was linked to the lower 6.2kb *Msp*I polymorphism in this family (Clinical service result, Fig. 32) but, as the grandmother (I-2) was homozygous for this polymorphism, carrier detection could not be offered to her daughters; II-4 and II-6. The daughter of II-4 (III-5) was excluded from being a carrier as she had inherited the 6.6kb allele from her potential carrier mother.

On SSCP analysis of exon 8 PCR product (Fig. 33), I-2 was found to be heterozygous for an SSCP variant and to have passed this on to her daughters II-2 and II-4, but not II-6. I-2 had also passed the variant pattern onto an apparently asymptomatic son, II-7, who it has not been possible to investigate further.

These results were confirmed by sequencing of exon 8 PCR product (Fig. 34). The mother (I-2) and sister (I-4) of II-2, who were predicted to be carriers on SSCP analysis, as heterozygotes, had both the C and T bands, while the clinically undetected male (II-7) had only the mutant T band. The remaining members of the family, specifically the second sister (II-6) and the daughter of II-4 (III-5), had only the wild
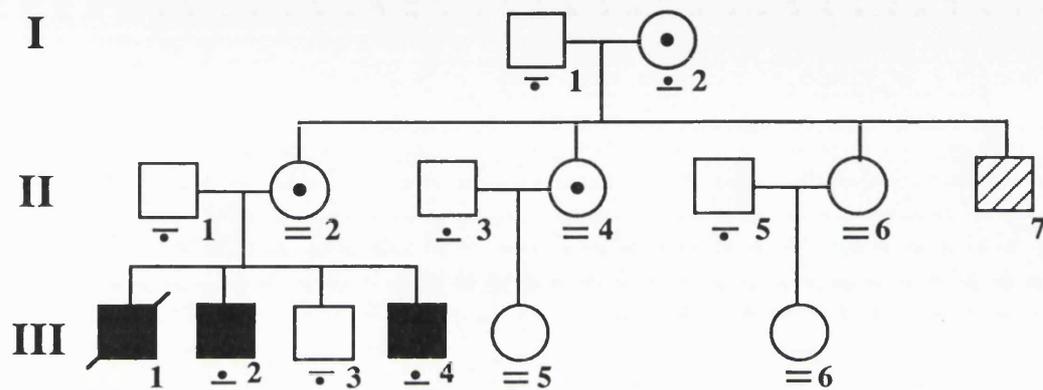
FIGURE 32: The results of the analysis of the OCT gene upper *Msp*I (6.2/6.6 kb) Polymorphism in the extended FAR family. Symbols are as for Figure 1, where the partially shaded shape indicates a clinically undetected individual.
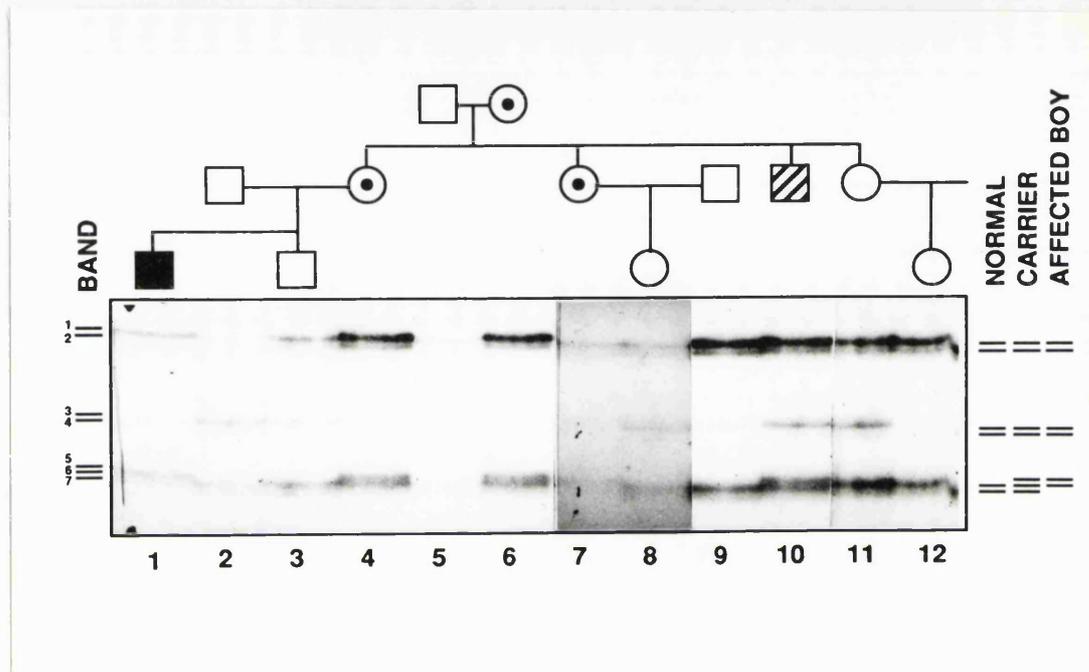
FIGURE 33 SSCP analysis of the 166bp exon 8 PCR product from the extended FAR family (Fig. 13B). Analysis was on a 6% nondenaturing polyacrylamide gel containing 5% glycerol at 4°C for 16 hours. Tracks 1-12: III-2, II-1, III-3, II-2, I-1, I-2, II-4, III-5, II-3, II-7, II-6, III-6. Bands 1-4, 6 and 7, and 1-4, 5 and 6 represent the wild type and mutant patterns respectively. The affected male (III-2) and the asymptomatic male (II-7) had only the mutant pattern, individuals; III-3 (unaffected male), II-1 (father), II-6 (aunt), I-1 (grandfather), III-5 (cousin), II-3 (uncle), III-6 (cousin) had only the normal pattern and I-2 (grandmother), II-2 (mother) and II-4 (aunt) had both patterns and hence were carriers. Symbols are as for figure 1 and the partially shaded shape indicates the clinically undetected male.

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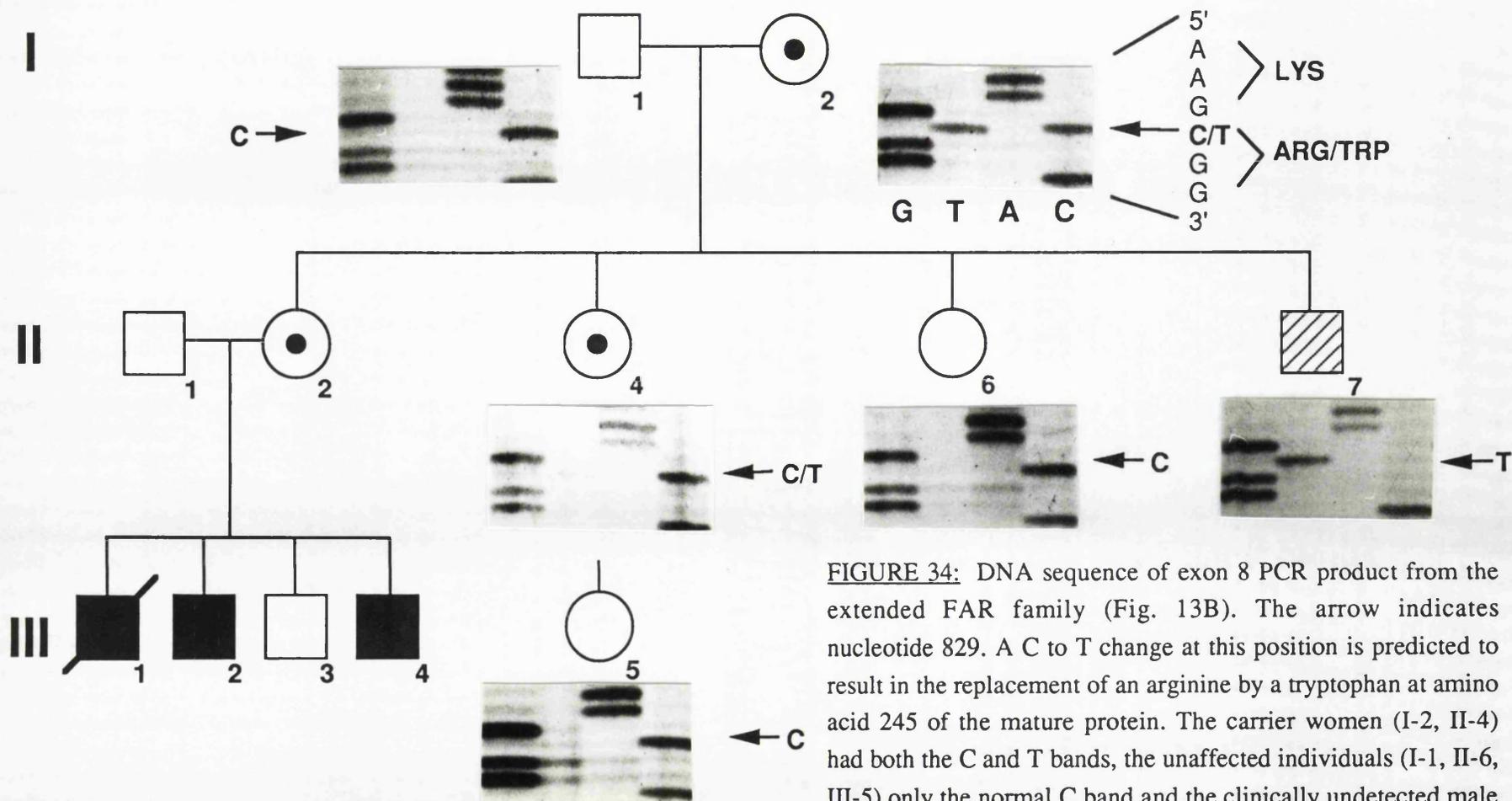
FIGURE 34: DNA sequence of exon 8 PCR product from the extended FAR family (Fig. 13B). The arrow indicates nucleotide 829. A C to T change at this position is predicted to result in the replacement of an arginine by a tryptophan at amino acid 245 of the mature protein. The carrier women (I-2, II-4) had both the C and T bands, the unaffected individuals (I-1, II-6, III-5) only the normal C band and the clinically undetected male (II-7) only the mutant T band. Symbols are as for Figure 1 and the partially shaded shape indicates a clinically undetected individual.

type C band confirming their noncarrier status.

The Microgenie package was used to determine if this change resulted in the creation or loss of a restriction site. The program predicted that the C to T change would abolish a *Fnu*4HI (GCNGC) site and the usefulness of this change for future family analysis was investigated by screening the extended family for the loss of this site. In the unaffected individuals (III-3, III-5 and II-6), the 166bp exon 8 PCR product was digested to 105 and 61bp fragments, while the carrier women (II-2, II-4 and I-2) showed partial digestion and the affected males (III-2 and III-4) and the clinically undetected male (II-7) showed no digestion (Fig. 35).

BAND

SIZE (BP)
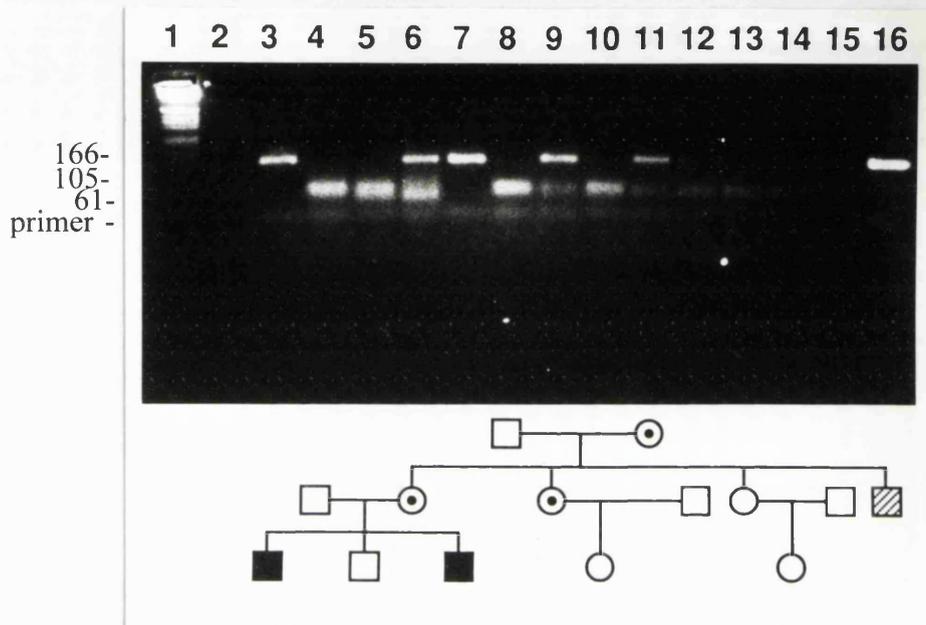
FIGURE 35 Analysis of *Fnu*4HI digested exon 8 PCR product from the extended FAR family (Fig. 13B) on a 4% Nusieve-GTG-agarose gel. The 166bp wild type DNA is digested to 105 and 61bp fragments, while mutant DNA shows no digestion. The 105 and 61 bands were not resolved as separate bands. Track 1 contains lambda kb ladder molecular weight marker, track 2 undigested product (faint), tracks 3-16 digestion products from III-2 (affected male), II-1 (father), III-3 (unaffected male), II-2 (carrier mother), III-4 (affected male), I-1 (grandfather), II-4 (carrier aunt), III-5 (cousin), I-2 (carrier grandmother), II-3 (uncle), II-6 (aunt), III-6 (cousin), II-5 (uncle) and II-7 (asymptomatic adult male) respectively.

Symbols are as for figure 1 and the partially shaded shape indicates a clinically undetected individual.

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4.0.0. <u>RESULTS: PELIZAEUS-MERZBACHER DISEASE</u>

4.1.0   **CASE STUDIES**

4.1.1   **PEDIGREE AND REFERRAL DETAILS**

Fourteen families with a suggested diagnosis of Pelizaeus-Merzbacher disease were selected for analysis (Table 12). Families CB, AB, HJ, PP, RR, WM, PA and BU were assessed and referred by Dr Baraitser of the Clinical Genetics Unit at Great Ormond Street Childrens' hospital, London. Family SA was referred by Professor Gardiner of University College Hospital, London, family LV by Dr Cross of the City Hospital, Nottingham. Families DD and MDJ were referred by Dr Willems of the Centre for Medical Genetics, University of Antwerp. Family OC was referred by Dr Wilkie of the Heath hospital Cardiff, family LM by Dr Tolmie of Glasgow University.

The mode of inheritance in 5 families was X-linked, though in four families this was based on the presence of an affected relative in the family pedigree in whom the diagnosis could not be verified. Four of remaining families had pedigrees with 2 affected male siblings, but no additional family history. Transmission in these families was consistent with X-linked, but also autosomal recessive inheritance. In the remaining 5 families the proband was a sporadic male. No affected females or manifesting carriers were reported. The carrier status of all mothers, excluding the families with apparent X-linked inheritance, was unknown.

The clinical phenotype was consistent with a diagnosis of PMD in 10 families (CB, MDJ, AB, DD, WM, HJ, PP, SA, OC and PA) and probable PMD in 2 further families (LV and LM). The clinical phenotype in the remaining 2 families (BU, RR) was atypical.

FAMILY	PEDIGREE	INHERITANCE	DIAGNOSIS
CB		X-linked	PMD
MDJ		X-linked	PMD
AB		2 affected brothers	PMD
RR		Sporadic male	PMD ?
DD		2 affected brothers	PMD
WM		2 affected brothers	PMD
HJ		X-linked	PMD
PP		2 affected brothers	PMD
BU		Sporadic male	PMD ?
SA		X-linked	PMD
OC		Sporadic male	PMD
LV		X-linked	PMD ?
PA		Sporadic male	PMD
LM		Sporadic male	PMD ?

TABLE 12: Pedigrees, diagnoses and inheritance patterns of the 14 Pelizaeus–Merzbacher families investigated in this study. In all cases, excepting WM and CB, only family members directly relevant to the inheritance of the disorder are shown.

4.1.2   CLINICAL DETAILS OF FAMILIES IN WHICH A SEQUENCE CHANGE WAS IDENTIFIED.

FAMILY CB

The pedigree of family CB is given in Table 12. III-1 was born with a normal birth weight at 37 weeks gestation after a normal pregnancy. In his first weeks of life his parents noticed that he was a snuffly baby. At the age of 5 weeks laryngeal stridor was noted by the general practitioner. Over the next few weeks there were problems with abdominal colic and the child did not gain weight properly. He was admitted to hospital at the age of 12 weeks. Laryngoscopy at that stage showed a floppy larynx. He was noted to have pale discs and was thought to have nystagmus. From the age of 12 months there was no social or motor development. He remained profoundly handicapped. A maternal uncle (II-2) died at the age of three years, having a very similar illness, with laryngeal stridor and failure of development. Autopsy was performed in Bristol and confirmed the diagnosis of PMD. Carrier detection and prenatal diagnosis was requested by II-3.

FAMILY AB

The pedigree of family AB is give in Table 12. II-1 was born to nonconsanguineous parents after an uneventful pregnancy which went to term. He was their firstborn child and it was of note in the family tree that the mother had a brother who died at age 9 months and who was said to have been mentally and physically handicapped. On further inquiry there was evidence from the hospital notes that this male was hydrocephalic, had pale optic discs and was quadriplegic. It must therefore remain uncertain whether he had the same condition as his two nephews. II-1 weighed 8lb 6oz at birth and his early development seemed normal in that he smiled at 6 weeks and fed well.

At the age of 10 weeks he was admitted to the local hospital because of inspiratory stridor and at that time his mother noted jerky eye movements. He was referred to the Hospital for Sick Children, Great Ormond Street, where, on examination, he was found to be

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floppy and to have nystagmus and a diagnosis of mild laryngomalacia was made to explain the stridor. After discharge his stridor settled, but he made poor developmental progress. At the age of 13 months he was readmitted for further investigation. At that stage he had truncal hypotonia but had brisk reflexes with extensor plantar responses. A computed-tomography brain scan showed minor cerebralatrophy, but his brain stem auditory evoked responses revealed only wave 1, which is often found where central myelination is poor. All other investigations, including those of chromosomes and a variety of metabolic tests, were normal.

By the age of 3 years, II-1 was still developmentally behind and a MRI scan was undertaken. This showed gross undermyelination throughout the white matter of both hemispheres and the posterior fossa. There was no evidence of any normal myelin formation and a diagnosis of PMD was made.

II-2, the brother of II-1, was born when II-1 was 21 months of age. The pregnancy was uneventful and II-2 had a good birth weight. It had been noted antenatally that the fetus had a left renal tract abnormality and postnatally this was shown to be duplex kidney on one side. He was jittery at birth, and, like his brother, he developed stridor. His progress was poor and nystagmus was noted early in life. The optic discs were pale and further investigations revealed abnormal brainstem evoked responses identical to those of his brother. At 22 months of age he is still floppy and has few words. Clinically, II-2 is identical to II-1 and further investigations were not undertaken.

<u>FAMILY MDJ</u>

The pedigree of family MDJ is shown in Table 12. The proband (III-1) was diagnosed as suffering from classical PMD. The disorder in this family was predicted to be X-linked as 2 maternal uncles had died in infancy with comparable symptoms.

The pedigree of family RR is shown in Table 12. The affected boy was born after an uneventful pregnancy and after a difficult labour was placed in the special care baby unit for 24 hours, although no further treatment was required.

He developed seizures on day one, which, whilst initially occurring daily, increased with age until by age 9 months he was having 3 bouts a day of approximately 50 of these episodes. These have been controlled pharmaceutically with variable success.

On examination at 20 months, he was visually inattentive and exhibited coarse nystagmus, truncal hypotonia, moderate and symmetrical hyperflexia, extensor plantar responses, sustained ankle clonus and microcephaly. The visual evoked responses were abnormal suggesting some impairment of function in the visual pathways to the brain on both sides. EEG showed frequent runs of discharges and a poverty of normal activity for his age.

On MRI a marked degree of hypomyelination, consistent with an age of less than 4 months was noted. Subsequent analysis at 30 months showed a slight progress in myelination, with a level equivalent to an age of 7 months.

Development was delayed from the outset, though no regression was noted, and he continues to learn new skills, albeit very slowly. Diagnosis is undetermined and suggestions have included microcephaly, epilepsy and mental retardation, and PMD.

4.2.0 CHARACTERISATION OF RESTRICTION FRAGMENT LENGTH POLYMORPHISMS AT THE PROTEOLIPID PROTEIN LOCUS

The frequency of the intragenic 4/4.5kb *Msp*I polymorphism (Fig. 10) was investigated in 10 PMD families and in a control population of 11 unrelated women.

The 4.5kb allele has been reported to occur with a frequency of 0.08 (Wu et al. 1987) in the North American population, but was not seen on analysis of 17 unrelated chromosomes from PMD families. Subsequent analysis of a control population representing 22 X-chromosomes identified a single 4.5kb of North European origin, giving a frequency in this population of 0.025.

Twelve PMD families were analysed to determine the frequency of the intragenic *Aha*II polymorphism (Fig. 11) reported by Trofatter et al. (1991) to occur with a frequency of 0.26. In the current study 7/24 unrelated X-chromosomes carried the rarer *Aha*II+ polymorphism, giving a frequency of 0.29 which supported this figure. Of 11 females investigated, 3 were informative for this polymorphism, 1 was homozygous for the *Aha*II+ allele and 7 were homozygous for the *Aha*II- allele.

4.3.0   TESTING FOR PROTEOLIPID PROTEIN GENE INVOLVEMENT IN PELIZAEUS-MERZBACHER DISEASE BY COSEGREGATION OF MARKERS FROM THE Xq21.3-q22 REGION

Two families were investigated for the cosegregation of markers from the Xq21.3-q22 region and hence the PLP gene, with PMD. The first family (WM) consisted of 3 brothers, 2 of whom had PMD, while the second family (AB) consisted of 2 affected brothers. The inheritance of opposing alleles of a polymorphism by affected brothers, or the same allele by both affected and unaffected brothers, would, barring recombination, support the involvement of a second X-linked or autosomal locus.

Seven polymorphisms detected by probes from the Xq21.3-Xq22 region (Table 9) were analysed. These were: p19-2 (*Taq*I; 3+2/5kb, *Msp*I; 4.4/12kb), p212-9 (*Taq*I; 1.8/3.2kb), CX52.5 (*Msp*I; 7.5/7.7kb), pRL1 (*Msp*I; 4.0/4.5kb), pXG-12 (*Pst*I; 6.5/7.2kb), and S21 (*Taq*I; 2.0/2.2kb). In addition the intragenic *Aha*II polymorphism in exon 4 of the PLP gene was analysed in each family.

In family WM (Fig. 36) the mother (I-2) was heterozygous for two polymorphisms; p212-9 (TaqI; 1.8/3.2kb) and CX52.5 (MspI; 7.5/7.7kb). In each case the 2 affected brothers (II-1, II-3) had inherited the 3.2 and 7.7kb alleles, while their unaffected brother (II-2) had inherited the opposing 1.8 and 7.5kb alleles.

In family AB (Fig. 37) the mother (I-1) was heterozygous for the TaqI polymorphism (2.0/2.2kb) detected with probe S21. Both affected brothers (II-1, II-2) had inherited the 2.0kb allele.

In both families affected status cosegregated with the markers which supported X-linked inheritance and the involvement of a gene, possibly PLP, from the Xq21.3-q22 region in PMD.

4.4.0   SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS

SSCP analysis of exons 1-7 of the PLP gene identified a variant band pattern in exon 4 in 3 families; CB, MDJ, RR and in exon 5 in one family; AB. No changes were identified in an additional 10 families screened (Table 13).

In each case analysis was carried out at both 4°C and 20°C to increase the probability that a change, if present, was detected. Exon 1, 3 and 4 PCR products were analysed both undigested and after digestion with MspI (exons 1 and 3) or RsaI (exon 4) to bring the fragments analysed closer to the optimal size of 200bp (Hayashi. 1991).

In family CB (Table 12), SSCP analysis of undigested exon 4 PCR product at 4°C identified a variant band pattern which segregated with carrier status. The SSCP variant, which is indicated by the arrow in figure 38, was inherited by both daughters (II-1, II-3) from their obligate-carrier mother (I-2). This variant was not present in the unaffected father (I-1) or the fetus (III-2) of II-3. The frequency of the mutant allele was estimated by screening exon 4 PCR products from a control population of 51 X-chromosomes.

KEY

p19-2	(*Taq*I; 3+2/5)
p19-2	(*Msp*I; 4.4/12)
p212-9	(*Taq*I; 1.8/3.2)
CX52.5	(*Msp*I; 7.5/7.7)
pRL1	(*Msp*I; 4.0/4.5)
Exon4/PLP	(*Aha*II; 0.044+0.183/0.277)
pXG-12	(*Pst*I; 6.5/7.2)
S21	(*Taq*I; 2.0/2.2)

I₁

I₂

3+2	3+2
4.4	4.4
1.8	**3.2**
7.5	**7.7**
4.0	4.0
0.44+ 0.183	0.44+ 0.183
6.5	6.5
2.0	2.0

II₁

3+2
4.4
3.2
7.7
4.0
0.44+ 0.183
6.5
2.0

II₂

3+2
4.4
1.8
7.5
4.0
0.44+ 0.183
6.5
2.0

II₃

3+2
4.4
3.2
7.7
4.0
0.44+ 0.183
6.5
2.0
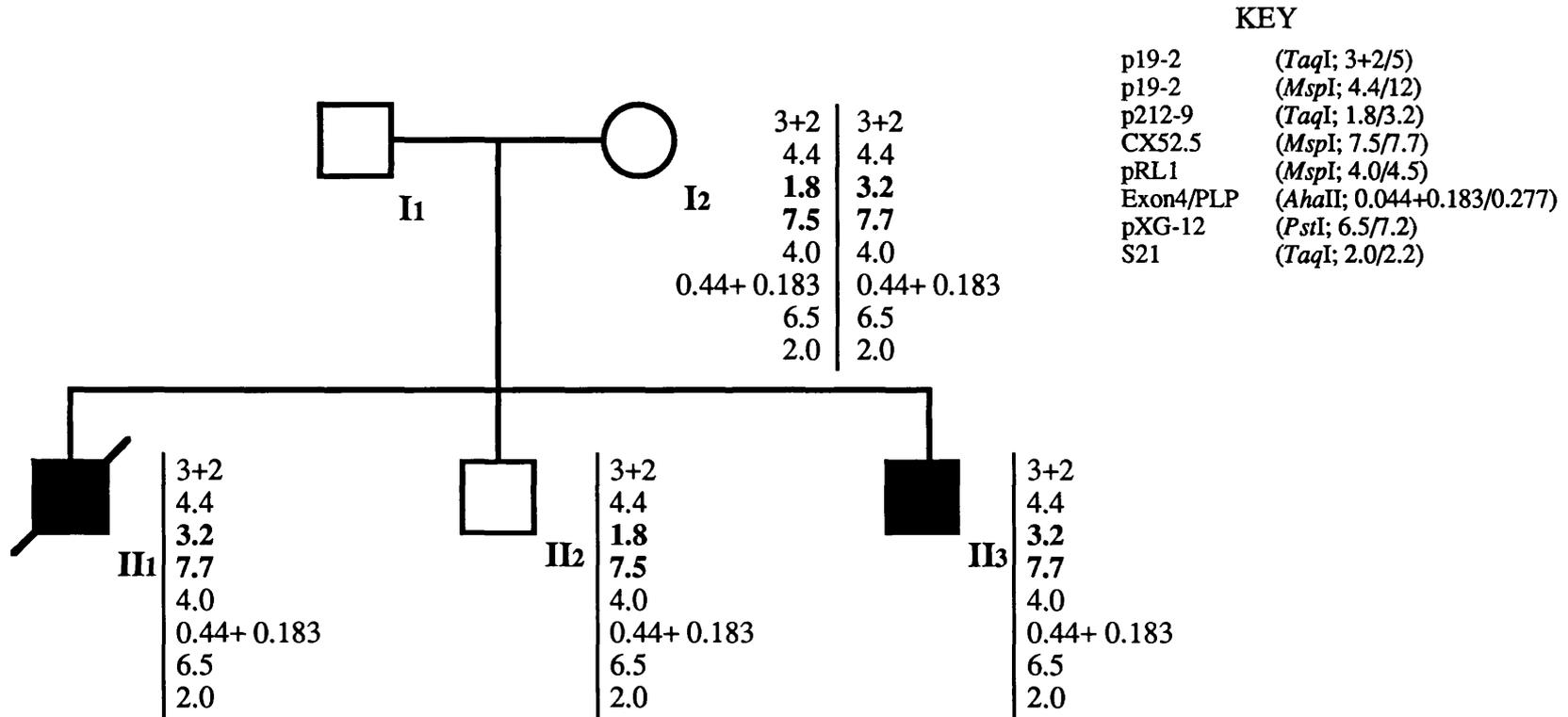
FIGURE 36: The results of the analysis of intragenic and linked polymorphisms from the Xq21.3-q22 region in family WM. Squares and circles represent males and females respectively, where completely shaded shapes represent affected individuals.

KEY

p19-2	(*Taq*I; 3+2/5)
p212-9	(*Taq*I; 1.8/3.2)
Exon4/PLP	(*Aha*II; 0.044+0.183/0.277)
S21	(*Taq*I; 2.0/2.2)

I₁

3+2 | 3+2
3.2 | 3.2
0.277 | 0.277
2.0 | 2.2

II₁

3+2
3.2
0.277
2.0

II₂

3+2
3.2
0.277
2.0
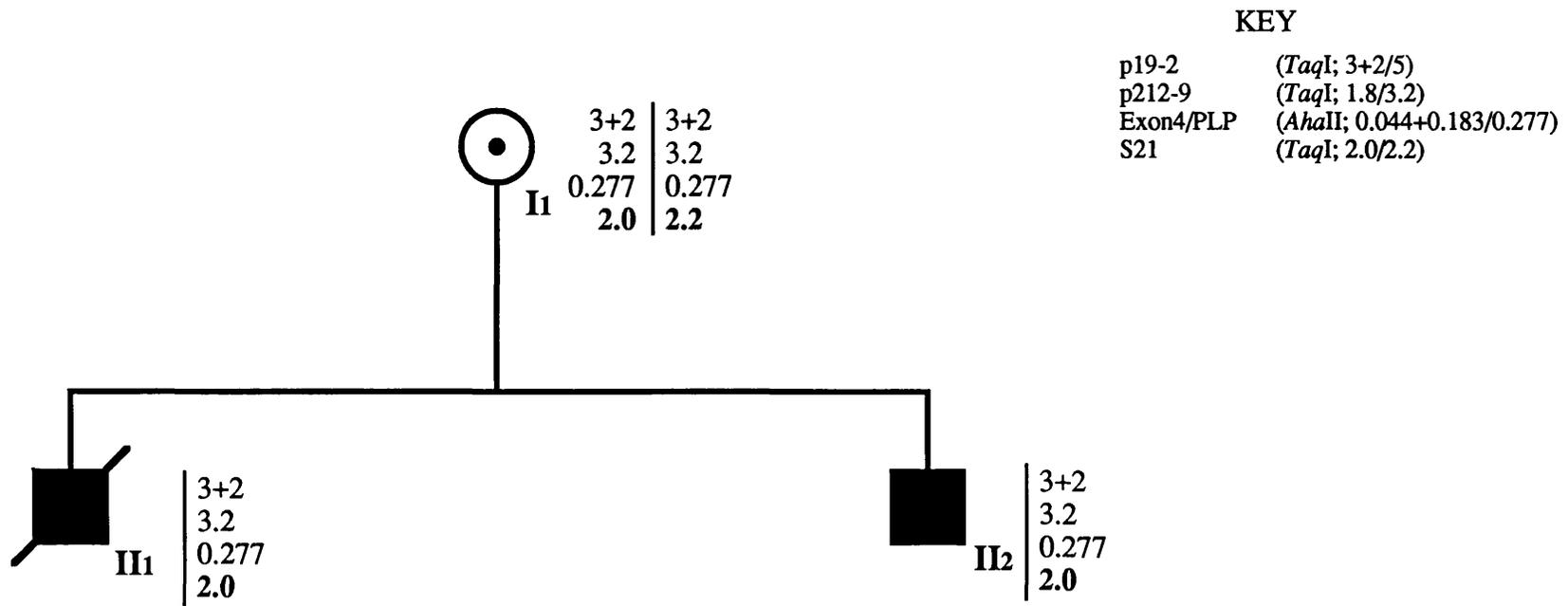
FIGURE 37: The results of the analysis of intragenic and linked polymorphisms from the Xq21.3-q22 region in family AB. Squares and circles represent males and females respectively, where completely shaded shapes represent affected individuals and the dotted circle a carrier identified by DNA analysis.

EXON	1				2		3				4				5		6		7	
STATUS	UN DIGESTED		DIGESTED		UN DIGESTED		UN DIGESTED		DIGESTED		UN DIGESTED		DIGESTED		UN DIGESTED		UN DIGESTED		UN DIGESTED	
CONDITIONS / FAMILY	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C
CB	−	−	−	−	−	−	−	−	−	−	+	−	+ −	−	−	−	−	−	−	−
MDJ	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
RR	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−
AB	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
PP	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LM	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
OC	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
LV	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BU	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
PA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
DD	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
WM	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HJ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 13: The results of SSCP analysis of exons 1 – 7 of the proteolipid protein gene in 14 families with Pelizaeus – Merzbacher disease.
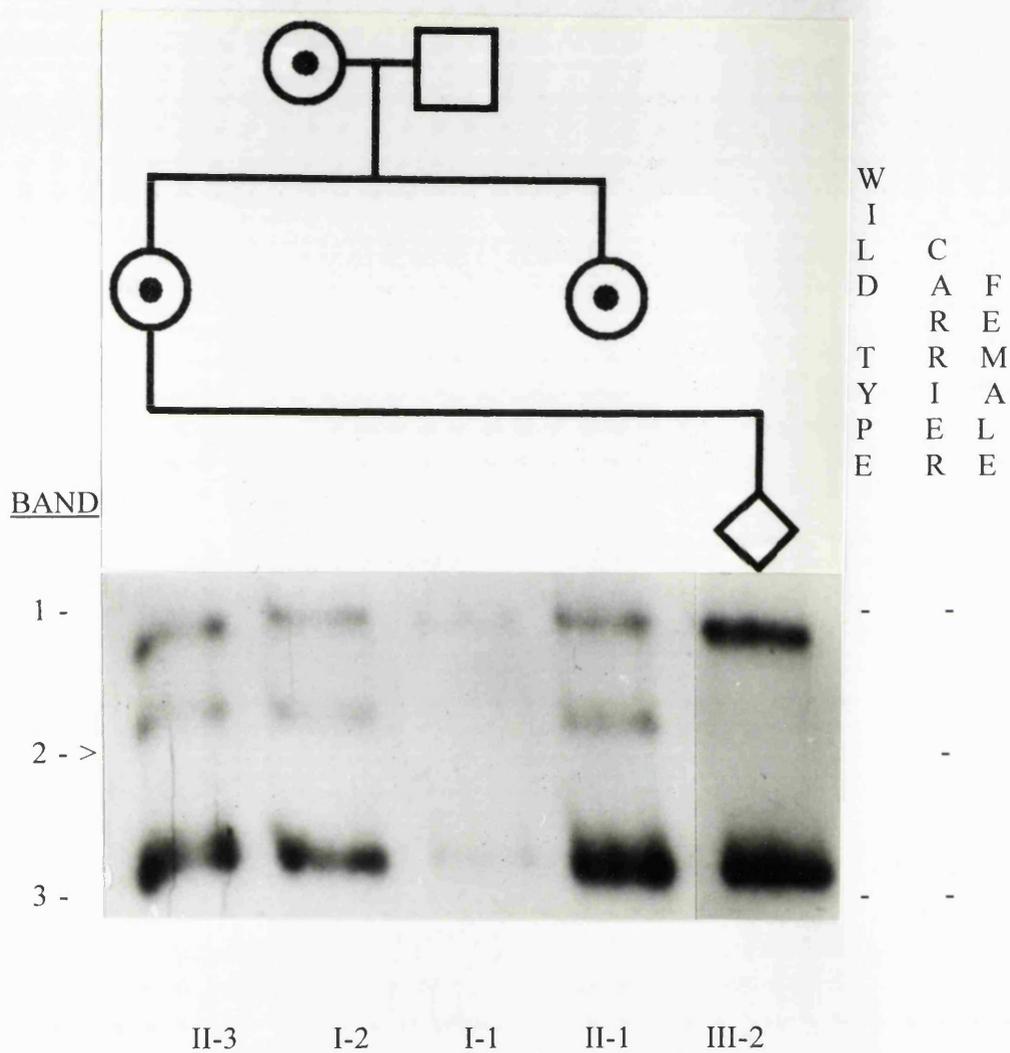
FIGURE 38 SSCP analysis of undigested exon 4 PCR product from family CB (Table 12) on a 6% nondenaturing polyacrylamide gel at 4°C for 16 hours. Bands 1 and 3 represent the wild type pattern which was present in the father (I-1) and the CVS (III-2). Band 2 represents the SSCP variant which was present in the carrier mother (I-2) and her daughters (II-3 and II-1) who, as heterozygotes, had both the wild type and the mutant bands.

In all cases only the wild type pattern was detected. An SSCP variant was also detected on analysis of digested product at 4°C, but not on analysis of digested and undigested product at 20°C (Section 4.6.0, Fig. 46).

A second band shift was identified in family MDJ (Table 12) on analysis of digested PCR product at both 4°C and 20°C. The affected boy (III-1) had only the variant pattern, as compared to the wild type control (Section 4.6.0, Fig. 46). This variant was not seen on examination of the remaining PMD families or on analysis of undigested product from the proband at either 4°C or 20°C (Fig. 46).

The third exon 4 band shift was identified in the affected boy (II-1) from family RR (Table 12) (Fig. 39). An SSCP variant was detected on analysis of both digested and undigested product at 4°C, but not at 20°C. The variant pattern was not seen on examination of the remaining PMD families.

In family AB (Table 12), SSCP analysis of exon 5 PCR product at 4°C identified a variant band pattern which segregated with affected status. The variant was present in both affected boys (II-1 and II-2), who each had only the variant bands (1 and 3), and in the carrier mother (I-1), who had both the wild type (2 and 4) and variant (1 and 3) bands (Fig. 40). An SSCP variant was also detected on analysis at 20°C (Fig. 47). The frequency of the mutant allele was estimated by screening exon 5 PCR products from a control population of 51 chromosomes. In all cases only the wild type pattern was detected.

4.5.0   SEQUENCE CHANGES IN THE PROTEOLIPID PROTEIN GENE

Sequencing of exon 4 PCR products from the members of family CB (Table 12), including the fetus of II-3 (III-2), identified an A to C change at nucleotide 541 in the 3 carrier women (I-2, II-1, II-3) (Fig. 41). This is predicted to result in a threonine (ACC) to Proline (CCC) change at amino acid 181. As heterozygotes, the carrier women had both A and C bands, while the CVS (III-2) and the unaffected father (I-1) had only the normal A band.

WT II-1   WT II-1

A   B
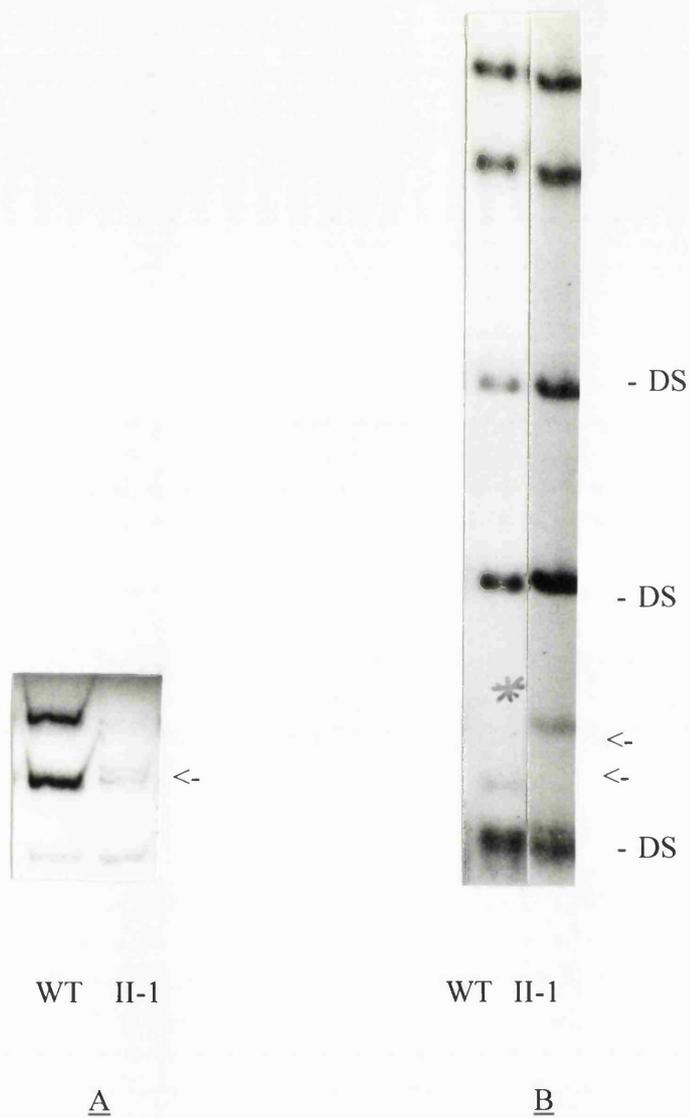
FIGURE 39 SSCP analysis of exon 4 PCR product from the affected male (II-1) in family RR. Analysis was on a 6% nondenaturing polyacrylamide gel for 16 hours. (A) Undigested product at 4°C (B) Digested product at 4°C. In each case the arrows indicate the variant bands, whilst double stranded DNA is indicated by DS and wild type control DNA is indicated by WT.
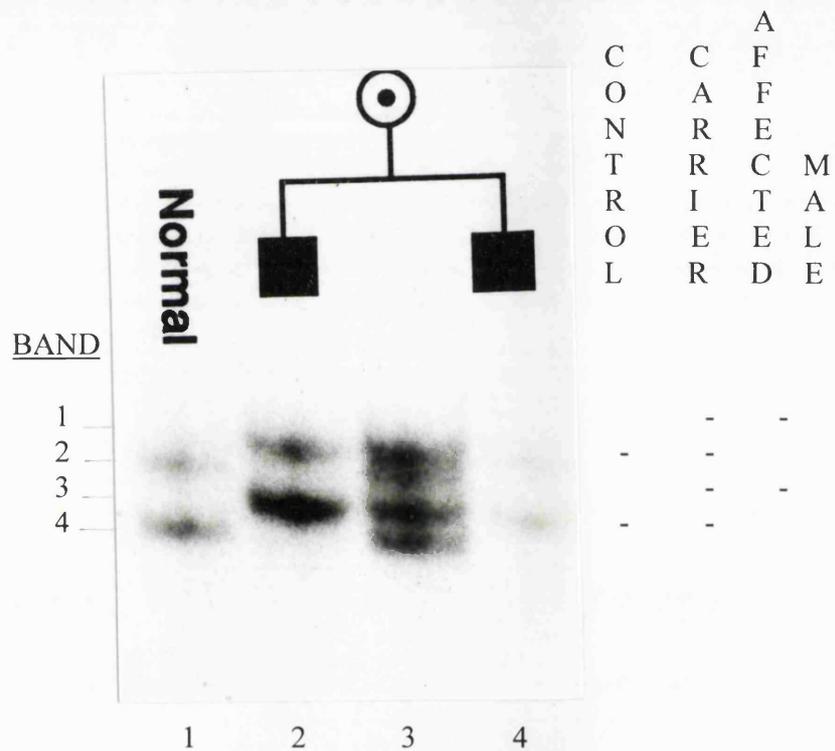
FIGURE 40 SSCP analysis of exon 5 PCR products from family AB on a 6% nondenaturing polyacrylamide gel at 4°C for 16 hours. Track 1 contains control DNA, tracks 2-4 DNA from: II-1 (affected boy), I-1 (carrier mother) and II-2 (affected boy) respectively. Bands 2 and 4 represent the wild type pattern, bands 1 and 3 the mutant pattern. Symbols are as for figure 1.
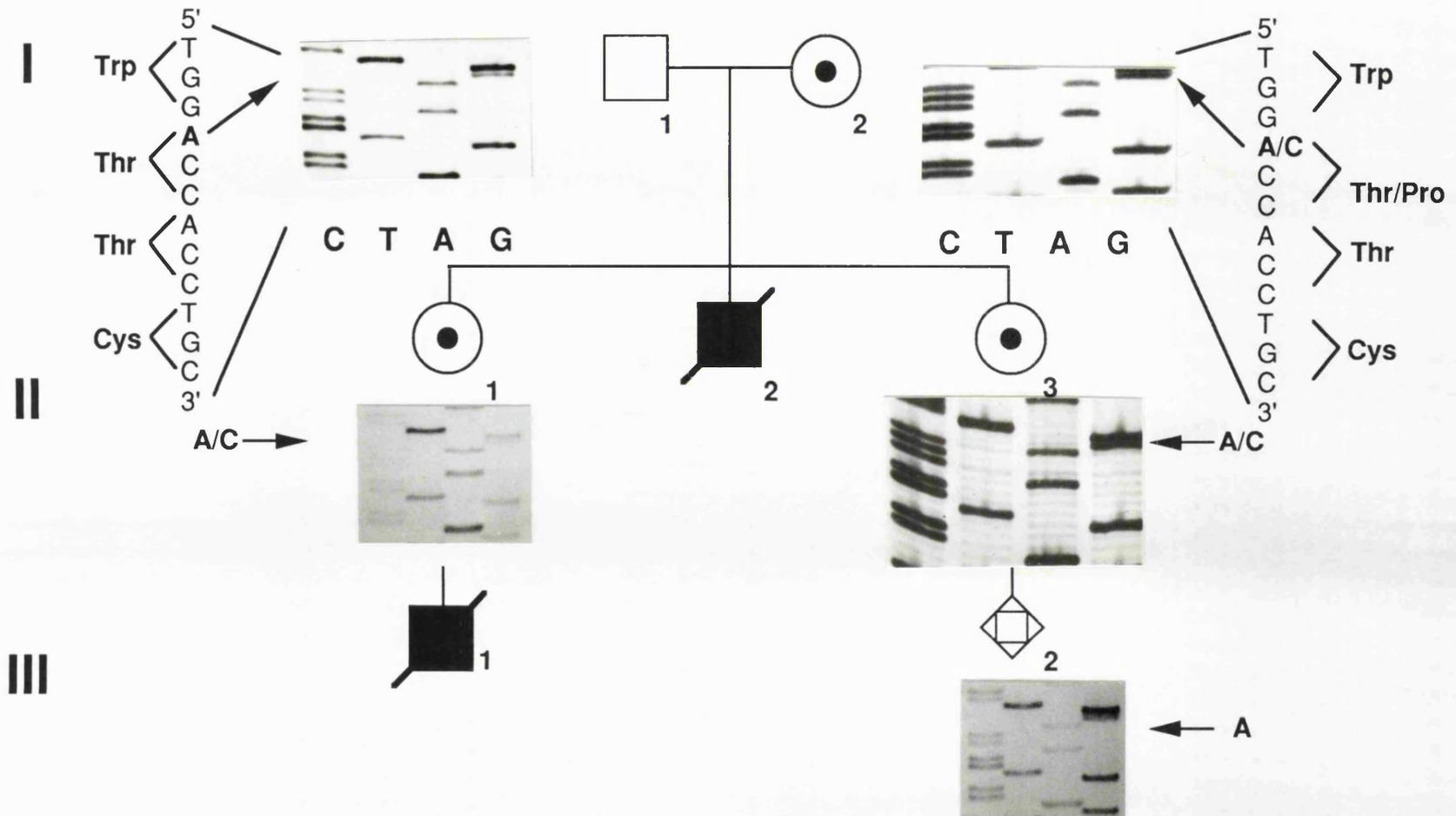
FIGURE 41: DNA sequence analysis of exon 4 PCR products from family CB (Table 12). The arrow indicates nucleotide 541. At this position an A to C change is predicted to result in the replacement of a threonine by a proline at amino acid 181. The carrier females (I-2, II-1, II-3), as heterozygotes had both A and C bands, the fetus of II-3 (III-2) and the unaffected father (I-1) had only the wild type A band. Symbols are as for Figure 1.

In family MDJ (Table 12), sequence analysis of exon 4 PCR products identified a G to T change in the proband (III-1) and his obligate carrier mother (II-3) and grandmother (I-1), who as heterozygotes had both the G and T bands (Fig. 42).

This change, at position -1 of the 3' acceptor splice site of exon 4, removed the usually invariant G of the AG dinucleotide at the 3' end of intron 3 (Fig. 43). A comparison of the statistical estimates for the probable use of the mutant and wild type splice sites, using the method of Shapiro and Senapathy (1987), suggested a decrease of 25%, from 77.76% to 52.78%, in the efficiency of this site in mediating splicing.

In family RR (Table 12), DNA from the affected male (II-1) failed repeatedly to sequence and is currently under analysis by a collaborating group. The change, if a coding mutation, is predicted to be located within the amino acid stretch 174-207. This is based on a comparison of the results of SSCP analysis of RsaI digested exon 4 PCR product in this family with those obtained in families MDJ and CB, who have changes in the 5' and 3' regions of exon 4 respectively (Section 4.6.0, Fig. 46).

The 335bp exon 4 PCR product contains 2 RsaI restriction sites, at amino acid 174 and 12bp into intron 4, and on digestion the product is cut into 3 fragments of 143, 81 and 111bp (Fig. 44). On analysis, fragments 3' and 5' to the exonic cut site were resolved to different portions of the gel and were mapped according to which showed an altered pattern in each family. The variant in family RR mapped to the same region as that in family CB indicating a change in either the 111bp fragment, containing amino acids 174-207, or the intronic 81bp fragment.

In family AB (Table 12), sequence analysis of exon 5 identified a T to C change at nucleotide 668 (Fig. 45). The carrier mother (I-1) had both C and T bands, while her affected sons (II-1 and II-2) had only the mutant C band. This is predicted to result in a leucine (CTT) to proline (CCT) change at amino acid 223.

5'
a
t
t
t
t
a
t ->
T
T
G
3'
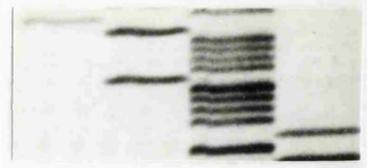
C   A   T   G

<u>AFFECTED MALE (III-1)</u>

5'
a
t
t
t
t
a
g/t ->
T
T
T
G
3'

C   A   T   G
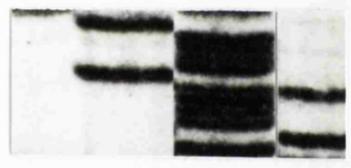
<u>CARRIER MOTHER (II-3)</u>

5'
a
t
t
t
t
a
g ->
T
T
T
G
3'

C   A   T   G

<u>WILD TYPE CONTROL</u>

165

FIGURE 42 DNA sequence analysis of exon 4 PCR product from the affected male (III-1) and the carrier mother (II-3) in family MDJ (Table 12), and a wild type control. The arrow indicates the G to T change at the -1 position of the 3' acceptor splice site. The proband (III-1) had only the mutant T band while the carrier mother had both the both G and T bands and the wild type control had only the G band. Exonic bases are in capitals, intronic bases in lower case lettering.

                    -EXON 4-   -EXON 4---

5' c a t t t t a g T T T 3' -> 5' c a t t t t a t T T T 3'

--INTRON 3---   ---INTRON 3----

FIGURE 43 A schematic representation of the intron 3/exon 4 boundary of the PLP gene showing the G to T change in family MDJ. This occurs at the last base of intron 3, at the -1 position of the 3' acceptor splice site. The exonic bases are in capitals, the intronic bases in lower case lettering.

FIGURE 44: A schematic representation of exon 4 of the PLP gene showing the location of *Rsa*I sites and the mutations in families MDJ and CB.

```
         5'
       / A        C    T    A    G
ASN      A
       \ C
       / C
LEU      T  ->
       \ T
       / C
LEU      T
       \ G
         3'
```

WILD TYPE CONTROL


```
             5'
           / A        C    T    A    G
ASN          A
           \ C
           / C
PRO/LEU   C/T ->
           \ T
           / C
LEU          T
           \ G
             3'
```

CARRIER MOTHER (I-1)

168
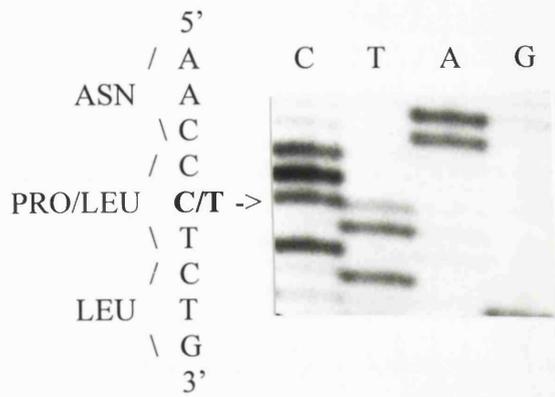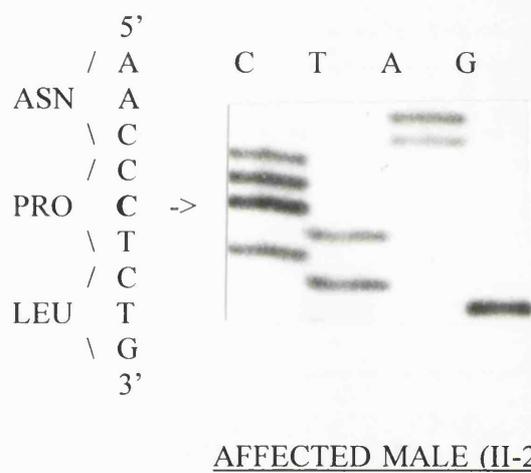
FIGURE 45 DNA sequence of exon 5 PCR product from a wild type control and the carrier mother (I-1) and affected male (II-2) from family AB (Table 12). The arrow indicates the T to C change which substitutes a proline for a leucine at amino acid 223.

4.6.0   <u>VARIATION OF SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS CONDITIONS</u>

The sensitivity of SSCP analysis was estimated by examining the range of conditions at which given sequence changes, either reported in the literature or identified during this study, were detected as band shifts and whether a band shift occurred in one or both strands. The changes investigated were in exon 4: the A->C Thr(181)Pro change in family CB, the G->T change at the -1 position of the 3' acceptor splice site in family MDJ and the T->C *Aha*II polymorphism at Asp202. In exon 5, the T->C Leu(223)Pro change in family AB was analysed. The results of the exon 4 and 5 analyses are given in Figures 46 and 47 respectively and are collated in Table 14.

In the case of exon 4, each sequence change was analysed under 4 sets of conditions: undigested (335bp) and digested (111, 143, 81bp) PCR product at 4°C and 20°C. Considering both strands, 8 opportunities to detect a band shift existed for each change.

The analysis (Table 14) showed that no sequence change was detected under all experimental conditions and the optimal conditions for each mutation varied greatly despite occurring in the same exon. The ideal size for SSCP analysis is reported to be 200bp or less (Hayashi 1991). In exon 4, the effect of digestion was found to depend on the sequence change. Digestion enabled the detection of the mutation in family MDJ, which could not be identified on analysis of undigested product, but abolished the detection of the *Aha*II polymorphism, which was visible on analysis of undigested product. Digestion had no effect on the detection of the change in family CB.

Despite this, the sensitivity of SSCP analysis was increased by decreasing the fragment size. Three of the six potential changes (on analysis of digested product at both 4°C and 20°C) were detected on analysis of digested samples, as opposed to two of the six for undigested samples.

The sensitivity was increased by analysis at 4°C. Three of the six potential mutations
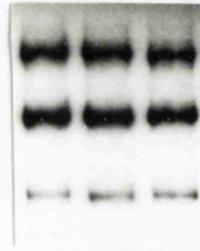
A- MDJ A+

A; Undigested exon 4 PCR product analysed at 4°C
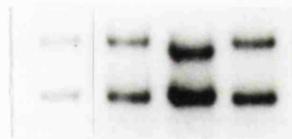
CB MDJ A+ A-

B; Undigested exon 4 PCR product analysed at 20°C
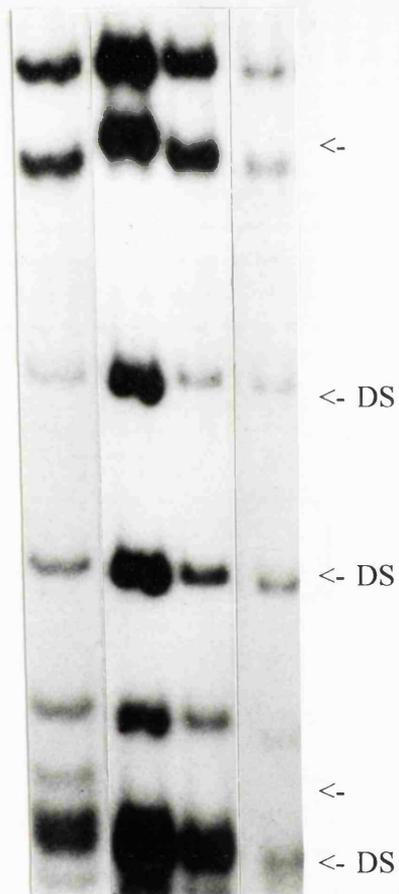
CB MDJ A- A+

C; *Rsa*I digested exon 4 PCR product analysed at 4°C

172
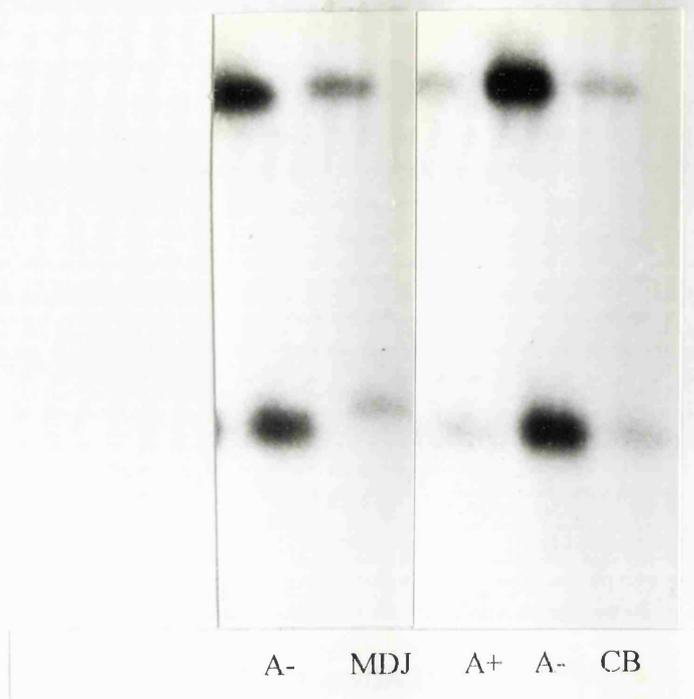
D; *Rsa*I digested exon 4 PCR product analysed at 20°C

FIGURE 46 A comparison of the sensitivity of SSCP analysis in detecting exon 4 sequence changes as band shifts under varying experimental conditions. Two parameters were altered: fragment length (undigested: 335bp or *Rsa*I digested: 111, 143 and 81bp) and temperature (4°C or 20°C). Three known sequence changes: a G-> T at the -1 position of the 3' acceptor splice site in family MDJ, an A->C Thr(181)Pro change in family CB and the T->C *Aha*II polymorphism (A+/A-) at Aspartate 202, were analysed.

In each case analysis was on a 6% nondenaturing polyacrylamide gel at 380-400V. The arrows indicate the variant bands and double stranded DNA is indicated by DS.

The result of SSCP analysis of undigested product from family CB at 4°C is shown in Figure 38.

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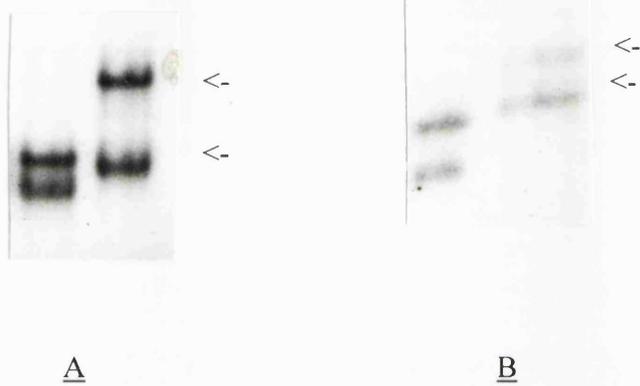
FIGURE 47 A comparison of the sensitivity of SSCP analysis in detecting a known sequence change, the C->T Leu(223)Pro mutation in family AB, as a band shift under the varying experimental temperatures: 4°C (A) and 20°C (B). In each case the arrows indicate the variant bands.

		STATUS	UNDIGESTED		DIGESTED	
		CONDITIONS	4°C	20°C	4°C	20°C
EXON	MUTATION	SEQUENCE CONTEXT				
F O U R	-1 position, 3' splice site Family MDJ	CATTTTAGTTTGTG ↓ T	–	–	+ (1)	+ (1)
	Thr (181) Pro Family CB	ACCACCTGGACCACC ↓ C	+ (1)	–	+ (1)	–
	*Aha*II+ Asp (202)	TGTGCTGATGCCAGA ↓ C	–	+ (1)	–	–
F I V E	Leu (223) Pro Family AB	TCCAACTTTCTGTCC ↓ C	+ (2)	+ (2)	▨	▨

TABLE 14: The effect of temperature and fragment size on the sensitivity of SSCP analysis in detecting known sequence changes in the proteolipid protein gene. The number of strands altered is indicated in brackets.

(digested and undigested product at 4°C) were detected on analysis at 4°C, as opposed to two of the six, at 20°C.

For each change detected, only a single altered band was present. Of the 8 potential changes (the 2 complementary strands at 4 conditions), 2/8 (25%) were detected in families CB and MDJ, while the *Aha*II polymorphism was detected in only one case (12.5%).

Finally if only digested or undigested product was analysed at one temperature, the probability of detecting a change dropped to 1/3 (33%) for all but one condition; digested DNA at 4°C, at which 2/3 (75%) of the changes were still detected. To detect all three mutations, analysis of both digested and undigested products at 4°C and 20°C was required.

Conversely the analysis of undigested (236bp) exon 5 PCR product showed that the change in family AB was detected at both 20°C and 4°C with both strands altered in each case, that is with 100% sensitivity.

Family RR was not included in this analysis as the molecular basis of this change remains to be defined.

4.7.0 PORENATAL DIAGNOSIS IN PELIZAEUS-MERZBACHER DISEASE

Carrier detection and prenatal diagnosis was requested by II-3 in family CB. In this family PMD is transmitted in a manner characteristic of X-linked recessive inheritance. The carrier status of II-3 was unknown but her sister (II-1) and mother (I-2) were obligate carriers, having had an affected son and brother and an affected son and grandson respectively. At this stage II-3 had a 50% risk of having inherited her mother's defective gene and thus of being a carrier..

RFLP analysis of the intragenic 4/4.5kb *Msp*I polymorphism was uninformative and the

family were analysed using the linked markers: p19-2 (*Taq*I; 3+2/5, *Msp*I; 4.4/12), p212-9 (*Taq*I; 1.8/3.2), CX52.5 (*Msp*I; 7.5/7.7), pXG-12 (*Pst*I; 6.5/7.2) and S21 (*Taq*I; 2.0/2.2). The results are given in Figure 48.

Three of these: S21 (*Taq*I), 19-2 (*Taq*I), and CX52.5 (*Msp*I) proved partially informative. In each case the obligate carrier mother (I-2) was heterozygous, which enabled her mutant and normal chromosomes to be distinguished. The mutant chromosome was the one she had passed on to her obligate carrier daughter (II-1), in this case identified by the 2.0, 2+3 and the 7.5kb alleles detected by probes S21, 19-2 and CX52.5 respectively.

As II-3 had also inherited these alleles from her obligate carrier mother, she was predicted to be a carrier. On the basis of the results of the flanking markers; S21 and 19-2, and taking account of the possibility of recombination, using recombination fractions of 0.08 between p19-2 and PLP and 0.04 between S21 and PLP, II-3 was given a 98.7% risk of carrier status.

As the father (I-2) was hemizygous for the 2.0, 2+3, 7.5kb alleles, his daughters (II-1 and II-3) were homozygous for these probes and prenatal diagnosis was not possible. At this stage II-3 and II-1 had a 50% risk of having an affected boy and their only options were to terminate all male fetuses or to accept this risk.

Further analysis of the intragenic exon 4 *Aha*II polymorphism showed that the mother (I-2) was heterozygous, having both the 227bp and the 183 and 44bp fragments, but, as for the linked probes, II-3 was homozygous having inherited the *Aha*II- allele from both parents.

Subsequent SSCP analysis of exon 4 of the PLP gene identified a variant band pattern in this family (Section 4.4.0, Fig. 38) which was present in the obligate carrier females (I-2, II-1) and in II-3. This result confirmed the carrier status of II-3, as predicted by RFLP analysis, and, as the mutant and normal alleles could now be distinguished, prenatal diagnosis was possible.

KEY

p19-2	(*Taq*I; 3+2/5)
p19-2	(*Msp*I; 4.4/12)
p212-9	(*Taq*I; 1.8/3.2)
CX52.5	(*Msp*I; 7.5/7.7)
pRL1	(*Msp*I; 4.0/4.5)
Exon4/PLP	(*Aha*II; 0.044+0.183/0.277)
pXG-12	(*Pst*I; 6.5/7.2)
S21	(*Taq*I; 2.0/2.2)

I1

3+2
–
–
7.5
–
–
2.0

I2

3+2	5.0
4.4	4.4
3.2	3.2
7.5	7.7
4.0	4.0
0.183 + 0.044	0.277
6.5	6.5
2.0	2.2

II1

3+2	3+2
–	–
7.5	7.5
–	–
–	–
–	–
2.0	2.0

II2

II3

3+2	3+2
4.4	4.4
3.2	3.2
7.5	7.5
4.0	4.0
0.277	0.277
6.5	6.5
2.0	2.0
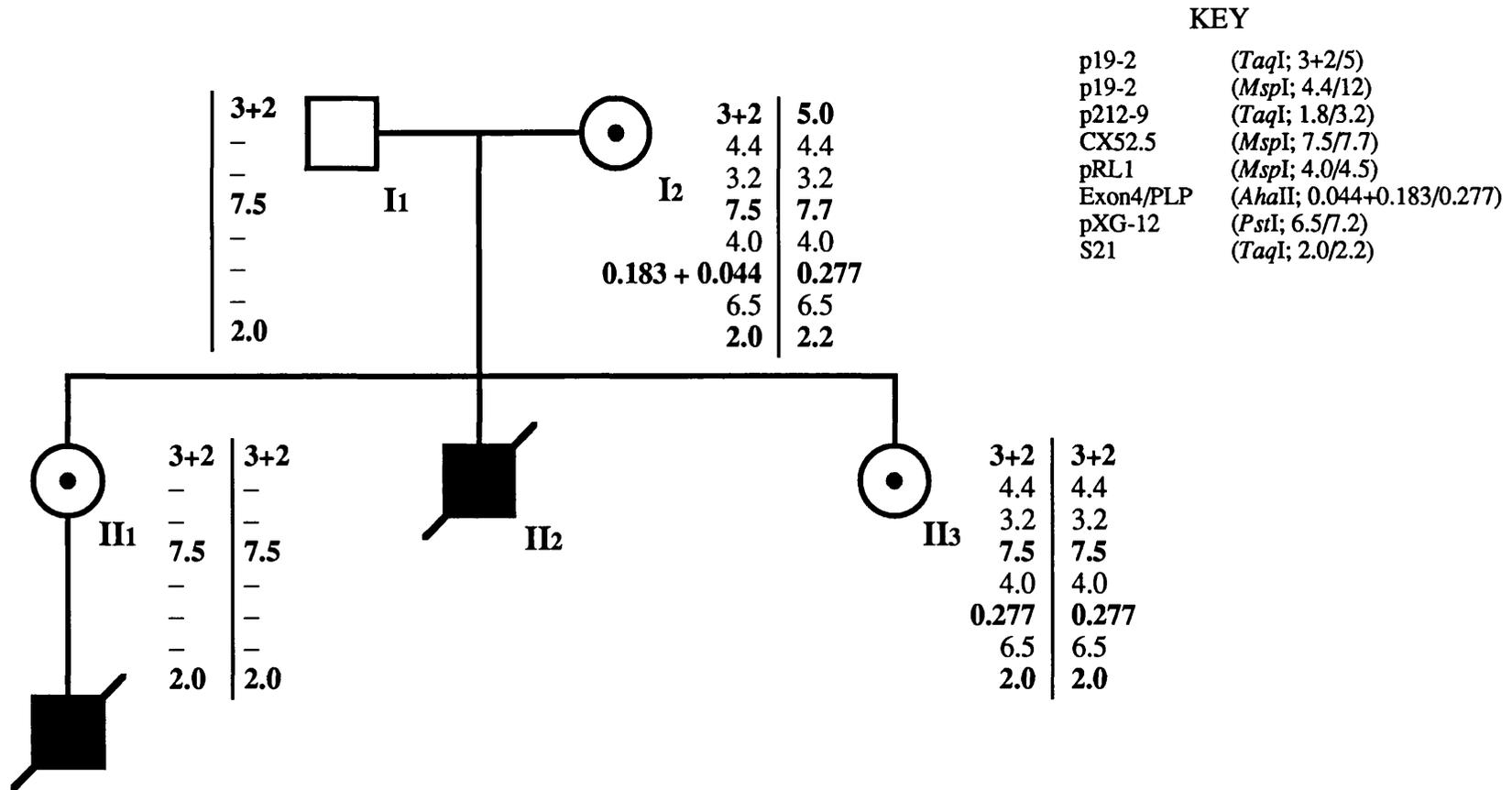
FIGURE 48: The results of the analysis of linked and intergenic polymorphisms from the Xq21.3-q22 region in family CB. Squares and circles represent males and females respectively, where completely shaded shapes represent affected individuals and dotted circles represent carriers identified by family history and/or DNA analysis

On fetal sexing of CVS material, the fetus (III-2) of II-3 was shown to be male and thus at risk of PMD. SSCP analysis of exon 4 (Section 4.4.0, Fig. 38) showed that the fetus had not inherited the variant SSCP pattern and was hence at low risk of developing PMD. Taking into consideration the possibility of genetic heterogeneity (2.5%) and the use of an as yet novel technique (2.5%), II-3 was given a 95% probability of having an unaffected child.

On sequencing of exon 4 PCR product from the members of family CB, including the CVS (III-2), a threonine to proline change at amino acid 181 was identified in the carrier women (I-2, II-1 and II-3) but not the CVS (III-2) (Section 4.4.0, Fig. 41). This supported the SSCP analysis result and the pregnancy was continued. The risk to the fetus was predicted to be reduced below the 5% quoted to the family after SSCP analysis and was probably less than 1%.

The pregnancy and birth proceeded uneventfully and the child (III-2) is developing normally.

5.0.0 <u>DISCUSSION</u>

5.1.0   **THE LIMITATIONS OF INDIRECT ANALYSIS**

This study has investigated the efficacy of direct mutation detection in 2 X-linked disorders: ornithine carbamoyl transferase deficiency and Pelizaeus-Merzbacher disease.

Indirect analysis using restriction fragment length polymorphisms (RFLPs) or variable number tandem repeats (VNTRs) has proved very useful in carrier detection and prenatal diagnosis of genetic disorders. However, its usefulness is limited by the need for family studies and the requirement for DNA from an affected individual to establish phase. Individuals may also be uninformative; the disorder may be heterogeneous with one or more genes involved; analysis can be complicated by the possibility of nonpaternity; and recombination between the marker and the mutation is possible, especially for large genes and intergenic probes.

Germline mosaicism (Hall. 1988), such that a proportion of germ cells bear a mutation while the rest are normal, can result in uncharacteristic patterns of inheritance and may severely complicate analysis. This has been reported, for example, in several cases of Duchenne muscular dystrophy (van Essen et al. 1992).

In X-linked disorders analysis is complicated by the high proportion of new mutations, estimated to be at least 1/3 and 2/3 in sporadic males and females respectively (Section 1.1.2). In the absence of a family history this can result in the misdiagnosis of a noncarrier as a carrier and the termination of unaffected pregnancies. Additional biochemical, clinical or genetic analyses are generally required to identify carriers before gene tracking is used.

5.1.1   **ORNITHINE CARBAMOYL TRANSFERASE   DEFICIENCY**

Carrier detection and prenatal diagnosis in OCT deficiency are typically provided by a combination of family history and biochemical and linkage based analyses.

RFLP analysis of the *Msp*I and *Bam*HI polymorphisms has been found to be informative

in only 60-80% of women (Rozen et al. 1985, Fox et al. 1986.) and for this reason, as well as the need for family studies and DNA from affected individuals to establish phase, linkage based analysis is typically not possible in a proportion of OCT deficiency families. Further due to the possibility of germline mosaicism and the high incidence of new mutations, it should only be used for carrier exclusion or after a woman has been diagnosed as a carrier by biochemical analysis or family history.

As the number of cases with a positive family history are limited, carrier detection is predominantly by biochemical analysis. This, though significantly improved, is not 100% accurate (Hauser et al. 1990, Pellet et al. 1990) and while a positive test result strongly suggests carrier status, a negative result does not eliminate it. Pelet et al. (1990) calculated that despite a negative allopurinol test result, the mother of an affected boy retains a significant 30% risk of being a carrier.

Biochemical analysis is limiting in four main groups. These are carriers of the mild form of late onset OCT deficiency who may give normal or borderline test results; young girls in whom the test has not been completely verified and who represent the predominant group presenting for testing; and favourably lyonised carriers. Carrier females exhibit a wide variation in the proportion of mutant X-chromosome which is active and, in cases where the normal X is predominantly active, this may result in borderline or even normal protein load results. The final category are mosaics whose tissues contain cells with either the normal or the mutant genotype. If the hepatic tissues contain few or no mutant cells but these are present in the germ cell population, protein load results may be normal, but the female will still be at risk of passing on the mutant gene to her offspring.

In several cases of late onset OCT deficiency asymptomatic males have been identified (Finkelstein et al. 1990a, Hata et al. 1991, this report) who, without direct mutation detection, can be identified only by an invasive liver biopsy and enzyme analysis. Diagnosis of such males is especially important as any daughters are obligate carriers for a potentially lethal disorder.

Direct mutation detection in these situations would enable unequivocal diagnosis, carrier detection and prenatal diagnosis.

5.1.2   **PELIZAEUS-MERZBACHER DISEASE**

In Pelizaeus-Merzbacher disease carrier females are generally asymptomatic and no biochemical means of carrier detection exists. Magnetic imaging has been used (Boltshauser et al. 1988) but has been shown to be inaccurate in young girls, the group presenting predominantly for testing (Pratt et al. 1991).

Carrier detection has been carried out in families with X-linked PMD using RFLP analysis, based on the assumption that the disorder was caused by a mutation at the PLP locus (Maenpaa et al. 1990, Bridge et al. 1991). But this approach has been thrown into question by the failure to find mutations in 75% of PMD cases in the PLP coding, control and splice site regions (Hudson et al. 1989a, Pham-Dinh et al. 1991a, Pratt et al. 1991, Doll et al. 1992) and hence the possibility that a second X-linked gene involved in PMD exists. Further as many cases are sporadic and manifesting females have been reported, the possibility of autosomal recessive inheritance remains (Begleiter and Harris. 1989).

Diagnosis of this disorder is complicated by the fact that both the clinical symptoms and the MRI evidence of a dysmyelinating disorder are common to a range of diseases and as the limits of clinical presentation have not been determined, uncharacteristic cases may be missed. The only definitive diagnostic element was, until recently, considered to be a scarcity of mature oligodendrocytes but with the discovery of the rumpshaker mouse mutant (Schneider et al. 1992), in which defective myelination and oligodendrocyte maturation are independent, the clinical criteria have been brought into question.

The direct identification of the causative mutation in each family provides the only accurate means of diagnosis, carrier detection and prenatal diagnosis in this disorder. Further this will enable the determination of the range of phenotypes associated with a mutation at the PLP locus.

5.2.0   **DIRECT MUTATION DETECTION**

Direct mutation detection by DNA sequence analysis is the most desirable course of action for the reasons given above. The ideal template is mRNA which, in addition to coding changes, allows the detection of mutations within intronic splice site sequences which result in exon skipping or the activation of cryptic splice sites, as well as changes which create cryptic splice sites.

In the case of tissue specific genes such as PLP and OCT, which are expressed in the nervous tissues and the liver respectively, mRNA is not readily available. In these cases mRNA analysis would involve an invasive biopsy. Instead, the analysis of these genes routinely involves the individual sequencing of each exon. Ectopic expression of tissue specific genes has been reported (Chelly et al. 1992) but remains to be verified for OCT and PLP.

Although sequencing provides an unequivocal means of mutation detection, the analysis of the individual exons of a gene, long stretches of DNA, or large numbers of samples is expensive in terms of both labour and cost. To overcome this problem a range of prescreening techniques has been developed. These are designed to localise a mutation before sequence analysis and include SSCP analysis (Orita et al. 1989a, 1989b), DGGE (Myers et al. 1985a, 1988), chemical mismatch analysis (Cotton et al. 1988) and the analysis of cytosine-guanine dinucleotide sites (Grompe et al. 1991).

In this study, the individual exons of the PLP and OCT genes were prescreened using SSCP analysis, followed by direct sequencing of those exons which produced an altered band pattern. Patients with classical and late onset OCT deficiency were also screened for major deletions and rearrangements by Southern blot analysis and for mutations at coding region 5' CpG 3' dinucleotide sites by restriction enzyme digestion.

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5.2.1 SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS: EXPERIENCE AND LIMITATIONS

This technique has been used in a wide range of disorders and its main advantage lies in its simplicity and the ability to screen large numbers of samples rapidly and inexpensively. The only requirement is sufficient sequence knowledge to design PCR primers for the region under analysis. Its limitations lie in the variable sensitivity. Several groups have shown that the technique can potentially detect the majority of sequence changes. Suzuki et al. (1990) analysed 10 point mutations in 6 sequences from the RAS family of oncogenes and identified a mobility shift in all cases, in at least one of the strands. Michaud et al. (1992), using a combination of three conditions, detected 100% (20/20) of known mutations and 79% (19/20) of unknown changes in the ornithine delta-aminotransferase gene.

The ability of a mutation to effect a conformation change depends on its sequence context. The sensitivity of the technique varies for each DNA segment analysed and for each individual sequence change. As the conformation is also affected by local environmental factors including; temperature, ionic strength and glycerol concentration, the sensitivity will vary according to these.

Changes in particular sequence contexts have been shown not to result in conformation changes. For example, the common intron 12 donor splice site mutation found in phenylketonuria was not detected by SSCP analysis despite the use of a range of conditions (Labrune et al. 1991).

On analysis of the PLP gene (Table 14) under a range of experimental conditions, the sensitivity of the technique in detecting exon 4 sequence changes varied between 12.5-25% (1/8-2/8), depending on the change under analysis. One change, the AhaII polymorphism, was detected at only one condition and as a change in only one strand. Conversely the exon 5 change was detected with 100% sensitivity, that is as a detectable mobility shift in both strands and at all experimental conditions.

A survey of the sequence context of the PLP changes analysed in this study (Table 14) showed no correlation with the ease of detection.

On analysis of the OCT gene, SSCP variants were detected at both 4 and 20°C in one family (CFA) and at either 20°C or 4°C in families AD and FAR respectively.

A consequence of the variable sensitivity is the fact that while few false positives occur, a negative screen does not eliminate the possibility of a mutation within the sequence. While this is less important in a single gene disorder such as OCT deficiency, the usefulness of this technique in PMD, in which the differentiation of cases with and without a PLP mutation is essential due to the possibility of genetic heterogeneity, is limited. Techniques such as GC-clamped DGGE or chemical mismatch analysis in which a negative screen corresponds to a high probability that no change is present may, barring direct mRNA sequencing, prove more applicable.

Several groups have attempted to determine the optimum conditions for SSCP analysis. Spinardi et al. (1991) varied the temperature (4°C, 20°C), the ionic strength (0.5 x TBE, 1 x TBE) and the glycerol concentration (5%, 10%) and found that optimal results were seen on room temperature gels in 0.5 x TBE and 5% glycerol, though for certain sequences, a second run at 4°C without glycerol was informative. Similarly Orita et al. (1989b) and Labrune et al. (1991) reported optimal resolution on room temperature gels with glycerol.

Analysis of the PLP gene showed that the optimum conditions for SSCP analysis are sequence dependent and vary for each mutation. Overall sensitivity was found to be maximal at 4°C on gels containing no glycerol.

Recently, Fraser et al (1992) have reported a good correlation between experimental results and computer generated secondary structure predictions, suggesting that computer programs may become available to predict the individual optimal conditions for a given sequence or mutation, overcoming these limitations.

As SSCP analysis will potentially detect any sequence variations, whether polymorphisms or mutations, it is usually necessary to identify the underlying base change by sequencing. In PMD, in which both additional autosomal or X-linked genes may be involved and in which the diagnosis itself is unclear, only a deleterious change can be used for family analysis. Conversely in OCT deficiency, a single gene disorder, an SSCP variant can be used as a marker to track the diseased gene through a family without establishing that it is the causative mutation. Though for analysis of extended family members and genotype and phenotype correlation, variants should be sequenced.

5.2.2   <u>CYTOSINE GUANINE DINUCLEOTIDE SITE ANALYSIS</u>

Systematic studies of both X-linked and autosomal diseases have shown that the dinucleotide 5' CpG 3' is highly prone to mutation to 5' TpG 3' and 5' CpA 3' and changes at these sites account for over a third of human polymorphisms and disease mutations (Koeberl et al. 1990, Cooper and Krawczak. 1990). For example, in haemophilia B of the 80-90% of cases reported to be due to single base substitutions , 38% are CG to TA transitions within 5' CpG 3' dinucleotides , and a 24-fold enhancement of mutation at 5' CpG 3' dinucleotides has been estimated in this gene.(Koeberl et.al. 1990)

In contrast 5' CpG 3' is not a mutation hotspot in the alpha and beta globin genes (Antonarakis et al. 1985), in which the greatest number of single base mutations have been delineated, though this may reflect the paucity of 5' CpG 3' sites in the beta globin gene as well as ascertainment bias, heterozygote advantage, founder effect and location (Koeberl et al. 1990). In the alpha globin gene the frequency of 5' CpG 3' and 5' GpC 3' dinucleotides is equal suggesting that 5' CpG 3' is not methylated in the germ line (Bird. 1986). An avoidance of methylation appears associated with genes which possess CpG rich islands; primarily constitutively expressed genes and a proportion of tissue specific genes, and a lower level of mutation at 5' CpG 3' dinucleotides is predicted at these loci.

Mutations at 5'CpG 3' sites make up 63% of reported base changes in OCT deficiency. In addition to the inherent hypermutability of these sites, this figure reflects detection bias, in that a proportion of 5'CpG 3' changes are readily detected as altered band patterns on Southern blot analysis, and the fact that many studies have undertaken the analysis of these sites prior to mutation detection or direct sequencing.

The OCT gene coding region contains 18 5'CpG 3' sites and mutations have been reported at 8 of these (Table 1). This study reports a further 5 mutations, 2 at a previously unreported site.

The identification of the Arg(109)Gln change in family PD brings the number of mutations reported at this site to 9 independently recurring incidences. This site represents a mutation hot spot in OCT deficiency and additional, as yet unidentified, mechanisms may be involved in promoting deamination at this position. The site may be especially prone to the mutagenic effects of chemicals such as bisulphites (Koeberl et al. 1990) or mutability at these sites may be affected by adjacent nucleotides, as reported by Bains and Bains (1987), although no such difference was noted by Cooper and Krawczak (1989) or Koeberl et al. (1989).

The Arg(109)Gln change has been shown by Lee and Nussbaum (1989) to abolish enzyme activity during expression studies and represents the first report of this mutation in a manifesting female. The proband (II-3) in this family is mildly affected and is predicted to show unfavourable X-inactivation in her hepatocytes.

Two additional recurring mutations are reported. These are the second report of a Pro(193)Leu change associated with classical OCT deficiency and the fourth report of an Arg(245)Trp change associated with a mild form of late onset OCT deficiency.

Mutations at 5'CpG 3' dinucleotides may also result from processes other than deamination as, in addition to CG:TA transitions, an excess number of other mutations have been reported at these positions. In the mutation survey conducted by Cooper and

Krawczak (1990), 10% of the changes reported at 5'CpG 3' sites were atypical and Sommer et al. (1992) reported that transversions (between C or T and A or G) at 5'CpG 3' dinucleotides were elevated 8 fold relative to those at non 5'CpG 3' dinucleotides, in a study of haemophilia B.

Atypical G to T changes have been reported in 2 cases of OCT deficiency (Feldmann et al. 1992, Grompe et al. 1991 / Spence et al. 1989) and result in Gly(18)Ter and Arg(288)Leu changes respectively. The identification of the novel Arg(97)Leu change in exon 4, which results from a G to T change, represents the third occurrence of this transversion at a 5' CpG 3' site and supports the existence of a second, less common, mutation mechanism.

In contrast to OCT deficiency, no changes were identified at 5'CpG 3' sites in the PLP gene. This contains 10 5'CpG 3' sites within the coding region: 7 in exon 3, 2 in exon 10 and 1 in exon 4, which potentially give rise to 12 amino acid changes. These include 2 arginine codons (CCG, CGA) which give rise to glutamine, threonine and a termination codon. The remainder of the changes are the replacement of an acidic by an amide group and the replacement of aliphatic amino acids with methionine, serine or other aliphatic amino acid groups. The lack of mutations at these sites suggests that either the gene may not be methylated in the germline or, that as indicated by the distribution of mutations, changes occurring in exons other than 4 and 5 will only lead to PMD if they result in a major disruption of the PLP protein structure and hence its organisation in the myelin membrane.

This is supported by an analysis of the reported changes in exons other than 4 and 5 (Tables 5 and 6). Four of the 5 changes resulted in the removal or introduction of the alpha-helix breaker proline (Trofatter et al. 1989, Boison et al. 1989, Nadon et al. 1990) or the introduction of a charged amino acid into a hydrophobic domain (Doll et al. 1992) and were predicted to have severe structural implications. The fifth, in the Jimpy[msd] mouse mutant, was an apparently conservative alanine to valine change, but may reflect a size effect in a tightly constrained alpha-helical region.

5.2.3   DELETION ANALYSIS

Deletions have been reported to account for some 10-20% of OCT deficiency cases (Grompe et al. 1991, Suess et al. 1992, Liechti-Gallati et al. 1991, Rozen et al. 1985). In this study a Southern blot pattern consistent with a deletion was identified in one of 29 families analysed.

This result may be explained by considering that deletions are identified as altered band patterns involving the loss of a band and / or the creation of a novel smaller band. In cases where a novel band is not produced or is coincident with a band in the normal pattern, the deletion is detectable only in an affected male. In a carrier female the mutant pattern is masked by the normal pattern from the unaffected allele.

In this study, due to the high incidence of families with a sporadic symptomatic female and the scarcity of DNA from affected male probands, only 7 of the 29 cases analysed were male, the remainder consisted of symptomatic and asymptomatic female carriers. Thus in 75% of the cases only deletions creating a novel band pattern could be detected.

In family TM, Southern blot analysis of *Taq*I digests identified a novel 3.1kb band with partial loss of the 4.6kb band in the affected boy and carrier mother. The 4.6kb band corresponds to exons 2 or 4 (Grompe et al. 1991) and as both exons amplified the change may represent a predominantly intronic deletion or, conversely, the creation of a new *Taq*I site within the 4.6kb fragment. The latter hypothesis is supported by the normal *Msp*I pattern on Southern blot analysis.

The high incidence of both partial and total gene deletions as the underlying basis of this disorder may reflect the presence of frequent repeat elements within the OCT gene as well as the limited proportion of base substitutions capable of eliminating OCT activity.

5.3.0   **LATE ONSET ORNITHINE CARBAMOYL**
               TRANSFERASE DEFICIENCY

Although OCT deficiency in boys is usually fatal in the first few months of life, some 10% of cases (Finkelstein et al. 1989a) are of late onset, with presentation in infancy, childhood or adult life.

In two cases, this has been shown to be due to somatic mosaicism for partial gene deletions (Legius et al. 1990, Maddalena et al. 1988a) and Arg(62)Thr, Leu(272)Phe and Glu(87)Lys point mutations have been reported in exons 3 and 9 (Tuchmann et al. 1992, Feldmann et al. 1992). A fourth point mutation, an Arg(245)Trp change, has been reported to recur independently in 3 families with mild late onset OCT deficiency (Finkelstein et al. 1990b, Hata et al. 1991).

SSCP analysis of 9 of the 10 exons of the OCT gene in 5 families with late onset deficiency identified variants in 3 families (AD, FAR and CFA). On sequencing two novel mutations were identified. These were a G to T substitution at the -1 position of the 5' donor splice site of exon 4, which was predicted to result in an Arg(97)Leu substitution, and an Ala(176)Thr change in exon 6. The third change was Arg(245)Trp which represented the fourth independent report of this mutation in late onset OCT deficiency.

No changes were detected on SSCP analysis of the remaining 2 cases. This may be as a result of the limited sensitivity of SSCP analysis, the nonscreening of exon 3, or the inability due to a lack of intronic sequence, to analyse splice site junctions and complete coding sequences. Of 18 splice sites only 1, the 5' donor splice site of exon 1, could be completely analysed. Due to the limited sequence data suitable for primer design some coding sequences were also lost. These were 40, 11, 47, 2, 9 and 14bp from exons 2, 4, 5, 6, 7+8 and 10 respectively.

Alternatively as both cases were sporadic they may be somatic mosaics for a molecular deletion, as reported previously in 2 cases of late onset deficiency (Maddalena et al.

1988a, Legius et al. 1990). As the wild type allele would amplify normally such a deletion would, in most cases, be undetectable on SSCP analysis.

5.3.1 A REVIEW OF THE CHANGES IDENTIFIED IN FAMILIES AD, CFA AND FAR

FAMILY AD

In family AD, a G to T change was identified at the last base of exon 4. This was predicted to result in the substitution of an arginine by a leucine at amino acid 97 of the mature protein and to remove the G at the -1 position of the 5' donor splice site.

A consensus sequence for the 5' donor splice site; **A G** <u>G T</u> A/G A G T, where exonic bases are indicated by bold type, has been established by the analysis of a large number of eukaryotic splice junction sequences. A systematic analysis of 5' splice site sequences of eukaryotic protein coding genes using the GENBANK database (Shapiro and Senapathy. 1987) has shown that most (78%) exhibit a guanosine as the last base of an exon. The possible consequence of the removal of this guanosine on the function of the splice site was assessed by comparing the statistical estimates for the probable use of mutant and normal splice sites (Shapiro and Senapathy. 1987). Based on the -3 to +6 consensus sequence for a 5' splice site, the G to T change resulted in a decrease in the likelihood that this site is used of 12.2%, from 71.7% to 59.5%. As most sites give a probability of 70 and higher, this change was predicted to result in defective splicing.

This is supported by the sparse fur^{-ash} murine model of OCT deficiency in which a G to A change at this position has been identified as the causative mutation (Hodges and Rosenberg. 1986). This change affects the efficiency of splicing at the 5' donor splice site and results in the production of 2 mRNA precursors. A normally spliced mRNA bearing an Arg(97)His point mutation is produced at 5% of normal levels and an elongated mRNA is produced by the activation of a cryptic splice donor site, 48 bases into the intron 4. This precursor is also translated at 5% of the expected level and is not assembled into the

mature protein.

This result can be explained by considering the exon definition model of splice site selection (Robberson et al. 1990). The 3' acceptor splice site is recognised by specific factors binding to the consensus sequence, the spliceosome complex is established and a scanning mechanism is proposed to operate across an exon to locate the 5' donor splice site. Splice site studies have shown that the best downstream site within 300 nucleotides is selected and that in cases were the effectiveness of a site is reduced by mutation, a cryptic site providing a better match to the mutated consensus sequence is activated. In cases were an alternative site is not available the exon is not defined and exon skipping results.

Splice site calculations for the G to A change suggest an 11.7% reduction, from 71.7% to 60%, in the probability that this site is utilised. This is virtually identical to that predicted in family AD. A reduction in splicing efficiency as opposed to an amino acid substitution has been shown to be the main cause of OCT deficiency in the murine model. Thus the G to T change in family AD is predicted to result in incorrectly processed mRNA and a lack of functional protein, but as an analysis of available human intronic sequence found no comparable cryptic site within 300 nucleotides, in man this change is predicted to result in the skipping of exon 4 in 95% of splicing events.

In OCT deficiency splice site changes have been identified in 6 previous cases (Table 2). This report represents the seventh independent splice site change in OCT deficiency and suggests that in addition to deletions (10-20%) and 5'CpG 3' dinucleotide site changes (30-40%), splicing errors may form a significant subgroup of defects in OCT deficiency. In 3 cases (Grompe et al. 1989, Hodges and Rosenberg. 1986, this report) the changes occurred within 5'CpG3' dinucleotides.

The importance of splice site mutations in OCT deficiency is supported by the fact that only a minority of splice sites can be readily investigated. Primer sequences, due to limited intron data or unsuitable sequences, cover splice site junctions in most cases and

cDNA analysis is limited by the scarcity of liver material. These results together with evidence from other disorders, including haemophilia B in which splice sites changes account for 13% of mutations, indicate that the division of genes into exons presents a significant genetic cost to the organism (Koeberl et al. 1990).

The Arg(97)Leu change in family AD was shown, during the PCR analysis of 5'CpG 3' dinucleotide sites, to abolish digestion at the *Msp*I site in exon 4. This change was not seen on digestion of PCR product from an additional 29 families, supporting the hypothesis that this is the causative mutation in this family. A comparison of the protein structures of OCT and aspartate transcarbamylase (Horwich et al. 1984) revealed extensive matching between residues 96 to 103 of the mature OCT protein. This suggests that these have been conserved through evolution and hence that the Arg(97)Leu change is likely to affect protein function. The effects of this change could be verified by sequencing of cDNA from the affected individual, though as OCT is expressed only in the liver and ectopic expression has not been verified, this would require an invasive liver biopsy.

<div align="center">

FAMILY CFA

</div>

The second novel mutation, a G to A change, was identified in a very mildly affected male in family CFA. This was predicted to result in the nonconservative replacement of alanine by the hydroxylated amino acid threonine at amino acid 176 and was identified during both SSCP and 5'CpG3' site analysis. The change was not seen on SSCP analysis of the remaining 4 late onset cases or on *Cfo*I digestion of exon 6 PCR product from 33 families, supporting its role as the causative mutation in this family.

Despite the mildness of the condition, the carrier mother (I-2) and sister (II-2) gave allopurinol load results clearly in the carrier range, but were uninformative on RFLP analysis. Identification of a sequence change in this family has enabled prenatal diagnosis to be made available to II-2 and carrier detection is now be possible in the extended family.

As OCT deficiency is a single gene disorder, should this change represent a neutral polymorphism and not the causative mutation, it can still be used as a linked marker to track the defective gene through the immediate family.

FAMILY FAR

The third mutation, a C to T change at the first nucleotide of codon 277, is predicted to substitute a tryptophan for an arginine at amino acid 245 of the mature protein in a family with three affected brothers. Although one boy died at 16 months after a short encephalopathic illness, the remaining two affected boys are well on a low protein diet, at ages 17 years and 12 years, indicating that the disease is relatively mild in this family.

The role of Arg(245)Trp as the disease causing mutation in family FAR is strongly supported by the associated phenotype; a similar clinical progression and the occurrence of asymptomatic males in the 4 families in which this change has been reported. It is a nonconservative amino acid change which alters both shape and charge and this residue is conserved across species being found in; *E.coli*, yeast, aspergillus, rat and human genes (Huygen et al. 1987). A comparison of the predicted secondary structures of mutant and normal protein sequences (Hata et al. 1991) localised Arg 245 to the boundary of an alpha-helix and a beta-sheet region and showed that tryptophan shifted this boundary as well as significantly increasing the hydrophobicity of the region. They predicted a significant conformational change in the resulting protein which may affect both its affinity for substrates and / or its stability.

This report represents the fourth independent incidence of this change in late onset OCT deficiency and suggests that in the same way as Arg 109 represents a mutation hotspot in classical OCT deficiency, so Arg 245 represents a mutation hotspot in late onset OCT deficiency.

5.3.2   AN EXTENDED FAMILY STUDY IN A CASE MILD LATE ONSET ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY

Prior to the identification of the Arg(245)Thr change, it was not known if the mother (II-2) in family FAR had a new mutation or if the disorder was familial and thus that her sisters (II-4, II-6) and her niece (III-5) were also at risk of being carriers.

Family FAR was partially informative for the upper (6.2/6.5kb) *Msp*I polymorphism. The mutant allele was linked to the 6.2kb band which was inherited by the 2 affected boys (III-2, III-4), but not their unaffected brother (III-3). This enabled prenatal diagnosis to be offered to their mother (II-2) who, barring germline mosaicism, was an obligate carrier.

As the grandmother (I-2) was homozygous for the 6.2kb band, carrier detection was not possible for her other 2 daughters (II-4, II-6) or her granddaughter (III-6) who had inherited her mother's 6.2kb band. A second granddaughter (III-5) was excluded on the basis that she had inherited her mother's 6.6kb band.

Following the identification of the SSCP variant in family FAR, the extended family were analysed in an attempt to resolve this problem. The mother (I-2) and one sister (II-4) of II-2 were identified as carriers, while the second sister (II-6) and the daughter of II-4 (III-6) were excluded. The apparently asymptomatic brother (II-7) of II-2 was also shown to carry the SSCP variant. These results were confirmed by sequencing.

Sequence analysis predicted that the change would abolish an *Fnu*4HI site. Analysis of the extended family by digestion of exon 8 PCR product confirmed the sequencing results and indicated that *Fnu*4HI digestion will provide a rapid and simple means of carrier detection and prenatal diagnosis in this family.

In both families reported by Hata et al. (1991) a further, apparently asymptomatic male, was found to carry the mutation. A clinically undetected adult male (II-7) was similarly identified in family FAR. He carried the Arg(245)Trp change, as shown by SSCP,

sequence and *Fnu*4HI analysis but, barring transient symptoms in infancy, had remained well. Identification of such asymptomatic males is especially important as any daughters will be obligate carriers for a potentially lethal disorder as severity in these families is not uniform. For example, in family FAR one child is deceased two are doing well through management, and an adult is asymptomatic. In their study Hata et al. (1991) concluded that dietary management, after the diagnosis of an affected boy, may explain an asymptomatic sibling. In family FAR the asymptomatic male is the middle aged uncle of the probands. Protein management as a result of family diet or a noted, but uninvestigated, reaction to excess protein may be responsible. Additional factors affecting susceptibility to infection and dietary protein tolerance may be involved in modulating the severity of the symptoms in these families. It has not been possible to investigate this subject further.

The frequency of this base change in late onset cases makes *Fnu*4HI digestion of exon 8 PCR products an advisable preliminary screen in all late onset cases.

5.4.0   **PELIZAEUS-MERZBACHER DISEASE**

SSCP analysis of the 7 exons of the PLP gene in 14 PMD families identified variant patterns in four families. On sequencing three novel changes were identified in families CB, AB and MDJ, including the first report of a splice site mutation, in man, in this disorder. The fourth family (RR) is currently under analysis by a collaborating group.

The diagnosis in the 3 families was classical PMD, with 2 families (CB, MDJ) showing typical X-linked inheritance of the disorder. The third family (AB) had no previous family history but consisted of 2 affected male siblings. Inheritance in this family was consistent with X-linked, but also autosomal, inheritance. In all three families the affected individuals showed severe hypomyelination of the CNS, developmental and psychomotor retardation, laryngeal stridor, nystagmus and pyramidal tract signs. In the fourth family (RR) the proband was a sporadic male with atypical symptoms (M. Baraitser, personal communication). He was referred on the basis of severe hypomyelination on MRI, coarse nystagmus, muscle hypotonia and developmental delay.

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As shown by Doll et al. (1992), who analysed 24 patients with leukodystrophies of unknown etiology and identified a mutation in the PLP gene in a previously undiagnosed case, the range of clinical phenotypes for mutations at the PLP locus remain to be determined. Thus despite the atypical presentation, family RR may have a PLP mutation. Alternatively, the disorder may be distinct from PMD in which case the SSCP variant identified in the affected boy (II-1) represents a neutral polymorphism.

The difficulties in sequencing DNA from the proband (II-1) in family RR, the localisation of the SSCP variant to the 3' region of the PCR product and the fact that the sequencing primer was located in this region, suggested that a change in the intronic primer sequence may be involved. As for the factor VIII gene (Naylor et al. 1993) this may still result in aberrant splicing and hence be the causative mutation in this family.

No changes were identified in the remaining 10 families despite screening the coding and promoter regions and the splice sites of each exon by SSCP analysis under 4 different conditions. This is unlikely to reflect the low sensitivity of SSCP analysis due to the extensive analysis and supports the results of Pham-Dinh et al. (1991a), Pratt et al. (1991), Hudson et al. (1989a) and Doll et al. (1992), who sequenced the entire coding region and the 3' and 5' untranslated regions of the PLP gene and found no changes in 26 cases of PMD-14 of which were consistent with X-linked inheritance. This suggests the existence of an additional X-linked gene involved in PMD. This may be closely linked to the PLP locus as no crossovers with closely linked and intragenic probes have been reported (Maenpaa et al. 1990, Johnson et al. 1991). Alternatively, the causative mutations may occur in the untranslated regions as, for example, in the factor VIII gene (Naylor et al. 1993) in which intronic mutations result in abnormal splicing patterns.

The involvement of noncoding regions or an additional closely linked gene is supported by the analysis of markers from the Xq21.3-q22 region in the 2 classical PMD families (AB and WM). Each family has 2 affected male siblings and in one case, in family WM, an unaffected male sib. Allele segregation was consistent in each case with the involvement of a gene, possibly PLP, from this region in PMD. In both families the

affected siblings had inherited identical alleles and in family WM, the unaffected brother (II-2) had inherited the opposing alleles to his affected siblings.

5.4.1 A REVIEW OF THE CHANGES IDENTIFIED IN FAMILIES CB, AB AND MDJ.

The changes identified in the three families were: a Thr(181)Pro change in exon 4, a G to T change at position -1 of the 3' acceptor splice site of exon 4 and a Leu(223)Pro change in exon 5. These are believed to be deleterious mutations and not neutral polymorphisms based on the following facts.

The PLP gene has been highly conserved through evolution. At the coding level, murine and human DNA shows 92% and 90% homology in the coding and 3' and 5' noncoding regions respectively (Macklin et al. 1987). At the protein level there is no difference in the amino acid sequence between the rat, mouse and human protein (reviewed by Popot et al. 1991) and only one conservative change with dog (Nadon et al. 1990) and 4 with cow (Stoffel et al. 1984).

This extreme conservation shows that the gene has been bound by strong selective restraints to maintain its integrity through evolution and indicates that little variation is tolerated. Hence most amino acid substitutions may be expected to be detrimental. This is supported by the association of the conservative changes; Val(218)Phe, Thr(155)Ile and Ala(242)Val with PMD and by the scarcity of polymorphisms in the PLP gene. There are only 3 reported polymorphisms; 2 noncoding *Msp*I polymorphisms (Wu et al. 1987, Raskind et al. 1992) and an *Aha*II polymorphism in exon 4 (Trofatter et al. 1991). No polymorphisms were identified in this study. In comparison, in the X-linked disorder Anderson-Fabry disease which is caused by mutations at the 12kb alpha-galactosidase A gene, 5 polymorphisms were identified during a single SSCP screen (Davies et al. 1993).

Considering the individual changes. Both the Thr(181)Pro and Leu(223)Pro changes are nonconservative amino acid changes. The Thr(181)Pro change introduces a proline into

a highly hydrophobic domain which is predicted in most topological models (Diehl et al. 1986, Popot et al. 1991, Hudson et al. 1989b) (Fig. 49) to form an alpha-helix and span the myelin membrane. Proline due to its inflexible ring acts as an alpha-helix breaking amino acid, being capable of turning the direction of an amino acid chain. While it occurs frequently at the start of a helix it is generally excluded from within, being the least likely amino acid to occur there (Brandl and Deber. 1986, Richardson and Richardson. 1988). The Thr(181)Pro change is predicted to prevent the correct folding of the protein, possibly preventing its insertion into the membrane, and to result in the disruption of the structural organisation and hence the stability and function of the sheath.

The Leu(223)Pro change introduces a proline into a region predicted in most models (Diehl et al. 1986, Laursen et al. 1984,) (Fig.49) to form an extracytosolic loop. This may similarly affect the structure of the region and hence its putative role in promoting the adhesion and compaction of the extracelluar faces of the membrane layers.

Both mutations result in a lethal disorder and as a defective myelin sheath has been shown in the rumpshaker mouse (Schneider et al. 1992) to be compatible with survival, the primary effect of these changes is predicted to be on the ability of the protein to function as a signal for oligodendrocyte differentiation. In these families the hypomyelination would be predicted to be attributable, primarily, to the deficit of mature oligodendrocytes.

An analysis of the distribution of PLP mutations shows that out of 16 point mutations reported (including this report), 11 (69%) occurred in exons 4 and 5, including the conservative Val(218)Phe and Thr(155)Ile changes. This highlights the importance of these regions in PLP function, possibly as critical determinants in the function of DM-20, and strongly supports the roles of Thr(181)Pro and Leu(223)Pro as the causative mutations in families CB and AB. In both cases these changes were not seen on analysis of a further 51 unrelated chromosomes.

The third change, in family MDJ, occurs at the last base of intron 3 and removes the generally invariant G at the -1 position of the 3' acceptor splice site of exon 4.

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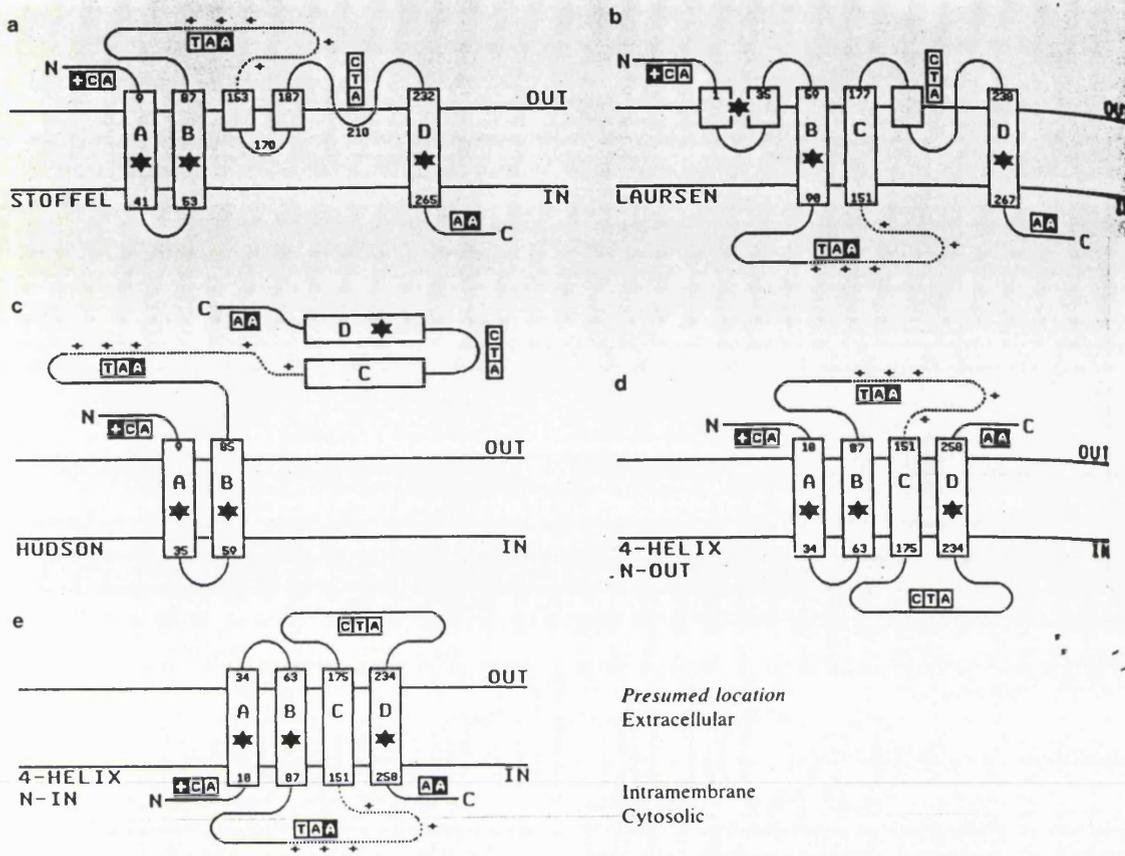
FIGURE 49   A schematic representation of five models for PLP transmembrane topology taken from Popot et al. (1991). Models a-c summarize the proposals of Stoffel et al. (1984), Laursen et al. (1984) and Hudson et al. (1989b), respectively. Models d and e, the 4-helix model of Popot et al. (1991) in its 2 possible orientations. The models are based on evidence derived from : Tryptic cleavage (T), immunolabelling (A), Hydrophobic labelling (star), the location of disulfide bridges (C) and the orientation of the N-terminal as predicted by the distribution of charges on either side of the first helix (cross). Regions predicted to face an aqueous phase are indicated by symbols on white (extracellular) or black (cytosol). The region missing in DM-20 is indicated by a dashed line.

Consensus sequences for the 3' acceptor and 5' donor splice sites have been established by the analysis of a large number of eukaryotic splice junction sequences. The 3' acceptor splice site has a consensus sequence of (T/C)x2 T T (T/C)x6 N C <u>A G</u> **G**, where N is any nucleotide and exonic bases are indicated by bold type. Within this sequence the last two (3'AG) bases of each intron are virtually invariant and are required for correct splicing (Breathnach and Chambon. 1981, Mount. 1982).

A comparison of the statistical estimates for the probable use of the mutant and wild type splice sites, using the method of Shapiro and Senapathy (1987) (Appendix), suggested a decrease of 25%, from 77.76% to 52.78%, in the probability that this site is utilised. This significant reduction, together with the fact that most splice sites give a probability of 70 or higher, predicts that this change will result in a splicing defect. It may either result in exon skipping as seen in the case of the antithrombin III gene (Berg et al. 1992) in which a G to T change at the -1 position resulted in skipping of the adjacent exon or, alternatively, in the activation of a cryptic splice site in the neighbouring sequence as seen, for example, in OCT where an A to T substitution at the -2 position resulted in the activation of a cryptic exonic splice site (Carstens et al. 1991).

Exon 4 of the PLP gene was screened for potential cryptic splice sites. Using the scoring system of Shapiro and Senapathy (1987) (Appendix) the highest score, 70%, was obtained for the sequence ATTGCCTTCC C C<u>AG</u>C. The utilisation of this site would result in an exon of only 50bp, with 117bp deleted and a resulting frameshift from this point.

The effects of the change in family MDJ remain to be verified by sequencing of cDNA from the affected individual, though as PLP is expressed only in the brain and the peripheral nervous system and ectopic expression remains to be verified, this would necessitate an invasive biopsy.

The change was not seen on SSCP analysis of 15 other X-chromosomes from female carriers and affected males supporting the hypothesis that this is the causative mutation in this family.

CARRIER DETECTION AND PRENATAL DETECTION
IN A FAMILY WITH CLASSICAL PMD.

The pedigree of family CB suggested X-linked recessive PMD. Carrier detection and prenatal diagnosis was requested by II-3 who had a 50% risk of having inherited the defective gene from her obligate carrier mother (I-2). RFLP analysis using linked and intragenic markers from the Xq21.3-q22 region was used to track the PLP gene in this family and showed that II-3 had inherited the same allele as her obligate carrier sister (II-1). Based on the assumption that PLP was the defective gene in this family II-3 was given a 98.7% risk of carrier status but, due to homozygosity, prenatal diagnosis could not be offered.

On SSCP analysis a variant band pattern was identified in exon 4 in the 3 women (I-2, II-1, II-3) which confirmed the carrier status of II-3. A prenatal diagnosis was requested by II-3. Fetal sexing identified the fetus as male and hence at risk of PMD but on SSCP analysis it was shown not to have inherited the variant band and was given a 95% probability of being unaffected. The pregnancy was continued on the proviso that the variant, which could still reflect a polymorphism in a genetically heterogeneous disease, was defined by sequencing. At this time only one case of X-linked PMD (Hudson et al. 1989a) had been reported in which a mutation had not been identified in the PLP coding region.

Sequencing of exon 4 identified a Thr(181)Pro change in the three carrier women but not the fetus and unaffected father, which confirmed the prenatal diagnosis. The pregnancy was continued and resulted in a healthy male.

This example, summarised in figure 50, illustrates the importance of direct mutation detection in PMD, where it provides the only clear means of diagnosis in an otherwise clinically and genetically heterogeneous disorder. Identification of the causative mutation enabled unequivocal identification of carriers and affected individuals in this family.

II-3

Mother obligate carrier
of X-linked PMD

50% carrier risk
Parental diagnosis not possible

RFLP analysis

98.7% carrier risk
Prenatal diagnosis not possible
Fetal sexing and termination of all males only option

SSCP analysis

Prenatal diagnosis possible with 95% accuracy
Risk of genetic heterogeneity remains

Sequence

Mutation defined
Accurate carrier detection and
prenatal diagnosis possible in this family

FIGURE 50: Carrier detection and prenatal diagnosis in family CB.

Further the identification of each mutation enables a more comprehensive picture of the clinical manifestations to be defined, which in turn will enable increasingly more accurate diagnosis in future cases.

5.5.0   **FUTURE APPROACHES**

5.5.1   **ORNITHINE CARBAMOYL TRANSFERASE DEFICIENCY**

We conclude, in agreement with Grompe et al. (1991), that the digestion of PCR products using restriction enzymes containing 5'CpG 3' in their recognition sequences is a valuable first step in analysis. Thus a productive strategy for mutation detection in those families where RFLP analysis and biochemical testing is inconclusive would be to test the PCR products of exons 1, 3, 5 and 9 for *Taq*I digestion and the products of exons 7, 4 and 2 for *Msp*I digestion. Additionally all cases should be screened for deletions and late onset cases for *Cfo*I, *Msp*I and especially *Fnu*4HI mutations in exons 6, 4 and 7 respectively.

5.5.2   **PELIZAEUS-MERZBACHER DISEASE**

The current approach to PMD is, in a clinically characteristic case, to screen or sequence the entire coding region of the PLP gene, exon by exon. RFLP analysis has been used in the past but this approach has been thrown into question by the finding that 75% of cases do not have a change at the PLP locus. Despite this we have found no evidence for the involvement of an additional gene, either X-linked or autosomal, in Pelizaeus-Merzbacher disease.

Recent work has shown that while it is not incorporated into the myelin membrane PLP is expressed at reduced, but nevertheless significant, levels in the PNS. A future approach would be to carry out peripheral nerve biopsies to obtain mRNA samples, followed by reverse transcription and direct sequencing. This would avoid the need to analyse individual exons. Alternatively, in cases where this is not possible, the initial sequencing of exons 4 and 5 could be carried out before proceeding to sequence the remainder of the

gene.

Additionally, the routine screening of leukodystrophies of unknown aetiology, as described by Doll et al. (1992), using a screening technique such as DGGE or chemical mismatch analysis would be useful in that a number of these may be due to PLP mutations. This would enable counselling to be offered to these families and would enable the range of phenotypes to be more comprehensively determined for this disorder.

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6.0.0 <u>APPENDIX</u>

The formula for scoring a 5' splice site is:

Probability of using a site =

100 (t-mint) / (maxt-mint)

where the score is between 0 and 100 and mint and maxt are the
are the maximum possible totals i.e. the sum of the lowest and
highest percentages in each of the eight positions, and t is
the total of the percentages for the eight nucleotides being
scored.

The formula for scoring a 3' splice site:

Probability of using a site =

100 [(t_1-l_1) / (h_1-l_1) + (t_2-l_2) / (h_2-l_2)] / 2

t_1 = sum of best 8 of 10 percentages at pyr. positions
t_2 = sum of 4 percentages at CAGG positions
l_1 = sum of lowest 8 of lowest 10 percentages at pyr.
positions
l_2 = sum of lowest 4 percentages at CAGG positions
h_1 = sum of highest 8 of the highest 10 at pyr.positions
h_2 = sum of highest 4 percentages at CAGG positions

t_1, t_2 refer to the sequence being scored, l_1, l_2, h_2, h_2 to the
percentages for each base at each of the 10 positions.

Nucleotide percentages at the 5' donor splice sites

	A	C	G	T
-2	58	13	15	15
-1	10	4	78	8
+1	0	0	100	0
+2	0	0	0	100
+3	57	2	39	2
+4	71	8	12	9
+5	5	6	84	5
+6	16	15	22	47

Nucleotide percentages at the 3' acceptor splice sites

	A	C	G	T
-14	9	31	15	45
-13	9	33	13	45
-12	7	31	11	51
-11	7	35	7	51
-10	10	35	7	47
-9	10	35	11	44
-8	7	43	7	42
-7	9	41	8	42
-6	6	39	6	48
-5	6	40	8	46
-4	23	29	23	24
-3	3	74	1	22
-2	100	0	0	0
-1	0	0	100	0
+1	28	13	49	10

8.0.0 <u>PUBLICATIONS</u>

Arginine 109 to glutamine mutation in a girl with ornithine carbamoyl transferase deficiency

S STRAUTNIEKS, P RUTLAND, S MALCOLM

Arginine 109 to glutamine mutation in a girl with ornithine carbamoyl transferase deficiency

Sandra Strautnieks, Paul Rutland, Sue Malcolm

Abstract
We studied DNA from 29 families with at least one member with ornithine carbamoyl transferase (OCT) deficiency and have found a mutation in the TaqI site within exon 5 of the OCT gene in a female presenting at the age of 21 months. Hybridisation with site specific oligonucleotides shows that the mutation is a C to T substitution resulting in a glutamine for arginine substitution at amino acid 109.

Ornithine carbamoyl transferase deficiency is an X linked disorder caused by mutation of the urea cycle enzyme ornithine carbamoyl transferase. Heterozygous females have very variable manifestations, some being virtually asymptomatic while others have severe protein intolerance and recurrent hyperammonaemia.[1] The reason for this variation is not known although it is usually assumed that it is a result of unequal X inactivation. A survey of mutations found in symptomatic females is needed to clarify the variation in clinical symptoms.

A number of mutations have been defined in males with OCT deficiency. These include deletions and point mutations.[2-7] Of particular interest is the finding of several independent mutations having arisen at codon 109 within a TaqI restriction enzyme site.[4 5 8] Mutations of C to T at this position in the coding strand change arginine to a stop codon[4 5] and arginine to glutamine when the change occurs on the complementary DNA strand.[5] Cell transfection studies confirmed that this latter change abolishes enzyme activity.[8] The arginine to stop mutation has already been found in a mildly affected female patient[4] and we now report the other, arginine to glutamine, change arising independently for a second time, in this case in an affected female.

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Materials and methods

CLINICAL DETAILS
The female proband is $3\frac{1}{2}$ years of age and presented at the age of 21 months with developmental delay (around the 9 to 10 month level) and poor growth ($-3\cdot62$ SD). Increased urine glutamine and orotic acid confirmed OCT deficiency. On a low protein diet, sodium benzoate, and arginine she has done well and her growth has caught up somewhat (now being only $-1\cdot96$ SD). At $3\frac{1}{2}$ years of age her development is now borderline normal. There was no previous family history. Chromosomal analysis was not performed.

We examined DNA from a further 28 families with at least one member, male or female, manifesting OCT deficiency, who had been referred to the Institute of Child Health for DNA testing for carrier detection or prenatal diagnosis.

OLIGODEOXYRIBONUCLEOTIDE SYNTHESIS
Oligonucleotide primers and probes were synthesised on an Applied Biosystems 381A DNA synthesiser.

TaqI RESTRICTION ENZYME ANALYSIS
DNA was prepared from the nuclei of peripheral leucocytes by guanidinium hydrochloride extraction,[9] digested with TaqI according to the manufacturer's instructions (NBL), and the fragments separated by electrophoresis on a 0·8% agarose gel. After denaturation of the gel with 0·4 mol/l NaOH, 1·5 mol/l NaCl, the DNA was blotted directly onto Hybond N+ (Amersham International) and fixed by rinsing with 0·4 mol/l NaOH followed by two washes with 2 × SSC.

HYBRIDISATION PROBE
The probe used was a 1·5 kb human cDNA insert isolated from Nuseive-GTG agarose or whole plasmid and labelled by random hexanucleotide primer extension with a ^{32}PdCTP. Prehybridisation and hybridisation were carried out in 10 × Denhardt's solution, 4 × SSC, 50 µg/ml sonicated salmon sperm DNA, and 0.1% SDS for 16 hours each. Filters were washed for 3 × 20 minutes in 3 × SSC,

0·1% SDS at room temperature followed by a final wash in 0·5 × SSC, 0·1% SDS for 30 minutes at 65°C.

The probe covers exons 1 to 10 and recognises *Taq*I restriction fragments of 4·6, 3·8, 2·7, 1·9, 1·8, 1·4, 0·9, and 0·8 kb.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS

Exon 5 (for the proband's family) and exon 1 (for an affected subject or known carrier from each family) were amplified using PCR. Primers for exon 5 have been previously reported.[4] Exon 1 primers were, on the 5' side, 5'TCACTGCAACTGAACA-CATTTCTTA3' and, on the 3' side, 5'CCTAAATCAAACCCAAGTCTCTGACC3'. The sequences were obtained from Dr M Grompe (personal communication).

Genomic DNA (500 ng) in a total volume of 100 μl was denatured at 94°C for four minutes; 2·5 units of *Taq*I polymerase (Promega) were added and the mix held at 50°C for five minutes and then 30 cycles of one minute at 72°C, one minute at 94°C and 30 seconds at 50°C for exon 5 and one minute at 72°C, one minute at 94°C, and 30 seconds at 56°C for exon 1.

*Taq*I RESTRICTION ENZYME ANALYSIS OF PCR PRODUCTS

Twenty microlitres of each amplified product were digested with *Taq*I according to the manufacturer's instructions (NBL) and visualised by electrophoresis in 4% Nuseive-GTG-agarose gels and ethidium bromide staining.

ALLELE SPECIFIC DETECTION OF MUTATION

Sixty microlitre samples of amplified exon 5 from the proband's family were denatured with 540 μl of 0·4 mol/l NaOH for 15 minutes before loading 100 μl into the wells of a Minifold Tm filtration manifold (Schleicher and Schuell) containing a Hybond N+ membrane presoaked in 2 × SSC. The wells were washed with 20 × SSC and filters fixed in 0·4 mol/l NaOH.

Three identical blots were prepared. One was hybridised with the wild type oligodeoxyribonucleo-tide probe as described in Hata *et al*[4] and the other two with the two possible mutant sequences. All probes were end labelled with gamma ³²P deoxy-adenosine triphosphate. Blots were hybridised for 16 hours at 47°C before washing three times in 3 × SSC, 0·1% SDS at room temperature and once at either 53°C (mutant probe hybridisation) or 57°C (wild type probe hybridisation) for 30 minutes before autoradiography for 16 hours.

Results

*Taq*I RESTRICTION ENZYME ANALYSIS

A preliminary analysis of 29 patients was carried out using Southern blotting. DNA from seven male and 22 female patients was digested with *Taq*I and probed with the cDNA probe for OTC. A new band at 3·4 kb was observed in the proband. No such band was observed in her mother. This band would correspond to a band predicted if the *Taq*I restriction site normally giving rise to bands 1·8 and 1·6 kb within exon 5 were mutated. Mutations of the *Taq*I sites in exons 3 and 9 would also give altered bands, but none such was observed. Only one other altered band was observed after *Taq*I digestion. This was found in a mother and affected son but must have arisen as the result of the creation of a new *Taq*I site and its origin remains to be discovered.

PCR ANALYSIS OF EXON 1

The polymerase chain reaction was used to amplify exon 1 from all 29 patients, as Southern blotting does not readily detect the change in the *Taq*I site found in exon 1. All amplified bands were digested with *Taq*I, the resulting products separated on Nuseive agarose gels and visualised by ethidium bromide staining (fig 1). In each case the 217 bp amplification product was digested to a band of 172 bp showing that there were no mutations at the *Taq*I site in this exon. The other 45 bp band was too small to detect.

PCR ANALYSIS OF EXON 5

Genomic DNA from the proband and her mother was amplified using primers specific for exon 5. *Taq*I digests of the amplified products showed two bands in the case of the proband (fig 2), the expected digestion product of 121 bp from the unaffected X chromosome and an undigested fragment of 156 bp from the other X chromosome, confirming that the *Taq*I site has been mutated. Again the other digestion product of 35 bp was too small to detect.

DETECTION OF POINT MUTATION USING ALLELE SPECIFIC OLIGONUCLEOTIDE HYBRIDISATION

The amplified exon 5 products from the proband and her mother were tested for both predicted C to T mutations by hybridisation to radiolabelled oligonucleotides corresponding to the sense strand of the normal sequence, and to the sense strands of both mutations. The proband showed clear hybridisation to the normal oligonucleotide but with reduced intensity, as expected for a heterozygote, and to the oligonucleotide corresponding to the arginine to glutamine change (fig 3). Her mother showed only hybridisation to the normal sequence.
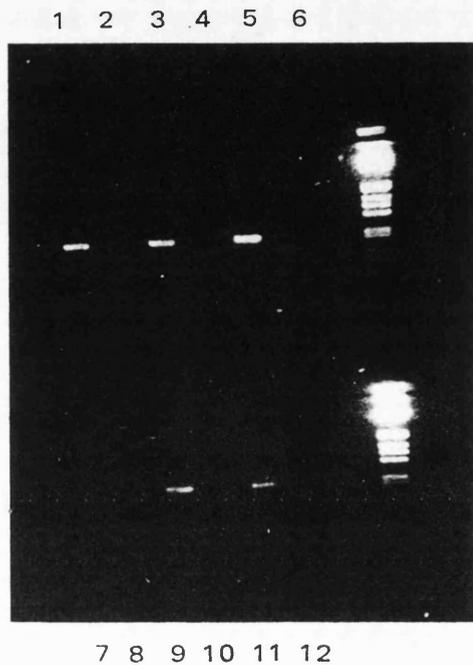
1 2 3 4 5 6

7 8 9 10 11 12

Figure 1 PCR amplification products of exon 1 digested with TaqI. Tracks 1, 3, 5, 9, and 11 undigested product. Tracks 2, 4, 6, 10, and 12 samples digested with TaqI. Tracks 7 and 8 blanks. The band size is reduced from 217 bp to 172 bp when the TaqI site is present. The 45 bp band is too small to detect.

1 2 3 4 5 6

7 8 9 10
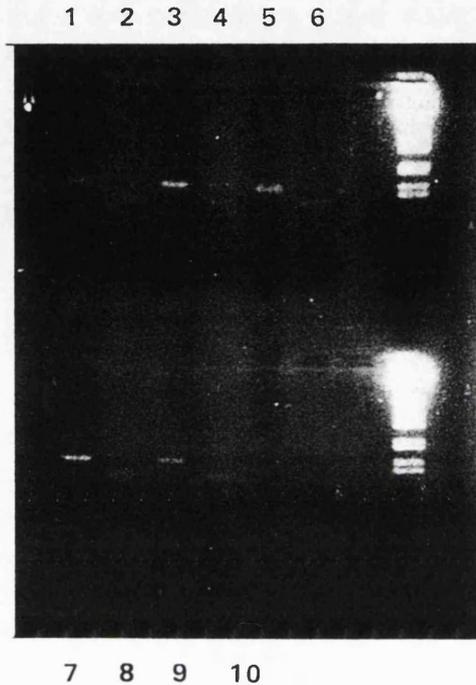
Figure 2 Analysis of amplified DNA from exon 5 in the family of the proband. Each pair of tracks shows the amplified product undigested on the left and digested with TaqI on the right. Tracks 1 and 2 are from the father, tracks 3 and 4 the affected girl, tracks 5 and 6 an unaffected sister, tracks 7 and 8 an unaffected brother, and tracks 9 and 10 the mother.

Discussion

The coding sequence for ornithine transcarbamylase contains four restriction enzyme sites for *Taq*I. Systematic studies in other diseases, both X linked and autosomal, show that the dinucleotide $5'CpG^{3'}$ is particularly prone to mutation to $5'TpG^{3'}$. Examples have been found in the analysis of the factor IX gene in haemophilia B[10 11] and the phenylalanine hydroxylase gene in phenylketonuria.[12] The reason for this is believed to be deamination of methyl C to form T, followed by repair of the resulting G.T mismatch to A.T. As the sequence is symmetrical this can happen in either strand resulting in C to T or G to A. A small proportion of CpG sequences will fall within *Taq*I sites (recognition site TCGA) and may be directly detectable on Southern blots. The mutation reported here falls within that class. However, the mutation within exon 1 is difficult to detect because of coincidence of band sizes. *Taq*I digestion of PCR amplified exon products provides a quick and clear screening method.

We have examined 29 X chromosomes giving rise to OCT deficiency at four *Taq*I sites. We have only
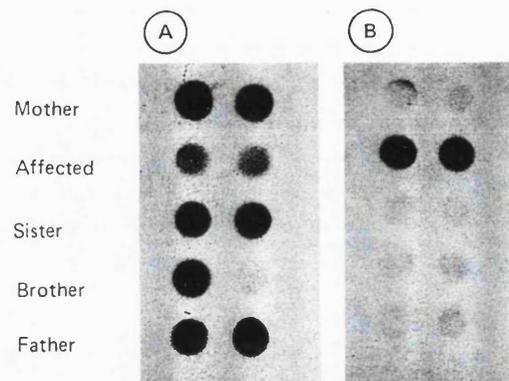
Figure 3 Detection of point mutation using allele specific oligonucleotide hybridisation. (A) DNA from family members hybridised with wild type oligonucleotide. Duplicate samples were applied in each case except for the brother. (B) DNA from family members hybridised with oligonucleotide detecting Arg-Gln mutation.

found one mutation and have found no deletions in eight male samples analysed.

A further 15 CpG sites[13] are found within the OCT coding region.[4] They remain to be screened. Five other independent mutations in OCT males have been reported.[3 7] They were initially detected either by chemical mismatch cleavage of mRNA[3] or by denaturing gradient gel electrophoresis.[7] Two of the five are produced by mutations within CpG dinucleotides.

This report describes the second mutation found in a female patient. So far they both reproduce mutations found in severely affected males. Further reports are awaited in order to deduce whether the severity in females is connected in any way with the mutation or whether the proportion of X inactivation is the only critical factor.

As the patient's mother showed no alteration within her *Taq*I site in exon 5 it is very likely that the patient represents a new mutation. However, a small possibility of mosaicism within the germ cells of the mother remains[2] and she could be offered prenatal diagnosis based on PCR amplification of exon 5 and *Taq*I digestion if required.

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Pelizaeus-Merzbacher Disease: Detection of Mutations Thr[181]→Pro and Leu[223]→Pro in the Proteolipid Protein Gene, and Prenatal Diagnosis

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Summary

A family with an apparent history of X-linked Pelizaeus-Merzbacher disease presented for genetic counseling, requesting carrier detection and prenatal diagnosis. RFLP analysis using the proteolipid protein (PLP) gene probe was uninformative in this family. A prenatal diagnosis on a chorionic villus sample (CVS) was carried out using single-strand conformation polymorphism (SSCP) analysis of a variant in exon 4 of the PLP gene. The fetus was predicted to be unaffected. Sequencing of the exon from the CVS, the predicted-carrier mother, and the obligate-carrier grandmother revealed an A-to-C change at nucleotide 541 in the two women but not in the fetus. As this change results in a Thr-to-Pro change at amino acid 181 in a region of the gene predicted to be part of a transmembrane segment, it was concluded that this was the mutation causing the disease in this family. In addition, in a second family, an exon 5 variant band pattern on SSCP analysis was shown by sequencing to be due to a T-to-C change at nucleotide 668. This results in a Leu-to-Pro change in a carrier mother and in her two affected sons. These results provide further examples of mutations in PLP that cause Pelizaeus-Merzbacher disease and illustrate the value of SSCP in genetic analysis.

Introduction

Pelizaeus-Merzbacher disease (PMD) causes loss of myelinating cells and myelin in the central nervous system. It is genetically and pathologically heterogeneous (Scheffer et al. 1991), but molecular genetic studies have recently demonstrated the role of the proteolipid protein (PLP) gene in the classical X-linked form of the disease. Five independent point mutations of the PLP gene have been reported (Gencic et al. 1989; Hudson et al. 1989; Trofatter et al. 1989; Pham-Dinh et al. 1991; Pratt et al. 1991), but an additional 14 cases reported by Pham-Dinh et al. (1991), 11 with an apparent X-linked pedigree, failed to reveal any sequence changes except for one silent change. This

leaves the possibility that there may be another X-linked gene involved. Evidence for autosomal inheritance in some families has also been presented (Begleiter and Harris 1989), particularly in the connatal form of the disease.

Carrier detection is especially difficult in PMD, as female carriers are asymptomatic and no biochemical tests exist. Magnetic imaging has been used but is inaccurate in young females (Boltshauser et al. 1988). As with any X-linked lethal disorder, there is the complication of new mutations, and in addition, in PMD there is the possibility of genetic heterogeneity.

If a PLP mutation is assumed in an apparently X-linked pedigree, then an RFLP for the gene can be followed through the family. The PLP gene is highly conserved. Two polymorphisms have been reported. An *Msp*I polymorphism has an allele frequency of .92/.08 (Wu et al. 1987) and is relatively uninformative. A second polymorphism arising from a single-base silent substitution in aspartate codon 202 has allele frequencies of .74/.26 (Trofatter et al. 1991).

As the PLP gene has been assigned to Xq22 by somatic cell hybrids and linkage (Willard et al. 1987), closely linked polymorphic markers can also be useful, although this introduces the possibility of error due to recombination.

In view of these difficulties it is clear that direct mutation detection by DNA sequencing is highly desirable in families requiring carrier detection or prenatal diagnosis. As messenger RNA is not readily available and as the gene has seven exons, a preliminary exon screen to identify the most likely site of mutation will simplify this task.

Single-strand conformation polymorphism (SSCP) is a convenient method of screening coding regions for changes (Hayashi 1991) and has been used to identify mutations in several genes, including those involved in familial polyposis coli (Groden et al. 1991) and DRASH syndrome (Pelletier et al. 1991). It is based on the principle that single-stranded DNA under non-denaturing conditions will adopt a unique sequence-dependent conformation and that this will determine its mobility on electrophoresis. In practice, radiolabeled PCR products are denatured by heating and are rapidly cooled and run on 6% nondenaturing polyacrylamide gels. We have applied this technology to two families with PMD.

Subjects and Methods

Clinical Details

Family 1.—III-1 was born with normal birth weight at 37 wk gestation after a normal pregnancy. In his first few weeks of life his parents noticed that he was a "snuffly" baby. At the age of 5 wk laryngeal stridor was noted by the general practitioner. Over the next few weeks there were problems with abdominal colic, and the child did not gain weight properly. He was admitted to hospital at age 12 wk. Laryngosocopy at that stage showed a "floppy" larynx. He was also noted to have pale optic discs and was thought to have nystagmus. From the age of 12 mo there was no social or motor development. He remained profoundly handicapped. A maternal uncle (II-2) died at the age of 3 years, having had a very similar illness, with laryngeal stridor and failure of development. Autopsy was performed in Bristol and confirmed the diagnosis of PMD. Carrier detection and prenatal diagnosis was requested by II-3.

Family 2.—II-1 was born to nonconsanguineous parents after an uneventful pregnancy which went to term. He was their firstborn child, and it was of note in the family tree that the mother had a brother who died at the age of 9 mo and who was said to been mentally and physically handicapped. On further inquiry there was evidence from the hospital notes that this male was hydrocephalic, had pale optic disks, and was quadriplegic; and it must remain uncertain whether he had the same condition as his two nephews. II-1 weighed 8 lb 6 oz at birth, and his early development seemed normal in that he smiled at 6 weeks and fed well.

At 10 wk of age he was admitted to the local hospital because of inspiratory stridor, and at that time his mother noted his jerky eye movements. He was referred to the Hospital for Sick Children, Great Ormand Street, where, on examination, he was found to be floppy and to have nystagmus, and a diagnosis of a mild laryngomalacia was made to explain the stridor. After discharge, his stridor settled, but he made poor developmental progress. At the age of 13 mo he was still unable to sit, and he was readmitted for further investigation. At that stage he had truncal hypotonia but had brisk reflexes with extensor plantar responses. A computed-tomography brain scan showed minor cerebral atrophy, but his brain-stem auditory evoked responses revealed only wave I, which is often found where central myelination is poor. All other investigations, including those of chromosomes and a variety of metabolic tests were normal.

By the age of 3 years II-1 was still very developmentally behind and, an MRI scan was undertaken. This showed gross undermyelination throughout the white matter of both hemispheres and the posterior fossa. In fact, there was no evidence of any normal myelin formation, and a diagnosis of PMD was made.

II-2 is the brother of II-1, and he was born when II-1 was 21 mo of age. At that age a diagnosis in his brother had not been arrived at.

The pregnancy was again uneventful, and II-2 had a good birthweight. It had been noted antenatally that the fetus had a left renal tract abnormality, and postnatally this was shown to be a duplex kidney on one side. He was jittery at birth, and, like his brother, he developed stridor. His progress was poor, and nystagmus was noted early in life. The optic disks were pale, and further investigations revealed abnormal brainstem evoked responses identical to those of his brother. At 22 mo he is still floppy but has a few words. Clinically, II-2 is identical to II-1, and further investigations were not undertaken.

Chorionic Villus Sampling (CVS)

DNA was prepared from a CVS sample by using standard procedures. Fetal sex was established by direct karyotyping culture and by DNA analysis.

Restriction-Enzyme Analysis

DNA was prepared from the nuclei of peripheral leukocytes by guanidinium extraction (Jeanpierre 1987). TaqI, MspI, and PstI digestions were carried out according to the manufacturer's (NBL) instructions. The resulting fragments were separated by electrophoresis on 0.8% agarose gels, either for 24 h at 95 mA or for 35 h at 95 mA, to separate closely sized fragments for hybridization with CX52.5 (DXS101), 19-2 (DXS3), S21 (DXS17), and PLP cDNA probes. Gels were denatured with 0.4 M NaOH, 1.5 M NaCl, were blotted directly onto Hybond N$^+$ (Amersham International), and were fixed by being rinsed with 0.4 M NaOH, followed by two washes with 2 × SSC.

Hybridization

The probes were labeled by random hexanucleotide primer extension with ^{32}P dCTP. Prehybridization and hybridization each were carried out in 10 × Denhardt's solution, 4 × SSC, 50 μg sonicated salmon sperm DNA/ml, 0.1% SDS for 16 h at 65°C in a rotating hybridization oven (HYBAID). Filters were washed for 3 × 20 min in 3 × SSC, 0.1% SDS at room temperature. Final washes for all probes except CX52.5 were carried out at 65°C for 10 min in 0.5 × SSC, 0.1% SDS, followed by a 0.2 × SSC, 0.1% SDS wash if background persisted. CX52.5 filters were washed in 3 × SSC, 0.1% SDS for 10 min at 65°C.

Oligonucleotide Primers

Primers, from the introns of the PLP gene, for the PCR were provided by Dr. L. Hudson (Gencic et al. 1989). Further syntheses were carried out on an Applied Biosystems 381A DNA synthesisor. 5′ Biotinylation was carried out during oligonucleotide syntheses using DMT-biotin-C-6-PA (CRB) according to the manufacturer's instructions. Primer sequences, including rested primers for sequencing and PCR product sizes, are given in table 1.

SSCP Analysis

Exons 1–7 of the PLP gene were individually amplified by PCR. Reactions were carried out in a 50-μl volume by using 100–200 ng of genomic DNA; 25 pmol of each primer; 20 nM dCTP, 200 nM each of dATP, dCTP, and dGTP; 0.1 μl of P^{32}-dCTP, reaction buffer (PROMEGA), and 10^{-5} M TEMAC.

Reactions were overlaid with approximately 50 μl of mineral oil and denatured at 94°C for 10 min and were cooled to 55°C (annealing temperature), and 1.5–2 units of Taq polymerase (Promega) were added. Twenty-five cycles, each comprising 72°C (1 min), 94°C (1 min), and 55°C (30 s) were carried out, followed by 10 min at 72°C on a Techne PHC-2 machine. PCR products were checked by running 10 μl on a 1.5% agarose gel, followed by ethidium bromide staining.

For SSCP analysis 4 μl of each PCR product were mixed with an equal volume of stop solution (U.S.B. Sequenase kit). Samples were denatured at 94°C for 10 min, placed in a dry-ice/ethanol bath, microfuged briefly, and placed on dry ice again. One to one and one-half microliters of each sample were loaded onto a 6% 37.5:1 acrylamide:bis nondenaturing polyacrylamide (Protogel, Lablogic) gel, and electrophoresis carried out at 4°C for 24 h at 380–400 V, on a BRL model 2 sequencing system. The gel was blotted onto Whatman paper, dried under vacuum at 80°C, and exposed to X-ray film (Kodak) for 24–120 h at -70°C.

Sequencing of PCR Products

The PCR reaction was performed using 5 pmol of each primer, one of which (5′ side) was biotinylated at the 5′ terminus, 200 nM of each dNTP, 10^{-5} M TEMAC, reaction buffer (Promega), and 200–500 ng of genomic DNA in a total volume of 100 μl. Reaction conditions and product analysis were as described previously for SSCP, except that the annealing temperature was 52°C and 30 cycles were performed.

To produce single-stranded DNA, 50 μl of PCR product (avoiding oil) and 30 μl of magnetic Dynal M-280 streptavidin (Dynal UK) beads were mixed and incubated for 5 min at 20°C with gentle agitation, and the reaction tube was placed in a magnet (Promega). The beads with bound double-stranded PCR product collected to one side, and the supernatant containing unincorporated nonbiotinylated primer and nucleotides was removed. The reaction was removed from the magnet, incubated with 0.15 M NaOH for 5 min at 20°C to denature the double-stranded product, and replaced in the magnet, and the supernatant containing the unbiotinylated PCR strand was removed. The single-stranded product was washed in turn
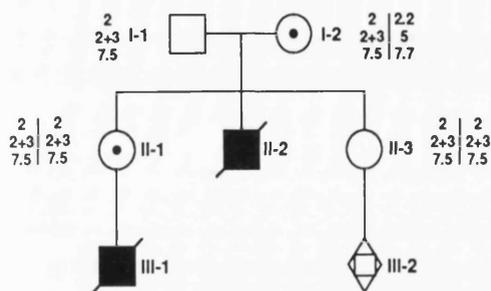
Table I

Positions and Sequences of PCR and Sequencing Priemrs

Exon and Position	Sequence	Size (bp)
1:		
Intronic 3'...............	5' CCC GAA CCC CAA AGT TGG AA 3'	511
Intronic 5'...............	5' AAG TTT CCA GGA CAT CTTCA 3'	
2:		
Intronic 3'...............	5' TGC TAG TTA CTA AGG TAC TG 3'	362
Intronic 5'	6' TGA GCA CAG GGC CTG GCA GA 3'	
3:		
Intronic 3'...............	5' ACG AGG CCA CAG ACT CGC GC 3'	412
Intronic 5'...............	5' ACC TCA CTT ATG TCG GGA AA 3'	
4:		
Intronic 3'	5' ACC ACC CTC CTT ACA CTA AG 3'	335
Intronic 5'	5' ACT CCA GGA TCT CCC AGT TT 3'	
Intronic 3'	5' CTT CCA TAG TGG GTA GGA GA 3'	Nested sequencing primers
Intronic 5'	5' GAT CTG CAG GCT GAT GCT GA 3'	
5:		
Intronic 3'	5' ATT GAA GGC CAT GGG TGT AA 3'	236
Intronic 5'	5' AGA GAT GGA AGA AGG GCT CT 3'	
Intronic 3'	5' TGG TAT TAG CTA CTC CCT TG 3'	Nested sequencing primers
Intronic 5'	5' CCC CGT AAC TCC ATA AAG CT 3'	
6:		
Intronic 3'	5' TTG CCT TTC AGA ATA GCT GT 3'	239
Intronic 5'	5' AAA GAT ATC AAC ACA TTC AG 3'	
7:		
Intronic 3'	5' GGT GGC AAA GGC AAA GAG TT 3'	261
Intronic 5'	5' TTT TAT TTC TAC CCT TCC TC 3'	

with 100 μl TES ([10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl] × 2) and 100 μl of water by adding the solution and mixing, placing it in the magnet, and removing the supernatant before resuspending it in 7 μl of water. The sequencing reaction was carried out using the 3' side sequencing primer according to the Sequenase kit (U.S.B.) protocol. The labeled strands were then separated from the beads by denaturing at 65°C for 5 min, placing them in the magnet, and removing the supernatant. The supernatant was heated to 85°C for 2 min, 2 μl were loaded onto a 6% polyacrylamide (Acugel; Lablogic) denaturing gel (8.3 M urea), and electrophoresis was carried out at 55–60 W on a BRL model 2 sequencing apparatus for 2–2.5 h. The gel was fixed in 10% methanol, 10% acetic acid for 30 min before the procedure was continued in the manner used for SSCP gels.

Results

Pedigree 1 (fig. 1) shows the characteristic pattern of X-linked recessive inheritance. II-3, whose brother

(II-2) and nephew (III-1) have died of PMD, presented for genetic counseling, requesting carrier detection and prenatal diagnosis. Her mother (I-2) and sister (II-1) are obligate carriers, having had an affected son and grandson and affected son and brother, respectively. No DNA from either affected male was available, and at this stage II-3 had a 50% risk of being a carrier. Pedigree 2 (fig. 2) shows that the mother (I-1) is an obligate carrier, having had two affected sons (II-1 and II-2).

RFLP Analysis

RFLP analysis of the *MspI* polymorphism, with alleles of 4 and 4.5 kb, proved uninformative in families 1 and 2. Linked markers from the Xq22 region were used, and three of these S21 (*TaqI*), 19-2 (*TaqI*), and CX52.5 (*MspI*) proved partially informative in family 1. In each case (fig. 1), I-2, the obligate carrier mother, was heterozygous, which enabled her mutant and normal chromosomes to be distinguished. The mutant chromosome is the one she has passed on to her obligate carrier daughter (II-1), in this case identified by

Figure 1 Partial pedigree of family 1, showing RFLP results with S21(*Taq*I), 19-2(*Taq*I), and CX52.5(*Msp*I) probes. ■ = Affected males; □ = normal males; ⊙ = carrier females; ◈ = male fetus; and ○ = females of normal or undetermined carrier status.

Figure 2 Partial pedigree of family 2, showing RFLP results with S21(*Taq*I) and PGX-12(*Pst*I) probes. Symbols are as in fig. 1.

the 2.0-, 2 + 3-, and 7.5-kb alleles detected with probes S21, 19-2, and CX52.5, respectively.

As II-3 had also inherited these alleles from her mother, she was predicted to be a carrier. On the basis of S21 and 19-2 probe results, and with account taken of the possibility of recombination when recombination fractions of .08 between DXS3 and PMD and .04 between DXS17 and PMD are used, II-3 was given a 98.7% carrier risk.

The grandfather (I-1) is hemizygous for the 7.5-, 2.0-, and 2 + 3-kb alleles. Thus, II-1 and II-3 were both homozygous for these probes, and prenatal diagnosis was not possible. At this stage II-3 had a 50% risk of having an affected boy, and her only options were to terminate all male pregnancies or accept this risk.

Subsequent to the prenatal diagnosis carried out in family 1 its members were tested for the exon 4 RFLP, detected by *Aha*II, reported by Trofatter et al. (1991). The polymorphism is visualized by digesting a 227-bp PCR product, from exon 4, with *Aha*II; this product digests to two fragments—of 183 and 44 bp—when the cutting site is present. I-2 was heterozygous for the presence of the *Aha*II site but passed on the *Aha*II⁻ form to both daughters, who were, therefore, uninformative for the polymorphism.

In the case of family 2, the mode of inheritance could not be determined from the pedigree, leaving the possibility of autosomal inheritance, and thus prenatal diagnosis could not be offered in this family, even though the mother (I-1) was heterozygous for S21 (*Taq*I) and pXG12 (*Pst*I). Only direct detection of a deleterious mutation would establish the mode of inheritance and enable counseling to be given to this family.
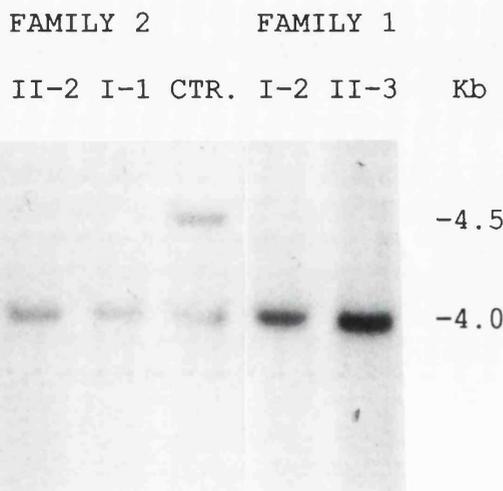
SSCP Analysis

SSCP analysis of exons 1–7 of the PLP gene showed a variant band pattern in exon 4, which segregated with carrier status in family 1 (fig. 3). The arrow in figure 3 represents the variant band, which II-3 and II-1 have inherited from their obligate-carrier mother. The mutant and normal chromosomes could now be distinguished, and prenatal diagnosis was now possible in this family. The variant band was not observed in 51 unrelated chromosomes used as controls.

Under the conditions used, only one strand of the mutant chromosome shows a conformation suffi-
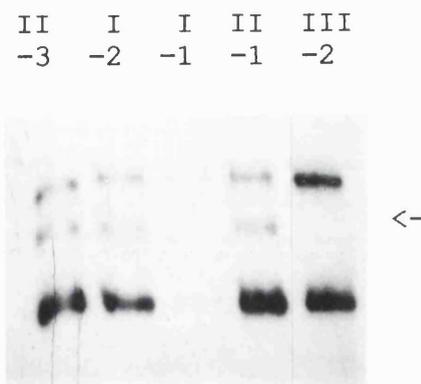

Figure 3 SSCP analysis of exon 4 in family 1. Radiolabeled PCR product was mixed with stop solution, denatured, and electrophoresed at 4°C on a 6% polyacrylamide gel at 380–400 V. The arrow indicates the variant band, which is present in all the carrier women (II-1, II-3, and I-2) but not in the normal (I-1) or CVS (III-2) samples.

ciently altered to modify mobility on electrophoresis. On fetal sexing of CVS material, the fetus of II-3 was shown to be a male. SSCP analysis of exon 4 (fig. 3) showed that it did not have the variant band pattern, and a 95% prediction of normality was given. The 5% risk allowed for both 2.5% based on the possibility of genetic heterogeneity and another 2.5% for the use of an as yet novel technique.

SSCP analysis of exons 1–7 of family 2 showed a variant band pattern in exon 5 (fig. 4). The mother (I-1) had both the variant band and the normal band, and the affected boys (II-1 and II-2) had only the variant. The variant band was not seen in 51 unrelated chromosomes used as controls.

The PCR products from three of the larger exons were digested with suitable restriction enzymes which cut within the exon-coding sequence, in order to increase the chance of observing a shift when SSCP was used. Exon 1 products were digested with *Msp*I, exon 3 products with *Msp*I, and exon 4 products with *Rsa*I. No additional shifts were seen.

Sequencing

Sequencing in I-1, I-2, II-1, II-3, and the CVS sample (III-2) from family 1 showed an A-to-C change at nucleotide 541 in the three carrier women (I-2, II-1, and II-3) (fig. 5). This results in a threonine (ACC)-to-proline(CCC) change at amino acid 181. In each case, because of heterozygosity, both the A and C bands were present. The CVS and I-1 (grandfather) had only the normal A band, supporting the SSCP analysis result. The pregnancy is continuing. The risk to the fetus is now considered to be reduced below the 5% quoted
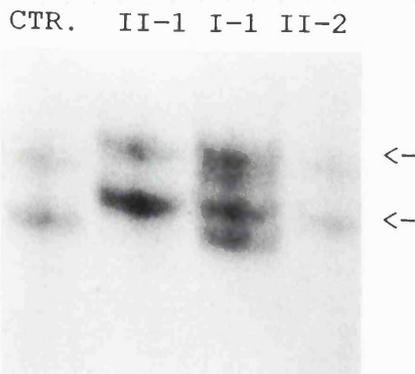

Figure 5   DNA sequence of exon 4 PCR product from a normal control (CTR.) and a carrier female (II-3) in family 1. The arrow indicates the A-to-C change which substitutes a proline for a threonine at amino acid 181.

Figure 4   SSCP analysis of exon 5 in family 2. Conditions were as in fig. 3. The arrows indicate the variant bands. The carrier mother (I-1) is heterozygous, while both affected boys (II-1 and II-2) are hemizygous for the variant bands. CTR = unaffected control.
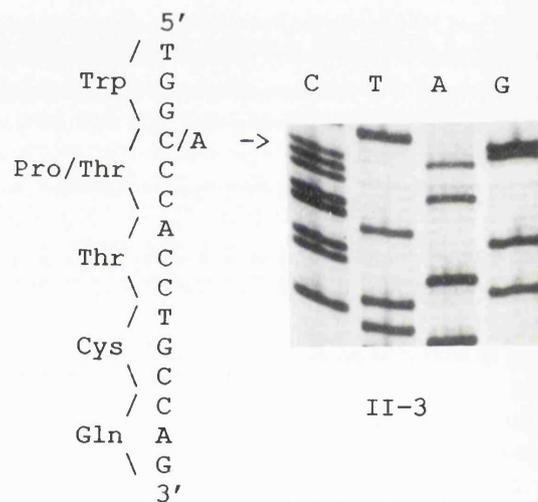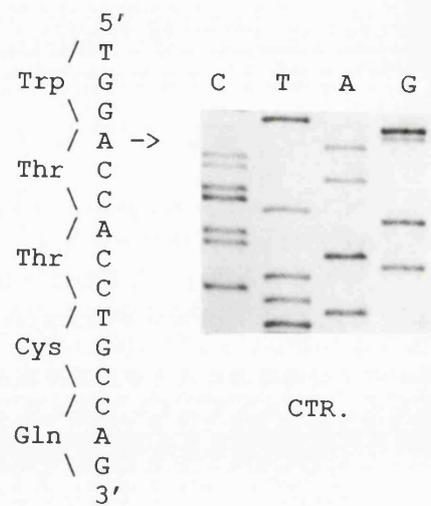
to the family after SSCP analysis and is probably less than 1%.

Sequencing of exon 5 in family 2 showed a T-to-C change at nucleotide 668 (fig. 6). The mother (I-1) had both C and T bands, while her affected sons (II-1 and II-2) had only the mutant C band. This results in a leucine(CTT)-to-proline(CCT) change at amino acid 223.
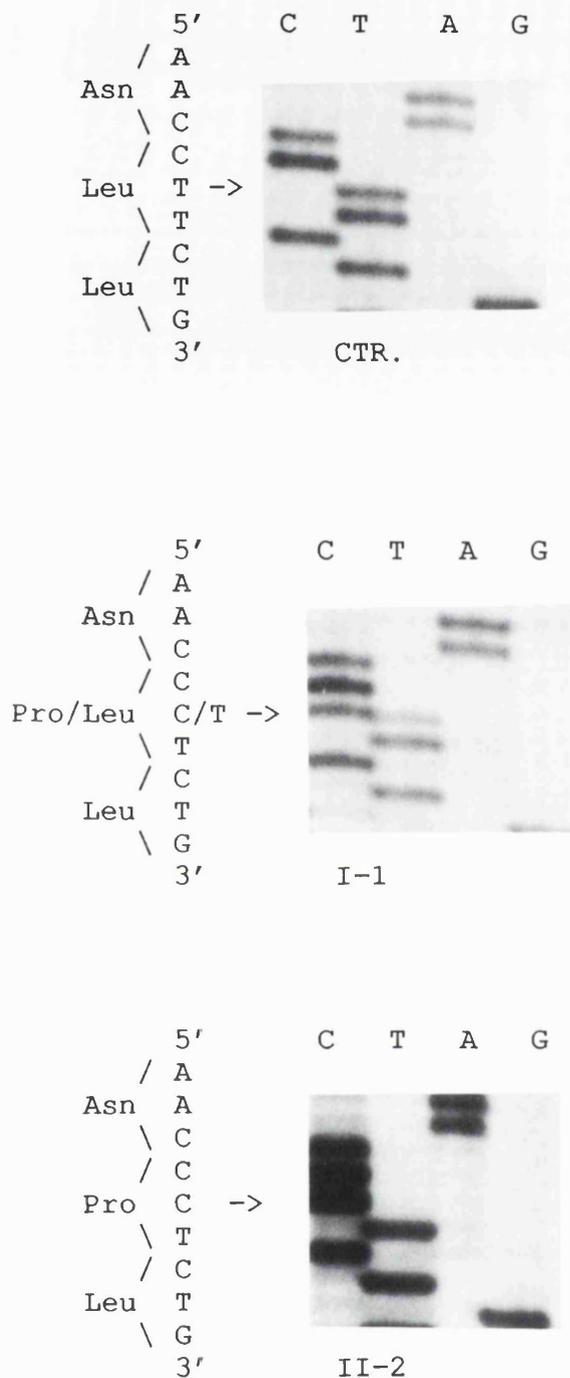
Figure 6   DNA sequence of exon 5 PCR product from a normal control (CTR.), the carrier mother (I-1), and an affected (II-2) boy in family 2. The arrow indicates the T-to-C change which substitutes a proline for a leucine at amino acid 223.

Discussion

Mutations in the PLP gene clearly result in classical X-linked PMD. However, as the PLP gene has been sequenced, in a number of cases, without evidence of a mutation, the possibility of further genes causing PMD, either X-linked or autosomal, is still unresolved. This complicates carrier detection and prenatal diagnosis using genetic markers in or around the PLP gene. The examples presented here illustrate the importance and usefulness of direct sequencing of the exons. The risk to a male fetus was initially raised from the pedigree risk of 25% to around 50% when it was shown, on the basis of linked probes, that the mother was likely to be a carrier. This risk was reduced substantially after SSCP analysis—and even further after sequencing. The finding of a Thr181-to-Pro change in a membrane-spanning segment established that the mutation causing PMD in this family is almost certainly in the PLP gene (see below) and that the fetus did not carry it.

SSCP analysis failed to detect the AhaII polymorphism present in the mother. The altered band found could not be a result of this polymorphism, as the mother alone was heterozygous, whereas the altered SSCP band was shared by the mother and both daughters.

As DNA sequencing becomes more straightforward, particularly as automated sequencing is introduced, it may be more efficient to sequence directly. However, at the present time many clinical molecular genetic laboratories will find that SSCP provides a straightforward and fast method of screening for sequence changes. It involves no special primers or equipment and is likely to be equally effective for small deletions or point mutations.

SSCP will also pick up changes which are silent or polymorphic. A silent change within the PLP gene has been described. Pham-Dinh et al. (1991) and Trofatter et al. (1991) found a frequent (.26) silent substitution (GAT to GAC) in aspartate codon 202. This falls within exon 4, but we failed to observe it by SSCP analysis. The exon 4 PCR product was 335 bp, which is slightly longer than the optimum reported for SSCP detection. We digested the products with RsaI, which cuts at amino acids 30 and 67 to give three fragments closer to the optimal size of 200 bp or less (Hayashi 1991). The AhaII polymorphism falls within the central portion of 110 bp. However, an SSCP change was still not observed.

Other easy screening methods are increasingly being reported. For example, Hydrolink gels appear to be efficient in detecting heteroduplexes (Tassabehji et al. 1992) and may in future be used in conjunction with SSCP.

As polymorphisms detected by SSCP may not be

disease-causing changes, sequencing of the altered exon is necessary. However, in family 1 the grandmother is an obligate carrier in the pedigree, and new mutation is not an issue, so the SSCP change could be used as a variant to follow the mutant PLP gene through the family, even if it has not been established that it reflects the disease-causing mutation. This is particularly useful for genes, such as PLP, where there are very few polymorphisms. SSCP polymorphisms are easy to detect and use PCR technology. They are likely to be widely applicable, as illustrated here for the CVS sample, but may be replaced as sequencing becomes more automated.

We report here two previously unreported mutations of the PLP gene in cases of PMD. A model of the protein structure and function has been proposed (Stoffel et al. 1984). In this model the mutation we find in exon 4 would arise inside an alpha-helical transmembrane segment, and the presence of a proline residue which has been shown to be an alpha-helix breaker makes it very likely that this is the disease-causing mutation. The mutation found in exon 5 also involves a nonconservative amino acid change, in this case Leu to Pro at amino acid 223. Amino acid 223 is predicted to be in an extracytosolic loop. We cannot predict how the altered amino acid would change function, but PLP gene structure is very highly conserved across species. In addition, as we have not seen either of these changes in 51 other X chromosomes studied, we feel that it is very likely that they are the disease-causing mutations.

Acknowledgments

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