MOLECULAR PATHOLOGY DETECTION STRATEGIES FOR THREE AUTOSOMAL DOMINANT NEURODEGENERATIVE DISEASES

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DECLARATION

I certify that this thesis does not contain material previously published or written by any other person, except where referred to in the text. The results in this thesis have not been submitted for any other degree or diploma.

A. E. Elshafey

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LIST OF ABBREVIATIONS

μg	Microgram
μl	Microlitre
μM	Micromolar
^{32}P	Radioactive phosphorus
^{35}S	Radioactive sulphur
AIRS	Artificial introduction of restriction site
ALS	Amyotrophic lateral sclerosis
ARMS	Amplification refractory mutation system
ASO	allele specific oligonucleotide
ATR-16	16 α-thalassemia/mental retardation syndrome
bp	Base pair
CCM	Chemical cleavage of mismatch
cDNA	Complementary copy DNA
cm	Centimetre
CNS	Central nervous system
cpm	Count per minute
CSF	Cerebrospinal fluid
dCTP	2'Deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
ddNTP	dideoxyribonucleoside triphosphate
DEPC	diethyl pyrocarbonate
DGGE	Denaturing gradient gel electrophoresis
dH_2O	distilled water
DM	Myotonic dystrophy
DMAHP	Myotonic dystrophy locus associated homeodomain protein

DM-PK	Myotonic dystrophy protein kinase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacitic acid
EMC	Enzyme mismatch cleavage
FALS	Familial amyotrophic lateral sclerosis
FISH	Fluorescent in situ hybridisation
FR	Free radical
FRAX	Fragile X
g	Gravitational (centrifugal) force
GAP	GTPase-accelerating protein
GDP	Guanine diphosphate
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
hnRNA	Heteronuclear ribonucleic acid
Ig	Immunoglobulin
kb	Kilobase
kd	Kilo dalton
l	Litre
LMN	Lower motor neurone
M	Molar
Mb	Megabase
mg	Milligram
ml	Millilitre
mM	Millimolar
MND	Motor neurone disease

mRNA	Messenger ribonucleic acid
MTJs	Myotendinous junctions
NF	Neurofilament
nm	Nanometer
NMDA	N-methyl-D-aspartate
NMJs	Neuro muscular junctions
^{o}C	Degree centigrade
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
pmol	Pico mole
poly(A)$^{+}$	Polyadenylate
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
sALS	Sporadic amyotrophic lateral sclerosis
SMA	Spinal muscular atrophy
SOD-1	Cu/Zn superoxide dismutase
SSCP	Single strand conformation polymorphism
TAE	Tris-acetate ethylenediaminetetraacitic acid
Taq	Thermus aquaticus
TBE	Tris-borate ethylenediaminetetraacitic acid
TE	Tris- ethylenediaminetetraacitic acid
TEMED	N,N,N,N-tetramethylethylenediamine

TGGE	Temperature gradient gel electrophoresis
T_m	Melting temperature
Tris	tris(hydroxymethyl)amino methane
TSC	Tuberous sclerosis complex
u	Unit
UMN	Upper motor neurone
UV	Ultraviolet

SUMMARY

The present study aimed to optimise mutation detection strategies for three autosomal dominant neurological diseases, myotonic dystrophy (DM), amyotrophic lateral sclerosis (ALS) and tuberous sclerosis complex (TSC).

(A) Myotonic dystrophy: After exploring different methods that have been used for the detection of CTG repeat expansions in DM patients, a strategy was chosen, optimised and applied to screen 49 DM families (86 DM affected and 96 apparently normal individuals). Using published primer sets, both radiolabelled and non-radiolabelled PCR amplification of the area containing the CTG repeat were optimised using the published conditions as a starting point. After PCR optimisation, DM alleles carrying up to 90 CTG repeats were properly amplified however, amplification of > 90 CTG repeats was not possible due to PCR limitations. To detect such expansions, Southern blot analysis using the *BglI* enzyme and the p5B1.4 DNA probe was optimised. The running time and the voltage used for gel electrophoresis were modified to get clear separation of the second band that represents the expanded CTG repeats. Meanwhile, normal individuals showed only a single band so that they were not confused with the affected persons. Using this strategy, all CTG repeat expansions between 50 and several thousands were detected. Analysis of the results obtained revealed the following: **1)** A correlation between the intergenerational repeat expansion and the patient phenotype giving legitimacy to the phenomenon of anticipation. **2)** Two cases of reduction of the repeat size upon paternal transmission. Since the age of both asymptomatic daughters were younger than the age at onset of the disease in their fathers, it was not possible to anticipate their clinical outcome. **3)** Lastly, a severely affected child with mental retardation and onset in infancy was found to be paternally transmitted.

(B) Amyotrophic lateral sclerosis: Two mutations in the superoxide dismutase (*SOD-1*) gene, Ala4Val and Ile113Thr, were previously shown to be prevalent among

familial amyotrophic lateral sclerosis (FALS) patients. These two mutations changed restriction enzyme recognition sites, so that restriction digestion of the appropriate PCR products was optimised and used to screen for their presence in the studied amyotrophic lateral sclerosis patients. SSCP analysis was also optimised and applied as a screening method for detection of unknown mutations in the *SOD-1* gene using DNA samples from 2 familial and 67 sporadic amyotrophic lateral sclerosis (ALS) patients. Any experiment with a positive SSCP screening result was repeated and a few false positive results due to PCR errors and/or errors in the gel preparations, loading or electrophoresis were detected.

A reproducible SSCP band shift was detected in exon 4 from one of the two familial cases. Sequencing of that exon revealed a G277→C point mutation which caused a Gly93Arg missense change. This mutation was confirmed to be present in all affected members of that family. Gly93 is a neutral and polar amino acid and is highly conserved among 18 different species and it was substituted by the basic arginine. Mapping of Gly93 to the crystallographic structure of the *SOD-1* gene revealed that it is one of the critical glycine residues that allow main chain conformation and packing interactions so that a mutation affecting this residue should have a deleterious effect on the conformation and stability of the enzyme dimer. In this FALS family, the affected members showed an early age of onset of the disease (26-40 years). Analysis of the results obtained revealed that: **1)** In the two FALS families screened one *SOD-1* mutation was detected in the two screened FALS patients. **2)** No *SOD-1* mutations could be detected in any of the sporadic cases. These results suggested that other gene(s) may be involved in the familial form of the disease and make it unlikely that *SOD-1* mutations are major determinants of sporadic ALS.

(C) Tuberous sclerosis: In order to screen the tuberous sclerosis complex (TSC) patients for point mutations within the *TSC2* gene, chemical cleavage of the mismatch (CCM) analysis was optimised and applied to four RT-PCR amplified (from 22 patients)

and two DNA PCR amplified (from 32 patients for one segment and from 10 patients for the other segment) *TSC2* segments. These segments were chosen as they include proposed important functional parts of the gene. Using this approach, nine cleavage products were detected from the screened patients. Sequence analysis of the corresponding cDNA and/or DNA segments revealed three missense mutations in three sporadic TSC patients and three polymorphic changes. No mutations could be detected in the screened promoter area of the gene.

The first mutation A4822→G produced a missense Met1602Val change in the GAP-3 related domain which is proposed to be an important domain of the gene. Both methionine and valine are neutral and hydrophobic amino acids and this change would normally be considered a conservative one. However, there are known examples of conservative missense mutations that can result in a disease phenotype if they occupy sites in the protein that are key determinants of stability or function.

The second mutation T5161→A produced a missense Ser1715Thr change. In spite of the fact that both serine and threonine are neutral and polar amino acids this mutation was associated with a severe phenotype. The third mutation C5176→T caused a missense Arg1720Trp change which changed the basic arginine to the neutral and hydrophobic tryptophan which may affect the protein structure and function. Moreover, both Ser1715 and Arg1720 are in exon 40 of the gene which was found to be conserved among different species.

The three missense mutations modified restriction enzyme sites. This was used to confirm for their presence in the corresponding patients. None of these missense mutations could be detected upon the screening of 100 normal chromosomes, in view of this and the expected effect on protein they were predicted to be responsible for the TSC phenotype in these patients. Until more is known about the function and structure of tuberin it will be difficult to speculate about the exact function of these mutations and their effects both on tuberin and on patient phenotype.

Three polymorphisms were also detected in the screened cohort of TSC patients. Two of them C4098→T and G5346→C were silent. They did not change known restriction sites, so that screening of the normal chromosomes for their presence was not performed. G5346→C was detected in four different patients and it may worth designing a technique to screen normal chromosomes for its presence. This may provide useful information for a linkage study analysis of the *TSC2* gene. The third polymorphism was due to an AA deletion at positions 5433 and 5434 in one of the polyadenylation signals of the gene. It was detected in one of the familial cases but failure to detect it in other affected family members and its detection in one normal control led to the conclusion that it was a polymorphic change.

In conclusion, the most appropriate screening strategy for the detection of molecular pathology is influenced by the expected nature of the mutation, size and structure of the gene in question and the availability of mRNA. Therefore, for each of the three studied disorders, a different mutation strategy was chosen and optimised. SSCP analysis suited the small, well characterised *SOD-1* gene. On the other hand, RT-PCR/CCM analysis allowed screening of a large part of the coding sequences of the *TSC2* gene even before the complete genomic structure was known. For myotonic dystrophy the nature of the underlying molecular pathology of the expanded CTG repeats which could be detected as bands of different sizes made the PCR/Southern blot approach the appropriate mutation detection procedure.

CHAPTER ONE

INTRODUCTION

(1) INTRODUCTION

1.1 Genes and single gene disorders

Genes are the units of heredity. Genetic information is stored in the nucleus of cells in deoxyribonucleic acid (DNA) which is packaged into 23 pairs of chromosomes. Each gene is a nucleic acid sequence which determines the amino acid content of an enzyme or other proteins. Genes are situated at specific sites, or loci, on chromosomes. Like the chromosomes, genes exist in homologous pairs one from each parent.

Inheritance of single gene disorders follows a simple Mendelian form of transmission in families and it can be divided into autosomal dominant, autosomal recessive, sex linked dominant, sex linked recessive and mitochondrial inheritance (Thompson et al., 1991).

1.2 Molecular pathology of single gene disorders

An alteration of the structure of a gene is called a mutation. As a result of mutation individual genes may exist in alternate forms, or alleles, only two of which can be present in one individual. The mutation may be present on only one chromosome of a pair (heterozygous) or on both chromosomes of the pair (homozygous). In either case, the cause is a single critical error in the genetic information.

1.2.1 Types and levels of abnormal gene expression

Gene action is mediated by a regulated flow of information reflecting the transcription of structural genes into messenger RNA (mRNA) precursors, a complicated series of steps involving processing of the large precursor molecules into definitive mRNAs and finally cytoplasmic translation of mRNA into a protein

product. Mutations may manifest themselves at any of these levels. That is, there may be a reduced rate of transcription of a gene, a variety of abnormalities involving the processing of mRNA precursors, defects of initiation, translation or termination of the synthesis of the protein product on the cytoplasmic mRNA template, or an abnormality in the structure of the gene product.

Mutations that produce disease are manifest in two ways. First, a single base substitution or other rearrangement in a gene can lead to an abnormal protein product. The second group of mutations are those that cause a reduction or absence of a particular protein product. This kind of disorder may result from mutations that involve transcription or processing of mRNA or that act at the translation level by interfering with initiation, elongation or termination. Unfortunately no classification is entirely satisfactory. For example, it turns out that some disorders that appear to result from defective synthesis of a particular protein are actually caused by the production of a structurally abnormal protein which is so unstable that its level in the cell is markedly reduced.

(I) Mutations causing synthesis of an abnormal gene product

Most of these mutations are due to single base substitutions (point mutations) in the parent genes. There are a few examples of structural variants that are caused by major rearrangements that lead to the formation of fusion genes which code for novel protein products. The first example of this kind of protein was haemoglobin Lepore.

Point mutations, on the bases of codon change, can be classified into ***silent, non-sense, missense,*** and ***sense*** mutations. A silent mutation is a mutation that causes no change in the amino acid present in the corresponding protein. A missense mutation causes the substitution of one amino acid for another in a protein. The result of single amino acid substitutions in proteins or their subunits vary depending on the type of the amino acid that is substituted and the site of the substitution in the particular protein. Also, most proteins are folded into a complex tertiary structure and the substitution of a charged for an uncharged amino acid can disrupt this structure

and lead to molecular instability. On the above basis, missense mutations may or may not affect the function of the gene product. Sickle cell anaemia, an autosomal recessive disorder leading to a form of haemolytic anaemia and intravascular thrombosis is a result of a missense mutation in codon 6 of the beta globin gene changing GAG (Glu) to GUG (Val). Another missense change in the same codon GAG (Glu) to AAG (Lys) results in a condition known as haemoglobin C which is associated with mild clinical symptoms. A harmless haemoglobin variant (haemoglobin Makassar) results from missense change in the same codon changing GAG (Glu) to GCG (Ala) (Weatherall, 1991).

(II) Mutations causing reduced output of a gene product

Many genetic disorders are caused by a reduced output of an enzyme or other type of protein and in some cases no product can be detected. These disorders result from mutations of the structural genes and the resulting defects are manifest at the levels of transcription, mRNA processing, translation or post- translational stability.

(A) Mutations that cause defective transcription: These mutations comprise:-

(i) Gene deletions and variation in gene number : Examples of this group are provided by the α thalassaemias. Normally, there are two closely linked α globin genes on chromosome 16. In many forms of α^+ thalassaemia there is a deletion involving this chromosome which leave a single functional α gene. In most types of α^o thalassaemia both α globin genes are lost.

(ii) Fusion genes : These result from chromosomal misalignment and abnormal crossing over. Examples of this are the genetic abnormalities that underlie red-green colour blindness. These appear to have resulted from unequal crossing-over between the red and green pigment genes that lie in a tandem array on the X-chromosome on which there is a single red pigment gene and variable numbers of green pigment genes.

(iii) Inversions : This term signifies that a region of DNA is back-to-front with respect to its normal orientation in the genome. A good example of this type of mutation has been shown in patients with δβ thalassaemia and in haemophilia A patients where half the serious cases have an intron 22 inversion.

(iv) Insertions : This mechanism underlies the molecular pathology of several varieties of Lesch-Nyhan syndrome, one form of Marfan's syndrome, lipoprotein lipase (LPL) deficiency and in several other conditions.

(v) Promoter box mutations : Examples of this kind of mutation have been found in several forms of β-thalassaemia and in haemophilia B Leyden type where mutations were found upstream from these genes either within or adjacent to promoter boxes. These mutations are associated with variable reductions in output from the adjacent gene loci.

(B) Mutations that cause defective mRNA processing: The primary mRNA transcript has to be processed by the removal of introns, joining together of exons and by polyadenylation. Normal splicing of mRNA is dependent on the presence of GT and AG dinucleotides at the 5' and 3' intron-exon junctions. Splice site mutations that affect the 5' donor (GT) or the 3' acceptor (AG) sequences will interfere with the normal splicing, resulting in an mRNA that retains an intron or is missing an exon. Examples of this are known in β^o thalassaemia, phenylketonuria, acute intermittent porphyria, neurofibromatosis type 1, retinoblastoma and more other conditions. In addition to the GT/AG junctional sequences there are highly conserved sequences at the boundaries between introns and exons that must also be involved in splicing of mRNA. Several forms of β^+ thalassaemia have been described which result from the production of cryptic splicing sites within these consensus sequences (Weatherall, 1991).

Polyadenylation signal site mutations also interfere with the normal processing of mRNA. Cleavage of the 3' end of the mRNA and addition of the poly A tail is controlled at least in part, by an AATAAA consensus sequence approximately 20

base pairs before the polyadenylation site. For example a single base change AATAAA → AATAAT, found in α globin genes of patients with α thalassaemia drastically reduces the production of α globin chains from the α_2 globin gene. Some genes have a number of alternative polyadenylation sites, selection among which may influence the stability of the resulting mRNA and thus the steady-state level of the mRNA (Thompson et al., 1991).

(C) Mutations causing abnormal translation: These include:-

(i) Initiation codon mutations : Several mutations have been observed in patients with α thalassaemia which involve either the initiation codon itself (ATG) or the sequences immediately adjacent to it leading to no α chain production from the affected α globin gene. Other examples have been seen in pseudohypoparathyroidsm and Tay-Sachs disease.

(ii) Non-sense mutations : Non-sense point mutations create stop codons and premature termination of translation with shortened gene products. These have been detected in a variety of genetic disorders such as Duchenne muscular dystrophy and neurofibromatosis type 1.

(iii) Frameshift mutations : Since proteins are encoded by a triplet code the loss or insertion of any number of nucleotides which are not three or its multiples will alter the reading frame of the message downstream of the change. The result is an anomalous amino acid sequence that is added to a normally initiated chain. Sometimes the altered base sequence generates a new termination codon leading either to premature termination of translation or elongation of the abnormal mRNA. Examples of this kind of mutation have been seen in β thalassaemia, Duchenne muscular dystrophy (DMD), haemophilia A and Christmas disease.

(iv) Termination codon mutations : Termination codon mutations were first described in the α globin genes. These mutations produce a longer than normal protein by changing a termination codon into one that codes for amino acid. In haemoglobin Constant Spring the α- chain is elongated at its C- terminus due to

single base mutation in the α- chain termination codon UAA to CAA (Gln) (Weatherall, 1991).

(v) Mutations that are distant from the structural genes : Most of the mutations that interfere with the production of peptide chains involve either the structural genes themselves or important regulatory sequences in their immediate flanking regions. However, there are other sequence elements that can markedly alter the efficiency of the transcription. The best characterised of these activating sequences are called enhancers. These are sequence elements that can act at quite a distance (often several kilobases) from a gene to stimulate transcription. Specific enhancer elements function only in certain cell types and thus appear to be involved in establishing the tissue specificity of many genes (Thompson et al., 1991). A so-called dominant control region (DCR) has been identified upstream from both the β like and α globin gene clusters. Several deletions that involve this sequence have been found to inactivate the structurally normal α globin genes in the same chromosome (Weatherall, 1991).

1.2.2 Mechanisms of mutagenesis

(I) Cytosine methylation and hot spots for point mutations

Point mutations have so far been the most common type of mutation in coding DNA sequences. The category of mutations that includes deletions and insertions accounts for 5 to 10 % of all known mutations (Cummings, 1994). Nucleotide changes that involve the substitution of one purine for the other (A↔G) or one pyrimidine for the other (T↔C) are called transitions. The replacement of a purine for pyrimidine or vice versa is called transversion. If nucleotide substitutions were random, there should be twice as many transversions as transitions. In fact there is a higher frequency of transitions than transversions among a collection of mutant alleles. The excess of transitions can be explained with the finding that the major form

of DNA modification in the human genome involves methylation of cytosine residues to form 5-methylcytosine, specifically when they are located immediately 5' to guanine i.e. as a dinucleotide 5'-CG-3'. Spontaneous deamination of 5' methylcytosine to thymidine in CG doublet gives rise to C→T or G→A transitions (depending on which strand of DNA the 5-methylcytosine is mutated). More than 30 % of all single nucleotide substitution detected in many inherited disorders are of this type (Thompson et al., 1991). Thus the CG doublet represents a true hotspot for mutation in the human genome (Cooper and Krawczak, 1990).

(II) Insertions, deletions and gene duplication

Alterations of gene structure by insertions or deletions have been described in numerous inherited disorders. The observed frequency of such mutations differs markedly among different genetic diseases. Some disorders are characterised by a higher frequency of detectable deletions, whereas in others deletion is a very rare cause of mutation.

A frequent cause of mutation involves a large deletion or duplication mediated by recombination between highly similar DNA sequences. Many genes exist as members of multigene families (e.g. the α and β globin gene clusters, immunoglobulin superfamily and colour vision gene family). When the members of such a gene family are located in a head-to-tail tandem fashion in the same chromosomal region, they sometimes misalign and pair out of register either in meiosis (when two homologues pair) or in mitosis after replication (when two sister chromatids often exchange DNA). Recombination with unequal crossing over occurring between mispaired chromosomes or sister chromatids can lead to gene deletion or duplication.

Recombination between homologous non-coding DNA sequences of the Alu family has been documented as the cause of duplication of several exons in the low density lipoprotein receptor gene in familial hypercholesterolaemia (Lehrman et al., 1987). A similar example is the mutational event in a case of XX maleness resulting

from aberrant exchange between an Alu repeat on the short arm of the X chromosome and one on the Y chromosome (Rouyer et al., 1987).

Examples of deletions of <20 bp and insertions of <10 bp of DNA sequences into human gene coding region were analysed by Cooper and Krawczak (1991). They stated that these events are not random and appear to be highly dependent on the local DNA sequence context. The majority of insertions can be explained by an endogenous replication mechanism of mutagenesis. This may be understood in terms of slipped mispairing due to direct repeats, runs of single bases, palindromes (inverted repeats) or the presence of symmetrical elements. Direct repeats are also a feature of a number of recombination, replication or repair based models of deletional mutagenesis. A significant excess of symmetrical sequence elements was found at sites of single base deletions. These elements were seen to possess an axis of internal symmetry (e.g. CTGAAGTC, GGACAGG) and varied between 5 base pairs and 11 base pairs in length. In addition a consensus sequence proposed to be a hot spot for deletions was drawn up : (TGA/GA/GG/TA/C).

(III) Expansion of trinucleotide repeats

The discovery of trinucleotide repeat expansion and instability in several inherited psychomotor disorders has provided a molecular explanation of the phenomena of anticipation where the disease shows increased clinical severity over successive generations in pedigrees (Richards and Sutherland, 1994). So far, expansion of trinucleotide repeat motifs, also referred to as dynamic mutations, have been found to be causally involved in several human genetic disorders including Fragile X syndromes (FRAXA & FRAXE) (Fu et al., 1991 and Knight et al., 1993); Myotonic dystrophy (DM) (Fu et al., 1992); Huntington disease (HD) (The Huntington's disease collaborative research group, 1993); Spinocerebellar ataxia type I (SCA1) (Orr et al., 1993); Spinal and bulbar muscular atrophy (SBMA, Kennedy disease) (La Spada et al., 1991); Dentatorubral and pallidoluysian atrophy (DRPLA) (Nagafuchi et al., 1994), Haw River syndrome (HRS) (Burke et al., 1994) and

Machado-Joseph disease (MJD) (Kawaguchi et al., 1994). In each case, tracts of trinucleotide repeats undergo expansion to produce the disease phenotype with expansions of either CCG/CGG (FRAXA & FRAXE) or CAG/CTG (All the remaining diseases) core sequences. These repeat tracts occur in the coding (HD, SBMA, DRPLA/HRS, MJD) as well as non-coding (DM and FRAX) regions of the gene. The mechanisms by which these trinucleotide repeat expansions produce their phenotypic effects are not clear. In FRAXA & FRAXE syndromes the full mutations are associated with fragile mental retardation 1 (*FMR1*) gene methylation leading to transcriptional suppression and an absence of the encoded protein (McConkie-Rosell et al., 1993). In DM there are conflicting reports of decreased (Fu et al., 1993 and Hofmann-Radvanyi et al., 1993) and increased (Sabourin et al., 1993) steady state of mRNA levels. In other disorders, where the repeat $(CAG)_n$ codes for polyglutamine, the expansion may confer some gain of function to the protein involved (Housman, 1995).

When compared to other types of single gene defects the properties of dynamic mutation diseases afford distinct advantages and disadvantages for diagnosis. The dynamic mutation disorders are remarkably homogeneous. Apart from fragile X syndrome, no other mutations in the genes involved in dynamic mutation disorders have been recorded. This is a great advantage for the diagnostic laboratories where the primary diagnosis of these disorders can now be made with confidence. The major disadvantages relate to uncertainty over the relationship between genotype and phenotype. Somatic variation can mean that copy number, determined from peripheral blood lymphocytes, is not an accurate determination of the size of the repeat in the affected tissue(s). In addition (perhaps as a consequence) there can be overlap in the copy number for the different phenotypic categories. For example, in the Huntington's disease about 2% of mutant chromosomes have copy numbers at the top of the normal range (Sutherland and Richards, 1993).

Recently a GAA repeat expansion is detected within an intron of a novel gene (*X25*) for Friedreich's ataxia (Campuzano et al., 1996). Unlike previously described

neurological disorders involving triplet repeat expansions, in which a dominant mode of inheritance is the norm, Friedreich's ataxia is an autosomal recessively inherited disorder. As such, there is no evidence of anticipation within families and carriers of the Friedreich's ataxia mutation show no obvious adverse consequence.

DISORDERS STUDIED

1.3 Myotonic Dystrophy (DM, Steinert's muscular dystrophy)

This disease is so called because patients who present to hospital on account of muscle weakness have a distinctive type of muscular dystrophy which is accompanied by myotonia of the tongue and hands (Bundey, 1992).

1.3.1 Clinical features

Myotonic dystrophy is the commonest adult form of muscular dystrophy, with an estimated incidence of 1 per 7,500, although this is likely to be an underestimate because of the difficulty of detecting minimal affected individuals (Harley et al., 1992). The clinical features of this disease are myotonia, weakness, muscle wasting, frontal baldness, cataracts, hypogonadism and ECG changes. Typically, symptoms become evident in middle life. Initial symptoms are insidious and by time the patient is severely disabled, retrospective questioning will reveal that the disease has been present for 20 to 40 years. Moreover, some patients, particularly those with onset late in life, may be without muscular symptoms or signs and have only lenticular opacities (Bundey, 1992).

Unlike other muscular dystrophies, DM initially involves the distal muscles of the extremities and only later affects the proximal musculature. The muscle weakness affects firstly the face, sternomastoids, muscles of the forearms and tibialis anterior muscle leading to foot drop. Atrophy of masseters, sternocleidomastoids and the temporalis muscles produces a characteristic haggard appearance. Myotonia or delayed muscular relaxation following contraction is most frequently apparent in the

tongue, forearm and hand. Myotonia is rarely as severe as in myotonia congenita and tends to be less apparent as weakness progresses (McKusick et al., 1994).

Mild endocrine and bony changes have been observed in DM patients. Males, particularly those with early onset, develop primary testicular atrophy and may present with impotence or infertility rather than with muscle symptoms. Females tend to suffer from menstrual irregularities, and they are predisposed to obstetric complications, such as prematurity, ante- and post-partum haemorrhage (Bundey, 1992). DM patients may develop personality changes such as irresponsibility or aggression, and if onset is early in life they are often mentally retarded (Bundey, 1992).

Complications affecting different organs have, also, been reported. Schwindt et al. (1969) claimed that 25 to 50% of patients have abdominal symptoms due to cholelithiasis. Brunner et al. (1992a) pointed out that there are many reports of familial occurrence of specific complications of DM e.g. cardiac conduction disturbances, focal myocarditis, miteral valve prolapse, polyneuropathy, normal pressure hydrocephalus and urinary tract dilatation.

Congenital myotonic dystrophy (CDM) is an unusual and severe type of myotonic dystrophy which occurs in about 10% of cases. Symptoms are present in the neonatal period and the ultimate prognosis is poor. It is characterised by respiratory distress and poor feeding after birth. A typical facial appearance due to bilateral facial paralysis and ptosis is present in the newborn. Hypotonia and talipes equinovarus are additional features. Respiratory difficulties are frequent and are often fatal (McKusick, 1994). The diagnosis is at times difficult if the family history is not known as the more characteristic manifestations of the disease (myotonia and cataracts) might not develop until much later. Those that survive the neonatal period initially follow a static course, eventually learning to walk but with significant mental retardation in 60 to 70% of cases. By the age of 10 year they develop myotonia and in adulthood develop the additional complications described for the adult onset disease. The mean IQ for those cases with neonatal respiratory distress was not

different from those without distress, suggesting that the mental retardation is unlikely to be related to anoxia (Baraitser, 1990). The inheritance of the congenital form differs markedly from orthodox Mendelian ratios in that, with only rare exception, it is the mother who transmits the disease.

1.3.2 Genetics

Myotonic dystrophy is an autosomal dominant disorder with high penetrance and rare new mutations (Harper, 1989a; Meiner et al., 1995). Various studies agree in demonstrating that 50% of patients had developed the disorder by around 20 years of age, and that a significant number do not develop it until after 50 years of age. Many obligatory gene carriers are asymptomatic and Harper (1973) found that 18% of asymptomatic first-degree relatives showed unequivocal abnormalities on clinical and slit-lamp investigation illustrating the inaccuracy of relying on age at symptomatic onset as a basis for penetrance analysis. Variation in clinical picture between a pair of monozygous twins has been reported (Harper, 1989b). Unfortunately, there are few reports of twins with myotonic dystrophy and further twin data would be particularly valuable.

Ives et al. (1989) described possible homozygosity for the DM gene. The possible homozygotes were more severely affected than the heterozygotes. On the other hand, Cobo et al. (1993b) studied a consanguineous French-Canadian family in which two sisters possessed two alleles with repeat sizes normally seen in minimally affected patients but were asymptomatic and showed no evidence of myotonic dystrophy on extensive clinical examination.

The myotonic dystrophy locus was assigned to chromosome 19 (Eiberg et al., 1983). Harley et al. (1991) concluded that the *DM* gene lies in region 19q13.2-q13.3. Linkage studies by Cobo et al. (1992) established the D19S63 marker as useful for prenatal and presymptomatic diagnosis and, as the closest marker to DM, in isolating the gene.

1.3.2.1 Identification of an expanded triplet repeat:

Harley et al. (1992) isolated a human genomic clone that detected novel restriction fragments specific to persons with myotonic dystrophy. A 2-allele *Eco*RI polymorphism was seen in normal persons, but in most affected individuals one of the normal alleles was replaced by a larger fragment, which varied in length both between unrelated affected individuals and within families. The unstable nature of this region was thought to explain the characteristic variation in severity and age at onset of the disease.

The causative mutation in myotonic dystrophy has been found to be an expansion of unstable tandem repeat of the sequence CTG, located in the 3' untranslated region of a gene, with strong homology to the protein kinase family, on chromosome 19q13.3 (Harley et al., 1992; Buxton et al., 1992; Aslanidis et al., 1992; Brook et al., 1992; Mahadevan et al., 1992 and Fu et al., 1992). In unaffected individuals the $(CTG)_n$ repeat number is polymorphic and ranges from 5 to 37 repeats (Brunner et al., 1992b) and is stably inherited. In DM, at least 50 copies are present in the minimally affected patients (Brook et al., 1992) with a dramatic increasing to an estimated 2000 copies in severely affected individuals (Fu et al., 1992 and Mahadevan et al., 1992). Expanded repeats are much more unstable, and there is a positive correlation between the length of the repeat and its instability (Lavedan et al., 1993a). Overall, there is an estimated 93 to 94% chance that the expanded allele will show an intergenerational enlargement on transmission from an affected parent to an affected child (Wieringa, 1994). Relatively stable behaviour of the repeat is most frequently found with alleles of less than 80 CTGs (Barcelo et al., 1993) and this could explain the persistence of an autosomal dominant disease such as DM, despite the presence of anticipation and low reproductive fitness of the severe DM phenotype. There is a relative paucity of alleles in the high normal range for the DM locus (Fu et al., 1992) and to date no transmission from a normal allele to a premutation allele has been observed.

The factors affecting trinculeotide repeat stability, normal allelic variation and the generation of new disease alleles are not fully understood. Both meiotic expansion and contraction events may normally occur in the DM allele but expansion is favoured once a threshold size is reached (O'Hoy et al., 1993). Hypotheses of both meiotic and mitotic instability have been proposed to explain the intergenerational variation in the CTG repeat (Jansen et al., 1994 and Wieringa, 1994). At the higher allele range, loss of interrupting motifs within tracts of trinucleotide repeats leads to greater instability and predisposes alleles to expansion (Chung et al., 1993; Hirst et al., 1994 and Leeflang and Arnheim, 1995).

Regressed DM Allele: A reduction in CTG repeat number to within the normal range was reported in DM kindreds by many investigators (Shelbourne et al., 1992; Brunner et al., 1993; O'Hoy et al., 1993 and Jansen et al., 1994). This can be either due to non-reciprocal crossovers or to gene conversion events that have been originally described in fungi (Orr-Weaver and Szostak, 1985). Cobo et al. (1993a), Lavedan et al. (1993a) and Ashizawa et al. (1994b) in their series of parent-child pairs showed that contraction rather than amplification of the CTG repeat was seen in the children of fathers with a repeat of 1 kb or greater. They also, concluded that for parents with large amplifications (1.5 kb or more), the likelihood that there will be further amplification as the gene is passed to affected offspring is less for fathers than for mothers. The tendency towards contraction of alleles on transmission through the male germline may be caused by selection of spermatozoa bearing smaller repeats (Giordano et al., 1994 and Jansen et al., 1994). Ashizawa et al. (1994b) also showed that the cases with the CTG repeat contraction clustered within sib sets more frequently than expected but the mechanism of this phenomenon is unknown.

Sex-related effect on CTG intergenerational expansion: At higher CTG-lengths the maternal transmission results in the larger average intergenerational increments (Harley et al., 1993). In contrast, careful inspection of the published data suggests that expansions of repeats at the lower end of the length spectrum (<100 CTG) are more exaggerated when inherited from males (Brunner et al., 1993 and

Wieringa, 1994). An apparently unexplained excess of male transmitters has been found in the ancestors' generation by many authors (Harper, 1989a; Harley et al., 1993; Lavedan et al., 1993a; Brunner et al., 1993). This excess was observed whenever the disease was inherited from the father or from the mother. Thus, there appears to be a male bias in the generation of new alleles in DM (both contractions and expansions). Although ascertainment bias can not be rejected, this male excess could be due to the following biases : first, women with neonatal cases will not appear as grandmothers of affected patients; second, there are more children born to an affected male than to an affected female (Lavedan et al., 1993a); and, third, it may simply reflects the larger number of cell divisions during spermatogenesis (Wieringa, 1994). In females the ovum-to-ovum sequence involves approximately 30 cell divisions, whereas the number of the cell divisions for male gamete production ranges between 50 and several hundred during the effective fertile life span (Edwards, 1989).

Mosaicism: The amplified CTG repeat region shows both meiotic and mitotic instability. The differences in size within one generation of a DM family may be explained by meiotic instability of germline mosaicism (Meiner et al., 1995). On the other hand, smears of hybridisation in Southern blot analysis, reflecting somatic instability, have been reported for the majority of DM patients with a CTG repeat length >1kb, in males as well as in females (Mahadevan et al., 1992 and Fu et al., 1992). Somatic instability has been demonstrated among a number of different tissues. Compared to leukocytes larger expansions have been described in several tissues including skeletal muscle, liver, testis, brain and may also be found in skin (Anvert et al., 1993; Ashizawa et al., 1993, Lavedan et al., 1993a; Zatz et al. 1995 and Wohrle et al., 1995). Lavedan et al. (1993a) and Brunner et al. (1993) showed that somatic instability is not only limited to the larger DM alleles and the PCR analysis of the expanded alleles, up to 90 CTG repeats, appeared on the polyacrylamide gel as a mosaic pattern of DNA fragments differing by one or a few trinucleotide repeats. Wong et al. (1995) showed that somatic heterogeneity is a continuous process and seen to be age and size dependent. Martorell et al. (1995)

confirmed that the repeat length in peripheral blood cells of patients increase over a time span of five years indicating continuing mitotic instability of the repeat throughout life. They also, stated that repeat length progression does not appear to be indicative of clinical progression but age probably is. The degree of size heterogeneity correlates with the initial repeat size, however, obvious size heterogeneity is not observed in congenital cases, regardless of the size of the expansion (Wong et al., 1995 and Martorell et al., 1995). This heterogeneity of expansions between tissues indicates that repeat expansion in vivo might be related to cell proliferation. Tissue-specific differences in the efficiency or availability of DNA repair systems may also account for the heterogeneity of expansions (Wohrle et al., 1995). Somatic heterogeneity in vivo could explain the overlapping in fragment sizes of the different clinical groups, especially in adults with classical DM.

Size of the unstable CTG repeat in relation to phenotype: There is a consensus among those working on DM that the size of CTG expansions in blood lymphocytes correlates to a reasonable degree with the age of onset and severity of the disease, although this is more readily observed within rather than between pedigrees (Redman et al., 1993). As seen from the typing of repeat sizes in blood from comprehensive cohorts of patients, minimally affected patients have repeat sizes of <0.45 kb (150 CTGs). Congenital cases have on average the largest repeat sizes, expansion of 1.5 to 6 kb or more with the majority in the 4.5 to 6 kb range (1500-2000 CTGs), and the classical cases with highly variable manifestation of clinical signs and age at onset in the 2nd to 3rd decade, have intermediate expansions. This correlation is by no means absolute, however, and overlap between clinical groups was present so that the CTG-length typing can not be reliably used as a diagnostic or prognostic criterion to predict the clinical status of patients (Hunter et al., 1992; Buxton et al., 1992; Mahadevan et al., 1992; Harley et al. 1993; Shelbourne et al., 1993; and Lavedan et al., 1993a). Zatz et al. (1995) analysed the CTG expansion in muscle as compared to lymphocyte DNA in a sample of DM patients of different ages and degrees of clinical severity. Results from their study showed that in contrast to

lymphocytes, no significant correlation was found between the size of the CTG expansion in muscle and age at onset of the disease. In addition, large expansions were observed in muscle from all adult symptomatic patients independently of the presence of muscle weakness, which raised the question of the value of analysing CTG expansions in muscle for predicting the severity of the phenotype.

1.3.2.2 Anticipation

The term anticipation has been used to denote the progressively earlier appearance of a disease in successive generations, generally with increasing severity (Harper, 1989b). Clinically, anticipation has been a strikingly consistent phenomenon in a large number of myotonic dystrophy families (Howeler et al., 1989 and Ashizawa et al., 1992). A positive correlation is seen between earlier onset/greater severity and increasing CTG repeat number (Buxton et al., 1992; Tsilfidis et al., 1992 and Lavedan et al., 1993a) and is considered to be the molecular basis for anticipation in DM.

Ashizawa et al. (1994b) showed that in about half of the reported cases of intergenerational contraction of the CTG repeat observed in lymphocyte DNA, clinical anticipation still occurred, despite the contraction and it was proportionally more frequent with maternal transmission than with paternal transmission. The most striking examples were the two cases in which anticipation resulted in congenital DM in offspring with contractions of the CTG repeat. The authors suggest that the presence of factors other than the CTG repeat size are responsible for the severity of the phenotype.

1.3.2.3 Genetics of congenital myotonic dystrophy

Congenital myotonic dystrophy (CDM) occurs in about 10% of cases and appears to be quite distinct from severe adult onset DM. Neither the mostly maternal transmission of the mutation nor the early onset of that form are fully explained. CDM is usually associated with large CTG triplet expansions in the mother or child or

both. Tsilfidis et al. (1992); Harley et al. (1993) and Redman et al. (1993) found some congenital cases with alleles in the 500 to 999 repeats range and an overlap between the CDM and non-CDM patients in the 1300 to 2300 repeats range. The lack of CDM in paternal transmissions has been attributed to the limited expansion of the CTG repeat through the paternal line (Lavedan et al., 1993b and Mulley et al., 1993). However, there is a considerable overlap in the length of the expansion in the blood between cases with congenital and adult onset forms (Harley et al., 1993) and males do sometimes pass on expansions that, if they were transmitted through the maternal line, would probably have led to expression of CDM in the child (Ashizawa et al., 1994b; Passos-Bueno et al., 1995). These findings indicate that size of the expanded CTG repeat sequence is not the only feature determining congenital onset of the disease.

Although genomic imprinting is one of the mechanisms that could explain the effect of parental sex in CDM, methylation patterns do not differ in offspring of maternal and paternal transmissions (Shaw et al., 1993a and Ashizawa et al., 1994a) and the DM kinase mRNA of paternal and maternal origins were equally expressed in CDM patients (Jansen et al., 1993). The picture is further complicated by the reports of CDM with a large CTG repeat of paternal origin (Nakagawa et al., 1994 and Ohya et al., 1994) and by the observations that a large expansion of a maternally transmitted CTG repeat does not always result in CDM (Abeliovich et al., 1993; Lavedan et al., 1993a and Redman et al., 1993). Furthermore, Cobo et al. (1993a) reported a case in which CDM is inherited despite a 3 kb contraction on transmission from mother to son. The finding that CDM can be paternally transmitted, even if the incidence of such cases is very low, casts doubts upon the interaction with intrauterine factors as possible mechanism previously postulated to explain the maternal transmission of the congenital form (Harper and Dyken, 1972). Poulton et al. (1995) have not found evidence that mtDNA is involved in CDM. The possibility that CDM may operate with a pathologic mechanism different than that in adult onset DM can not be excluded. Bundey (1982) and Lavedan et al. (1993a) put forward the

hypothesis that the propensity to have neonatally affected offspring is familial. They noticed that transmitting sisters, whatever their clinical status or the repeat length, always gave birth to children affected with the same clinical type of disease i.e. mild form, adult form or congenital form.

1.3.2.4 Founder chromosome and the origin of the expansion mutation in DM

Haplotype analysis of DM chromosomes has detected a striking total linkage disequilibrium in both Caucasian and Japanese patients, between the DM mutation and a two-allele insertion/deletion polymorphism located 5 kb upstream from the repeat, suggesting a single origin of the mutation (Harley et al., 1992; Mahadevan et al., 1992; Yamagata et al., 1992 and Lavedan et al., 1994). This finding was unexpected for a dominant disease which, in its severe forms diminishes or abolishes reproductive fitness. Such diseases are in general characterised by a high level of new mutations, which compensate for the loss of abnormal alleles due to the decreased fitness. Further work revealed that normal chromosomes with five and 19-30 repeats only carry insertion alleles, while those with 11-13 repeats are almost exclusively associated with the deletion alleles (Imbert et al., 1993). These results led to a model being proposed to describe the origin of the DM mutation. The initial predisposing event(s) leading to the formation of the DM chromosomes were proposed to be a limited number of duplication steps of a $(CTG)_5$ allele that resulted in generation of CTG ($n \geq 19$), with the lack of a predominant allele in the CTG ($n = 19$-30) range. The heterogeneous class of CTG ($n = 19$-30) alleles, which was found to have an overall frequency of about 10%, may constitute a reservoir for recurrent DM mutations. The CTG expansion beyond a certain threshold, approximately 50 repeats, would confer a rapid and irreversible instability, leading to increased severity or earlier onset of the disease (anticipation), or both. This model has been supported by the finding that differences between the frequency of large alleles in the high normal

range of the allele size distribution (CTG ≥ 19) in global human population are congruent with observed variation in prevalence rate of the disease (Watkins et al., 1995). DM has a very low prevalence rate among ethnic Africans (Ashizawa and Epstein, 1991) and Goldman et al. (1994) reported fewer large DM alleles in South African Negroids than in Japanese or white subjects. Further support of the model proposed by Imbert et al. (1993) came from a report of a Japanese family (Yamagata et al., 1994) where the father (which is clinically normal) of the proband (with early adolescent onset DM) was found to have alleles containing 29 and 46 repeats and the grandmother had alleles of 5 and 44 repeats. From the evidence of the last case it seems that a premutation (as few as 44 repeats) can in two generations expand to give early adolescent onset DM and this finding is supporting the multistep model for the maintenance of the DM mutation in the population. The insertion allele is not a prerequisite for CTG expansion beyond 19 repeats or into the pathogenic range and suggest that the proposed increased mutability of large normal repeats is simply a function of their length. Moreover, it is possible that $(CTG)_5$ allele gave rise to $(CTG)_{11-13}$ alleles as the latter alleles found associated with the insertion polymorphism in African populations with the additional possibility that the $(CTG)_{19-30}$ chromosomes may have been derived from the $(CTG)_{11-13}$ chromosomes and the deletion event occurred on a $(CTG)_{11-13}$ background.

1.3.2.5 Myotonic dystrophy gene(s)

Three groups of collaborating DM-researchers simultaneously and independently recognised that the unstable CTG repeat element is in the 3' non-coding segment of a gene that belongs to the serine/threonine protein kinase family (Mahadevan et al., 1992; Brook et al., 1992; Fu et al., 1992). This gene is now commonly referred to as DM-protein kinase (*DMPK*) or myotonin protein kinase (Mahadevan et al., 1992; Fu et al., 1992).

Characterisation of the DM region in mouse revealed another active gene designated (*DMR-N9*) that appeared to have a human homologue in close proximity to 5' prime end of the *DMPK* gene. Shaw et al. (1993b) described the human homologue of the mouse *DMR-N9* gene and called it *59* gene. Transcripts of these genes, mainly expressed in brain and testis, possess a single, large open reading frame, but the function of its protein product is unknown. Two regions of the predicted protein show significant homology to tryptophan/aspartic acid (WD) repeats, highly conserved amino acid sequences found in a family of proteins engaged in signal transduction or cell regulatory functions. This led Jansen et al. (1995) to concluded that the *DMR-N9* gene is a candidate for being involved in the manifestation of mental and testicular symptoms in severe cases of DM. Strikingly, the combined pattern of tissue specific expression of both *DMPK* and *DMR-N9* genes from the DM region corresponds exactly to those sites where the clinical manifestations in DM patients are most prominent.

At the 3' end of the *DMPK* gene, there is a CpG island (Shaw et al., 1993b; Boucher et al., 1995) where the unstable CTG repeat is located within it. Extensive homology between the murine and human sequences 3' to the CTG repeat supported the hypothesis that another gene lies in this region and allowed Boucher et al. (1995) to identify candidate exons. A highly significant homology with the homeodomain-containing protein genes has been identified. Consequently, the gene was named DM locus-associated homeodomain protein (*DMAHP*). Using RT-PCR with primers from the putative homeodomain-encoding exonic sequences showed that *DMAHP* gene is widely expressed in a number of human tissues, including skeletal muscle, heart and brain. The identification of a gene immediately downstream of the CTG repeat supports a model of symptom development in which as the expansion increases in length, broader gene dysfunction occurs, correlating with clinical severity. This could involve *DMPK, 59, DMAHP* and possibly other as yet uncharacterised gene(s). It has been proposed that level of expression of the DM-kinase isoforms and/or the *DMR-N9* (*59*) product(s), as regulated by cis influences on transcription, translation or

mRNA stability, is affected differentially by the variation in length of the repeat, and so the clinical manifestation of DM. It is not inconceivable that other, yet unknown, gene(s) in the immediate vicinity of the CTG repeat are also affected at the level of expression (Jansen et al., 1992).

***DMPK* gene:** *DMPK* gene encodes a protein of 624 amino acids with a highly conserved kinase domain encoded by exons 2 through 8. A region showing significant homology to the α-helical (coiled-coil) domains of myofibrillar and filamentous protein is encoded by exons 9 through 12, with the strongest homology being contained in exons 11 and 12. Exon 15, the last exon, contains a relatively short region encoding for a hydrophobic, possibly transmembranous domain, and also contains the CTG repeat in the 3' untranslated region. Exons 13 and 14, which show no strong homology to any proteins or protein domains, are alternatively spliced out in several cDNA clones isolated from a human heart cDNA library. As a consequence of a shift in the open reading frame, an earlier termination codon (5 bp after the beginning of exon 15) is used. This results in loss of the hydrophobic region. Immediately following the region encoding the kinase domain there is a cryptic splice donor site that, if used, takes out five additional amino acids (VSGGG) from the human and mouse proteins. This peptide sequence forms an imperfect (and putative) glycoseaminoglycan addition site. Any potential function of this segment will remain elusive until more is known about the cellular location of the each of the different *DMPK* isoforms (Mahadevan et al., 1993; Shaw et al., 1993b; Wieringa, 1994). Fu et al. (1993) has proposed two alternative N-terminal forms both of which were shorter than those proposed by Mahadevan et al. (1993) and Shaw et al. (1993b). It remains to be seen which initiators are actually used in vivo. As shown from the above different *DMPK* mRNAs carry open reading frames ending in regions which differ in length and use of reading frame. As a consequence, different hydrophobic tail pieces are predicted for the putative protein kinase. The finding of alternatively spliced and species specific mRNAs with different 3' coding information make it tempting to speculate that the variable C-terminus of the protein has a regulatory function as a

kinase inhibitor site (Soderling, 1990) or functions in the differential anchoring of the kinase to cellular structures. A similar situation has been shown for muscle myosin heavy-chain gene (George et al., 1989).

Until now, not much data were available which address the structure-function relationship of the individual domains in the *DMPK* gene product(s) directly. The putative kinase may play a specific role in the regulation of excitation-contraction coupling or maintenance of cellular physiology via regulation of protein-protein interactions in ion channels (Roherkasten et al., 1988; Catterall, 1991) or insulin receptor signalling (Moxley et al., 1984). Abnormalities in phosphorylation of membrane proteins have been reported to be a characteristic finding in DM (Roses and Appel, 1974) and the application of protein kinase C modifier can evoke myotonia in mouse muscle (Brinkmeier and Jockusch, 1987). No clear evidence has been provided to indicate that the product of *DMPK* gene is the affected protein in DM, though the expression patterns of the gene (Jansen et al., 1992) suggest it is the most likely candidate. Antisera have been developed to peptide immunogene and fusion protein of the myotonin protein kinase. The antipeptide antibody detected 52-55 kd protein species (Fu et al., 1993; Brewster et al., 1993; van der Ven et al., 1993). Brewster et al. (1993) showed that one of the antisera they developed also recognised a dominant 42 kd protein in brain. This smaller protein may result from alternative splicing or post-translational processing and be a genuine product of the DM-kinase gene. Van der Ven et al. (1993) found that the DMPK 53 kd protein is markedly concentrated at the sites of specialised membrane regions like neuromuscular junctions (NMJs) and myotendinous junctions (MTJs) in skeletal muscle, intercalated discs in the heart and dense plaques in smooth muscle cells. They also showed that Purkinje cells and several other neurones of the cerebellum and other parts of the brain have been stained by the anti-peptide antibody. The antifusion protein antibodies of the DMPK detected prominent protein species in heart, brain and skeletal muscle that were larger than the previously detected 52-55 kd species. These species (~74 and 82 kd) were more consistent with the size of the *DMPK*

expressed in both bacteria and in insect from a full length cDNA. It is likely that the lower molecular weight species are detected through cross reactivity with the abundant kinases present in muscle and other tissues. This is supported by the fact that antibodies raised against the expressed C-terminal half of the DMPK only detect the larger species (Johnson and Siciliano, 1995). Using immunofluorescence, Whiting et al. (1995) found that the larger species of the DMPK were localised post-synaptically at the neuromuscular junctions of skeletal muscle, at intercalated discs of cardiac tissue and at the apical membrane of the ependyma and choroid plexus. Synaptic localisation of the DMPK in the cerebellum, hippocampus, midbrain and medulla was also noted. These results suggest that DMPK plays a specialised role in intercellular communications. While this is consistent with the abnormalities noted in the heart, the relationship between DMPK localisation at neuromuscular junctions and myotonia is uncertain. A selective action of this kinase within the central nervous system function has been suggested. However, what the function of DMPK may be in the brain is still a matter for speculation.

Studies of *DM* kinase gene expression in myotonic dystrophy have provided discordant results. Both over-and under-expression, as well as unaltered *DMPK* expression have been reported (Jansen et al., 1993; Fu et al., 1993; Hofmann-Radvanyi et al., 1993; Roses et al., 1992; Sabourin et al., 1993; van der Ven et al., 1993; Koga et al., 1994). Hofmann-Radvanyi et al. (1993) noted also decreased (20-30% of normal) expression of the unaffected allele.

Wang et al. (1995), using a new method for RNA quantitation and myopathic controls, suggested that the CTG expansion mutation has only a minor effect in the transcription and accumulation of the *DM* kinase hnRNA from the diseased allele. Instead the mutation dramatically alters the ability of the mutant RNA to be processed into poly $(A)^+$ mRNA. More importantly, the same expansion-containing hnRNA seems to affect the accumulation of the normal allele mRNA in trans. This interpretation suggests a dominant-negative RNA mutation model which is consistent with the dominant inheritance pattern observed in DM. Moreover, if the mutant RNA

can affect alteration in the accumulation of the normal DM kinase mRNA, then it is possible that it can alter the accumulation of other species of mRNA sharing similar regulatory elements. This model could explain the tissue and development specific features of myotonic dystrophy. Each tissue has populations of stage specific RNAs, which could be affected differently by the expanded DM kinase RNA. However, a contrasting study has suggested that while post-transcriptional processing of the expansion allele mRNA is impaired, this has little effect on overall *DMPK* mRNA levels in heterozygous patient material (Krahe et al., 1995b).

It is still unclear if the *DMPK* gene is the only gene involved in DM. Although the mutation in the great majority of DM patients is expansion of the CTG repeat in the 3' region of the *DMPK* gene, there are a few cases in which this does not appear to be the case (Shaw and Harper, 1992). To my knowledge, there is no reported mutation in *DMPK* gene in such cases. Meiner et al. (1995) reported four such families, that fulfilled the criteria of DM diagnosis without CTG repeat expansion in the affected persons. Upon full sequencing of the coding region of *DMPK* gene in two of them they could not find any abnormal sequence alterations.

1.4 Motor neurone disease (MND) [Amyotrophic lateral sclerosis (ALS)]

ALS is a progressive neurodegenerative disorder of adults resulting from variable combined degeneration of the lower motor neurones (LMN) and upper motor neurones (UMN). It is referred to as motor neurone disease (MND) in the UK and in some European countries, as amyotrophic lateral sclerosis (ALS) in the USA and as Charcot's disease in France. At least three types of the disease are recognised : classical sporadic disease, familial and usually dominantly inherited disease and the type seen in the high-incidence foci in the western Pacific Ocean (Guam, Kii Peninsula of Japan and West New Guinea) (Tandan, 1994).

1.4.1 Clinical features of MND [Amyotrophic Lateral Sclerosis (ALS)]

About 90 to 95% of cases of adult onset ALS that occur in Europe and USA are isolated. Males are twice as often affected as females. The annual incidence rate of classical sporadic MND has varied between 0.5 and 2.4 per 100,000 population, and the prevalence rate between 2.5 and 7 per 100,000 population world-wide in different studies (Kurtzke, 1991). There are no significant racial differences in the incidence, prevalence or death rate of the disease (Tandan, 1994). Onset is generally between 50 and 60 years though with a wide range, and mean duration of illness is three to four years. The early symptoms usually occur in the limbs or shoulders (Bundey, 1992).

Degeneration of the LMN typically produces focal or multifocal and often asymmetric muscle weakness and atrophy, cramps, prominent fasciculations, fatigue, dysarthria and dysphagia. Clinical involvement from UMN degeneration results in spasticity, pathological hyperreflexia, Babiniski sign, brisk jaw jerk and emotional lability. By the time of presentation, however, features of combined LMN and UMN degeneration are seen in the majority of classical MND patients.

Motor involvement is frequently asymmetrical, and at onset is more common in the hands and arms than in the legs and bulbar muscles. With eventual progression of the disease, bulbar weakness develops in almost 50% of patients, and respiratory muscle weakness occurs almost universally (Tandan, 1994).

About 5 to 10% of cases of ALS are familial (de Belleroche et al., 1995). The empirical risk of recurrence in a first degree relative is 1 to 2%, that is about 1000 times commoner than in the population (Bundey, 1992). Horton et al. (1976) concluded that at least three forms of familial ALS (FALS) exist, each inherited as an autosomal dominant. In the most usual type, patients have a very similar illness to that seen in non-familial ALS, except that onset tends to be earlier (20 to 40 years), duration shorter (2 to 3 years), onset is generally with symptoms in the lower limbs rather than in the upper, and sensory symptoms are more frequent (Li et al., 1988).

The second type, is about one-fifth as common as the first one, with identical clinical features but with more extensive pathological features. The third type is similar to the second type except for a much longer survival (usually beyond 10 and often 20 years) (Alberca et al., 1981). The initiation of the disease is usually focal and asymmetrical, lower motor neurone involvement is usually conspicuous in most cases whereas involvement of upper motor neurones is less marked.

There is incomplete penetrance being 0.8 at the age of 85 years. It is therefore not uncommon to see obligate carriers in a family who died without manifesting the disease (de Belleroche et al., 1995).

Phenotypic heterogeneity was seen in both sporadic and familial ALS. For example the age of onset may vary over 30 years within a family as can duration of illness (for example, 0.5 to 5 years) and signs at onset.

Although the overall median survival in classical MND is usually about 3 years from onset of the disease, early diagnosis and more aggressive management have led to increased survival (Caroscio et al., 1987). It is generally agreed that, independent of the clinical variant of MND present, survival is greater in patients with onset of symptoms before the age of 50 years than after (Tandan, 1994). On the other hand Pradas et al. (1993) found that sex and age at clinical onset did not affect the deterioration rate in their studied group.

1.4.2 Genetics of ALS

1.4.2.1 Genetic linkage study of FALS

It is estimated that 5-10% of ALS cases have a familial aetiology. Siddique et al. (1989) presented preliminary data from genetic linkage analysis in 150 families with FALS. Two regions of possible linkage were identified on chromosome 11 and 21. In 1991, a FALS locus was identified on human chromosome 21q22.1-q22.2 (Siddique et al., 1991). Tests for heterogeneity in these families revealed a significant probability of locus heterogeneity. Therefore, it is clear that at least one other FALS

gene is present in human genome. In 1993, a gene within this region encoding the protein cytosolic copper-zinc superoxide dismutase (*SOD-1*) was found to be in tight genetic linkage with FALS. Given this linkage and the potential role of free-radical toxicity in neurodegenerative disorders, Rosen et al. (1993) investigated *SOD-1* as a candidate gene in FALS and identified 11 different missense mutations in different FALS families. Other workers failed to find linkage to chromosome 21 loci in their FALS families (King et al., 1993). This is to be expected because of the recognised heterogeneity. Moreover, the lack of mutations in *SOD-1* gene in a number of FALS families confirm the genetic heterogeneity.

1.4.2.2 Copper-zinc superoxide dismutase gene (*SOD-1*) and its product

Cu/Zn SOD-1 is a cytoplasmic enzyme which is responsible for the conversion of the toxic free radical superoxide anion O_2^- to molecular oxygen and hydrogen peroxide. *SOD-1* gene is one of three superoxide dismutase genes, each with a unique subcellular localisation; a second one Cu/Zn-containing SOD functions extracellularly and an Mn-containing SOD is found in mitochondria.

(1) Genomic structure of *SOD-1* gene : The gene locus for human *SOD-1* was assigned to chromosome 21q2.2 (Tan et al., 1973). It has been shown that the *SOD-1* gene has multiple polyA-addition signals and that there are two predominant mRNA species of 0.7 and 0.9 kb found in a variety of human cells. The major 0.7 kb species is approximately four times more abundant than the minor 0.9 kb mRNA (Sherman et al., 1983; Groner et al., 1986). Isolation of the human *SOD-1* gene revealed that the two mRNAs are transcribed from a unique *SOD-1* active gene on chromosome 21q2.2, and that sequence at the 3' untranslated region account for the difference between them (Hallewell et al., 1986). This has been further confirmed by detecting only one region of human DNA with nucleotide sequences identical to *SOD-1* cDNA (Groner et al., 1986).

SOD-1 gene, which is present as a single copy per haploid genome, is known to have five exons spanning approximately 11 kb. of genomic DNA of chromosome 21q2.2 and is interrupted by four introns. In the donor sequence of the first intron a T to C transition occurred and hence it deviates from 5' GT...AG 3' consensus, but few cases of such violation of the 5' GT...AG 3' consensus have been reported (Levanon et al., 1985). At the 5' end of the gene there are the 'TATA' and 'CAT' promoter sequences as well as four copies of the -GGCGGG- hexanucleotide. Two of these -GC- elements are contained within a 13 nucleotide inverted repeat that can fold into a stem-loop structure (Groner et al., 1986). In some cases such sequences appear to activate transcription (Hallewell et al., 1986).

(2) *SOD-1* related pseudogenes: Four *SOD-1*-related pseudogenes have been described by Groner et al. (1986). All these four sequences are devoid of introns, which typifies pseudogenes of the processed type (Lewin, 1990). Although the overall sequence homology of two of them to the *SOD-1* gene was extensive yet they contain multiple genetic lesions, such as insertions, deletions and base substitutions resulting in-frame termination codons, that preclude the translation of the normal SOD-1 polypeptides. These processed pseudogenes do not reside on chromosome 21.

(3) SOD-1 enzyme: Superoxide dismutases are thought to be an important component of the cellular defence mechanisms against oxidative damage mediated by superoxide radicals produced as a by-product of oxygen metabolism (Groner et al., 1986). SOD-1, which is a cytoplasmic enzyme, is a dimer of 32 kilo dalton (kd) composed of two identical non-covalently linked subunits. Each subunit contains one zinc and one copper atom, the latter being directly involved in the dismutation reaction as an electron acceptor.

(A) SOD-1 enzyme and the free-radicals: A free-radical (FR) is an atom, molecule or other chemical species that is capable of independent existence and which

has one or more unpaired electrons (Pall, 1994). They are usually short-lived as a consequence of being highly reactive, an effect resulting from their intrinsic need to gain an additional electron or to lose the unpaired one and hence achieve a stable electronic configuration. The oxygen-containing FRs include the superoxide ($O_2^{\bullet -}$) and the hydroxyl ($OH^{\bullet}$) radicals. Hydrogen peroxide (H_2O_2) is a reactive oxygen metabolite that is not a FR but which is capable of producing reactive FRs. Many biomolecules including DNA, polyunsaturated fatty acids and catecholamines are damaged by superoxide radicals (Halliwell and Gutteridge, 1989). Dismutation is the term applied to the reaction in which one superoxide radical reduces another to peroxide, itself becoming oxidised to oxygen in the process. This appears to be the function of the SOD enzymes. The hydroxyl radical ($OH^{\bullet}$) can also be produced from superoxide via hydrogen peroxide by low molecular weight/loosely bound complexes containing copper, iron and possibly manganese (fenton reaction). The superoxide anion can also react with nitric oxide to form peroxynitrite (Koppenol et al., 1992) (Figure 1.1).

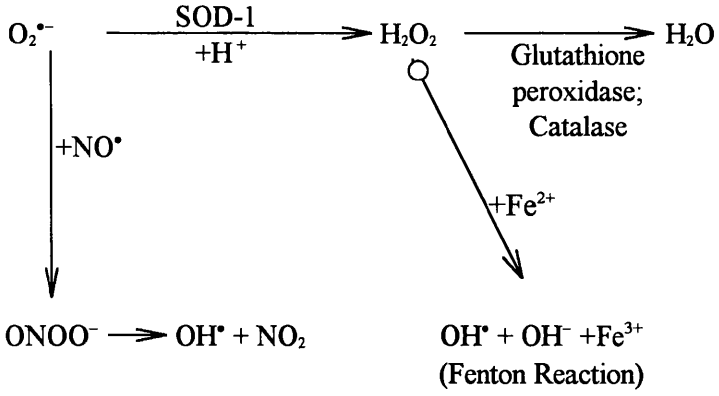


Figure 1.1 (Top) The superoxide anion ($O_2^{\bullet-}$) can react with SOD-1 to be detoxified to form hydrogen peroxide(H_2O_2) that, in turn, is converted to water through the action of catalase and glutathione peroxidase. Superoxide may also combine with nitric oxide ($NO^\bullet$) to form peroxynitrite ($ONOO^-$) which may then breakdown non-enzymatically to produce hydroxyl radicals ($OH^\bullet$). $OH^\bullet$ may also be generated from hydrogen peroxide via Fe^{2+} (Fenton Reaction).

These reactive oxygen species may cause oxidative degradation of DNA, lipids and proteins (Bottom).

(B) Crystallographic structure of the SOD-1 enzyme: Human SOD-1 is a dimeric enzyme with ellipsoidal dimensions of about 30x40x70 Å. Each identical subunit contains 153 residues, one copper and one zinc ion. The two metal ions are located in the bottom of a channel formed by the topography of the molecular surface. The polypeptide fold of the SOD subunit consists primarily of two large loops and eight extended antiparallel β-strands. The overall topology is characterised as a Greek-key β barrel (Getzoff et al., 1986). Greek-key β barrel structures tend to be structurally quite stable. Overall, there are seven loops in SOD, numbered in sequence order. Loops I and V are short β-hairpin connections between adjacent β-strands. Loop II (residues 24-27) form the β-hairpin containing the two residue insertion relative to bovine SOD. Loops III (residues 37-40) and VI (residues 102-114) form the two Greek-key β-barrel connections. The active site channel with its bound metal ions is formed between electrostatic loop VII (residues 121-144), implicated in substrate attraction, and loop IV (residues 49-84), made up of the disulphide and the zinc ligand subloop regions. Superposition of the human SOD-1 and bovine SOD-1 structures indicated that the sequence changes do not alter the β-barrel diameter, strand angles, or loop conformations except in the region of the two amino acid insertion at sequence position 25 (Parge et al., 1992).

At critical position within or near the loops, 14 sequence-conserved, structurally- conserved (Parge et al., 1992) side chains appear to play important roles in loop conformation and interactions. Most of the dimer contacts are made between adjacent strands around the barrel (Getzoff et al., 1986). Cross sections through the dimer interface showed the complementarity of fit between the two buried surfaces (due to dimer contact), the interdigitation of side chains from one subunit to the other, and the twist of the dimer contact. By pulling the two subunits of the dimer apart hydrophilic regions contributed by oxygen and nitrogen atoms are scattered throughout the interface. Electrostatic potentials calculated from partial charges assigned to each of the atoms in the structure can also be mapped into the molecular surface. At the dimer interface, the centre of the pattern is electrostatically neutral and

only the edges show significant electrostatic potential. The surfaces are electrostatically complementary to one another and essentially seal the edges of the dimer contact at protein-water interface (Getzoff et al., 1986). The copper and zinc ions are in high peaks of electron density and one unique feature of this enzyme active site is the bridge formed between the zinc and copper ions by the His-63 side chain (Getzoff et al., 1986; Parge et al., 1992). This bridge can be seen to be made and broken as the reaction proceeds.

Finally, reconstructing the enzymatic dimer and showing the electrostatic fields around the two active sites in the dimer emphasises that the enzyme is extremely efficient in attracting superoxide radicals (Getzoff et al., 1986).

1.4.2.3 *SOD-1* gene and ALS

Of all cases of ALS, 5-10% are familial. Of the familial cases, fewer than 20% map to the *SOD-1* gene, so there is locus heterogeneity (Rowland, 1995). The *SOD-1* gene was found to be mutated in several FALS families (Rosen et al., 1993). Although new mutations must occur, remarkably few have been identified in people with the sporadic disease (Jones et al., 1993; Jones et al., 1994). At present at least 31 ALS missense mutations and one deletion have been characterised in the *SOD-1* gene. Over the last two years, several studies have begun to define the effects of the FALS mutations on SOD-1 function, although many critical questions remain unanswered. It is now clear that the mutations reduce total cellular activity of the enzyme by 25 to 70%, as analysed in red blood cells, lymphoblastoid cell lines and cerebrospinal fluid. (Deng et al., 1993; Bowling et al., 1993; Robberecht et al., 1994; Orrell et al., 1995b).

The original demonstration of *SOD-1* mutations led Rosen et al. (1993) to hypothesise that FALS could raise either by an increase in SOD-1 monomer activity (dominant gain of function) or the heterozygous mutation could cause mutant monomers to be functionally defective and inhibit wild-type monomers in the

heterodimer (Dominant negative effect). A third possibility was that the mutation could cause a simple loss of function without any effect on the wild type monomer.

The elucidation of the structure of SOD-1 dimer by X-ray crystallography permits the localisation of the mutations in FALS (Denge et al., 1993). The majority of mutations detected to date lie in regions outside the active site affecting conserved regions of the enzyme at turns in the backbone of the protein (beta strands, Greek-key connections, turns of loop V) or in regions involved in the dimerisation of the two subunits. These mutations will affect the conformation or stability of the enzyme dimer (Deng et al., 1993). Overexpression of the normal *SOD-1* gene inserted into transgenic mice resulted in increased lipid peroxidation (Elory-Stein et al., 1986) and distal tongue and hind limb motor deficits (Avraham et al., 1992) in such mice. These changes, which were not the classical FALS picture, may have been caused by overproduction of H_2O_2 by SOD with subsequent paradoxical increase in $OH^{\bullet}$ synthesis. In other mice transgenic for *SOD-1* with more than 2-fold overexpression of normal mouse SOD activity, paralytic disease has not been seen in animals followed to the adult life (Gurney et al., 1994).

Gurney et al. (1994) used transgenic mice to introduce either of two human mutant SOD-1 enzymes. The human enzyme was expressed, but at 50% of normal activity. The mouse genes also continued to function and, in one line of mice, there was overexpression of total SOD-1 nevertheless, the animals developed a clinical syndrome of hind limb paralysis, with histological signs of degeneration and loss of motor neurones in spinal cord. Similar results have been obtained by other investigators (Borchelt et al., 1994; Price et al., 1994). Ripps et al. (1995) supplemented Gurney et al. (1994) observations. By using site-directed mutagenesis, they introduce a missense mutation, Gly86Arg, which corresponds to a human mutation observed in codon 85 (Rosen et al., 1993; Denge et al., 1993), into transgenic mice. SOD enzyme activity in these animals did not reveal a diminution of activity. However, in two lines of mice, that produce high levels of transgene mRNA

in the CNS, motor paralysis developed and was associated with degenerative changes of motor neurones within the spinal cord, brain stem and neocortex.

More interesting has been the observation that in the case of some mutations enzyme activity is only minimally affected despite a normal clinical presentation of the disease (Esteban et al., 1994). In one of these mutations, Gly37Arg, it has been shown from transient expression of this mutant in primate cells that the mutation leads to full activity, in contrast to other SOD mutants studied under similar conditions (Borchelt et al., 1994). However, in all cases, including Gly37Arg, polypeptide stability of the mutant subunit was found to be reduced. Moreover, a mutation has been identified at the active site in codon 125 (Enayat et al., 1995) which led to a major charge effects. However, it is associated with a classical form of FALS with no evidence that this location of mutation has a greater effect on the course of the disease. A mutation in exon 2, His46Arg, was associated with a more benign form of the disease and had only slightly reduced (20%) levels of SOD-1 enzyme activity (Ogasawara et al., 1993). This mutation was the first mutation to be detected in the active site, the residue histidine being important in copper binding which is essential to the catalytic activity of the enzyme. Recently, Enayat et al., (1995) reported a mutation His48Gln. Both His46 and His48 are highly conserved residues and are important for copper binding. However, His48 mutation was associated with a relatively severe nature of the disease which contrasts with the benign course of the His46 mutation.

The Ile113Thr mutation has been detected in three out of 56 sporadic cases in the population based study of Scotland (Jones et al., 1993). A single case of an exon 1 mutation, Glu21Lys, has been detected in this cohort as well (Jones et al., 1994). There are no other published reports of *SOD-1* mutations in sporadic cases, although several hundred cases have been screened in North America and the possibility of incomplete penetrance within the families together with incomplete family history can not be ruled out in these cases (de Belleroche et al., 1995).

How do *SOD-1* mutations cause ALS?. Gurney et al. (1994) and others (Gurney, 1994; Rowland, 1995; de Belleroche et al., 1995; Brown, Jr., 1995) suggested some mechanisms to explain the pathogenetic effect of *SOD-1* mutations on FALS. All the mechanisms they suggest were consistent with the idea that mutations in *SOD-1* cause an acquisition of injurious properties by the mutant enzyme. The first explanation they suggest is that *SOD-1* mutations potentiate catalysis of normally unfavourable side reactions to which motor neurones are selectively vulnerable. As it is known, in addition to the dismutation reaction $2H^+ + O2^{\bullet-} \rightarrow H_2O_2$, SOD also catalyses several alternate reactions including : (1) the formation of the hydroxyl radical from hydrogen peroxide and (2) the nitration of proteins on tyrosine residues by peroxynitrite. Such side reactions might be facilitated by mutations, and to a lesser extent, by high expression of the wild-type enzyme. The rate limiting step in reactions (1) and (2) may be the access of reactants to the copper catalytic centre at the bottom of the active site channel. By relaxing constraints on the size of the active site channel, the mutation found in affected families might cause a "gain-of-function" by facilitating one or more of these alternative reactions. If this is true, then high expression of the wild-type enzyme may cause subclinical pathology (Gurney, 1994), but more work will need to be done to prove this.

Alternatively, the mutations might adversely affect the binding of copper and that cation, in local excess, might have toxic consequences (Rowland, 1995). Thirdly, the mutant enzyme may be so unstable that it precipitates to form toxic cytoplasmic aggregates. There may be more than one mechanism or a combination of mechanisms, some causing peroxidation but others having nothing to do with free radicals.

Glutamate, excitotoxicity and motor neurone selectivity: The selective vulnerability of motor neurones in ALS may depend on specific features of these neurones. Motor neurones are seen to possess very high levels of *SOD-1* mRNA (Tsuda et al., 1994). These neurones which may be critically dependent on SOD-1 for protection against superoxide may potentially become susceptible targets for additional adverse effects of the protein. On the other hand, an important feature

distinguishing motor neurones from dorsal root neurones, which do not degenerate in ALS, is the presence of excitatory synapses on the former. Glutamatergic excitatory synaptic input was suspected to enhance the sensitivity of motor neurones to free radical injury. One popular hypothesis is that the activation of nitric oxide synapse by N-methyl-D-aspartate (NMDA) glutamate receptors leads to the generation of nitric oxide (NO) which reacts readily with superoxide to produce the toxic species peroxynitrite, which in turn releases hydroxyl free radicals (Lafon-Cazal et al., 1993). Following stimulation of NMDA-sensitive glutamate receptors, $O2^{\bullet -}$ activated cell death was dependent on calcium (Ca^{2+}) and release of arachidonate and was reduced by trapping $O2^{\bullet -}$ (Lafon-Cazal et al., 1993). The motor neurones may be particularly sensitive to increase in cytosolic calcium levels because, by comparison with other types of neurones, it is relatively poor in some calcium binding proteins (Ince et al., 1993).

1.5 Tuberous sclerosis (Bourneville disease; Epiloia; TSC)

1.5.1 Clinical manifestations

Tuberous sclerosis complex (TSC) is a disease that affects all tissues. It is characterised by the growth of benign tumours (hamartomas) and malformations (hamartias) in one or more organs. Its prevalence is between 10 to 14 in 100,000 persons, more common than previously thought (Hunt and Lindenbaum, 1984; Sampson et al., 1988; Sampson et al., 1989a), making it one of the most common autosomal dominant disorders. The common clinical picture of TSC is that of a mentally retarded epileptic patient with facial angiofibromas (adenoma sebaceum). However, milder manifestation may occur and, indeed, the manifestation of TSC may be very variable (Bundey, 1992). The organs most frequently involved , in addition to the brain and retina, are the skin, kidneys, heart and lungs.

The full clinical picture evolves gradually and differences are to be expected in the presentation in children and adults. Skin lesions due to their accessibility often lead to diagnosis of TSC. Hypopigmented macules, which fluoresce under Wood's (UV) light are the earliest signs of TSC. They are probably present at birth in most cases. They vary in shape and are only occasionally in the form of mountain ash leaf as described by Fitzpatrick et al. (1968). With the passage of time pigmentation does occur within the macules (Baraitser, 1990).

Facial angiofibromas (previously misnamed as adenoma sebaceum) are dome-shaped papules symmetrically distributed on the nasolabial folds, cheeks and chin, but with spare of the upper lip and philtrum. Only 50% of patients have this sign (Gomez, 1991). This hamartoma, rarely found before 3 years of age, is usually present by 5 years and rarely appears after puberty. Two other hamartomas, the periungual fibromata and shagreen patches (flat or slightly elevated, flesh-coloured and wrinkled, like pigskin), do not develop until the second decade in approximately 20% and 40% of patients respectively, and both do not necessarily occur in the same patient. The ungual fibroma is pathognomonic, but the shagreen patch is not (Gomez, 1991). Pigmented nevi and sublingual fibromata are other cutaneous manifestations seen in TSC patients. If, by puberty, no skin lesions have appeared they are unlikely to develop in adolescence or adult life (Baraitser, 1990). Fibrous forehead, eyelid, cheek, or scalp plaque, can be found at a younger age than can the facial angiofibroma, and unlike the facial angiofibroma, it is often seen at birth. The large ones on the scalp tend to calcify after many years (Gomez, 1991).

Epilepsy (usually beginning with infantile spasms) occurs in about 80% and mental retardation occurs in 60-70% of TSC affected individuals. It is a useful rule of thumb that seizures might occur without mental retardation, but the diagnosis is unlikely in those with mental retardation without seizures (Baraitser, 1990). Mental retardation is of variable severity and may be profound and its pathogenesis is obscure. Although, Gomez (1979) suggested that there is a relationship between the severity of the seizures and mental retardation this might not be so simple. Central

nervous system lesions are of two types (Baraitser, 1990), pale, hard gliotic areas in the convolutions known as tubers, and multiple tumour-like nodules which have a predilection for the subependymal region (giant cell astrocytoma) and project into the ventricles giving the radiological picture of candle guttering. The subependymal nodules usually calcify and are demonstrable by CAT scan in the brains of some 80% of affected individuals (Houser and Nixon, 1988).

Retinal phakomatoses are present in about half of the gene carriers at birth (Baraitser, 1990). They are glial cell hamartomas which may be flat or nodular. The nodular variety frequently calcify but significant visual impairment is unusual (Sampson, 1990).

Kidney lesions are in the form of angiomyolipoma (Anderson and Tannen, 1969). Indeed, Van Baal et al. (1989) found renal angiomyolipomas in 23 of 38 patients with proven tuberous sclerosis. Renal cysts are less commonly seen (15% of the cases) than renal angiomyolipomas (Gomez, 1991). Multiplicity and bilateral localisation were important differences between the TSC cases and the isolated, usually solitary, cases. Renal lesions are more common in females and usually asymptomatic but cystic disease, when florid, can lead to hypertension or chronic renal failure (Sampson, 1990).

Single or multiple (usually) rhabdomyomas occur in 30% of TSC patients coming to post-mortem (Baraitser, 1990). The lesions seem to regress by age and serious morbidity or mortality due to their presence is unusual after early childhood (Sampson, 1990). Most patients seem to be asymptomatic but it may lead to mechanical and conductive problems in infancy.

Pulmonary involvement is infrequent and almost exclusively confined to women with TSC in the third or fourth decade of life (Gomez, 1991). Angiolipomas of the liver and spleen which can be detected by ultrasound or CAT scan have been reported (Sampson, 1990).

1.5.2 Genetics

A sufficient number of multigenerational families have been reported to make it clear that TSC is an autosomal dominant condition (Bundey and Evans, 1969; Gomez, 1991). Sixty to 90% of cases are isolated and many of these are likely to be new mutants. The mutation rate of TSC is estimated to be 2.5×10^{-5} mutation per gene per generation (Hunt and Lindenbaum, 1984; Sampson et al., 1989a).

1.5.2.1 Phenotypic variability

TSC is notable for its phenotypic variability, which can range from clinical normality to dysfunction of multiple organs. Moreover, there is great variability within families, and many examples where a single lesion is the only manifestation of the disease. In addition, there have been reports of clinically normal parents who have had normal CAT scans, but who have two affected children (Wilson and Carter, 1978; Connor et al., 1986). The probable explanation for those parents who have one sign only, or who have no signs but two affected children, is that they have mosaicism of their germ cells, with or without mosaicism of somatic cells (Hall and Byers, 1987). Webb and Osborne (1991) reported an instance of apparent non-penetrance in two successive generations, between a great-grandfather and his great-grandson. The great- grandfather developed a single fleshy ungual fibroma on 1 little toe as the only clinical sign; on echocardiography, he showed 2 probable rhabdomyomata in the right ventricular wall and right ventricular outflow tract. His daughter had no discernible features of the disorder.

1.5.2.2 Linkage studies and locus heterogeneity

Linkage studies have demonstrated locus heterogeneity for TSC. In 1987 a TSC locus (now termed *TSC1*) was assigned to 9q34 (Fryer et al., 1987). This finding had been confirmed by different groups of investigators (Sampson et al., 1989b;

Haines et al., 1991a&b; Janssen et al., 1991; Northrup et al., 1992). The uncertain level of locus heterogeneity made it extremely difficult to exclude the possibility of subgroups of families linked to particular chromosome regions. Clark et al. (1989) described a t(11q23.3;22q11.1) unbalanced translocation in liveborn infant with TSC. A study indicating linkage between TSC and markers on distal 11q seemed to support an 11q locus (Smith et al., 1990). Subsequently more extensive studies under different models of heterogeneity proved inconclusive, leaving the provisional assignment in doubt (Kandt et al., 1992; Short et al., 1992). Fahsold et al. (1991) reported the association of TSC with a balanced translocation t(3;12)(p26.3;q23.3) followed by a report of linkage between TSC and PAH gene locus on 12q. Short et al. (1992) found little or no evidence for a TSC locus on 11q, 12q, or 14q. Sampson et al. (1992) collated data on 1,622 members of 128 tuberous sclerosis families. They estimated that the locus on 9q34 accounts for approximately 50% of families and concluded that there was no evidence of major loci on 11q or 12q. Meanwhile, indisputable evidence for linkage between TSC and marker at 16p13.3 was established by investigation of five large families in which TSC was clearly unlinked to chromosome 9 (Kandt et al., 1992). Confirmation of a tuberous sclerosis locus on chromosome 16 was provided by Pericak-Vance et al. (1992), Short et al. (1992) and Smith et al. (1992). With the growing consensus that there is no form of TSC encoded by either chromosome 11 or chromosome 12, it seemed desirable to refer to the chromosome 16 form of tuberous sclerosis as TSC2. It was estimated that linked TSC families are evenly divided between these two loci (Kwiatkowski et al., 1993).

1.5.2.3 TSC genes as tumour suppressor genes

TSC is an autosomal dominant condition characterised by tumour-like malformations of different organs and tissues. Another autosomal dominant condition with some resemblance to TSC is neurofibromatosis type 1 (*NF1*) characterised by benign tumours of peripheral nerves, pigmented skin lesions and retinal hamartomas. The

gene for NF1 has been cloned and a somatic deletion of the normal allele has been described in neurofibrosarcoma from a familial case of *NF1* (Legius et al., 1993).This gave rise to the suggestion that NF1 gene acts as a tumour suppressor (Seizinger, 1993) which fits with the Knudson's two-hit hypothesis (Knudson, 1971). There has been speculation that the lesions in TSC might arise in a similar way (Comings, 1980). Loss of heterozygosity (loss of alleles at constitutionally heterozygous loci near the genes; LOH) at *TSC1* and *TSC2*-associated markers has now been demonstrated in hamartomatous lesions of some TSC patients (Green et al., 1994a&b; Carbonara et al., 1994). The pattern of LOH at 16p13.3 (*TSC2* locus) is only 30 kb from the cloned *TSC2* gene (The European chromosome 16 Tuberous Sclerosis Consortium). On 9q34 (*TSC1* locus), LOH is consistent with the map position of *TSC1* defined by haplotype analysis in TSC families and the segregation analysis showed that the 9q34 haplotype lost carried the putative normal *TSC1* gene product in one family (Kwiatkowski et al., 1993; Carbonara et al., 1994). These data, together with the discovery of germline deletions in the *TSC2* gene in 5% of TSC patients, support the hypothesis that *TSC1* and *TSC2* genes act as growth suppressor genes, analogous to the traditional tumour suppressor genes. Due to the well known benign, slow growth of the hamartomatous cells and because TSC is not considered as cancer-prone disease, the definition of *TSC1* and *TSC2* genes as anti-oncogenes seems inappropriate, and terms such as stem cell growth suppressors should better represent their putative function (Carbonara et al., 1994). Moreover, in the same astrocytoma (Carbonara et al., 1994) an additional region of LOH is present at 9p21. The possibility of the presence of another gene controlling tumour progression, differentiation and/or stem cell proliferation in that region needs further investigations.

1.5.2.4 Positional cloning and characterisation of the *TSC2* gene

(1)positional Cloning : Linkage studies have established locus heterogeneity with disease-determining loci on chromosomes 9q34 and 16p13.3, leading to apparently indistinguishable phenotypes. The Genome Data Base Nomenclature Committee agreed that the loci on chromosomes 9 and 16 should be termed *TSC1* and *TSC2*, respectively. It was considered that as the *TSC* genes probably act as cell growth suppressors, and if a two-hit mechanism, as proposed by Knudson (1971), does apply to TSC, then constitutional deletions might be associated with the TSC phenotype in a proportion of cases, the same as that seen in the case of NF1 gene (Viskochil et al., 1990). TSC has not been noted in individuals with the chromosome 16 α-thalassaemia/mental retardation syndrome (ATR-16), who have terminal deletions of 16p which extended into the *TSC2* area. This suggested that *TSC2* was localised to the proximal 300 kb of the candidate region. Using pulsed field gel electrophoresis (PFGE), The European Chromosome 16 Tuberous Sclerosis Consortium (1993) identified five tuberous sclerosis-associated deletions at 16p13.3. These were mapped to a 120-kb region that was cloned in cosmids and from which 4 genes were isolated. One gene, designated *TSC2*, was interrupted by all 5 PFGE deletions, and closer examination, using *TSC2* cDNA subclones as hybridisation probes, revealed several intragenic mutations, including one de novo deletion. In this case, Northern blot analysis identified a shortened transcript, while reduced expression was observed in another TSC family, confirming the *TSC2* as the chromosome 16 *TSC* gene.

(2) Characterisation of *TSC2* gene : A zoo blot containing genomic DNA from various animal species revealed that the *TSC2* gene was conserved throughout the higher vertebrates. *TSC2* is widely expressed and both fibroblasts and lymphoblastoid cell lines represent good sources of RNA. The *TSC2* transcript is composed of ~ 5.5 kb covering a genomic region of ~ 43 kb. The cDNA contains an open reading frame extending from nucleotide 1 to 5370. The inframe AUG start

codon was found at nucleotide position 19. At the 3' end are two potentially overlapping polyadenylation signals (AATAAA-TAAA) at nucleotide 5425. The occurrence of this doublet may cause differential polyadenylation, since there have been detected polyadenylation sites that differ by up to 15 bp in four different cDNA clones. In one family in which TSC has been shown to co-segregate with chromosome 16p13.3 markers, but in which the deletion in *TSC2* locus has not been identified, the affected members showed clearly reduced levels of *TSC2* transcripts (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993).

Maheshwar et al. (1996) characterised the exon/intron boundaries of the *TSC2* gene. It has been found that the gene comprises 41 exons, including the alternatively spliced exon 31 which was absent from the originally described human *TSC2* transcript. This exon has been found in one human foetal brain cDNA clone but was absent in RNA extracted from human lymphoblastoid cell line. Comparative analysis of the *TSC2* gene in human and pufferfish (*Fugu rubripes*) showed 65% sequence homology at the nucleotide level.

(3)Tuberin, the protein product of the *TSC2* gene : The total length of the predicted protein is 1784 (plus 23 from the alternatively spliced exon 31) amino acids, with a calculated molecular mass of ~198 kd. Four potential transmembrane domains and four potential glycosylation sites were observed downstream of the last putative transmembrane domain. No sequence at the amino terminus of the predicted protein matched the signal peptide structure as defined by von Heijne (1985). However, the occurrence of several transmembrane domains without an apparent signal peptide was noted in cystic fibrosis-related protein (Riodran et al., 1989). A periodic array of leucine residues (the leucine zipper), a structure associated with protein-protein interaction was also observed. Of considerable interest, but undetermined functional significance, is a small region of homology to the GTPase activating protein rap1GAP (GAP3), which suggests that tuberin may itself have GAP activity, consistent with its proposed function as a tumour or a stem cell growth suppressor (Green et al., 1994a; Carbonara et al., 1994). After optimal alignment the protein product of the *TSC2*

gene in Fugu and humans revealed 60% identity of amino acid residues, with 79% similarity if conservative changes were included (Maheshwar et al., 1996). Four regions of high conservation were identified. These include the GAP-related domain (human residues 1593-1631) and the sequences flanking this, two small regions between residues 750 and 1100, and the N-terminal portion of the molecule. Using anti-sera against the N-terminal and the C-terminal portions of the tuberin, Wienecke et al. (1995) specifically recognised a 180 kd protein. A wide variety of human cell lines express the 180 kd tuberin protein, and subcellular fraction revealed that most tuberin is found in a membrane/particulate fraction. Small differences in migration rate of tuberin were observed in some cell lines. This might reflect post-translational modification or alternative splicing. Immunoprecipitates of native tuberin contain an activity that specifically stimulates the intrinsic GTPase activity of Rap1a. Tuberin does not stimulate GTPase activity of Rap2, Ha-Ras, Rac, or Rho. These results suggest that the loss of tuberin leads to constitutive activation of Rap1 in tumours of patients with tuberous sclerosis (Figure 1.2).

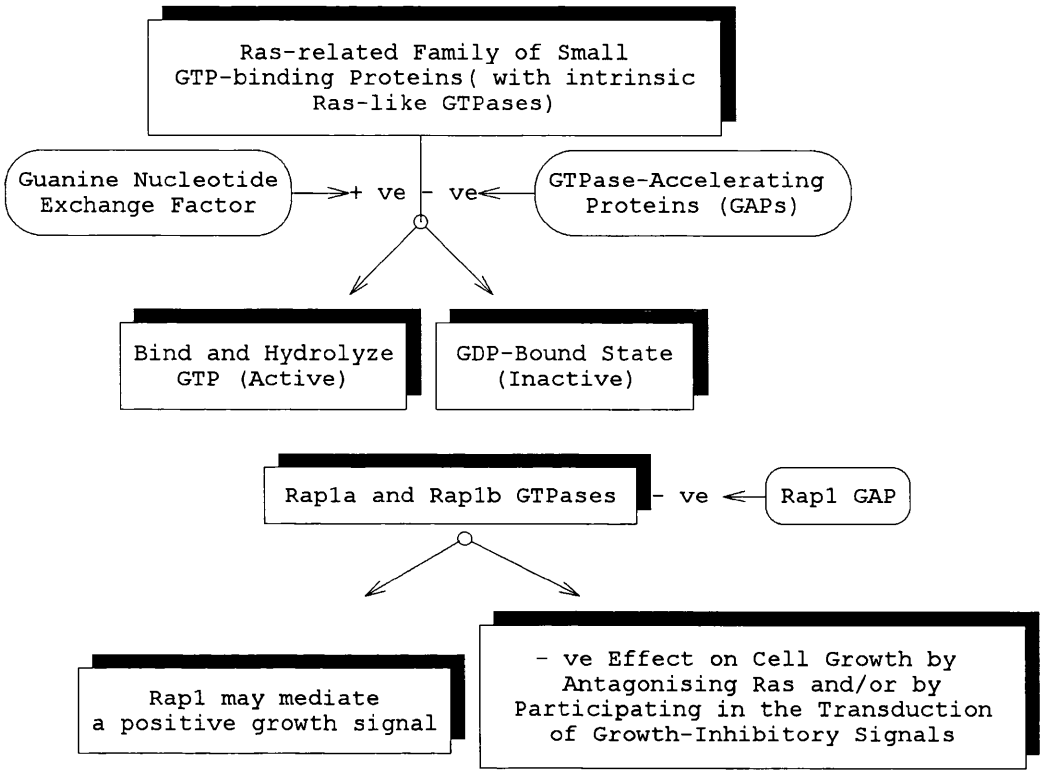


Figure 1.2 (Top) Ras-related family of small GTP-binding proteins bind and hydrolyse GTP. They are active when bound to GTP and inactive in the GDP-bound state. Cellular regulatory proteins regulate guanine nucleotide binding Ras-related GTPases. GAP proteins act by stimulating the intrinsic GTPase of the GTP-binding proteins, keeping them in the inactive, GDP-bound state.

(Bottom) Rap1a and Rap1b GTPases were suggested to have negative effect on cell growth either by antagonising Ras through competitive binding of Rap1 to Ras effector molecules or through transduction of growth-inhibitory signals. In accordance with this scenario, tuberin might function as an effector protein (as well as a GAP) for Rap1, and loss of tuberin expression would prevent transmission of the growth-inhibitory signals originating from Rap1. Alternatively, there is evidence that Rap1, like Ras, may mediate a positive growth signal and because tuberin is suggested to have a Rap1-GAP activity it may bind to the Rap1 preventing it from interacting with its mitogenic effectors. Loss of tuberin expression in cells would free up Rap1, thereby allowing it to transmit a positive signal through its effectors.

1.6 Methods for mutation detection

Detection of mutations and polymorphisms in DNA is an important feature of the investigation of gene structure and function. Furthermore, easy identification of specific sequences and sequence changes plays a central role in the diagnosis of human inherited diseases. Procedures for mutation detection can be separated into two distinct groups. The first consists of techniques which efficiently identify known disease alleles, e.g. population screening for carriers of the common cystic fibrosis mutations. The second group consists of methods to scan sequences for unknown mutations.

1.6.1 Methods to detect known sequence alterations

Once mutations or polymorphisms have been described they can be searched for using one of the following conditions:-

(1) Allele-specific oligonucleotide (ASO)

This is a hybridisation based method where sequence variants are distinguished by taking advantage of the difference in stability of hybrids formed between target sequence (usually amplified by PCR) and oligonucleotide probes that are perfectly matched or mismatched to the target sequence (Wallace et al., 1979). The original method involved probing separated bands which had been transferred to a membrane, but more recently the sample has been directly transferred to the membrane (the dot blot). Short oligonucleotide probes of about 19 nucleotides corresponding to a particular region of a gene are prepared. Two probes are made; one has the normal sequence, while the other is identical except for a single altered base corresponding to particular mutation. By carefully regulating hybridisation conditions, it is possible to arrange things so that the normal probe hybridises to the normal but not to the mutant DNA, while the mutant probe hybridises to the mutant but not to the normal DNA. Both radiolabelled and fluorescently labelled probes have

been used. In the reverse blot (Saiki et al., 1989) different probes were spotted onto the membrane and the biotin-labelled target sample applied. The probes forming duplexes without mismatches and retaining the target became stained with HRP avidin. The main advantage of the current method is that it does not require electrophoresis and the method is non-radioactive. The main disadvantage is that two primers have to be synthesised for amplification and two more, mutant and wild-type, for mutation detection.

(2) Amplification refractory mutation system (ARMS)

The technique was first described by Newton et al. (1989). It is based on the concept that PCR primers with the 3' end complementary either to a mutant or a normal nucleotide sequence can be used to selectively amplify one or the other allele. This is due to the lack of 3' exonucleolytic proof reading activity of *Taq* DNA polymerase. Two target DNA samples, from each patient, were PCR amplified with a wild-type or mutant primer and a common primer. The mutant and the wild-type primers differ at their 3' ends by a single base which corresponds to the wild-type and mutant alleles. As a control, internal primers are added in the same reaction. Upon gel electrophoresis of the two target samples the presence of a band defines the presence of the corresponding allele. The main advantage of this method is that it requires only a single PCR reaction and a result is obtained in a matter of hours. Also, the technique can be automated for screening of large number of samples. This technique is very useful in screening for a common mutation in a gene such as ΔF508, the common cystic fibrosis mutation. PCR amplification of multiple specific alleles (PAMSA) has been shown to be possible by the use of primers that generate PCR products of different lengths. Mistry et al. (1992) have been used PAMSA technique to screen for 6 Gaucher's alleles in 12 patients.

(3) Artificial introduction of restriction sites (AIRS)

When a mutation changes a restriction site, it can readily be sought in a genome. Unfortunately, most mutations do not change a restriction site. Cohen and Levinson (1988) developed a strategy to introduce a restriction site by mismatched primers in the region of a mutation for either the wild type or mutant sequence. In this method an artificial restriction site is introduced, by a primer that has one base mismatch near to the site of mutation. Presence of the mutation will make the proper recognition site for a specific restriction endonuclease. Digestion of the PCR products using this enzyme followed by gel electrophoresis will enable the identification of the absence or presence of the mutation in the sample. This method has been successfully used by researchers (e.g. Ng et al., 1991; Taroni et al., 1993; and Grau and Griffais, 1994) to screen for common mutations in different genes.

1.6.2 Methods to scan sequences for unknown mutations

While several useful technologies for the detection of sequence heterogeneity exist, no single method is applicable for all situations. The most appropriate screening technology is influenced by the expected nature of the mutation, size and structure of the gene in question, degree of sensitivity required and resources available. The spectrum of mutations ranges from cytogenetically visible chromosome re-arrangements to micro-deletions and insertions and finally single base alterations.

1.6.2.1 Detection of large gene alterations

Large gene alterations are mutations in which substantial portions (>500 bp) of the gene are deleted, duplicated or otherwise rearranged (Grompe, 1993). Techniques used for this kind of mutation detection includes cytogenetic techniques, Southern blotting, pulsed field gel electrophoresis (PFGE) and multiplex PCR.

(1) Cytogenetic techniques

Extremely large (> 4 megabases (Mb)) deletions and insertions can be detected by high resolution cytogenetics. The use of fluorescent ***in situ*** hybridisation (FISH) has improved the power of cytogenetic analysis. FISH uses fluorescently labelled DNA probes, which are hybridised to chromosome spreads, to detect both numerical and structural chromosome aberrations not only in metaphase, but also in interphase nucleus. FISH is particularly suited for the detection of aneuploidy, microdeletions or duplications and complex rearrangements (Lichter and Cremer, 1992).

(2) Southern blot hybridisation

The Southern blotting technique, developed in 1975 by E. Southern, is the standard way of analysing the structure of DNA cleaved by restriction enzymes and agarose gel electrophoresed. It remains one of the fastest methods to quickly screen for mutations. No detailed knowledge of the structure and sequence of a gene is required and a preliminary screen can be carried out with a probe of interest immediately after its isolation. Large deletions and insertions may be detected by the presence of junction fragments or changes in band intensities (in case of autosomal dominant conditions and mutation carriers). Point mutations may be also detected, if they alter restriction sites. By using appropriate restriction enzymes and probes Southern blotting has been used successfully to detect trinucleotide repeat expansion in patients with FRAXA and myotonic dystrophy where a massive expansion of the repeats occurs in patients with full blown clinical presentation (Fu et al., 1991 and Harley et al., 1992).

(3) Pulsed field gel electrophoresis (PFGE)

The gels that have been used for traditional southern blotting can only separate DNA fragments of about 20 kb or less. Molecules of this size have linear dimensions comparable to the pore size of the gel. On the other hand very large

molecules, of 100 to 500 kb for example, are much larger or longer than the pores. However, even they can move into a gel by finding a path that involves many pores simultaneously. In this type of migration the forward force is proportional to the electric charge of the molecule, that is its length, and the drag due to friction is proportional to the number of pores through which the large molecule is passing, again being related to its length. The overall effect is that large molecules of different sizes move at the same rate and are not separated (Weatherall, 1991).

The principle of PFGE is periodically to change the orientation of the electric field. Every time this happens the large extended molecules must re-orientate and find a path through the gel matrix in other direction. This process is size dependent, that is very large molecules will take more time to re-orientate than shorter ones. This allows the resolution of DNA fragments of 100 to 1000 Kb in size and, in some cases, even larger. The large fragments for PFGE are generated by using restriction enzymes which have infrequent cutting sites such as *Not*I and *Sfi*I. The resultant gels can be Southern blotted and probed by standard methods. This technique allows the identification of major deletions and major chromosomal rearrangements in a single test (Weatherall, 1991).

(4) Multiplex PCR for the detection of deletions

If a locus of interest is prone to deletions and if its genomic sequences are known, the simultaneous PCR amplification of several sequences throughout the gene is the most rapid and practical method for their detection. Deletions are indicated by the absence of some of the bands in the multiplex pattern in homozygotes. In heterozygotes, in spite of being technically difficult, deletions are seen as 50% reduction of band intensities in a quantitative analysis of the multiplex PCR reaction (Abbs and Bobrow, 1992). The introduction of dye labelled primers and automated computer analysis of the multiplex PCR products facilitates this technique. This method has been used widely in screening for deletions in Duchenne muscular dystrophy, in which 60% of cases represent deletions (Grompe, 1993).

1.6.2.2 Detection of single base changes and small sequence alterations

Single base substitution and small sequence alterations are the most common type of mutation at most loci. A number of methods may be used for detecting these subtle changes. All the methods used are polymerase chain reaction (PCR) based detection methods using either genomic DNA and/or mRNA as a starting material for mutation analysis (Table 1.1). PCR is a technique for the in vitro amplification of specific DNA or RNA sequences. The PCR method was devised and named by Mullis and Faloona (1987) at the Cetus Corporation, although the principle has been described in details by Khorana and colleagues over a decade earlier (Taylor, 1993). In brief, PCR is based on the enzymatic amplification of a fragment of DNA that is flanked by two short oligonucleotides "primers" that hybridise to the opposite strands of the target sequence. A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. Twenty cycles of PCR yields about a million fold amplification of the specific DNA fragment.

Table 1.1 Genomic DNA versus mRNA as a starting material for mutation analysis (Grompe, 1993)

	Genomic DNA	mRNA
Advantages		
	Easily accessible (blood)	Long segment of peptide coding region can be analysed
	In autosomal loci both alleles are equally represented	Gene structure information not needed
	Mutations in the promoter and intronic splice junctions can be detected	Fewer PCR reactions
	First choice in autosomal dominant traits	Aberrant mRNA size can be seen
		First choice in X-linked traits
Disadvantages		
	Genomic sequence and gene structure information are needed	Gene may not be expressed in accessible specimens
	Only small segments of coding region (exons) are analysed	In autosomal loci only one allele may be represented
	More PCR reactions	Mutations in promoter and intronic splice junctions are not detected

Some important strategies for mutation detection of unknown single base changes and/or small sequence alterations are as follows :

(1) Single strand conformational polymorphism (SSCP) analysis

Of the various methods applied for the detection of unknown mutations SSCP is by far the easiest and cheapest procedure presently available. In 1989, Orita et al. first report the use of SSCP to detect mutations. Wild type and mutant target DNAs are amplified by PCR, denatured and then electrophoresed side by side through a non-denaturing polyacrylamide gel. The two single-stranded DNA molecules from each denatured PCR product assume a three-dimensional conformation which is dependent on their primary sequence. If a sequence difference (mutation) exists between wild-type and mutant DNA, this may result in differential migration of one or both of the mutant strands. PCR products with altered migration patterns can then be analysed by DNA sequencing to determine the exact nature of the alteration. In most published studies the amplification products are rendered radioactive by the addition of ^{32}P dCTP to the PCR reaction (Glavac and Dean, 1993). However, non-radioactive detection by ethidium bromide staining and silver staining (Grade et al., 1994) have been successfully used. No adequate theoretical model is available for predicting the three dimensional structure of single stranded DNA under a given set of conditions. Accordingly, the effects of mutations on DNA mobility in an SSCP gel cannot be reliably predicted.

Different data concerning the efficiency of SSCP have been reported. In general, SSCP analysis detects 70 to 95% of mutations in PCR product of 200 bp or less (Grompe, 1993). The sensitivity of the method decreases with the size of the PCR product and is less than 50% when fragments of > 400 bp are analysed. Some of the size limitations of the method may be overcome by restriction digestion of a larger amplification product prior to electrophoresis (Iwahana et al., 1992). The use of RNA generated by in vitro transcription of PCR products also appears to improve detection of mutations in larger fragments (Sarkar et al., 1992a). This modification of SSCP

requires longer primer (to include phage RNA promoters) and extra experimental steps, so it may be less desirable for general mutation detection. There are many parameters other than the size of the PCR product that can affect the informativeness of SSCP method. Physical factors such as temperature, type of the gel matrix and ionic strength is very important such that sequence changes that have little or no effect on conformation in one set of conditions can have a dramatically different effect under other conditions (Hayashi and Yandell, 1993). A useful consequence of this is that it enables empirical optimisation of SSCP sensitivity for a given fragment by varying these conditions.

Based on the experience of a number of laboratories, electrophoresis at room temperature (20-25^{o}C) with 5-10% glycerol or 4^{o}C without glycerol seems to be a good starting point for detection of most mutations (Hayashi, 1991).

Choice of the gel matrix is a second important area for optimisation. Complementary single strands are separated more effectively using a polyacrylamide matrix containing a low percentage of crosslinker (%C) (the percentage of N,N'-methylenebisacrylamide in the total acrylamide monomer). Most investigators have found that SSCP gels with 2% C or less are best for detection of single nucleotide substitution (Hayashi and Yandell, 1993). New gel products (e.g. MDE^{TM} gel, AT Biochem.) are now available and seems to be more appropriate for SSCP analysis.

Overloading of the gel sometimes results in abnormal migration of the bands and reduced resolution. For this reason, radioisotopic or fluorescent labelling of the PCR products to a high specific activity is highly recommended. Samples can then be diluted so that DNA concentrations are sufficiently low in the loading solution, yet detectable either by autoradiography in a reasonable exposure time (few hours to overnight) or with fluorescent DNA sequencer (Hayashi and Yandell, 1993).

The way the bands are shifted by mutation can be different depending on the flanking sequences, and changing primer positions bracketing the region suspected to have a mutation is one effective way of enhancing mobility shift.

Sarkar et al. (1992b) have proposed a dideoxy-termination reaction using PCR-amplified fragments followed by SSCP analysis. In this method, DNA fragments having various sequences flanking the mutated site can be examined in a single gel. This approach, however, involves extra steps of experiments and may be not suitable for general mutation detection.

(2) Heteroduplex analysis

This technique is based on the differing electrophoretic mobility between a perfectly matched duplex DNA fragment compared with a similar DNA fragment that contains a mismatch (a heteroduplex) when analysed side by side. Heteroduplexes formed of one mutant strand and one wild type strand migrate at a different rate through regular polyacrylamide gels, because the region of mismatch forms a "kink" in the DNA. Thus, a heteroduplex frequently appears on the gel as a distinct band, separate from the homoduplex DNA (Nagamine et al., 1989). New gel matrices (Hydrolink and MDE™ from AT Biochem.) have become available which markedly enhance the ability to detect mutation induced mobility shifts in heteroduplex molecules (Grompe, 1993). This simple technique was reported to be able to identify different types point mutations and minor deletions or insertions in PCR fragments of different size. Studies indicate a level of sensitivity similar to SSCP analysis (80-90%) in small DNA fragments (<300bp) (Perry and Carrell, 1992; White et al., 1992). This technique is simple to perform, does not require complex chemical or temperature gradients, bands can generally be detected without radioactive labels and has been applied successfully to the study of a number of human genetic disorders (Grompe, 1993; Friedle et al., 1993 and Schreiber et al., 1995).

(3) Denaturing gradient gel electrophoresis (DGGE)

The denaturing gradient gel electrophoresis is acknowledged to detect almost 100% of mutations (Dianzani et al., 1993). The mutation-resolving power of DGGE relies on a physical separation between similar DNA fragments differing in melting

properties due to difference in nucleotide composition. The separation is based on the fact that DNA molecules differing by a single base change have slightly different melting properties, which cause them to migrate differently in a polyacrylamide gel containing a linear gradient of DNA denaturants (urea and formamide) (Fischer and Lerman, 1983 and Myers et al., 1987). There are two types of denaturant gradient gels : **(I) Parallel gels**, which contain a linearly increasing gradient of DNA denaturants from top to bottom in the gel and **(II) perpendicular gels**, which contain a linear gradient of denaturants from left to right across the gel. Both gel types can be used independently to screen for point mutation in PCR-amplified DNA products. Parallel denaturing gradient gels have the advantage of being useful for analysis of multiple samples in a single gel (Sheffield et al., 1990). Two modifications greatly increased the sensitivity of the technique : (1) Attachment of a thermostable GC-clamp to one PCR primer (Sheffield et al., 1989) and (2) Analysis of heteroduplex molecule i.e. hybrids formed between mutant and wild-type strands (Myers et al., 1985a). Mutations can be found most reliably when sequence heterogeneity lies within a domain of relatively low melting temperature. This can be achieved for virtually any sequence of interest by the use of computer programs to predict theoretical melting profiles and design PCR primers (Grompe, 1993). Base changes in the region of the highest melting temperature normally can not be resolved since melting of this domain leads to the generation of single strands with loss of the sequence-dependant mobility. Introduction of a GC-clamp at one end of the sequence, leads to an improved sensitivity of about 90% of all DNA polymorphisms. As in SSCP analysis, DGGE detects the presence of sequence difference between mutant and wild type DNA fragments but not its location within the fragment, which has to be determined by sequencing.

The denaturing gradient can also be generated by temperature. **Temperature gradient gel electrophoresis (TGGE)** allows the separation of molecules depending on their different melting behaviour in temperature gradients and has been successfully applied to separation of HLA alleles (Meyer et al., 1991) and screening

of Tp53 mutations (Scholz et al., 1993). Like DGGE, after parallel TGGE wild-type conformation is characterised by one single band, whereas mutations are demonstrated usually by the presence of four bands corresponding to the four different double strands (one homoduplex wild-type, one homoduplex mutant and two heteroduplex mutant/wild-type). Using thermal instead of a chemical denaturant gradient, the preparation of gradient gels is avoided and only one gel condition is sufficient for TGGE (Scholz et al., 1993).

Both DGGE and TGGE require a special apparatus to control gel temperature and long PCR primers which include a 30 to 50 bp high melting temperature GC-clamp (Grompe, 1993).

(5) Cleavage of mismatch analysis

The principle of mismatch cleavage is based upon the cleavage of heteroduplexes between strands of nucleic acid mismatched at one or more nucleotides. Three different techniques adopting this strategy are now known, chemical cleavage of mismatch (CCM), RNaseA cleavage and enzyme mismatch cleavage (EMC).

(A) Chemical cleavage of mismatch (CCM) : This technique has been described by Cotton et al. (1988). In CCM, a heteroduplex between a radiolabelled wild-type DNA molecule and mutant DNA (or RNA) is created by boiling and reannealing. Hydroxylamine and osmium tetroxide react to modify mismatched or unmatched cytosine or thymine residues, respectively. Sites modified by hydroxylamine and osmium tetroxide are more susceptible to cleavage by piperdine than unmodified base pairs. Cleavage occurs in one strand of the helix only. Products are resolved by denaturing polyacrylamide gel electrophoresis to allow the identification and location of the mutation sites. CCM is very sensitive, detecting more than 95% of mismatches when only the wild type DNA are labelled and 100%, when both wild-type and mutant DNA are labelled (Grompe, 1993). It was shown to

be an excellent method for the detection and location of mutations, particularly because it can easily scan up to 1.7 kb segments of DNA at a time, DNA or RNA templates may be used and the precise location and nature of the change is also indicated by the size of the cleavage band and the cleaving reagent (Grompe, 1993). Improved resolution of larger cleavage products is possible by using of ^{35}S-labelled dATP probes rather than ^{32}PdCTP-labelled probes. ^{35}S labelled cleavage products are more discrete and therefore more easily identified than those obtained with ^{32}P (Saleeba and Cotton, 1991). Saleeba et al. (1992) described a method for unlabelled CCM which detect the cleavage products by silver staining. This procedure is thought best applied to heteroduplexes < 600 bp long (Cotton, 1993)

The main drawbacks of the technique is that many manipulations need to be performed in the fumehood to reduce exposure to toxic chemicals, the use of radioactive probes and the need for a two-step reaction and treatment with three reagents.

(B) RNase A cleavage : This was first described by Myers et al. (1985b). The method is based on the observation that RNase A is frequently able to cleave a mismatched base in an RNA probe hybridised to an experimental target containing a point mutation. As originally described, the substrates for RNase A digestion were RNA/DNA duplexes made by hybridising a radiolabelled wild-type RNA probe to a double-stranded DNA target. The enzyme will recognise and cleave single-stranded RNA at the points of mismatch. The reaction is analysed by denaturing polyacrylamide gel electrophoresis and autoradiography. RNase A can only detect ~50% of mismatches (Grompe, 1993). It has therefore been largely replaced by the similar CCM technique. The recent non-radioactive version developed by Ambion laboratories may improve the popularity of the technique. Two different transcription promoters are used to produce both sense and anti-sense strands of an RNA duplex which gives each mutation two chances of being detected but the detection rate is still to be determined.

(C) Enzyme mismatch cleavage (EMC) : Mashal et al. (1995) and Youil et al. (1995) have described a method that may prove superior to existing techniques in the search for an elusive mutation. Bacteriophage resolvases, whose function ***in vivo*** is to cleave branched DNA, have the property of recognising mismatched bases in double stranded DNA and cutting the DNA at the site of mismatch. Radiolabelled DNA is cleaved by the resolvases at the site of mismatch in heteroduplex DNA and digestion products are resolved on a denaturing polyacrylamide gel. Thus, both the presence and the estimated position of an alteration is revealed. EMC has the potential to be as easy and inexpensive as SSCP and as sensitive as DGGE, with the added advantages of predicting the precise position of an alteration and of being applicable to fragments 1 kilobase or larger. Both research groups (Mashal et al., 1995 and Youil et al., 1995) analysed all possible mismatch types as well as several deletion mutations and obtained very comparable results with a 94% detection sensitivity. However, several improvements need to be made before EMC replaces existing methods.

(6) Protein truncation test (PTT)

Having made the effort to identify the mutation, the question often still remains :"Does this mutation actually cause disease?". This is where the functional assays come into their own right and for some genes they are now considered as the first mutation screening test to be performed. The PTT identifies mutations that result in premature termination of protein synthesis. This method has been reported by Roest et al. (1993) and the technique is based on the combination of RT-PCR, in vitro transcription and translation with incorporation of ^{3}H-leucine to detect translation products after SDS-polyacrylamide gel electrophoresis. This technique is mainly applied to the disorders where protein termination mutations account for a significant proportion of mutations. For example the dystrophin gene, four genes for hereditary non-polyposis colon cancer, the breast cancer gene (*BRCA1*) and the gene for familial colorectal cancer ((*APC*), in which translation terminating mutations

account for about 70% of the mutation identified (Miyoshi et al., 1992)). Compared to other point-mutation detection techniques, PTT allows the analysis of relatively large stretches of coding sequences of 2.4 kb or even more. The site of the mutation is identified so that only a small part of the gene needs to be sequenced.

(7) Direct sequencing

Sequencing can be used either as a screening and/or a diagnostic method. Sequencing defines precisely the location and nature of the change and therefore is a necessary final step of any mutation detection method. Two methods are used. One uses end labelled probe and partial chemical cleavage is achieved at all bases with particular chemicals. Size of fragments and the chemicals which produced them can then be used to define the sequence (Maxam and Gilbert, 1980). The other method uses enough nucleotide analogue (dideoxy) to allow some chain termination at each base, and again the base used and the length of the fragments defines the sequence (Sanger et al., 1977). The latter method is most frequently used.

In order to sequence PCR products successfully by the conventional dideoxy termination protocol, it is essential to convert the double-stranded PCR product into a single-stranded sequencing template. Several methods have been described to achieve this. In the first technique, termed asymmetric PCR (Gyllensten and Erlich, 1988) one primer is 1 : 50 to 1 : 100 diluted and used for a second round PCR. In the second method one biotinylated PCR primer is used to create a 5' biotinylated DNA strand which is then magnetically captured on an avidin-coated magnetic beads (Gibbs et al., 1990). In a third approach termed genomic amplification with transcript sequencing, the original PCR primers carry T7 RNA polymerase binding sites and ***in vitro*** transcription is then used to generate single-stranded RNA template for sequencing (Stoflet et al., 1988).

Cycle sequencing is a newly developed sequencing technique where the DNA template is simultaneously amplified and sequenced by the addition of dideoxy terminators to a PCR reaction. Recently, a new protocol based on cycle sequencing

and fluorescence detection technology has been developed (Rosenthal and Charnock, 1992). Fluorescently labelled dideoxy terminators are used with different fluorescent dye coupled to each of the four dideoxy nucleotide triphosphate (ddNTPs). A computer software analysis of fluorescent cycle sequencing data is used. This method appears especially useful when high throughput automated sequencing is available (Grompe, 1993).

1.7 Aims of the present project

The aims of the present project were to optimise and apply strategies for detection of mutations in three different autosomal dominant neurodegenerative conditions, myotonic dystrophy (DM), amyotrophic lateral sclerosis (ALS) and tuberous sclerosis complex (TSC). These diseases were chosen because of their differences with regard to known molecular pathology (CTG trinucleotide repeat expansion in DM patients, point mutations in ALS patients and point mutations and gene deletions in TSC patients), available gene information (only cDNA sequence of the *TSC2* gene was available) and/or the availability of study materials (RNA was available from TSC patients only). These constraints made it necessary to optimise different mutation detection strategies for each of them.

Specifically this involved:

(1) Optimisation of PCR and Southern blot analysis to screen for CTG repeat expansion in DM patients

(2) Optimisation of PCR , restriction digestion and SSCP analysis to screen for both known and unknown point mutations in the *SOD-1* gene in both familial and sporadic ALS patients.

(3) Optimisation of PCR, RT-PCR of the *TSC2* gene cDNA, Southern blot and chemical cleavage of the mismatch analysis to screen for mutations in the *TSC2* gene in patients with tuberous sclerosis complex.

(4) Characterisation of the detected point mutations with direct sequencing of cDNA and/or genomic DNA.

CHAPTER TWO

MATERIALS AND METHODS

(2) MATERIALS AND METHODS

2.1 Patients

Forty nine myotonic dystrophy (DM) families (86 DM patients and 96 apparently normal individuals) were studied for the status of the CTG repeat expansion in the 3' untranslated part of the *DM* locus on chromosome 19q13.3. All the families were ascertained by the clinical diagnosis of the proband; an expanded *DM* allele was identified afterward in each of these individuals.

Two patients from ALS families and 67 sporadic ALS cases were studied for the presence of mutations in the copper/zinc superoxide dismutase (*SOD-1*) gene on chromosome 21q2.2. All patients were diagnosed clinically by the presence of a progressive neurodegenerative disease of combined upper and lower motor neuron features and confirmed by doing some electrophysiologic studies of the muscle and nerve.

12 familial and 20 sporadic patients with tuberous sclerosis were studied for the presence of mutations in the *TSC2* gene on chromosome 16p13.3. The linkage status for all the familial cases, except one, are unknown. One familial case was found, later on, to be linked to chromosome 9 markers. All patients were diagnosed according to the revised Gomez criteria (Gomez, 1991).

2.2 DNA extraction

From all the patients studied, DNA was extracted from peripheral blood leukocytes using a modification of the method described by Kunkel et al. (1977).

In 50 ml Falcon centrifuge tubes, 40 ml of cold lysis buffer was added to each 10 ml of patient's blood and mixed gently. Tubes were then centrifuged for 15 minutes at $4^{o}C$ and 2500 rpm in an IEC DPR-6000 centrifuge. The resulting pellets were then resuspended in 3 ml nuclei lysis buffer, 200 µl 10% SDS and 100 µl proteinase K (10 mg/ml) and incubated at $37^{o}C$ for overnight. After incubation, 1 ml of 6M sodium

chloride was added to each tube and shaken vigorously for few seconds and then centrifuged for 15 minutes at 15°C and 2500 rpm. Carefully the supernatant was transferred, using a plastic pastette, to a fresh 5 ml Falcon tube and gently mixed, by inversion, for 1 minute, with 1 ml buffered phenol/chloroform followed by centrifugation at room temperature and 2500 rpm for 15 minutes. The upper aqueous phase was transferred to a 20 ml universal tube and the DNA was precipitated by addition of two volumes of absolute ethanol. The DNA was then spooled out using a sealed glass Pasteur pipette, washed in 70% ethanol, air dried and suspended in 500 µl autoclaved T.E. buffer and kept overnight at 4°C to insure its complete dissolution.

Determination of DNA concentration

To determine the concentration of the DNA samples, optical density (O.D.) reading at 260 nm, using dual beam spectrophotometer, was performed. An O.D. reading of 1 corresponds to 50 µg/ml of DNA.

2.3 Total cellular RNA extraction from peripheral blood lymphocytes

RNA was extracted from 22 patients with tuberous sclerosis using two different methods.

2.3.1 Acid-guanidinium thiocyanate method

A modification of Chomczynski and Sacchi (1987) acid-guanidinium thiocyanate method was used to extract RNA from peripheral blood lymphocytes. The technique involved initial separation of lymphocytes from whole blood, followed by the acid-guanidinium thiocyanate phenol/chloroform extraction. The RNA must be protected from degradation by ribonucleases. Therefore, all the solutions used (except organic solutions) were prepared with 0.1% diethyl pyrocarbonate (DEPC) in distilled water and then autoclaved. The tubes and tips required for handling and storing of the

RNA were treated in 0.1% DEPC in water for 14-16 hours before being autoclaved. All plastic-ware were kept aside from general use. Gloves were changed frequently during the steps of RNA extraction. Isolated RNAs were stored in aqueous solution at -70^{O}C and repeated freeze and thaw cycles were avoided.

(a) Separation of lymphocytes from whole blood: 5 ml of heparinised or EDTA treated, freshly collected, whole blood was carefully layered over 5 ml of Histopaque (Sigma) in a 20 ml universal tube. Tubes were centrifuged at room temperature and 1400 rpm for 30 minutes. The opaque (buffy) interface was transferred to a fresh tube and washed, twice, in 15 ml of cold phosphate buffered saline (PBS, Gibco BRL), then pelleted by centrifugation at 1400 rpm and 4^{O}C for 15 minutes and the supernatant was discarded.

(b) The acid guanidinium thiocyanate phenol/chloroform extraction: The pellet was re-suspended in 500 µl solution D (37 µl 2-mercaptoethanol, Sigma, was added to each 5 ml solution D just before use) by repeated slow pipetting and transferred to an autoclaved, DEPC treated, 1.5 ml Eppendorf tube on ice. To the dissolved pellet the following ice-cold solutions were added: 50 µl 2 M sodium acetate (pH 4), 500 µl water saturated phenol and 100 µl chloroform/isoamyl alcohol (98 : 2). Tube contents were mixed by inversion after each reagent addition and the final mix was shaken vigorously for 15 seconds and kept on ice for 15 minutes. The tubes were centrifuged for 20 minutes at 4^{O}C and 14000 rpm. The upper aqueous phase was carefully transferred to another fresh 1.5 ml Eppendorf tube and to it 600 µl of isopropanol was added and mixed by repeated inversion. Tubes were incubated at -20^{O}C for a minimum of one hour then centrifuged for 20 minutes at 4^{O}C and 14000 rpm. The supernatant was discarded and the pellet was dissolved in 300 µl solution D. To the dissolved pellet 300 µl isopropanol was added and the tubes were kept at - 20^{O}C for at least one hour. Tubes were then centrifuged for 20 minutes at 4^{O}C and 14000 rpm. The supernatant was discarded and the pellet was washed in 500 µl 75% cold ethanol (diluted by DEPC treated autoclaved distilled water) and air dried. The pellet was dissolved in 50 µl DEPC treated water and stored at - 70^{O}C.

2.3.2 TRIzol™ Reagent extraction

TRIzol™ reagent (Life Technologies) is a ready-to-use reagent for isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is used as a modification to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). Lymphocytes were separated as before and only once washed with cold PBS. The pellet was dissolved in one ml of TRIzol reagent by repetitive pipetting and then transferred to 1.5 ml DEPC treated Eppendorf tube and kept for 5 minutes at room temperature permit the complete dissociation of nucleoprotein complexes. After the incubation 0.2 ml of chloroform was added to the dissolved pellet and the tube was shaken vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were then centrifuged at no more than 12000 g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and to it 0.5 ml of isopropanol was added and mixed well. The sample was incubated at room temperature for 10 minutes and centrifuged at no more than 12000 g for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was once washed with 1 ml of 75% ethanol by brief vortexing and then centrifuged at no more than 7500 g for 5 minutes at 4°C. The pellet was then briefly air dried for 5-10 minutes and dissolved in 50 µl of DEPC treated water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 60°C. Dissolved RNA samples were then stored at -70°C.

Determination of RNA concentration

To determine the concentration of the RNA samples, an optical density (O.D.) reading at 260 nm, using a dual beam spectrophotometer, was taken. An O.D. reading of 1 corresponds to 40 µg/ml of the RNA. The integrity of the RNA was assessed by comparing the ratio of O.D. at 260/280 nm. A good quality preparation should give a value of 2.

Qualitative assessment of the RNA

To assess the quality of the extracted RNA 1 µl of it was checked by agarose gel electrophoresis. The RNA checking gel was prepared by boiling 0.45g agarose in 22 ml distilled water (dH_2O) until dissolved. The evaporated amount of water was restored and the gel was cooled to 55°C. Under the fume hood, 5 ml of 37% formaldehyde and 3 ml of 10X MOPS were added to the gel which was then rapidly poured and left to set. The RNA sample was prepared by mixing 1 µl total RNA with 1.3 µl dH_2O, 5 µl formamide, 1.7 µl 37% formaldehyde and 1 µl 10X MOPS. The sample was heated in a 60°C water bath for 10 minutes and rapidly quenched on ice. To this 2 µl of 10X RNA loading buffer was added and the sample immediately loaded alongside 1 µg of E. coli ribosomal RNA (Boehringer Mannheim) as a marker and electrophoresed in 1X MOPS buffer at 70 to 80 volts for 40 to 60 minutes (till the bromophenol dye reached the bottom of the gel). The gel was then rinsed in water to wash out the formaldehyde and stained for 5 minutes in 5 µg/ml solution of ethidium bromide. The gel was destained in water for 2 to 16 hours and viewed under the U.V. light to check for the integrity of ribosomal RNA bands.

2.4 Oligonucleotide design

Oligonucleotides were designed to amplify both DNA and cDNA sequences. Seven sets of primers were designed to amplify exon 3 of the *SOD-1* gene and exon 1, and exons 38-41 (as a single amplification product)of the *TSC2* gene. Four cDNA segments of the *TSC2* gene were also amplified. Moreover, six sequencing primers were designed. The computer program Oligo™ version 3.4 (Medprobe) was used for primer designing with the following guidelines: **1)** The length of the primer was in the range of 18 to 30 bases. **2)** PCR primers should be free of significant complimentarity at their 3' termini as this promotes the formation of primer-dimer artifacts that reduce product yield. **3)** Avoidance of hairpin loop forming primers (self-complementarity). A hairpin loop forming primer is troublesome when its 3' end is "tied up," since this

can cause internal primer extension. Hairpins near the 5' end, however, do not significantly affect the PCR. **4)** The ability of the primer to form a stable duplex (by calculating the free energy of duplex formation, ΔG) with the specific site only on the target DNA. **5)** The T_m (melting temperature) difference between both primers and between the template and the less stable primer is kept to a minimum. **6)** Avoidance of runs of C's or G's at the 3' ends of the primers.

2.5 Synthesis, deprotection and purification of oligonucleotides

All primers, either published or newly designed, were synthesized in-house on an ABI 391-DNA synthesizer with "Trityl off". When synthesis was finished, the product exists as phosphate-protected, base-protected phosphotriesters. Complete deprotection was necessary to produce biologically active oligonucleotides.

Cleavage and phosphate deprotection

Following synthesis, the primer remains covalently attached to the support and it has to be cleaved by a one hour treatment with fresh, concentrated ammonium hydroxide and collected in a vial fitted with a Teflon-lined cap. Phosphate deprotection (removing of cyanoethyl protecting groups) requires treatment with ammonium hydroxide and occurs at the same time as cleavage.

Base deprotection

Base deprotection is an ammoniolysis reaction and the benzoyl and isobutyryl base protecting groups are removed by placing the vial containing the oligonucleotide at 55^{o}C for 8 to 15 hours. After completion of deprotection, the ammonium hydroxide-oligonucleotide solution is cooled at room temperature for 30 minutes. Primers are stored in ammonia eluant, which stays liquid at -20^{o}C, enabling the dispensing of them without repeat freeze-thawing.

Purification of the primer

To remove the ammonia, Aliquots from the ammonia stock were left in opened tubes under the fume hood for 16-24 hours. Alternatively, the butanol extraction method was used. 100 µl of the ammonia stock was mixed with 1 ml n-butanol in a 1.5 ml microcentrifuge tube, vortexed vigorously for 20 seconds and then centrifuged at full speed microcentrifuge for 10 minutes. The resulting supernatant was discarded, the pellet was air dried and dissolved in 50 µl sterile double distilled water and its OD was detected by UV spectroscopy at 260 nm. As a useful approximation, 1 OD unit of single-stranded oligonucleotide consists of about 33 micrograms, by mass. A micromole of oligonucleotide has a number of OD units equal to 10 times the number of bases e.g., a micromole of a 20 mer would be 200 OD units.

2.6 Reverse transcriptase PCR (RT-PCR)

2.6.1 Reverse transcription

Because RNA cannot serve as a template for PCR, reverse transcription of RNA followed by the polymerase chain reaction is an extraordinarily sensitive method to detect as few as 1-100 copies of a specific RNA. Reverse transcription was accomplished in a final volume of 20µl. A 2X reverse transcription master mix was prepared containing multiples of: 4 µl 5X first strand buffer (Gibco BRL), 2 µl dNTP's mix (10 mM each), 2 µl of the 0.1M DTT (dithiothreitol), and 1 µl of M-MLV reverse transcriptase (200 u/µl; Gibco BRL). 1 µg of total RNA was made up to 9 µl by DEPC treated water, denatured at 95^{o}C for 5 minutes then kept on ice. 2 µl (20 pmol) of the downstream primer or 2 µl (200 ng) of oligo $(dt)_{12\text{-}18}$ (Pharmacia Biotech.) was added to the denatured RNA, the mixture was heated at 65^{o}C for 10 minutes and quenched on ice for 5 minutes to anneal the primer. To the annealed RNA-primer mix (11 µl), 9 µl of the reverse transcription master mix was added,

pipetted up and down several times to mix and incubated at 42°C for one hour. After reverse transcription tubes were heated at 95°C for 5 minutes to inactivate the M-MLV enzyme then stored until used for PCR at -20°C.

2.6.2 Second-strand synthesis and PCR amplification

PCR was performed in a final volume of 50 µl. A PCR master mix was made consisting of multiples of: 5 µl 10X GeneAmp PCR buffer (Perkin Elmer/Cetus), 4 µl dNTP's mix (200 µM of each dNTP, Boehringer Mannheim), 3 µl of each primer (30 pmol each), 0.3 µl (1.5 units) Ampli-*Taq* polymerase (Perkin Elmer/Cetus) and sterile double distilled water up to 45 µl. To each 0.5 ml Eppendorf tubes 45 µl of the PCR master mix was aliquoted and covered by 50 µl mineral oil. 5µl of the reverse transcription reaction mixture was added under the oil to the PCR master mix and mixed by pipetting up and down several times and then centrifuged briefly to obtain a clean oil/aqueous interface and to remove air bubbles. Tubes were transferred to a pre-heated PCR block at 94°C and incubated for 3 minutes for the initial denaturation. 35 PCR cycles were then started at Ta°C for 1 minute annealing (Ta determined for each primer set), 72°C extension for 1.5 minutes and 94°C denaturation for 1 minute and a final extension step of 72°C for 10 minutes.

2.7 Methods for mutation screening

2.7.1 Southern blot analysis

(A) Probe preparation for Southern blotting

(1) Probe p5B1.4 to detect CTG expansion in myotonic dystrophy patients

The DNA probe p5B1.4 is a 1.4 kb *Bam*HI fragment in a pBluescript plasmid. This probe was kindly supplied as plasmid DNA by Dr. Kevin Kelly, Department of Medical Genetics, Aberdeen Royal Hospitals NHS Trust. This probe detects a human genomic *Bgl*I fragment of 3.4 kb from individuals with the normal range of CTG

repeats. Individuals with expansions of the CTG repeats (50 repeats or more) showed extra bands with varying sizes. The probe was transformed into bacterial cells, grown up and stored as a glycerol stock.

Transformations of the plasmid DNA to competent cells

Plasmid DNA (pBluescript) containing the p5B1.4 probe was transformed to DH5∝^{TM} (Gibco BRL) competent E. Coli cells according to the supplier protocol with some modifications. The competent cells were removed from -70^{o}C and thawed on wet ice. The required number of autoclaved 1.5 ml Eppendorf tubes were placed on ice. The cells were gently mixed and 50 µl were aliquoted into each chilled tube using chilled, sterile pipette tips. 10 µl of the plasmid DNA (containing 10 ng of DNA) were added to all tubes except the negative control by moving the pipette tip through the cells while dispensing. Tubes were gently shaken for 5 seconds immediately after addition of DNA and then were incubated on ice for 30 minutes. The cells were then heat-shocked at 42^{o}C water bath for 2 minutes without shaking and then rapidly placed on ice for 2 minutes. 800 µl of SOC medium was added to all tubes and the cells were incubated for one hour at 37^{o}C with mild shaking (100 r.p.m.). 200 µl of the reaction were spread onto LB agar plates (with 100 µg/ml ampicillin) and left at room temperature until the excess moisture has been absorbed. The plates were then inverted and incubated at 37^{o}C oven for 12-16 hours then stored in 4^{o}C (cold room) for up to one month. Discrete colonies were observed in all plates except the negative control.

Isolation of recombinant plasmid DNA and formation of glycerol stocks

The agar plates were removed from the cold room and a single colony was picked by autoclaved pipette tip and thrown into 5 ml of LB with ampicillin (100 µg/ml) in a universal tube. The colony was grown for overnight at 37^{o}C shaker (225 r.p.m.). Glycerol stock was made by adding 0.7 ml of the growth to 0.3 ml glycerol, mixed by brief vortexing and immediately stored at -70^{o}C. To recover bacteria from

the glycerol stock, the frozen surface of the stock was scraped with sterile inoculating loop and streaked out into LB/ ampicillin agar plate to obtain isolated colonies. The remaining amount of the culture was used to isolate recombinant plasmid DNA using the Insta- Prep™ kit.

Isolation of recombinant plasmid DNA using the INSTA-PREB™ kit (5 Prime→3 Prime, Inc.®)

The unopened INSTA-PREB™ tube was prepared by centrifuging in a microcentrifuge at full speed (12,000 g or greater) for 10 seconds to pellet the INSTA-PREB™ gel. To a sterile 1.5 ml Eppendorf tube 1.5 ml of the bacterial culture was transferred and centrifuged for 30 seconds at 12,000 to 16,000 g. The supernatant was discarded and additional 1 ml of the culture was added to the bacterial pellet and centrifuged as before. Virtually all the supernatant was aspirated carefully taking care not to disturb the pellet. The pellet was resuspended into 50 µl of sterile T.E. (10 mM Tris-Cl, 1 mM EDTA, pH 8) by brief vortexing. 300 µl of shaken PCI solution (phenol:chloroform:isoamyl alcohol, 50:49:1) was added to the tube containing the resuspended pellet and mixed by repeated inversion. The entire aqueous and organic contents of the tube were carefully transferred to the pre-spun INSTA-PREB™ tube using a large bore pipette tip. The INSTA-PREB™ tube (containing bacterial lysate) was then centrifuged in a microcentrifuge at full speed (12,000 g or greater) for 30 seconds at speed. 300 µl of CI solution (chloroform:isoamyl alcohol, 49:1) was then added to the spun tube and mixed briefly by repeated inversion then centrifuged for another 30 seconds at full speed in a microcentrifuge. The topmost phase (containing the plasmid DNA) was recovered by pipetting to a fresh sterile microcentrifuge tube and stored at -20°C until used.

Releasing insert probe from plasmid

To release the insert probe from plasmid DNA a 50 µl digest was set up. 14 µl of plasmid DNA, obtained by using INSTA-PREP™, along with 4 µl *Bam*HI

restriction endonuclease, 5 µl 10X enzyme buffer and 27 µl ddH_2O were mixed by pipetting and incubated at 37°C water bath for 2 hours. The digest was loaded alongside 1 kb ladder (Gibco BRL) on 1% low melting agarose gel (SeaKem agarose, FMC bioproducts) in 1X TAE buffer and electrophoresed at 100 volts for ~ 2 hours. The gel was visualised on a UV transilluminator and the 1.4 kb insert band was cut by a sterile scalpel blade and placed in a pre-weighed Sarstedt 1.5 ml tube. The weight of the insert was then determined and to it a volume of sterile T.E. buffer 2 times the weight was added. The insert was then boiled for 5 minutes and stored at -20°C until required.

(2) Probes E1.6 and E2.5 to detect major structural rearrangements in patients with tuberous sclerosis

Two *TSC2* cDNA probes were kindly supplied by Dr. M. Nellist, Institute of Medical Genetics, University Hospital of Wales, Cardiff. These probes were *Eco*RI fragments (1.6 kb and 2.5 kb) in pBluescript SK- plasmid. The E2.5 probe contains the 3' end of the *TSC2* gene while the E1.6 probe covers a 1.6 kb of the *TSC2* cDNA starting about 0.5 kb from the 5' end of the gene. The probe E1.6 detects 4 and 18 kb fragments of *Eco*RI digested normal genomic DNA while the E2.5 probe detects 3 and 7.5 kb fragments of *Hin*dIII digested normal genomic DNA. Both probes were supplied as cDNA clones in agarose and needed only to be grown up. These two probes were grown up, glycerol stocks were made and recombinant plasmid DNAs were isolated using the same protocol as for probe p5B1.4. The inserts were released from the plasmid DNAs by digestion with *Eco*RI as previously mentioned for probe p5B1.4.

(B) Southern blotting

(1) Digestion of genomic DNA

7 to 10 µg of genomic DNA was digested using the appropriate restriction endonuclease in a total volume of 40 µl containing 1X appropriate enzyme buffer and

40 units of the specific enzyme. The digests were incubated for overnight in a water bath set at the recommended temperature. Digests were either loaded on an agarose gel or stored at -20^{O}C for later use.

(2) Agarose gel electrophoresis

The digests were resolved in 20 cm 0.8% agarose gels (FMC bioproducts) prepared in 1X TAE buffer and containing 0.5 μg/ml ethidium bromide (10 mg/ml). Samples were prepared by adding 8 μl of 6X agarose gel loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in 1X TBE buffer) to the 40 μl digest. Samples were then loaded alongside 1 kb ladder (Gibco BRL) in the gel and electrophoresed at 2 volts/cm for 18 hours. After electrophoresis the gel was visualised and photographed under UV light and the wells were then cut off. A ruler was always photographed alongside the gel so that the mobility of the marker fragments can be easily determined. The gel was then transferred to a plastic box, upside down and rinsed several times in water. it was then treated in a depurination solution for 15-20 minutes then rinsed in water. The gel was then transferred to the denaturation solution for 30 minutes then rinsed in water and neutralized in two changes of neutralization solution for 15 minutes each.

(3) Setting up the transfer apparatus and Southern transfer of digested DNA

The transfer apparatus comprised a tray with a raised platform for the gel to set on. The tray was half filled with 1.5 liters of 10X SSC. The platform was covered by double layer of 3MM Whatman paper wick with its ends dipped into the SSC. The 3MM paper was left to soak the SSC and became completely wet. Any air bubbles between the paper and the platform were smoothed out. After the neutralisation step the gel was placed on the wick on the platform and again any air bubbles between the gel and the 3MM wick paper were smoothed out. The gel was surrounded by a plastic wrap which let out to cover the transfer apparatus. A gel-size Hybond N membrane was cut, marked using a permanent pen, wet in 2X SSC and layered on to the gel

taking care to get rid of any air bubbles that were trapped between the membrane and the gel. A gel-size double sheet of 3MM paper was wet in 2X SSC and layered on to the membrane followed by a stack of absorbent paper (paper towels). A glass plate and a weight of ~ 500 g were laid on top of the absorbing paper stack. The blotting was carried out for 12-16 hours at room temperature. After the transfer was complete the gel was stained in ethidium bromide (0.5 μg/ml in water) to check the efficiency of transfer. The Hybond N membrane was rinsed in 5X SSC to remove residual agarose and placed between two sheets of 3MM paper and backed for 3 hours at 80°C oven to crosslink the DNA fragments to the Hybond N membrane. Filter was then wrapped in a Saran wrap and stored in the cold room (4°C) until hybridization.

(C) Hybridization of DNA blot

(1) Prehybridization of filter

The filter was soaked in 2X SSC and placed, face to face, onto a mesh then rolled up and placed in a hybridization bottle containing 5 ml of 2X SSC. The bottle was closed securely, laid flat and gently rolled to unfold the mesh and the filter so as they stuck to the wall of the bottle. The bottle was then opened and the SSC was discarded. To a pre-warmed 10 ml of prehybridization solution (at 65°C oven for few minutes till it became clear) a 100 μl denatured (by boiling for 10 minutes) sonicated salmon sperm DNA (10 mg/ml) was added. The mixture was then added to the filter in the bottle and placed in 65°C hybridization oven for a minimum of 6 hours. This step of prehybridization was carried out to prevent non-specific binding of radiolabelled probe to the filter.

(2) Probe labelling

While prehybridization of the filter, the probe was radioactively labelled using the random primed DNA labelling kit (Boehringer Mannheim) according to the manufacturer protocol. 24 μl of the probe (in low melting agarose) was boiled for 6 minutes then incubated at 37°C for one minute. The probe was then added to a mix of

2 µl each of dATP, dGTP and dTTP, 4 µl of the reaction mix, 1.5 µl of Klenow enzyme and 5 µl (50 µCi) of α-^{32}P dCTP (Amersham) and mixed by repeated pipetting. The tube was then incubated at 37^{O}C for two hours and the reaction was then stopped by addition of 2 µl of 0.5M EDTA. The labelled probe was then separated from the unincorporated α-^{32}P dCTP using NICK^{TM} columns (Pharmacia Biotech.) which were prepacked columns containing sephadex G-50 DNA grade. The column was rinsed once by 3 ml 1X SSC by allowing them to drip through by gravity. The labelled probe (~ 40 µl) was added to the top of the suspended column and then 400 µl 1X SSC was added to the column and allowed to drip through. A Sarstedt tube was then placed under the column and a further 400 µl 1X SSC was added to the top of the column and collected. This had the labelled probe after its separation from the unincorporated α-^{32}P dCTP.

(3) Hybridization of filter

After at least 6 hours of prehybridization of the filter the prehybridization solution from the hybridization bottle was poured into a universal tube and to it the denatured (boiled for 6 minutes, and kept on ice for 2 minutes) probe was added. The contents of the universal tube were mixed gently and transferred back into the hybridization bottle and the bottle was returned to the hybridization oven at 65^{O}C for overnight incubation.

(4) Washing the filter after hybridization

After hybridization the hybridization solution was discarded and the filter was rinsed in 2X SSC/0.1% SDS while inside the bottle. The filter was washed once by adding 10 ml of 2X SSC/0.1% SDS to the bottle and returning it to the 65^{O}C hybridization oven for 10 minutes. The filter was then taken out from the bottle and placed in a plastic tray with 0.5X SSC/0.1% SDS and washed by shaking at 65^{O}C for a further 5 minutes. The filter was monitored using a series 900 minimonitor and if a signal greater than 10 cpm (count per minute) was detected it was then washed at

increasing stringency (65^{o}C, 0.25X SSC/0.1% SDS then 0.1X SSC/0.1% SDS) taking care not to over wash it (filter was frequently monitored every 3 minutes).

(D) Autoradiography

After washing the filter, it was briefly dried and covered by a plastic wrap. It was then placed in an autoradiography cassette with intensifying screens and exposed to Kodak Diagnostic AR Imaging film with the DNA side of the filter facing the film. The position of the upper border of the filter was marked on the film surface so that a ruler could be used to estimate the size of the bands detected. The cassette was stored in a -40^{o}C freezer for 2 to 7 days before developing the film.

2.7.2 PCR amplification of the unstable CTG repeat in the *DM*-Kinase gene and CAG repeat in the androgen receptor gene from genomic DNA

(A) Radiolabelled PCR

Radiolabelled PCR was optimised and used to amplify DNA containing CTG repeats in the *DM*-Kinase gene from patients with myotonic dystrophy (DM). PCR reactions were performed in a 15 µl PCR reaction mix containing 1X GeneAmp PCR buffer, 100 µM of each dNTP, 15 pmol of each primer (409 and 410, Table 2.1), 100 ng genomic DNA and 2 µCi α-^{32}P dCTP (Amersham). The reaction was overlaid with 25 µl mineral oil, vortexed and centrifuged briefly. Tubes were heated to 95^{o}C for 5 minutes then held at 90^{o}C where 1 unit of Ampli*Taq* DNA polymerase was added to each tube under the oil. Immediately after *Taq* polymerase was added 32 PCR cycles were started at 95^{o}C for 1 minute, 65^{o}C for 30 seconds and 72^{o}C for 1 minute each and was then followed by a final extension step of 72^{o}C for 5 minutes.

(B) Non-radiolabelled PCR

Non-radiolabelled PCR reactions to amplify both genomic DNA that contains the CTG repeats at the 3' end of the *DM*-Kinase gene in DM patients (primer set

409/406; Mhadevan et al., 1992) and the CAG repeat (primer set 1/2; Allen et al., 1992) in the first exon of the androgen receptor gene in patients with amyotrophic lateral sclerosis (Table 2.1) were optimised. PCR reactions were performed in 30 µl PCR reaction mix containing 1X GeneAmp PCR buffer, 200 µM of each dNTP, 25 pmol of each primer and 1 µg genomic DNA. The reaction was overlaid with 50 µl mineral oil, vortexed and centrifuged briefly. Tubes were heated to 95°C for 5 minutes then held at 90°C where 2 units of Ampli*Taq* DNA polymerase was added to each tube under the oil. Immediately after the addition of *Taq* polymerase PCR cycles were started as follows: **1)** For CTG repeat amplification, 32 cycles of 95°C for 1 minute, 64°C for 1 minute and 72°C for 1.5 minutes followed by a final extension step of 72°C for 5 minutes. **2)** For CAG repeat amplification, 30 cycles of 95°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute without the final extension step. Non-radiolabelled PCR products were resolved using agarose gel electrophoresis.

Table 2.1 Primers used for PCR amplification

Primer Name	Primer Sequence	Comments
409 (Forward)	5'-GAAGGGTCCTTGTAGCCGGGAA-3'	For CTG repeat
410 (Reverse)	5'-AGAAAGAAATGGTCTGTGATCCC-3'	For CTG repeat
406 (Reverse)	5'-GGAGGATGGAACACGGACGG-3'	For CTG repeat
Primer 1	5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'	For CAG repeat
Primer 2	5'-TCCAGAATCTGTTCCAGAGCGTGC-3'	For CAG repeat

(C) Polyacrylamide gel electrophoresis

(1) Gel preparation

To detect the CTG repeat expansion in DM patients, radiolabelled PCR products were resolved on 8% denaturing polyacrylamide gels (20cm x 50cm x

0.4cm) with 7M urea prepared and electrophoresed in 1X TBE buffer. 30 µl TEMED and 300 µl freshly prepared 10% ammonium persulphate were added to the prepared gel solution (60 ml) to allow its polymerisation. The gel was then rapidly poured in a pre-assembled, clean and bottom sealed Sequigen sequencing gel apparatus (Biorad) and a 20 well, 0.4 cm comb was inserted in place. The gel was left to polymerise for a minimum of one hour before electrophoresis.

(2) Labelling a DNA size marker

The 1 kb ladder (Gibco BRL) was radioactively end labelled at the 5' ends using T4 polynucleotide kinase (Pharmacia Biotech) and [γ-^{32}P] ATP. A 10 µl mix of 1 µl ladder (~ 1 µg), 1 µl One-Phor-All Plus buffer (Pharmacia Biotech), 6 µl sterile distilled water, 1 µl [γ-^{32}P] ATP (10 µCi of 3000 Ci/mmol) and 1 µl of T4 kinase enzyme, was made in 0.5 ml microcentrifuge tube and incubated at 37^{O}C for 45 minutes. 80 µl of formamide loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol) was then added to the reaction. The tube was stored at -20^{O}C and the labelled ladder can be used for up to 15 days.

(3) Sample preparation, gel loading and gel electrophoresis

After the gel has set, it was assembled within the gel kit and the well forming comb was removed. Buffer chambers were filled with 1X TBE buffer and the wells were rinsed thoroughly with buffer. 1 µl of the PCR product was mixed with 5 µl formamide loading mix and denatured at 96^{O}C for 2 minutes and immediately quenched on ice for few minutes. 3 µl of labelled 1 kb ladder were denatured in the same way and loaded alongside prepared samples. Samples were electrophoresed at 2000 volts for about 2 hours (till the xylene cyanol dye moved 2/3 of the gel length).

(4) Autoradiography

After gel electrophoresis, the glass plates were opened and the gel was transferred to 3MM Whatmann paper, covered with Saran wrap and dried under

vacuum for 30 minutes at 80°C on a gel dryer (Biorad model 583). The gel was autoradiographed in an autoradiographic cassette with intensifying screens using X-Omat diagnostic AR imaging film (Kodak), with the DNA side facing the film for 5 to 16 hours at -40°C.

(D) Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to detect both CTG and CAG repeat expansions after non-radiolabelled PCR amplification of the repeat. 1.5%, 15 cm agarose gels were prepared in 1X TBE buffer with 0.5 μg/ml ethidium bromide. 30 μl of PCR product was mixed with 6 μl of 6X agarose gel loading buffer and loaded alongside 1 kb and 100 bp (Gibco BRL) ladders. Gels were electrophoresed at 170 volts for 3 hours and then visualised and photographed under UV light.

2.7.3 Single strand conformational polymorphism (SSCP) analysis

SSCP analysis allows the detection of a single base change. Wild type and mutant target DNAs are amplified by PCR, denatured and then electrophoresed side by side through a non-denaturing polyacrylamide gel. The two single-stranded DNA molecules from each denatured PCR product assume a three-dimensional conformation which is dependent on their primary sequence. If a sequence difference (mutation) exists between wild-type and mutant DNA, this may result in differential migration of one or both of the mutant strands.

(A) PCR amplification of the *SOD-1* gene exons from genomic DNA

Radiolabelled PCR amplification of the *SOD-1* gene exons 1, 2, 4 and 5 were optimised and carried out using primer sets designed by Rosen et al. (1993) and Deng et al. (1993). To amplify exon 3 a primer set was designed using the computer program Oligo™ (Medprobe) and the PCR conditions for this primer set was optimised. Table 2.2 shows the sequence of the used primer sets. 15 μl PCR reaction

mix was set up containing 1X GeneAmp PCR buffer, 50μM of each dNTP, 15 pmol of each primer, 100 ng genomic DNA and 2 μCi $\propto$-^{32}P dCTP. The reaction was overlaid with 25 μl mineral oil, vortexed and centrifuged briefly. Tubes were heated to 95°C for 2 minutes then held at 90°C where 1 unit of Ampli*Taq* DNA polymerase was added to each tube under the oil. Immediately after the addition of *Taq* polymerase, 28 PCR cycles were started at 95°C for 1 minute, 61°C for 1 minute and 72°C for 45 seconds each and was then followed by a final extension step of 72°C for 6 minutes.

Table 2.2 PCR primers used in analysis of the *SOD-1* gene

Exon	Primer Sequence (5' to 3' end)	Product Size	Reference
1 'F'	TTCCGTTGCAGTCCTCGGAA	158 bp	Deng et al.,
1 'R'	CGGCCTCGCAACACAAGCCT		(1993)
2 set a 'F'	ACTCTCTCCAACTTTGCACTT	132 bp	Rosen et al.,
2 set a 'R'	CCCACCTGCTGTATTATCTCC		(1993)
3 'F'	TAAATAGGCTGTACCAGTGCA	130 bp	Designed for
3 'R'	ATGAACTCCAGAAACGTATCG		this study
4 set a 'F'	CATATAAGGCATGTTGGAGACT	214 bp	Rosen et al.,
4 set a 'R'	TCTTAGAATTCGCGACTAACAATC		(1993)
5 'F'	AGTGATTACTTGACAGCCCA	216 bp	Deng et al.,
5 'R'	TTCTACAGCTAGCAGGATAACA		(1993)

'F' stands for forwards and 'R' stands for reverse primers.

(B) Sample preparation

After thermal cycling, 1 μl PCR product was mixed with 9 μl formamide loading buffer, heat denatured at 95°C for 3 minutes then quenched on ice for at least 5 minutes before loading 3 μl of it into SSCP gel.

(C) Gel types and conditions of electrophoresis used in SSCP analysis

(1) MDE™ gel (FMC Bioproducts) electrophoresis:

The MDE™ gel is a polyacrylamide-drived matrix designed to improve the resolution of conformationally different DNA molecules. 0.5X MDE™ gel (20 cm x 40 cm x 0.4 cm) containing 5% glycerol was prepared in 0.6X TBE buffer. The gel was left to set for at least one hour before sample loading. 3 μl of each prepared sample was loaded and electrophoresis was carried out at 5 watts for 16 hours at room temperature.

(2) Polyacrylamide gel electrophoresis (PAGE):

Three different conditions using 8% non-denaturing PAGE in 1X TBE buffer were tried. The first was by running gels containing 5% glycerol at 5 watts at room temperature. The second was by running gels containing 10% glycerol at 8 watts at room temperature and the third by running gels without glycerol at 12 watts in cold room (4°C).

(D) Autoradiography

Autoradiography was carried out by placing the dried gel with X-Omat diagnostic AR film (Kodak) in a cassette with intensifying screens. The gel was autoradiographed for 5 to 16 hours at -40°C.

2.7.4 Screening for known mutations by digestion of PCR products

Patients with amytrophic lateral sclerosis (ALS) were screened for three known mutations in the *SOD-1* gene using restriction endonuclease digestion of PCR products. These mutations were the Ala4Val in exon one, Gly93Ser and Ile113Thr in exon four. Moreover, 100 normal chromosomes were screened for the presence of three missense mutations in the *TSC2* gene, M1602V (*TSC2* cDNA segment III), S1715T and R1720W (*TSC2* cDNA segment IV).

(A) Non-radiolabelled PCR

Non-radiolabelled PCR amplifications were optimised and carried out for both exons 1 and 4 using the above mentioned primer sets (Table 2.2). A 50 µl reaction mix was set up containing 1X GeneAmp PCR buffer, 200 µM of each dNTP, 25 pmol of each primer and 1 µg genomic DNA. The reaction was overlaid with 50 µl mineral oil, vortexed and centrifuged briefly. Tubes were heated to 95^{o}C for 2 minutes then held at 90^{o}C where 2 units of Ampli*Taq* DNA polymerase was added to each tube under the oil. Immediately after the addition of *Taq* polymerase, 32 PCR cycles were started at at 95^{o}C for 1 minute, 61^{o}C for 1 minute and 72^{o}C for 1 minute each and was then followed by a final extension step of 72^{o}C for 6 minutes. *TSC2* cDNA segments III and IV were amplified as described before (2.6).

(B) Testing the non-radiolabelled PCR

After completion of the PCR reaction 5 µl of the PCR product was aliqouted from each tube, mixed with 1 µl loading mix (0.25% bromophenol blue, 40% (w/v) sucrose in 1X TBE buffer) and resolved on a 1% agarose minigel containing 0.5 µg/ml ethidium bromide and prepared and electrophoresed in 1X TBE buffer at 100 volts for 30 minutes. The gel was visualised on a UV transilluminator to check for the efficacy of the PCR amplification. Single, correctly sized bands were detected from

most of the PCR reactions. If otherwise, the PCR reaction for the specific product was repeated.

(C) Ethanol precipitation of the PCR products

Ethanol precipitation of the PCR products was carried out for the remaining 45 µl PCR reaction. 4.5 µl 3M Na acetate (pH 5.2), 90 µl absolute ethanol and 2 µl glycogen (20 mg/ml) were added to each 45 µl PCR reaction and mixed by brief vortexing. Tubes were kept at -70^{O}C for 15 minutes then centrifuged in a microcentrifuge at full speed (12,000 g or greater) for 15 minutes. Pellets were washed in 70% ethanol and centrifuged for 5 minutes as before. Ethanol was then discarded and the pellet air dried for 5 to 10 minutes then dissolved in 15 µl ddH_2O.

(D) Restriction endonuclease digestion of the PCR products

10 µl of the ethanol precipitated PCR amplification products were digested using the appropriate restriction endonuclease according to the supplier recommended buffer and temperature, for 2 hours. For one of them, *Bsr*I enzyme, after 2 hours of incubation at 65^{O} C, an extra 10 units of the enzyme were added to each reaction tube and the whole reaction was incubated at 65^{O} C for overnight. The digested products were resolved by 8% or 12% polyacrylamide gel electrophoresis in 1X TBE buffer for 2.5 to 6 hours at 280 volts (constant) then either silver or ethidium bromide stained and photographed.

(E) Silver staining

During all the steps the gel was gently shaken. It was first fixed in two changes of solution 1 (10% ethanol, 0.5% acetic acid) each for 5 minutes. To stain the gel, it was incubated for 15 minutes in freshly prepared 0.1% silver nitrate (solution 2). The gel was rinsed briefly with distilled water and developed by incubation in solution 3 (1.5% NaOH, 0.1% formaldehyde, mixed just before use) for 20-30

minutes. It was then treated with 0.75% Na_2CO_3 solution for 10 minutes, sealed in a plastic bag and photographed.

2.7.5 Chemical cleavage of mismatches (CCM) analysis

To screen for mutation sites within the amplified PCR products from *TSC2* cDNA and DNA, the CCM method described by Cotton et al. (1988) was used with some modifications. In this technique, mutant DNA was allowed to form a duplex with a radiolabelled wild type control DNA (probe) obtained by PCR amplification. Where mutations were present, mismatches were chemically modified and cleaved leading to detection of different-sized radioactive fragments. The protocol involved the following steps:-

(A) Preparation of test DNA

(1) RT-PCR and DNA-PCR

Four RT-PCR segments were optimised and amplified from the *TSC2* cDNA using four home-designed sets of primer (Table 2.3) and the previously mentioned method (2.6).

Two DNA-PCR segments were also optimised and amplified from the same gene. The first one gave amplification product from exon 1, 325 bp from the upstream promoter area and part of intron 1. The second segment produced amplification products from exons 38 to 41 with the intervening intronic sequences. The primer sets used for the DNA-PCR were home-designed (Table 2.3) and the PCR amplification was accomplished in a 50 µl reaction mix using standard PCR protocol (2.7.4-A) with 32 cycles and a final 5 minutes extension step at 72^{O}C.

Table 2.3 PCR primers used in amplification of DNA and cDNA segments of the *TSC2* gene. (F= forward, R= reverse)

Segment/exon	Primer Sequence (5' to 3' end)	Annealing Temp.	Product size (bp)
Seg. I 'F'	5'-CATCACCAGGCTCATCAAGC-3'	60 ^{O}C	837
Seg. I 'R'	5'-AGAAACAGGAAGTCAAAGGC-3'		
Seg. II 'F'	5'-CCTGGACGGGGAAAGTGCTG-3'	59 ^{O}C	602
Seg. II 'R'	5'-TGGCGATGTGGAAGACGGCT-3'		
Seg. III 'F'	5'-ATCGCCGTCCTGTATGTTGG-3'	60 ^{O}C	663
Seg. III 'R'	5'-TAGGCTGGGGTTGGAGTAGG-3'		
Seg. IV 'F'	5'-TCACCCCGCTGGACTACGAG-3'	63 ^{O}C	505
Seg. IV 'R'	5'-ATCTGTGCCTCTATGTCTGTGC-3'		
Exon 1 'F'	5'-GGGTAGAGGAGAGACGGCAA-3'	60 ^{O}C	663
Exon 1 'R'	5'-CACCCAGATCCTGACTTTCG-3'		
Exon 38-41 'F'	5'-CCCCAGCAATTAGAGGTGTC-3'	63 ^{O}C	1120
Exon 38-41 'R'	5'-GCACCAAGCAGACAAAGTCA-3'		

(2) Identification of PCR products

10 µl aliquots from PCR products were electrophoresed on 1% agarose gels in 1X TAE buffer with 0.5 µg/ml ethidium bromide. They were visualised with a UV transilluminator to confirm the size and to detect any abnormal alterations in it (for RT-PCR, where exon skipping and small intragenic insertions or deletions could cause alteration of the PCR product size). The bands were then excised from the gels, covered with 50 µl sterile double distilled water and stored at -20°C for later use. The remaining 40 µl PCR products were cleaned for use in CCM analysis.

(3) Cleaning of PCR products

Geneclean™ purification kit (Bio 101 Inc.) was used to purify DNA amplification products (the remaining 40 µl) directly from the PCR mixture. If there was non-specific amplification products, the whole PCR product was electrophoresed on 1% low melting point agarose gel (NuSieve) in TAE buffer and the required band was cut out and the DNA was recovered using the same kit. The cleaning procedure was as follows:-

To the PCR product (or the excised band) 3 volumes of 6M NaI (supplied with the kit)was added. If purifying DNA from excised gel band, tubes were kept at 55°C for 5 minutes, with mixing after one minute, to ensure gel dissolving. 7 µl glassmilk (silica matrix in water) was added to each tube, vortexed briefly every two minutes for ten minutes at room temperature then pelleted by centrifugation in a microcentrifuge at full speed for 5 seconds. The resulting pellet was washed two times with 300 µl NEW wash solution (14 ml of the NEW concentrate, 280 ml dH_2O, 310 ml 100% ethanol) Pellet was then dissolved in 10 µl double distilled water and incubated at 65°C water bath for 10 minutes. DNA was recovered by centrifugation in microcentrifuge at full speed for 2 minutes and the supernatant (containing the recovered DNA, ~ 10 µl) was transferred to a fresh tube and stored at -20°C until used. 1 µl of the recovered DNA was tested on 1% agarose gel to judge for the efficacy of the recovery. The purification results in removal of both PCR primers and unincorporated nucleotides

which are likely to affect mismatch signal by serving as additional targets for chemical modification.

(B) Preparation of labelled probes

A probe is a PCR product obtained from wild-type DNA for the segment of interest. Internal labelling of the PCR was carried out by direct incorporation of 2 μCi [α-^{32}P] dCTP in the PCR reaction mix. PCR was carried out in 50 μl reaction mix using standard PCR conditions and PCR cycles as the non-radiolabelled one. The labelled probe was isolated from low-melting-point agarose using Geneclean™ purification and its specific activity was determined (approximately 10^7c.p.m./μg).

Alternatively, 5' end labelling of the probe (both strands) was done by mixing 100 ng of the probe DNA (after Geneclean™ purification), 10 μCi [γ-^{32}P] ATP, 1X One-Phor-All buffer Plus (Pharmacia Biotech), ~10 units of polynucleotide kinase FPLCpure (Pharmacia Biotech.) and up to 10 μl ddH_2O. The mixture was incubated at 37°C for 45 minutes then stored at -20°C until later use. Probes were stored for a maximum of two weeks before radiolysis rendered them impractical.

(C) Formation of the hybrids (heteroduplexes)

To minimise the formation of probe homoduplexes, hybridisation between probe and target were set up in which the target DNA was present in 10 to 20 fold molar excess over probe DNA. Approximately 5 ng of probe DNA per target sample per modification reaction was needed. A premix containing 1X hybrid buffer and the appropriate quantity (~10 ng/ test DNA sample) of labelled probe in $T_{0.1}E$. buffer was made. 9 volumes of this premix was added to one volume of test DNA (~100 to 150 ng) in 0.5 ml Eppendorf tubes, topped with 50 μl mineral oil and then placed in a boiling water bath for 5 minutes. Immediately, after boiling, the tubes were transferred to 65°C water bath and incubated for 5 to 16 hours to allow hybrid formation to occur. Tubes were then transferred on ice and the aqueous phase from each tube (containing the hybrids) was equally divided between two 1.5 ml siliconized

Eppendorf tubes (one for each chemical modification). To each tube, 3 µl of 20 mg/ml mussel glycogen (Boehringer-Mannheim) and 750 µl of the stop precipitation mix was added, mixed well and chilled on dry-ice for 10 minutes. Tubes were spun in a microcentrifuge at full speed (~ 14000 g) for 10 minutes. Supernatants were discarded and the pellets were washed in 70% ethanol, air dried and resuspended in 7 µl $T_{0.1}E$ buffer. Samples were either used immediately or stored for no more than 16 hours at -20°C for later use.

(D) Mismatch analysis

(1) Chemical modification using hydroxylamine and osmium tetroxide

Approximately 6.5M solution of hydroxylamine hydrochloride (BDH) pH 6 (adjusted with diethylamine, BDH) was made and stored at 4°C for up to one week. 1.39 g of hydroxylamine was dissolved in 1.6 ml double distilled water and its pH was adjusted to 6 by approximately 1.5 ml diethylamine. 20 µl of this solution was added to 7 µl hybrid, mixed well and incubated at 37°C for 2 hours.

Fresh solution of osmium tetroxide (Aldrich) and pyridine (BDH) was made on ice by mixing 6.75 µl pyridine with 1.5 µl osmium tetroxide (4% solution stored at 4°C for up to 2 months) and 154 µl $T_{0.1}E$ buffer. 18 µl of this solution was added to the other 7 µl hybrid, mixed well and incubated at 37°C for two hours.

Modification reactions were stopped and precipitated by adding 750 µl stop/ precipitation solution to each tube, mixing them well, incubating the tubes on dry ice for 10 minutes and centrifugation in a microcentrifuge at full speed (~14000 g) for 10 minutes. The pellets were washed in 70% ethanol and air dried.

(2) Piperdine cleavage of the chemically modified mismatches

1M (10%) freshly made solution of piperdine (Fluka) was made in ddH_2O and 50 µl of it was added to each pellet and the tubes were vortexed for 1 minute to resuspend the pellet then briefly centrifuged. Tubes were incubated at 90°C for 30 minutes then kept on ice for few minutes and precipitated by 750 µl stop/precipitation

solution as above. The pellets were washed in 70% ethanol, air dried and resuspended in 10 µl formamide loading buffer (95% deionized formamide, 10mM EDTA, 10 mg/ml bromophenol blue and 10 mg/ml xylene cyanol).

(3) Denaturing polyacrylamide gel electrophoresis and autoradiography

6% denaturing polyacrylamide gel (20cm x 50cm x 0.4cm) with 7M urea was prepared in 1X TBE buffer and cast in a sequencing gel apparatus (Biorad) as before. Samples were denatured by heating at 95°C for 5 minutes and quenched on ice for few minutes then loaded (5 µl) alongside a labelled ladder (1 kb ladder) and electrophoresed at 2000 constant volts for approximately 3 hours (until the bromophenol blue dye reached the bottom of the gel). Gel was dried and autoradiographed for 12 to 16 hours as before.

2.8 Sequencing of the PCR products

After screening for point mutations or minor sequence alterations using either SSCP or CCM analysis, samples that gave positive screening results were subjected to sequencing of the PCR products from the corresponding exon(s) or cDNA segments to detect any sequence alterations. Three different sequencing strategies were used in this study. (1) Direct sequencing of the asymmetric PCR products, (2) PCR sequencing using PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer) and (3) Sequencing of cloned PCR products. Any alterations in the normal sequence were confirmed by sequencing of the opposite DNA strand.

2.8.1 Direct sequencing of the asymmetric PCR products

In this technique asymmetric PCR amplification of the genomic DNA or cDNA template to be sequenced was followed by dideoxynucleotide sequencing of

the PCR products (Sanger et al., 1977) using US Biochemicals sequanase version 2.0 kit.

(A) Asymmetric PCR

Asymmetric PCR was performed according to the protocol developed by Mgone et al. (1992). The templates used for asymmetric PCR reaction were the PCR products of the corresponding regions. DNA or cDNA was amplified normally by PCR. PCR products were resolved by agarose gel electrophoresis and bands were cut out of the gels, placed in 1.5 ml Eppendorf tubes with 50 µl TE buffer and frozen at -20^{o}C for overnight. 5 µl aliquots were then used for asymmetric PCR amplification using standard protocol of 40 cycles, unequal primer ratios (1/50 to 1/100) and the same conditions as symmetric PCR for that region. Two PCR reactions were performed for each segment where the second reaction used a reversed primer ratio so that both single strands could be generated. 5 µl aliquots from each reaction was tested by agarose gel electrophoresis alongside a DNA size marker and a double stranded control PCR. The remaining PCR volumes were cleaned prior to sequencing to remove excess dNTPs, salt and primers. Equal volume of 4M ammonium acetate and two volumes of isopropanol were added to each reaction, mixed by brief vortexing and incubated at room temperature for 10 minutes then centrifuged in microcentrifuge at full speed (~14000 g) for another 10 minutes. Pellets were washed in 70% ethanol, air dried and resuspended into 7 µl of ddH_2O and used directly in sequencing reaction.

(B) Sequencing protocol using USB sequenase version 2.0 sequencing kit

T7 DNA polymerase (sequenase version 2.0) was diluted by mixing the whole amount of the enzyme (25 µl) with 25 µl inorganic pyrophosphatase and 150 µl of glycerol enzyme dilution buffer. This diluted the polymerase 8 folds to its working concentration of 1.6 units/µl.

7 µl of the cleaned asymmetric PCR product was mixed with 2 µl 5X sequenase buffer to give a final concentration of 40 mM Tris-HCl pH 7.5, 50 mM Na Cl and 20 mM $MgCl_2$ and 1 µl (1 pmol) of sequencing primer complementary to the synthesised single strand. Primer concentration was determined using the arbitrary formula: Concentration (pmol/µl)= primer OD at 260 nm/ (0.01 x number of primer bases). The tube was vortexed and centrifuged briefly then incubated at 65°C in a PCR block for 2 minutes and slowly cooled to less than 35°C over 15 to 30 minutes then quenched on ice for few minutes. To each tube 1 µl of 0.1M DTT, 2 µl of a 1/5 dilution of labelling mix (1.5 µM of each dNTP except dATP), 0.5 µl of [α-^{35}S]dATP (1000 Ci/mmol; 10µCi/µl) and 2 µl of a 1/8 dilution of T7 DNA polymerase were added, mixed well, briefly centrifuged and incubated at room temperature for 2-5 minutes. Once the labelling reaction was completed 3.5 µl of the mix was transferred to a prewarmed (at 42°C) 2.5 µl of each termination mix and continued the incubation at 42°C for 5 minutes. There were four termination mixes contained 80 µM of each dNTP and 8 µM of the appropriate dideoxyribonucleoside triphosphate (ddNTP). The reaction was then terminated by the addition of 4 µl formamide dye stop solution. Tubes were then stored at -20°C until further need.

(C) Gel electrophoresis

8% denaturing polyacrylamide gels containing 7M urea were prepared and run in 1X TTE buffer (glycerol tolerant buffer) as before (2.7.2). Gels were pre-run for variable periods of time (30 to 60 minutes) at 2500 volts to heat it to 50°C then the volts was reduced to keep this temperature constant. Samples were denatured at 75°C for 3 minutes, quenched on ice and 2.5 µl from each termination reaction was loaded. Electrophoresis was carried out at 50°C for 2-4 hours depending how far was the area of interest. After electrophoresis the gel was taken out and dried in a vacuum gel dryer then exposed to Kodak X-Omat AR film using a cassette with intensifying screens for 16 to 40 hours at -40°C.

(D) Sequencing close to the primer

To read sequences close to the primer (few bases) some modifications were done in the sequencing protocol. One of these was to add 1 µl of Mn buffer (0.15M Na isocitrate, 0.1M $MnCl_2$) to the labelling mix before adding sequenase enzyme. Mn reduces the average length of DNA synthesised in the termination step, intensifying the sequencing ladder close to the primer. Mn buffer is supplied with the USB sequencing kit. Other modification was to use a higher dilution of the labelling mix (1/10 or 1/20) instead of the ordinary 1/5 dilution. It was important to have a sufficient quantity of the PCR template and to double the amount of the sequencing primer to read sequences close to the primer. The gel running time of such samples was around two hours.

(E) Sequencing far from the primer

To read sequences more than 200 bp from the primer it was essential to make some modifications in the sequencing protocol. These changes were recommended by the 'Step-by-Step' protocol 9th edition supplied with the sequanase version 0.2 kit, by using more nucleotides in the labelling reaction, by adding undiluted labelling mix and by lengthening the labelling time to 5 minutes. Altering the nucleotide mixture in the termination mix was another recommendation. This was achieved by using the sequence extending mix. 1.5 µl of the termination mix was mixed with 1 µl of the extending mix to replace the usual 2.5 µl termination mix. Gels were usually run at a lower temperature (40^{o}C) for a longer time (8 hours).

2.8.2 Automated sequencing of PCR products using PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkin Elmer)

(A) DNA preparation and cycle sequencing

Double stranded PCR products were obtained from areas of interest as mentioned before. The products were cleaned using the Geneclean™ kit (2.7.5) or by ethanol precipitation (2.7.4). A 10.5 µl PCR mix was made containing 2 µl of the cleaned PCR product, 3.2 pmol of one of the primers used for the initial PCR and ddH_2O and kept on ice. 9.5 µl of the terminator premix (Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, Perkin Elmer) was added to each 10.5 µl PCR mix on ice, mixed well, overlaid with one drop of mineral oil (~40 µl) and placed on 96°C preheated PCR block (Perkin Elmer/Cetus PCR model 480). Immediately a 25 PCR cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes was started. The PCR cycles were then followed by a rapid thermal ramp to 4°C and held.

(B) Purification of the extension products

At the end of thermal cycling 80 µl of ddH_2O was added to each 20 µl reaction volume under the oil and mixed by pipetting. The whole 100 µl volume was transferred from under the oil to a fresh 0.5 ml Eppendorf tube. The terminators were extracted two times with 100 µl phenol:water:chloroform (68:18:14) at room temperature. The extension products were precipitated by adding 15 µl of 2M Na acetate, pH 4.5, and 300 µl of absolute ethanol followed by incubation at -70°C for 15 minutes and centrifugation in microcentrifuge at full speed for another 15 minutes at room temperature. Pellets were washed in 70% ethanol and vacuum centrifugation dried for 5 minutes.

(C) Running of the sequencing reaction on the automated sequencer (ABI Prism 373 automated sequencing apparatus)

6% denaturing polyacrylamide gels with 8M urea was prepared and run in 1X TBE buffer according to the parameters suggested by the ABI. Gel plates were carefully cleaned to avoid smearing and background noise which could interfere with data analysis. The plates were scanned for any signal artifacts caused by dirt. If such artifacts were present, the gel-scanning area was cleaned once with a damp tissue then re-scanned. The gel was pre-run before loading for 10 minutes and re-scanned for signal artifacts as before. 4 μl of gel loading mix was added to each sample pellet. Samples were denatured at $92^{O}C$ for 2 minutes, rapidly quenched on ice then loaded into the gel.

(D) Result analysis

Analysis of the results obtained from each gel run was done by the Mackintosh computer attached to the prism 373 DNA sequencer system using 373A software version 1.2.1 (ABI). The sequencer which has a fluorescence detection system sends the collected data to the computer. The computer processes the data and presents it as a chromatogram with four coloured peaks, each representing one of the four bases (A,T,C & G). Base designation is also given above each peak. Heterozygosity is represented by two superimposed peaks and designated as an 'N'. Heterozygosity was always confirmed by sequencing the other DNA strand.

2.8.3 Sequencing of cloned PCR products

(A) Cleaning of the PCR products

PCR products were obtained as previous. To prepare the PCR products for cloning, each product was mixed thoroughly with SDS and EDTA, pH 8.0 to a final concentrations of 0.5% and 5 mM respectively in a 0.5 ml microcentrifuge tube. Proteinase K was added to each tube to a final concentration of 100 μg/ml and the

tubes were incubated for 30 minutes at 56°C. DNA was precipitated by 0.1 volume 3M Na acetate, pH 7.0 and 2 volumes ethanol at -20°C for 15 minutes. DNA was recovered by centrifugation at 12000 g for 15 minutes at 4°C in a microcentrifuge. Pellets were washed with 70% ethanol at 4°C and recentrifuged. Pellets were then air dried and redissolved in 30 µl sterile T.E. buffer, pH 7.6.

(B) PCR cloning using pGEM®-T vector systems (Promega)

The pGEM®-T vector is prepared by cutting Promega's pGEM®-5Zf(+) vector with *Eco*R V and adding a 3' terminal thymidine to both ends. Ligation of these single 3'-T overhangs at the insertion site takes advantage of the non-template dependent addition of a single deoxyadenosine to the 3' end of PCR products by certain thermostable polymerases. A 1:1 molar ratio of the pGEM®-T vector to a PCR product was used in the ligation reaction. The pGEM®-T vector is about 3 kb in length and to calculate the appropriate amount of PCR product the following equation was used:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ x insert:vector molar ratio} = \text{ng of insert}$$

Following the protocol supplied by Promega, the pGEM®-T vector and pGEM®-T vector control DNA tubes were briefly centrifuged to collect contents at the bottom of the tube. A 10 µl ligation reaction was set up in two 0.5 ml microcentrifuge tubes, one tube for the control reaction and the other for the standard reaction as follows:

	Standard Reaction	Control Reaction
T4 DNA ligase 10X Buffer	1 µl	1 µl
pGEM®-T vector (50 ng)	1 µl	1 µl
PCR product (or pGEM®-T vector control)	x µl	2 µl
T4 DNA ligase (1 Weiss unit/µl)	1 µl	1 µl
dH_2O to a final volume of	10 µl	10 µl

The reactions were incubated overnight at 15°C and 2 µl of each reaction was used for the transformation reaction as described in 2.7.1 with only one difference. The transformation culture was grown on LB/ampicillin/IPTG/X-gal plates equilibrated to room temperature prior to plating. 100 µl of 0.1M IPTG and 20 µl of 50 mg/ml X-Gal were spread over the surface of LB/ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use. Insertional inactivation of the α-peptide coding region of the enzyme β-galactosidase of the ligation vector allowed recombinant clones to be directly identified (white colonies) by colour screening on indicator plates. Colonies containing β-galactosidase activity (usually has no insertion of the PCR fragment) will produce blue colonies.

(C) Isolation of recombinant plasmid DNA

Recombinant plasmid DNA was grown up and isolated using INSTA-PREB™ kit as described in 2.7.1 with only one modification where the plasmid DNA was extracted with an additional 200 µl of PCI solution. After the first PCI extraction, 200 µl of PCI solution was added to the spun tubes, mixed briefly by repeated inversion and spun again. This was followed by the standard addition of 300 µl of CI solution and the standard protocol was continued. Recombinant plasmid DNA was isolated from at least 10 single white colonies for each target PCR DNA.

(D) Double stranded sequencing of the recombinant plasmid DNA

Prior to sequencing, using the USB sequenase version 2.0 sequencing kit, 4 µg of plasmid DNA was alkaline-denatured. 20 µl of plasmid DNA (~4 µg) was mixed with 2 µl of 2M NaOH/2mM EDTA solution and incubated at 37°C for 30 minutes. Denatured DNA was then precipitated by 0.1 volume 3M Na acetate, PH 4.5-5.5 and 3 volumes 100% ethanol at -70°C for 15 minutes. DNA was recovered by centrifugation in a microcentrifuge at full speed for 15 minutes. Pellets were washed with 70% ethanol, air dried and redissolved in 7 µl ddH_2O. Sequencing and sequence

analysis were carried out as described before (2.8.1). For each insert-PCR, sequencing of 10 colonies was carried out and any sequence abnormalities were confirmed by sequencing the opposite DNA strand.

CHAPTER THREE

RESULTS

(3) RESULTS

3.1 Screening for the CTG repeat expansion in DM families

3.1.1 Radiolabelled PCR amplification of the DNA sequence containing the $(CTG)_n$ repeat

The status of the DM locus was surveyed in 49 DM families (86 DM patients and 96 apparently normal individuals). All the families were ascertained by clinical diagnosis of the proband; an expanded DM allele was identified afterwards in each of these individuals. Primer set 409/410 (Mahadevan et al., 1992) was used and the PCR conditions were optimised to amplify the region of the myotonic dystrophy protein kinase (*DMPK)* gene that contains the unstable CTG repeat sequence. ^{32}P dCTP was used in the PCR reaction mix to internally label the PCR products. The products of the PCR reactions were resolved in 8% denaturing polyacrylamide gels. Gels were exposed to Kodak X-AR5 film and autoradiographed at -70° C for 16 hours. PCR amplified products from subjects of the same family were electrophoresed in the same gel to avoid electrophoresis variability.

The size of the amplified products from the normal chromosome varies between 64 bp (for the 5 repeats) and 140 bp (for the 37 repeats). Expanded alleles containing up to ~83 repeats could be amplified from DM chromosome. The expanded alleles always show a multiple band pattern which may reflect somatic mosaicism or stuttering during the PCR reaction (Figure 3.1)

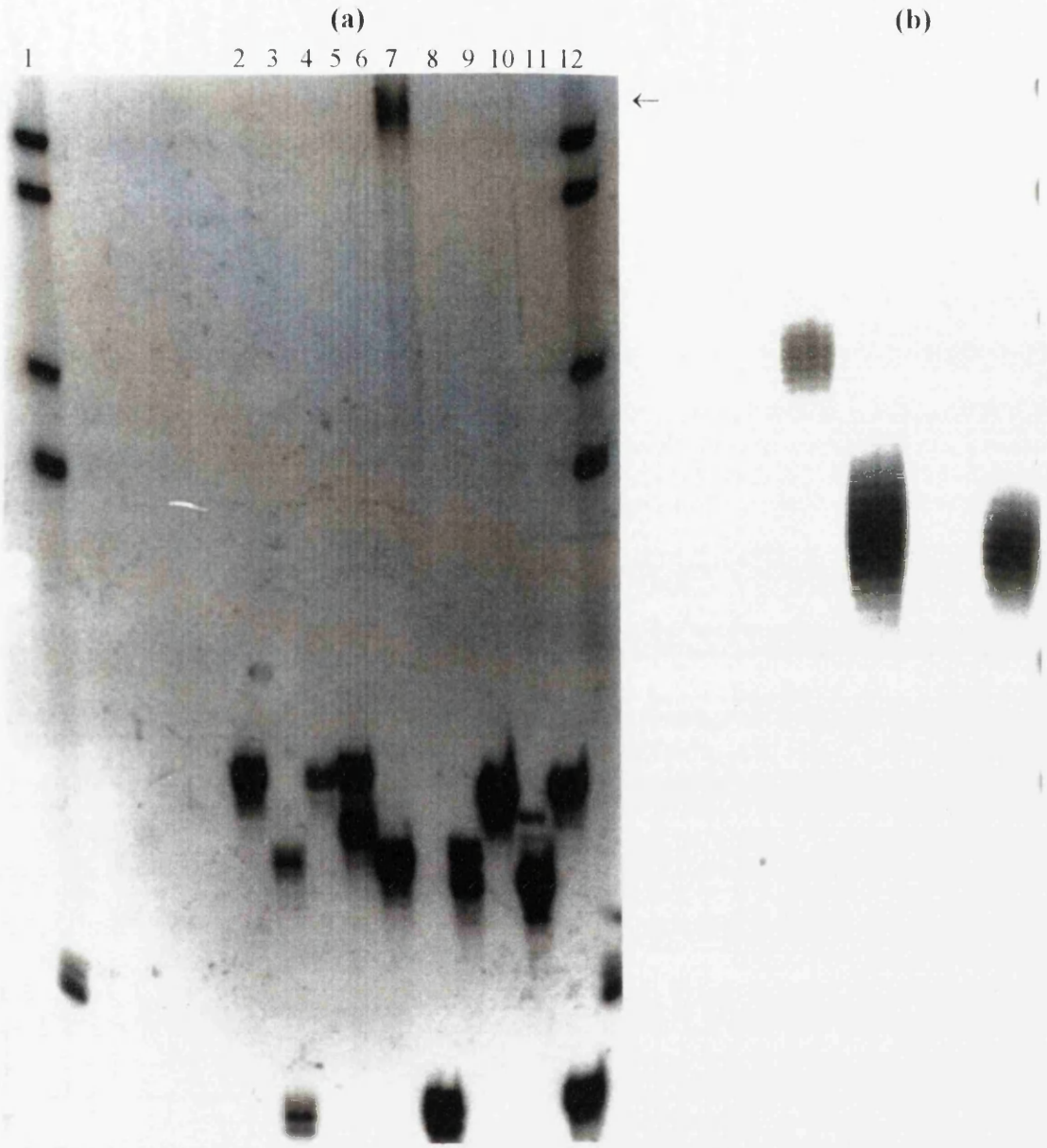


Figure 3.1

(a) Size distribution of CTG repeats in a DM family using ^{32}P-labelled PCR products analysed by denaturing polyacrylamide electrophoresis. Lanes 1 and 12 are radiolabelled 1 kb ladder. Lanes 2, 4, 6, 9 and 10 show PCR products from individuals with one allele (either normal homozygous for that allele or heterozygous with the second allele too large to amplify). Lane 7 shows PCR products from a DM patient with one normal allele (5 repeats) and one expanded (~ 59 repeats) allele (arrow).

(b) Zoom-in of some CTG expanded alleles to show the multiple band pattern of amplification.

3.1.2 Non-radiolabelled PCR amplification of CTG repeats

Non-radiolabelled PCR amplification of DNA sequences containing CTG repeats was optimised in a subset of DM families using primer set 406/409 (Mahadevan et al., 1992). PCR products were resolved in 1.5% agarose gels stained with ethidium bromide then visualized and photographed under UV light. The size of the amplified products from the normal chromosome varied between 149 bp (for the 5 repeats) and 245 bp (for the 37 repeats). Expanded alleles containing up to 90 repeats were detected (Figure 3.2).

3.1.3 Southern analysis to detect CTG expansion in DM chromosome

CTG repeats of more than 90 repeats are not usually visible upon PCR amplification although the unaffected alleles are readily amplified. These large expansions can be seen on Southern blots of *BglI* digested genomic DNA hybridized with the p5B1.4 genomic probe. Some of the expanded alleles were seen as diffuse hybridization signals (Figures 3.3 & 3.4).

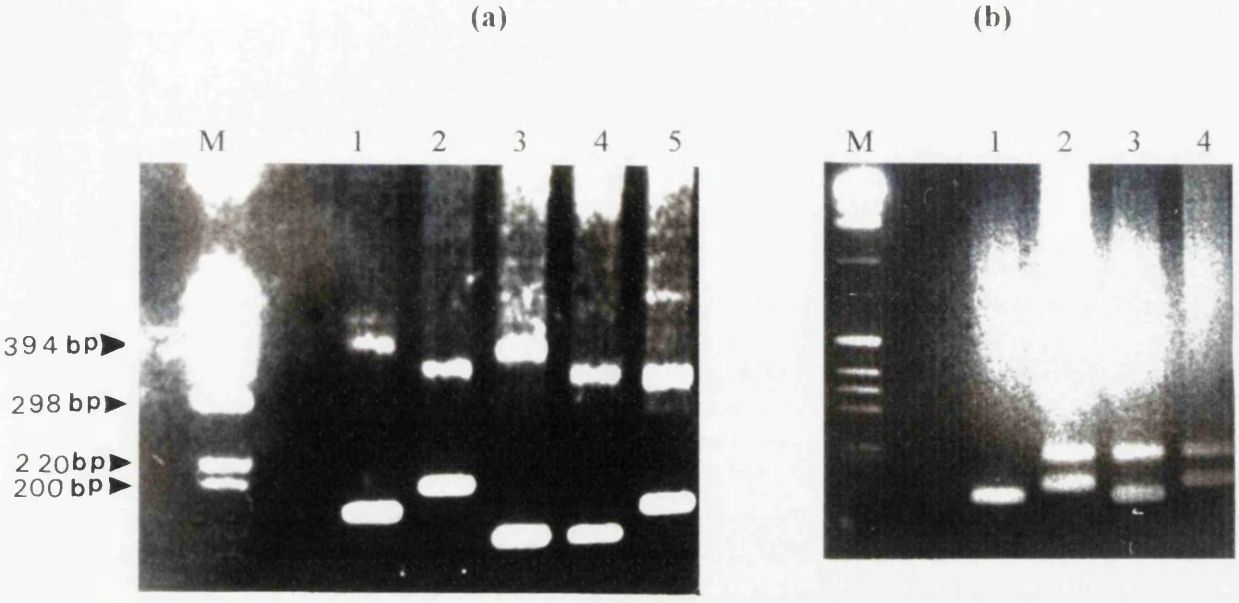


Figure 3.2

Example of PCR products resolved in 1.5% agarose gels and stained with ethidium bromide. **(a)** Products obtained by PCR amplification of DNA from DM patients with minimal symptoms. The upper bands represent expanded DM alleles (lane 1= ~90 repeats, lane 2= ~71 repeats, lane 3= ~87 repeats, lanes 4&5= ~70 repeats). The lower bands are the normal alleles in these individuals (~15, 25, 5, 5 and 21 repeats respectively).

(b) Products obtained by PCR amplification of DNA from a DM family. Lane 1 shows products from an individual with one normal allele (~5 repeats, either normal homozygous for that allele or heterozygous with the second allele too large to amplify). The other lanes(2-4) show normal heterozygous individuals (~13/28, 5/28 and 13/28 repeats respectively). M= 1 kb ladder.

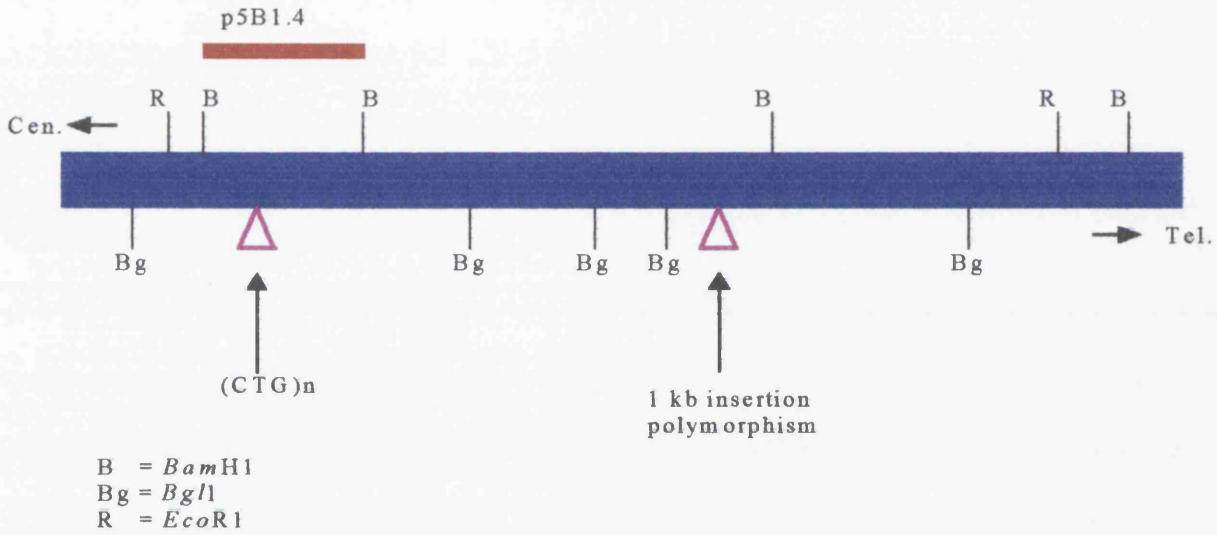


Figure 3.3

A restriction map of the region containing the CTG polymorphism, showing the position of the genomic probe p5B1.4 and the relevant restriction sites (Shelbourne et al. 1992).

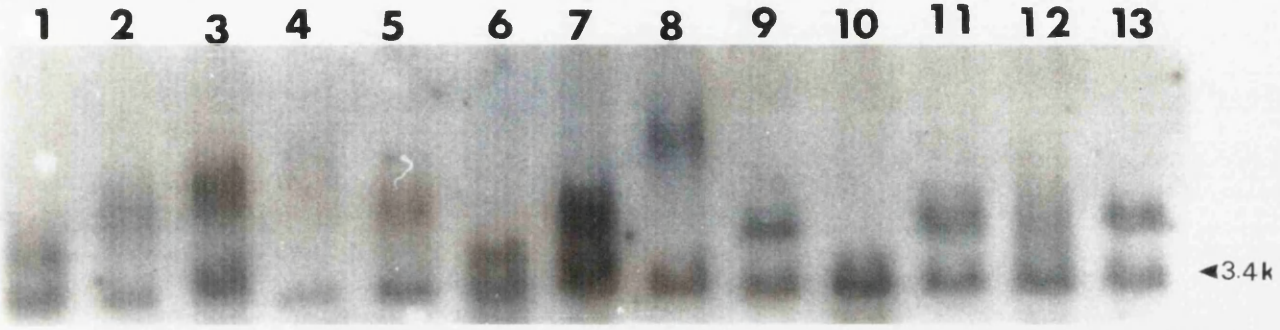


Figure 3.4

Example of Southern blot analysis, using *Bgl*I enzyme and the p5B1.4 probe, showing varying degrees of allelic expansion (upper bands) in DM-affected individuals. The lower band (~3.4 kb) shows the normal size allele in these individuals. Lanes 1,2 and 3 are from affected siblings with variable degrees of allelic expansions (~330, 1200 and 1320 repeats respectively). Lanes 8 and 9 are from a father (lane 8) carrying an expanded allele of ~1900 repeats and a daughter carrying ~580 repeats (lane 9) where a reduction in repeat size occurred during paternal transmission. Lanes 10, 11 and 12 are from a father (lane 12) with a smear of expansion (~370 repeats), a daughter (lane 11) with an expansion of ~400 repeats and a granddaughter (lane 10) who did not inherit the expanded maternal allele, she only carries normal alleles of ~3.4 kb.

3.1.4 Size of the expanded CTG repeat sequence in relation to phenotype

The clinical status of the DM patients was classified according to the method of Harley et al. (1992b). Five categories of the patients can be recognized (1) mild disease, in individuals presenting in later life with minimal features of the disease (e.g. cataracts) and asymptomatic obligate carriers (grandparents); (2) classic adult-onset type; (3) childhood type; (4) young carriers without symptoms and (5) congenitally affected individuals. In all of the studied patients the DM expansion mutation was associated with the disease. Table 3.1 shows the range of repeat sizes in each clinical category, separated by the sex of the individual. The only obvious difference observed between the sexes was in the minimally affected group, with an excess of males having the smallest repeat size (<0.25 kb; ~85 repeats), which is not statitstically significant ($p>0.05$). The correlation between the disease severity and CTG repeat size is weak in spite of the general trend of the more severe the phenotype, the larger the repeat size ($r = 0.42$, $p < 0.01$, 95% confidence limits = 0.23 to 0.58) and overlapping between the various clinical groups does exist (Figure 3.5).

Figure 3.6 shows the relationship of repeat size to apparent age at onset. A significant correlation was observed when repeat size was plotted on a logarithmic scale ($r = -0.62$, $p < 0.001$, 95% confidence limits = -0.48 to -0.75).

Table 3.1 Range of CTG repeat sizes in kilobase (kb) among different clinical categories of myotonic dystrophy (DM) patients divided by the sex of the affected individual.

	Male	Female
Minimal Change DM		
0.15-0.25 kb	9	3
0.25-0.35 kb	1	1
Classical DM		
0.25-2.0 kb	14	18
2.0-3.5 kb	4	7
3.5-6.0 kb	5	5
Childhood onset DM		
1.1 kb	2	-
3.0 kb	2	1
Young asymptomatic DM		
0.5-1.4 kb	3	2
1.5-2.0 kb	1	1
Congenital DM		
1.7-2.9 kb	1	2
2.9-6.0 kb	2	2

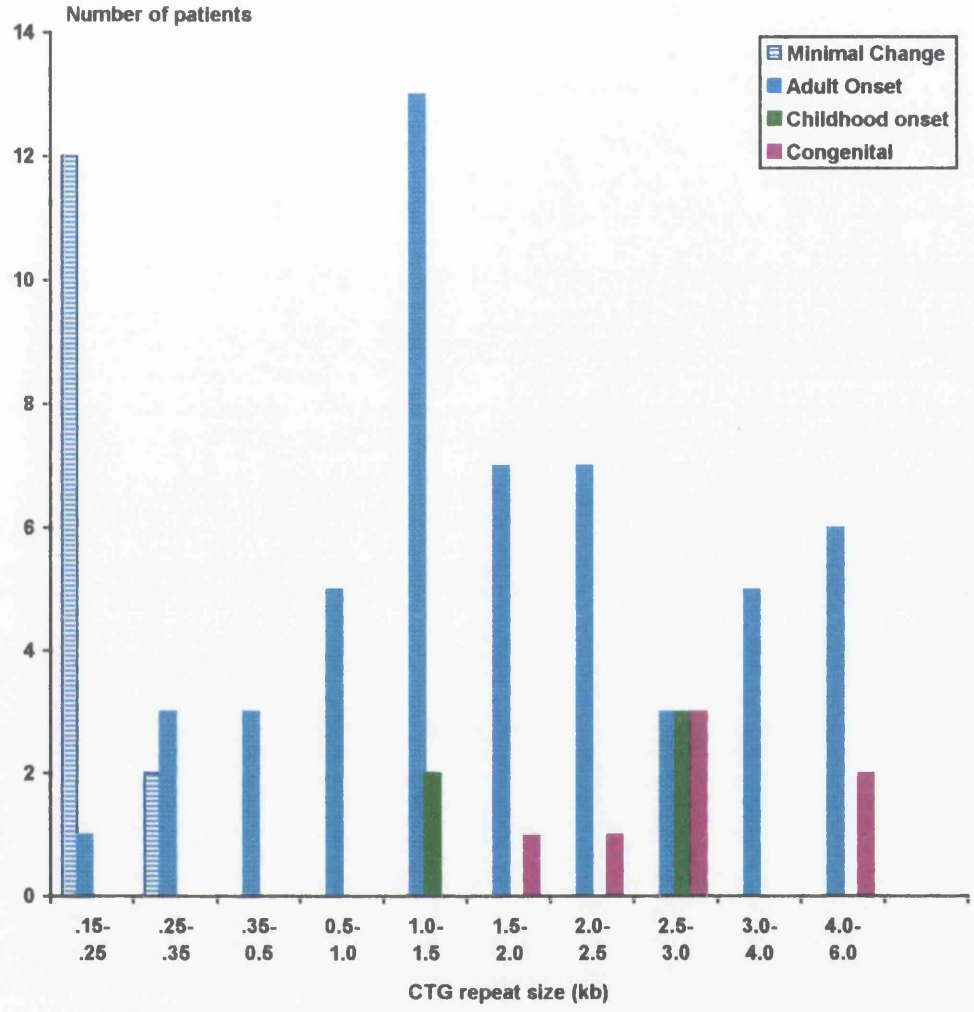


Figure 3.5

Size distribution of the CTG repeat expansion in different DM phenotypes. An overlap between different clinical groups is clearly present.

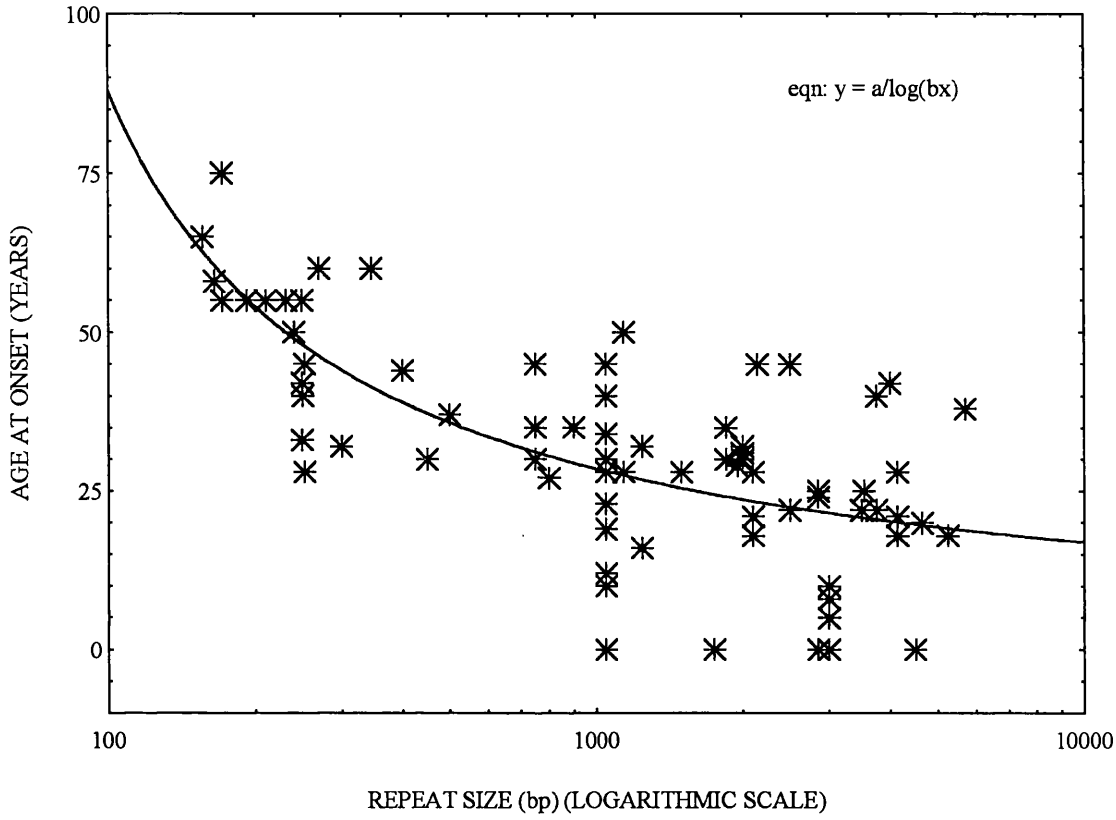


Figure 3.6

Age at onset for DM patients, plotted against CTG repeat length (logarithmic scale)

3.1.5 Intergenerational instability of the CTG repeats

To study the intergenerational instability of the CTG repeats 31 parent-child pairs, 10 maternal-child and 21 paternal-child pairs, were analyzed. In most cases there was an increase of the repeat size during parental transmission with only two exceptions (Figure 3.7 and Figure 3.8)

There were two cases where the repeat size has decreased during paternal transmission. The first one (Figure 3.9) showed a decrease from the paternal 5.7 kb (1900 repeats) to 1.7 kb (~565 repeats). The second one (Figure 3.10) showed a decrease from the father (DNA tested in another laboratory, and shown to have a moderate expansion, but was not available for this study) to his daughter (0.27 kb; 90 repeats). The CTG repeat inreased again during the transmission to the grand son (350 CTG repeats) and the grand daughter (450 CTG repeats).

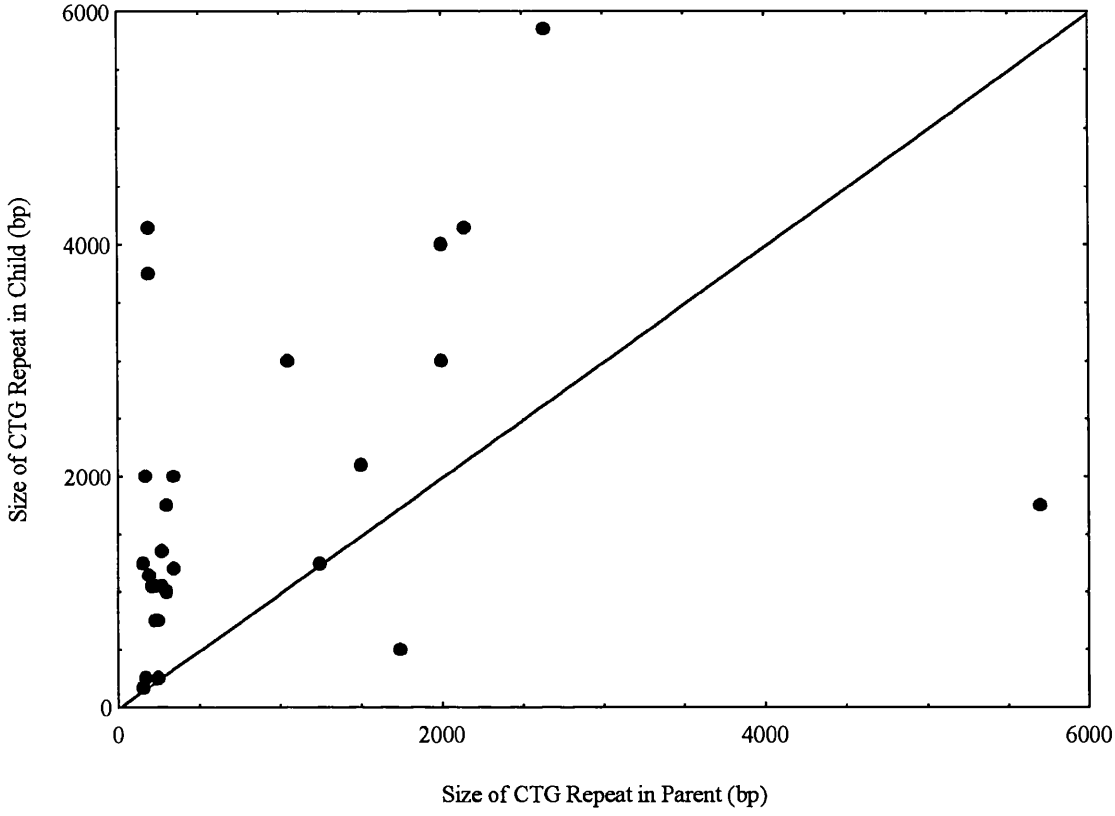


Figure 3.7

CTG repeat size for 31 parent-child pairs. Points above the diagonal indicate repeat size greater in child than in parent.

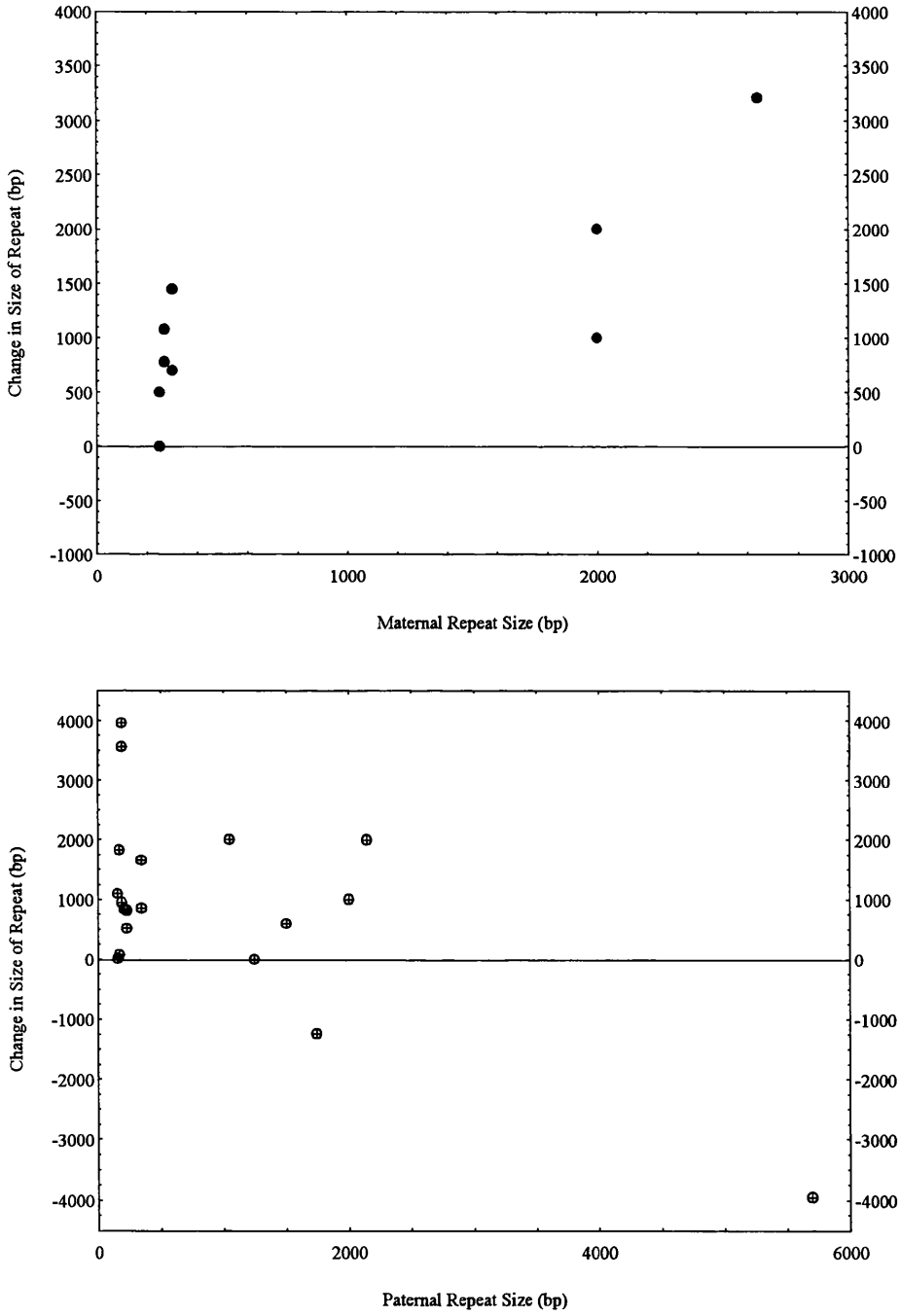
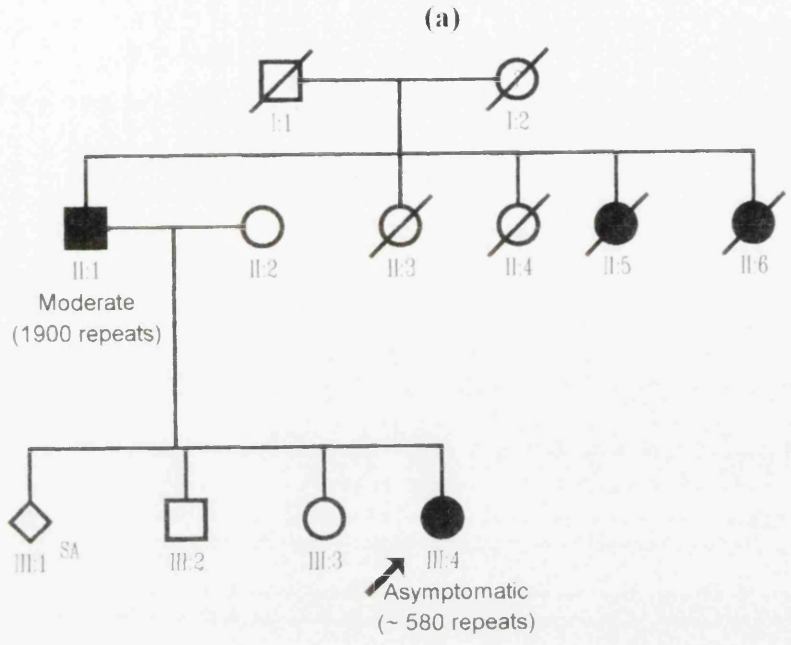


Figure 3.8

Change in repeat size (in bp) on transmission from mothers to 10 offspring (top) or on transmission from fathers to 21 offspring (bottom) plotted against repeat size of parent.



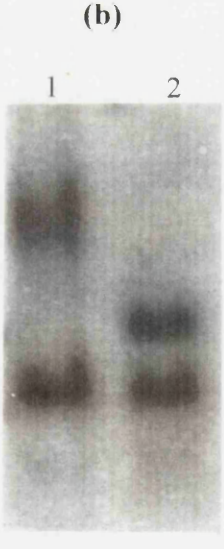


Figure 3.9

Reduction of the CTG repeat length during paternal transmission. **(a)** Family pedigree, **(b)** Southern analysis of the father, II:1 (lane 1, ~3.4/5.7 kb) and the daughter, III:4 (lane 2, ~3.4/5.14 kb) using *Bgl*I enzyme and p5B1.4.

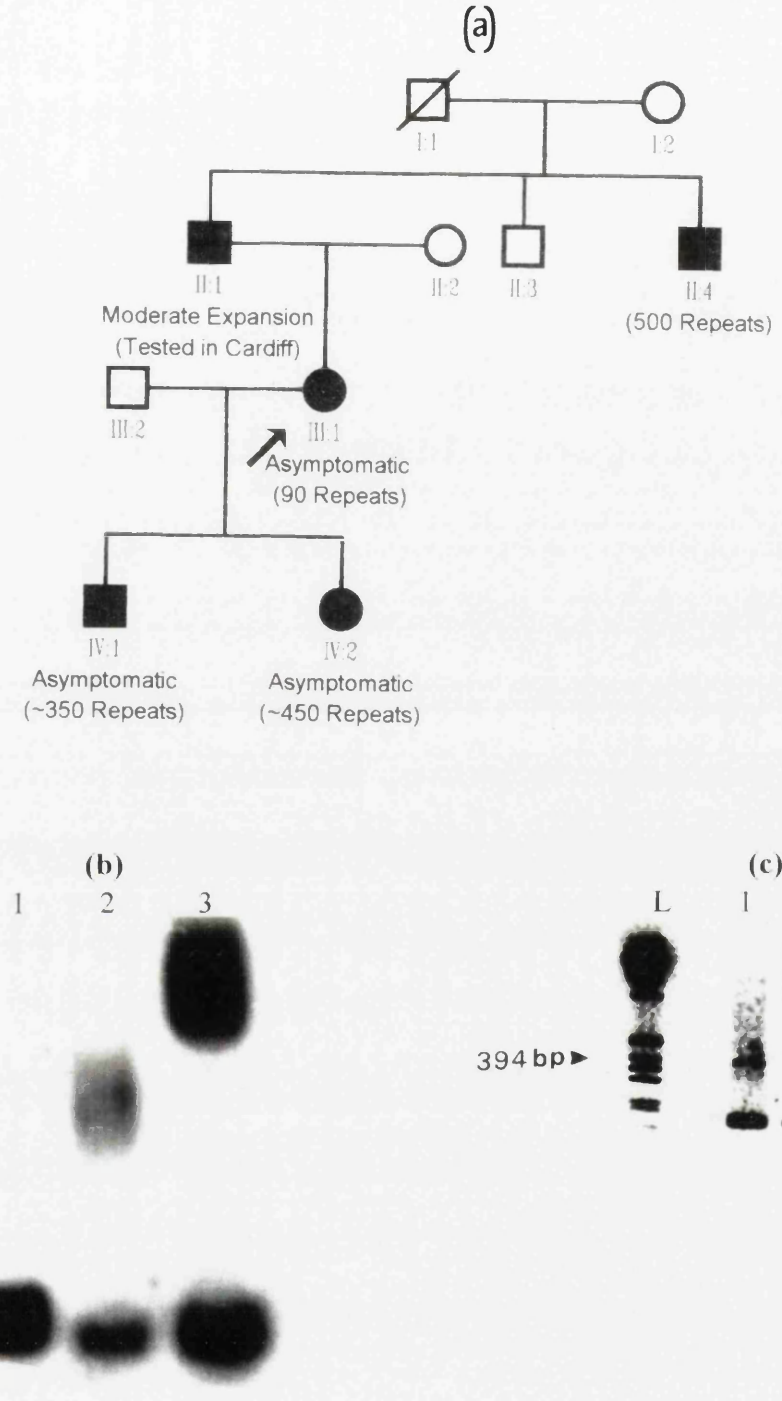


Figure 3.10

Reduction of the CTG repeat length during paternal transmission followed by increase of the CTG repeat upon transmission from the daughter to the grand offspring. **(a)** Family pedigree, **(b)** Southern analysis of III:1 (lane 1, ~3.4/3.7 kb), IV:1 (lane 2, ~3.4/4.4 kb) and IV:2 (lane 3, ~3.4/4.7 kb) using *Bgl*I enzyme and p5B1.4 probe. **(c)** PCR amplification of this family. Lane 1 shows III.1 alleles of 15 and 90 repeats, lanes 2 and 3 show IV.1 and IV.2 normal alleles (15 and 13 repeats) only, their expanded alleles are to big too be amplified by PCR. L = 1kb ladder.

One family showed a stable transmission of the CTG repeat in two generations (Figure 3.11a&b). The grand mother had cataracts at the age of 60 years, died at the age of 80 years from head injury, (DNA not available). Her daugther inherited an allele with 63 repeats (~0.2 kb) and had cataracts only at the age of 55 years. The latter had passed her 63 repeat (~0.2 kb) allele unchanged to her son and a slightly increased allele (73 repeats; ~0.22 kb) to her daughter.

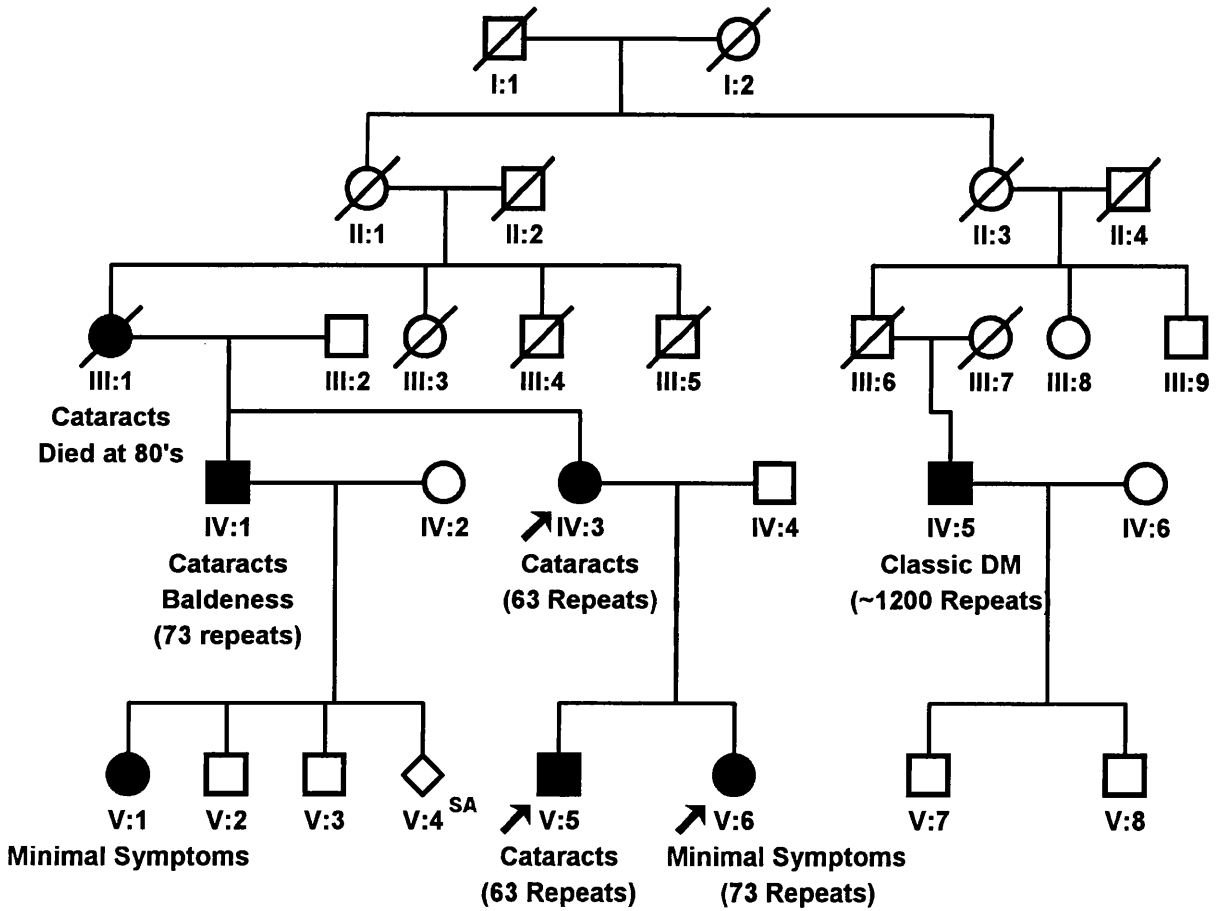


Figure 3.11a DM family shows stable transmission in one of its branches

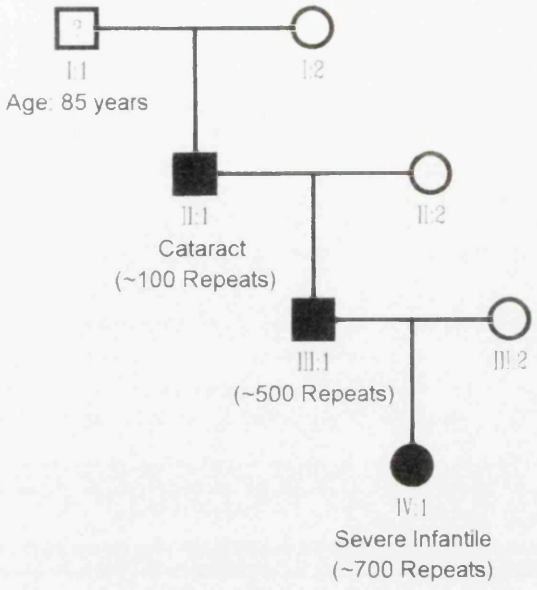
Figure 3.11b

^{32}P-labelled PCR product from the previous family (Figure 3.11a) showing the expanded alleles (arrows). Lane 1 (V:6), lane 2 (V:5) and lane 4 (IV:3). Lane 5 shows labelled 1 kb ladder.

3.1.6 Severe infantile myotonic dystrophy of paternal origin

A case of severely affected mentally retarded child with onset in the first year of life was found to be due to paternal transmission of an expanded CTG allele (500 repeats) to his daughter who carried a 700 CTG allele (Figure 3.12).

(a)



(b)

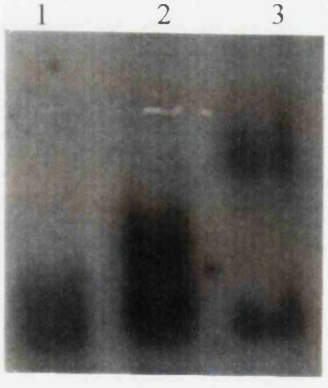


Figure 3.12

Paternal transmission of severe infantile onset DM. **(a)** Family pedigree. **(b)** Southern analysis of II:1 (lane 1, ~3.4/3.7 kb), III:1 (lane 2, ~3.4/4.9 kb) and IV:1 (lane 3, ~3.4/5.5 kb) using *Bgl*I enzyme and the p5B1.4 probe.

3.2 Screening of ALS patients for mutations in the *SOD-1* gene

3.2.1 PCR amplification of the *SOD-1* gene in ALS patients

DNA samples from 2 familial and 67 sporadic ALS cases were subjected to polymerase chain reaction (PCR) amplification of the all five exons of the *SOD-1* gene (Figure 3.13a). For exon 3 a primer set was designed using the computer program Oligo™ (Medprobe) and the PCR reaction was optimised by trying different concentrations of DNA, primers and dNTPs and by applying different annealing temperatures. Published primer sets (Rosen et al., 1993; Deng et al., 1993) were used for PCR amplification from the remaining exons (1, 2, 4, and 5) after optimisation of the published conditions. Non-radiolabelled PCR products from the five exons were checked on 1% agarose gel in 1X TBE buffer (Figure 3.13b).

3.2.2 Screening for the Ile113Thr mutation in ALS patients

Ile113Thr mutation creates a new restriction site for the enzyme *Bsr*I so that the PCR product from the normal *SOD-1* exon 4 allele will be digested into two fragments of 124 and 90 bp while the mutant one will be digested into three fragments of 113, 90 and 11 bp respectively (Rosen et al., 1993). This enzyme was used to digest the PCR amplification products of the *SOD-1* exon 4 from all the studied ALS

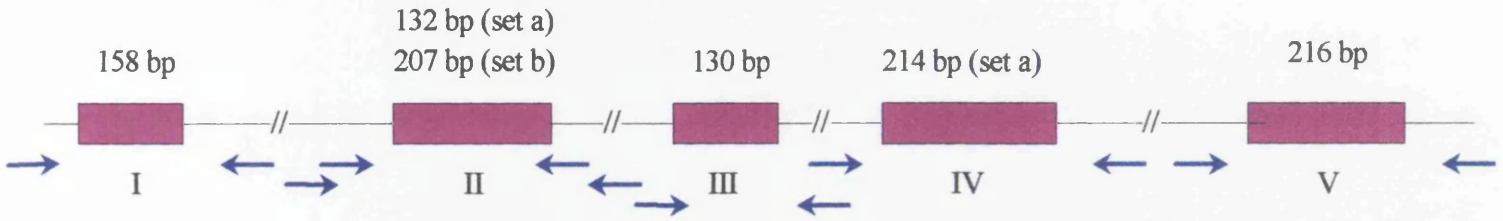


Figure 3.13a

Diagrammatic representation of the *SOD-1* gene. Filled boxes represent the five exons (numbered I to V) of the gene. The interrupted lines connecting the boxes represent the introns. The arrows represent the primer sets used for PCR-amplification of the *SOD-1* exons. Numbers above each exon represent the size of the PCR amplification products.

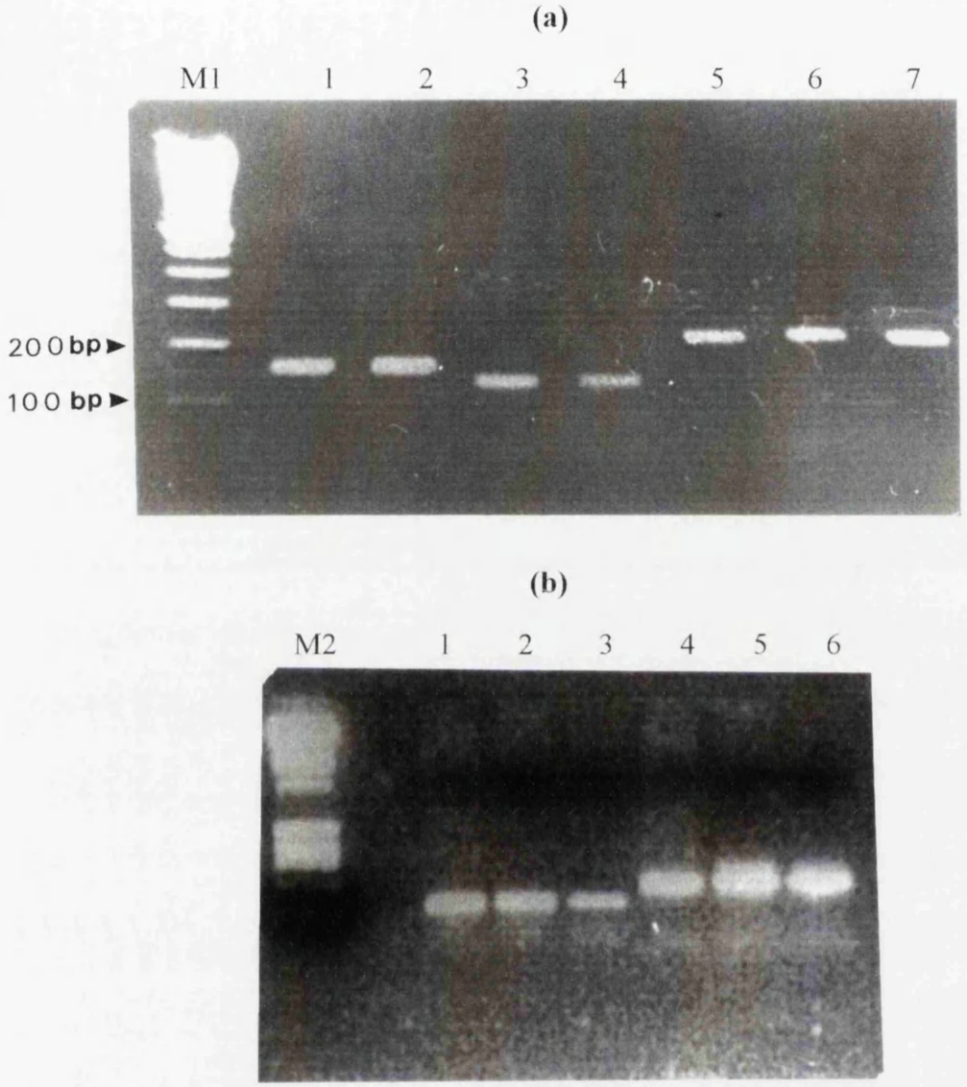


Figure 3.13b

PCR amplification of exons 1 to 5 in the *SOD-1* gene. **(a)** Amplification products from: exon 1 (lanes 1&2, 158 bp), exon 3 (lanes 3&4, 130 bp), exon 4 set a (lanes 5&6, 214 bp) and exon 5 (lane 7, 216 bp). **(b)** Amplification products from: exon 2 set a (lanes 1-3, 132 bp) and exon 2 set b (lanes 4-6, 207 bp). M1= 100 bp ladder, M2= 1 kb ladder.

patients. The digestion products were resolved by denaturing 8% polyacrylamide gel electrophoresis in 1X TBE buffer alongside a ladder marker for 4 hours at 280 constant volts then silver stained. Only normal digestion products were present in the screened patients (Figure 3.14).

3.2.3 Screening for the Ala4Val mutation in ALS patients

Ala4Val mutation abolishes a *Hae*III restriction site thus producing an additional larger restriction fragment of 49 bp in the heterozygous mutants. PCR amplifications of *SOD-1* exon 1 from all studied-ALS patients have been subjected to *Hae*III restriction endonuclease digestion. The digested products were resolved by running them on 8% denaturing polyacrylamide gel alongside a ladder marker for 2.5 hours at 280 constant volts then silver stained. This mutation could not be detected either in the two familial or the 67 sporadic screened ALS patients (Figure 3.14).

3.2.4 Screening for the CAG repeat expansion in exon 1 of the androgen receptor gene

CAG repeat expansion in the exon one of the androgen receptor gene has been reported in patients with spinal and bulbar muscular atrophy (SBMA, Kennedy disease) (La Spada et al, 1991). A primer set flanking the CAG repeat site in exon 1 of the androgen receptor gene (Allen et al., 1992) was used and the PCR conditions were optimised to amplify DNAs from sporadic ALS patients. The products of PCR amplification were resolved by running on 1.5% agarose gels in 1X TBE buffer. Amplification from the normal chromosomes gave PCR products of ~280 to 310 bp. Abnormal chromosomes, with CAG repeat expansion, produce PCR products of ~350 to 390 bp with no overlap between normal individuals and affected patients. No expansion of the CAG repeat could be detected in the tested (67) DNA samples (Figure 3.15).

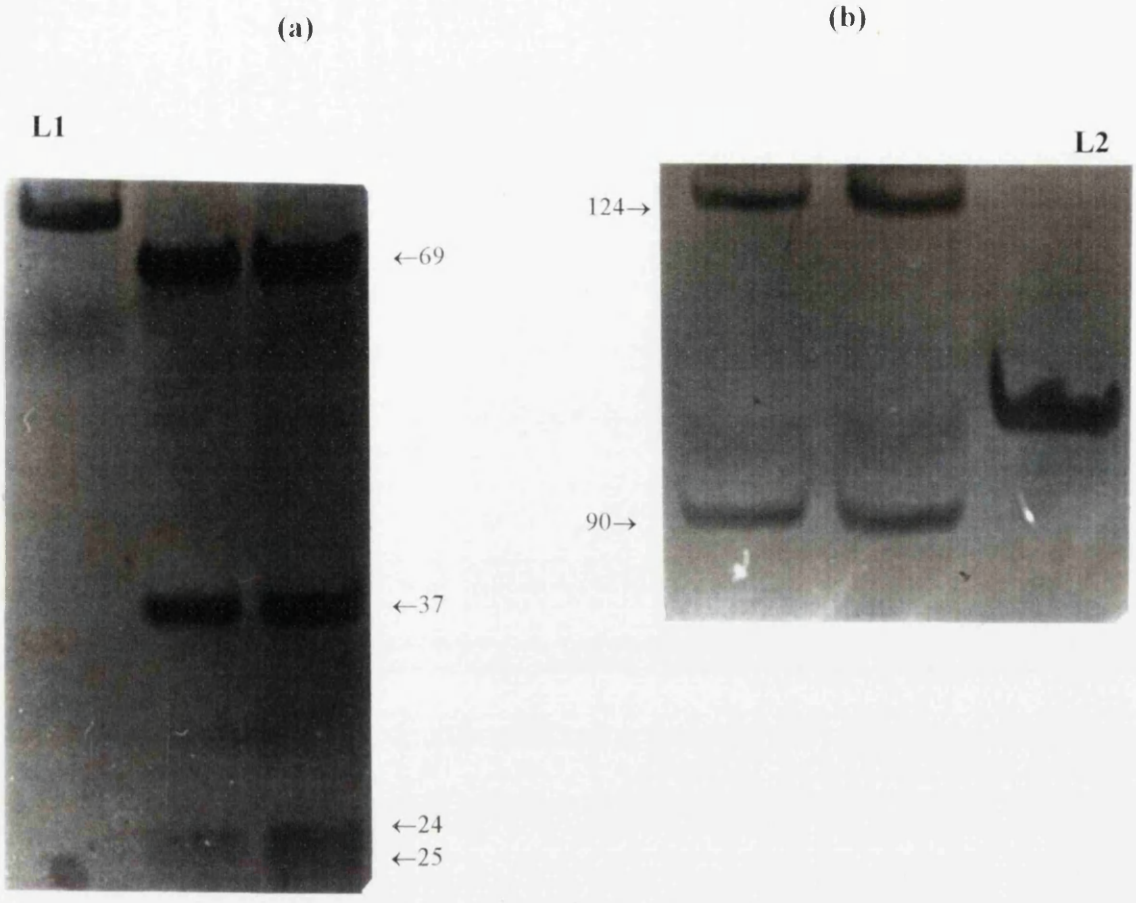


Figure 3.14

Screening for the Ile113Thr and the Ala4Val mutations. **(a)** Screening for the exon 1 Ala4Val mutation using restriction enzyme (*Hae*III) digestion of the PCR products. No abnormal (49 bp) product has been seen. L1 = 75 bp band of 1 kb DNA marker. **(b)** Screening for the exon 4 Ile113Thr mutation using restriction enzyme (*Bsr*I) digestion of the PCR products reveals a normal digestion pattern (no abnormal 113 bp product could be seen). L2 = 100 bp band of the DNA marker. Arrows point to the digestion products. Numbers beside the arrows indicate the size of the products in base pair (bp).

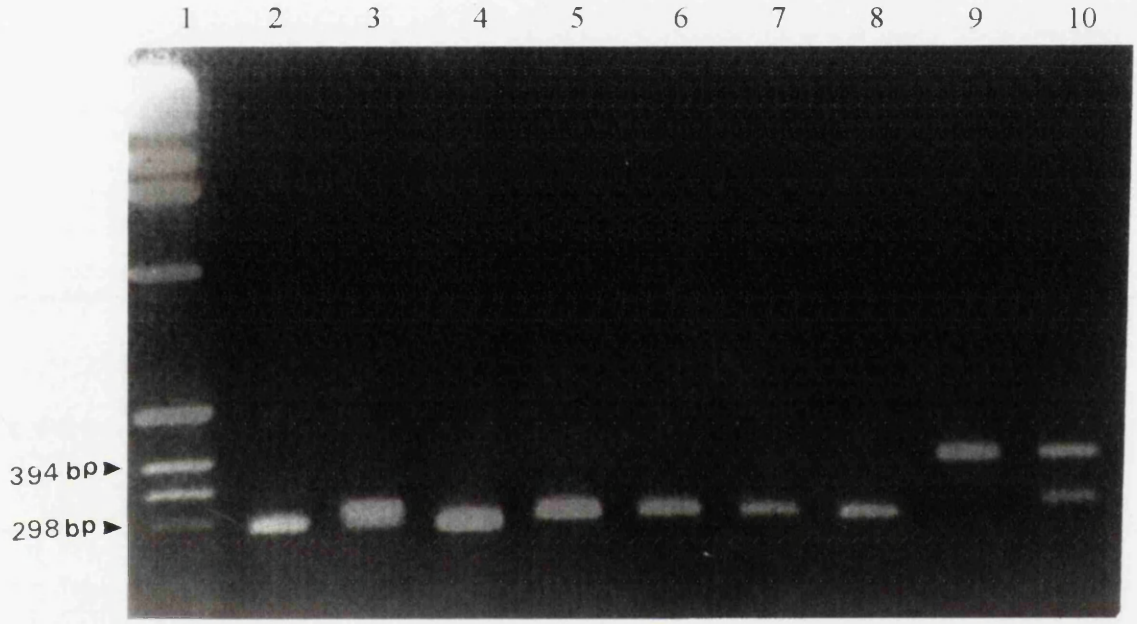


Figure 3.15

Screening for the CAG repeat expansion in exon 1 of the androgen receptor gene in sporadic ALS patients. No CAG repeat amplification could be detected in the screened patients (lanes 2 to 8). Lanes 9 and 10 show the CAG repeat expansion (~390 bp) in an affected Kennedy's disease male patient and in a female carrier (arrow). Lane 1 shows 1 kb DNA ladder.

3.2.5 PCR-SSCP screening for mutations in the *SOD-1* gene in ALS patients

In this technique radiolabelled PCR-amplified products were obtained from the five exons of the *SOD-1* gene. The *SOD-1* gene was screened in two familial and 67 sporadic ALS patients. With each radiolabelled-PCR set of reactions (20 reactions) two non-radiolabelled PCR reactions were done and tested on 1% agarose gel to be taken as a guide for the condition of the radiolabelled reactions. Aliquots of 1 µl PCR product were mixed with 9 µl SSCP loading mix, denatured by heating for 3 minutes at 95° C, quenched on ice for at least 5 minutes and then 3 µl of aliquots were loaded into: **(1)** 0.5 X MDE gel in 0.6 X TBE buffer. The gels were run at 5 watts for 16 hours at room temperature. **(2)** 8% non-denaturing polyacrylamide gel with the following conditions: ***(a)*** 5% glycerol, running at 5 watts for 16 hours at room temperature, ***(b)*** 10% glycerol, running at 8 watts for 12 hours at room temperature and ***(c)*** without glycerol, running at 12 watts for 12 hours at 4°C.

A patient from one of the two studied FALS families (Figure 3.16a and table 3.2) showed an altered migration of the *SOD-1* exon 4 PCR product on SSCP analysis. The same altered pattern was detected in all affected family members as compared with controls (Figure 3.16b).

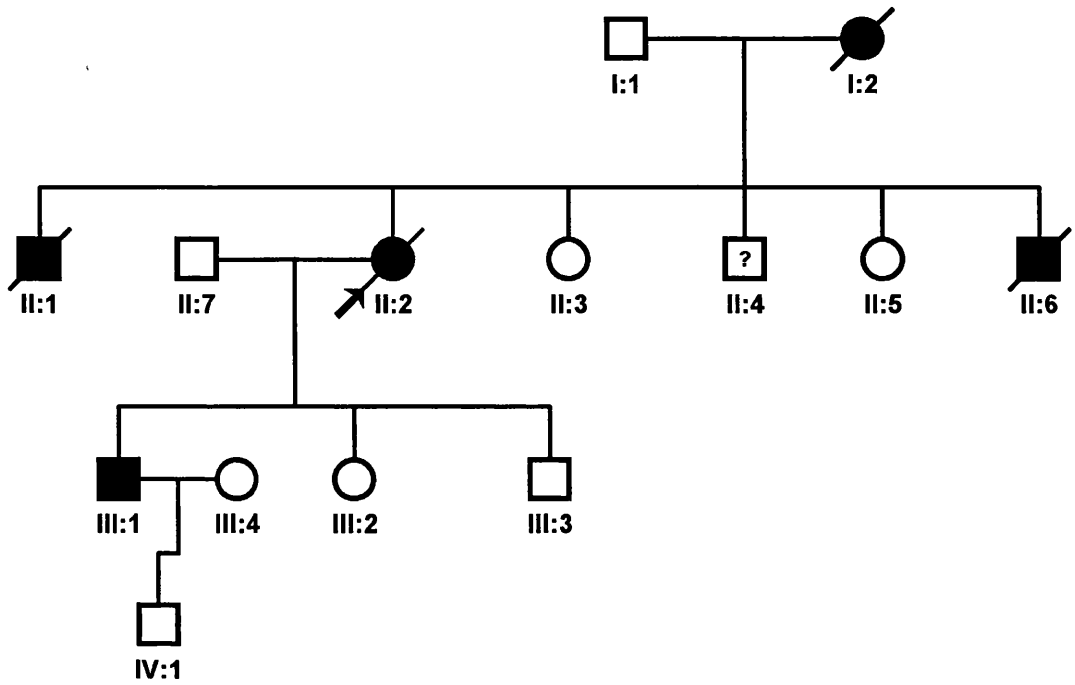


Figure 3.16a

Diagrammatic representation of the FALS family where an altered SSCP pattern of the *SOD-1* exon 4 has been observed in the affected family members. Arrow points to the proband. DNAs were tested from all the affected members. Individual II:4 showed some manifestations of ALS but refused to be further investigated.

Table 3.2

The age at onset, age at death and duration of the disease process in FALS family members who showed the Gly93Ser mutation in exon 4 of their *SOD-1* genes.

Patient's Number	Age at onset	Age at death	Duration of the disease
I.2	36 years	48 years	12 years
II.1	40 years	43 years	3 years
II.2	36 years	39 years	3 years
II.6	28 years	33 years	5 years
III.1	26 years	----------	1 year

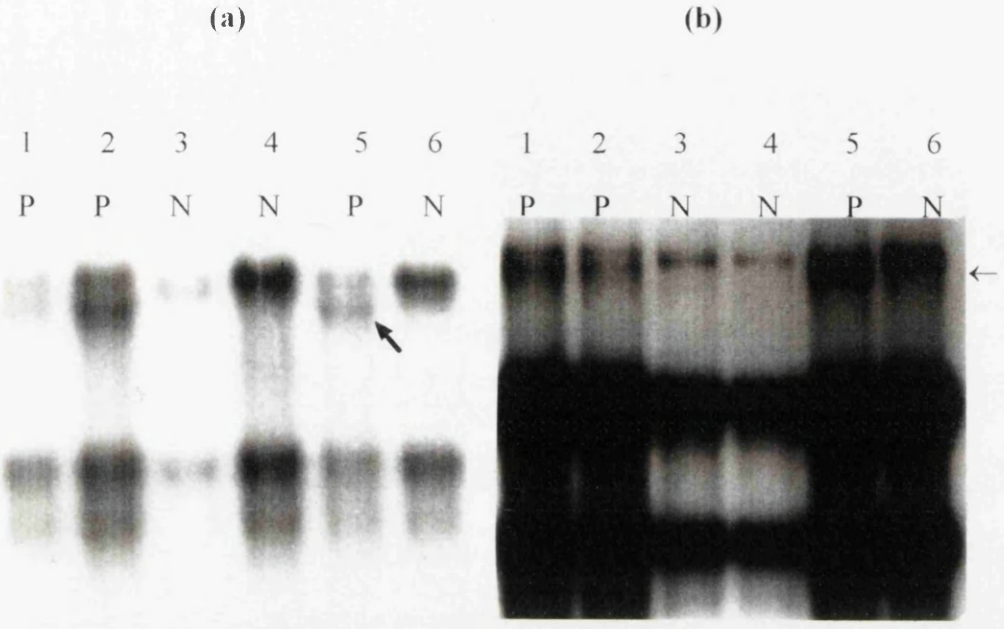


Figure 3.16b

(a) The result of SSCP analysis of *SOD-1* exon 4 in one FALS family (Figure 3.16a) using MDE gel. Lane 1 shows results obtained from individual I:2, lane 2 from individual II:1 and lane 5 from individual II:2. Lanes 3, 4 and 6 are from individuals II:3, II:5 and a normal control respectively. **(b)** The same analysis using 8% non-denaturing polyacrylamide gel run at 12 watts for 12 hours at $4^{O}C$. The arrow indicates the presence of a band shift in the affected family members. P = patient; N = normal.

3.2.6 Sequencing of the *SOD-1* exon 4 from FALS patients showing the G277→C mutation which caused the missense Gly93Arg change.

To detect the cause of the band shift in SSCP analysis of this family, direct sequencing of both DNA strands of the asymmetric PCR products from the affected family members and a normal control were performed. A point mutation (G277 to C) was detected in all affected family members but not in a normal control. This caused a missense mutation Gly93Arg (Figure 3.17).

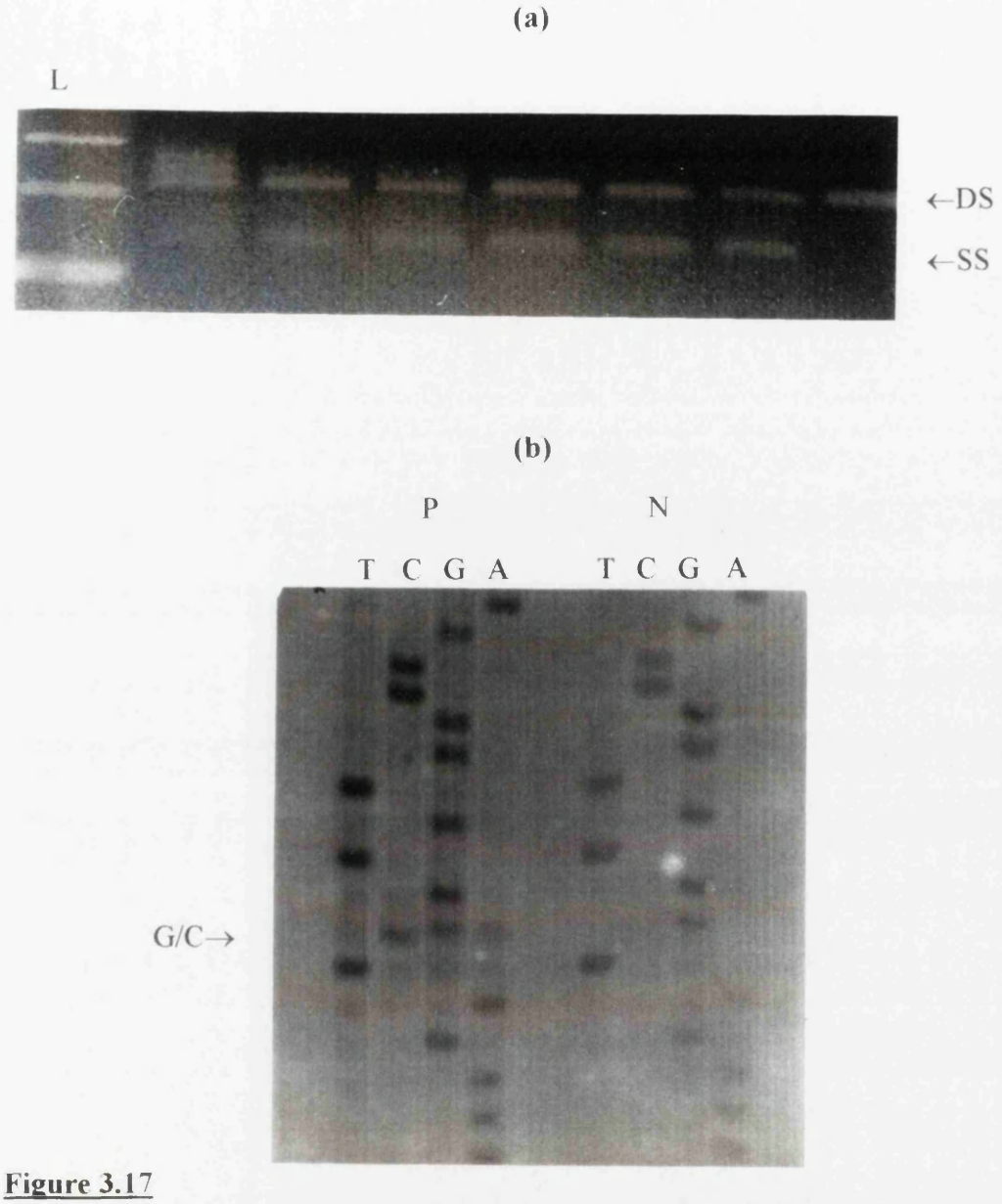


Figure 3.17

(a) Asymmetric PCR amplification products from exon 4 of the *SOD-1* gene from a FALS patient and a normal control. Reactions which give good single strand yield (arrow) have been used for subsequent sequencing procedure. SS = single strand; DS = double strand; L= DNA ladder. **(b)** Sequence analysis of the *SOD-1* exon 4 in a FALS patient who showed a band shift with SSCP analysis. The arrow indicates the presence of the G277 to C change in the patient. P = patient; N = normal control.

3.2.7 The G277 to C change in *SOD-1* exon 4 creates a new restriction site for the enzyme *Sau*3AI

To find if the G277 to C mutation caused changes in the restriction map of *SOD-1* exon 4, the GCG package from the Unix computer was used. It showed that this mutation has creates a new restriction site for the enzyme *Sau*3AI. PCR amplification products from the normal chromosome were cut into two fragments of 135 bp and 79 bp while that from the mutated chromosome were cut into three fragments of 135 bp, 44 bp and 35 bp (Figure 3.18).

To screen for the presence of the G277 to C mutation in other ALS patients PCR amplification products of *SOD-1* exon 4 from 67 sporadic and one familial ALS have been subjected to restriction enzyme (*Sau*3AI) digestion. The digestion products were resolved in 12% acrylamide gels using 1X TBE buffer. The gels were stained using ethidium bromide, visualised and photographed under UV light. No such change could be detected in any of the screened patients (Figure 3.18)

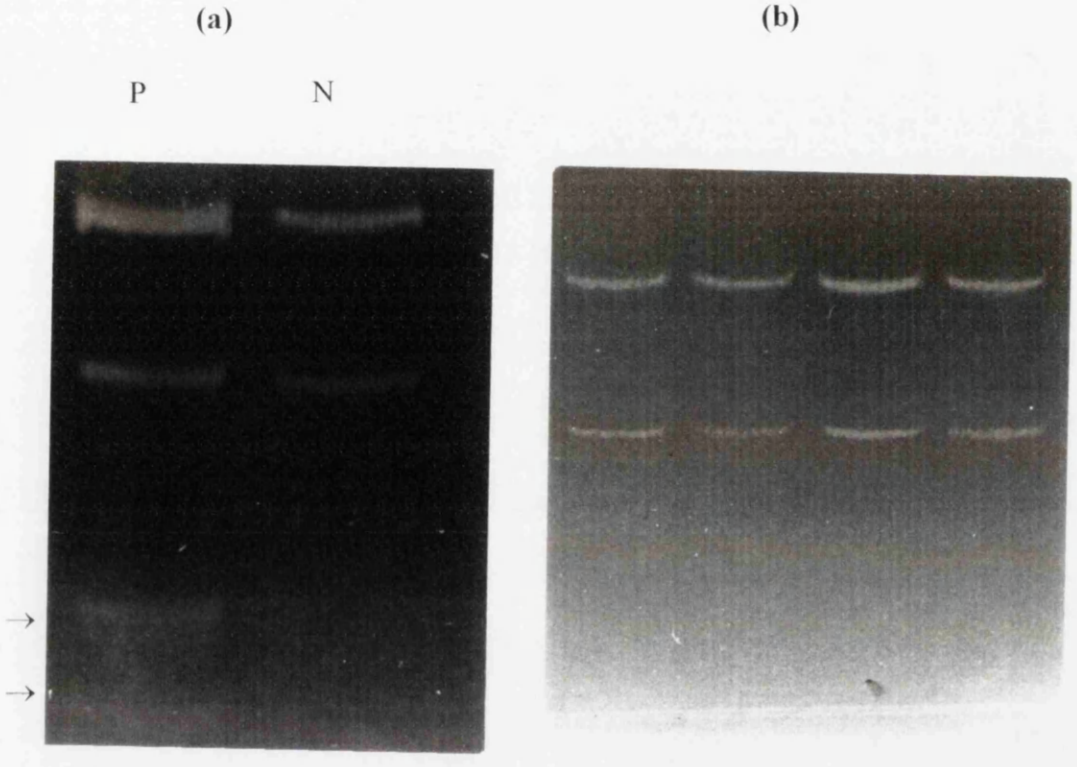


Figure 3.18

(a) *Sau*3AI digestion of the *SOD-1* exon 4 PCR amplification products from a G277 to C mutant patient and a normal control. Arrows point to the presence of two extra digestion product from the patient. P = patient; N = normal control. **(b)** Screening for the G277 to C mutation in other ALS patients using the *Sau*3AI restriction enzyme digestion. No abnormal products could be detected.

3.2.8 SSCP analysis showing band shifts due to electrophoresis and/or PCR errors

When abnormal SSCP band shifts were observed in a patient the analysis was repeated for confirmation. In some instances the abnormality would disappear upon the second gel run (Figure 3.19).

In one sample the abnormality was expected to be due to PCR error (band shift disappeared upon second gel run of newly made PCRs from the whole pannel of patients (20 patients) tested before but not from the old one), sequencing of the abnormal PCR product detected a PCR incorporation error (Figure 3.20). Subsequent sequencing of a newly amplified PCR product failed to detect this abnormality.

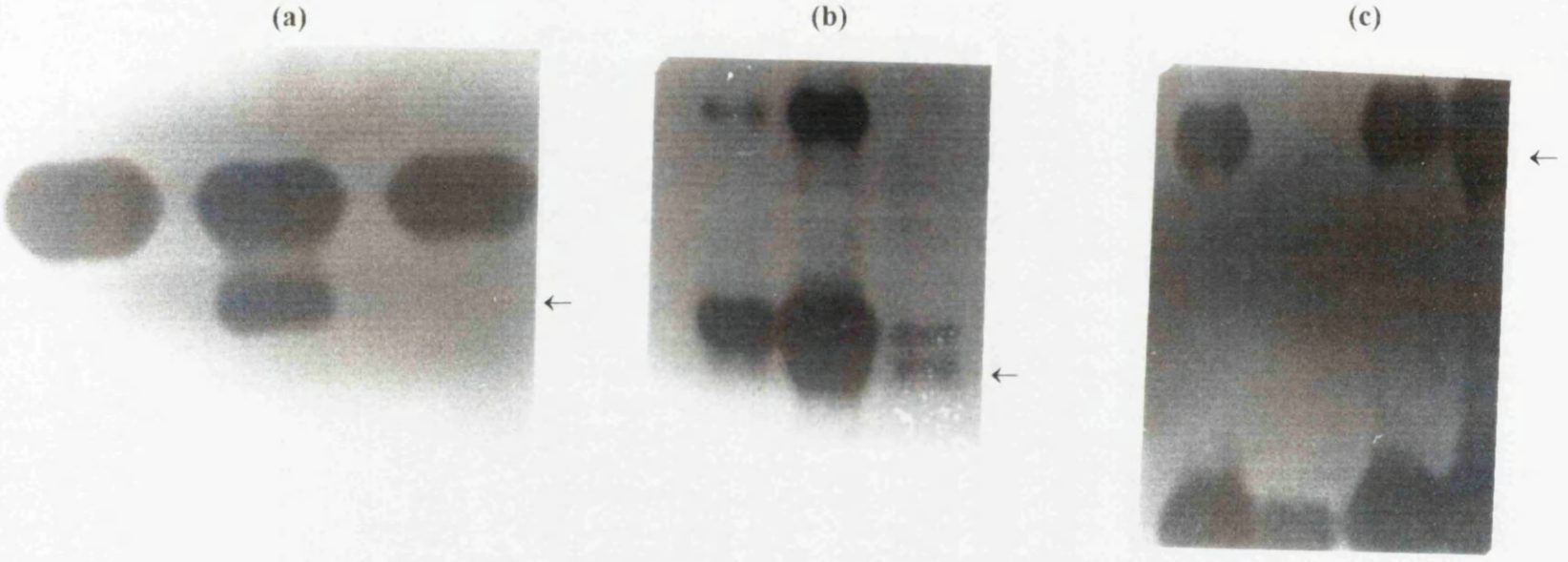


Figure 3.19

Examples of SSCP analysis band shifts (indicated by arrows) due to errors in sample loading, electrophoresis or PCR mis-incorporation. These photographs show only the single strands. **(a)** Abnormal SSCP pattern in the exon 1 of the *SOD-1* gene. **(b)** Abnormal SSCP pattern in the exon 2 of the *SOD-1* gene. **(c)** Abnormal SSCP pattern in the exon 5 of the *SOD-1* gene

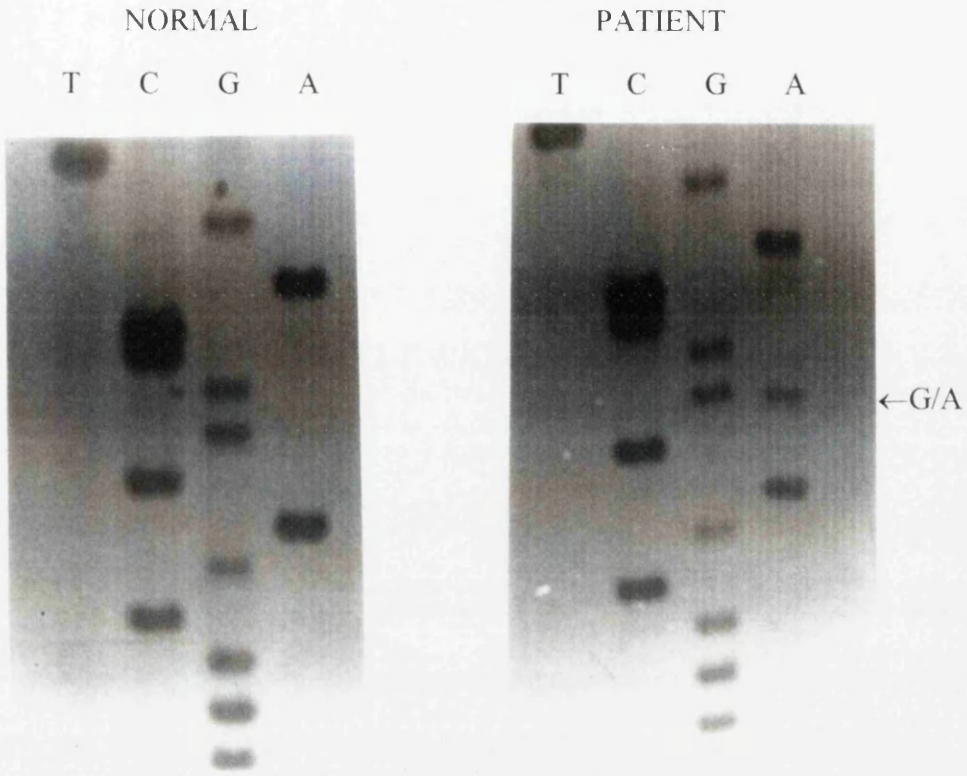


Figure 3.20

Sequence analysis of the PCR product which gave abnormal SSCP band shifts. Sequence analysis of exon 1 of the *SOD-1* gene shows a G34 to A mutation in the patient but not in the normal control. This mutation, if real would lead to a missense Gly12Ser change.

3.3 Screening for germline mutations in the *TSC2* gene

32 patients with tuberous sclerosis (TSC), including both sporadic and inherited cases were analysed for germline mutations within ~ 40% of the *TSC2* coding sequence and 325 bp of the upstream promoter area. All patients were diagnosed as TSC according to the revised Gomez criteria (Gomez, 1991).

3.3.1 Screening for structural rearrangements by Southern blot analysis

To screen for major structural rearrangements (deletions and/or insertions) all patients' DNAs were digested twice by *Hin*dIII and *Eco*RI restriction endonucleases and transferred to Hybond N nylon membranes. The *Eco*RI membrane was probed using the cDNA probe E1.6 and the *Hin*dIII membrane was probed using the cDNA E2.5 probe kindly supplied by Dr. M. Nellist, Institute of Medical Genetics, University Hospital of Wales, Cardiff. No major structural rearrangements were detected.

3.3.2 Amplification of the *TSC2* cDNA by RT-PCR

Total cellular RNA from whole blood lymphocytes of TSC patients (22 patients) was used to synthesise cDNA using oligo dT or oligonucleotide primers with nucleotide sequences complementary to various regions of the *TSC2* mRNA. Four sets of PCR primers were designed and PCR conditions were optimised to amplify four segments of the *TSC2* coding sequence. The first segment spans positions 1041 to 1857 of the cDNA and contains a possible membrane-spanning regions. The second to the

fourth segments span positions 4215 to 5471 of the cDNA and contain the GAP3-related domain and the polyadenylation signals (Figure 3.21 & 3.22a).

3.3.3 Amplification of exon 1 and exons 38-41 of the *TSC2* gene from genomic DNA

One PCR primer set was designed and PCR conditions were optimised to amplify exon 1, part of intron 1 and 325 bp of the promoter area of the *TSC2* gene from all patients' DNAs (32 samples). For a subset of patients (10 patients) only DNAs were available and another PCR primer set was designed and optimised to amplify *TSC2* exons 38-41 (numbering according to Maheshwar, et al. 1996) as one amplification segment of 1120 bp (Figure 3.22b).

Figure 3.21

Diagrammatic representation of the *TSC2* cDNA with the four RT-PCR amplified segments. Segment I spans cDNA positions 1041-1857, segment II spans positions 4215-4806, segment III spans positions 4582-5243 and segment IV spans positions 4967-5471.

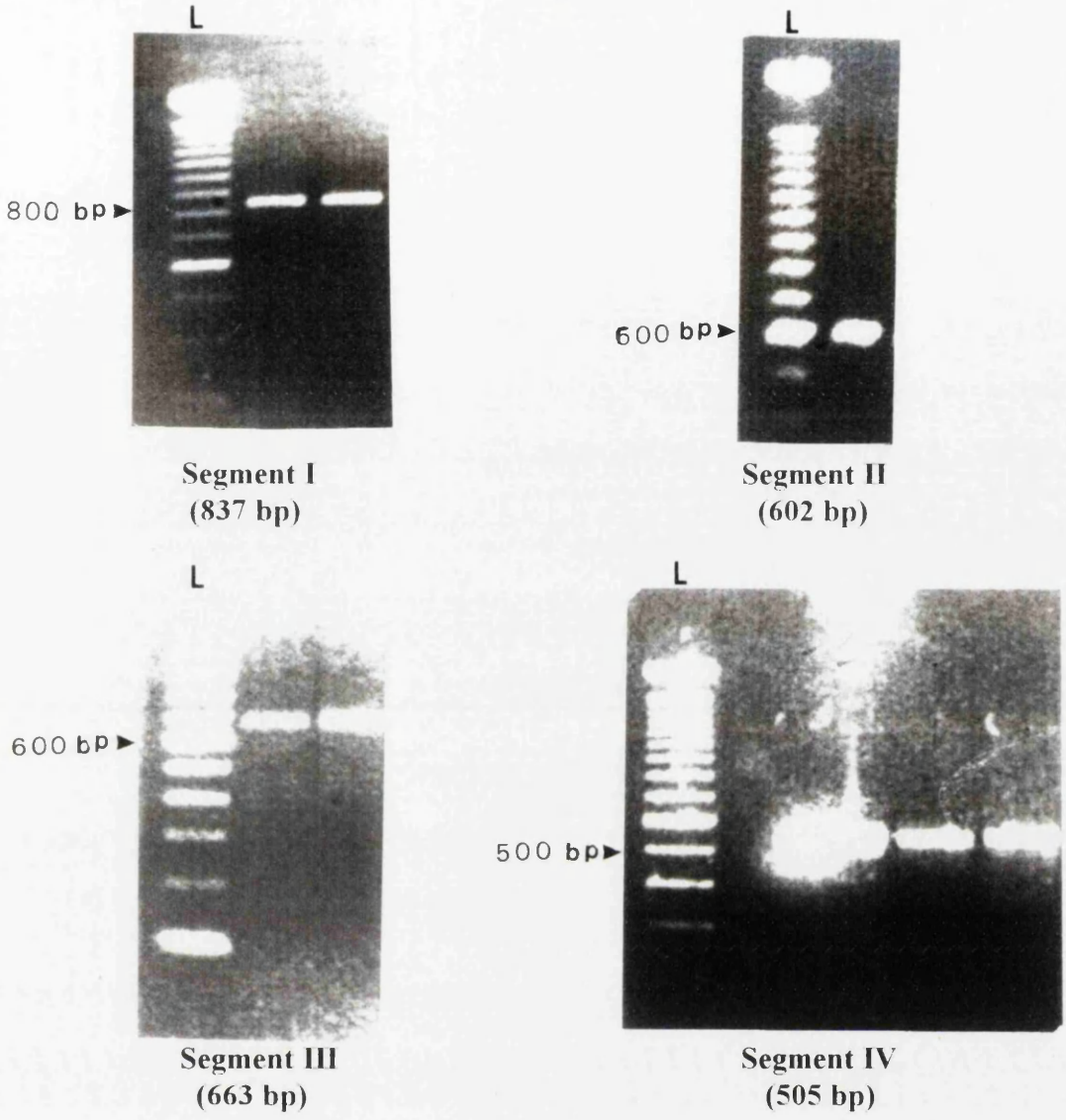


Figure 3.22a

RT-PCR amplification products (arrows) from the *TSC2* cDNA. Four sets of PCR primers were used to amplify 2.072 kb of the cDNA as been shown in the previous figure (Figure 3.21). The PCR products were run on 1% agarose gels, stained with ethidium bromide and visualised under the UV light. L= 100 bp DNA ladder.

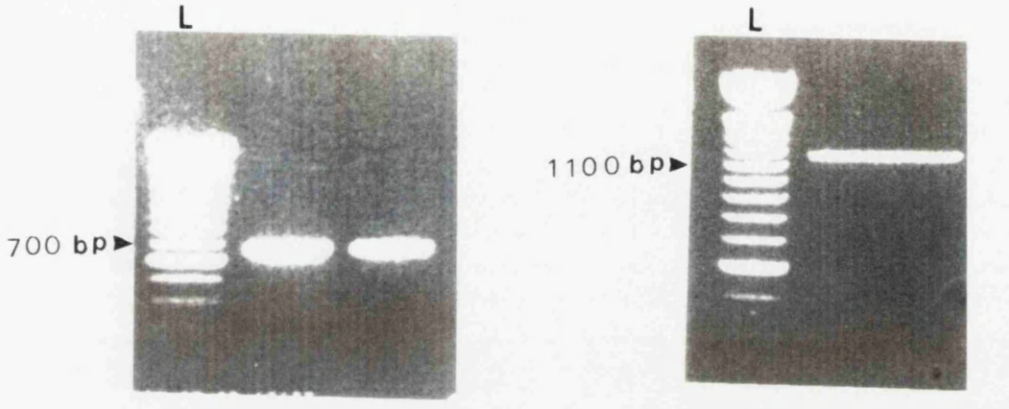


Figure 3.22b

PCR amplification of exons 1 and 38 to 41 of the *TSC2* gene. L= 100 bp DNA ladder.

3.3.4 Mutation screening using chemical cleavage of mismatch (CCM) analysis

After initial amplification by PCR, the products were electrophoresed on 1% agarose gels to check for any abnormal size alterations. If no alteration was seen, the amplification products were analysed by CCM to detect and locate the presence of point mutations and small sequence alterations within the amplified parts of the *TSC2* gene. CCM analysis was used to screen all cDNA fragments (I to IV), exon 1 (with 325 bp of the promoter area and part of intron 1) and exons 38-41 (with their introns) from patients with tuberous sclerosis (TSC). Figure 3.23 shows examples of positive screening results.

3.3.5 Sequencing of the samples that showed positive screening results

Any mismatch detected by CCM analysis was then confirmed by sequencing of the corresponding exon or cDNA segment. Three different sequencing techniques were used. Asymmetric PCR amplification of the segment or exon of interest and direct sequencing by the Sanger dideoxy chain termination method using USB sequenase version 2 sequencing kit was performed to characterise most of the detected mutations. For few samples direct sequencing of the PCR, using PRISM™ ready reaction Dyedeoxy™ terminator cycle sequencing kit and ABI prism 373 automated sequencer and/or double stranded sequencing of cloned PCR segments were performed. Each change found on sequencing was confirmed by sequencing the complementary strand. Any change found on sequencing the cDNA was confirmed, if possible, by sequencing the corresponding exon at the DNA level. For cloned PCR, sequencing of 10 colonies

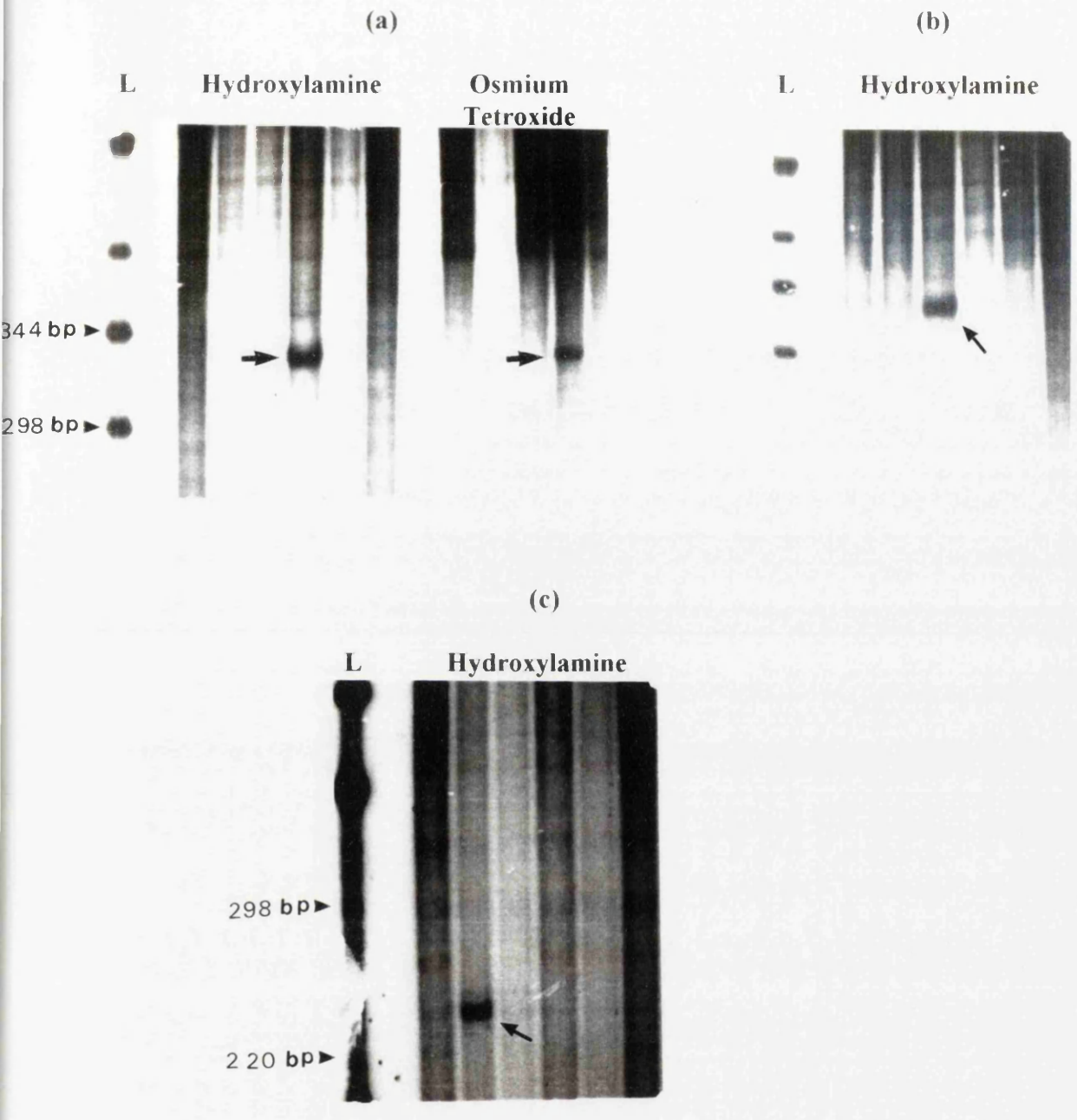


Figure 3.23

An example of chemical cleavage of mismatch analysis. Osmium tetroxide and hydroxylamine are the chemicals used to modify either T or C mismatches respectively. "L" stands for labelled 1 kb ladder. **(a)** Cleavage products of ~330 bp (arrows) from PCR products of *TSC2* cDNA segment IV upon both hydroxylamine and osmium tetroxide modifications. **(b)** Cleavage product of ~320 bp (arrow) from a PCR product of *TSC2* cDNA segment III. **(c)** Cleavage product of ~240 bp (arrow) from a PCR product of *TSC2* exons 38-41.

was done and the change was confirmed in four of them. If sequence alteration was detected in familial case, analysis of other family members for this change was also done. Every sequence change seen was analysed, using the GCG package 'MAP' program, to see if it created or abolished a restriction enzyme site. If alteration of a restriction site was found it has been used as a further confirmation of the sequence change.

Using this technique 5 point mutations and one 2 base pair deletion were characterised. Of these, three point mutations caused missense amino acid changes and the other three mutations were proposed to be polymorphisms.

3.3.6 S1715T missense mutation in exon 40 of the *TSC2* gene

One sporadic, mentally handicapped, TSC patient showed a cleavage product of his RNA segment IV upon osmium tetroxide modification. To characterise the cause of this cleavage, asymmetric PCR of the patient's cDNA (segment IV) and DNA (exons 38-41) were performed followed by direct sequencing using the USB sequenase version 2 sequencing kit. The cleavage was due to a T5161→A base change converting serine to threonine (S1715T) (Figure 3.24). This mutation creates new restriction sites for the enzymes *Pfl*MI and *Dra*III. The screening of 100 normal chromosomes with these restriction enzymes revealed no further examples of this mutation.

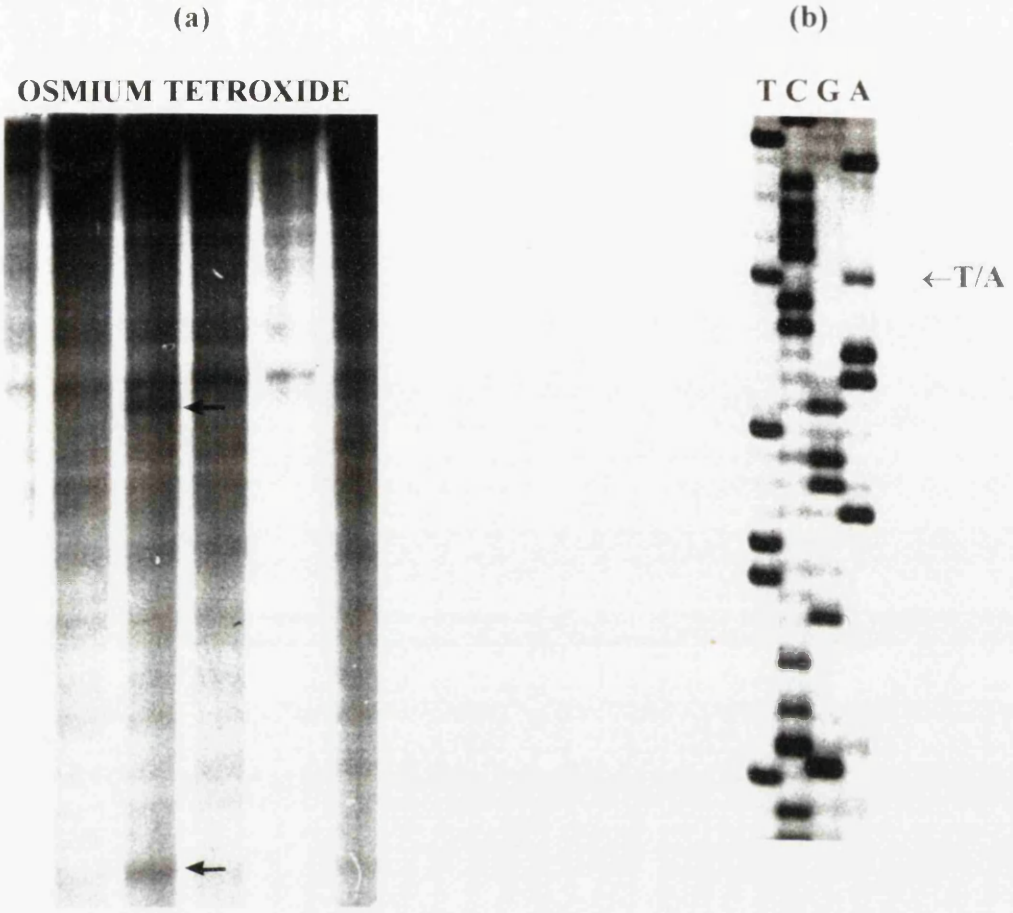


Figure 3.24

(a) Chemical cleavage analysis of cDNA segment IV revealed a cleavage product (arrow) upon osmium tetroxide modification. **(b)** sequencing analysis of an asymmetric PCR product from segment IV revealed a T5161→A (arrow) change which produces a S1715T missense mutation.

3.3.7 R1720W missense mutation in exon 40

A second missense change was detected, in a sporadic TSC patient, a few amino acids downstream of the previous one. Cleavage products were detected upon hydroxylamine modification of cDNA segment IV. To characterise the cause of this cleavage three sequencing techniques were used. First by direct sequencing of the PCR product using the PRISM™ ready reaction Dyedeoxy™ terminator cycle sequencing kit and testing the sequencing products on an ABI prism 373 automated sequencer. Secondly, the PCR product from patient's cDNA segment IV was cloned in a plasmid vector followed by double stranded sequencing by the Sanger dideoxy chain termination method using the USB sequenase version 2 sequencing kit. Thirdly, asymmetric PCR amplification of cDNA segment IV and exons 38-41, followed by direct sequencing using the USB sequenase version 2 sequencing kit. All three techniques revealed the same result. Cleavage was due to a C5176→T base change producing a C mismatch of the wild-type DNA producing a cleavage product upon hydroxylamine modification (Figure 3.25). This change causes a missense R1720W mutation and creates a new restriction site for the enzyme *Eco*RII. Using this enzyme to screen for the presence of that mutation in 100 normal chromosomes revealed no further examples of this change.

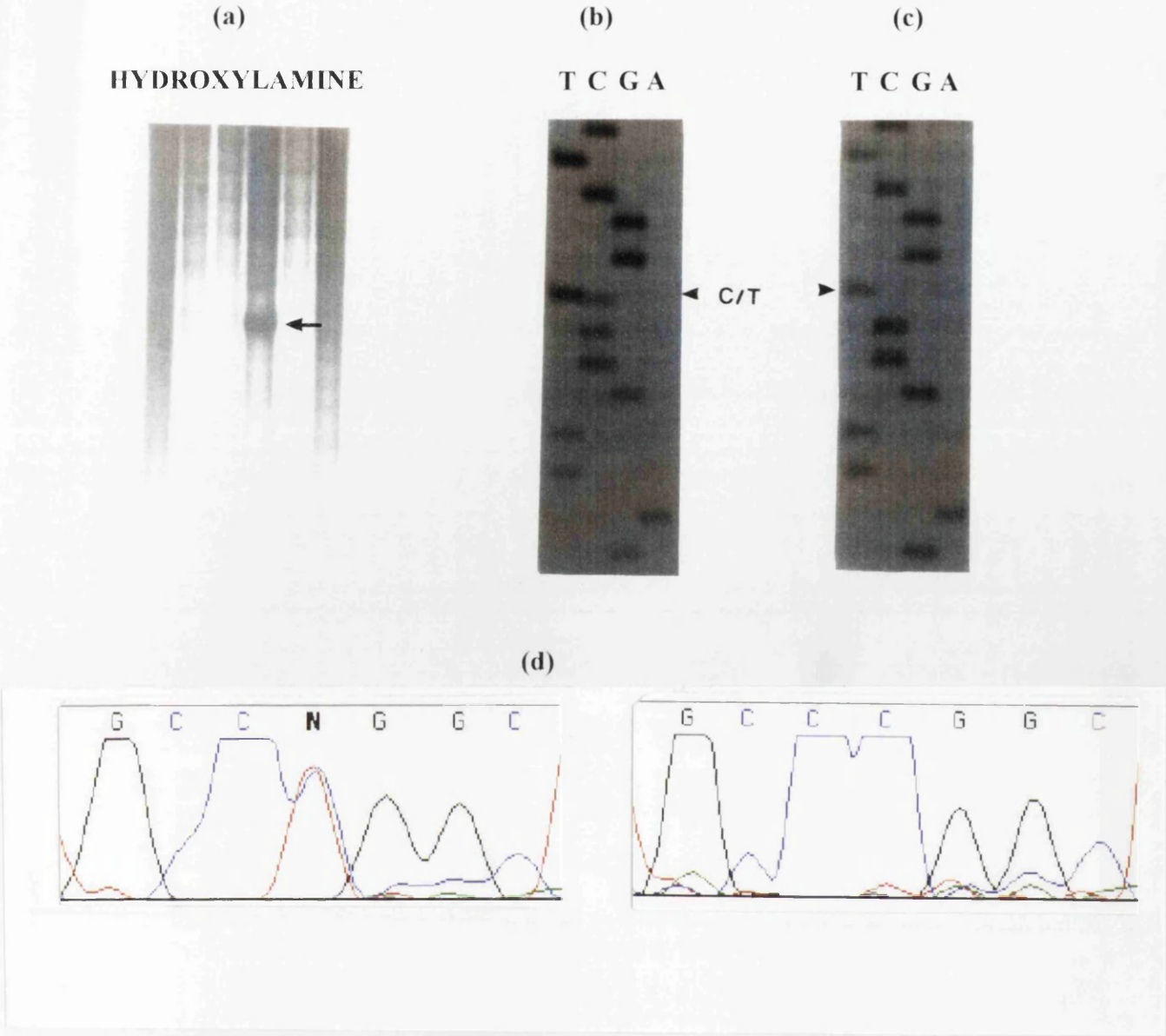


Figure 3.25

(a) Chemical cleavage analysis of cDNA segment IV revealed a cleavage product (arrow) upon hydroxylamine modification. ***(b)*** Sequencing analysis of an asymmetric PCR product from segment IV revealing a C5176→T change (arrow) and producing the missense mutation R1720W. ***(c)*** Double stranded sequencing of the cloned PCR segment IV shows T5176 instead of C at that position. ***(d)*** Automated DNA sequencing of the PCR product from segment IV of the patient and normal control. At position 'N' of the patient's sample, a C (blue) to T (red) change is observed.

3.3.8 Missense mutation M1602V in exon 37 of the *TSC2* gene

Screening of segment III by CCM revealed a cleavage product in a 25 years old sporadic, mildly affected TSC patient. Cloning of the patient's segment III PCR product followed by sequencing of the plasmid with the insert showed an A4822→G change and produced a missense M1602V mutation (Figure 3.26). This is the only mutation detected in the GAP3-related domain of the *TSC2* gene and it creates a new restriction site for the enzyme *Eco*RII. The screening of 100 normal chromosomes with the enzyme revealed no further examples of this mutation.

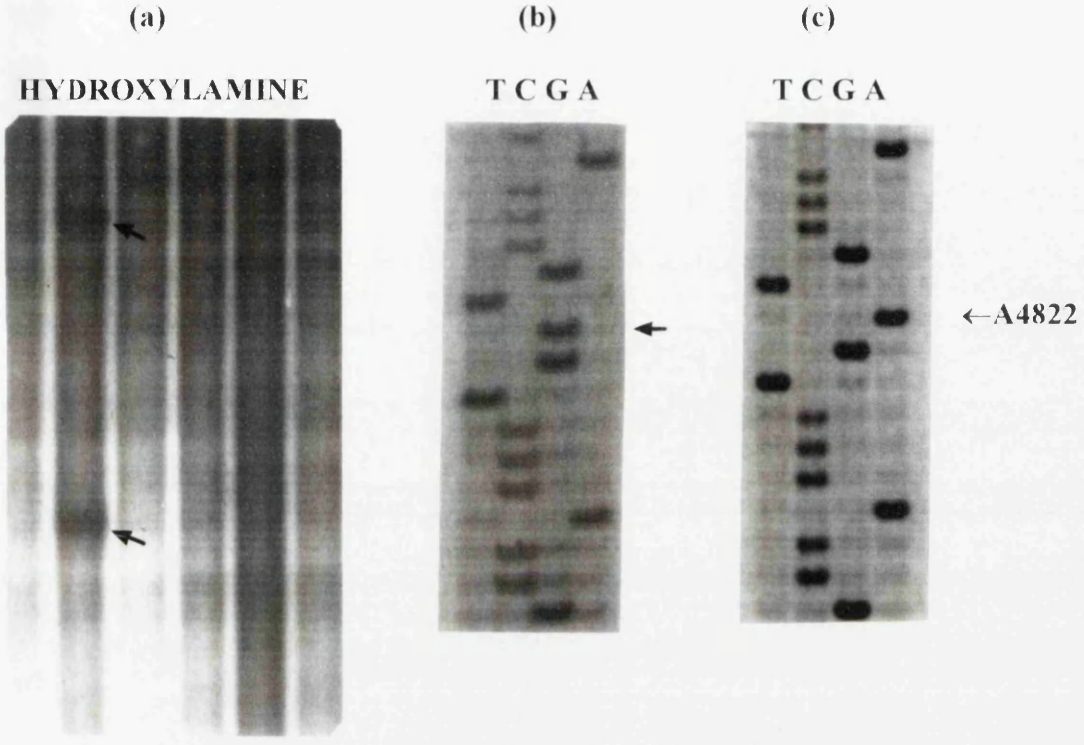


Figure 3.26

(a) Chemical cleavage analysis of cDNA segment III revealed cleavage product (arrow) upon hydroxylamine modification. **(b)** Sequencing analysis of the cloned PCR product from segment III revealed a G instead of A4822 (arrow) which produces an M1602V missense mutation. **(c)** Sequencing analysis of the same segment from normal control.

3.3.9 Two base pair deletion in one of the polyadenylation signals

One patient from a TSC family showed a cleavage product on modification with osmium tetroxide in RNA segment IV. Direct sequencing of the asymmetric PCR from that segment revealed a complex sequencing pattern. Analysis of this pattern revealed that it was due to a two base pair deletion (AA numbers 5433 & 5434) of the second polyadenylation signal of the *TSC2* gene (Figure 3.27). This change was also confirmed by sequencing PCR product from exons 38-41 of that patient. This family has been proved to be linked to TSC locus on chromosome 9 (data not shown) and sequencing of other affected family members did not reveal such change confirming that it is a polymorphic one. This change does not change any restriction sites, so screening of normal chromosome was done by direct sequencing of asymmetric PCR which detected this change in one normal sample.

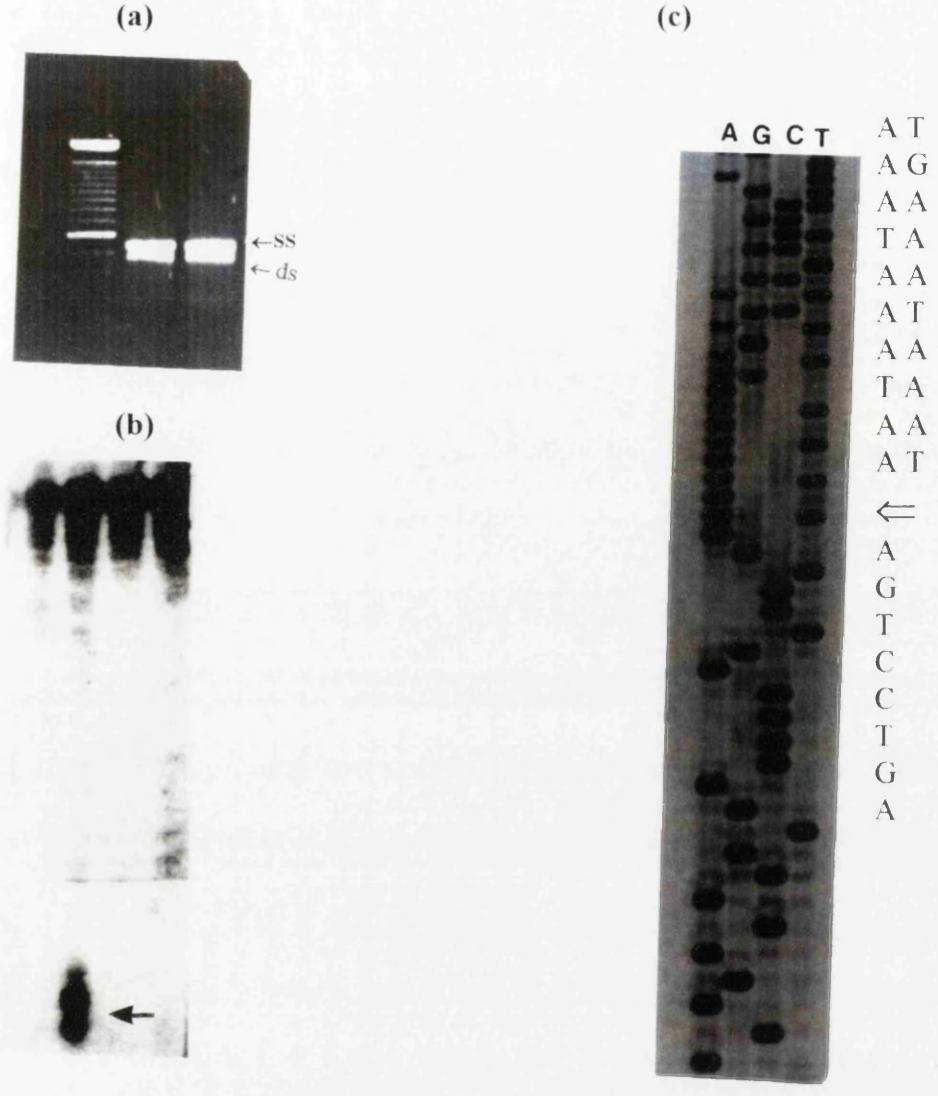


Figure 3.27

(a) Asymmetric PCR of RNA segment IV from a TSC patient who showed a CCM cleavage product. ss= single strand; ds= double strand **(b)** Chemical cleavage analysis of the patient's cDNA segment IV on modification with osmium tetroxide. Cleavage product is indicated by an arrow. **(c)** Direct sequencing of the asymmetric PCR revealed a complex sequencing pattern due to an AA deletion (indicated by arrow) of one of the *TSC2* polyadenylation signals.

3.3.10 Silent mutations in exons 37 and 41

Two silent mutations affecting two different serine residues (S1630 and S1776) were detected upon screening of segments III and IV of the *TSC2* cDNA by CCM analysis. The first one (S1630 in exon 37) was detected in one patient and is due to a C4908→T change (Figure 3.28). The second one (S1776 in exon 41) was detected in four (~12%) out of the screened 32 patients and is due to a G5346→C change (Figure 3.29).

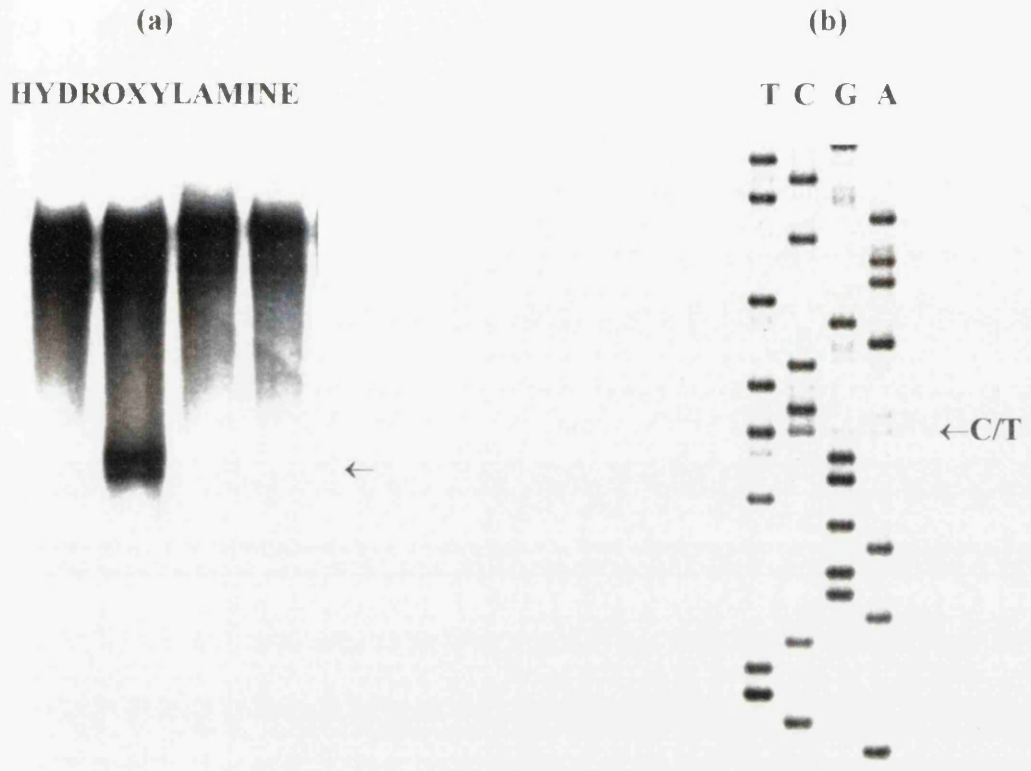


Figure 3.28

(a) Chemical cleavage analysis of cDNA segment III revealed a cleavage product (arrow) upon hydroxylamine modification. **(b)** Sequencing analysis of an asymmetric PCR product from segment III revealed a C4908→T (arrow) mutation which is silent (S1630).

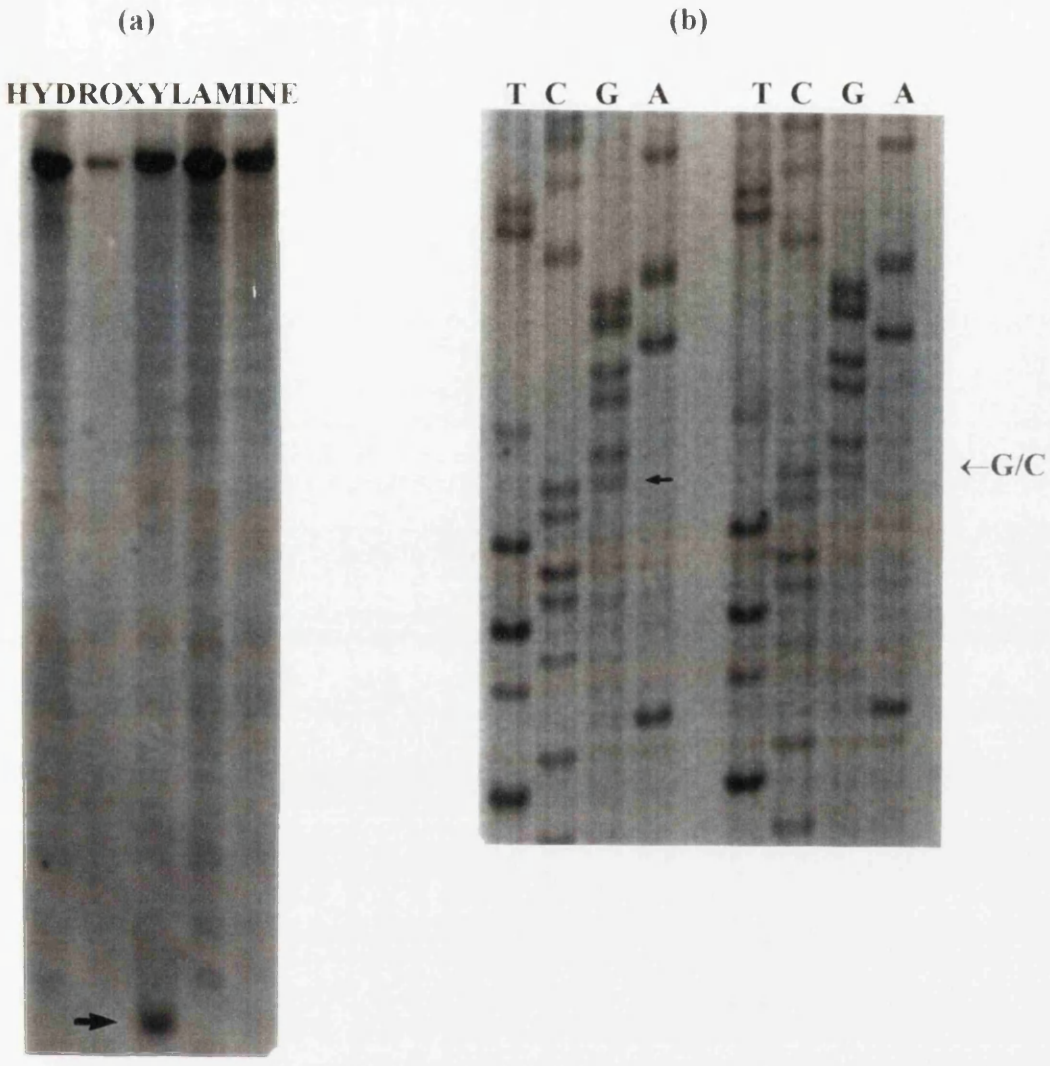


Figure 3.29

(a) Chemical cleavage analysis of cDNA segment IV revealed a cleavage product (arrow) upon hydroxylamine modification and was detected in four different patients.

(b) Sequencing analysis of asymmetric PCR products from two different patients revealing a G5346→C mutation (arrows) which is silent (S1776).

CHAPTER FOUR

DISCUSSION

(4) DISCUSSION

Easy identification of specific sequences and sequence changes plays a central role in the diagnosis of human inherited diseases. In the present study three autosomal dominant neurodegenerative diseases (myotonic dystrophy, amyotrophic lateral sclerosis and tuberous sclerosis), with different underlying molecular pathologies, were studied. To each one of them a different mutation detection strategy was optimised and applied to screen for the underlying molecular pathology.

4.1 $(CTG)_n$ repeat instability in patients with myotonic dystrophy

Myotonic dystrophy (DM) is the most common form of adult muscular dystrophy. The disease is transmitted in an autosomal dominant manner with the phenomenon of anticipation. The *DM* locus was mapped to the chromosome 19q13.3 and an expanded $(CTG)_n$ trinucleotide repeat in the 3' untranslated region of a protein kinase (*DMPK*) gene family member has been identified as the mutation that causes myotonic dystrophy.

In the present study, a screening strategy for 49 DM families, based on PCR testing followed by Southern blot for the suspected cases was adopted.

For the DM families who sought genetic counselling, DNAs of the family were screened by PCR amplification of the 3' untranslated region of the *DMPK* gene that had the polymorphic CTG repeats. At the beginning of that study radiolabelled PCR using the primer set 409/410 (Mahadevan et al., 1992) was optimised and used as a screening test. PCR products were resolved by running on 8% denaturing polyacrylamide gels and autoradiographed by exposure to Kodak AR film for 5 to 16 hours. Normal chromosomes (carrying 5 to 31 repeats) gave PCR products in the range of 64 bp to 152 bp and were detected. Chromosomes carrying minimally expanded CTG repeats (~56 to 83 repeats) were easily distinguished from those carrying the upper normal CTG repeats.

These expanded CTG alleles always appeared on the gel as mosaic pattern of DNA fragments. Similar results were obtained by different groups of investigators and the mosaic pattern of the expanded CTG repeat was attributed to be due to the presence of somatic instability of the expanded repeat (Lavedan et al., 1993a; Brunner et al., 1993). In spite of the reliability and accuracy of that test, it required handling of hazardous materials (radioisotopes and polyacrylamide) and it was time consuming (~ 24 hours to get the result). To overcome these drawbacks, non-radiolabelled PCR using the primer set 409/406 (Mahadevan et al., 1992) was optimised and applied to screen 22 DM families. First, the test was applied to some previously radiolabelled-screened samples and it gave the same conclusions, reached previously, regarding an individual CTG repeat expansion status. Using non-radiolabelled PCR followed by agarose gel electrophoresis, the discrimination between the upper normal CTG repeat size and the smallest detected CTG repeat expansion was not confusing. The technique was easy to perform, cheap, less time consuming (results obtained within few hours) and did not deal with any hazardous materials. The only difference was that, by using non-radiolabelled PCR less accurate sizing (± few repeats) of the normal or expanded CTG repeats was achieved. But as stated by Harper et al. (1992), "We currently have no knowledge of the risk of genetic instability in the offspring of minimal mutation carriers, nor do we know the likelihood of such individuals later developing cataract or other clinical features". Such reduced accuracy should not affect the result of the patient's counselling so that it seemed reasonable to adopt the non-radiolabelled PCR screening as a first step in the screening strategy of DM families. Comparable results were obtained by different group of investigators using the same or different primer sets and using either radiolabelled or non radiolabelled technique (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992; Barcelo et al., 1993; Abeliovich et al., 1993; Ashizawa et al., 1994b; Goldman et al., 1995; Krahe et al., 1995a).

Limitation of PCR amplification of the expanded CTG repeats was detected. It preferentially amplifies alleles that carry smaller CTG repeat expansions but reasonable amplification could not be achieved of alleles with more than 90 repeats. Similar limitation has been reported by other investigators who could not efficiently amplify alleles with large CTG repeat expansions (between 80 and 150 repeats) (Brook et al., 1992; Barcelo et al., 1993). For individuals who showed only one band within the normal range upon PCR amplification of their DNAs, it was not possible to tell if they were normal homozygous for that allele size or affected heterozygous with the second expanded allele too large to amplify. To solve this ambiguity, the second step of the screening strategy was to perform Southern blot analysis to detect the presence or absence of the expanded allele. To avoid ambiguity, it is preferable that the resolution of the system cannot distinguish between normal alleles of the CTG polymorphism but can distinguish these from minimal expansions (50 CTG repeats and above).

In the present study *Bgl*I digested genomic DNA from all DM family members were analysed by Southern blot using p5B1.4 ^{32}P-labelled genomic probe. Unambiguous discrimination between normal homozygous and DM affected heterozygous was achieved. Normal individuals showed only one band while affected members showed second band of varying degrees of expansion (~56 to several thousand repeats).

It has been demonstrated that Southern analysis using *Eco*RI, *Hin*dIII and *Nco*I-digested DNA does not detect small DM-specific expansions (Brook et al., 1992; Aslanidis et al., 1992 and Fu et al., 1992). Using enzymes generating smaller target fragments containing the CTG polymorphism should increase the resolution of the technique. Shelbourne et al. (1992) compared the resolution efficacy of three enzyme systems. They used *Eco*RI, *Bam*HI and *Bgl*I to digest DNAs from normal individuals and DM patients with varying repeat sizes. Minimal expansions of the CTG repeats were detectable in *Bgl*I and *Bam*HI digests but not in *Eco*RI digests. They described two drawbacks of using *Bam*HI digests. First, its ability to clearly distinguish between two

normal CTG alleles when one of them is in the upper normal range. This may lead to confusion especially in the diagnosis of isolated cases. Second, many of the large expansions were detected as smears with both enzymes, but in *Bam*HI digests, these smears were barely distinguishable from background hybridisation. The large fragments generated in *Bgl*I digests were unambiguously detected.

Goldman et al. (1994) reported a *Bgl*I polymorphism, in normal Southern African Negroids, which could lead to erroneous diagnosis of DM in people from this population. To exclude the possibility of the presence of such a polymorphism in the DM families in the present study, Southern analysis using *Eco*RI digested genomic DNA from patients who showed an expanded allele, upon *Bgl*I digestion, was performed. No single case was found to have such a polymorphism. It is possible therefore that this polymorphism is specific to Negroes (Goldman et al., 1994).

After applying this screening strategy to all studied DM families collected data were analysed. All DM affected members showed a varying degree of CTG repeat expansion. CTG repeat sizes in DM patients revealed that minimally affected patients have repeat sizes of < 0.4 kb (~130 CTG repeats). Congenital cases had on average the largest repeat sizes (3.3 ± 1.4 kb) while the adult onset cases (classical DM) showed on average intermediate expansions with a wide range of CTG repeat sizes (1.99 ± 1.37 kb) which overlapped with the repeat sizes of all other clinical phenotypes. Moreover, there was a significant correlation between the size of the CTG repeat expansion and the age at onset of the disease. The larger the repeat the earlier the age at onset ($r = -0.62$; $p<0.001$). These data are in accordance with the previously published data. Nearly all cases of DM (98%; Mahadevan et al., 1992) displayed expansion of the CTG repeat region. Moreover, in DM patients the size of CTG expansion in blood lymphocytes correlates to a reasonable degree with the age at onset and severity of the disease but it is by no means absolute and cannot be reliably used as a single diagnostic or prognostic

criterion to predict clinical status of the patient (Harley et al., 1993; Nokelainen et al., 1993 and Lavedan et al., 1993a).

Somatic mosaicism due to mitotic instability was observed in the majority of DM patients (Mahadevan et al., 1992; Lavedan et al., 1993a; Wong et al., 1995). Smears of hybridisation in Southern blot analysis and the presence of multiple PCR alleles(observed in the present study and previous studies) are reflections of this mosaicism. Tissue-specific differences in the efficiency or availability of DNA repair systems may account for the heterogeneity of CTG expansions (Wohrle et al., 1995). Somatic heterogeneity could explain the overlapping in fragment sizes of the different clinical groups, especially in adults with classical DM. Lavedan et al. (1993a) raised the possibility that somatic mosaicism may explain the absence of a strict correlation between the size of the mutation observed in patient lymphocytes and the severity of the disease and it is that different degrees of expansion may cause varying phenotypic effects in other more relevant tissues (Harley et al., 1993). On the other hand, Zatz et al. (1995) showed that no significant correlation was found between the size of the CTG expansion in muscle and age at onset of the disease. Moreover, large expansions were observed in muscle from all adult symptomatic patients independently of the presence of muscle weakness. More studies will be needed to solve this dilemma.

In the present study an apparently unexplained excess of male transmitters was found in the ancestors' generation. This has been reported before by many authors (Harper, 1989b; Harley et al., 1993; Lavedan et al., 1993a; Passos-Bueno et al., 1995). Although ascertainment bias can not be rejected, this male excess could be because women with neonatal cases will not appear as grandmothers of affected patients.

A reduction in repeat size upon paternal transmission was detected in two child/paternal pairs in the present study. In one of them, the father had an adult onset classical DM (age at onset 35 years) and an expanded CTG allele of 5.7 kb (~1900 repeats). His daughter had inherited a reduced allele of 1.7 kb (~565 repeats) and was

asymptomatic at the age of 25 years. In the second case, the father, also, had a classical adult onset DM (age at onset 42 years). His DNA was tested in Cardiff, before the discovery of the CTG repeat instability as the molecular pathology underlying DM, and was shown to have a moderately expanded allele. At that time his daughter's DNA did not show this expansion and she was told that she did not inherit the affected father's allele. In the present study, PCR amplification of the daughter's DNA revealed an expansion of ~ 90 CTG repeats which was confirmed by Southern analysis using *BglI*/p5B1.4 system. She, also, requested testing of her 9 years old asymptomatic son and 7 years old asymptomatic daughter. Both of them showed expanded alleles of 350 and 450 repeats respectively. It seemed that the paternal regressed allele had re-acquired its ability to expand when transmitted through his daughter's line to his grandchildren. Since the two affected fathers had late onset and their daughters are still young, it is not possible to assess if their clinical picture will be milder than that observed in their DM fathers or not.

Both meiotic expansion and contraction events may occur in the *DM* allele but expansion is favoured (Jansen et al., 1994). A reduction in CTG repeat number to within the normal range was reported before by many investigators (Wieringa et al., 1994). It was mostly seen upon paternal transmission especially in fathers with a repeat of 1 kb or greater (Ashizawa et al., 1994b), as was seen in the aforementioned cases. The tendency towards contraction of alleles on transmission through the male germline may be caused by selection of spermatozoa bearing smaller repeats (Jansen et al., 1994).

Ashizawa et al. (1994b) reported that clinical anticipation still occurred in about 50% of cases that showed intergenerational CTG repeat contraction. On the other hand, Harley et al. (1993) and O'Hoy et al. (1993) reported few cases where the reduction of repeat size was accompanied by a later age at onset or less severe phenotype.

In the present study six child/parent DM pairs showed either no change (3 cases) or minimal increase (< 9 CTG repeats) upon transmission of the CTG repeat from the

parent to his/her offspring. In four of these pairs (2 child/maternal and 2 child/paternal) the parent repeat sizes were between 52 and 83 repeats. In the other two pairs (parent were fathers) the parental repeat sizes were 1.1 (~365 repeats) and 1.25 (~417 repeats) and showed no change upon transmission. Relatively stable behaviour of the repeat was observed with transmission of alleles less than 80 CTGs (Barcelo et al., 1993) and this could partially explain the persistence of this autosomal dominant disease in the population despite the presence of anticipation and low reproductive fitness.

A case of uncertain diagnosis of congenital myotonic dystrophy (CDM) was seen to be paternally transmitted. She was a 12 years old girl with mental and physical retardation, hypotonia, sluggish reflexes and weak musculature. She was an outcome of a normal pregnancy with no complications. At birth, she presented with cleft palate, a heart murmur and mild talipes deformity of the right foot but there has been no respiratory problems or severe hypotonia. She has been slow to reach milestones. She could roll over and sit if supported by the age of 10 months and she did not walk until she was almost two and half years old. The absence of facial weakness, neonatal hypotonia and neonatal respiratory distress in that case weaken the diagnosis of CDM. On the other hand the presence of neonatal right talipes and delayed milestones in the first year of life are concomitant with the diagnosis of CDM. These data put that case on the border line between congenital and early onset DM.

Her mother is normal but her father, who seemed to be an active man and not aware of having any health problems, was found to have a mild DM. The grandfather was a 65 years old male who had cataract but no muscle weakness or myotonia either clinically or by EMG. DNA analysis from this family revealed that the grandfather had a small repeat expansion (~100 repeats), the father had an expansion of 1.5 kb (~500 repeats) and the affected daughter had a 2.1 kb expansion (~700 repeats). These were detected by Southern blot analysis using the *BglI*/p5B1.4 system.

Congenital myotonic dystrophy (CDM) is the most severe form of the phenotype which results in significant foetal loss and death in the perinatal period (Harper, 1989b). Transmission of that form of the disease has been nearly exclusively maternal with few exceptions (Wieringa, 1994). CDM patients usually have large CTG triplet expansions although some congenital cases with alleles in the 500 to 999 repeats range were reported (Tsilfidis et al., 1992; Harley et al., 1993 and Redman et al., 1993). The finding that CDM can be paternally transmitted, even if the incidence of such cases is very low, casts doubt upon certain mechanisms previously postulated to explain the maternal transmission of the disorder e.g. imprinting and the presence of an intrauterine factor. The possibility that CDM may operate with a pathologic mechanism(s) different than that adult onset DM can not be excluded and more studies are needed to solve this dilemma.

4.2 Mutation detection in the *SOD-1* gene in patients with amyotrophic lateral sclerosis (ALS)

Screening for mutations in the *SOD-1* gene in 2 familial and 67 sporadic ALS cases was performed. Single strand conformation polymorphism (SSCP) analysis was optimised to screen the 5 exons of the gene in all cohort of the studied patients. Different types of gel matrices and different running conditions were used for the analysis. 0.5X MDE™ gel with 5% glycerol running at room temperature and 8% polyacrylamide gels with or without 5-10% glycerol running at different watts and temperatures (at room temperature and at 4°C) were used along the study. A band shift was detected upon analysis of exon 4 from one familial patient. It was detected upon MDE™ analysis and on polyacrylamide gel without glycerol running at 12 watts and 4°C. Sequencing of the asymmetric PCR of that exon from the corresponding patient revealed a G277→C mutation which caused a missense Gly93Arg change and created a new restriction site for the enzyme *Sau*3AI. This SSCP pattern and its causative mutation were confirmed to be

present in all other affected family members while it could not be detected upon SSCP analysis and restriction enzyme screening of the remaining familial and 67 sporadic ALS cases.

Of the various methods applied for the detection of unknown mutations, SSCP is by far one of the easiest and cheapest procedures presently available. This technique was efficiently used by many investigators to screen for *SOD-1* mutations in ALS patients (Rosen et al., 1993, Deng et al., 1993, Jones et al., 1995, Deng et al., 1995) where ~ 20% of the screened familial cases showed point mutations, nearly all of them caused missense amino acid changes. In general, SSCP analysis detects 70 to 95% of mutations in PCR product of 200 bp or less (Grompe, 1993). The *SOD-1* gene is a single copy gene which is known to have five small exons encoding for 153 amino acids. This makes it ideal to use single stranded conformational polymorphism (SSCP) analysis as a screening method for point mutations or minor sequence alterations in that gene. There are many parameters other than the size of the PCR product that can affect the efficacy of the technique. Physical factors such as temperature, type of gel matrix, and ionic strength is very important such that sequence changes that have little or no effect on conformation in one set of conditions can have dramatically different effects under other conditions. A new gel matrix (MDE™, AT Biochem.) was used by most of the investigators who screened the *SOD-1* gene for mutations in ALS patients. This matrix offered a good detection rate and did not require many gel condition variations. Overloading of the gel sometimes results in abnormal migration of the bands and reduced resolution. For this reason, radioisotopic or fluorescent labelling of the PCR products to a high specific activity is highly recommended so as samples can then be diluted so that DNA concentrations are sufficiently low in the loading solution, yet detectable (Hayashi and Yandell, 1993). In the present study a mutation was detected in exon 4 of the *SOD-1* gene using the MDE™ gel and polyacrylamide gel running at $4^{o}C$ but not at room temperature. This emphasises the importance of using different gel conditions if

polyacrylamide gel is going to be used for SSCP analysis in the *SOD-1* gene as had been stated by different investigators for other genes (Grompe, 1993).

In the present study, a missense Gly93Arg change was detected in the *SOD-1* gene in one of the two familial patients with ALS. Mutations in Gly93 were previously detected in familial ALS patients where Gly93Ala and Gly93Cys were described in one report (Deng et al., 1993). This amino acid was shown to be highly conserved among 18 different species (Pramatarova et al., 1995). Moreover, mapping of the Gly93 to the crystallographic structure of the SOD-1 enzyme revealed that it is one of the critical glycine residues that allow main chain conformation and packing interactions, closing one end of the β barrel structure of the enzyme (Deng et al., 1995). Mutations affecting this amino acid should have a deleterious effect on the conformation and stability of the enzyme dimer. These together with the detection of the Gly93Arg mutation in all affected family members and not in the other screened patients make it to the most probable cause of the disease in that family.

All family members carrying the previously determined missense mutation (Gly93Arg) showed an earlier age at onset of the disease (26-40 years) compared to an average of about 50 years seen for other mutations. On the other hand, while the disease was rapidly progressive in three of them (3 to 5 years till death), one member showed a slower disease course (10 years). In screening erythrocytes for SOD-1 enzyme activity in 27-UK families with FALS (the aforementioned family was one of them), Orrell et al. (1995a) found that the previously discovered Gly93Arg mutation showed evidence of a dominant negative effect, where the tested proband had only 30% of wild-type enzyme levels.

Previous studies showed a variable degrees of reduction in SOD-1 enzyme levels associated with different *SOD-1* mutations. Moreover, it was apparent that there was no relation between the disease phenotypes and the level of the SOD-1 enzyme and considerable phenotypic variation occurs within a family among affected members bearing

the same mutation (Ogasawara et al., 1993; Esteban et al., 1994; Borchelt et al., 1994; Gurney et al., 1994; Enayat et al., 1995; de Belleroche et al., 1995). The mechanism(s) by which *SOD-1* mutations could cause FALS disease is not well understood. The most acceptable explanation among investigators working in the *SOD-1*/FALS relationship is that FALS is caused by a toxic property present in the Cu/Zn SOD-1 enzyme which is produced by the mutations (Gurney et al., 1994; Rowland, 1995; de Belleroche et al., 1995; Brown, Jr., 1995). In the present case, the very low enzyme level together with the earlier age at onset of the disease arose by probably two, at present indistinguishable, mechanistic possibilities for Gly93Arg: reduced enzyme activity is contributing to the disease or Gly93Arg is more toxic than the other mutants and the reduced activity is incidental. But, if disease severity was a simple function of SOD-1 toxicity, more toxic mutants would be expected to have early onset and short duration. The finding that onset and duration appear to act independently suggests that another property of the mutant proteins, in addition to toxicity, is involved in the disease mechanism.

Artefacts detected during SSCP analysis

During this study, in some instances, variant bands, which were interpreted as mobility shifts, were detected upon SSCP analysis of the PCR products from different exons of the *SOD-1* gene. In all of them, except one, reloading of the same PCR product on a newly made gel was associated by the disappearance of such bands. There is no clear explanation for this observation, but errors in sample loading (over loading), gel polymerisation, improperly flushed gel wells, uneven gel running or a combination of these may play a role in producing such observations. Differentiation among polymorphic molecules by SSCP is not entirely predictable and the method can result in false negatives, ambiguous results and experimental artefacts (Sheffield et al., 1993).

In one case, the abnormal band shift pattern persisted upon re-running of the same PCR product while it disappeared upon electrophoresis of a newly amplified product

from the same exon from the same patient. To assess for the cause of this condition, the radiolabelled PCR which showed this band upon SSCP analysis was recovered out of low melting point agarose gel and stored until its radioactivity decayed to a safe level. After that 1μl aliquots were used in asymmetric PCR amplification reactions to create single stranded DNA for sequencing. Sequencing of this product revealed the mis-incorporation of a single base, most probably due to a PCR error. The DNA polymerase currently used in most PCR assays is isolated from *Thermus aquaticus*. It lacks 3' to 5' proof-reading activity and has an error rate of approximately 1/10 000 bases (Shibata, 1992). In the previous case, such a PCR error must be produced early in the PCR amplification to make sufficient PCR products detectable by SSCP analysis and DNA sequencing.

SOD-1 mutations in sporadic ALS patients

Because both sporadic and familial ALS have a very similar phenotype with minor differences, it was thought that the molecular pathology operating in both of them might be similar. In that respect it was decided to screen the present cohort of 67 sporadic ALS patients for both unknown and previously known common mutations in the *SOD-1* gene. For the unknown mutations, SSCP analysis was used as described before. For two known mutations, Ala4Val and Ile113Thr, screening of the PCR products from the corresponding exons using restriction enzyme digestion was performed. No abnormal SSCP pattern suggestive of the presence of unknown sequence alterations or abnormal restriction digestion products were detected in the cohort of the studied patients.

Ala4Val was known to be the most common familial ALS mutation detected in *SOD-1* exon one (Siddique et al., 1993). This mutation abolishes a *Hae*III restriction site producing an additional restriction fragment upon the digestion of the PCR product of that exon. Using *Hae*III to screen for that mutation should be very sensitive with no possibility of false negative results. The Ile113Thr mutation in *SOD-1* exon 4 is the second most frequent mutation identified in FALS patients (Deng et al., 1995).

Moreover, it was identified in 3 apparently sporadic ALS patients (Jones et al., 1995). This mutation creates a new restriction site for the enzyme *Bsr*I so that the PCR product from the normal *SOD-1* exon 4 allele will be digested into two fragments while the mutant one will be digested into four fragments. The presence of a *Bsr*I recognition site in the normal exon 4 PCR product served as an internal control for the efficacy of the digestion make it unlikely that the digestion products due to such a mutation could be missed.

SSCP analysis is a sensitive technique and it was already used to detect mutations in ~ 20% of the familial ALS cases studied by different investigators. Moreover, it detected a mutation in one of two studied familial cases in the present study.

No data has been published regarding the detection of *SOD-1* mutations in sporadic ALS although several hundred cases have been screened in North America (de Belleroche et al., 1995). The only exception is the published data by Jones et al. (1993; 1994 and 1995). They detected the Ile113Thr mutation in three apparently sporadic ALS patients and the Glu21Lys in one other patient. Ile113Thr was described before to be the second most common mutation in FALS and is known to have a low penetrance estimated to be less than 50% by the age of 60 years (Suthers et al., 1994). Because of this, Ile113Thr probably masquerades as a new mutation in apparently sporadic ALS, especially when the family history is incomplete (Deng et al., 1995). The inability to detect mutations in any of the screened 67 sporadic ALS patients, in spite of using reasonable screening techniques, and in other sporadic ALS patients from different centres raises the possibility that the molecular pathology is different for the two forms of the disease i.e. sporadic and familial ALS. Linkage studies of FALS families revealed locus heterogeneity with at least one other FALS gene present in the human genome. It may be also the case for sporadic ALS or it may be of non-genetic aetiology. Investigators are searching for other candidate genes for ALS and in that respect

Figlewicz et al. (1994) described mutations in the C-terminal region of the human gene for the neurofilament heavy subunit in five patients with sporadic ALS.

4.3 Screening for mutations in the *TSC2* gene in patients with tuberous sclerosis (TSC)

In the present study, 32 patients with either apparently sporadic TSC or from families non-informative for linkage analysis (except one that was later confirmed to be chromosome 9 linked) were screened for the presence of germline mutations in the *TSC2* gene using two-step strategy. To screen for deletions and/or other structural rearrangements, all patients included in this study were examined by Southern blot hybridisation with two *TSC2* cDNA probes. Second, to screen for point mutations and/or minor sequence alterations, part of the *TSC2* gene, expected to be functionally important (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993), was examined by chemical cleavage of the mismatch (CCM) analysis. Four cDNA segments (spanning positions 1041 to 1857 & 4215 to 5471) and one DNA segment (325 bp of the promoter area, exon 1 and part of intron one) were amplified by PCR from 22 patients. In another 10 patients, only DNA was available and from them two DNA segments (exons 38-41 as one segment and 325 bp of the promoter area, exon 1 and part of intron 1 as another segment) were PCR amplified. All PCR amplification products were analysed by CCM analysis.

Using this strategy, no deletions or other structural rearrangements were detected on Southern analysis while CCM analysis revealed nine mismatch bands due to differences between the target and the normal reference probes. Sequencing of the appropriate PCR products from the observed CCM mismatch bands showed three missense mutations (in three sporadic TSC patients), two silent mutations (in five sporadic TSC patients) and two base pair deletion in one of the polyadenylation signals (in one TSC patient).

Some 5% of germ-line mutations in the *TSC2* gene appear to be deletions detectable by pulsed field gel electrophoresis (PFGE) or conventional Southern analysis (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). In the present study, only 32 patients were screened for the presence of such mutations so it was expected to find 1-2 patients with such deletions. This is a quite small number and may not be present among the studied group because of chance. Moreover, big deletions that could be easily detected upon PFGE will not be detected using conventional Southern analysis due to deletion of the whole probe area from the affected chromosome.

At commencing of the present study (1994), only cDNA sequence of the *TSC2* gene was published and there was no available information regarding the number of the exons and the exon/intron boundaries. Moreover, there were no reports of point mutations in that gene and areas that are hot spots for such mutations. An approach, using RT-PCR amplification of ~40% of the expressed coding sequence of the gene combined with chemical cleavage of mismatch (CCM) analysis, was adopted as a mutation screening strategy. Approximately 1.3 kb from the 3' end and 0.8 kb from the 5' end of the *TSC2* gene were RT-PCR amplified in four segments. The 5' end segment was expected to have a possible membrane-spanning regions and the 3' end segments contain the GAP3-related domain and the polyadenylation signals. These RT-PCR amplified segments were expected to represent critical areas of the gene toward which mutation analysis could be directed.

The principle of mismatch cleavage is based upon the cleavage of heteroduplexes between strands of nucleic acid mismatched at one or more nucleotides. Chemical cleavage of mismatch (CCM) analysis was known to be very sensitive mutation screening technique, detecting > 95% of mismatches when only wild-type DNA is labelled and 100%, when both wild-type and mutant DNA are labelled. It was shown to be an excellent method for the detection and location of mutations as it can easily screen up to 1.7 kb segments of DNA at a time (Grompe, 1993). Using this strategy to screen for

mutations in the *TSC2* gene in 22 TSC patients, 3 missense mutations and three polymorphisms were detected. TSC shows locus heterogeneity with two disease-determining genes on chromosome 9q34 (*TSC1*) and on chromosome 16p13.3 (*TSC2*). Estimates from linkage studies in families suggest that about 50% of the TSC cases are related to each gene (Kwiatkowski et al., 1993). If this distribution is the case in the studied patients as well, then it was expected to detect 11 mutations if the whole *TSC2* gene was screened. As only 40% of the gene was screened and as there was no apparent clustering of mutations within the *TSC2* gene according to recently published data (Wilson et al., 1996), so it was expected to find ~4 mutations, if the detection rate is 100%. The RT-PCR/CCM approach adopted in the present study seemed to be effective (detected 3 of the assumed 4 mutations). A small percentage of mutations is expected to be missed when this approach is used. This may be due to one of the following causes: **1)** Some mutations might affect RNA expression or stability, RT-PCR approach would only amplify the normal mRNA and not detect the mutant form. **2)** Some of the mutations lies outside of the coding sequence that has been amplified by the RT-PCR.

Additional mutations in the *TSC2* gene might be detected by screening each exon from genomic DNA, but unfortunately, the complex genomic structure of the human *TSC2* gene with many small exons will make any exon screening approach to mutation identification labour-intensive.

When some information of the exon/intron boundaries of the *TSC2* gene was kindly supplied by Dr. A. Green, Addenbrooke's NHS Trust, Cambridge, screening for mutations in the promoter area of the gene was adopted. 663 bp segment was PCR amplified from the DNA of 32 patients (including the 22 patients previously screened by RT-PCR). This segment included the *TSC2* exon 1, part of intron 1 and 325 bp of the promoter area. CCM analysis was used to screen for mutations in this segment as before but no sequence changes could be detected. In general, mutations in the promoter area

are not common and it may be necessary to screen more patients to detect such mutations.

In 10 patients, only DNA was available as a study material. A primer set was designed and the PCR conditions were optimised to amplify the *TSC2* exons 38-41 (numbering according to Maheshwar, et al. 1996) as one segment of 1120 bp. The amplified PCR products were screened by CCM analysis and two silent nucleotide changes were detected, resulting in no change to the predicted amino acid. No conclusions could be derived from these results as the number of the screened patients were small and only ~12% of the their *TSC2* gene was screened.

Missense mutations in the *TSC2* gene

In the present study, three missense mutations were detected. First (M1602V), in the GAP3-related domain and second and third (S1715T and R1720W), in the N-terminal of the tuberin, the *TSC2* gene product. These mutations are novel and the M1602V is the first mutation to be detected in the GAP3-related domain of the protein. Comparison of the predicted human and pufferfish (Fugu rubripes) peptide sequences identified four regions of high conservation. These include the GAP3-related domain (human residues 1593-1631) and an area of ~200 amino acid residues which surrounds this, two small regions between residues 750 and 1100, and the N-terminal portion of the molecule (Maheshwar et al., 1996). This placed these missense mutations in an important part of the gene. Moreover, as these changes were not found upon restriction enzyme screening of normal controls (100 chromosomes), these mutations likely contribute to the TSC phenotype in these patients.

In contrast to what might be expected, M1602V mutation in the GAP3-related domain was not associated with severe phenotype. GAPs act by stimulating the intrinsic GTPase activity of the Ras-related encogens, keeping them in the inactive, GDP-bound state (Lowy and Willumsen, 1993). The GAP3-related homology of the tuberin suggested

that tuberin may itself have GAP activity, consistent with its proposed function as a tumour or a stem cell growth suppressor (Green et al., 1994a; Carbonara et al., 1994) and mutations in that part should be associated with the severe phenotype. This patient had the skin manifestations of TSC (fibrous forehead plaque, adenoma sebaceum, peri-ungual fibromata) and seizures which is now under control and patient's recent EEG was normal. This mutation changed methionine to valine and both are neutral and hydrophobic amino acids. Such change may not have severe effects on the protein stability and function. If this is case it was expected that the second missense mutation R1720W, which changed the basic arginine to the neutral and hydrophobic tryptophan, will be associated with a more severe phenotype. This is still also not the case. The patient that carried R1720W was presented with mild phenotype with skin manifestations and seizures as it is the case in most TSC patients. Finally, the missense mutation S1715T, which is very close to the previous R1720W, was associated with the most severe phenotype. The patient who is 18 years old is mentally handicapped and he is a resident of a hospital for the mentally handicapped. The S1715T would normally be considered to be a conservative change, both serine and threonine are neutral and polar amino acids. However, there are known examples of conservative missense mutations that can result in a disease phenotype if they occupy sites in the protein that are key determinants of stability or function (Deng et al., 1993).

Until more is known about the function of tuberin and until additional mutations have been characterised pin-pointing critical areas, it is difficult to speculate about the exact effects these mutations have on tuberin's function. Moreover, until mutations are defined in a large set of patients, correlation between genotype and phenotype will be difficult to perform.

To date, the mutations that have been identified in the *TSC2* gene include the five large-scale deletions and five intragenic deletions defined in the original report describing the cloning of the *TSC2* gene (The European Chromosome 16 Tuberous Sclerosis

Consortium, 1993) and a further six patients that were described in a subsequent report with deletions including both the *TSC2* and the *PKD1* genes (Brook-Carter et al., 1994). In addition, 13 other mutations have been described (Table 4.1). A high percentage of apparently expressed missense mutations (50%) is apparent rather than the expected nonsense mutations with prematurely truncated protein products (Figure 4.1). The proportion of missense mutations is comparable to that seen in the *p53* tumour suppressor gene where missense mutations have been reported at a relatively high frequency (Chiba et al., 1990), but it is quite different from the mutations found in the other phakomatoses where truncation mutations are far more prevalent (Legius et al., 1993; Trofatter et al., 1993; Latif et al., 1993). In *p53* the wide spectrum of the missense mutations have been shown to disrupt both DNA binding and the stability of the protein which may also be the case for tuberin (Wilson et al., 1996).

Table 4.1 ***TSC2*** **mutations detected in different studies in both familial and sporadic TSC patients**

Serial	Sequence Change	Codon Change	Type of Mutation	Reference
1	5110 delA	M1698 fs →stop at codon 1801	Frame Shift (fs)	Kumar et al., 1995a
2	4590/4591 delC	V1524/L1525 fs→stop at codon 1553	Frame Shift	Kumar et al., 1995b
3	156+1G→A	Splice Junction	Splice mutation	Kumar et al., 1995b
4	C3616→T	R1199W	Missense	Wilson et al., 1996
5	G1365→C	M449I	Missense	Wilson et al., 1996
6	C5075→T	P1686L	Missense	Wilson et al., 1996
7	C5084→A	A1689E	Missense	Wilson et al., 1996
8	C1849→T	R611W	Missense	Wilson et al., 1996
9	C1531→T	R505Stop	nonsense	Wilson et al., 1996
10	1112/1113 del(TC)	I365 fs→stop at codon 385	Frame Shift	Wilson et al., 1996
11	4474/4476 del(TTC)	F1486	In Frame Deletion	Wilson et al., 1996
12	4519/4547 29 bp tandem duplication	L1510 fs→stop at codon 1541	Frame Shift	Wilson et al., 1996
13	A52→T	K12Stop	nonsense	Vrtel et al., 1996
14	T5161→A	S1715T	Missense	Present Study
15	C5176→T	R1720W	Missense	Present Study
16	A4822→G	M1602V	Missense	Present Study

Numbering of the bases and codons is given relative to the published cDNA sequence (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). fs= frame shift.

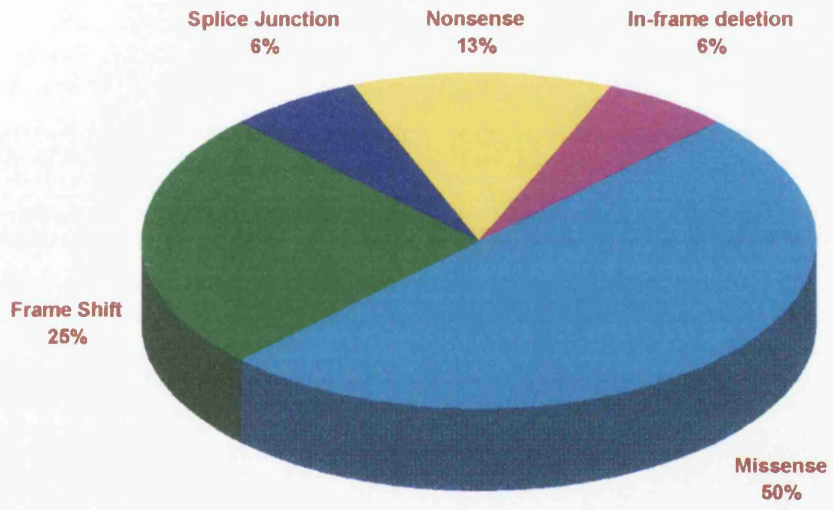


Figure 4.1

Percentage distribution of point mutations and minor sequence alterations within the *TSC2* gene.

Polymorphisms in the *TSC2* gene

Two silent mutations, C4098→T and G5346→C caused silent Ser1630 and Ser1776 changes respectively, were detected in five out of the screened 33 patients. One of these mutations, G5346→C change, was detected in four patients. This change seems to be frequent as it has been also reported in one TSC patient (Wilson et al., 1996). Unfortunately, this mutation does not change a commercially available restriction site so it was not able to screen the normal chromosomes for its presence. Designing a primer set to introduce a restriction site, use of ASO or ARMS analysis could be useful to screen normal population for that polymorphism which may be used later (if proved useful) for linkage studies in the *TSC2* gene.

The third polymorphism was due to an AA deletion at positions 5433 and 5434 in one of the two partially overlapping polyadenylation signals of the gene. It was detected in one of the familial cases, which proved, later on, to be linked to markers on the chromosome 9q34 (*TSC-1* locus). Sequencing of the relevant part of the *TSC2* gene in other affected family members failed to detect this mutation. Moreover, sequencing of 20 normal chromosomes detected this mutation in one of them. Kumar et al. (1995b) reported a polymorphic 4 base pair deletion, 5425del4, in the two partially overlapping polyadenylation signals in one TSC family and in six of 72 African-American control chromosomes examined. The role of polyadenylation signals at the 3' untranslated region of the genes is well documented. Deletion and/or substitution of any nucleotide in the conserved AATAAA polyadenylation signal can result in abnormal processing of mRNA (Lewin, 1990). The *TSC2* gene has two partially overlapping polyadenylation signals that may cause differential polyadenylation (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). As the AA deletion still leaves one completely functional polyadenylation signal and as it could not be detected in other affected family members but detected in one normal chromosome, it is reasonable to consider it as a polymorphic change.

4.4 Conclusions and future work

Mutation detection strategy is influenced by the expected nature of the mutation, size and structure of the gene in question, availability of mRNA and the degree of required sensitivity. Taking this into consideration, three different mutations strategies, each for a different gene, were optimised and proved to be useful screening techniques for the detection of mutations within the *DMPK*, the *SOD-1*, and the *TSC2* genes.

When the mutation in a gene like *DMPK* is due to the new molecular mechanism of trinucleotide repeat length expansion, PCR amplification to detect this change in length aided by Southern blot analysis, when the PCR fails to amplify large repeat expansions will, theoretically, be the ideal mutation detection method. Nearly all trinucleotide repeat expansions that are known to be associated with disease conditions are GC rich and using high annealing temperature and hot start for PCR will improve the PCR results. When accurate number of CTG repeats is important to know (e.g. in the upper normal range of the repeat size), radiolabelled PCR resolved by denaturing polyacrylamide gel electrophoresis is mandatory. On the other hand, non-radiolabelled PCR proved to be a useful first step screening technique. Southern blot analysis is needed to confirm PCR results and to detect alleles that carry more than 80-150 repeats. Choosing proper enzyme/probe system for Southern analysis is very important to provide an unambiguous detection of both normal (homozygous or heterozygous) and affected individuals.

The mechanism by which the expanded trinucleotide repeat in the 3' untranslated region of the *DMPK* gene leads to the clinical features is unclear. The DM region of chromosome 19 is gene rich, and it is possible that the repeat expansion may lead to dysfunction of a number of transcription units in the vicinity, perhaps as a consequence of chromatin disruption. Further work will be needed to study different genes in the vicinity of the CTG trinucleotide repeat to clarify the role of this repeat expansion in the disease

process. Moreover, intensive study of DM patients who did not show CTG repeat expansions (~2% of DM patients) might be of importance in that respect.

The *SOD-1* gene is a small well-characterised gene with few exons. It is known that nearly all expected mutations within the gene in FALS patients will be point mutations causing missense amino acid changes, so that using DNA as a template for PCR/SSCP analysis seems to be a reasonable mutation detection strategy. In the present study SSCP analyses of the *SOD-1* gene were performed to screen for mutations in both familial and sporadic ALS patients. Different gel matrices and different running conditions were tried and it was shown that using MDE™ gel with 5% glycerol running at room temperature was the most suitable choice to screen this gene for mutations. One missense mutation was detected in one familial ALS patient (out of two screened familial cases) but no mutations were detected in any of 67 screened sporadic ALS patients. In this study, false positive results upon SSCP analysis were obtained and careful result interpretation and repeating of the experiments which show positive band shifts are mandatory to exclude possible errors.

Only 5% of ALS cases are familial, and of these, fewer than 20% map to the *SOD-1* gene, so there is locus heterogeneity (Rowland, 1995). Failure to detect mutations in the sporadic ALS cases (67 patients) in the present study may be simply because the *SOD-1* gene is not the gene responsible for that disease phenotype. As this gene is very small, direct sequencing of the gene (100% detection rate) in sporadic ALS may prove or disprove such a possibility. Linkage study analysis of more FALS families is recommended to detect other genes responsible for the disease process. This will help in more understanding of ALS molecular pathology and will improve genetic counseling of ALS patients.

RT-PCR and CCM analysis for the detection of sequence variations in PCR fragments is an ideal mutation detection method for genes with big transcripts and multiple small exons, especially if the gene mRNA can be easily obtained from peripheral

blood lymphocytes and when the genomic organisation of the gene is not yet known. Using this strategy to screen the *TSC2* gene allowed the amplification and screening of a large area of the coding sequence of the gene (~40%) using only 4 sets of PCR primers. Moreover, by using mRNA as a template it was possible to begin screening for mutations in the *TSC2* gene before the genomic organisation was known (exon/intron boundaries were recently published, Maheshwar et al., 1996). CCM analysis allowed accurate localisation of the mutation thus enabling sequencing of a limited area instead of the whole product. Moreover, the nature of altered bases was predicted depending on the modifying chemical. Osmium tetroxide modifies T mismatches and hydroxylamine modifies C mismatches. This further confirms the nature of the mutation in sequencing. In the present study, 3 missense and two silent mutations and one two base pair polymorphic deletion were characterised and the adopted mutation detection strategy proved to be useful.

Since 60% of the gene is still to be screened, little is known about the function of tuberin and only few mutations were characterised so far, a clustering of mutations in the yet unanalysed part of the *TSC2* gene cannot be ruled out. Moreover, it will be difficult to speculate about the exact effects these mutations have on disease phenotype and on tuberin's function. Using CCM analysis to screen the whole *TSC2* cDNA in more patients is highly recommended to clarify some of these unknown queries. To decrease the hazards of radioactivity and to improve mutation detection rate from 95% to 100%, optimising a silver staining technique of CCM analysis is also recommended.

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APPENDIX 1

Solution used for DNA extraction from peripheral blood

(1) Lysis Buffer	0.32M Sucrose 10mM Tris-HCl, PH 7.5 5mM $MgCl_2$ 1% Triton X-100
(2) Nuclei Lysis Buffer	0.4M NaCl 10mM Tris-HCl, PH 8.2 2mM EDTA
(3) Phenol/Chloroform	Water Saturated Phenol one volume 1M Tris, PH 7.5 0.1% 8-hydroxyquinoline Chloroform one volume
(4) T.E. Buffer	10mM Tris-HCl, PH 7.5 1mM EDTA
(5) Protinase K (10mg/ml)	Boehringer Mannheim

Solutions used for RNA extraction

(1) Histopaque '1077'	Sigma
(2) Solution D	4M guanidinum thiocyanate (Fluka) 25mM Sodium citrate, PH 7.0 0.5% Sarcosyl (Sigma)
(3) 2M Na acetate, PH 4.0	
(4) Water-saturated phenol	
(5) Chloroform:isoamyl alcohol (49:1)	
(6) Phosphate buffered saline, PH 7.2 (PBS, Gibco BRL)	0.14M NaCl 3mM KCl 0.1mM sodium hydrogen phosphate 1mM potassium dihydrogen phosphate
(7) diethyl pyrocarbonate (DEPC), 0.1%	

Solutions for RNA sample electrophoresis

(1) 10X MOPS	200mM MOPS 50mM Na acetate, PH 7.0 10mM EDTA
(2) 37% formaldehyde solution	
(3) Formamide	
(4) Loading mix	2g Ficoll 1ml 2.5% bromophenol blue 1ml 2.5 xylene cyanol 0.2ml 0.5M EDTA 8ml water
(5) E. Coli ribosomal RNA	Boehringer Mannheim

Reagents used for reverse transcription

(1) 5X first strand buffer (Gibco BRL)	250 mM Tris-HCl, PH 8.3 375 mM KCl 15 mM $MgCl_2$
(2) DTT (0.1M dithiothreitol	Gibco BRL
(3)M-MLV reverse transcriptase	200 units/μl (Gibco BRL)
(4) oligo $(dt)_{12\text{-}18}$	Pharmacia Biotech.
(5) dNTPs (dTTP, dATP, dCTP, dGTP)	100mM each, Boehringer Mannheim.

Reagents used for PCR

(1) 10X GeneAmp PCR buffer (Perkin-Elmer/Cetus)	500mM KCl 100mM Tris-Cl, PH 8.3 15mM $MgCl_2$ 1000 μg/ml gelatin
(2) AmpliTaq DNA polymerase	5 units/μl, (Perkin-Elmer/Cetus)
(3) dNTPs (dTTP, dATP, dCTP, dGTP)	100mM each, Boehringer Mannheim.

Reagents used in plasmid work

(1) Luria broth (LB), 1 litre (PH 7.5, adjusted by 5M NaOH)	10g bactotrypton 5g bacto yeast extract 5g NaCl
(2) LB-agar	Like LB + 5g bacto agar/litre
(3) SOC Media, 1.020 litre (PH 7.0, adjusted by 5M NaOH)	20g bactotrypton 5g bacto yeast extract 0.5g NaCl 10 ml of 250mM HCl 20 ml of 1M filter sterile glucose

Reagents used for Southern blotting

(1) T.A.E. buffer 50X	242g Tris base 57.1 ml glacial acetic acid 100 ml 0.5M EDTA, PH 8.0 Up to 1 litre by dH_2O
(2) Depurination solution	0.25M HCl
(3) Denaturation solution	0.5M NaOH 1.5M NaCl
(4) Neutralization solution	3M NaCl 0.5M Tris-HCl, PH 7.4
(5) 20X SSC (standard saline citrate)	3M NaCl 300 mM Na citrate, PH 7.4
(6) 100X Denhardt's solution	2% (w/v) BSA (bovine serum albumin) 2% (w/v) Ficoll 2% (w/v) polyvinylpyrrolidone (PVP)
(7) Prehybridization solution	5X SSC 1% SDS (v/v) 5X Denhardt's solution
(8) 6X agarose gel loading buffer	0.25 bromophenol blue 40% (w/v) sucrose 1X TBE buffer

Reagents used for chemical cleavage of mismatches

(1)$T_{0.1}E$	10mM Tris-HCl, PH 7.4 0.1mM EDTA
(2) 10X hybrid buffer	3M NaCl 1M Tris-HCl, PH 8.0
(3) Stop/precipitation mix	63mM Na acetate 20μM EDTA 80% ethanol
(4) 10X One-Phor-All buffer plus (Pharmacia Biotech.)	100mM Tris-acetate 100mM Magnesium acetate 500mM potassium acetate
(5) T4 Polynucleotide kinase	~ 10 units/μl (Pharmacia Biotech.)

Solutions used for sequencing (all supplied with Sequenase version 2.0 kit, USB)

(1) 5X sequenase buffer	200mM Tris-HCl, PH 7.5 100mM $MgCl_2$ 250mM NaCl
(2)5X labeling mix (dGTP)	7.5μM dGTP 7.5μM dCTP 7.5μM dTTP
(3) Termination mix (ddG,T,C or ATP)	8μM ddG,T,C or ATP 80μM dNTPs 50mM NaCl
(4) Stop solution	95% formamide 20mM EDTA 0.05% bromophenol blue 0.05% xylene cyanol
(5) 20X T.T.E buffer	216 g Tris base 72 g taurine 4 g EDTA distilled water up to 1 litre

(6) Glycerol enzyme dilution buffer	20mM Tris-HCl, PH 7.5 2mM DTT 0.1mM EDTA 50% glycerol
(7)Sequenase version 2.0 T7 DNA polymerase	13 units/μl

Solution used for fluorescent automated sequencing

(1) Terminator premix (Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, Perkin Elmer)	1.58μM A-DyeDeoxy 94.47μM T-DyeDeoxy 0.42μM G-DyeDeoxy 47.37μM C-DyeDeoxy 78.95μM dITP 15.79μM of each dATP,dCTP & dTTP 168.42mM Tris-HCl, PH 9.0 4.21mM $(NH_4)_2SO_4$ 42.10mM $MgCl_2$ 0.42 units/μl AmpliTaq DNA polymerase
(2)Phenol:water:chloroform (68:18:14)	86 ml of water saturated phenol 14 ml chloroform
(3)gel loading mix	5 parts deionized water 1 part of 50mM EDTA, PH 8.0 30 mg/ml blue dextran (Sigma)
(4) 10X T.B.E. buffer	108 g Tris base 55 g boric acid 9.3 g EDTA Distilled water up to 1 litre

Reagents used for ligation of PCR products (all supplied by Promega)

(1) 10X ligase buffer	300mM Tris-HCl, PH 7.0 100mM $MgCl_2$ 100mM DTT 10mM ATP
(2) T4 DNA ligase	1 Weiss unit/μl

(3) pGEM^{R*}-T vector	50 ng/µl
(4) pGEM^{R*}-T vector control DNA	4 ng/µl
(5) IPTG stock solution (0.1M)	24 mg/ml
(6) X-Gal (100 mg) was dissolved in 2 ml of N,N' dimethylformamide	50 mg/ml