CHROMOSOME 21 AND AMYLOID PLAQUE PROTEINS IN ALZHEIMER'S DISEASE

Styliani Fidani

1993

A thesis submitted for the degree of Doctor of Philosophy

St. Mary's Hospital Medical School

BIBL. LONDIN. UNIV.

ABSTRACT

Alzheimer's disease, a neurodegenerative disorder, is known to be familial in some cases. In this genetic form linkage studies had shown (1987) that the disease co-segregated with markers on chromosome 21. In an attempt to develop new highly polymorphic markers on this chromosome in order to localise the gene more precisely, I have used the interspersed repeat sequence polymerase chain reaction on chromosome 21 specific somatic cell hybrids. Although the amplification of inter-Alu, inter L1 and inter Alu-L1 fragments of chromosome 21 was achieved, isolation and characterisation of one clone mapping on 21 failed to reveal any useful polymorphisms.

Evidence for genetic heterogeneity in Alzheimer's disease (1990), was followed by the discovery of mutations in the ß-amyloid protein precursor (APP) gene leading to the development of the disease (1991).

I designed primers for the coding regions of the APP gene and used single strand conformation analysis to screen a number of affected members of Alzheimer's disease pedigrees with early age of onset, I have identified one new family with the APP717 val->ile mutation and was able to detect all new APP alterations in exon 17 of the gene. Screening of the other 17 exons failed to reveal any new variation. These data support the notion that the APP717 val->ile mutation is pathogenic and also provide further evidence that the disease shows non-allelic genetic heterogeneity.

Following the evidence of genetic heterogeneity, I have tried the "candidate gene" approach to determine whether mutations in

another plaque core protein, α-1 antichymotrypsin, may be responsible for the disease in 10 early onset families with no known mutations in the APP gene. α-1 antichymotrypsin (like APP) is a serine protease inhibitor. I have developed two polymorphic markers tightly linked to the α-1 antichymotrypsin locus and used them in linkage studies in these 10 early onset Alzheimer's disease families. These results are presented, together with a discussion of the problems of linkage analysis in complex disorders such as Alzheimer's disease.

CONTENTS

Title	1
Abstract	2-3
Contents	4-7
List of tables	8
List of figures	9-10
Abbreviations	11-13
Acknowledgements	14-16

Chapter 1

Introduction 18-37

1.1 Introduction to Alzheimer's disease

1.2 Brief Introduction to positional cloning

1.3 History of AD genetics

1.4 My project

Chapter2

Materials and Methods 40-67

2.1 Dialyzed fetal calf serum (FCS) preparation

2.2 Routine tissue culture maintenance of hybrid cell lines

2.3 High Molecular Weight DNA preparation from cell lines

2.4 RNase treatment of DNA

2.5 Precipitation of nucleic acids

2.6 Preparation of plasmid DNA

a. Mini preparation

b. Midi preparation

2.7 Restriction enzyme digests

2.8 DNA agarose and polyacrylamide gel electrophoresis

2.9 Isolation and purification of DNA fragments

2.10 Quantification of nucleic acids by spectrophotometry

2.11 Preparation of competent cells

2.12 Cloning and ligations

2.13 Transformations

2.14 PCR (Polymerase Chain Reaction)

2.15 Labelling of DNA

a.end-labelling

b.random-hexamer labeling

2.16 Southern blotting- Nucleic acid hybridisation

2.17 Spun columns

2.18 Preparation of DNA from blood

2.19 Isolation of total human DNA from placental tissue

2.20 Determination of base sequence of DNA

a. Automated DNA sequence analysis

b. Dideoxynucleotide sequencing

c. Solid phase sequencing

2.21 Preparation of sequencing gel

Chapter 3

Interspersed repeat sequence polymerase chain reaction on chromosome 21 68-100

3.1 Introduction

3.1.1 Chromosome 21

3.1.2 Alzheimer's disease and chromosome 21

3.1.3 Generation of polymorphic markers and human genetic map

3.1.4 Somatic cell hybrids

3.1.5 Human repeat sequences

a. Alu family of repeats

b. L1 or Kpn1 family of repeats

3.1.6 IRS-PCR on chromosome 21

3.1.7 APP gene in Alzheimer's disease
3.2 Methods
3.2.1 Cell lines
3.2.2 High molecular weight DNA preparation
3.2.3 PCR amplification and primers
3.2.4 Agarose gel electrophoresis
3.2.5 Southern blot
3.2.6 Restriction enzyme digest
3.2.7 Random-hexamer labeling
3.2.8 Pre-reassociation of DNA
3.2.9 5' end labelling
3.2.10 Extraction of DNA from agarose
3.2.11 Cloning of IRS-PCR fragments
3.2.12 Automated sequencing
3.2.13 Double strand sequencing
3.3 Results
3.4 Discussion

Chapter 4

Screening for mutations in the open reading frame of the ß-amyloid precursor protein gene in familial Alzheimer's disease 102-137

4.1 Introduction
4.1.1 APP gene and Alzheimer's disease
4.1.2 APP gene: genomic organisation
4.1.3 Screening methods
4.1.4 The use of PCR-SSCP in the detection and identification of mutations in APP gene
4.2 Methods
4.2.1 DNA samples

4.2.2 PCR

4.2.3 SSCP

4.2.4 Solid phase sequencing

4.3 Results

4.4 Discussion

Chapter 5

Analysis of α-1 antichymotrypsin as a candidate gene for Alzheimer's disease 138-165

5.1 Introduction

5.1.1 Alzheimer's disease genetics: our strategy

5.1.2 AD families with no mutations in APP

5.1.3 AACT as a candidate gene for Alzheimer's disease

5.1.4 AACT gene

5.2 Methods

5.2.1 DNA samples

5.2.2 PCR primers and conditions

5.2.3 Generation of AACT marker

5.2.4 Linkage studies

5.3 Results

5.4 Discussion

Chapter 6

Discussion 166-180

Addendum 181

References 182-197

Published material

LIST OF TABLES

Chapter 1

Table 1.1 38-39

Chapter 4

Table 4.1 132-134

Table 4.2 135-137

Chapter 5

Table 5.1 162

Table 5.2 163-165

LIST OF FIGURES

Chapter 1

Figure 1.1 20

Figure 1.2 25

Chapter 2

Figure 2.1 54

Chapter 3

Figure 3.1 71

Figure 3.2 74

Figure 3.3 77

Figure 3.4 81

Figure 3.5 89

Figure 3.6 90

Figure 3.7 91

Figure 3.8 91

Figure 3.9 93

Figure 3.10 95

Figure 3.11 97

Figure 3.12 98

Figure 3.13 98

Chapter 4

Figure 4.1 103

Figure 4.2 115

Figure 4.3 115

Figure 4.4 116

Figure 4.5 116

Figure 4.6 117

Figure 4.7 117

Figure 4.8 118

Figure 4.9 118

Figure 4.10 119

Figure 4.11 119

Figure 4.12 120

Figure 4.13 120

Figure 4.14 121

Figure 4.15 121

Figure 4.16 122

Figure 4.17 122

Figure 4.18 123

Figure 4.19 123

Figure 4.20 124

Figure 4.21 125

Figure 4.22 126

Figure 4.23 128

Chapter 5

Figure 5.1 142

Figure 5.2-5.10 147-155

Figure 5.11 156

Figure 5.12 156

Figure 5.13 157

Figure 5.14 160

Chapter 6

Figure 6.1 167

Figure 6.2 169

Figure 6.3 173

Figure 6.4 175

Figure 6.5 176

ABBREVIATIONS

AACT	: α-1 antichymotrypsin
AD	: Alzheimer's disease
APP	: ß-amyloid precursor protein
APS	: Ammonium Persulfate
ASO	: Allele specific Oligohybridisation
ß-A4	: ß-amyloid
BLAST	: Basic Local Alignment Search Tool
BSA	: Bovine Serum Albumin
CFTR	: Cystic Fibrosis Transmembrane Receptor
CHO	: Chinese Hamster Ovary
Ci	: $3.70X10_{10}$disintegrations/second
CIP	: Calf Intestinal Phosphatase
CJD	: Creutzfeld-Jacob Disease
cM	: CentiMorgans
Cot½	: Half reaction time: the point in a reannealing experiment when half of the DNA is present in double stranded fragments
cpm	: Counts per minute
ddNTP	: Dideoxynucleotide triphosphate
DGGE	: Denaturing Gradient Gel Electrophoresis
DMEM	: Dulbecco's modified Eagle's medium
DMSO	: Dimethylsulphoxide
dNTP	: Deoxynucleoside triphosphate
DTT	: Dithiothreitol
EDTA	: Ethylene diamine-tetracetic acid
FCS	: Fetal Calf Serum

G/Q	: Giemsa/Quinacrine
GSS	: Gerstmann Straussler Syndrome
HCHWA-D	: Hereditary Cerebral Hemorrhage with Amyloidosis- Dutch type
HEPES	: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HSP70	: Heat Shock Protein
IPTG	: Isopropylthio-ß-D-galactosidase
IRS-PCR	: Interspersed Repeat Sequence Polymerase Chain Reaction
kD	:Kilodalton
LB	: Luria-Bertani
LMP	: Low melting point
MW	: Molecular Weight
NFT	: Neurofibrillary tangles
OD	: Optical Density
O/N	: Overnight
p	: Small arm of the chromosome
PBS	: Phosphate- Buffered Saline
PEG	: Polyethylene Glycol
PHF	: Paired helical filament
PI	: antitrypsin
PrP	: Prion
PVP	: Polyvinyl Pyrollidone
q	: Long arm of the chromosome
R	: Reverse
RF	: Recombination fraction
RFLP	: Restriction Fragment Length Polymorphism
RNase	: Ribonuclease

rpm	: Rotations per minute
Sarkosyl	: Sodium Sarcocinate
SDS	: Sodium Dodecyl Sulphate
SSC	: Standard Saline Citrate
SSCP	: Single Strand Conformation Polymorphism
TEMED	: NNN'N'-Tetramethylethylene-diamine
Tris	: 2-Amino-2-(hydroxymethyl)-1,3-propandiol crystalized
UV	: Ultraviolet radiation
VNTR	: Variable Number Tandem Repeats
v/v	: Volume/Volume
w/v	: Weight/Volume
X-gal	: 5-bromo-4-chloroindolyl-ß-D-galactosidase

ACKNOWLEDGEMENTS

After three years of work in the Department of Biochemistry of St. Mary's Hospital Medical School on a daily basis, I have been very lucky to experience how efficient and productive organised team work can be when all persons involved are willing to collaborate and support each other. I would like to express my appreciation to all, as with their contribution, the Department provides an excellent basis for scientific evolution.

Especially I would like to thank Gail Davies, for long scientific discussions, but above all for being a very good friend during those past three years.

I would also like to thank Nick Irving, Fiona Crawford, Henry Houlden, Chris Talbot, David Hughes, Louise James, Richard Crook, Matt Parfit, who were all very cooperative, and Hilary O'Donnell who put up with my tissue culture experiments.

Special thanks to Dr. Mike Owen, Dr. Marie-Christine Chartier-Harlin, Dr. Jerry Brown, Dr. Gareth Roberts, Joy Dexter and Jane Ashenden, Prof. Bob Williamson, who was always there with thoughtful advice and Dr. Alison Goate who supervised me during the first year of my work.

The screening of the Spanish individuals was done with the aid of Rosa Adroer. The linkage analysis was performed in collaboration with Dr. Mike Mullan, who has been helpful throughout my work.

Above all, I would like to express my gratitude to Prof. John Hardy, who has always been there for me, giving me not only his invaluable scientific knowledge and opininon, but also providing me with a brilliant example of what a gifted person can achieve when pursuing noble endeavours.

Data form this thesis contributed in the publication of the following papers:

1. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Goate A. et al., (1991) Nature, 349:704-706

2. Early onset Alzheimer's disease is caused by mutations in codon 717 of the ß-amyloid precursor protein. Chartier-Harlin M-C. et al., (1991) Nature, 353:844-846

3. Sequencing of exons 16 and 17 of the ß-amyloid precursor protein gene in 14 families with early onset Alzheimer's disease fails to reveal mutations in the ß-amyloid sequence. Crawford F. et al., (1992) Neuroscience Letters 133:1-2

4. Screening for mutations in the open reading frame and promoter of the ß-amyloid precursor protein gene in familial Alzheimer's disease: Identification of a further family with APP717 Val->Ile. Fidani L. et al., (1992) Human Molecular Genetics 1,3:165-168

5. Screening of the promoter and the ß-amyloid sequence of the APP gene for polymorphism in families with late onset Alzheimer's disease. Rooke K. et al., (1992) Neurodegeneration in press

6. One novel silent variantat codon APP711 and a variant at codon APP708 of the APP sequence detected in Spanish Alzheimer and control cases. Adroer R. et al., (1992) Neuroscience Letters in press

7. A locus for familial early onset Alzheimer's disease on the long arm of chromosome 14, proximal to α1-antichymotrypsin. Mullan M. et al., (1992) Nature in Genetics in press

To my parents

and my brother

CHAPTER 1

INTRODUCTION

1.1 Introduction to Alzheimer's disease

Alzheimer's disease is the most common cause of dementia in the elderly affecting 5% of the population over 65 years and is responsible for dementia in about 300,000 individuals in Britain. It is a progressive neurodegenerative disorder involving memory impairment, disorientation in time and place, and restlessness, evolving to apraxia, agnosia and dysphasia leading to global cognitive decline, loss of reasoning, deterioration of personality, and eventually to a vegetative state, and death (Breitner and Folstein, 1984). Definite diagnosis is difficult and requires neuropathological examination (biopsy or autopsy material). However, sophisticated neurological and psychiatric examinations offer a high degree of diagnostic certainty. The NINCDS/ADRDA task force definition of dementia provide criteria, sufficient for such a diagnosis (Tierney *et al*.1988).

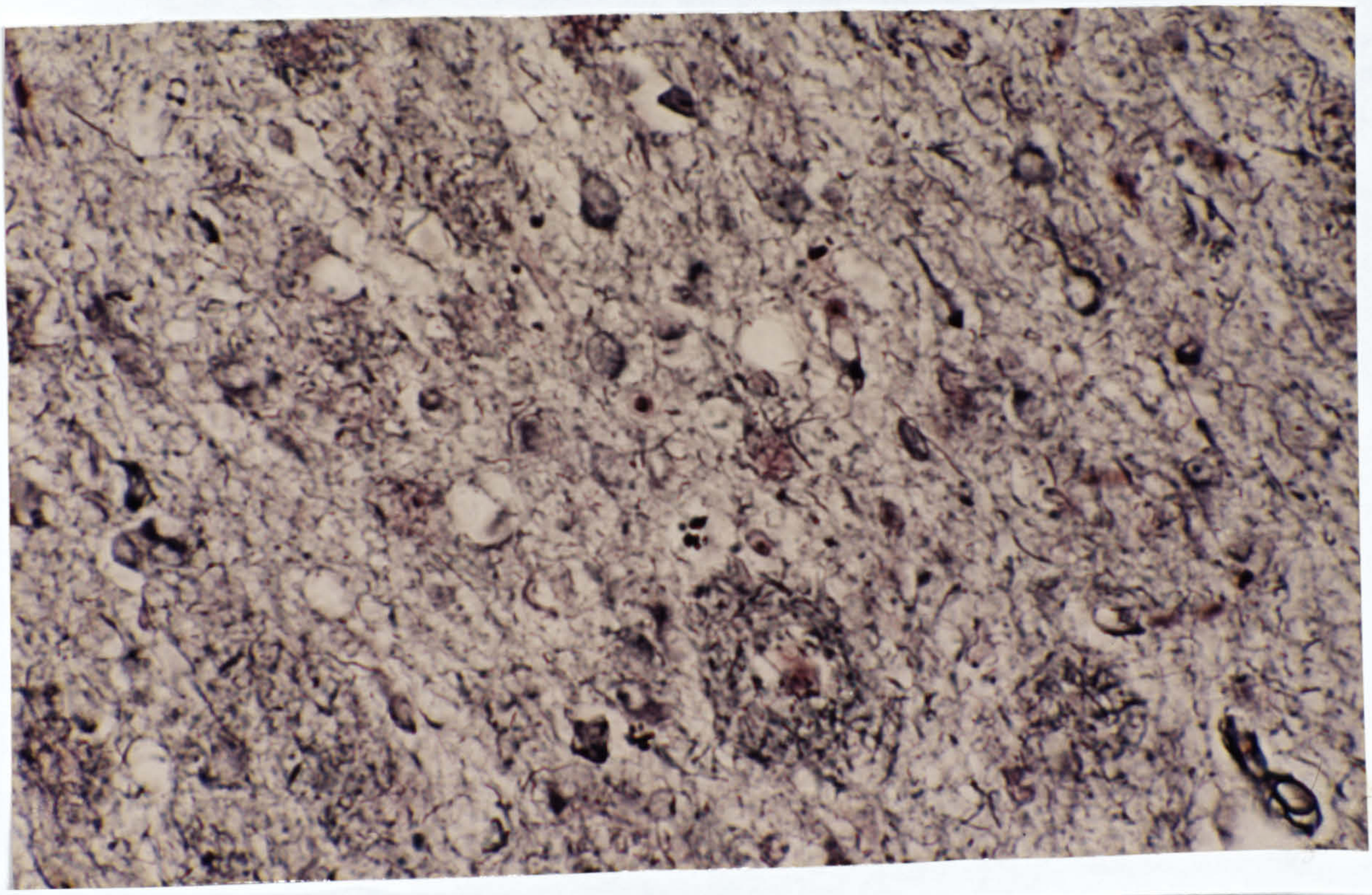
Alzheimer's disease is accurately defined by its pathology (Terry and Katzman, 1983), as clinical diagnoses are uncertain:-

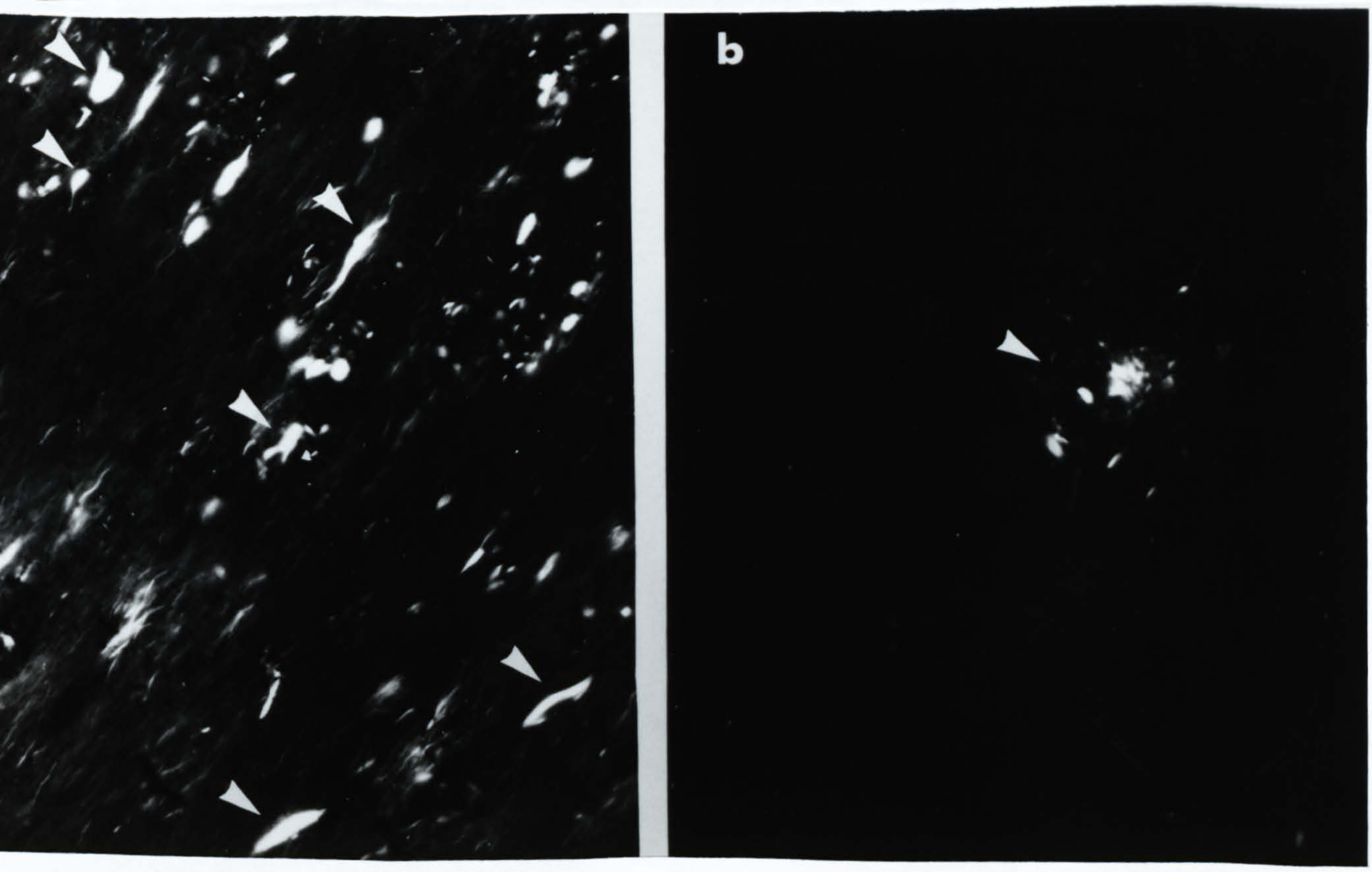
a) cerebral extracellular deposits of ß-amyloid protein, forming the senile plaque core which is surrounded by

degenerating nerve terminals. The senile plaques are spherical structures ranging in diameter from 5- 200μm, which can be easily detected by silver impregnation techniques, by thioflavine S fluorescence, and by the use of immunocytochemistry. ß-amyloid protein, when stained with Congo red shows green-red birefrigence under polarised light. This property is also used for plaque detection in Alzheimer's disease (Figure 1.1). The plaques develop from diffuse (Yamaguchi *et al*.1988), to a classical or mature plaque and finally to a burnt out or compact plaque mainly consisting of an amyloid core. Components of the amyloid core, apart from ß-amyloid, are α1-antichymotrypsin (Abraham *et al*.1989) and complement factors (Eikelenboom *et al*.1989). They are most dense in the hippocampus, the parahippocampal gyrus and the amygdala. Apart from the senile plaques, abnormal deposits of ß-amyloid are found in the walls of cerebral blood vessels (Glenner and Wong, 1984).

b) intracellular neurofibrillary tangles (NFT) containing the abnormal phosphorylated cytoskeletal protein tau (Lee *et al*.1991). The neurons do not survive and those with these intracellular inclusions disintegrate (Sumpter *et al*.1986). These changes can be visualised using the same techniques as for plaque detection. Tangles are made of a pair of helically twisted filaments each 10nm wide (PHFs) and are found in the same areas as plaque pathology (Figure 1.1). The PHFs are often associated with ubiquitin, a cellular protein used to maintain the metabolic integrity of cells, possibly as a response to the abnormal component of PHFs, over-phosphorylated tau (Perry *et al*.1987). Tangles are seen in a number of other disorders such

Figure 1.1





(Above) :Plaques and tangles as seen when stained with Bielschowsky silver stain and
(Below) a. Tangles and b. senile plaques, when Congo red stain is used.
Photograph was provided by Dr. G. Roberts.

as the Parkinsonian dementia complex of Guam, dementia pugilistica and post traumatic dementia (reviewed in (Hardy *et al*.1986)).

c) neuronal loss and atrophy in the hippocampus, neocortex, and amygdala. The loss of large pyramidal neurons of layers II and IV (Hubbard and Anderson, 1985) relates directly to the cognitive decline seen in Alzheimer's disease and a loss of synapses in affected cortical areas is probably the primary morphological basis of the cognitive deficits.

A number of minor neuropathological changes are also associated with the disease, such as granulovacuolar degeneration and Hirano bodies in the hippocampus, and Lewy bodies in the cerebral cortex.

It has not been possible to define the relationships between these aspects of the pathology. It was not clear whether they were independent events or if they are dependent, which is the order they follow. The same pathology is found in individuals with Down's syndrome (trisomy 21) after the 3rd decade of life (Mann *et al*.1984) and cerebrovascular ß-amyloid deposition is observed in patients with Hereditary Cerebral Haemorrhage with Amyloidosis (HCHWA-D) (van Duinen *et al*.1987).

Epidemiological studies (Heston *et al*.1981) have consistently revealed only two main risk factors for the occurrence of the disease: old age, as the best defined risk factor (the disease's prevalence rises over 20% at 85 years of

INSERT

1.1.1. Clinical genetics of Alzheimer's disease

It is not clear what the relationship between the undoubted occurence of a relatively small number of families with early onset, autosomal dominant inheritance and the observation that the disease in general shows familial clustering. Epidemiological studies consistently reveal that a fairly modest proportion of first degree relatives of unselected probands of Alzheimer cases develop the disease (Heston et al.,1981, Van Duijn et al.,1991, Mohs et al., 1987). If the disease in general was a simple autosomal dominant with full penetrance, one would expect that the risk to first degree relatives would approach 50% as it does for Huntington's disease.

However, the fairly modest familial clustering seen in Alzheimer's disease can be interpreted in several different ways. In particular it was suggested (Mohs et al.,1987) that the disease in general is an autosomal dominant, but that it is only fully penetrant by the age of 85 years. The relatively low proportion of familial cases, they suggest, is because a high proportion of people die before they develop the disease. In contrast, Heston et al., 1981 find a lower degree of familial clustering even by this advanced age. The problems with the interpretation of these data are many.

First, familiality might also reflect shared environmental influences.

Second, even in large studies, relatively few first degree

age) and family history (van Duijn *et al*.1991) in first degree relatives. Among other possible risk factors are, family history of Down's syndrome, previous head trauma, family history of Parkinson's disease, depression and maternal age (>40). * See INSERT (22a, 23a, 24a)

Besides the evidence for family clustering, there are also numerous reports of large pedigrees in which Alzheimer's disease segregates as an autosomal dominant disorder (St.George Hyslop *et al*.1989). Such families offer the prospect for positional cloning of the defect.

1.2 Brief Introduction to Positional Cloning

1.2.1 Genetic linkage

The use of normal DNA polymorphism in the generation of genetic markers has made it possible to find the location of a genetic variability responsible for a particular disorder. Recombinant DNA technology has provided the tools with which we can reach the precise locus on the chromosome and computer programmes designed to analyse the data thus generated, have also evolved rapidly in their power and efficiency (Lathrop *et al*.1984). As genetic markers, we use any DNA sequence variability which can be visualised by gel technologies. The inheritance of these sequences can thus be examined in families. Genetic linkage programmes allow the analysis of these genetic data and the construction of maps which make the positioning of any DNA sequence in the genome possible , if rather

relatives live to age 85 and assessed: in even fewer is the diagnosis (post mortem) of Alzheimer's disease (as opposed to dementia) possible.

Third and perhaps more serious, is the possibility that the risk to relatives (and indeed the risk to everybody) of developing Alzheimer's disease, continues to rise with age until it reaches 100%.. This possibility immediately brings up the relationship between Alzheimer's disease and normal aging (see below).

Farrer and colleagues (Farrer et al.,1990, Farrer et al.,1991) have recently come to the conclusion that the risk to first degree relatives of developing Alzheimer's disease reflects a more complex aetiology than simple autosomal dominance, and propose that the susceptibility to AD is determined, in part, by a major autosomal dominant gene with an additional multifactorial component. However, the origin of the great majority of AD cases (late onset and sporadic cases) remains controversial. Because of the possible complexities of the aetiology of (particularly) late onset Alzheimer's disease, our group have taken the pragmatic attitude that linkage analysis is likely to be useful only in families with a mean age of onset below 60 years (Hardy et al.,1989). For this reason, this thesis is concerned only with such families.

arduous.

The principle of genetic linkage analysis is that loci which are close together tend to cosegregate rather than segregate independently into individual gametes. If the disease gene and a genetic marker are close together they will be inherited together most of the time unless their distance is such as to allow recombination to occur in a specific fraction. If the marker and the disease are far apart or on different chromosomes, then they will be inherited independently and the number of recombinants will be the same as the number of non-recombinants. In this case the recombination fraction is 50%.

(RF= number of recombinants/number of total offspring).

In linkage analysis, the odds ratio of the probability that two loci are linked to the probability that two loci are unlinked is calculated. Commonly the *Lod score* is used for this calculation. This is the decimal logarithm of the odds ratio.

LOD score : Log_{10} [probability of occurrence of the phenotypes at a particular recombination fraction (θ)/ probability when θ is 0.5

A *Lod* score of 3 is taken as statistically significant evidence for linkage, while a *LOD* score of -2 is taken to provide evidence for independent assortment.

1.1.2. The relationship between Alzheimer's disease and "normal ageing"

The relationship between Alzheimer's disease and normal ageing has provoked a long and fruitless debate. The pathological features of the disease occur in the normal elderly and show the same distribution (Mann et al., 1984): the pathological differences between Alzheimer's disease and normal ageing are thus quantitative, not qualitative. The recent demonstration that ß-amyloid production is a physiological rather than a pathological process (Shoji et al., 1992), may explain in some way this conundrum. Instead of thinking of Alzheimer's disease as "accelerated ageing", perhaps it would be better to think of Alzheimer's disease as a biological process which occurs at different rates in all people and could be preventable or delayed once the causes for its manifestation are clear.

Multi-point linkage analysis enables testing of multiple loci simultaneously, thus maximising the informativeness of each mating as well as providing a more refined placement of the disease gene when the relative genetic order of the markers tested is known. In a homogeneous disorder, adding up the information deriving from each family tested results in a better estimate of the position of the defective locus.

When a genetic marker is found to be linked tightly with the phenotype of the disease, a variety of techniques can be used to localise precisely and clone the defective gene (Figure 1.2). This strategy has proved successful in a number of diseases, for example, cystic fibrosis.

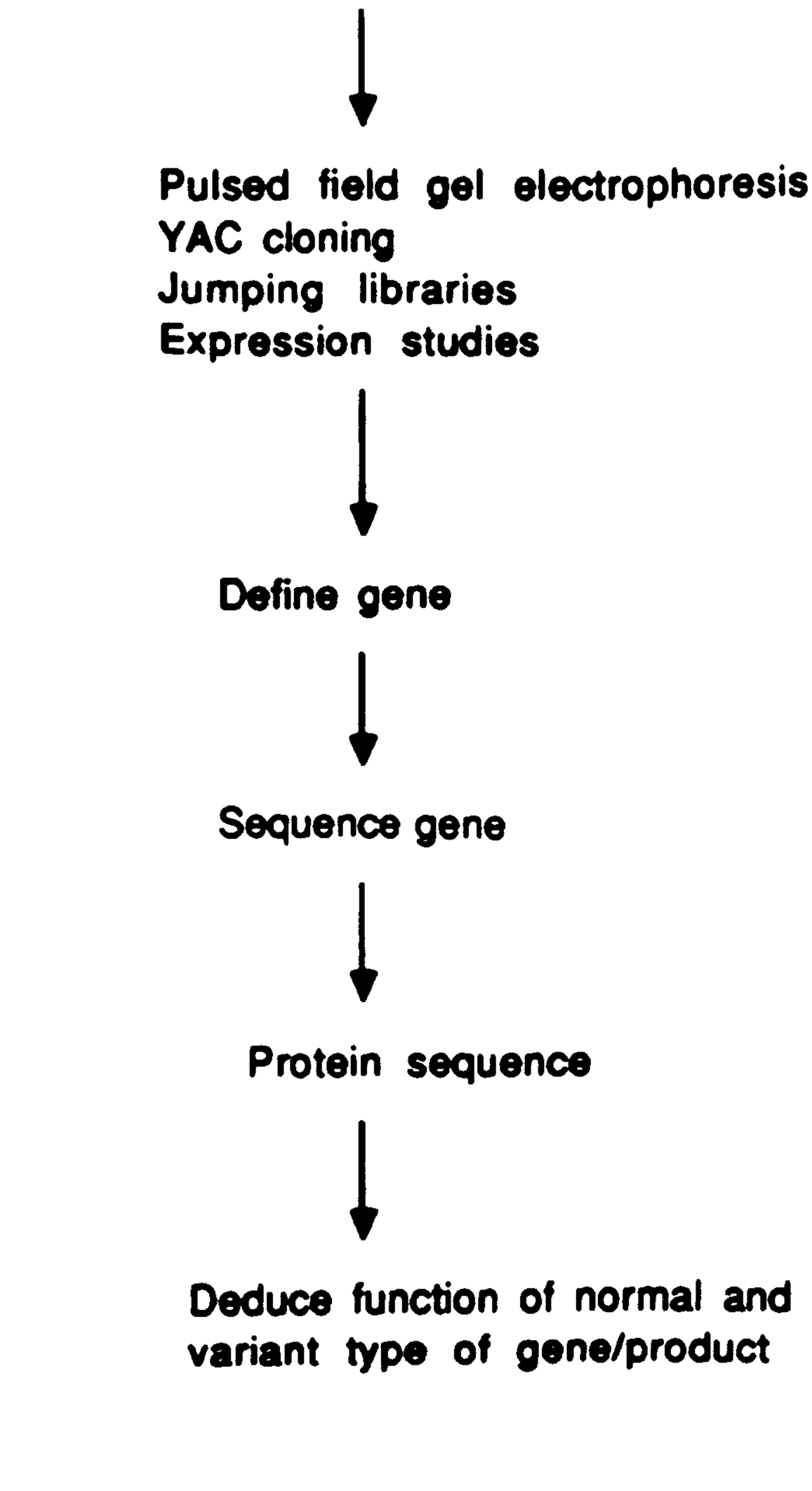
1.2.2 Examples of positional cloning approach

Using the above strategy it was found that the cystic fibrosis gene is on chromosome 7. Its position was further defined by two flanking markers (Beaudet *et al.*1986) and by subsequent mapping of many more markers to that area, a candidate gene was discovered. Sequencing then revealed point mutations in a gene (subsequently called CFTR) in individuals with the disease which are now known to be pathogenic (Rommens *et al.*1989).

The success with cystic fibrosis has not yet been repeated for Huntington's disease: an autosomal dominant disorder which presents some of the same challenges as Alzheimer's disease. In Huntington's chorea, although the gene is known to be on the

Figure 1.2

A schematic diagram of positional cloning



small arm of chromosome 4, genetic analyses have, as yet, failed to define the area precisely (Bates et *al*.1991). There are a number of reasons accounting for this slow pace (Pritchard et *al*.1991). First, diagnosis of Huntington's disease is not always evident, so misdiagnosis can mislead genetic studies since individuals can be scored wrongly as recombinants. Second, the late onset and fatal nature of the disease makes the collection of a large number of families with many affected individuals in different generations very difficult: the possibility of genetic non allelic heterogeneity augments the above problems and finally, there are no candidate genes known to map in the defined area. This illustrates both the strengths and weaknesses of the positional cloning strategy: while it is relatively straightforward to obtain genetic linkage, it can be very difficult to clone the pathogenic gene.

The other approach is the candidate gene approach, where the segregation of a gene whose product is involved in the pathology of the disease is examined. Prion diseases are an example of this. (Prusiner, 1991) Prion disease is a term used for any of the spongiform encephalopathies, including Creutzfeld-Jacob disease (CJD) and Gerstmann-Straussler syndrome (GSS) in man. Although these can be transmitted with infected tissue (probably by modified prion protein), often they involve an autosomal dominant inheritance. They are often characterised by brain plaques consisting of prion protein. The prion protein gene was an evident candidate gene and indeed different point mutations (Hsiao et *al*.1989) and a 144bp insertion were identified (Prusiner, 1991) and shown to segregate with the disease. Thus, in this case the candidate gene approach was very successful. However, when there is not

any substantial information about the pathogenesis of a disease and major contributors, as for example in many other neurodegenerative and psychiatric disorders, this strategy cannot be applied, and positional cloning presents the only alternative.

This summary illustrates the difficulties and assumptions of the positional cloning strategy. As I will show, a mixture of the positional cloning and candidate gene approach is what proved most useful for Alzheimer's disease.

1.3 History of Alzheimer's disease genetics

An important role for chromosome 21 in the pathology of Alzheimer's disease had long been assumed because of the development of the same neuropathology in the brains of trisomy 21 patients who survive until middle age (Oliver and Holland, 1986). Furthermore, the gene encoding the ß-amyloid deposits of Alzheimer's disease (Glenner and Wong, 1984; Masters *et al*.1985) was localised to this chromosome (Tanzi *et al*.1987; Goldgaber *et al*.1987).

The existence of large early onset Alzheimer's disease pedigrees, where the disease segregates as an autosomal dominant trait, raised the prospect that the chromosomal location of the Alzheimer's disease gene could be discovered by identifying co-segregation of a polymorphic marker with the disorder. The above data ensured that the candidate chromosome to begin genetic linkage studies on, was chromosome 21.

Genetic linkage was detected in Alzheimer's disease between chromosome 21 markers and the disease in four families affected by an autosomal dominant form of early onset Alzheimer's disease (St George Hyslop *et al*.1987).

Late onset Alzheimer's disease (>65), does not present a clear mode of inheritance, and is likely to involve a mixture of genetic predisposition and environmental effects. (Hardy et al., 1989) Thus, it does not provide a good model for a genetic localisation study given the probability of aetiologic heterogeneity, since multiply affected kindreds might include one or more members with environmentally induced illness (phenocopies), that if included in the analysis might falsely suggest recombination and result in a mislocalisation of the gene.

The fact that the gene coding for ß-amyloid protein, the major component of the senile plaques maps on chromosome 21, made it an obvious candidate gene. ß-amyloid is a 4kD breakdown product of a larger precursor, ß-amyloid protein precursor (APP) which has features of a glycosylated cell surface receptor (Kang *et al*.1987). The ß-amyloid peptide is found partially in the transmembrane domain (see figure 6.1). Other research at the time had appeared to show that sporadic Alzheimer's disease patients have a duplication of the APP locus (Delabar *et al*.1987) so APP looked more and more as the obvious candidate for the Alzheimer's disease locus. Excitement though, was soon over when recombinants were found between APP and the disease (Van Broeckhoven *et al*.1987; Tanzi *et al*.1987) and the report of APP gene duplication was not repeated and was later retracted (St

George Hyslop *et al*.1987). Sequencing of APP cDNAs from brains of sporadic Alzheimer's disease patients was also undertaken but the sequence was found to be normal (Vitek *et al*.1988).

Genetic linkage studies had suggested that the disease locus mapped close to the pericentromeric markers D21S16 and D21S13 (St George Hyslop *et al*.1987; Goate *et al*.1989). The first inkling of genetic heterogeneity in Alzheimer's disease came in 1988, when a group of researchers failed to detect linkage with chromosome 21 markers in their families (Schellenberg *et al*.1988). Two years later a large collaborative study in 48 Alzheimer's disease families provided additional evidence for genetic heterogeneity (St George Hyslop *et al*.1990). A proportion at least of early onset families were linked to chromosome 21 markers while late onset families were unlinked. There also appeared to be evidence that not one but two loci existed on chromosome 21 at either end of the most highly investigated area. This conclusion had to be regarded with skepticism, as an unlikely coincidence, and we considered alternative explanations particularly non-allelic genetic heterogeneity. Linkage analysis when there is non allelic heterogeneity, interprets falsely recombinants and although it permits the detection of linkage, it gives a false localisation.

In the meantime, in HCHWA-D, cosegregation was found between APP and the disease locus (Van Broeckhoven *et al*.1990) and a point mutation, a C to G transversion was identified, which changes a glutamic acid residue to a glutamine at codon 693 of the APP770 transcript (position 22 of the ß-amyloid protein),

suggesting that this is the cause of the specific amyloid deposition in the cerebral vessel walls of HCHWA-D (Levy *et al*.1990). This finding suggested for the first time, that mutations in APP can cause amyloid deposition.

The demonstration of genetic heterogeneity led us to use one single large, autopsy confirmed pedigree (F23) with an age of onset around 55 years of age, which showed linkage between the disease and chromosome 21 markers to localise the gene more precisely. In this family cosegregation was observed between APP and the disease suggesting that APP could, in fact, be the site of the defect in this particular family. Subsequent sequencing of exon 17 (which, with exon 16 encodes the ß-amyloid peptide part of APP) revealed a G to A transition changing a valine to an isoleucine at position 717 of the APP770 transcript (Goate *et al*.1991). This mutation is found in the transmembrane segment two residues beyond the C-terminal of the ß-amyloid peptide.

This mutation was shown to cosegregate with the disease in the two families in which it was identified, and was not found in a large number of unaffected chromosomes tested (>400) (Chartier Harlin *et al*.1991). Subsequently, it has been found in several other early onset Alzheimer's disease families of different ethnic background. Those facts make its pathogenicity very probable.

However, this particular mutation accounts only for a very small proportion of genetic cases of early onset Alzheimer's disease (van Duijn *et al*.1991; Hardy *et al*.1991; Schellenberg *et*

al.1991).

Subsequent investigations have identified more mutations in codon 717. One was found in a British family (age of onset: 59 years) involving a C to G transition changing the valine to a glycine (Chartier Harlin *et al*.1991) and the other one was found in an American family with an age of onset at 40s, involving a C to T base change and a valine to phenylalanine aminoacid change (Murrell *et al*.1991).

Recently, a new mutation has been identified in exon 16 of the APP gene, just outside the N-terminal of the ß-amyloid peptide (Mullan *et al*.1992). It seems that Alzheimer's disease mutations in some early onset genetic cases will be clustered in the two terminals of this peptide and somehow modify APP processing (see chapter 6).

Despite the increasing number of identified pathogenic mutations, it is clear that APP mutations account for a very small proportion of genetic cases of early onset Alzheimer's disease. Other aetiologies will appear with the identification of a new locus(i). These findings suggest that abnormal APP processing is involved in the pathogenesis of the disease and this idea directs research towards the search for candidate genes that are likely to take part in this process.

Genetic analysis has not yet allowed the location of the non APP locus to be determined: indeed there may be more than one other locus.

Although a large proportion of our non APP families do not show linkage with chromosome 21 markers, there is still a large American pedigree (FAD4) in which linkage on chromosome 21 exists and sequencing of the whole open reading frame of APP has not revealed any alterations (Tanzi *et al*.1992). There are a number of possibilities explaining these confusing results. First, there is indeed another locus on chromosome 21 responsible for the defect in this particular family. Second, an intronic mutation is causative in this pedigree and the recombinants between APP and the disease are explained by intragenic recombination due to the length of the APP gene (>180kb). Thirdly, a mix up in the pedigree might be responsible. Fourthly, the original linkage report may be wrong, even though it was confirmed by is Goate et al., 1989.

1.4 My project

I began to work on Alzheimer's disease in October of 1989 (Table 1.1). At that time, our group had confirmed the American linkage results in a study of 6 families suggesting that the disease locus was close to D21S16 and D21S13 near the centromere on the long arm of the chromosome (Goate *et al*.1989) (see Figure 3.1). The isolation of new, more informative markers was considered essential for the localisation of the gene and its eventual cloning. At the time, we considered that the early onset, familial Alzheimer's disease was most likely to be genetically homogeneous although we considered that allelic heterogeneity according to the age of onset in each family might be important (van Duijn *et al*.1991). Analysis of variance of the

age of onset within and between families with early onset Alzheimer's disease showed the age of onset to be constant within a family and variable between families. The only evidence of genetic heterogeneity at that time came from the analysis of an inbred population, the Volga Germans, where linkage studies did not detect cosegregation with markers on chromosome 21 (Schellenberg *et al*.1988). This study failed to draw our attention to the issue, since the more centromeric markers were not informative in the Volga German pedigrees and inbreeding might have resulted in failure to detect linkage.

Lod scores in a genetically homogeneous disorder can be added up between families and thus increase the amount of information they provide. In our study the major contribution of positive *Lod* score at recombination 0 came from one family (F23): 1.73 with D21S1/S11 and 1.38 with D21S16. Another pedigree (F74) in this study gave a *Lod* score of -2.66 with D21S1/S11 and 0 with D21S16 and was responsible for the apparent localisation in our study. (See figure 3.1).

We assumed genetic homogeneity among the pedigrees tested and took the localisation we achieved at face value. Our efforts were concentrated in trying to localise the defective gene, using the positional cloning approach. In order to achieve this, the generation of polymorphic markers in the pericentromeric region of chromosome 21 close to markers D21S16 and D21S13 was essential.

A new method for the rapid isolation of human DNA sequences from hybrid cell lines emerged in the literature and I tried to optimise it for chromosome 21. This technique was the interspersed repeat sequence polymerase chain reaction (IRS) PCR. Using a hybrid panel of eight chromosome 21 hybrids and a human Alu primer as well as an LI human primer I amplified the cell lines to get a specific pattern of amplification in each of them. This would allow a positioning of different gel bands to different parts of the chromosome. The idea of the generation of a library from such derived PCR clones resulted in the cloning and characterisation of one such fragment of 700bp, as cloning of PCR products proved very difficult. Subsequent experiments were carried out in order to make this clone, containing a Poly-A tail polymorphic.

While I was facing difficulties in this technique, a collaborative study proved the existence of genetic heterogeneity (St George Hyslop *et al*.1990) in Alzheimer's disease and we decided to concentrate our group efforts on one well characterised family (F23), which was giving an evident positive *Lod* score with markers on chromosome 21. In this pedigree we found cosegregation of the disease locus with APP. Sequencing of the APP gene revealed a valine to isoleucine change in exon 17 at codon 717.

As expected, this finding changed the direction of Alzheimer's research in general, and mine in particular. I started developing a screening protocol which I then used for the

detection of the 717 mutation and the identification of others simultaneously. I modified a single strand conformation polymorphism analysis protocol (Orita *et al*.1989; Orita *et al*.1990), and managed to detect all known variation in exon 17 as well as the majority of prion mutants (as a control experiment). I subsequently used this protocol to screen the whole open reading frame of APP gene, in an attempt to determine the proportion of genetic early onset Alzheimer's disease caused by mutations in this locus. The results agreed with those of our linkage studies in the fact that the majority of those cases are not caused by mutations in APP, since no variation was found in the families tested in any of the remaining 17 exons. One new family with a valine to isoleucine mutation was identified, sharing such phenotypic characteristics, as the age of onset, with the other 717 val->ile families.

As part of the screening procedure we screened a number of British sporadic, Spanish sporadic and familial early onset Alzheimer's disease individuals where a new variation of sequence was detected. Sequencing revealed a silent mutation in codon 711, whose importance in the development of the disease is, as yet, unclear. (Chapter 4).

Since it became evident from the above studies that other loci are involved in the pathogenesis of Alzheimer's disease, the identification of candidate loci became necessary since Alzheimer's disease families are in general too small to allow a complete genome search. Mutations in genes coding for proteins involved in APP processing, in plaque formation or modulation of APP expression may account for other cases of familial

Alzheimer's disease.

I decided, therefore, to test the segregation of α1-antichymotrypsin (AACT), a serine protease inhibitor, and a major component of the senile plaques as ß-amyloid, since a local excess of it, could potentially prevent a normal brain protease from cleaving the amyloid precursor protein, thus resulting in amyloidogenic fragments. The families that were used for this study did not show segregation with APP and did not show any variation from the normal sequence when screened with SSCP for all the exons of APP gene. Most of them are too small to form the basis for a complete genome search, and this is the reason we decided to use the candidate gene approach.

No evidence for linkage was demonstrated in any of the pedigrees and in two of them a reliable exclusion was observed. These data suggest that the deposition of α-1 antichymotrypsin is a secondary step in the formation of the senile plaques, and other loci should be tested in order to identify the other gene(s) for early onset Alzheimer's disease.

In Chapter 2, I describe general methods that I have used in my three year research, in Chapter 3, I present in detail my attempts to use IRS-PCR for the development of polymorphic markers on chromosome 21, in Chapter 4, I discuss the single strand conformation polymorphism data and the identification of new mutations and variations in APP, in Chapter 5 I present the exclusion results for α1-antichymotrypsin and finally in Chapter

6, I discuss our results in relation to the future of Alzheimer's disease research.

Table 1.1

Year	Paper	Interpretation	What I did
1987			
	Hyslop	ch21 Alzheimer's disease linkage	
	Goldgaber,Tanzi	APP maps on 21	
	Van Broeckhoven,Tanzi	ch21, but not APP	
1988			
	Schellenberg	not ch21, Heterogeneity, or near centromere.	
1989			
	Goate	ch21, near centromere	IRS-PCR for positional cloning of the gene
1990			
	Hyslop	ch21 but heterogeneous	

1991		
Goate	APP mutations in some	SSCP APP
Chartier-Harlin	genetic cases	screening
Murrell		
1992		
Hardy	Amyloid cascade hypothesis (what causes the occurrence of non-APP disease?)	AACT analysis

CHAPTER 2

MATERIALS AND METHODS

2.1 Dialysed fetal calf serum (FCS) preparation

Dialysis tubing is available from: Medicell International Ltd. (Dialysis-membranes-Visking). It is a partially permeable membrane with a molecular weight cut off in the range 12,000 to 14,000 and an approximate pore size of 2.4nm.

Fetal calf serum Globepharm

The tube was cut into pieces of convenient length (20cm), and heated in boiling water for 10 minutes. When it was cooled a double knot was tied in one end of the tubing and it was filled with 150mls of fetal calf serum. The tube was left in a tank with running water at 4C. Since fetal calf serum was dialysed and therefore expanded, space was left in the tube to allow it to do so. Dialysis was carried out for 24 hours. The volume was measured after dialysis and sodium chloride was added to 0.9% (w/v). Dialyzed FCS was filter-aliquoted and stored at -20C.

2.2 Routine tissue culture maintenance of hybrid cell lines

Cell-culture-materials

10% (w/v) PBS [0.1MSodium phosphate pH 7.5, 1.5M NaCl]

F12 (Ham) Nutrient Mixture with L-glutamine GIBCO BRL

Uridine	GIBCO BRL
Hepes Buffer Solution (1M)	GIBCO BRL
Glucose	GIBCO BRL
Versene 1:5000 in an isotonically buffered saline	GIBCO BRL
200 mM L-Glutamine	Imperial
10X PBS Dulbecco's phosphate buffer saline	GIBCO BRL

Dulbecco's Mod Eagle Medium without sodium pyruvate with 4500mg/L (GIBCO BRL)

DMEM media (supplemented) 200mls

DMEM(10X)	20mls
Hepes (1M)	5mls
5%$NaHCO_3$	3mls
Pen/Strep(100X)	2mls
200mM glutamine	2mls
FCS	20mls
pH with NAOH(1M)	2mls

Made up to 200 mls with H_2O

100X Penicillin/Streptomycin

500µg/ml Streptomycin

500units/ml Penicillin

Filter sterilised and aliquoted

50X Trypsin/Versene

Trypsin Sigma

0.5 trypsin in 20mls Versene

The media was changed on cells regularly, every other day. The old media was aspirated off, the remains were washed with Versene and 0.05% (w/v) of trypsin was used to remove the cells. Trypsin was neutralised by adding an equal volume of medium. A 1:20 dilution of cells was transferred into fresh media. The cells were trypsinized twice a week.

2.3 High Molecular Weight DNA preparation from cell lines (Gusella et al.1979)

Harvest of cells from 5 flasks (750mls)

After harvesting, the cells were spun down at 1800rpm, for 5 minutes and washed once with PBS. The pellets were resuspended in 1 ml PBS. Subsequently the cells were added carefully to a lysis mixture (10X lysis mixture:0.5 M EDTA pH:8.0, 10% (w/v) Sarkosyl (Sigma). Proteinase K (Sigma) was added to 1X lysis mixture to a final concentration of 20µg/ml and NaCl to a concentration of 100mM. This mixture was incubated at 60C-65C for 3 hours or 37C O/N.

An equal volume of phenol was added, mixed gently and centrifuged for 3 minutes at 1800 rpm. The phenol layer was

taken off, the extraction was repeated and the aqueous layer was kept. An equal volume of ethanol was added and DNA was precipitated by gentle inversion. The DNA glob was removed with a hooked pasteur and put into 25 mls of 70%(v/v) ethanol. After the final washing step the DNA glob was transferred to a 5ml sterilin tube, ethanol drained off and 1ml of TE (10mM Tris.Cl pH 7.4; 1mM EDTA pH 8.0) added.

2.4 RNase treatment of DNA

RNase A (Sigma) was at a final concentration of 10μg/ml. The diluted RNase was boiled for 10 minutes to lose any DNase activity and was added to the DNA at the equivalent concentration. The samples were incubated in 37C waterbath for 1/2 hour. One volume of phenol (Rathburn Chemicals Ltd.) was added and the samples were microfuged for 5 minutes. The phenol was discarded and a phenol-chloroform (BDH) extraction was performed. After 5 minutes of centrifugation the DNA was kept in new tubes. Another extraction same as the above followed with chloroform-isoamyl alcohol (BDH) (24:1) and finally the DNA was precipitated by the standard procedure. (See section 2.5)

2.5 Precipitation of nucleic acids (Maniatis *et al*.1982)

Two and a half volumes of ethanol and 1/10 volume of Sodium Acetate (3M pH5.2) are added to the DNA containing solutions and the samples are placed in dry ice ethanol bath for 15 minutes. A centrifugation at 4C for 15 minutes follows and the ethanol is discarded. The pellet is washed once with 70%$^{(v/v)}_{\wedge}$ethanol and is then centrifuged for 5 minutes. Finally the DNA pellet is left

to dry and is dissolved in H_2O or TE pH 8.0.

2.6 Preparation of plasmid DNA

Solutions:	I. 50mM glucose	II. 0.2M NaOH	III. KOH 180g
	25mM Tris pH:8.0	1% SDS	CH_3COOH 290mls
	10mM EDTA pH:8.0	H_2O	H_2O 300mls

a) Mini preparation

1.5 ml of an O/N cell culture was spun for 3 minutes, the supernatant discarded and pellet left to dry. 100µl of solution I was added, the mixture was vortexed and left at room temperature for 5 minutes. Then 200µl of solution II was added and the mixture was mixed gently until clear and left for 5 minutes on ice. Finally, 150 µl of solution III was added, vortexed and mixture left on ice for 5 minutes. Centrifugation (microfuge) followed at 4C for 5 minutes. The supernatant was kept and 2-3 phenol/chloroform extractions were performed. Finally, one chloroform extraction was performed and the DNA ethanol precipitated (Section 2.5)

b) Midi preparation

A 500 ml culture was grown in a shaking incubator (37C) overnight. The cultured cells were centrifuged at 5000 rpm for 15 minutes at 4C. (Autoclaved sterile centrifuge tubes were

used). The supernatant was discarded and the pellet was resuspended in 12 mls of solution I adding 1mg/ml of lysozyme (Sigma). Incubation in ice water for 10 minutes followed. 24 mls freshly made solution II was then added and mixed carefully. The mixture was incubated in ice water for 10 minutes and 15 mls of solution III was added and mixed carefully. The samples were incubated in ice water for 20 minutes and centrifuged at 10,000 rpm for 30 minutes at 4C. The supernatant was kept, 5µl RNase (10mg/ml) added, and incubated for 20 minutes at 37C. An equal volume of phenol: chloroform: isoamylalcohol was added and centrifuged at 10,000 rpm for 10 minutes. The extraction was repeated once and then the upper layer was transferred to another tube. Two volumes of ethanol were added and the tubes were left on ice for 30 minutes. A centrifugation step followed at 10,000 rpm for 20 minutes. The supernatant was discarded and the pellet dissolved in 3.2 mls H_2O. 0.8mls 4M NaCl and 4 mls 13% (w/v) PEG (MW 8,000) were added and mixed. The mixture was incubated in ice water for 60 minutes centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded. Finally the pellet was washed in 70%(v/v) ethanol and dissolved in TE buffer pH 8.0.

2.7 Restriction enzyme digests (Maniatis *et al*.1982)

BglII, BamH1, HindIII, EcoR1,	
Taq1,Bcl1	BRL
BstN1	New England Biolabs

Restriction enzymes are bacterial enzymes that possess an endonuclease activity directed to specific base sequences in double stranded DNA. Each endonuclease requires specific conditions in order to digest double stranded DNA. For digests of genomic DNA, 3μg of DNA were cut in a 30μl reaction volume of appropriate reaction buffer (provided by the enzyme company), 1μl of spermidine (Sigma) (100mM) and 1-10 units of enzyme. In cases of PCR product digests approximately 50 μl of the product were used in the digestion reaction.
Incubation at enzyme-specific temperatures for 4-15 hours resulted in a completely digested product.

2.8 DNA agarose and polyacrylamide gel electrophoresis

(Maniatis et al.1982)

DNA size markers

Lambda HindIII	GIBCO BRL
1 Kb Ladder	GIBCO BRL

Gel Loading Buffers

Xylene cyanol FF	BDH
Bromophenol blue	SIGMA
Orange G	SIGMA

Type II 10X buffer

(w/v)
0.25% bromophenol blue

0.25% (w/v) xylene cyanol

25% (w/v) Ficoll (type 400) in H_2O

Stored at room temperature

Orange G

6% (v/v) glycerol

0.025% (w/v) orange G

10mM EDTA

The size of DNA can be determined by measuring its electrophoretic mobility in gels of polyacrylamide or agarose. The mobility of each sample depends on its size and on the concentration of the gel. In an electric field, DNA moves at a rate inversely proportional to its size so that bigger fragments move slower than small ones. Nucleic acids are visualised by staining the gels with ethidium bromide (Sigma). Ethidium bromide has an intense fluorescence when excited by ultraviolet radiation and complexed with nucleic acids. Usually, agarose gels are used to separate fragments of 200bp up to 5 kb and polyacrylamide gels are used to separate smaller fragments.

Agarose gels were made with agarose powder (BRL) (desired concentration) in TAE buffer (40mM Tris, 20mM Acetate, 2mM EDTA at pH 8.0). The mixture was heated in a microwave oven until the agarose was in solution, cooled and then poured into the appropriate former.

Polyacrylamide gels were made using a 40% (w/v) solution of acrylamide (BDH) (19:1 acrylamide: N,N,methenebis-acrylamide) to the required concentration in TBE buffer (89mM Tris, 89mM Borate, 2mM EDTA pH:8.0), and ammonium persulphate (BRL) (10%) (w/v) and TEMED (BDH) were added as polymerisation initiator and catalyst respectively. The mixture was then poured between glass plates and allowed to set before use for about one hour. Gels were run usually at 50 volts (agarose) and 60 Volts (polyacrylamide).

2.9 Isolation and purification of DNA fragments

Geneclean Kit(BIO 101 Inc.)

The Geneclean kit contains a specially formulated silica matrix called *Glassmilk* that binds single and double stranded DNA without binding DNA contaminants. It is most efficiently used for DNA fragments between 500bp and 15kb. The procedure required is the following: three volumes of NaI stock solution is added in DNA and incubated for 5 minutes at 55C to dissolve agarose (LMP nusieve agarose, BRL). Then 5µl of *Glassmilk* is added and incubated for 5 minutes at room temperature. The *Glassmilk*/DNA complex is centrifuged for 5 seconds and the supernatant is removed. 200µl of new solution is added to the pellet which is washed with a pi pette. A brief centrifugation follows and the supernatant is removed. This step is repeated three times. The DNA is then eluted in 10µl of TE pH:8.0 and it is incubated at 55C for 2 minutes. After a 30 seconds centrifugation, the supernatant containing the eluted DNA is carefully removed and placed in a new tube. A second elution may result in a higher yield.

2.11 Quantification of nucleic acids by spectrophotometry

Spectrophotometer readings are taken at wavelength of 260nm and 280nm, for the quantification of DNA. An OD of 1 corresponds to approximately 50µg/ml for double stranded DNA and 20µg/ml for oligonucleotides. The ratio between the readings at 260nm and 280nm provides an estimate for the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} of >1.8. For oligonucleotide primers a formula was also used to estimate their concentration.

Concentration (M) =

OD X dilution factor/(Ax15200)+(Gx12010)+(Cx7050)+(Tx8400).

2.12 Preparation of competent cells

LB-broth: Bacto-tryptone 10g

(1lt) Bacto-yeast extract 5g

NaCl 10g

pH: 7.5

For the preparation of plates, in the above medium before autoclaving 15g of Bacto-agar were added.

Antibiotics

Ampicillin (50mg/ml), tetracycline (25mg/ml)

KCL LONDON UNIV.

XL1 cells	STRATAGENE
100 mM IPTG	BRL
2% (w/v) X-gal	BRL

X-gal is prepared in dimethyl-formamide and stored at -20C.

Cells used were the:-

XL1-blue -recA-(recA1, lac-, endA1, gyrA96, thi, hsdR17, supE44, relA1, [F'proAB, lacIq, lacZδM15, Tn10(tet)*R*)])

developed at Stratagene. This strain allows blue/white colour selection for pBluescript phagemids because of the lacZδM15 complementation on the F'episome. The colour selection may be seen when plating on L-broth plates containing 50μg/ml ampicillin, 80μg/ml of fresh X-gal and 20mM IPTG. Colonies containing phagemids without inserts will be blue after incubation for 12-18 hours at 37C. Colonies with phagemids containing inserts will remain white. An overnight culture of XL1-blue is grown on a plate and one single colony is used to make an overnight liquid culture (10mls of L-broth and appropriate antibiotic: in this case tetracycline). One half ml of this culture is inoculated into 50ml of medium in a 250mls flask. This culture is grown at 37C till it reaches an OD_{550}:0.3-0.4. Then it is centrifuged at 3000rpm, for 10 minutes at room temperature. The pellet is resuspended in 10ml of 50mM Calcium Chloride (ice cold) and placed on ice for 40 minutes. Finally it is centrifuged as above, resuspended in 1 mls 50mM Calcium Chloride (ice cold) and left at 4C overnight. 200μl of the cells were used for each transformation.

2.13 Cloning and ligations (Maniatis *et al*.1982)

Plasmid vector used:

pBluescript SKII	**STRATAGENE**
T4 ligase	**BRL**
Calf Intestinal Phosphatase (CIP)	**Boehringer**

In plasmid cloning the vector DNA is digested with an enzyme and joined *in vitro* to the foreign DNA. Marker genes allow the selection of the successful recombinants.

For ligation, the ideal ratio of insert to vector is variable: however, a reasonable starting point is 2:1 (insert:vector), measured in picomole ends.

$2X10^6$/#base pairs X660 = Picomole ends/micrograms of DNA.

During ligation DNA ligase can catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5' phosphate group and the other a 3' hydroxyl group (Sgaramella and Khorana, 1972). In order to eliminate the recircularisation of the vector, calf intestinal phosphatase is used to remove the 5' phosphates from both ends of the linear vector DNA.

Inserts and vector were digested with NotI (37C for one and a half hours). The digest was checked on a mini agarose gel and the enzyme was heat inactivated at 85C. The total volume was made up to 100μl for treatment with Calf Intestine Phosphatase (CIP) (4μl of CIP at 37C for 1/2 hour). Finally the DNA was ethanol precipitated, the pellets dissolved in the appropriate buffer supplied by the enzyme company and 1μl of T4 ligase was added. The ligations were left overnight at 15C.

2.14 Transformations

1μl of uncut vector was kept in order to be used for control transformations.

1-5ng of the ligation mixture was added to 200μl of competent cells which were then placed on ice for 30 minutes, heat shocked for 2 minutes at 37C and then placed on ice for 5 minutes. 1ml of L-broth was added and the mixture was incubated at 37C for 30 minutes. 100μl of 100mM IPTG and 40μl of 2% X-gal were spread on LB/Tet/Amp plates 30 minutes prior to plating the transformants. Subsequently, 400μl of cells were plated on those plates and left to dry. When dried, they were incubated at 37C O/N.

2.15 PCR (Polymerase Chain Reaction)(Saiki *et al*.1988)

Amplitaq DNA polymerase	Cetus and Cambio
Nucleotides(dNTPs)	Pharmacia

PCR buffer: 1.5mM $MgCl_2$, H_2O, 0.01% glycerol, 10mM Tris.Cl pH:8.0, 50mM KCl

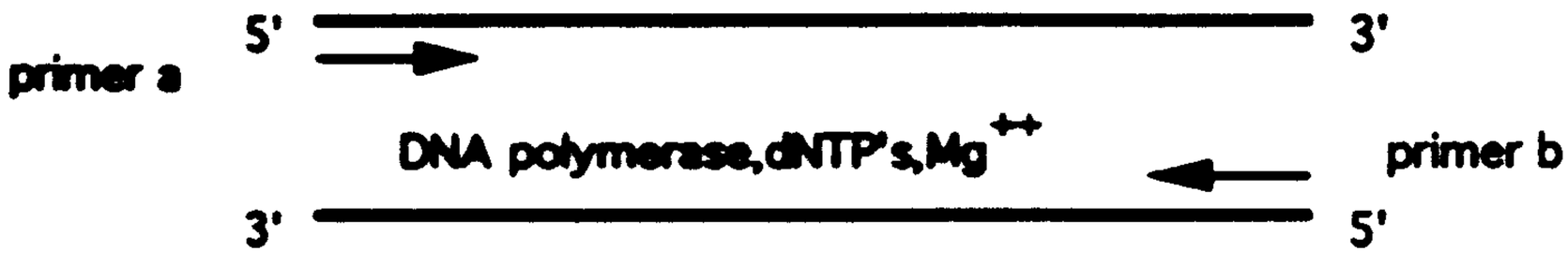
Glycerol	BDH
Melting Point Bath Oil	SIGMA

PCR involves the *in vitro* enzymatic synthesis of millions of copies of a specific DNA segment (figure 2.1). The reaction is based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA; after denaturation of the DNA each primer hybridises to one separate strand so that extension from each 3' hydroxyl end is directed towards the other. The annealed primers are then extended on the template strand with DNA polymerase. Those steps (denaturation, primer annealing and DNA synthesis) when repeated result in the exponential accumulation of a discrete fragment whose termini are defined by the 5' ends of the primers. After n cycles there will be 2^n copies of the region between the two primers. The primer design can be considered as a critical factor in the reaction. Primers should have a random base distribution with average GC content. Their secondary structure must also be checked in order to avoid complementarity especially in the 3' ends. Taq DNA polymerase which is used for the synthesis of DNA is sensitive to the concentration of magnesium ions and monovalent ions. Since dNTPs (nucleotides) can bind magnesium, the precise concentration that is required to activate the enzyme maximally is dependent on the dNTPs concentration. The temperatures applied for primer annealing and DNA synthesis vary with the enzymes used and depend also to some degree on the base composition of the primers and template DNA.

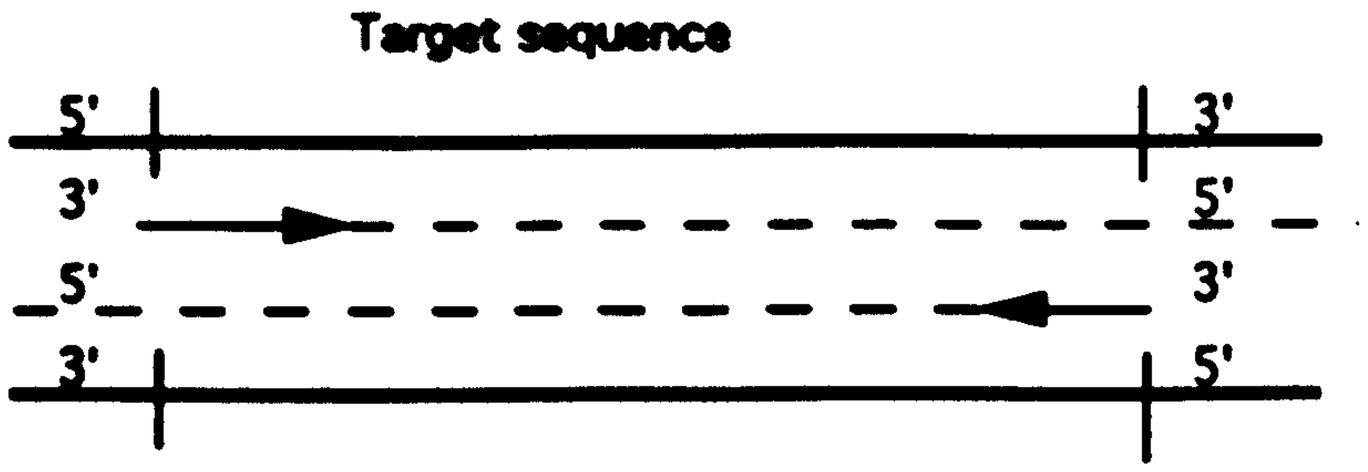
Figure 2.1

PCR-Polymerase chain reaction

A Schematic diagram



1.Denaturation
2.Annealing of primers to template DNA
3.DNA synthesis by primer extension



Exponential accumulation of the target sequence occurs, when process is repeated since both the template and the newly synthesized DNA strands serve as templates

Taq DNA polymerase, by allowing synthesis at elevated temperature, reduces the chances of unintended oligonucleotide priming by destabilising mismatch-pairing with unwanted target sequences.

A typical PCR is described next, although conditions frequently vary after optimisation for each set of primers used : 10 minutes initial denaturation at 94C followed by 35 cycles of 1 minute at 55C, 1 minute at 94C and 1 minute at 72C. The reaction volume usually used is 25 µl of PCR buffer with a final concentration of primers at 0.2µM. All nucleotides are in a final concentration of 200µM and 1 unit of Taq DNA polymerase is added last. One drop of oil on the top of the reaction mixture ensures that there is limited condensation.

2.16 Labelling of DNA. (Maniatis et al.1982)

Radioisotopes

[α-^{35}S] dATP	Amersham
[γ-^{32}P] dATP	Amersham
[γ-^{32}P] dCTP	Amersham

a) end-labelling (Berkner AND Folk, 1977)

T4 polynucleotide kinase (PNK) BRL

T4 polynucleotide kinase catalyses the transfer of the

γ-phosphate of a ribonucleoside 5'-triphosphate donor to the 5'-hydroxyl group of a polynucleotide, oligonucleotide or nucleoside 3'-phosphate. For end labelling [γ-^{32}P]dATP is used as a donor. 5'-end labelling is efficiently used for the labelling of oligonucleotides. 200ng are labelled in a 10μl total reaction volume in Kinase buffer provided by the manufactur· containing 1 μl of T4 PNK and 5μl of [γ-^{32}P] dATP at 37C for 30 minutes.

b) Random-Hexamer Labelling (Feinberg and Vogelstein, 1983)

OLB (Oligolabelling buffer)

OLB is made by adding solutions A, B and C in a ratio 100:250:150.

Solution A: 1ml solution of 1.25M Tris pH 8.0, 0.125M $MgCl_2$

18μl 2-mercaptoethanol

5μl dATP(100mM)

5μl dTTP(100mM)

5μl dGTP(100mM)

Solution B: 2M Hepes adjusted to pH 6.6 with NaOH

Solution C: Hexadeoxyribonucleotides each suspended in TE at 90 OD units/ml

Klenow fragment of DNA polymerase BRL

Random hexanucleotides have been used to prepare labelled

copies of DNA to a very high specific activity. Single stranded DNA was used as a template then random primers, Klenow polymerase, labelled and unlabelled nucleotide precursors were added in suitable oligolabelling buffer and the reaction allowed to proceed. The reaction was carried out at 37C for 4-12 hours. 20-30 ng of DNA were used in a 50 µl reaction of oligolabelling buffer using 2µl BSA and 5µl [γ-^{32}P] dCTP and 2 units of Klenow.

2.17 Southern blotting- Nucleic acid hybridisation (Southern, 1975)

Hybridisation mixture (SSC mixture)

7.5ml 20XSSC, 1.25ml 100XDenhardt's, 1.25 ml 10% (w/v) SDS, 15 mls dH_2O, 2.5g Dextran sulphate. Mixture is heated at 65C to dissolve.

Washing solutions (when SSC mixture is used)

2XSSC, 0.1%SDS (w/v) 20 minutes at 65C

1XSSC, 0.1%SDS (w/v) 10 minutes at 65C

0.1XSSC, 0.1%SDS (w/v) 10 minutes at 65C

100X Denhardt's solution [2% BSA,2% Ficoll,2% PVP (polyvinylpyrollidone)]

Hybridisation mixture (alternative:- Church buffer)

180mls Na_2HPO_4 1M

70mls NaH_2PO_4 1M

3.5g SDS

1ml 0.5M EDTA pH8.0

made up to 500mls with H_2O

Washing solution (when Church buffer is used)

Sodium Phosphate buffer pH 7.2

72 mls of Na_2HPO_4 1M

28mls of NaH_2PO_4 1M

100mM Sodium phosphate buffer for 30min at 65C

40mM Sodium phosphate buffer for 10min at 65C

Sonicated Salmon Sperm DNA (competitor) (1% w/v)

Hybond membrane N Amersham

20XSSC [3M NaCl, 0.3M Na_3citrate]

Denaturing solution [1.5M NaCl, 0.5M NaOH]

Neutralising solution [1.5M NaCl, 0.5 Tris-HCl pH 7.0, 0.001M EDTA]

Stripping solutions : 0.5% (w/v) SDS or 10mM NaOH

Double stranded DNA can be denatured by various procedures that break the hydrogen bonds linking the complementary base pairs. Denaturation is reversible and when cooled the complementary strands reassociate. This process is known as renaturation or annealing and can occur between DNA strands that

were not originally base-paired, provided they are sufficiently complementary. Specific sequences in the DNA are detected by hybridisation to radiolabelled probes of DNA. The binding of the probe can then be detected by autoradiography. The precise conditions under which hybridisation is performed may affect the extent of hybridisation with similar but not identical sequences. This is the basis for Southern blotting.

The gel is placed in denaturing solution for 30 minutes. This solution is replaced with neutralising solution for another 30 minutes. In the meantime, a transfer apparatus is set up. A large tray is filled with 20XSSC, 3MM filter paper is soaked in 20X SSC and covers a glass plate. The paper must be flat when gel is placed on it. The apparatus is covered with cling film apart from the gel's surface. A piece of Hybond N membrane soaked in 2XSSC is directly placed on top of the gel. On top of the membrane are put 4 pieces of 3MM paper cut to the same size as the gel. It is essential to remove any air bubbles trapped between the membrane or the papers which would impede the transfer. Finally a stack of paper towels, a glass plate and a 500g weight are placed on top of the blot which is left overnight. When dismantled, the gel loading slots are marked and the Hybond membrane is soaked in 2XSSC to remove any agarose. The filter is dried between two sheets of 3MM paper and then wrapped in cling film. To fix the DNA on the filter, the membrane is placed with DNA side down on UV transilluminator for 3 minutes.

The filter is placed in a hybridisation tube with 200mls

2XSSC and is smoothed to the sides. SSC is poured out and 5-10mls of hybridisation mixture are added. 100µl of salmon sperm DNA are then added to the mixture which is incubated in hybridisation oven for at least one hour. This prehybridisation mixture is poured off and the probe is denatured (10 minutes), placed on ice for 10 minutes and then added to hybridisation mixture which is poured in the tube. This time the incubation takes place for at least 4 hours (usually overnight). The labelled probe may be poured off and saved for another filter hybridisation or disposed down a radioactive sink. The tube is then filled with a washing solution. Washing proceeds until filter is sufficiently *cold* (as determined with a Geiger tube). Finally it is dried on Whatman paper, covered with cling film and exposed to X-Ray film at -70C either overnight or for longer periods if necessary.

2.18 Spun columns

Sepharose G-50, G-25 Pharmacia

1ml syringe barrels are used. One end is plugged with polymer wool, and Sephadex G-50 equilibrated in 3XSSC is added to fill syringe using a plastic pi pette. Syringes are placed inside 1.5ml plastic centrifuge tubes and are centrifuged for 10 minutes at 1600 rpm. If necessary, more Sephadex G-50 is added and centrifugation is repeated. The probe is loaded onto the column and is centrifuged for 5 minutes at 1600rpm. The columns are discarded and eppendorf tubes containing the probe are placed in counter (liquid scintillator). For a successful labelling >$5X10^6$ cpm are expected.

2.19 Preparation of DNA from blood

Washing solution: (autoclaved)

0.1% Nonidet N-P40

0.9% NaCl

Lysis buffer:

7M urea

0.3M NaCl

10mM EDTA

10mM Tris/HCl pH:7.6

Blood samples are kept at -70C. Samples are thawed and poured into 50ml tubes where an equal volume of washing solution is added. They are mixed and centrifuged for 15 minutes at 3000rpm. The supernatant is discarded and the pellet is resuspended in 10-15ml of washing solution. The samples are centrifuged at 3000rpm for 15 minutes, the supernatant is poured off and 1 ml of lysis buffer is added. After a gentle mix with an inverted plastic pippette, 9ml of lysis buffer is added and 2ml of 10%SDS$_{(w/v)}$. The samples are placed at 55C overnight. The following morning, 7.5ml of phenol and 2.5ml $CHCl_3$ are added. The samples are centrifuged at 3000rpm for 15 minutes and this step is repeated 2-3 times. The supernatant is kept in new tubes and 4ml $CHCl_3$ added. After a final centrifugation the supernatant is transferred to new tubes and the DNA is ethanol precipitated.

2.20 Isolation of total human DNA from placental tissue

Placental tissue (15g) is homogenised in 100mls of TE buffer. 150ml of the following lysis solution are added: 0.5M

EDTA (pH:8.0) 100µg/ml proteinase K and 0.5% (w/v) Sarcosyl. The suspension of lysed cells is placed in a 50C water bath overnight.

DNA is extracted 3 times with an equal volume of equilibrated phenol. It is centrifuged at 10,000rpm for 10 minutes and the aqueous layer is removed. The phenol is usually the upper layer due to the high salt concentration in the aqueous phase. Following the phenol extractions, two extractions with chloroform take place. After centrifugation at 10,000rpm for 10 minutes, the aqueous phase is removed. The chloroform is usually the lower layer. The DNA is added in two volumes ethanol with 1/10 volume of 3M sodium acetate. The DNA is spooled from the interface using a glass rod. A final wash in 70% (w/v) ethanol takes place and then the pellet is dried and redissolved in TE pH 8.0. If the DNA is to be used for competition it is boiled for 4-5 hours until fragment size is 300-600bp, or it is sonicated.

2.20 Determination of base sequence of DNA

Sequenase kit (Version 2.0) USB

a) Automated DNA sequence analysis (Smith et al.1986)

Pharmacia LKB A.L.F. DNA Sequencer

Cloned DNA (pBluescrpit or M13) is denatured after all the protein is removed. Concentration is adjusted so there is 15µg of DNA suspended in 32µl of water. 8µl of 2M NaOH are added and after a brief centrifugation, the mixture is incubated at room

temperature for 10 minutes. 12μl of 3M sodium acetate (pH4.8), 28μl of distilled water and 240μl of 100% ethanol (AnalaR, BDH) are added and the mixture is placed on ice for 15 minutes. Following a centrifugation for 10 minutes the supernatant is discarded and the pellet is washed in 70% ethanol. Subsequently the pellet is dried briefly under vacuum and is thus stored until use.

Fluorescence detection of the DNA fragments is accomplished by a fluorophore which is covalently attached to the oligonucleotide primer used in enzymatic DNA sequence analysis. A different colour fluorophore is used for each reaction, specific for A, C, G, T. Four fluorescent compounds with different excitation and emission wavelengths are coupled to separate samples of the primer. Then four sequencing reactions with dideoxynucleotides are performed as usual, each using one of the fluorescently labelled primers. The reactions are mixed and co-electrophoresed in a polyacrylamide gel. The fluorescent bands of DNA are detected at the bottom of the gel and the information is acquired directly by the computer.

b) Dideoxynucleotide sequencing (Sanger *et al*.1977)

Deionised Formamide

Formamide BDH

Analytical grade mixed bed resin AG 501-X8 (D) 20-50 mesh fully regenerated BIO-RAD

50 mls of formamide are mixed with 5g of mixed-bed, ion exchange resin. The mixture is stirred for 30 minutes and filtered through Whatman No.1 filter paper.

Stop buffer

98% deionised formamide

10mM EDTA (pH 8.0)

0.025% (w/v) xylene cyanol FF

0.025% (w/v) bromophenol blue.

This method uses a DNA polymerase to synthesise a complementary copy of a single-stranded DNA template. Chain elongation occurs at the 3' end of a primer DNA that is annealed to the template. The dideoxy sequencing method is based on the ability of the DNA polymerase to use 2'3'-dideoxynucleoside triphosphates as substrates. When a ddNTP is incorporated at the 3' end of the primer, chain elongation is terminated because the primer lacks a 3'-hydroxyl group and thus a phosphodiester bridge between the 3'-hydroxyl group at the end of the primer and the 5'-phosphate group of the incoming deoxynucleotide can not be formed.

The sequencing reactions were performed using the Sequenase kit from USB (Version 2.0) T7 DNA polymerase is used where the 3' 5' exonuclease activity has been totally removed.

Following the denaturing of the DNA to be sequenced as a

pellet, it is resuspended in 7μl of water 2μl of reaction buffer and 5ng of primer is added. Then it is incubated at 37C for 15 minutes to allow annealing. Meanwhile the labelling mixture is diluted and so is the enzyme. According to the number of DNA samples to be sequenced a quantity of labelling mixture is prepared contain DTT, [α-^{35}S] dATP, a labelling buffer supplied by the Kit, and the enzyme dilution. This mixture is added to the annealing mixture and is incubated for 5 minutes at room temperature. Eppendorf tubes with dideoxynucleotides are incubated at 37C for 10 minutes. The labelling reaction mixture is subsequently added to the 4 termination mixture (dideoxynucleotides) tubes and is incubated for 5 minutes at 37C Finally the reaction is stopped with the stop solution and samples can be stored at -20C.

c) Solid phase sequencing (Hultman *et al*.1989)

One strand of a double stranded DNA is biotinylated. The DNA molecule is then bound to streptavidin-ferromagnetic beads. The strands are denatured by treating the beads with alkali and the biotinylated strands are separated from the non-biotinylated strands using a magnet that traps the bead complex to which the biotinylated strands are bound. Subsequently sequencing reactions are performed.

Initially the PCR product is bound to the Dynabeads (M-280) 30μl of beads are washed first, three times in two volumes TE/1.0M NaCl per sample and are finally resuspended in 30 μl of TE/1.0M NaCl. Then they are added to PCR product and are incubated for 30 minutes at 28C with frequent mixing. Supernatant is

removed using the magnet and mixture is washed with 340μl of TE/0.1M NaCl. 20μl of 0.15M NaOH are added and mixture is incubated at 28C for 5 minutes. The supernatant is removed and the 0.15M NaOH incubation is repeated. Then, the beads are washed three times with 40μl of distilled water and are stored if necessary. Finally the sequencing reactions are performed as normal using the Sequenase kit.

Samples are denatured for 3 minutes at 80C and are loaded on the gel in a specific pattern.

2.22 Preparation of sequencing gel

6% acrylamide solution was used (Sequagel-National Diagnostics). (final concanhation)

Dimethyldichlorosilane solution BDH

In a total volume of 100mls the following solutions were added:

66mls of Diluent

24mls of Concentrate

10 mls of TBE buffer 10X

800μl of 10%APS

40 μl of TEMED

The glass plates are carefully washed with detergent and ethanol and the smaller one is siliconised.

The acrylamide mixture is then poured between the sequencing plates and is left to set for 1 hour. Sequencing gels are

normally prerun for 1/2 hour prior to the loading of the samples. DNA is usually electrophoresed for 3 hours at 60 Watts Then the gel is dried and exposed to film overnight.

CHAPTER 3

INTERSPERSED REPEAT SEQUENCE POLYMERASE CHAIN REACTION ON CHROMOSOME 21

3.1 Introduction

3.1.1 Chromosome 21

Chromosome 21 is the smallest human autosome, containing only about 1.7% of the human genome. Its genetic length is estimated at 158cM (Haldane function), using 27 markers of an average distance of 6cM (Petersen *et al*.1991) and its physical length about 5,4000kb (Gardiner *et al*.1990). However, these figures may be an underestimate since there are not sufficient markers on the short arm of the chromosome contributing to this information. From physical mapping data, the most proximally located known locus on 21q is D21S16 (Gardiner *et al*.1990) and the most distal locus in the linkage map is the Col6A1 gene (Petersen *et al*.1991).

Apart from its small size, which makes mapping a much easier task in the human genome project, this chromosome presents intriguing interest in human genetics, since it is involved in a number of disorders. Mapping efforts will hopefully facilitate the identification of genes responsible for those conditions.

Already a localisation of the gene responsible for some cases of familial amyotrophic lateral sclerosis has been achieved near the marker D21S58 in the 21q22.1 region of the chromosome (Siddique *et al*.1991), and genetic studies showed linkage of the gene for progressive myoclonus epilepsy (EPM1) of the Unverricht-Lundborg type to 21q22.3 (Lehesjoki *et al*.1991). Its close association with Down's syndrome, (when trisomic, chromosome 21 leads to the development of Down's syndrome phenotype) and the characteristic Alzheimer's like neuropathology that Down's syndrome patients develop when and if they live up to thirty years of age (Oliver and Holland, 1986), led scientists to assume a connection between Alzheimer's disease and genes on this chromosome.

3.1.2 Alzheimer's disease and chromosome 21

Alzheimer's disease displays an autosomal mode of inheritance in some families with an early onset of the disorder (St George Hyslop *et al*.1987). In four such pedigrees linkage to chromosome 21 markers D21S1/S11 and D21S16 was established (St George Hyslop *et al*.1987; Goate *et al*.1989). So research for the defective gene was focused at that time on this part of the chromosome, although other groups failed to repeat the same observation (Schellenberg *et al*.1988).

Under the assumption of genetic homogeneity, our group failed to correctly interpret the fact that the major positive *Lod* score in our analysis, was arising from one of the families studied, F23, and that one other family, F74, was clearly

* This map will eventually help in physical and genetic localisation of different genes underlying human traits on specific chromosomes (Botstein et al., 1980, White et al., 1985).

negative (Goate *et al*.1989). As we proved later, heterogeneity was mislocalising the defect towards the centromere (figure 3.1).

Although cloning of ß-amyloid precursor protein gene (APP) (Kang *et al*.1987) and its mapping to chromosome 21 (Goldgaber *et al*.1987; Robakis *et al*.1987; Tanzi *et al*.1987) made it an evident candidate at the time, it was soon ruled out as the defective gene in Alzheimer's disease since recombinants were found between APP and the disease locus (Van Broeckhoven *et al*.1987). One of our families, F23 generated a *Lod* score of 3 between several markers on chromosome 21 and the disease. We decided to use this family to test new and more informative genetic probes so that the region containing the Alzheimer's disease mutation could be defined more precisely. Therefore, we needed a fast and reliable technique to generate polymorphic markers around the centromere of chromosome 21.

3.1.3 Generation of polymorphic markers and human genetic map

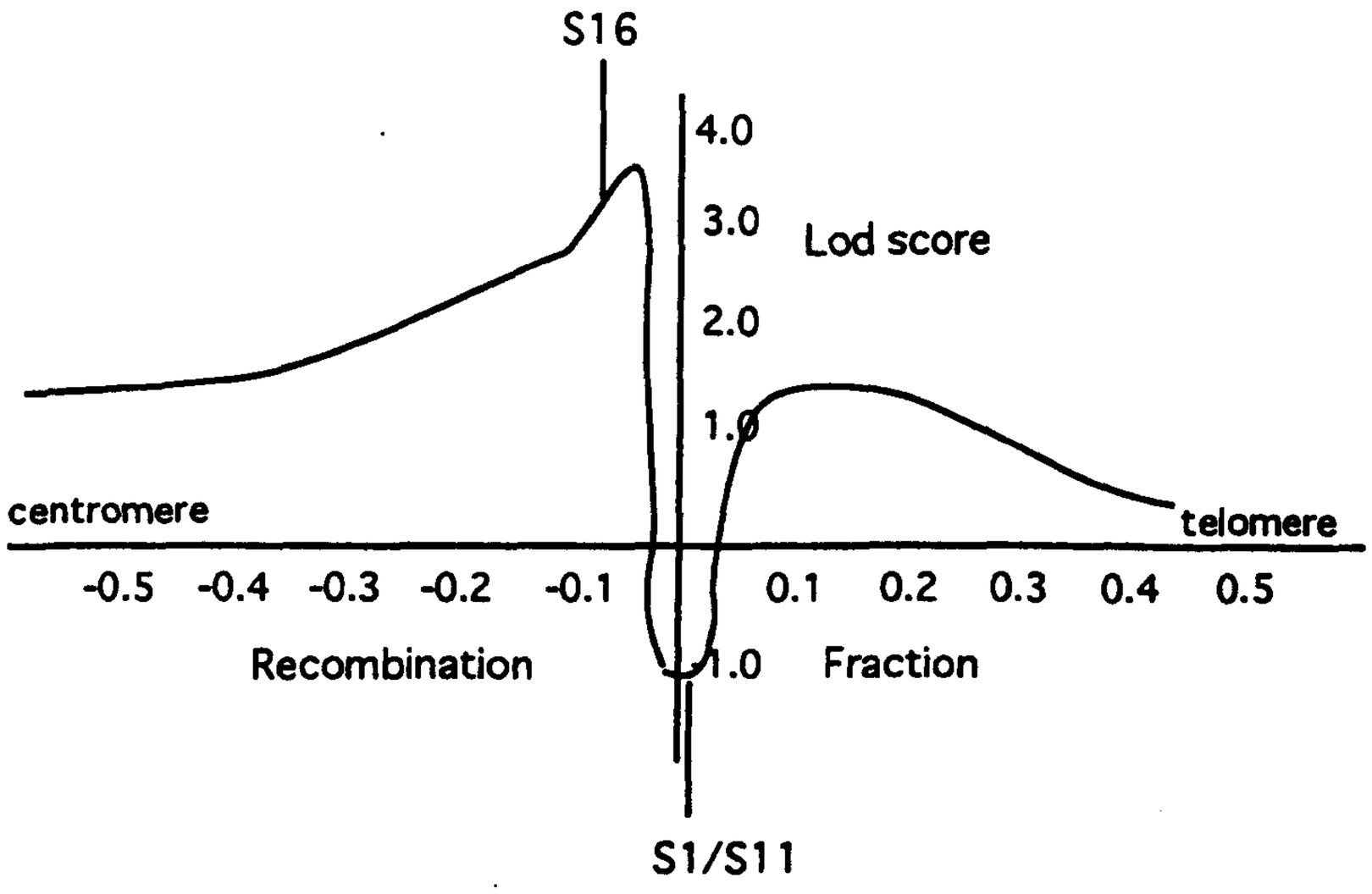
The identification of DNA sequence variation which does not result in any expressed phenotypic differences has revolutionised human genetics. Polymorphism in DNA sequence is common enough to be used in the form of informative genetic markers allowing the study of human heredity by constructing a detailed linkage map for the human genome.*

Genetic markers include:- a) restriction fragment length

Figure 3.1

Two-point Lod scores between Alzheimer's disease and D21S1/S11 and D21S16

	Recombination fraction				
D21S1/S11	0	0.1	0.2	0.3	0.4
Pedigrees					
1	0.38	0.27	0.17	0.08	0.02
2 (F23)	1.73	1.39	1.00	0.58	0.18
3	0.60	0.43	0.27	0.13	0.03
4	0.29	0.31	0.23	0.06	0.04
5	0.30	0.21	0.13	0.06	0.02
6 (F74)	-2.66	-0.45	-0.20	-0.08	-0.02
Totals	0.64	2.16	1.60	0.89	0.27
D21S16					
1	0.41	0.30	0.19	0.10	0.03
2 (F23)	1.38	1.12	0.82	0.50	0.18
3	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00
6 (F74)	0..00	0.00	0.00	0.00	0.00
Totals	1.79	1.42	1.01	0.60	0.21



Figures modified from Goate et al.,1989

polymorphisms (RFLPs) deriving from an alteration of base sequence, an insertion or a deletion of a DNA piece and resulting in the creation or abolition of a restriction enzyme site and b) Variable number of tandem repeats (VNTRs), as $(CA)_n$ repeats and poly-A tails (amongst others).

Repeat sequences represent an abundant class of DNA polymorphisms in the human genome and are therefore very useful for these studies. The interspersed repeat $(CA)_n$, has been identified as occurring approximately every 60kb in the human genome, and it shows frequent length polymorphism. The length polymorphisms can be detected via PCR, using primers flanking the repeat sequence (Weber AND May, 1989). Recently, the 3' ends of Alu and L1 elements were found to have long poly(dA) tracts which are often polymorphic (Economou *et al*.1990).

3.1.4 Human repeat sequences

a) Alu family of repeats.

The Alu family of repeat sequences was first described (Houck *et al*.1979) as 300 nucleotide duplexes sharing a cleavage site for the restriction enzyme AluI. This restriction site is located 170 nucleotides from one end. A typical Alu element corresponds to a head to tail tandem dimer approximately 300bp long, the right monomer containing an additional 31bp segment relative to the left monomer (figure 3.2). It has been proposed that both monomers arose from the 7S RNA through deletion of the central 7S-specific sequence. Alu elements are thought to have spread throughout the primate genomes via retrotransposition,

involving an RNA intermediate. Their transcription is controlled by the RNA polymerase III promoter located in the left half of the element. This RNA is used as a template by reverse transcriptase to generate a cDNA that is subsequently integrated to a new locus in the genome. This introduction produces short direct repeats that flank the element (Schmid AND Jelinek, 1982; Weiner *et al*.1986). Despite the variability, all Alu elements can be related to a consensus sequence (Deininger *et al*.1981). Alu is found in approximately 10^6 copies of a 300bp sequence spaced around every 4kb in the genome (Britten *et al*.1988).

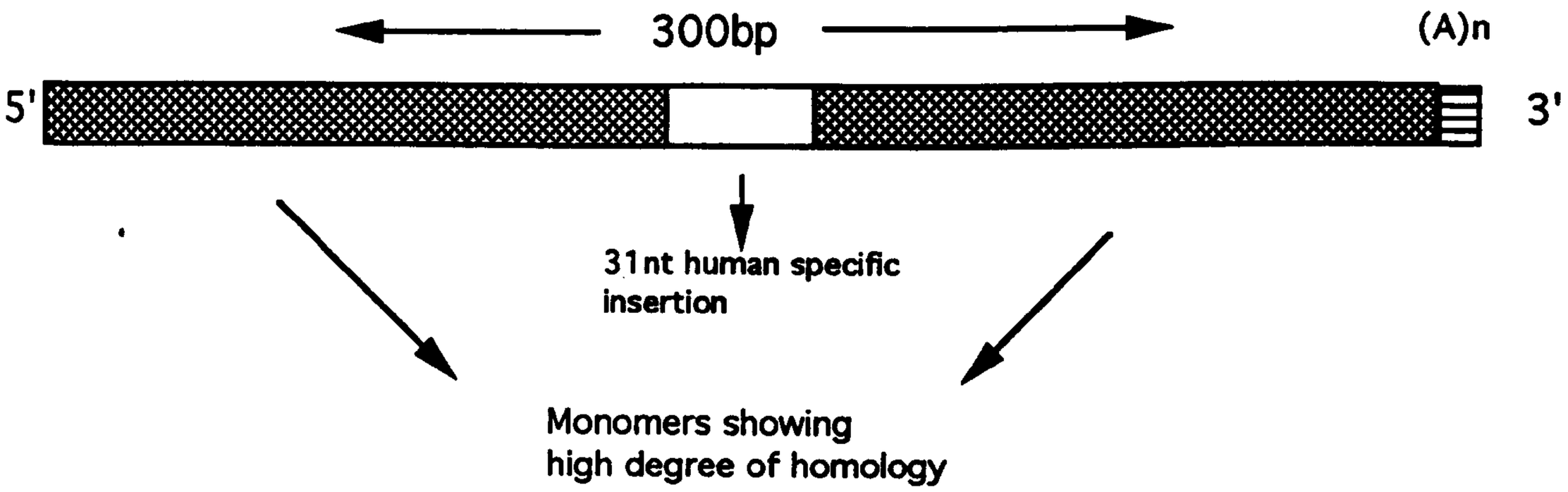
Although no function has been ascribed to this genetic material, its high genomic representation and its involvement in mutations causing genetic defects (Rouyer *et al*.1987) indicate a role in primate evolution. It has also been suggested that they may be involved in gene regulation, but up to now their function remains unclear.

b) L1 or KpnI family of repeats

The full length of their consensus sequence is 6.1-6.4kb and their structure resembles retroviral DNA. (Jelinek and Schmid, 1982; Weiner *et al*.1986). There are approximately 10^4 copies of L1 or Kpn1 in the human genome (Scott *et al*.1987). KpnI repeats are associated sometimes with flanking direct repeats that may be target site duplications, suggesting that some KpnIs may be mobile. They have polyadenylation signals at their 3' ends and some are truncated at the 5' end. Another notable feature of this family is the gradient of increasing homology among family members from the 5' end to the 3' end (Grimaldi *et al*.1984). L1

Figure 3.2

Schematic diagram of human Alu repeat consensus sequence



Schematic diagram of human L1 repeat sequence

sequences are also known to cause mutations in human disease (Kazazian Jr *et al*.1988) and are presumably dispersed by an RNA intermediate like Alu repeats.

Alu and L1 families have an inverse distribution on the human metaphase chromosome suggesting an inverse functional relationship (Korenberg and Rykowski, 1988). The G/Q bands, relatively poor in expressed genes are L1 rich, whereas R bands where Alu family dominates are rich in GC and thought to be the chromosomal regions in which active genes are concentrated (Korenberg and Rykowski, 1988).

Since chromosome 21 is approximately 54,000kb, 11250 copies of Alu and 140 copies of L1 are expected to be present. This is probably an overestimate because of the banding of the chromosome and the differential distribution of the repeats.

3.1.5 Somatic cell hybrids

Somatic cell hybrids containing human chromosomes isolated in a rodent cell background have served as powerful tools for gene mapping and for the isolation of chromosome specific sequences. The gene correcting a mutation causing a purine requirement in Chinese Hamster Ovary (CHO) cells (Gars gene) was assigned on chromosome 21 (21q22.1) (Chadefaux *et al*.1984). Thus, the presence of human chromosome 21 in somatic cell hybrids is sufficient to correct the purine requirement in these mutants and to cause the mutant cells to produce the enzyme (Patterson *et al*.1981). This has allowed retention of specific fragments of

human chromosome 21 containing the Gars gene region, in somatic cell hybrids on the basis of correcting this nutritional requirement. Such hybrids are useful in the construction of a detailed physical map of the long arm of chromosome 21 (Van Keuren *et al*.1986; Gardiner *et al*.1990). This has allowed the regional localisation of many genes to chromosome 21.

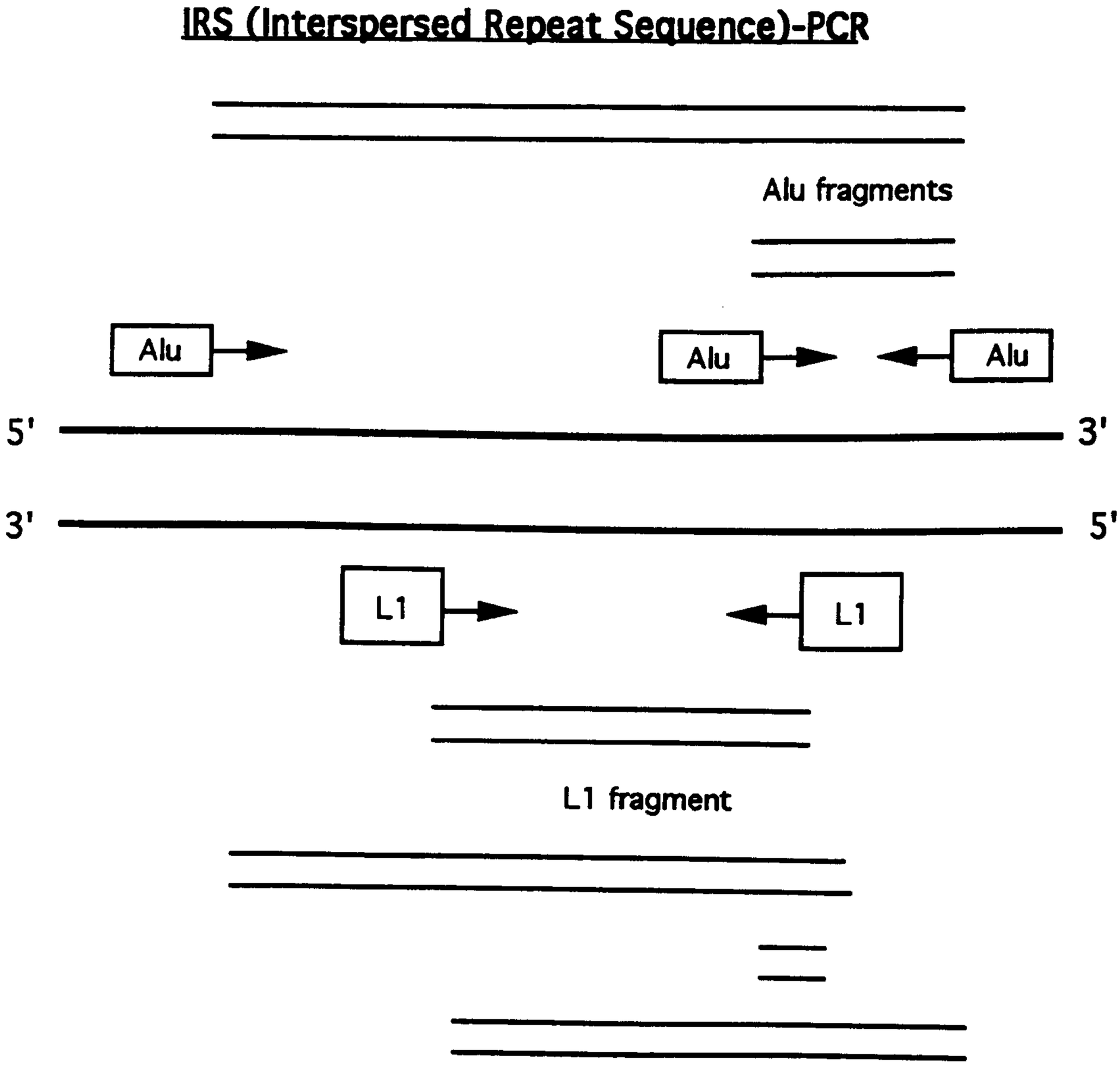
3.1.6 IRS-PCR on chromosome 21.

To isolate a number of human clones directly from cell hybrid DNA containing chromosome 21, I used oligonucleotides 3directed to the human specific portion of interspecific repetitive elements (Nelson *et al*.1989; Ledbetter *et al*.1990). This method is based on the fact that amplification products will be generated using PCR, between repeat sequences of inverted orientation (figure 3.3).

An Alu primer was used (Nelson *et al*.1989), which is located within a 31bp sequence that is unique to the second monomer of primate Alu repeats and although repetitive elements homologous to the human Alu repeat are also found in rodent genomes, there is sufficient sequence divergence to reduce cross hybridisation of human and rodent repeats. The L1 primer used is located close to the 3' end of the L1 human repeat within the 208bp region which has been described as constant among human sequences but not conserved across species (Scott *et al*.1987).

The IRS-PCR method obviates the requirement of cloned DNA

Figure 3.3


IRS (Interspersed Repeat Sequence)-PCR
Alu fragments
Alu
Alu
Alu
5'
3'
3'
5'
L1
L1
L1 fragment
Alu-L1 fragments

libraries for the isolation of human clones, through the use of human specific repeat sequence primers. It also allows access to sequences that may be underepresented in traditional DNA libraries, like centromeric or telomeric sequences. I decided to use this method as a quick way of generating polymorphic markers either in the form of $(CA)_n$ repeats or in the form of poly-A tails or other repeat patterns, which follow the 3' end of Alu or L1 repeat sequences (Economou *et al*.1990; Zuliani and Hobbs, 1990). These markers would have given us a more precise estimate for the location of the Alzheimer's gene on chromosome 21.

3.1.7 APP gene in Alzheimer's disease

Almost simultaneously as this technique was starting to give some first interesting results, our group discovered a mutation in exon 17 of the APP gene in F23 (Goate *et al*.1991) suggesting that mutations in APP can lead to Alzheimer's disease. It was thought probable that different mutations in the same gene account for the pathogenesis of the disease in other families with early onset Alzheimer's disease, showing linkage to chromosome 21. Since we could not exclude the possibility that in some pedigrees another gene proximal to APP may be responsible for its overexpression the isolation of fragments from chromosome 21 and the identification of new polymorphisms that may be potentially used for linkage analysis in Alzheimer's families was still a requirement, although not as much a priority as it had been previously.

In that respect, I changed the direction of my project in order to develop a protocol for mutation screening, in APP gene

and thus identify new, and estimate the number of mutations in APP gene. (Chapter 4)

3.2 Materials and Methods

3.2.1 Cell lines

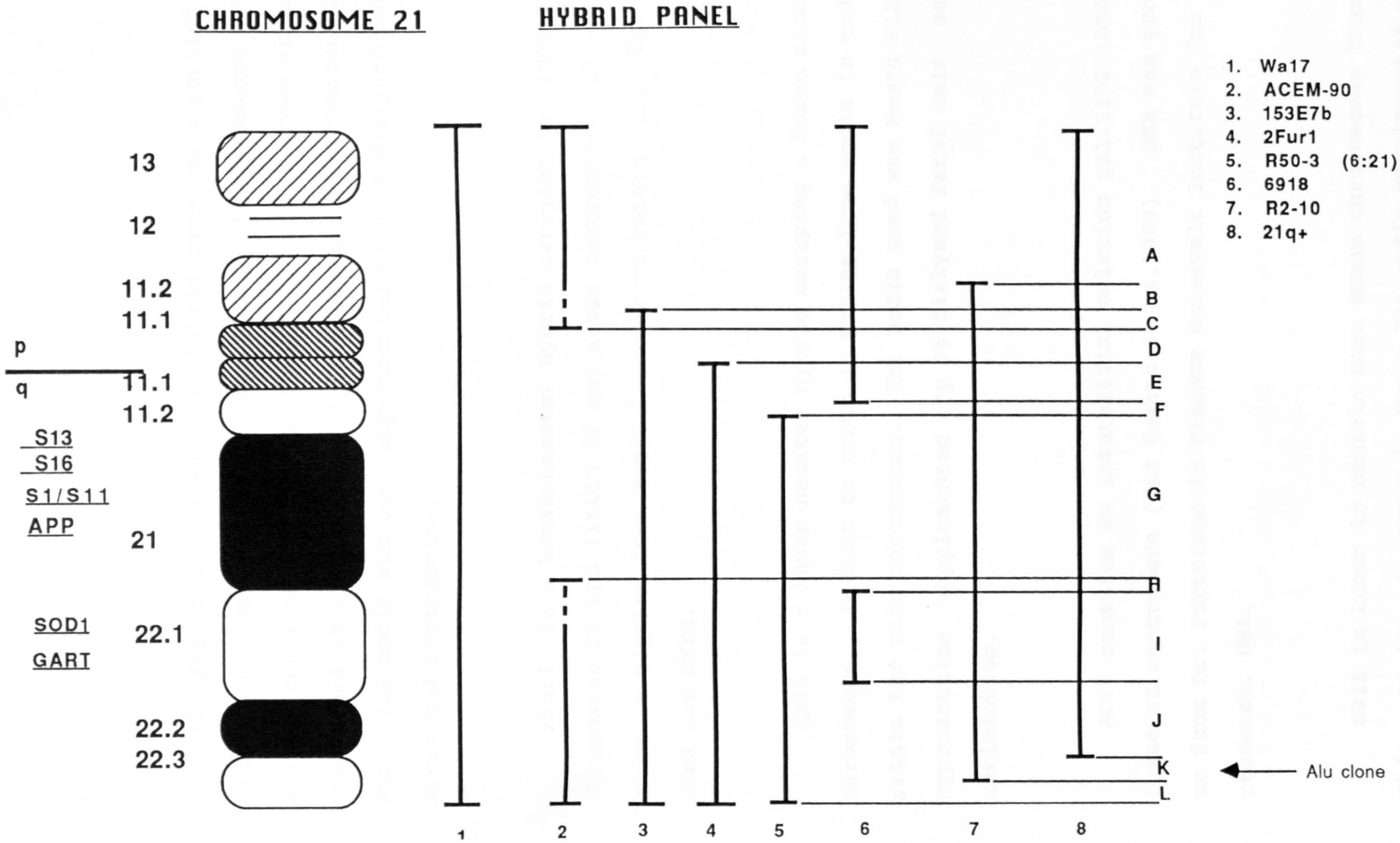
The complete panel of somatic cell hybrids that I have used in these studies, with the approximate regions that are retained in each hybrid is shown in figure 3.4. These regions were originally estimated by cytogeneticists and refinements were made after molecular analysis (Gardiner *et al*.1990; Van Keuren *et al*.1986).

Most hybrids are selected for growth and maintenance in F12D+8% fetal calf serum that had been dialysed to remove small molecules including purines. This medium does not allow growth of the CHO parental cells and thus selects for the maintenance of any human chromosome containing 21q22.1 where the Gars gene is known to map (Chadefaux *et al*.1984).

Wa17 is a mouse X human hybrid cell line with three copies of chromosome 21 with no known selection but appears to be stable in culture. This cell line is grown in DMEM media.

R50-3 is a 6;21 human-hamster hybrid cell line. It carries the derivative human chromosome 6 which has a portion of 21q translocated onto the p arm of the chromosome 6. There are other chromosomes present in the cell. R50-3 must be grown in purine-free media in order to retain the translocation. Since the CHO parent was a pyrimidine auxotroph, R50-3 requires uridine to grow

Figure 3.4


CHROMOSOME 21
HYBRID PANEL
1. Wa17
2. ACEM-90
3. 153E7b
4. 2Fur1
5. R50-3 (6:21)
6. 6918
7. R2-10
8. 21q+
13
12
11.2
11.1
p
q
11.1
11.2
S13
S16
S1/S11
APP
21
SOD1
22.1
GART
22.2
22.3
A
B
C
D
E
F
G
H
I
J
K
L
Alu clone
1
2
3
4
5
6
7
8
81

also.

153 E7b is a human-hamster hybrid carrying a portion of human chromosome 21 translocated onto a CHO chromosome. The long arm and centromere may be partial. It must be grown in purine free media in order to retain that portion of chromosome 21 so Ham's F12 media was used supplemented with 7% dialyzed fetal calf serum and antibiotics.

2Fur1, is a human-hamster hybrid carrying the long arm of chromosome 21 and little if any human centromere. It must be grown in glycine free media in order to retain 21q. The media used was DMEM.

R210 is a human-hamster hybrid carrying a human ring 21 chromosome. It must be grown in purine free media in order to retain the ring chromosome. The media used was Ham's F12 without hypoxanthine supplemented by 7% dialysed fetal calf serum and antibiotics.

ACEM contains an interstitial deletion q21.21+2 long arms fused at centromere (Van Keuren *et al*.1989). DNA was provided to us from Dr. Patterson in Eleanor Roosevelt Institute for cancer research. USA.

6918 is known to contain more human chromosomes than 21. This cell line is grown in Ham's F12 media supplemented with dialyzed fetal calf serum and antibiotics.

3.2.2 High molecular weight DNA preparation

This method was described previously (Section 2.3)

3.2.3 PCR amplification and Primers

Polymerase chain reaction was carried out in a total volume of 100μl using 250ng of DNA in 10mM Tris-HCl pH 8.0, 50mM KCl, 2.5 or 3.0mM $MgCl_2$ respectively for each primer Alu or L1, 0.01% gelatin, 300μM of each dNTP, 2.5 units Taq polymerase (Cetus Amplitaq). The final primer concentration was 1μM for both Alu and L1 primers. An initial denaturation of 10 minutes at 94C was followed by 35 cycles of denaturation at 94C for 1 minute, annealing at 60C (TC-65) or 50C (L1) for 45 seconds and extension at 68C for 4 minutes. The Alu PCR was optimised and carried out on an automated Perkin-Elmer DNA thermal cycler while both optimisation and PCRs with the L1 primer were carried out on a Hybaid machine.

TC-65 sequence: 5'-AAGTCGCGGCCGCTTGCAGTGAG-CCGAGAT-3' (It contains a Not1 cloning site.)

L1 sequence: 5'-CATGGCACATGTATATC-3'

LSC1 sequence: 5'-GCTAAATGAGAGAATGGG-3'

For the Poly-A tail PCR primers were used in a ratio 40nM:400nM TC-65: LSC1 PCR conditions were: 94C for 10 minutes,

followed by 30 cycles of 72C for 30seconds, 94C for 30 seconds, and 60C for 30 seconds. DNA was used at a concentration of 200ng, $MgCl_2$ was at a concentration of 1.5mM, dNTPs at 200μM and 1 unit of Taq. The PCR product was 172bp.

Primers were supplied by Oswell DNA Service and Mark Fagan and Dr. Howard Beswick in our Department at St.Mary's Hospital Medical School using an oligonucleotide synthesiser (Pharmacia, LKB, Gene Assembler plus).

3.2.4 Agarose gel electrophoresis

Aliquots (20 μl) from the reaction mixture were separated on a 2%$^{(\omega/\upsilon)}_{\lambda}$ agarose gel in TAE at 100V for 4 hours and were visualised with ethidium bromide (0.25μg/ml). The size marker used was 1kb ladder.

3.2.5 Southern blot

This method was described previously (Section 2.17)

3.2.6 Restriction enzyme digest

This method was described previously (Section 2.7)

3.2.7 Random-hexamer labelling

This method was described previously (Section 2.16b)

3.2.8 Pre-reassociation of DNA

To determine the best pre-reassociation strategy for reducing unwanted signal from a repeat whilst preserving the signal from low copy components we used the formula

:$Cot_{1/2}$(probe)=LX106

where L:the length of the probe in kb. The $t_{1/2}$ of the probe should be greater than the t of the driver DNA (Sealey *et al*.1985).

Labelled probe was competed with sonicated human placenta DNA at 60C until a Cot value of 100 for the Driver DNA was reached. The basic procedure was the following:

The probe was mixed with the driver DNA, 25μl of 20XSSC and TE was added till a volume of 100μl was reached. The mixture was denatured for 10 minutes and cooled in ice for 1 minute. Then it was incubated at 60C for the required time. Finally it was added in the prewarmed hybridisation mixture and hybridisation followed the normal procedure.

3.2.9 5' end labelling

This method is described previously (Section 2.16a)

3.2.10 Extraction of DNA from agarose

Extraction of DNA from agarose was performed using the

Geneclean kit (Section 2.7)

3.2.11 Cloning of IRS-PCR fragments

Alu PCR fragments were purified using Primerase columns (Stratagene), ethanol precipitated and resuspended in 20μl TE pH 8.0

The synthesised DNA was digested with Not1 and aliquots of 50ng were ligated to Not1 digested pBluescript vector (KS Stratagene) at a ratio 1:1 vector to insert. Ligations were carried out for 16 hours at 14C in a final volume of 10μl. 2μl, approximately 10-20ng were used to transform 200μl of XL1 competent cells (Section 2.12). The transformed cells were plated on Amp/Tet X-gal/IPTG- containing plates according to the manufacturer's recommendations (Section 2.14).

3.2.12 Automated sequencing

This method was described previously (2.21a). Primers used:

Reverse primer: 5'-AACAGCTATGACCATG-3'

M13-20 primer: 5'-GTAAAACGACGGCCAGT-3'

Automated sequencer was operated by Sukhi Mahal.

3.2.13 Double strand DNA sequencing

This method was described previously (2.21b).

Sequence specific primers used:

Reverse: 5'AAACTCCAGCTGCACAATTCCC-3'

Forward: 5'CTTTTTCAGGACAGTTACTCTTAG-3'

3.3 Results

The TC-65 primer was used to generate Alu-PCR fragments on chromosome 21. Amplification of total human DNA resulted in a smear on an ethidium bromide gel with few distinct bands (indicating a large number of PCR products). A discrete though amplification pattern, was observed for each hybrid (figure 3.5).

The L1 primer was designed after a search through the Databanks so that there is no cross homology with other species and it is found towards the 3' end of the L1 or Kpn1 human repeat. (6083-6098bp) (figure 3.6).

PCR conditions were optimised for each set of primers used. The optimum concentration of $MgCl_2$ used for each primer has been decided after titration (figure 3.6).

As part of the optimisation procedure different annealing temperatures as well as different extension times were tried to determine the best conditions to be used. Titration of DNA concentration and the amount of Taq polymerase used also showed the effect of these parameters on the PCR amplification. Both low annealing temperatures and high concentrations of Taq resulted in a very high background of unspecific amplifications (figure 3.7) while the amount of DNA and the extension time proved less critical (figure 3.8).

PCR of the chromosome 21 hybrid DNA with the Alu primer (TC-

Figure 3.5

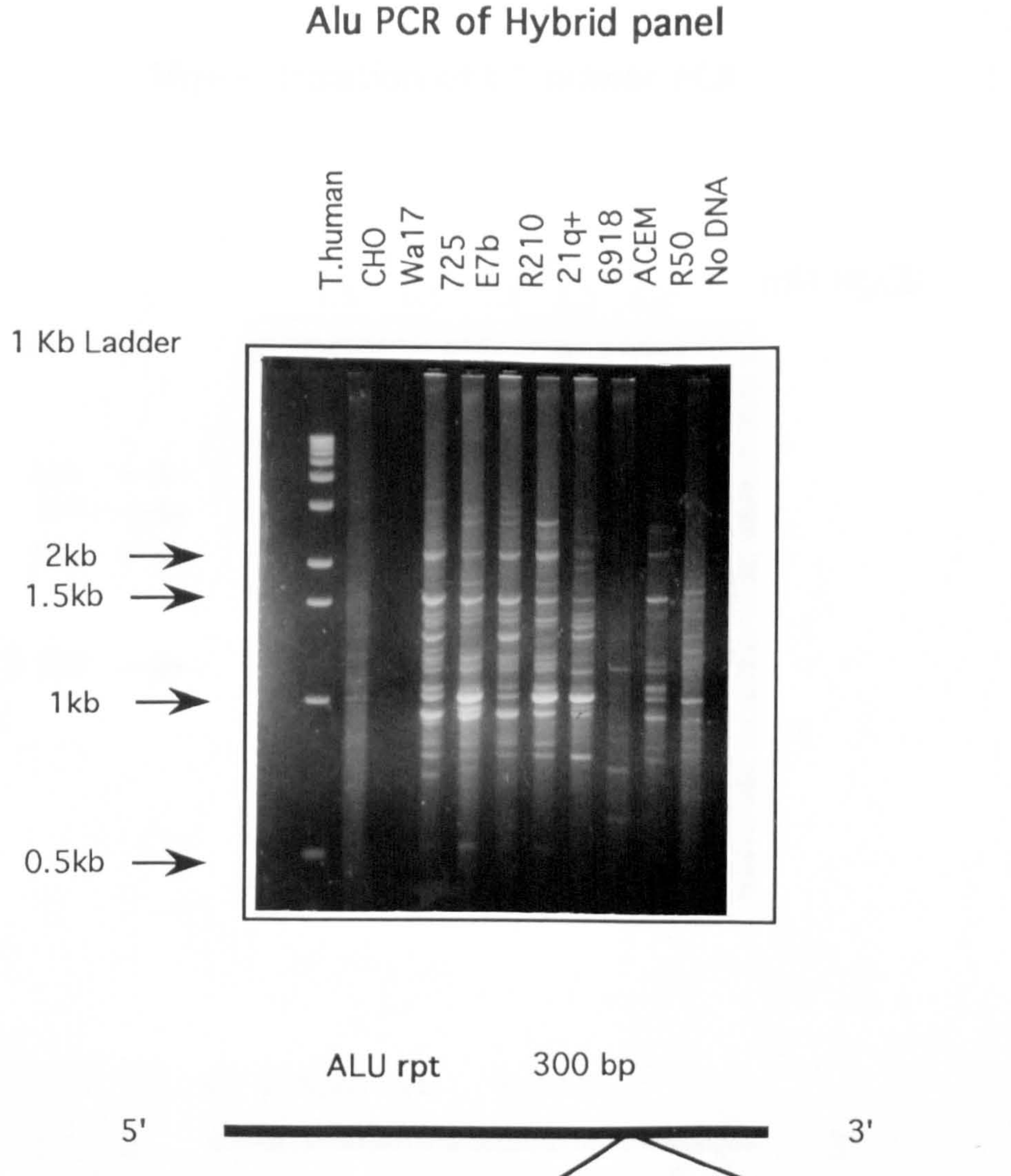


Figure 3.5

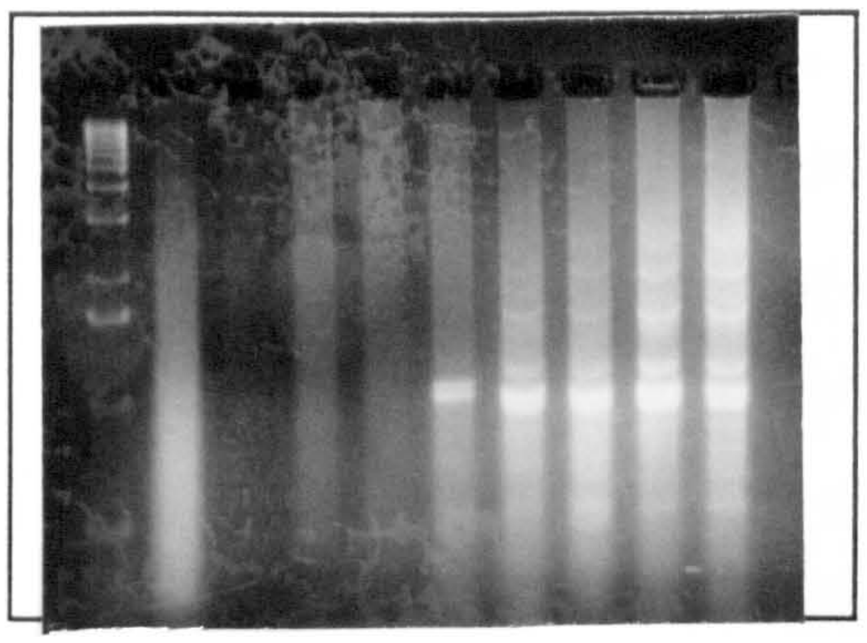
Amplification using standard conditions (3.2.3) of hybrid cell lines with Alu primer. Products are run on a 2% (w/v) agarose gel.

Figures 3.7 and 3.8

ALU-PCR optimisation procedures

a. Taq units

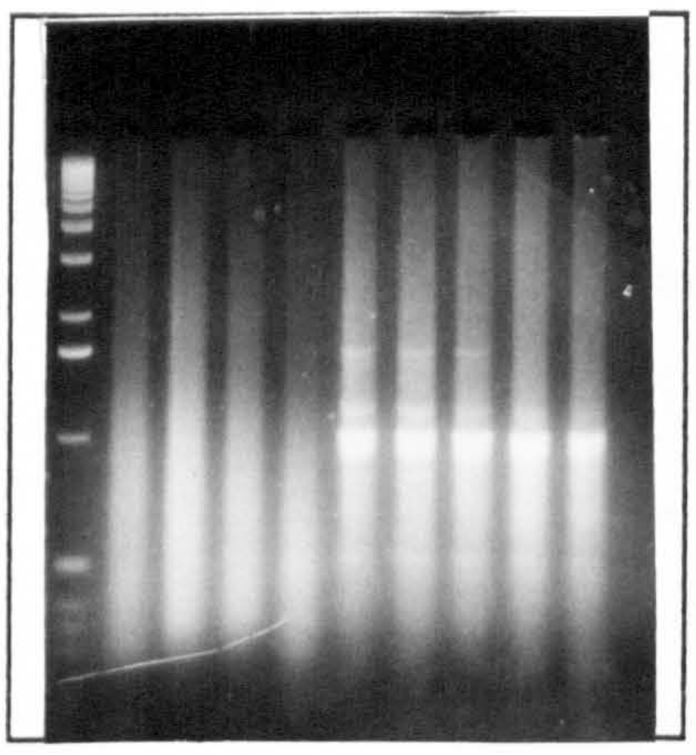
1 2 3 4 5 6 7 8 9 10 11



1. 1kb ladder
2. control: human DNA
3. control: hamster DNA
4. 0.25 unit of Taq
5. 0.5 unit of Taq
6. 1 unit of Taq
7. 2 units of Taq
8. 2.5 units of Taq
9. 4 units of Taq
10. 8 units of Taq
11. control: No DNA

b. extension time

1 2 3 4 5 6 7 8 9 10



1. 1kb ladder
2. Human DNA : 4 min.
3. Human DNA : 3 min.
4. Human DNA : 2 min.
5. Human DNA : 1 min.
6. Hybrid DNA : 5 min.
7. Hybrid DNA : 4 min.
8. Hybrid DNA : 3 min.
9. Hybrid DNA : 2 min.
10 Hybrid DNA : 1 min.

Figures 3.7, 3.8

Titration of Taq DNA polymerase units and optimisation of extension time. Reactions were performed as described in section 3.2.3.

65) yielded approximately 30 discrete bands, ranging in size from 500 to 3000bp (figure 3.5).

The frequency of amplification products when an L1 primer was used was less than with Alu: about 15 bands were generated (figure 3.6). Combination of a single Alu primer and the L1 primer yielded a number of products distinct from those observed with either primer alone. In this case, the relative amount of each primer used is of major significance for the number and size of bands produced (figure 3.9).

A background smear of DNA in the lane was much more pronounced in hybrids containing multiple human chromosomes such as 6918.

The amplified products from the cell hybrids exhibited a discrete and reproducible pattern of sizes (the amplification experiments were repeated five times) suggesting that a restricted set of inter repeat sequences has been amplified using the repeat primers. Similar results have been reported previously (Nelson *et al*.1989; Ledbetter *et al*.1990; Cotter *et al*.1990) using different cell hybrids.

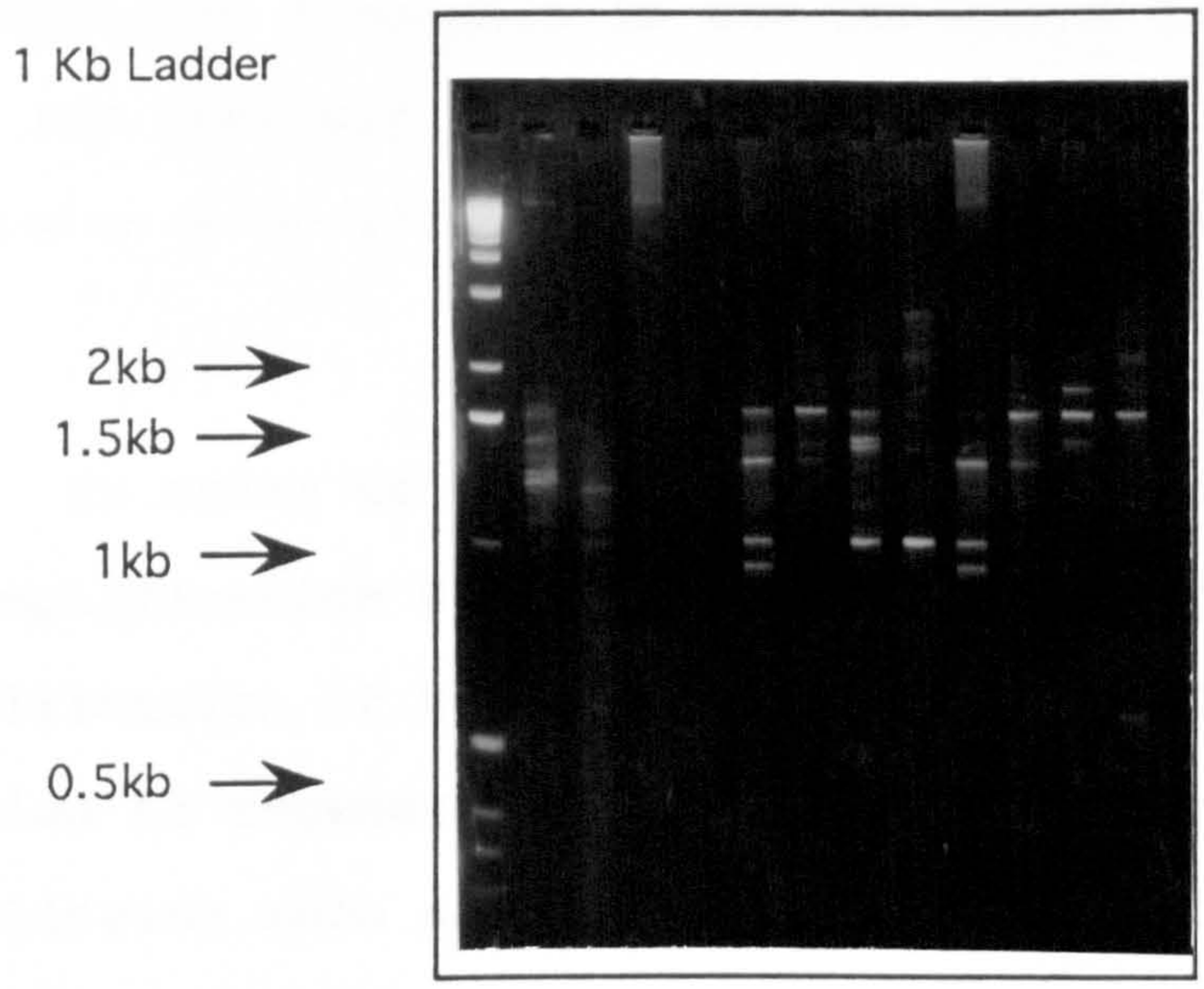
The panel of eight somatic cell hybrids used divides chromosome 21 into 12 regions (figure 3.4). The presence or absence of a particular band in different hybrids allows the regional assignment of bands to different parts of the

Figure 3.9

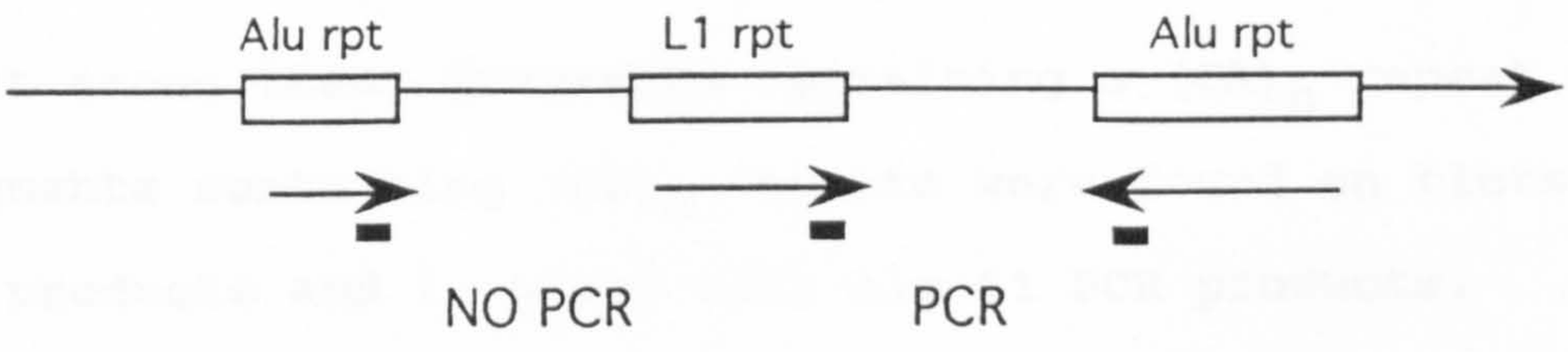
Amplification (3.2.3) of hybrid cell lines with a combination of Alu and L1 primers: 1. when 0.5μM of each primer was used and 2. when 1μM of each primer was used.

Figure 3.9

L1-ALU PCR of Hybrid Panel



1. 0.5μM of each primer
2. 1μM of each primer



chromosome. Confirmation of this localisation must be obtained by using the different bands to probe Southerns of the hybrid panel. Fragments that seemed to map in the region of interest for Alzheimer's disease, were isolated and their localisation was tested by hybridising them after competition with total human DNA, to Southern blots of digested hybrid DNA. These probes hybridised to multiple fragments in the Southerns making the mapping confusing and possibly suggesting sequence homology with loci on other chromosomes or with multiple loci in the same chromosome.

In order to maintain large amounts of a specific fragment, reamplification with the Alu primer was performed after purification of the specific agarose bands. Those attempts failed to regenerate the specific fragments and ended up amplifying much smaller ones (figure 3.10). A possible explanation is that the large fragments contain inverted Alu repeats within them, so with the PCR favouring the amplification of smaller fragments rather than large, smaller Alu fragments are generated from the specific substrate.

Our initial goal was to identify new polymorphic markers fast, preferably in the pericentromeric region of chromosome 21. In order to achieve this, Southern blots of the PCR amplification products were hybridised with GT oligo to test whether there exist among them, fragments containing a $(CA)_n$ repeat. Several fragments containing $(CA)_n$ repeats were found on blots with Alu PCR products and in blots with Alu-L1 PCR products.

Figure 3.10

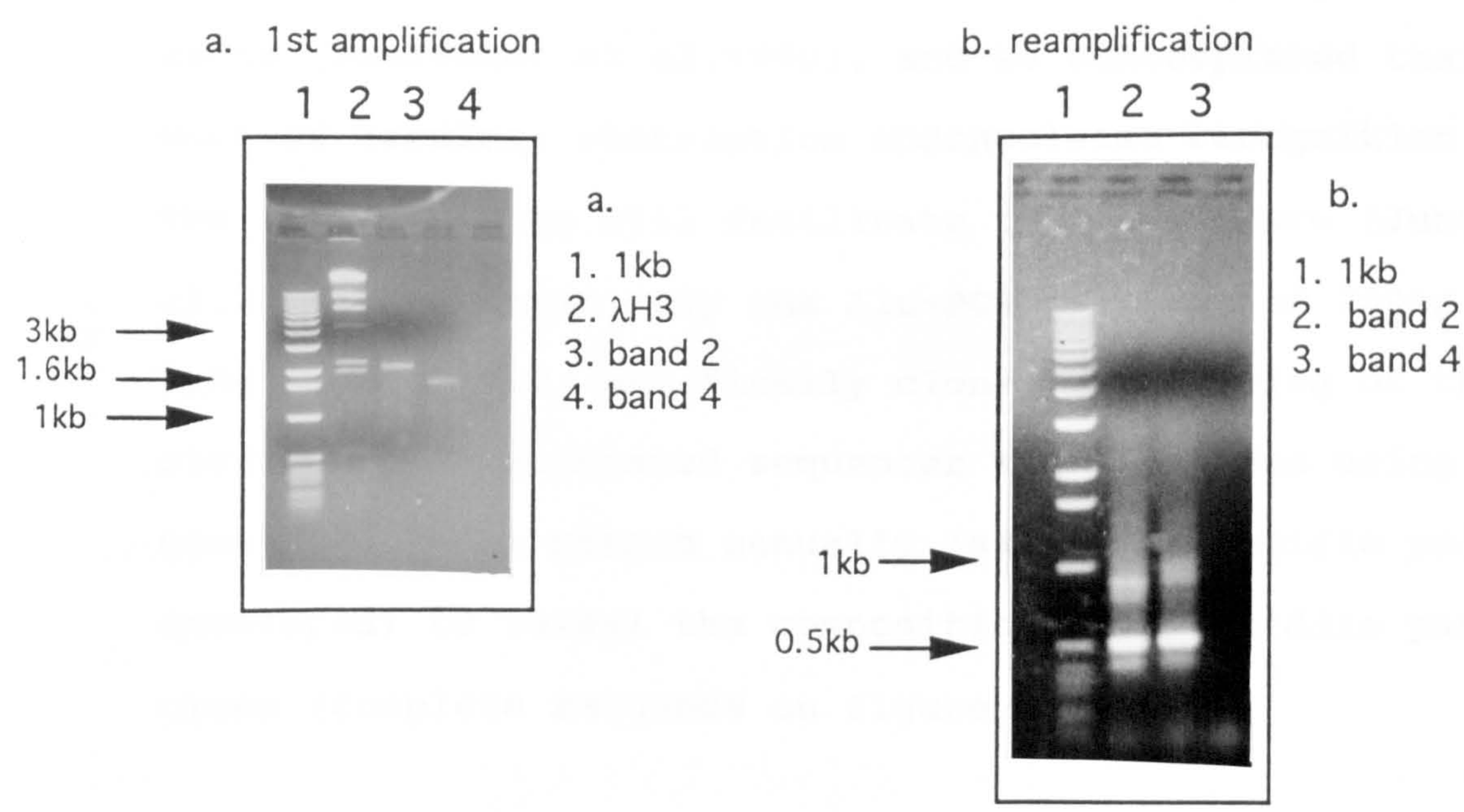


Figure 3.10

a. Isolation of 2 DNA fragments generated with the Alu primer on a 2% (w/v) agarose gel.

b. Reamplification results of the isolated bands with the Alu primer: a much smaller DNA fragment is thus generated.

Subsequent cloning of Alu-PCR DNA fragments has proved difficult. This is a well known fact, generally for PCR fragments (Shuldiner *et al*.1990), and we contemplated that the existence of terminal restriction endonuclease recognition sites in the PCR fragments will facilitate this procedure (Jung *et al*.1990). However only one Alu-PCR fragment of 700bp deriving from Wa17 hybrid, was finally cloned. Sequencing of the clone started on an automated sequencer and proceeded using the dideoxynucleotide method manually (sequence specific primers were developed) to reveal the composition of the middle part of the clone (complete sequence on figure 3.11).

A poly-A tail is present in one end of the sequence. In order to check whether this poly-A is polymorphic two single copy primers were designed at positions 123-144 and 139-157 of the cloned sequence and PCR conditions between the Alu and the new single copy primers were optimised. One primer was finally chosen for following studies (LSC1:139-157). The resulting PCR fragment is 172 bp long. This primer did not show any homology with known DNA sequence when Genbank was searched with BLAST. (Altschul et al., 1990)

This poly-A marker was used as a tool to map the Alu clone back to the chromosome 21 hybrids (figure 3.12).

Unfortunately, this marker was not found to be polymorphic in a number of unrelated individuals tested (figure 3.13).

Figure 3.11

Complete sequence of Alu-PCR generated cloned fragment

5'- GGTACCACTG CACCTGGGAG CTGGGCGAAA GAGTGAGACT

ATGTCTCAAA GAAAAAAAAA AAAAGTACTC GCCCAGAGAA ACCGACTCCC

CCGCCGCAGGT TTGTGTTACA CTGTGGGCAT CTCAACCCAG GCTCCATTTC_

CCATTCTCTC ATTTAGCACA GTATAAAACC CTACGTTACC TTGGAAAATG

AAACTCCAGC TGCACAATTC CCTGCCTCTG AGCAGGAAGC CTGAGGCCAG

TGTCTGAAGG CGGAGGCGAA AGTTCTGAGC CTGTGACTCA GGAGTGCCAG

ACGTGGTTTC TGGTCCCACC CAGCTGAGCT CCGTTAGTCA CCCCGCAGTC

ACGAGCTTCA CGCAGCCTCT AGGCCAGATTG TGTTCTTCCA CCTGCTTTAT

CAGCCAGAAC AGAGCCCTGA CTAATCTGAA GCCCTGGCCT CAGAAGAAAA

CAACGCTAAT GACTCAGAGA CCAGAGCTGA GACCAGACAG ACCGGTGTAG

CCACTCGGCC AGGCTGCTCT GCGAAGCTGC TGTTTCCTCT GCTGCCGAAG

CGGTTTCTTC CACCCAGGGC GGTGCGGTGG TTTGGCGACT TAGAGTTTGA

GGTCCCTTCT CCTAAGAGTA ACTGTCCTGA AAAAGCCCAA AACAAGAGCG

GCATGATCTT AAATCCTGGG GAGAGGACAC AGGCTGCCTG GAGGGGCCGA

GGCACACCTA GAGCTGGGGT TTTGTTCTCA CTCTGTTGCC CAGGCTGCAG

CACAGTGGTC CG-3'

Internal primers used are underlined

Figure 3.12

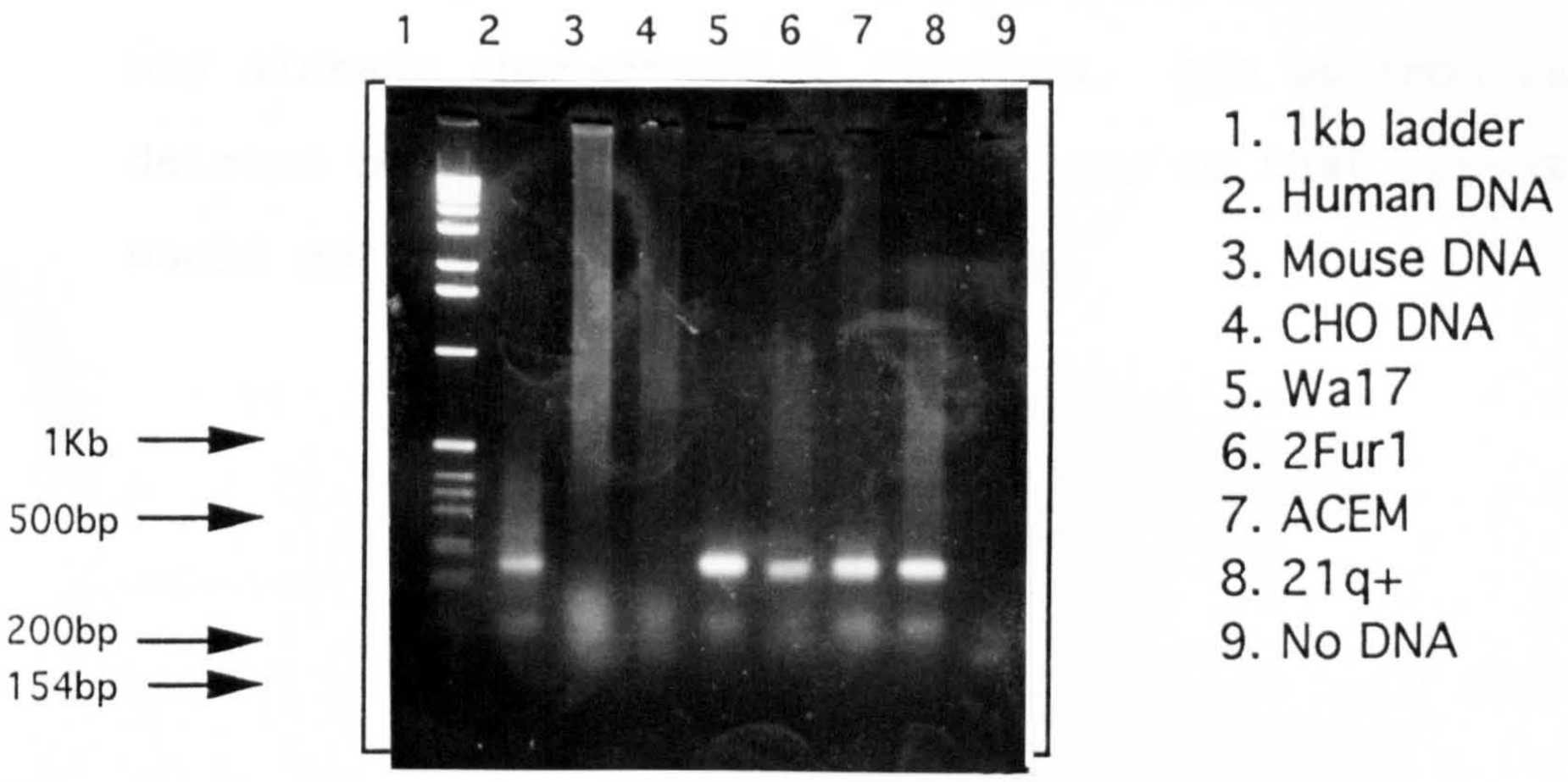


Figure 3.12

Amplification of the poly-A repeat on the chromosome 21 hybrid cell lines.

Figure 3.13

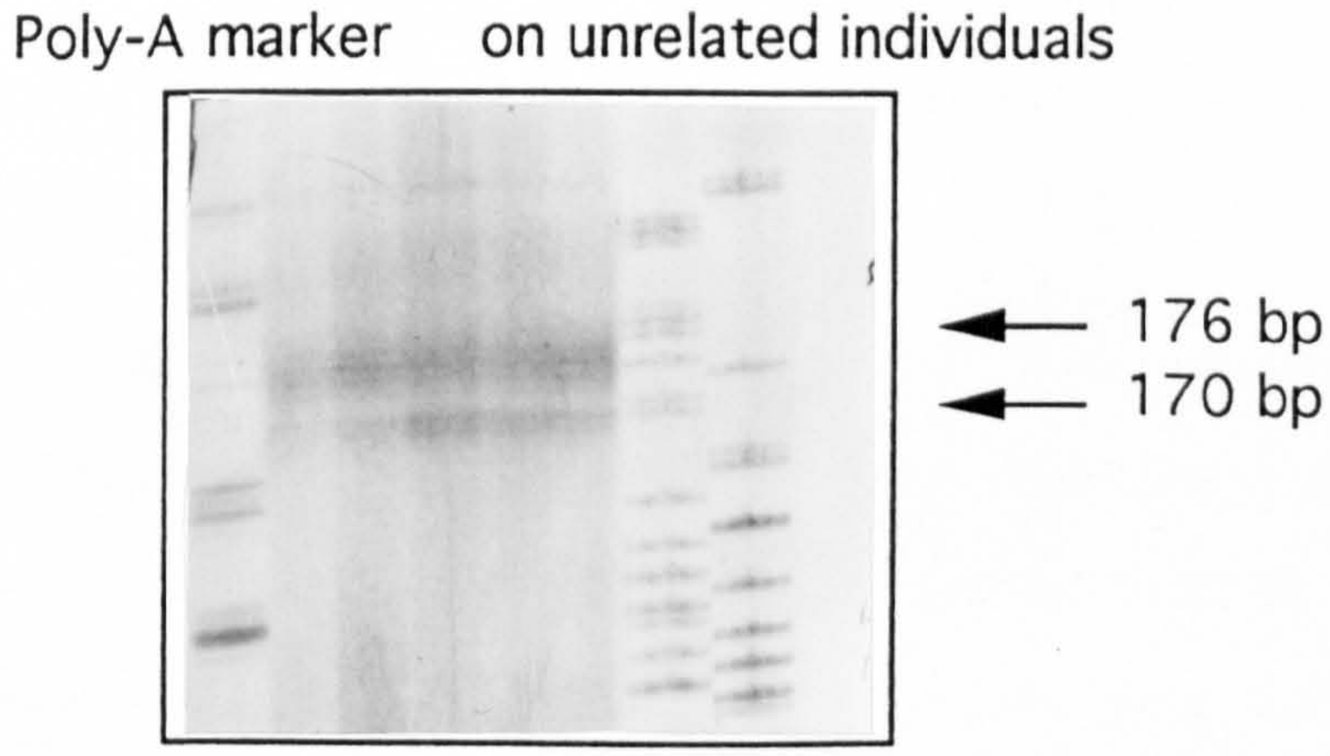


Figure 3.13

Poly-A marker showing lack of polymorphism on a 6% (v/v) polyacrylamide sequencing gel.

A Genbank search using BLAST (Altschul *et al*.1990) with the complete sequence of the clone did not reveal any homology with any already characterised sequence. 100 bp from each end were deleted before the search took place so that repeat sequences would not intervene in the search.

3.4 Discussion

It is clear from this study, that IRS-PCR can be successfully performed on chromosome 21 and result in the generation of distinct DNA fragments. The relative size range in Alu-PCR is expected, since Alu repeats closer than 500 bp are preferentially oriented in the same direction (Moyzis *et al*.1989). Thus the use of a single Alu primer tends to favour the amplification of larger inter Alu regions. A possible explanation of this may be that an A-rich bias extending for a short range on a single strand may for some unknown reason, influence the orientation of Alu insertions, or a postinsertion selection against that orientation takes place (Moyzis *et al*.1989; Cotter *et al*.1990). The fact that a smaller number of fragments were generated with the L1 primer is due to the much lower frequency of the L1 element in the genome. It is evident that in the combination Alu-L1 experiments the ratio of each primer indeed affected the size range of the products.

In subsequent mapping experiments single Alu fragments mapped to more than one location in the hybrids, suggesting that "one" band, may consist of two different Alu products, or that this fragment has regions of homology with multiple loci on the chromosome. Similar findings have been described previously (Benham and Rowe, 1992). A detailed characterisation of the map position of individual amplification products may provide additional insight into the relative distribution of the repeat elements.

More than half of Alu mediated PCR clones are thought to be single copy sequences, (Brooks-Wilson *et al*.1990) but it is

possible that the unique versus repetitive composition of inter-Alu fragments may vary from one region of the genome to another. This may stand as a possible explanation to our inability to identify a large number of $(CA)^n$ fragments on chromosome 21.

The use of Alu, L1 and Alu-L1 PCR was considered a revolutionary fast technique for the generation of chromosome specific markers, representative of all areas of the chromosomes, due to the inverse distribution of human repeats (Moyzis *et al*.1989; Sainz *et al*.1992).Centromeric heterochromatin regions are underepresented in Alu, in contrast with L1 repeats which occur relatively more frequently than Alu in the centromere. Thus, in combining the two, an overall representation can be expected.

However, cloning of fragments generated by IRS PCR has proved exceptionally difficult and consequently the generation of genetic markers, almost a fluke. In that respect this technique, although promising, cannot be considered as one of the best techniques for the rapid generation of markers. The need for further centromeric chromosome 21 genetic markers still exists in Alzheimer's disease as there appears to be pedigrees which are linked to chromosome 21 (Heston *et al*.1991), with no identified mutations in the APP gene (Tanzi *et al*.1992). Although this technique provides evident advantages over traditional methods, the practical drawbacks are such, that make other approaches more attractive.

CHAPTER 4

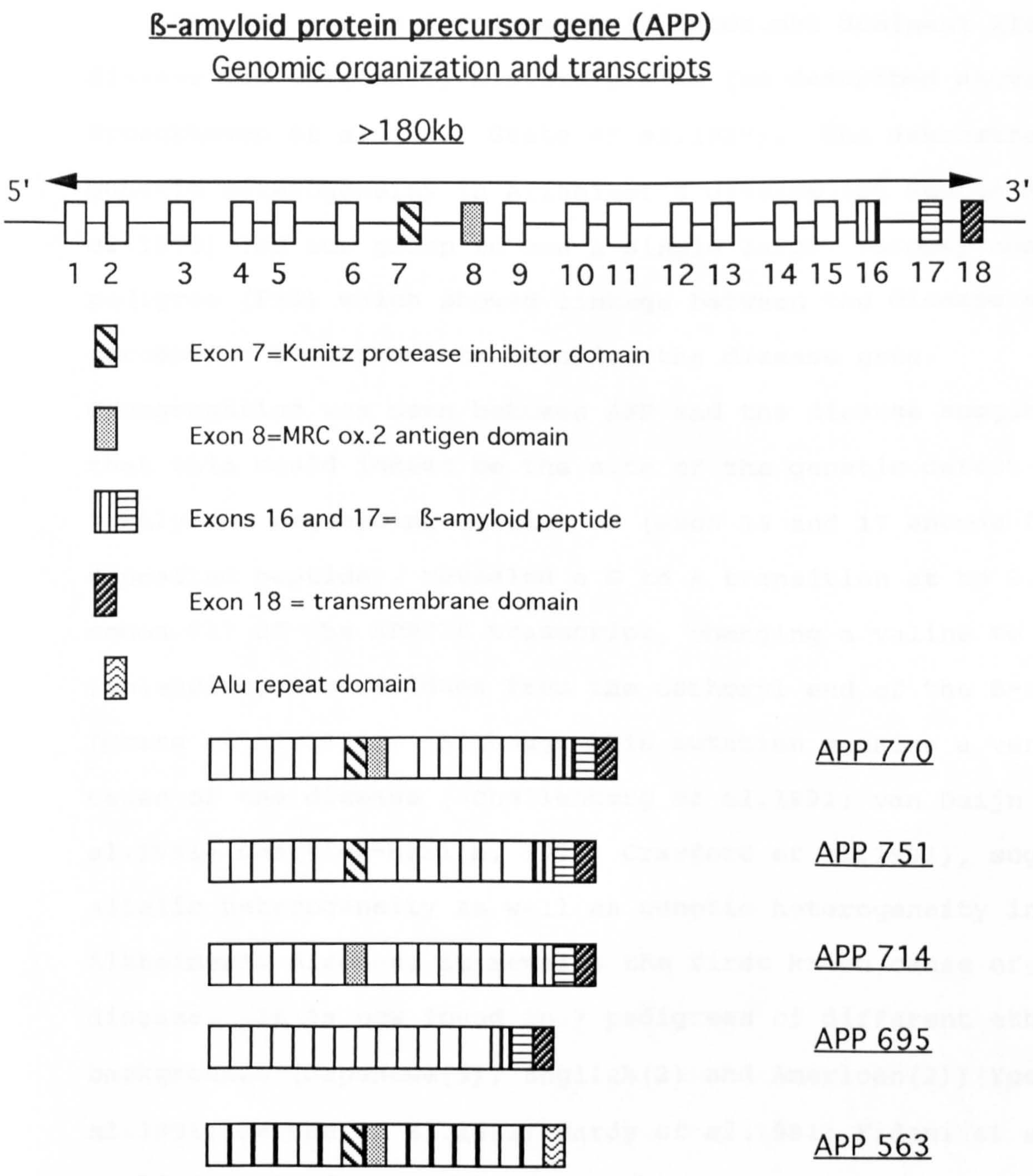
SCREENING FOR MUTATIONS IN THE OPEN READING FRAME OF THE β-AMYLOID PRECURSOR PROTEIN GENE IN FAMILIAL ALZHEIMER'S DISEASE

4.1 Introduction

4.1.1 APP gene : Genomic organisation

The amyloid precursor protein gene is greater than 180kb, contains 18 exons (Yoshikai *et al*.1990) and is found in chromosome 21, 21q21. Exons 16 and 17 encode for the ß-A4 protein. Exon 7 encodes for the Kunitz protease inhibitor domain, exon 8 for the Ox antigen and exon 18 encodes for the cytoplasmic domain. Five different transcripts have been identified which can be produced from the APP gene by differential splicing (figure 4.1). Two of these show homology to serine protease inhibitors (Ponte *et al*.1988; Tanzi *et al*.1988). The APP714 transcript (Golde *et al*.1990; Kang AND Muller Hill, 1990) has 57nt insert (exon8) but lacks the protease inhibitor domain and the APP563 transcript (Neve *et al*.1990; de Sauvage and Octave, 1989), lacks the ß-A4 domain and contains an Alu repeat domain, in the carboxyl-terminal. The protease inhibitor domain is not found in the major transcript (APP695) found in neurons. However, those transcripts containing the protease inhibitor domain have been shown to be increased in the brain tissue from Alzheimer's disease patients (Tanaka *et al*.1989), suggesting that levels of those transcripts may be important in the pathogenesis of the disease.

Figure 4.1


ß-amyloid protein precursor gene (APP)
Genomic organization and transcripts
>180kb
5'
3'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Exon 7=Kunitz protease inhibitor domain
Exon 8=MRC ox.2 antigen domain
Exons 16 and 17= ß-amyloid peptide
Exon 18 = transmembrane domain
Alu repeat domain
APP 770
APP 751
APP 714
APP 695
APP 563

4.1.2 APP gene and Alzheimer's disease

The APP gene's involvement in autosomal dominant Alzheimer's disease was originally misinterpreted (as described above) (Van Broeckhoven *et al*.1987; Goate *et al*.1989). The demonstration of genetic heterogeneity in Alzheimer's disease (St George Hyslop *et al*.1990) led our group to use a single large, autopsy confirmed pedigree (F23) which showed linkage between the disease and chromosome 21 markers to localise the disease gene. Cosegregation was seen between APP and the disease suggesting that this could indeed be the site of the genetic defect in this family and sequencing of exon 17 (exon 16 and 17 encode for the deposited peptide), revealed a G to A transition at bp 2,149 at codon 717 of the APP770 transcript, changing a valine to an isoleucine, two residues from the carboxyl end of the ß-amyloid (Goate *et al*.1991). Although this mutation remains a very rare cause of the disease (Schellenberg *et al*.1991; van Duijn *et al*.1991; Chartier-Harlin, 1991; Crawford *et al*.1991), suggesting allelic heterogeneity as well as genetic heterogeneity in Alzheimer's disease, it remains the first known cause of the disease. It is now found in 7 pedigrees of different ethnic backgrounds (Japanese(3), English(2) and American(2))(Yoshioka *et al*.1991; Naruse *et al*.1991; Hardy *et al*.1991; Fidani *et al*.1992; Karlinsky *et al*.1992).

4.1.3 Screening methods

Following the identification of the APP mutations we embarked on an attempt to develop a single protocol that would

detect the already identified mutations and would identify new ones in the open reading frame of the gene.

Techniques that allow the detection of single base changes in genomic DNA have had a major impact on our understanding of human genetic disease, both by identifying specific mutations that result in disease, or by identifying polymorphisms that are used in linkage studies as genetic markers. It was thus made possible to create pre- or post natal diagnostic tests for several human genetic disorders (Myers *et al*.1988). Some of these techniques are:

1) Restriction enzyme digests, which can be used in the cases where a specific mutation creates or abolishes a restriction site in genomic DNA. Not all base changes though alter a restriction site. Detects only known mutations.

2) RNase cleavage, which is based on the cleavage of mismatches in RNA:DNA duplexes with RNase A. Incomplete cleavage can make results difficult to interpret and preliminary steps include cloning of probe in RNA-polymerase vector, which may prove considerably time consuming. Detects all mutations.

3) Denaturing gradient gel electrophoresis (DGGE), which involves the electrophoresis of double stranded DNA fragments through a standard acrylamide gel, containing a linear gradient of DNA denaturants such as formamide, urea or temperature. This method although known to detect up to 99% of mutations needs a

considerable set-up investment and is quite expensive. An extension of this method using a GC clamp at each 5' end of the primers is known to increase its efficiency but also its cost (Abrams *et al*.1990). Detects most mutations.

4) Allele specific oligohybridisation (ASO), which involves Southern blots of PCR products which are hybridised subsequently with a mutation specific oligonucleotide (Bakker *et al*.1991). This technique presents all the drawbacks associated with Southern hybridisation. Detects only known mutations.

Taking into account all the above disadvantages of the techniques mentioned, I decided to use the single strand conformation polymorphism analysis method (Orita *et al*.1989), in order to screen the exons of APP gene for putatively new mutations as well as a tool for the detection of the already characterised ones in exon 17.

4.1.4 The use of PCR-SSCP in the detection and identification of mutations in APP gene

SSCA is based on the tendency of single stranded DNA molecules to adopt a sequence dependent secondary structure. These structures can be separated as they migrate through a nondenaturing polyacrylamide gel. Thus SSCA can be used as a sensitive indicator of sequence variation at the level of a single base pair, and provides a fast protocol to identify new mutations and also to screen familial and sporadic cases for already characterised mutations. Data suggest that over 80% of

mutations can be detected by SSCA under a single condition (Orita *et al*.1989; Orita *et al*.1990; Hayashi, 1991). SSCA has proved to be a successful method in identifying DNA variations in a number of diseases such as neurofibromatosis type 1 (Cawthon *et al*.1990), Marfan syndrome (Dietz *et al*.1991), Cystic fibrosis (Dean *et al*.1990), Phenylketonuria (Labrune *et al*.1991) and Haemophilia B (Fraser *et al*.1992).

The identification of mutations in the APP gene in families with early onset Alzheimer's disease has led to the hypothesis that mutations in this gene are pathogenic (Goate *et al*.1991; Chartier Harlin *et al*.1991; Murrell *et al*.1991). However, genetic data have suggested that the disease is heterogeneous (St George Hyslop *et al*.1990) and that the APP gene is not the only predisposing locus (Van Broeckhoven *et al*.1987; Tanzi *et al*.1987). It is often not possible to determine whether the APP locus is pathogenic in any particular family by linkage analysis because many pedigrees with early onset Alzheimer's disease are too small to obtain significant genetic information. Since evidence for linkage between Alzheimer's disease and the long arm of chromosome 21 is strongest in familial early onset Alzheimer's disease, we restricted our analysis to families with an onset of less than 65 years.

Sequencing of the region coding for the ß-amyloid peptide (exons 16 and 17 of APP (Yoshikai *et al*.1990)), had previously failed to reveal any alterations in a number of families with early onset Alzheimer's disease (Chartier Harlin *et al*.1991; Crawford *et al*.1991). Therefore, we decided to test the

hypothesis that other exons of the APP gene contain pathogenic mutations by screening these areas of the gene in families with early onset, familial Alzheimer's disease (see table 4.1) by SSCA (Orita *et al*.1989; Orita *et al*.1990) and to confirm that the exon 17 mutations we had previously reported are the only changes from the normal sequence in those families in which they occur.

4.2 Methods

4.2.1 DNA samples

DNA from an affected individual from each family multiply affected by early onset Alzheimer's disease, was used in the single strand conformation analysis (SSCA) (see Table 4.1). The age of onset was below 65 years in all families. A clinical diagnosis of Alzheimer's disease was confirmed by histopathology in 17 families. Drs. Bert Bakker, Christine Van Broeckhoven, Raymund Roos and Merrill Benson provided samples with APP693 and APP717Val->Phe mutations. Drs. David St.Clair and Lydia Hendriks provided samples with the APP713 and APP692 mutations. Samples from the FAD Alzheimer's disease families came from the Coriell Cell Repository. The Spanish Alzheimer's patients and controls were provided by Dr. Rafael Oliva.

4.2.2 PCR

Specific primers for PCR amplification of the 18 exons of the APP gene were designed (Yoshikai *et al*.1990). Intronic primers were chosen to give product sizes of 100-400bp whenever possible, since SSCA is believed to be most effective at detecting sequence variability in this range. For the efficient design of most primers a computer programme has been used (Lowe *et al*.1990). Conditions for the PCR reactions were optimised as shown in Table 4.2. Attempts to optimise the PCR using formamide (Sarkar *et al*.1990) or 7-deaza-2'-deoxyguanosine (McConlogue *et al*.1988) did not prove to be useful.

PCR conditions were: 10min at 94C initial denaturation then 35 cycles of 45sec at the optimum annealing temperature (Table 4.2), 2min at 72C, 1.5min at 94C, and a final cycle of 10min at 72C. The reaction volume used was usually 25μl with a final concentration of primers of 0.2μM. Final concentrations of 200μM of all nucleotides were used apart from dATP which was used at a concentration of 24μM, 0.5μl of [α-^{35}S]dATP (Specific activity 600Ci/mmol). 1 unit of Taq polymerase was added. PCR of exons 1, 5, 10, 11 and 14 was performed in a total volume of 50μl and PCR of exons 6 and 8, was performed in a total volume of 100μl. Intronic sequences for primers 1, 5, 10, 11 and 14 were provided by Dr. Rudi Tanzi.

Annealing temperature used for Prion primer set 1 was 52C and for Prion primer set 2, 60C. For Prion primer set 1, DMSO was used at 5% (v/v) to inhibit nonspecific DNA synthesis. Both reactions were performed using $MgCl_2$ at 1.5mM.

4.2.3 SSCP

The resolving power of non-denaturing acrylamide gels is determined by the acrylamide concentration and the proportion of bisacrylamide added as crosslinker. I optimised the conditions to give maximal separations of the APP693 (the pathogenic mutation for Hereditary Cerebral Haemorrhage with Amyloidosis) (Levy *et al*.1990) and APP717Val->Ile and APP717Val->Gly mutations (figure 18). This optimisation was performed by varying the ratio of acrylamide to bisacrylamide at either 25C or 4C and

running gels both with and without 10% (v/v) glycerol. As a result of this optimisation procedure we adopted the following as our standard protocol for all 18 exons.

5µl of the amplified product was mixed with gel loading buffer containing 98% (v/v) formamide, 0.01% (w/v) xylene cyanol, 0.01% (w/v) bromophenol blue, 10mM EDTA, heated to denature at 80C for 10min and placed on ice. The mixture was loaded and separated on 5% (v/v) polyacrylamide gel (acrylamide/N',N'-methylene bisacrylamide 100:1) in 90mM Tris-borate, 2mM EDTA and 10% (v/v) glycerol (pH 8.0). Electrophoresis was carried out at 30W for 7-8 hours at 25C. The gel was dried and exposed to X-ray film for 2-5 days.

In addition to this, our standard protocol, most samples were also run using different ratios of acrylamide to bisacrylamide (19:1, 49:1) at 4C with or without glycerol (10%)(v/v) and at room temperature with and without glycerol (10%)(v/v). Different protocols (Spinardi *et al*.1991) were also tested, however these resulted in no evident improvement. The conditions described above gave the clearest separation and sharpest focussing of the different variants of exon 17.

4.2.4 Solid Phase Sequencing

This method was described previously (Section 2.21c). (Specific conditions for exon 17 sequencing are discussed in (Adroer *et al*.1992))

4.3 Results

In all cases, PCR amplification resulted in a single product of the predicted size (see Table 4.2). The identity of the bands was confirmed by PCR amplification of a yeast artificial chromosome containing the APP gene which gave the same size bands in all cases (Talbot, Rooke and Goate: unpublished results).

The known APP717 mutations were resolved using our protocol as was APP693, APP692 and APP713 (Levy *et al*.1990) (Figure 4.18) (data for APP693, is not shown). One sequence variation detected during this screening effort was in a family, F172, which had an SSCA mobility consistent with that of the APP717 Val->Ile mutation. Direct sequencing (Crawford *et al*.1991) of exon 17 in this family confirmed the presence of this mutation.

No additional sequence variation in the open reading frame of APP was observed in samples from those families (F23 and F19) in which we have previously found mutations in exon 17.

A further variation of exon 17 was detected in an affected individual with early onset Alzheimer's disease from Spain (figure 4.22). This variation has proved to be, after sequencing a 711 silent mutation involving a C to A transition. The mutation was not found in any normal Spanish individual tested (200 chromosomes) and further details for the family of the affected individual remain to be collected before we can argue on its importance in the development of the disease. It is not

found though, in any of the children of the affected individual nor in one of his brothers. A silent variant APP708, already described in Swedish population (Balbin *et al*.1992) was identified in a Spanish normal control sample (figure 4.22).

No other variation was found in the samples tested in any of the exons of APP gene. (Figures 4.2-4.19)

Since we optimised our SSCA conditions using exon 17 wmutations, we cannot use our data from this exon to gain a realistic estimate of its sensitivity. However, we tested the same protocol on PCR products derived from characterised variants (single base changes) of the prion gene (figures 4.20-4.21).

The open reading frame of PRP gene is contained in a single exon. A point mutation at PrP codon 102 was found to be linked to GSS (Hsiao *et al*.1989) some Libyan and Tunisian Jews have a PrP point mutation at codon 200 (Goldfarb *et al*.1990) and other point mutations at codons 117, 178, and 198 also segregate with inherited Prion diseases (Prusiner, 1991). A polymorphism (non-pathogenic) has been identified at codon 129 resulting in the substitution of valine for methionine (Owen *et al*.1990).

Our protocol detected 9 out of 10 Prion point variants tested (figures 4.20-4.21).

To determine whether sporadic cases are also caused by those mutations, 100 sporadic cases of Alzheimer's disease were screened in which there were no variations identified. Screening of another 40 early onset familial cases for exon 16 and 17 also failed to reveal any alterations (table of screening summary).

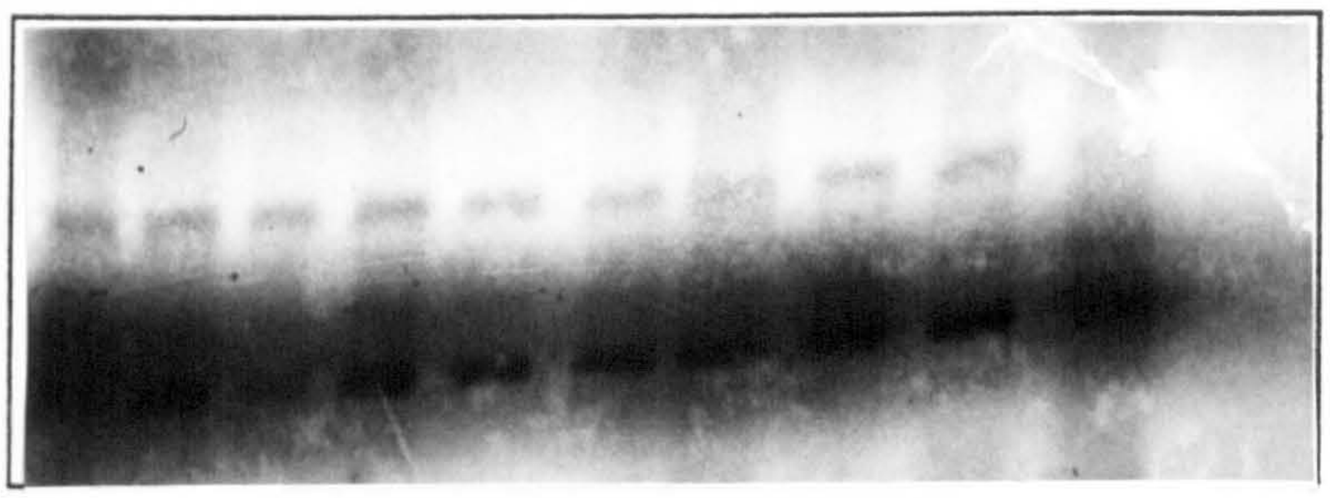
Figures 4.2-4.21

The different migration of the DNA strands is shown on 5% (v/v) SSCP gels for the 18 exons of APP gene and the coding region of the Prion protein gene in different affected individuals.

Figure 4.2

Exon 1

1 2 3 4 5 6 7 8 9 10



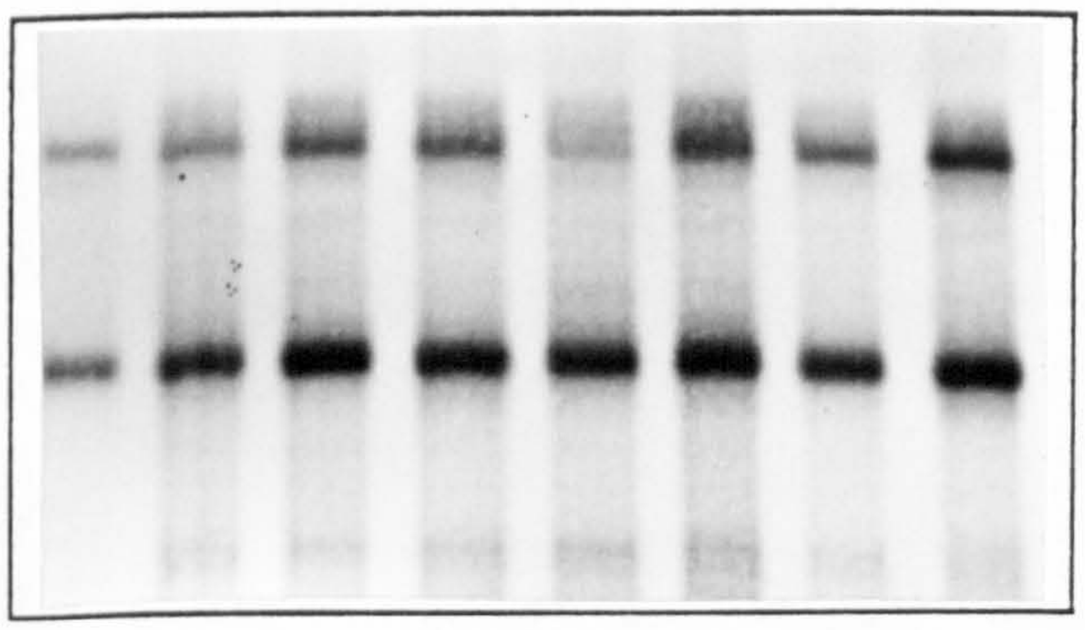
Individuals

1.23 2.9
2.53 3.2
3.148 2.7
4.134 3.5
5.105 2.12
6.121 3.8
7.Unaffected
8.126 3.4
9.168 3.1
10.84 2.6

Figure 4.3

Exon 2

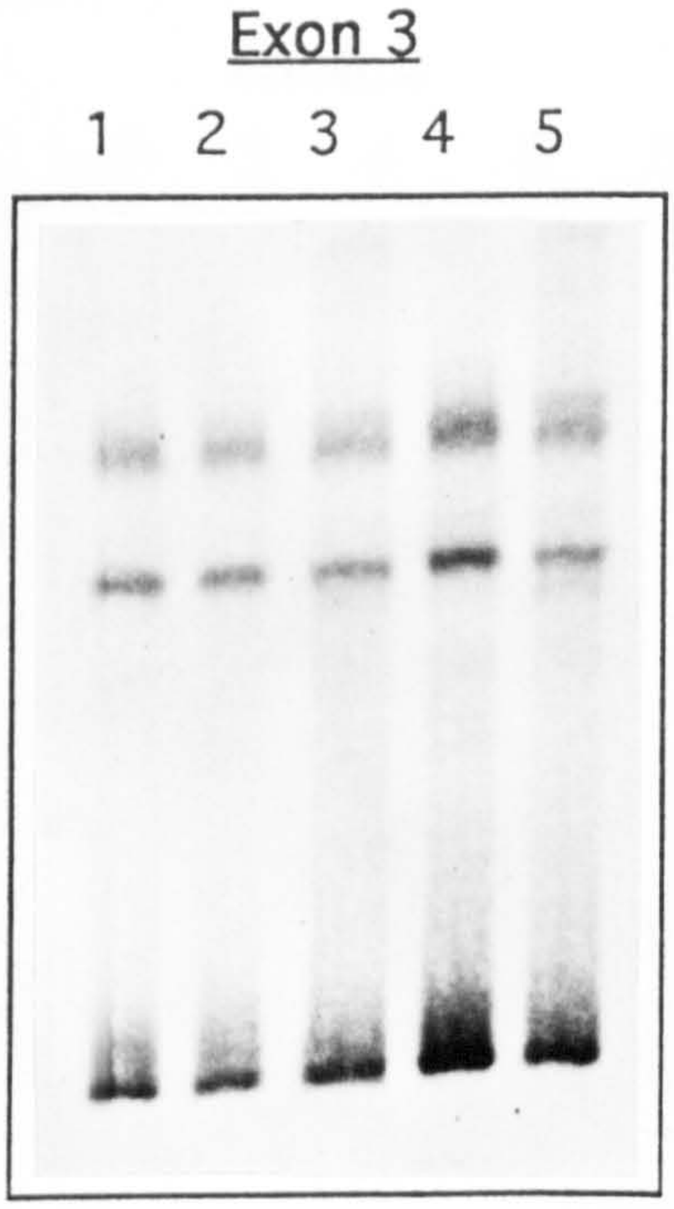
1 2 3 4 5 6 7 8



Individuals

1.148 2.7
2.19 3.17
3.23 2.9
4.84 2.6
5.74 2.4
6.Unaffected
7.53 3.2
8.168 3.1

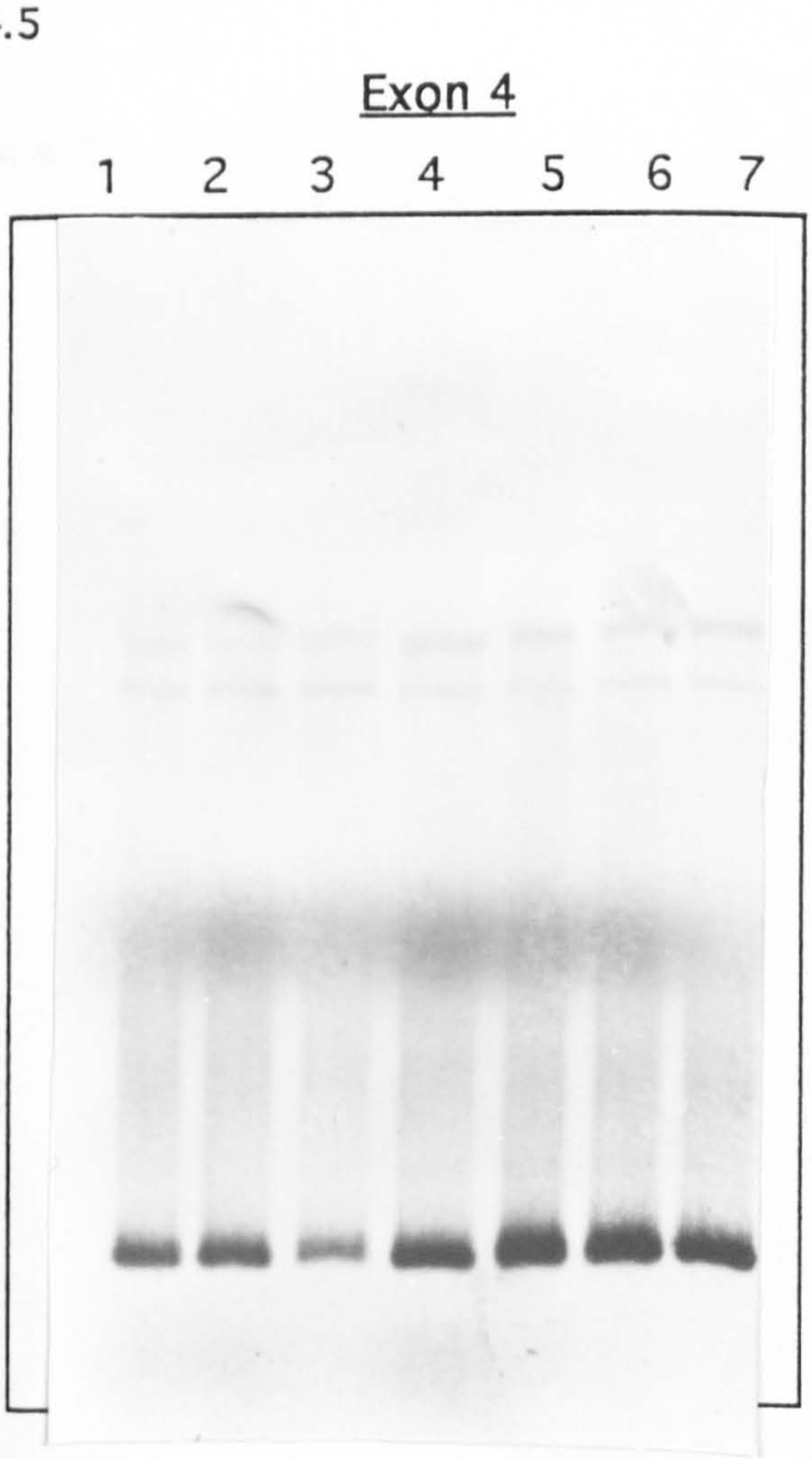
Figure 4.4



Individuals

1.74 2.4
2.105 2.12
3.121 3.8
4.134 3.5
5.148 2.7

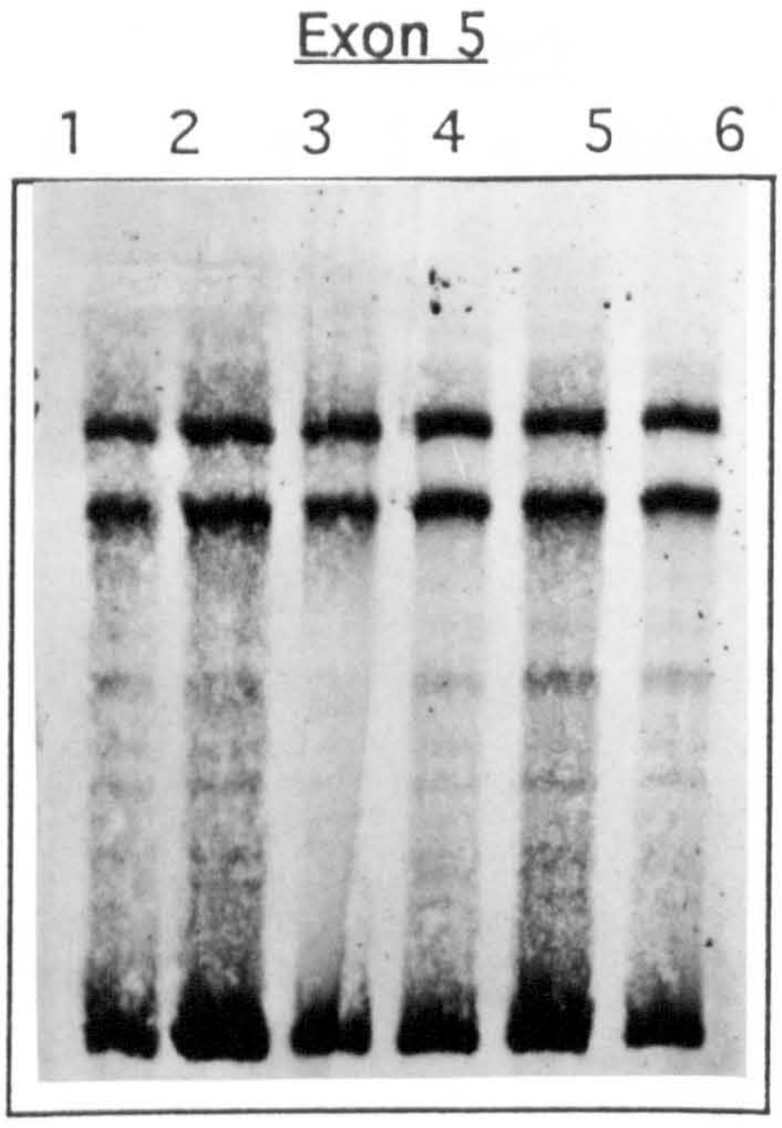
Figure 4.5



Individuals

1. 53 3.2
2. 74 2.4
3. 84 2.6
4. 105 2.12
5. 121 3.8
6. 126 3.4
7. 134 3.5

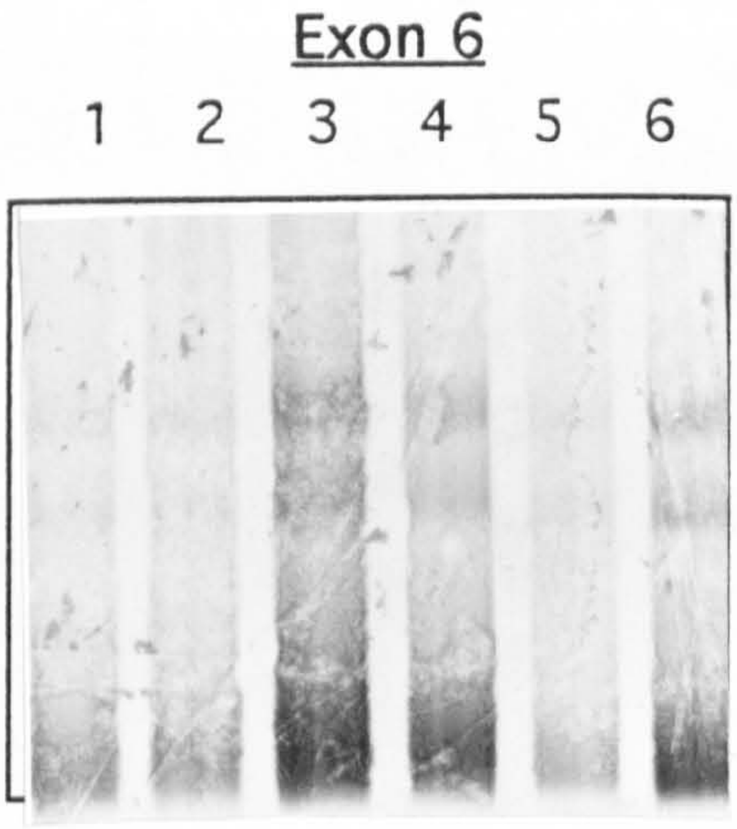
Figure 4.6



Individuals

1.23 2.9
2.53 3.2
3.148 2.7
4.134 3.5
5.121 3.8
6.105 2.12

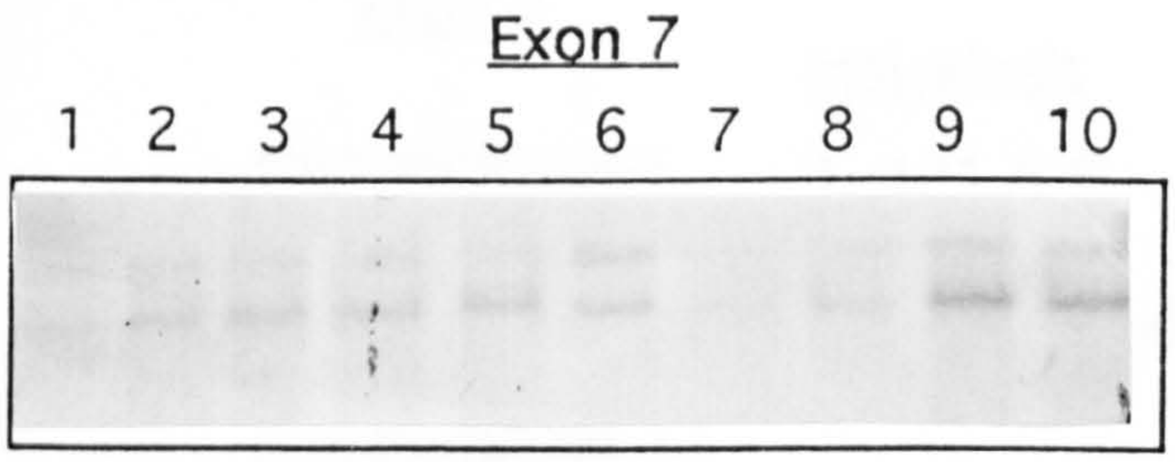
Figure 4.7



Individuals

1.105 2.12
2.121 3.8
3.126 3.4
4.168 3.1
5.134 3.5
6.Yac 17HC7

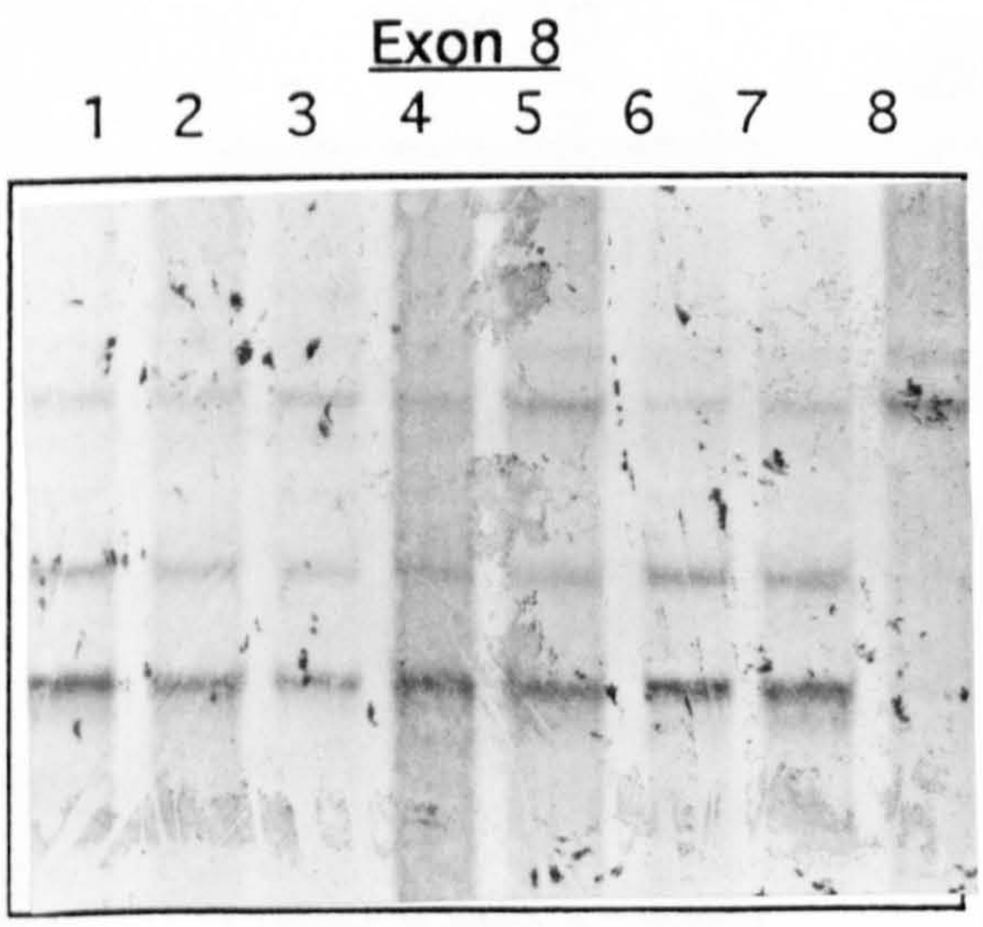
Figure 4.8



Individuals

1.148 2.7
2.97 2.4
3.19 3.17
4.23 2.9
5.75 3.1
6.Unaffected
7.84 2.6
8.15 1.3
9.168 3.1
10.Sp.32

Figure 4.9

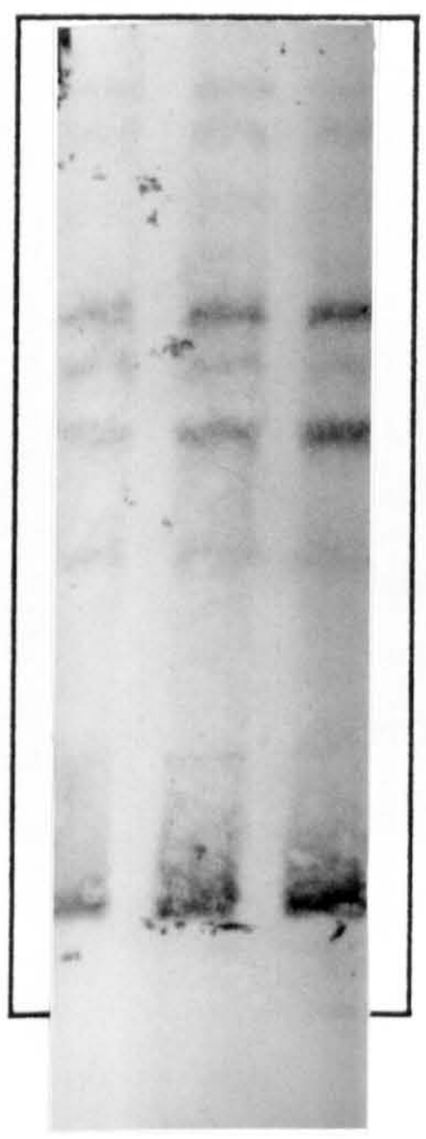


Individuals

1.105 2.12
2.121 3.8
3.74 2.4
4.126 3.4
5.168 3.1
6.134 3.5
7.148 2.7
8.Yac 17HC7

Figure 4.10

Exon 9

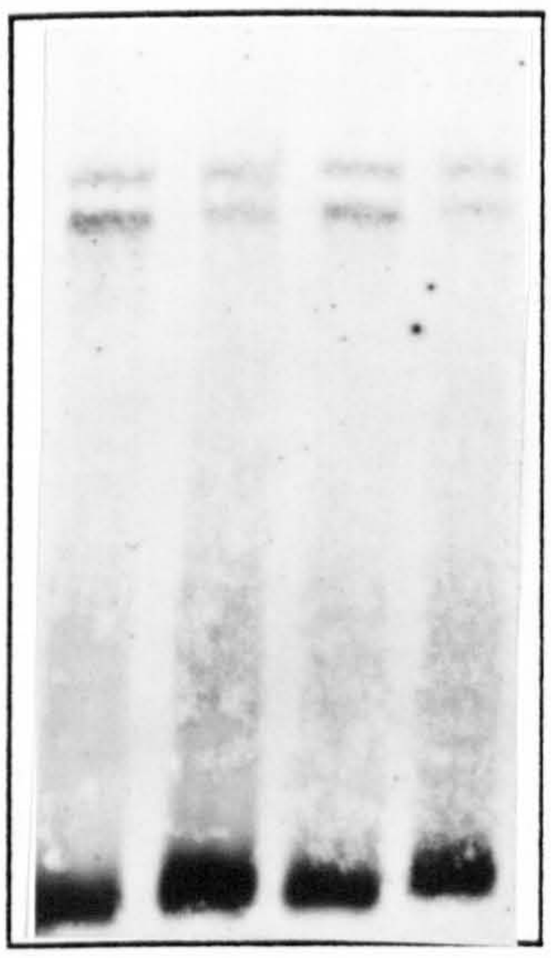


Individuals

1. 121 3.8
2. 97 2.4
3. Unaffected

Figure 4.11

Exon 10



Individuals

1.23 2.9
2.53 3.2
3.148 2.7
4.134 3.5

Figure 4.12

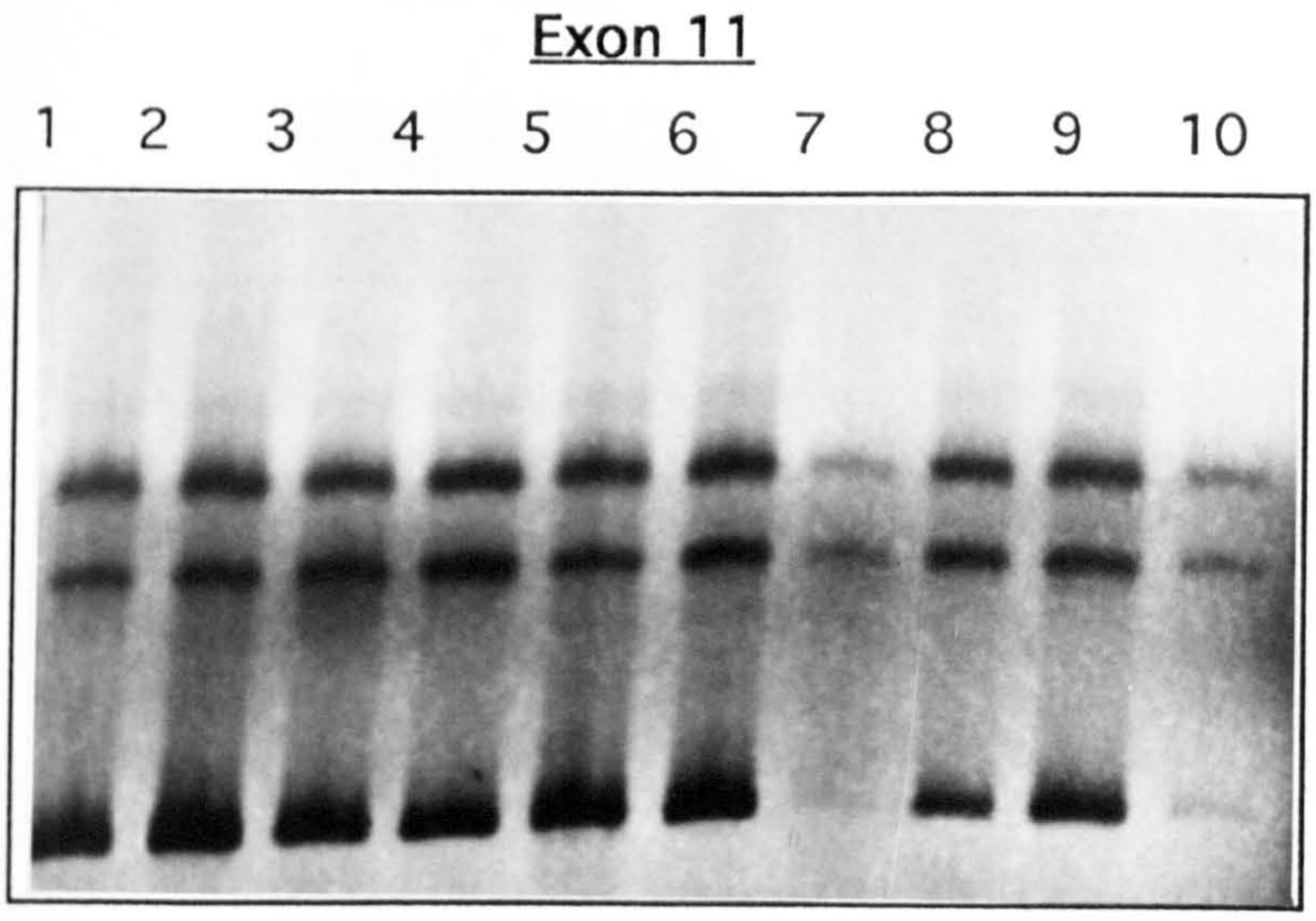


Figure 4.13

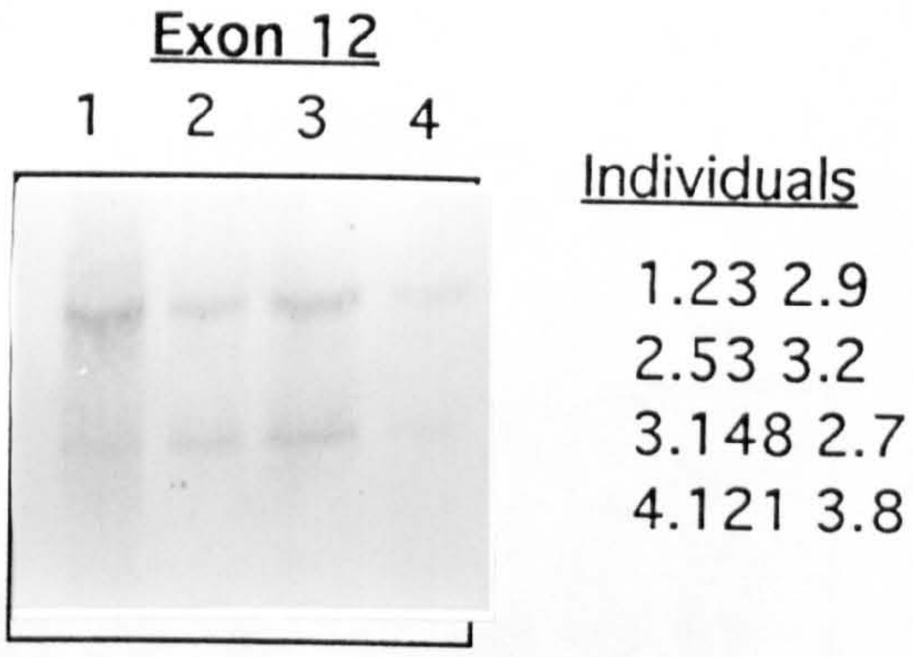


Figure 4.14

Individuals

1.84 2.6
2.134 3.5
3.Unaffected

Figure 4.15

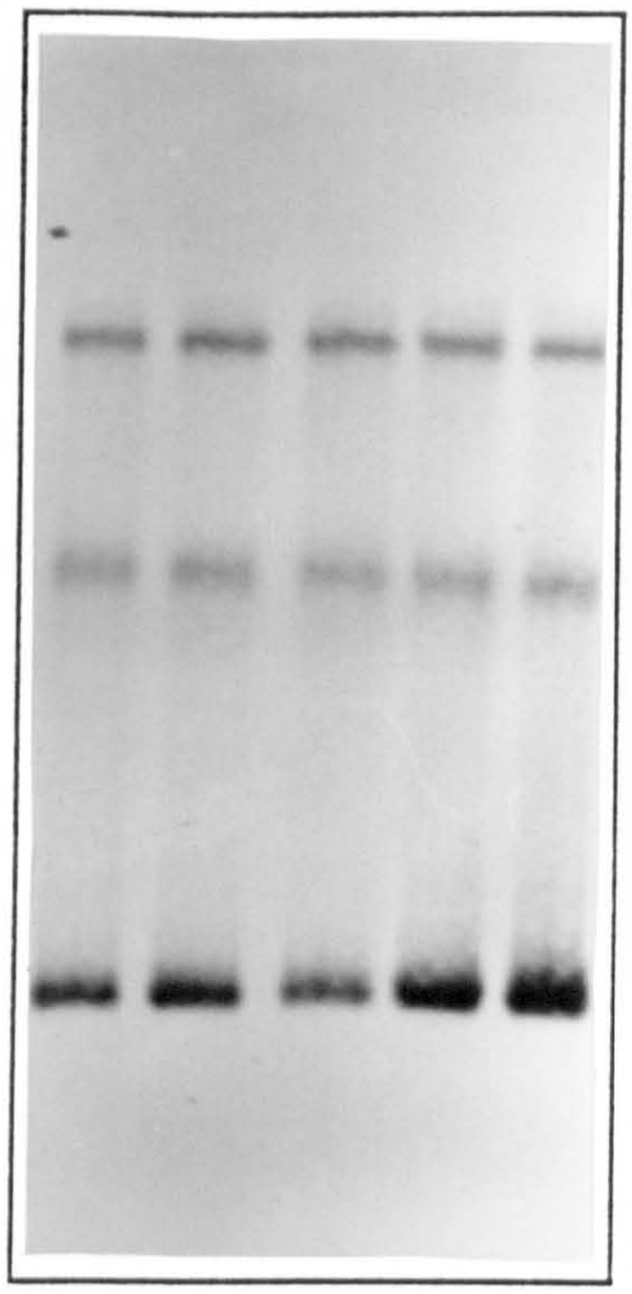
Individuals

1.23 2.9
2.53 3.2
3.148 2.7
4.134 3.5
5.121 3.8
6.105 2.12
7.84 2.6
8.126 3.4
9.168 3.1
10.Unaffected
11.19 3.17

Figure 4.16

Exon 15

1 2 3 4 5



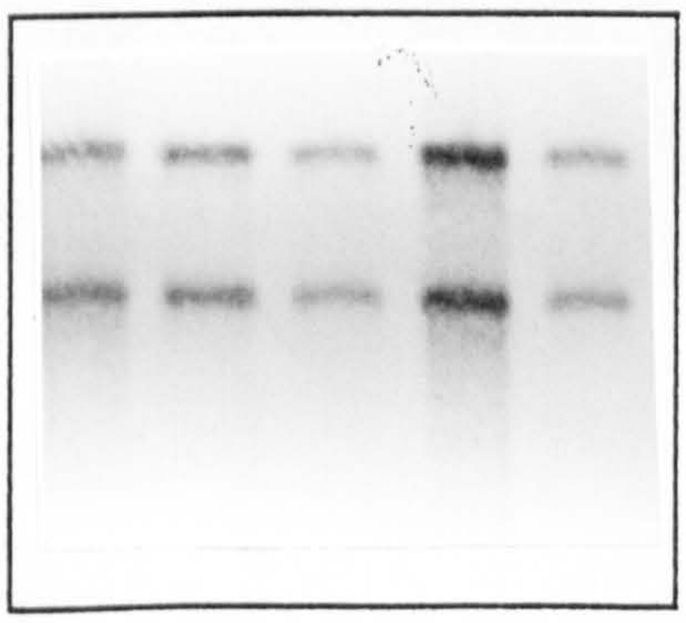
Individuals

1.53 3.2
2.74 2.4
3.84 2.6
4.105 2.12
5.121 3.8

Figure 4.17

Exon 16

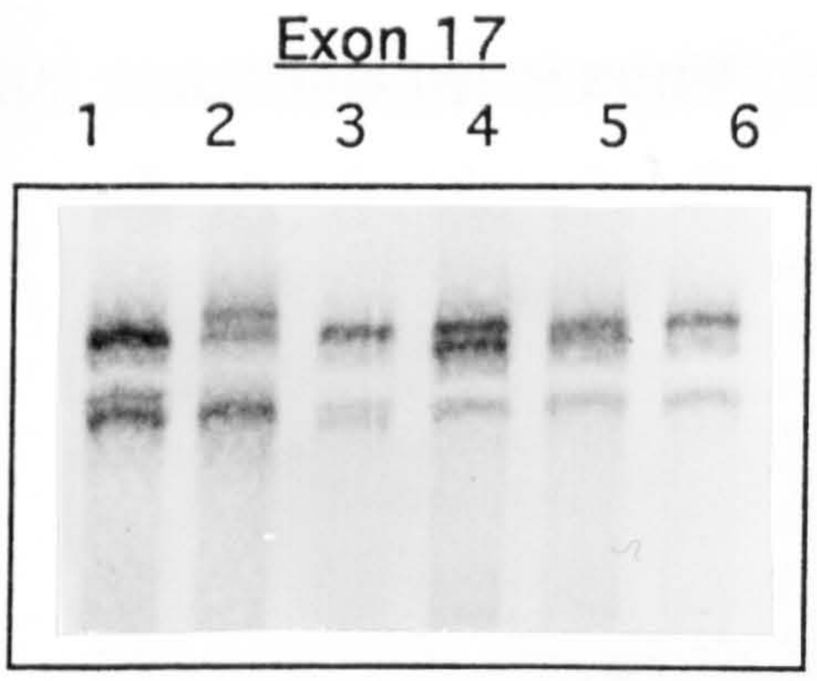
1 2 3 4 5



Individuals

1.23 2.9
2.53 3.2
3.148 2.7
4.121 3.8
5.Unaffected

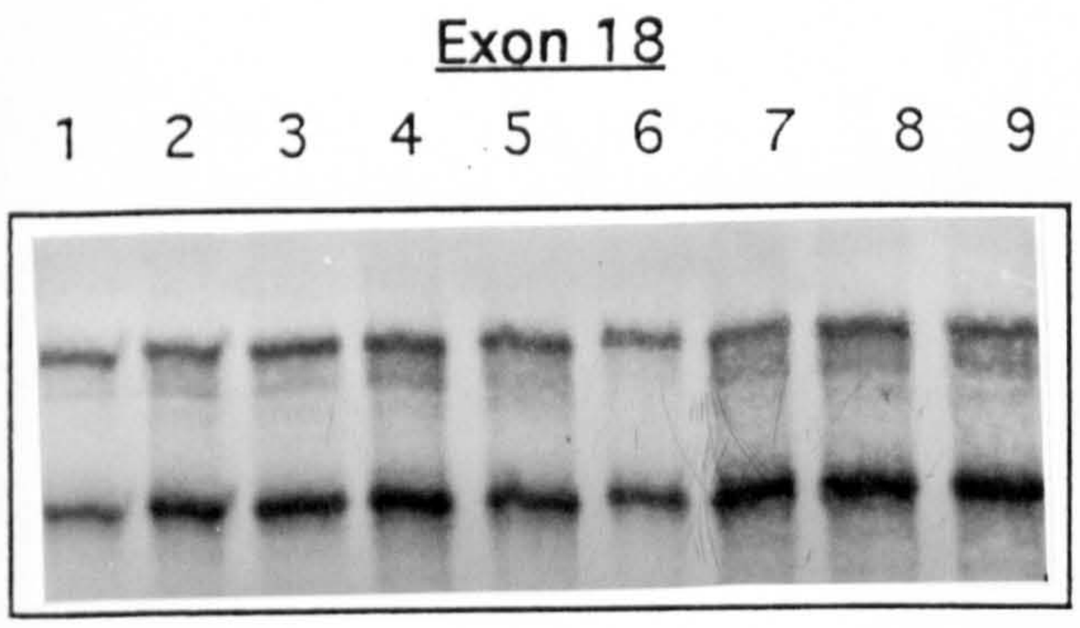
Figure 4.18



Individuals

1.APP713 Ala->Val (A->G)
2.APP692 Ala->Gly (C->G)
3.APP717 Val->Gly (T->G)
4.APP717 Val->Ile (G->A)
5.APP717 Val->Phe (G->T)
6.Unaffected

Figure 4.19

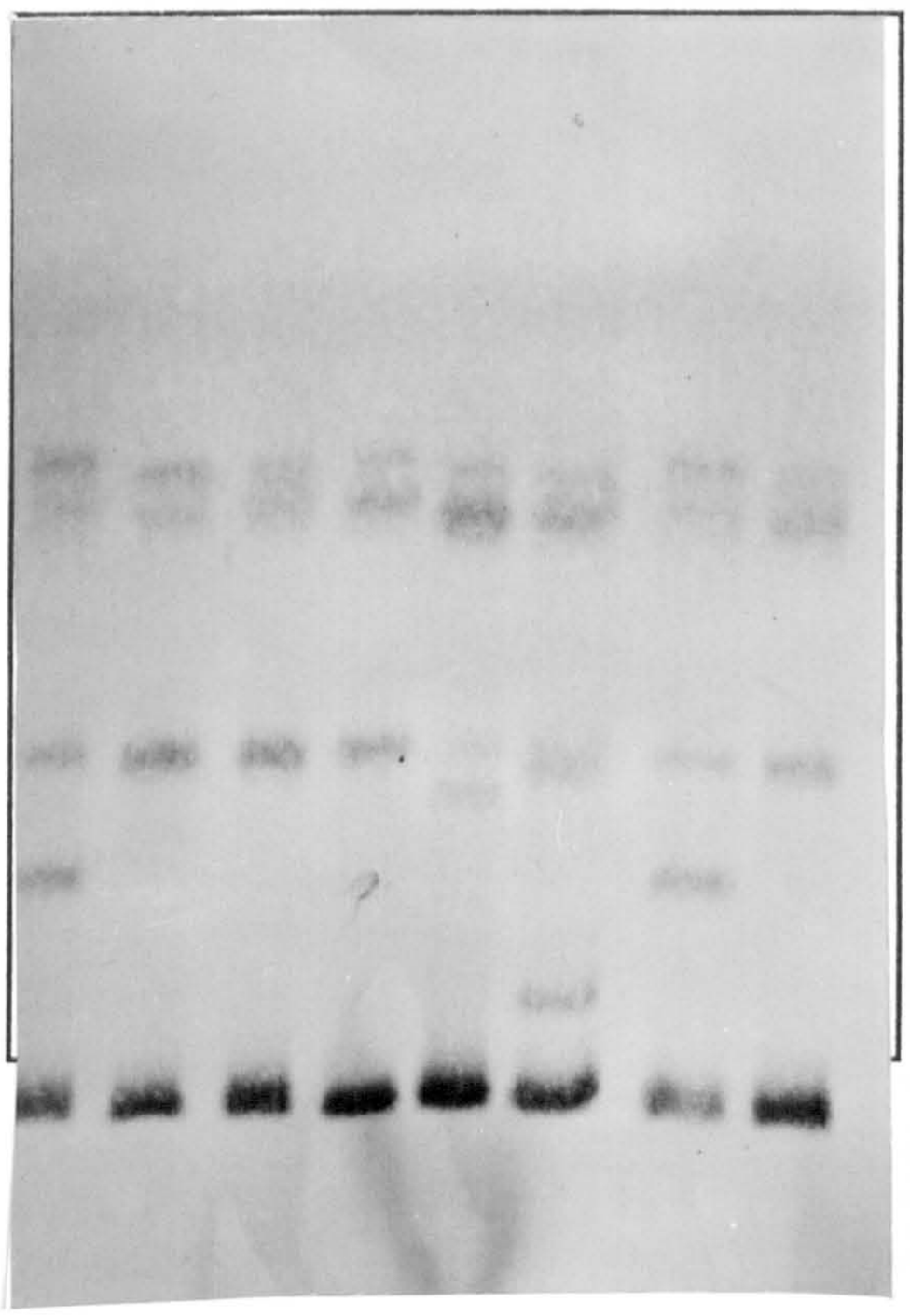


Individuals

1. 84 2.6
2. 97 2.4
3. 105 2.12
4. 126 3.4
5. 19 3.17
6. 168 3.1
7. Sp.53
8. Sp.32
9. Unaffected

Figure 4.20

Prion mutations using primer set No 1

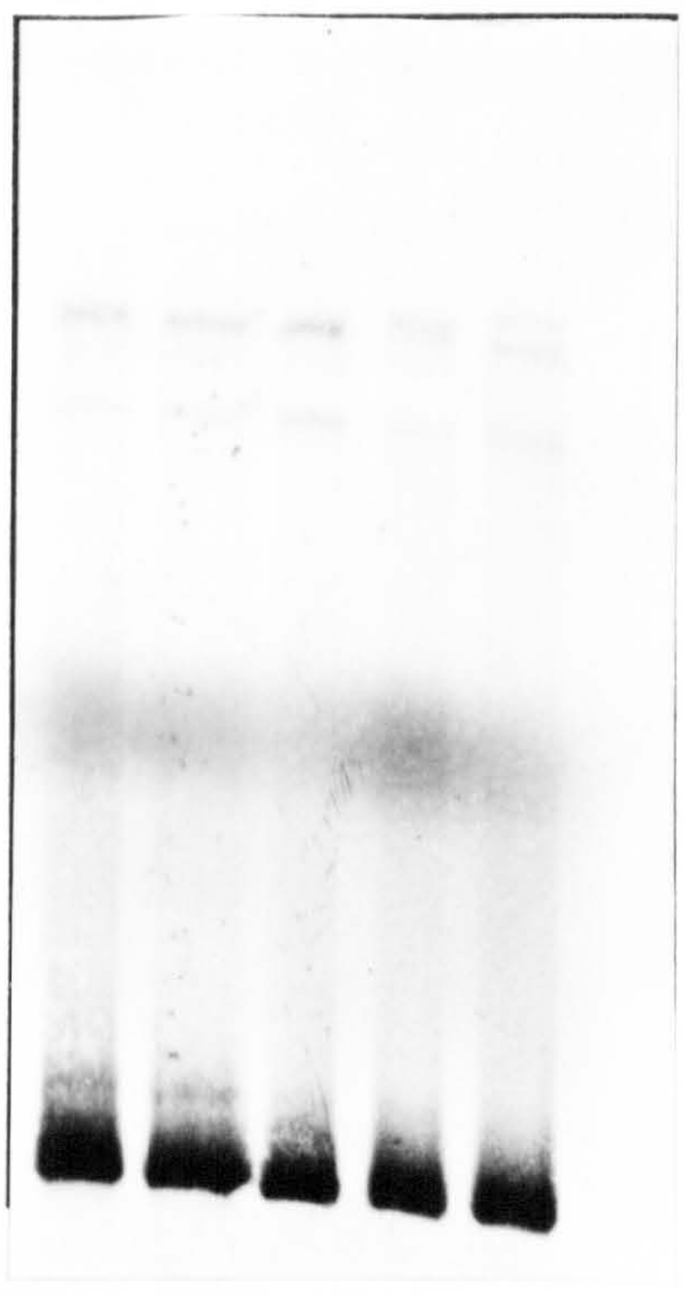


Genotypes

1. MM,A+/A+
2. MV,A+/A+
3. VV,A+/A+
4. MM,102 mutation
5. VV,117 mutation
6. VV,A+/A-
7. MV,A+/A-

Figure 4.21

Prion mutations using primer set No 2



Genotypes

1. Control
2. Control
3. Control
4. Codon 200 mutation
5. Codon178 mutation

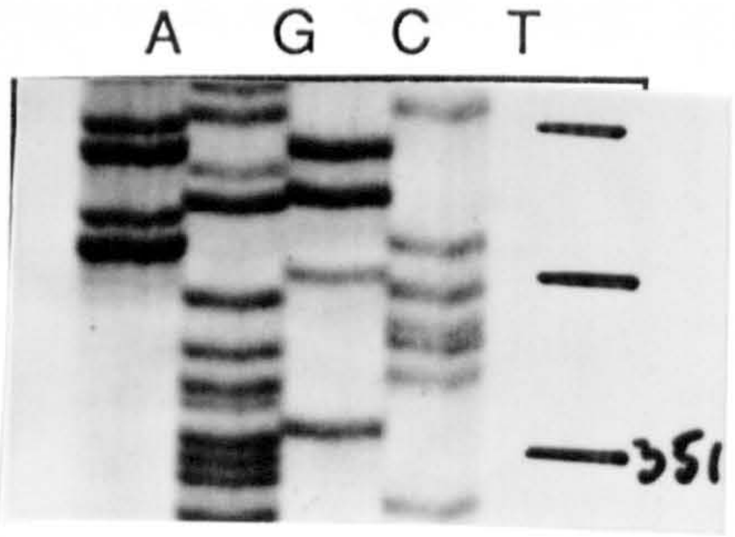
Figure 4.22

SSCP of exon 17 for APP708 and APP711 variants

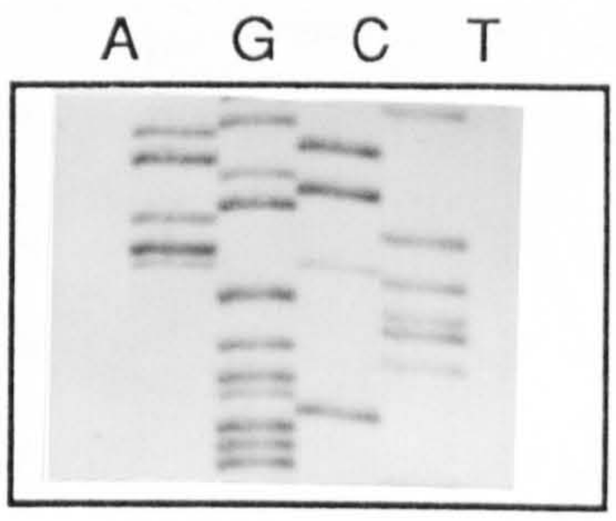
1. Normal conformation
2. APP 708
3. APP 711

Sequencing gel showing the C->A transition at codon 711

1.Normal



2.711



4.4 Discussion

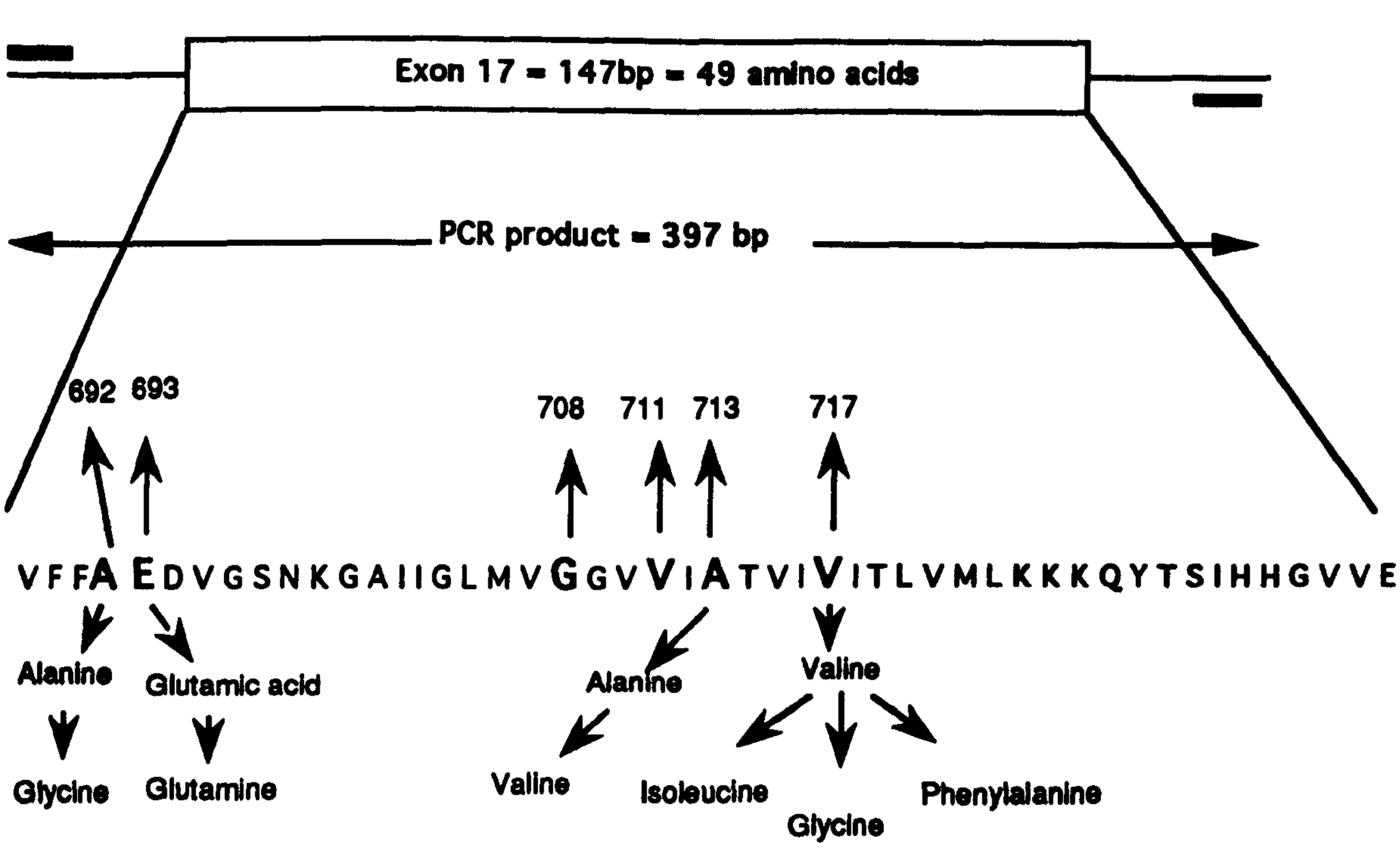
The screening system reported here is preferable to enzyme digestion because it is generally applicable. It is preferable to direct sequencing because of its ease and efficiency and yet the procedure has a high probability of detecting new mutations (Orita *et al*.1989; Orita *et al*.1990). However, the nature of mutations should be confirmed by direct sequencing.

The screening of the APP gene discussed here confirms the impression that exon 17 is the major site of APP mutations in Alzheimer's disease (figure 4.23), since the two changes we have found are: a new example of the original APP717Val->Ile mutation, and a silent mutation in codon 711. Genetic analysis of F172 indicates it is not related to any of the Caucasian families in which this mutation has been found (Houlden, Mullan and Hardy, unpublished observations). Thus, we have found APP mutations in 2 (F23 and F19) out of 11 families in which the whole gene has been screened, and in 2 (F172 and S52) out of (50) families in which we have only examined exon 17 and exon 16.

While these numbers are small, they are consistent with the notion that mutations in APP are a relatively rare cause of early onset familial Alzheimer's disease. It remains possible that there are mutations which have not been detected using the SSCA conditions employed herein. However, we regard this as unlikely since two reports have estimated that the technique detects 80%-90% of variation in a PCR product of 100-450bp by using a single protocol (Hayashi, 1991; Orita *et al*.1990). Our experience with

Figure 4.23

Location of mutations in exon 17 of the human amyloid protein precursor gene



screening known prion variants is consistent with this. In addition, most exons in most families have been screened by both the protocol described by Orita and colleagues and by our modifications of this protocol. The GC content of all exons of APP analysed and that of the two PrP products was calculated. The GC content of all PCR products except those of exon 1 (74%) are comparable with our control samples. PrP 1 and 2 have GC contents of 55 % and 50% respectively whilst exons 16 and 17 have a 37% GC content. The remaining exons have GC contents between 44.5-54.8%. The efficiency of detection of mutations in exons 2-18 should therefore be comparable with our control samples. Under these circumstances, we suggest that 80% must be a minimum estimate for the sensitivity of the procedure we have employed. The efficiency of detection of mutations in the exon 1 PCR products is unknown due to the high GC content and may be quite different.

However, because of this uncertainty and because of the possibility that there may be mutations in controlling elements of the gene which we have not screened, accurate estimation of the proportion of cases of early onset, familial Alzheimer's disease caused by mutations at the APP locus will probably not be possible until the other pathogenic loci are identified.

More clinical information and a detailed genetic analysis will determine the nature and the importance in pathogenicity of the silent 711 mutation identified. The position of this mutation is of interest because it is within the putative "stem

loop structure" proposed as of pathogenic importance in APP mutations (Tanzi and Hyman, 1991). However it does not alter the bonding because it changes one mismatched base for another. Moreover the fact that this individual does not have a convincing family history of the disorder, since only the mother of this man had shown evidence for cognitive decline towards the end of her 82nd year, and no other members of the family had had any evidence for dementia, suggests that the variant is unrelated to the disease pathogenesis. It remains possible that the mutation might have arisen de novo in the proband and be pathogenic for him and for any of his offspring had they inherited the variant.

The data on APP708 further supports the notion that it represents a rare polymorphism with no relevance to the disease pathology.

Despite the conservative nature of the APP717 Val->Ile mutation, evidence is accruing in support of its pathogenicity. Seven families with early onset Alzheimer's disease have now been identified carrying this mutation (Goate *et al*.1991; Naruse *et al*.1991; Yoshioka *et al*.1991; Hardy *et al*.1991) (this study) (Karlinsky *et al*.1992). All exons of the APP gene have now been screened for other mutations in two families with APP717 mutations and no other changes in other exons have been found. For these reasons, and because of the identification of two other mutations at this amino acid (Chartier Harlin *et al*.1991; Murrell *et al*.1991), we are confident that these mutations are pathogenic.

Full details of the clinical phenotype of F172 are not yet

available. However, our preliminary information suggests that the onset age is in the mid 50s, like the other families which have been reported with this mutation. F19 (APP717Val->Gly) has a mean onset in the late 50s and the Indiana family (APP717 Val->Phe) has a mean onset in the mid 40s. While more families with each mutation will have to be identified before firm conclusions can be drawn, it seems as if age of onset is in part determined by the nature of the pathogenic mutation. However, clearly there is considerable overlap in the onset age, both between families with different characterised mutations and families in which the APP gene is not the site of the pathogenic change.

Following our screening of 45 early onset genetic cases and 100 sporadic cases, as well as the screening of other groups (Schellenberg *et al*.1991; Tanzi *et al*.1992), it is clear that those mutations represent a very small proportion of Alzheimer's disease individuals. Their importance stands on the fact that they are the first identified cause of the disease, allowing us for the first time to make realistic speculations about the initiation and the progression of the disease.

TABLE 4.1

Clinical Samples Used

a) Whole gene screened

Family No	Age of onset	Affecteds (path. confirmed)	Mutations
F53	43-60	5 (1)	None
F74	39-50	7 (2)	None
F84	59-64	3	None
F105	36-39	14	None
F121	35-39	6	None
F126	48-63	4	None
F134	41-50	6 (2)	None
F148	42-46	5 (1)	None
F168	40-54	6 (1)	None
F19	48-58	10 (1)	APP717 Val->Gly
F23	53-61	11 (1)	APP717 Val->Ile

b) Mutation Controls: exon 17 tested only.

INDIANA	41-45	5		APP717 Val->Phe
HCHWA-D	40-60	Many		APP693 Gln->Glu
HCHWA-D/Alzheimer's disease		Many		APP692 Ala->Gly
Schizophrenia		Many		APP713 Ala->Val

c) Samples in which exon 16 and exon 17 were tested.

F172	44-64	10		APP717 Val->Ile
F161	50-55	3	(1)	None
F165	53-60	2		None
F175	36-42	3	(1)	None
F184	50-55	5		None
F196	32-38	5		None
F206	37-42	7		None
FAD1	Mean 52	Many	(Many)	None
FAD2	Mean 48	Many	(Many)	None
FAD4	Mean 40	Many	(Many)	None
F112	60	2		None
F96	50	1		None

F128	38	2(1)	None
F153	55	1	None
F171	45	6(1)	None
F175	38	3(1)	None
F178	63	6	None
F180	63	2	None
F202	60	4	None
F207	55	3	None
F241	55	1(1)	None
F242	55	1(1)	None

TABLE 4.2

Primer Sequences for APP Gene Amplification

Sequences (product size)		$MgCl_2$	An.Temp.
Exon 1:	5'-AGCAAGGACGCGGCGGATCC-3'		
(172)	5'-GTATCGCGTCCCCACCGTGC-3'	1.5mM	58C
Exon 2:	5'-GAATTC-TGCCTTGGAGCTATGGATAC-3'		
(262)	5'-GAATTC-ATCCAACGTGAATTGCTAGC-3'	2.0mM	58C
Exon 3:	5'-GAATTC-ATGCTTGTAAATGCCAGCCC-3'		
(262)	5'-GAATTC-TTTCTTCCCTCAAGACCAGG-3'	1.5mM	58C
Exon 4:	5'-CCTTGATGTCTTCTGGGGTA-3'		
(210)	5'-CAGGAAATCTTCACTTTGCA-3'	1.5mM	55C
Exon 5:	5'-ACACATCTACTCTAGCCAC-3'		
(399)	5'-ACTCCATACAAGATGTTTCAGC-3'	1.5mM	63C
Exon 6:	5'-GACGGCCGCTGTTTGCCTTCACAGTG-3'		
(484)	5'-GCGGCGCGCCCTTGGGATTTGCCAAGCAGC-3'	3.0mM	60C

Exon 7:	5'-GAATTC-TCATGCTAAATGTGGTTCCC-3'		
(282)	5'-GAATTC-ACAAAAGCAGAGTCAGTGGC-3'	1.5mM	60C
Exon 8:	5'-GAGCGCGCTTGGTTTTGTTGGAGGGACC-3'		
(250)	5'-GCCGATCGCTGGAGTTATGTGAACCCAA-3'	3.0mM	60C
Exon 9:	5'-GAATTC-TTCTCAAACCTCCTTCTC-3'		
(211)	5'-GAATTC-ATCGTAGGGCTGAATGATGG-3'	2.0mM	58C
Exon 10:	5'-AACATAATCATCCATCCTAT-3'		
(257)	5'-GCAGACATATTAAATGGATG-3'	1.5mM	53C
Exon 11:	5'-GCTGCTTCATCCTACTTATTC-3'		
(266)	5'-CTTACTGTCTGTGCTGTGAC-3'	1.5mM	55C
Exon 12:	5'-GAATTC-GCCAATGGAAGAAGCAGTGT-3'		
(262)	5'-GAATTC-CCAAGAAGCAAGAATCCTGG-3'	1.5mM	58C
Exon 13:	5'-GAATTC-ATTCCTACCCCCAGACATGT-3'		
(196)	5'-GAATTC-ACATGTCCATGTGCAGCATC-3'	1.5mM	58C

Exon 14: 5'-GTTACTCACCAAAGAGATGGT-3'

(282)

5'-CCCTGCTGGCTCAGGGGACTC-3' 1.5mM 55C

Exon 15: 5'-CGAATTC-CGACTTGTTTGGGAGCCAC-3'

(256)

5'-CGAATTC-CTGCAGTAAGTACAATTGAG-3' 1.5mM 55C

Exon 16: 5'-GGGTAGGCTTTGTCTTACAG-3'

(250)

5'-GGCAAGACAAACAGTAGTGG-3 1.5mM 58C

Exon 17: 5'-ATAACCTCATCCAAATGTCCCC-3'

(402)

5'-GTAACCCAAGCATCATGGAAGC-3' 1.5mM 60C

Exon 18: 5'-GAATTC-CTTCTCCATGTTCACCCTTA-3'

(313)

5'-GTTACAGCACAGCTGTCAAAAGGG-3' 1.5mM 60C

Primers used for the PrP PCRs:

1. JC11/33

5'-CACAGTCAGTGGAACAAGCCGA-3' 5'-TTTCACGATAGTAACGGTCC-3'

2. 34/35

5'-CTATGAGGACCGTTACTATCG-3' 5'-GTACTGGGTGATACACATCTGC-3'

The PrP primers were designed by Dr. John Collinge and Dr. Mark Palmer.

CHAPTER 5

ANALYSIS OF ANTICHYMOTRYPSIN AS A CANDIDATE GENE FOR ALZHEIMER'S DISEASE

5.1 Introduction

5.1.1 Alzheimer's disease genetics: our strategy

Alzheimer's disease has a complex etiology as discussed previously. In a few cases it is inherited as an autosomal dominant disease and in a subset of those cases it is caused by mutations in APP. It is evident now, after large collaborative screening efforts, (Schellenberg *et al*.1991; Fidani *et al*.1992; Tanzi *et al*.1992), that only a few early onset genetic cases are due to mutations in APP.

No features have yet been described which distinguish individuals in which the pathogenic mutation is in APP. There is, as yet, no evidence concerning the location or nature of the other locus(i) causing Alzheimer's disease.

For these reasons, we adopted a hierarchical approach to the analysis of the genetics of Alzheimer's disease. Our approach is to:-

a) collect families multiply-affected by early onset Alzheimer's disease.

b) screen for the known mutations within APP.

c) assess the segregation of APP in those without known mutations,

d) in those in which there is possible co-segregation of APP and Alzheimer's disease, screen the entire APP gene by single strand conformation polymorphism analysis (SSCP) and direct sequencing.

Using this approach I have determined by SSCA that our family collection includes at least nine families multiply affected by early onset Alzheimer's disease in which it is extremely unlikely that APP is the pathogenic locus (Fidani *et al*.1992). Genetic linkage analysis has confirmed this conclusion (Houlden and Mullan, unpublished data). These families are described on Table 5.1.

5.1.2 Families with no mutations in APP.

Such families in which there is no cosegregation of APP and the disease, or there is ambiguous segregation of APP but there are no mutations, are entered into the genome search programme to aid the identification of the other locus(i). A full genome search requires large, multiply affected pedigree(s) and many highly polymorphic markers covering all the genome in approximately equal and short distances. Our Alzheimer's disease families are fairly small (figure 5.2-5.10). Due to the relatively late onset of the disorder there are often a few living affected individuals in only one generation. Under these

circumstances, the candidate gene approach presents a number of definite advantages in the genetic analysis of Alzheimer's disease. Furthermore, the discovery of the APP mutations in some families with the disease points at a specific biochemical process as central to the etiology of the disease (Hardy and Allsop, 1991). This should help in the rational selection of candidate genes.

For these reasons, we have embarked on a candidate gene approach to the analysis of familial early onset Alzheimer's disease, rather than a positional cloning strategy.

5.1.3 AACT as a candidate gene for Alzheimer's disease.

I have decided to start by analysing the segregation of α1-antichymotrypsin (AACT) locus, since there are a number of facts supporting its involvement in the pathogenesis of Alzheimer's disease:

a) like ß-amyloid, it is a component of the amyloid plaque core (Abraham *et al*.1988).

b) like some transcripts of APP (Tanzi *et al*.1988; Kitaguchi *et al*.1988) it is a serine protease inhibitor (Travis *et al*.1978).

c) ß-amyloid deposition is believed to follow mismetabolism of APP by proteases. (Abraham et al., 1988) AACT may be involved in the regulation of these proteases through inhibition.

d) like APP it is an acute phase protein and as such, is likely to upregulate when there is neuronal injury (Vandenabeele and Fiers, 1991).

e) suggestive linkage on chromosome 14, where the AACT gene is found, has been reported (Weitkamp *et al*.1983).

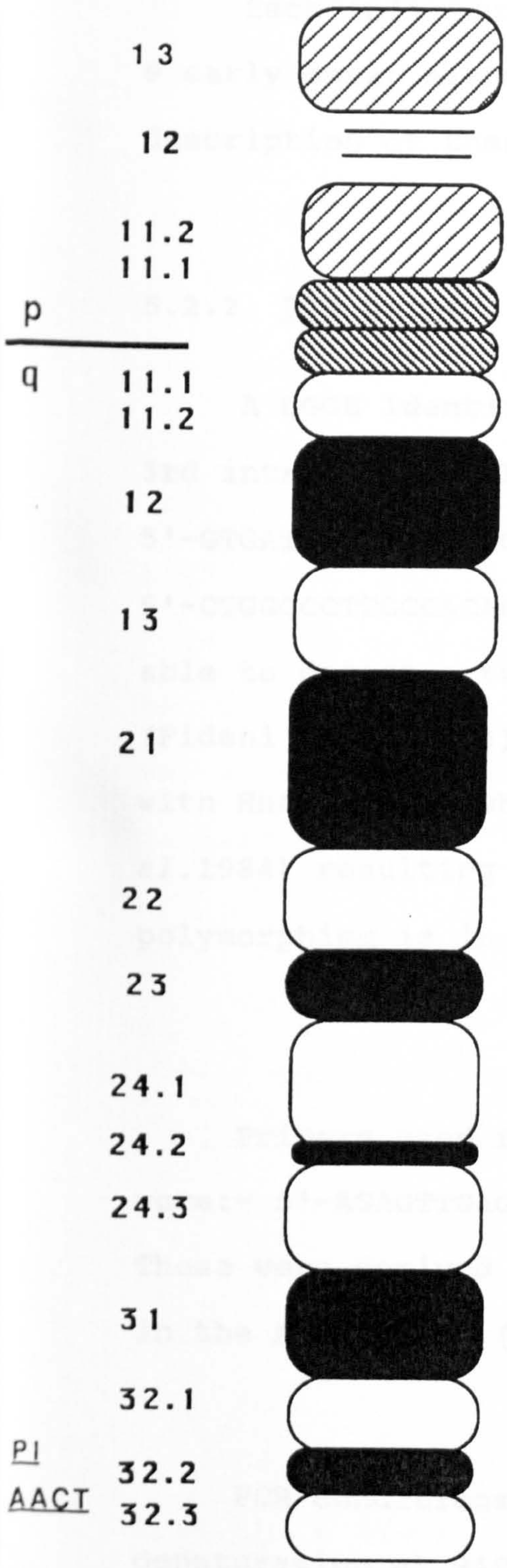
5.1.4 AACT gene

The AACT gene is found on chromosome 14q32-33 (Rabin *et al*.1986; Schroeder *et al*.1985) (figure 5.1). The gene is approximately 12kb long and contains five exons and four introns. Its organisation is homologous to that of antitrypsin (PI) gene, with which it shares a high degree of protein sequence homology, suggesting that the two loci arose by recent gene duplication (Bao *et al*.1987). AACT is a plasma serine protease inhibitor. Among its strongest target substrates is cathepsin G, a lysosomal enzyme, known to play an important role in the APP processing (Abraham *et al*.1991).

There are no characterised highly polymorphic markers in this locus and so we have used two two-allele markers: one single strand conformation polymorphism in the antitrypsin (PI) gene and a restriction site polymorphism in the signal peptide of the AACT gene. The PI and AACT genes are separated by 220kb and are oriented in opposite directions with their 5' ends pointing towards each other (Sefton *et al*.1990). Since they are so close to each other we have haplotyped the markers to get more information for our linkage analysis in the families.

Figure 5.1

CHROMOSOME 14



5.2 Methods

5.2.1 DNA samples

Each individual was sampled and DNA was extracted from the 9 early onset Alzheimer's pedigrees used. A limited clinical description of these families is found in Table 5.1.

5.2.2 PCR primers and PCR conditions

A DGGE identifiable polymorphism has been reported in the 3rd intron of the PI gene (Johnson *et al*.1991) using the primers: 5'-GTGATTCCCCAACCTGAGGGTGACC-3' and 5'-CTGGGCCTTGCCACACAGGCTCTTC-3'. Using these primers, we were able to detect a two-allele system using our SSCP protocol (Fidani *et al*.1992), after a digest of the PCR product (480bp) with Hha1 enzyme which cleaves once in the product (Long *et al*.1984) resulting in two fragments of 280bp and 200bp. The polymorphism is in the 280bp fragment (Figure 5.11).

Primers used for the detection of the AACT polymorphism were:- 5'-AGAGTTGAGAATGGAGAG-3' and 5'-GTCCACGTGTGTCCCTCG-3'. These were derived from the known sequence of the signal peptide in the AACT locus (Abraham *et al*.1988).

PCR conditions for both primers were:- 10 minutes initial denaturation at 94C followed by 35 cycles of 72C for 1min, 94 for 1min and 55C for 45sec. The reaction was performed in a total

volume of 50μl with a final concentration of primers of 0.2μM. The $MgCl_2$ optimum concentration for the PI primers was 1.0 mM and for the AACT primers 1.5 mM. Final concentrations of 200μM of all nucleotides were used apart from dATP in the PI PCR, which was used at a concentration of 24μM and 0.75μl of [α-^{35}S] dATP (Specific activity 600Ci/mmol). 2 units of Taq polymerase were added and DNA was at a concentration of 100ng per reaction.

5.2.3 Generation of the AACT marker

The PCR product generated with the AACT primers was 155bp giving after a digest with BstN1 enzyme at 60C, 3 fragments 84bp, 32bp and 33bp. The polymorphism is found at position 680 of the AACT signal peptide changing an alanine to a threonine abolishing a restriction site for BstN1 and resulting in the generation of two fragments of 116 and 33bp. Allele frequencies were estimated for alanine: 0.3 and for threonine: 0.7 (Faber *et al*.1991) (figure 5.12).

The digested samples were run on polyacrylamide gels of 8% (19:1) for 1 hour at 300Volts. Ethidium bromide was used to stain DNA and gels were visualised and photographed under U.V radiation.

5.2.4 Linkage studies

Linkage analysis was performed using the MLINK programme from the LINKAGE package (Lathrop *et al*.1984). Individuals were divided in 7 liability classes, according to age (Mullan, in preparation). Allele frequencies for the PI polymorphism were

estimated as allele 1: 0.3 and allele 2: 0.7 (40 individuals). Allele frequencies for the AACT polymorphism as allele 1:0.7 and allele 2:0.3.

Autosomal dominance was assumed, a disease frequency of .001, a phenocopy rate of .0001 and seven age dependent liability classes were assigned from the distribution of the known ages of onset in each family.

5.3 Results

In the families tested no co-segregation of the disease and the AACT locus was found (see Table 5.2). Individually, data from two families (74 and 206) excluded the locus at a recombination fraction of 0. The data from the remaining families when pooled, allowed exclusion but the data do not allow for definitive exclusion in any other individual family if there is further heterogeneity. When all the data are pooled they allow exclusion of 9cm from the AACT/PI assuming that there is only a single non-APP locus (figure 5.13). These data are not significantly altered if there is some recombination between the PI and the AACT loci (Table 5.2).

Figures 5.2-5.10

AD pedigrees used in this study with the genotypes of the two polymorphic markers. The top marker is the SSCP generated PI gene polymorphism and the one below the AACT gene polymorphism.

* indicates the availability of Blood and DNA samples

Square symbols represent male individuals and round symbols represent females. Shaded symbols are affected individuals and diagonal lines indicate death.

Family 74

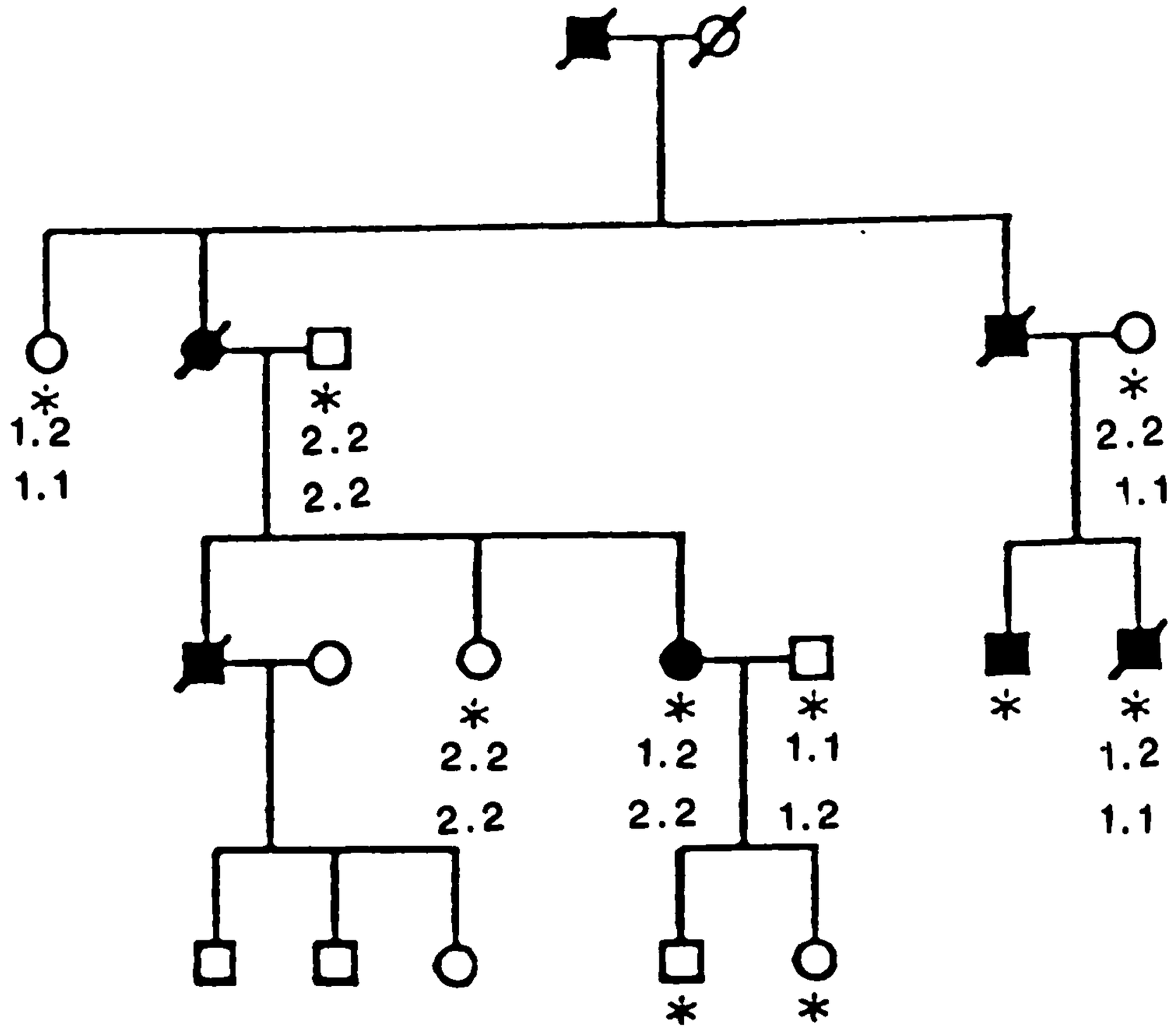


Figure 5.2

Family 134

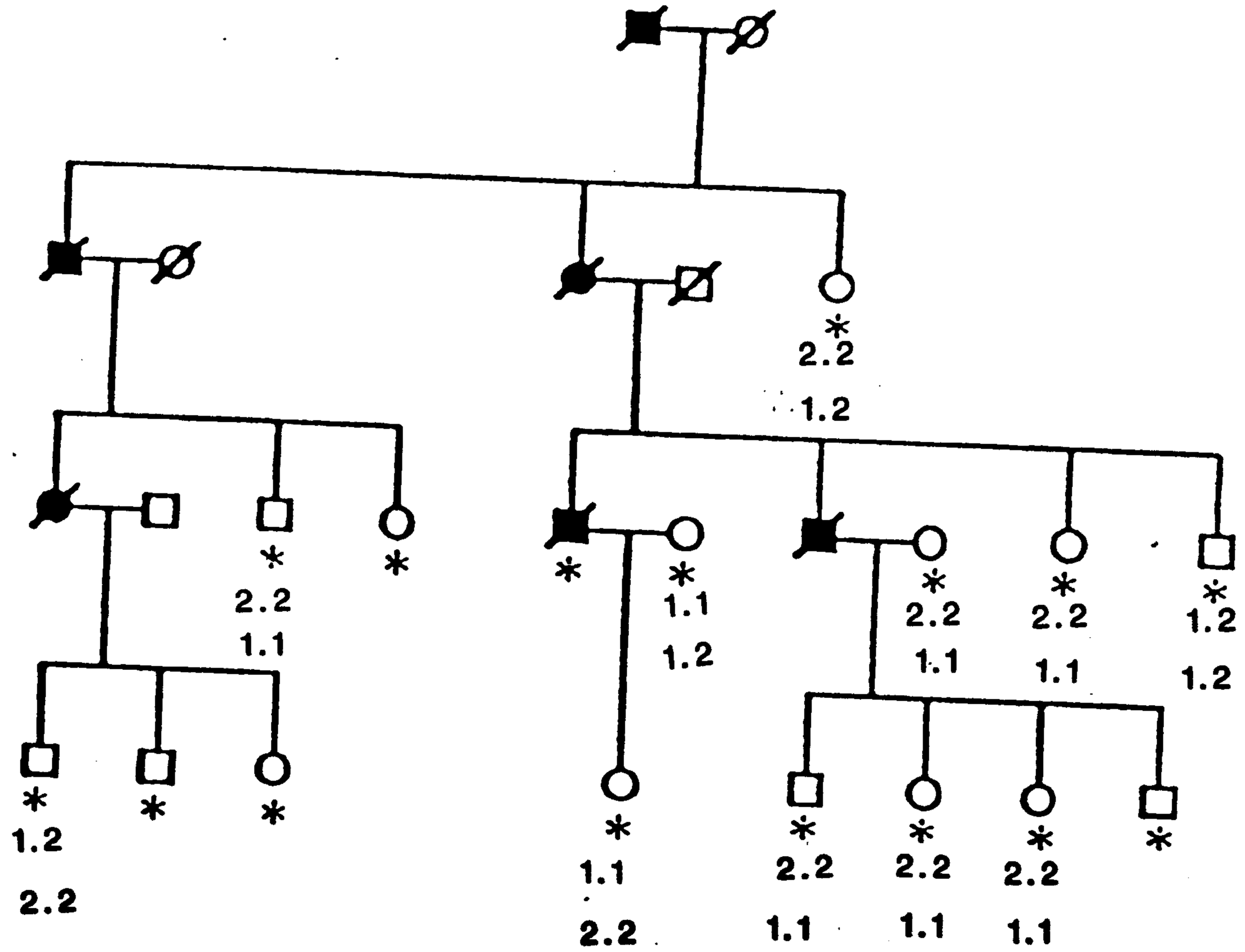


Figure 5.3

Family 206

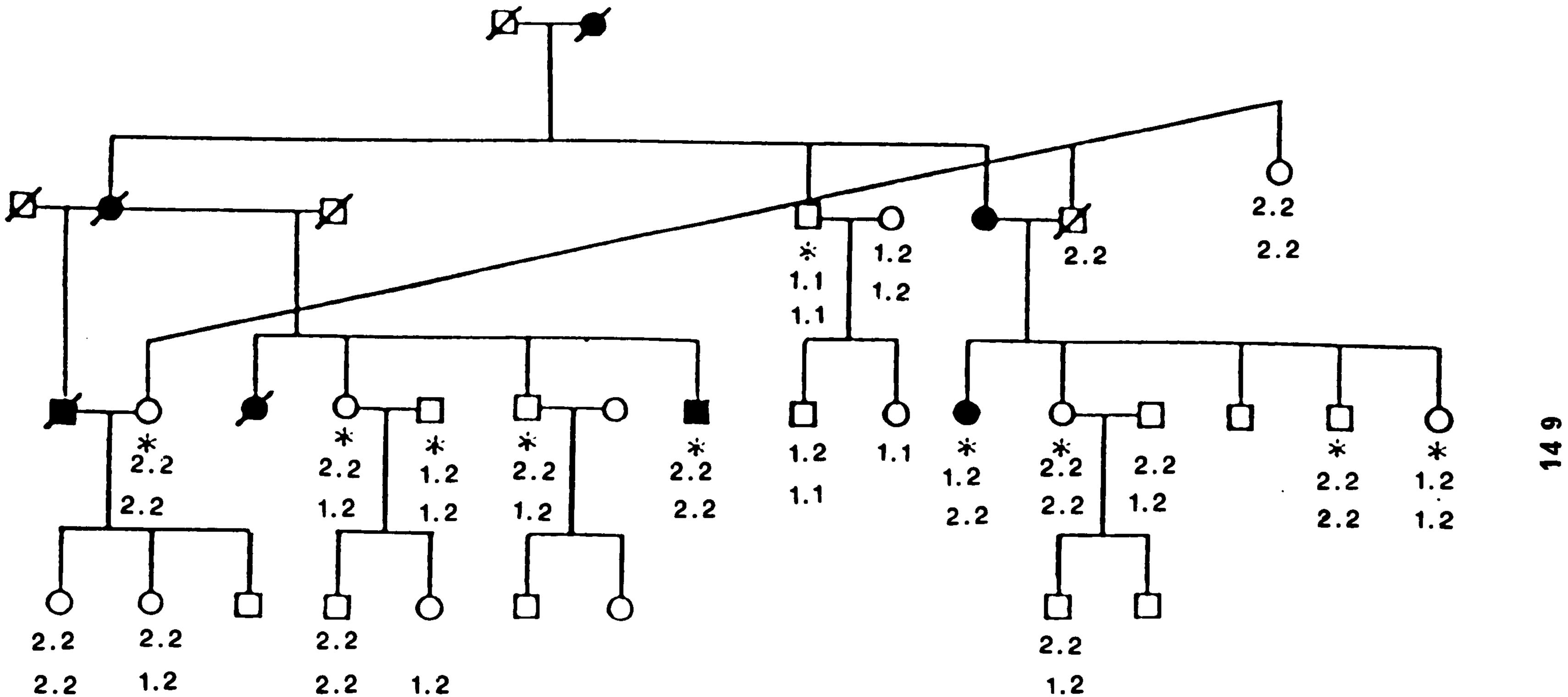


Figure 5.4

Family 84

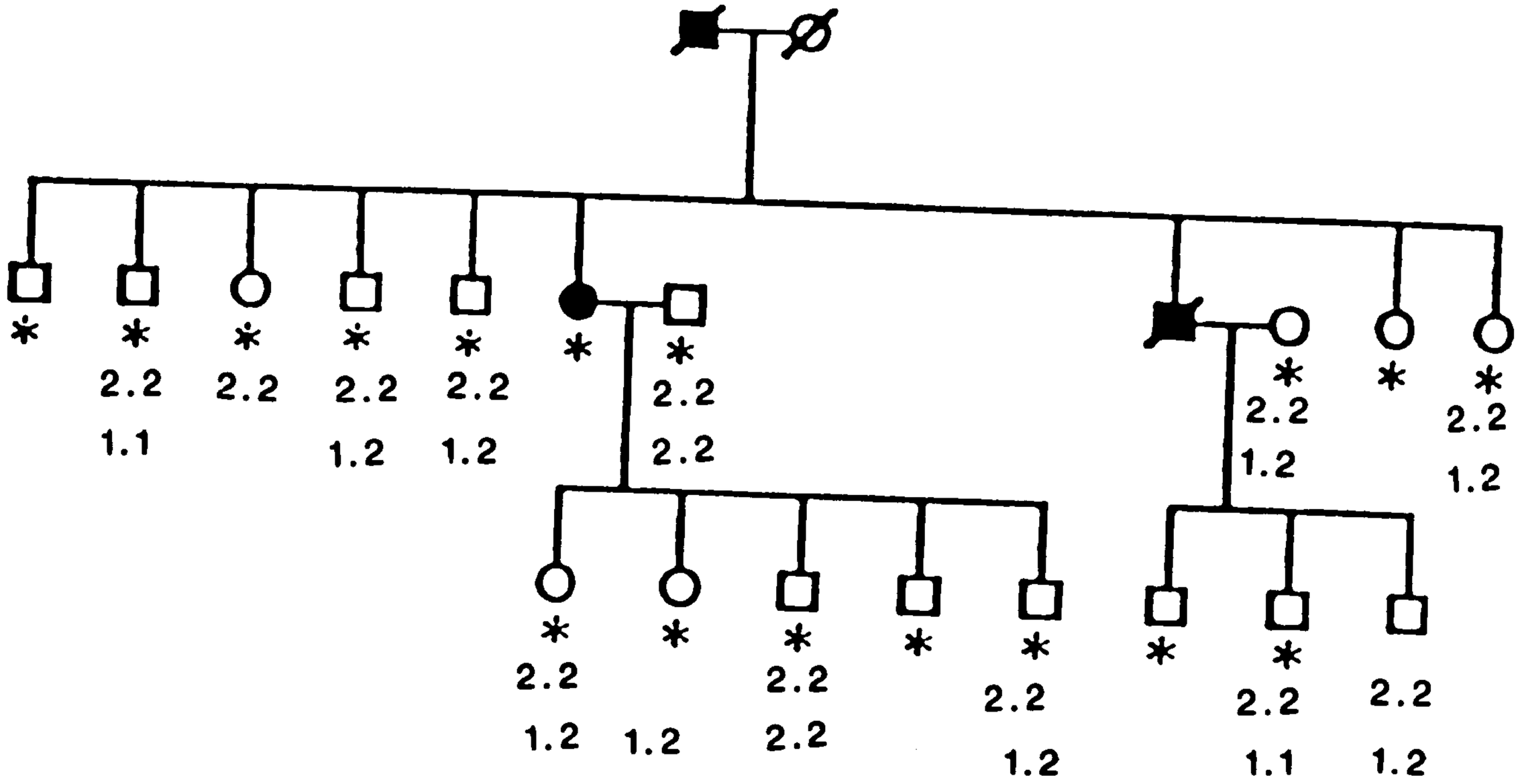


Figure 5.5

Family 105

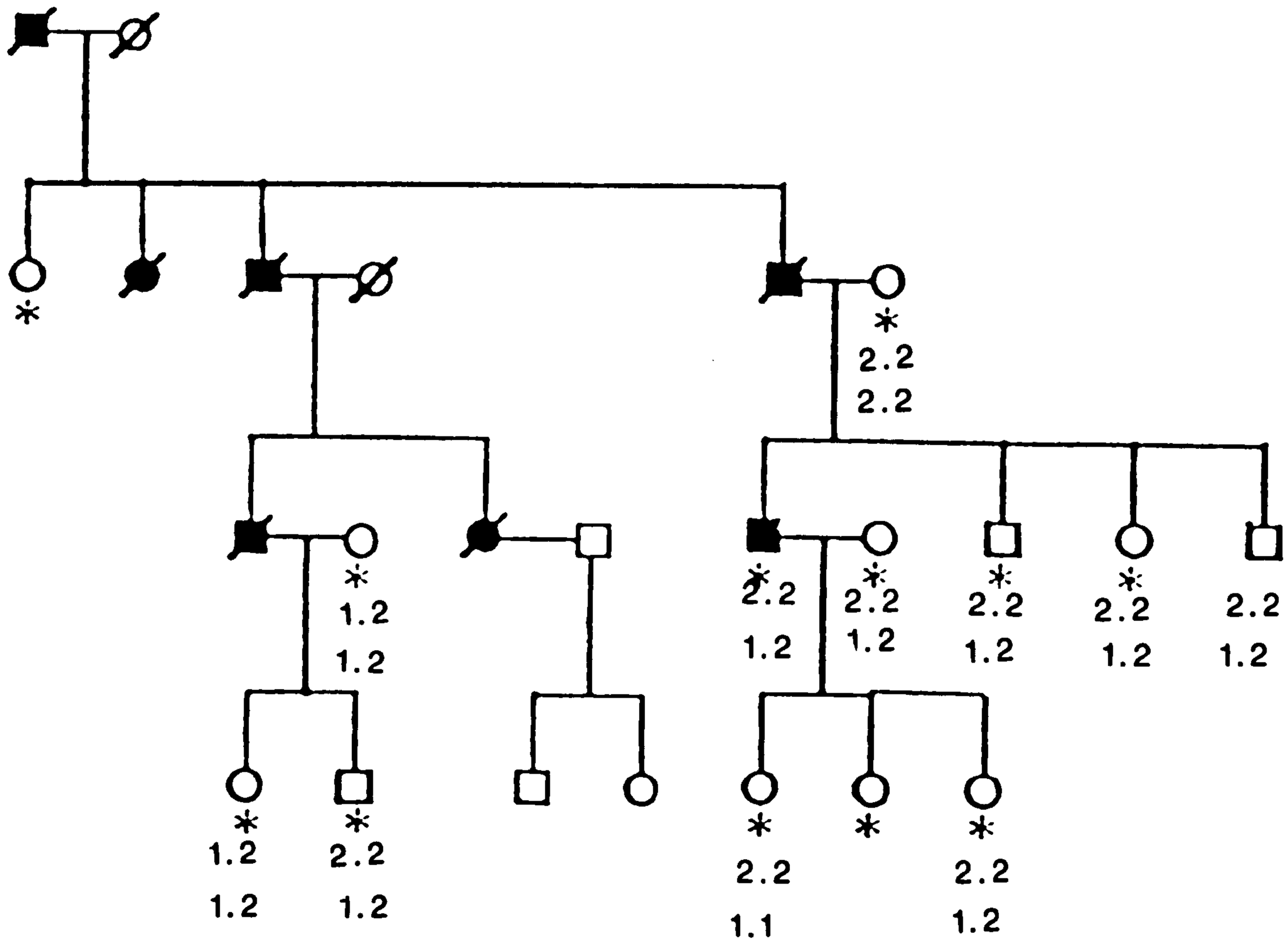


Figure 5.6

Family 53

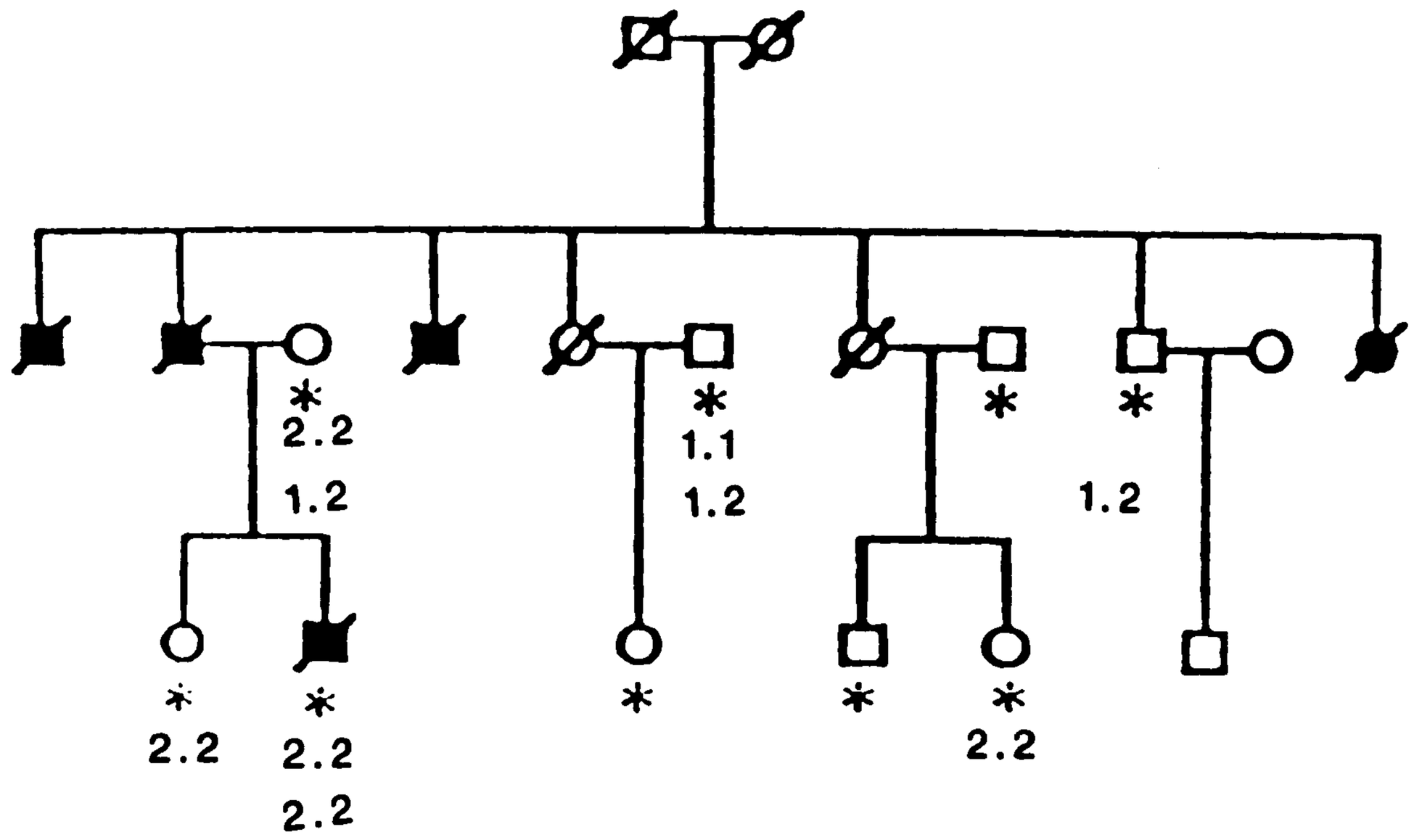


Figure 5.7

Family 148

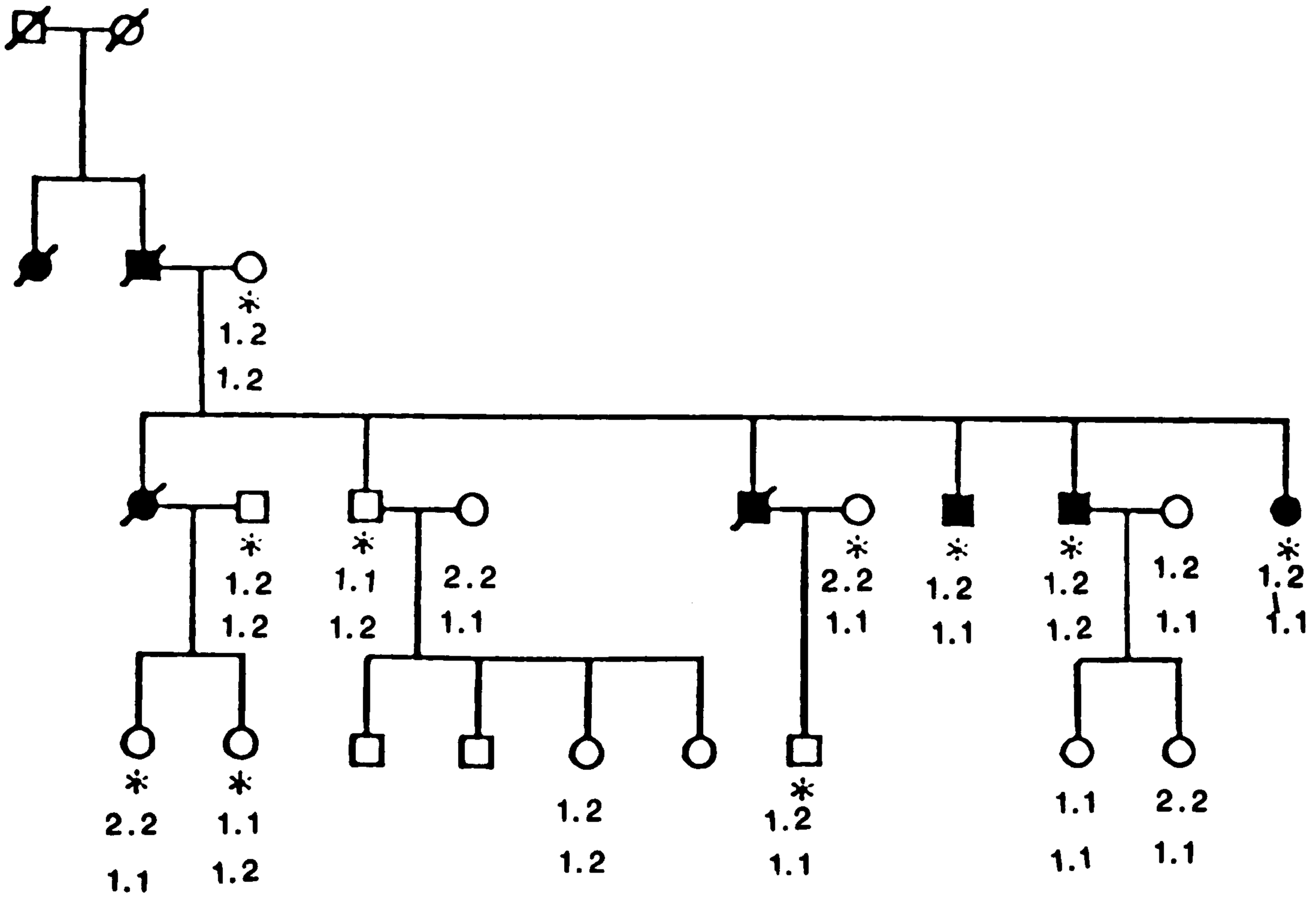


Figure 5.8

Family 121

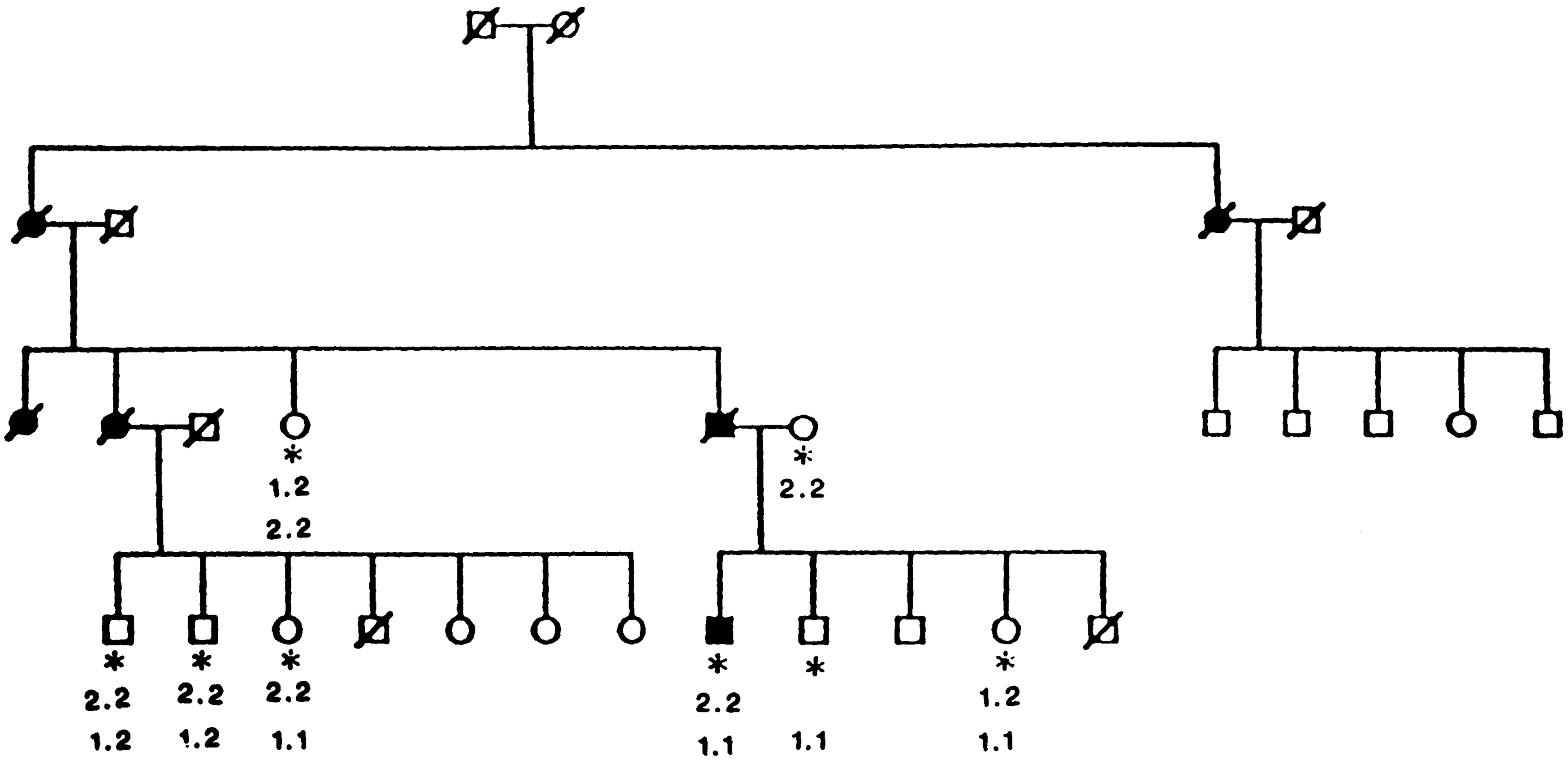


Figure 5.9

Family 126

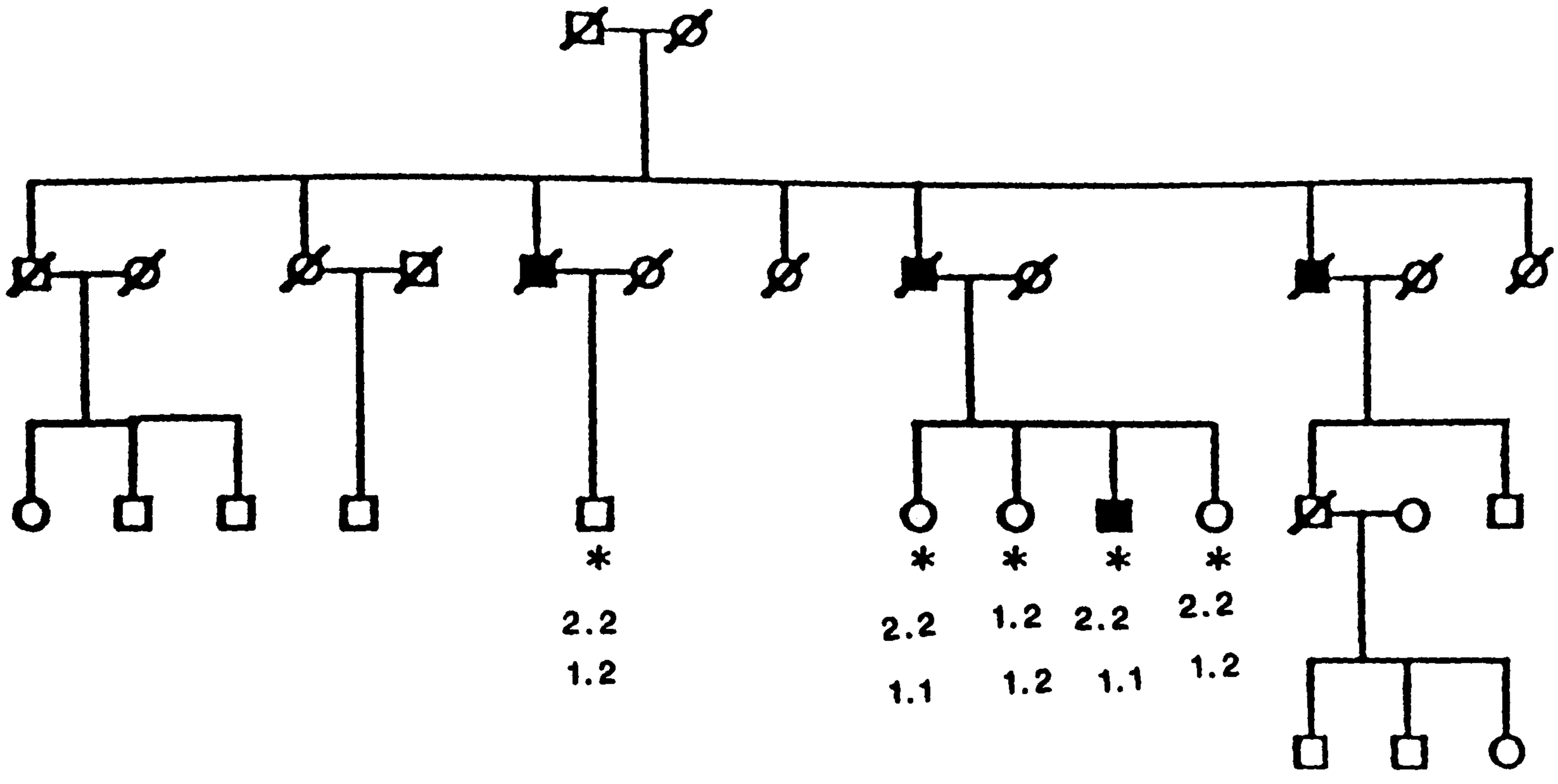


Figure 5.10

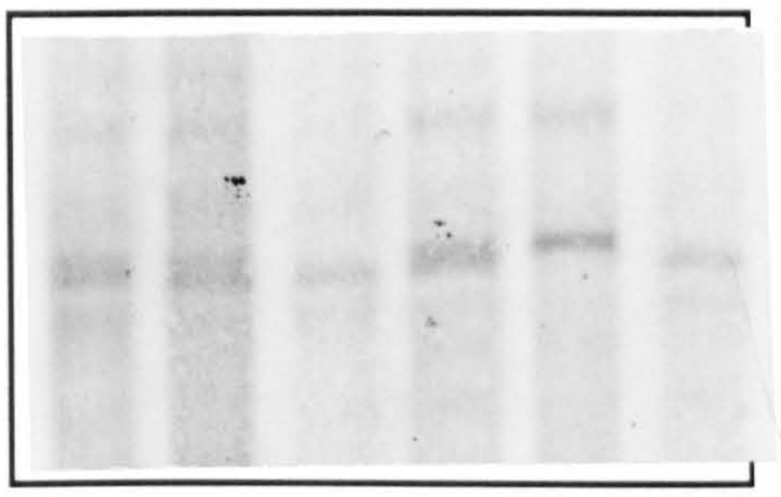
Figure 5.11

5%(v/v) SSCP gel, showing the different alleles of the PI gene polymorphism in some unrelated individuals.

Figure 5.11

SSCA marker for PI gene

1 2 3 4 5 6



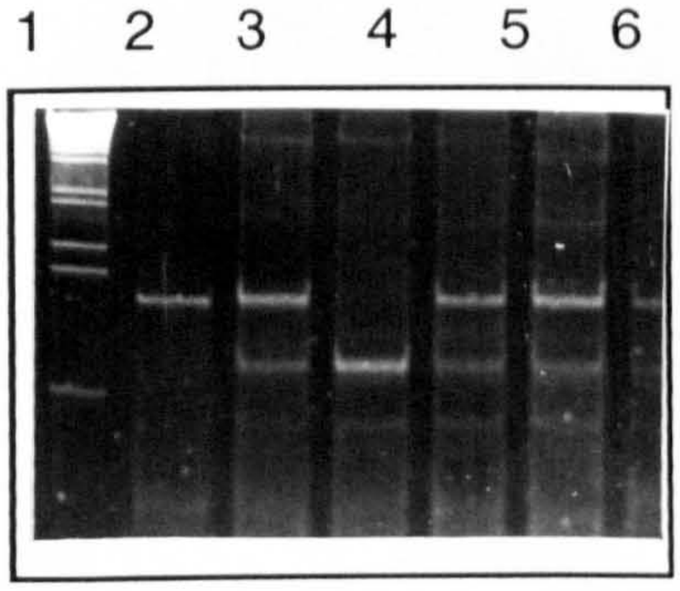
Genotypes

1. 1/2
2. 1/2
3. 2/2
4. 1/2
5. 1/1
6. 2/2

Figure 5.12

Restriction digest marker for AACT gene

1 2 3 4 5 6



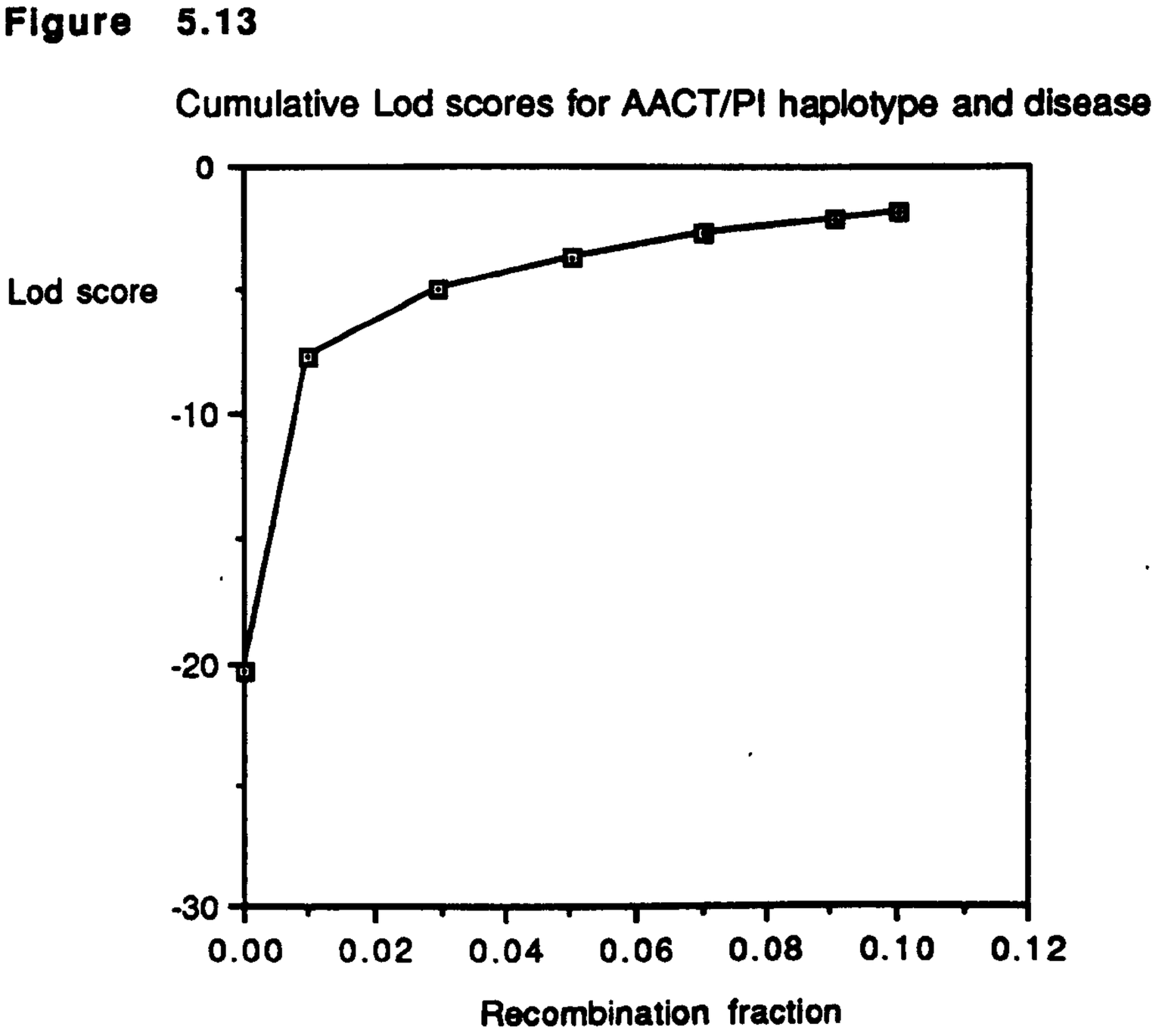
Genotypes

1. 1kb
2. 1/1
3. 1/2
4. 2/2
5. 1/2
6. 1/2

Figure 5.12

3% (w/v) agarose gel, showing the different alleles of the AACT ge
polymorphism in a number of unrelated individuals.

Figure 5.13


Cumulative Lod scores for AACT/PI haplotype and disease
Lod score
0
-10
-20
-30
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Recombination fraction

5.4 Discussion

There are a number of problems associated with genetic studies in Alzheimer's disease, the most prominent being genetic heterogeneity and others, rarity of phenotype, phenocopies (individuals of normal genotype displaying the mutant phenotype due to non genetic causes as in sporadic cases of Alzheimer's disease) and incomplete penetrance.

In families with inherited Alzheimer's disease, the disease may not show complete penetrance because some members die of other causes, before they display any symptoms. That way unaffected individuals will appear to be recombinants. Reciprocal to this problem is the potential misdiagnosis of unaffected individuals as affected. This problem becomes critical in psychiatric disorders, in general, where a wide range of behaviours are included among the diagnostic criteria.

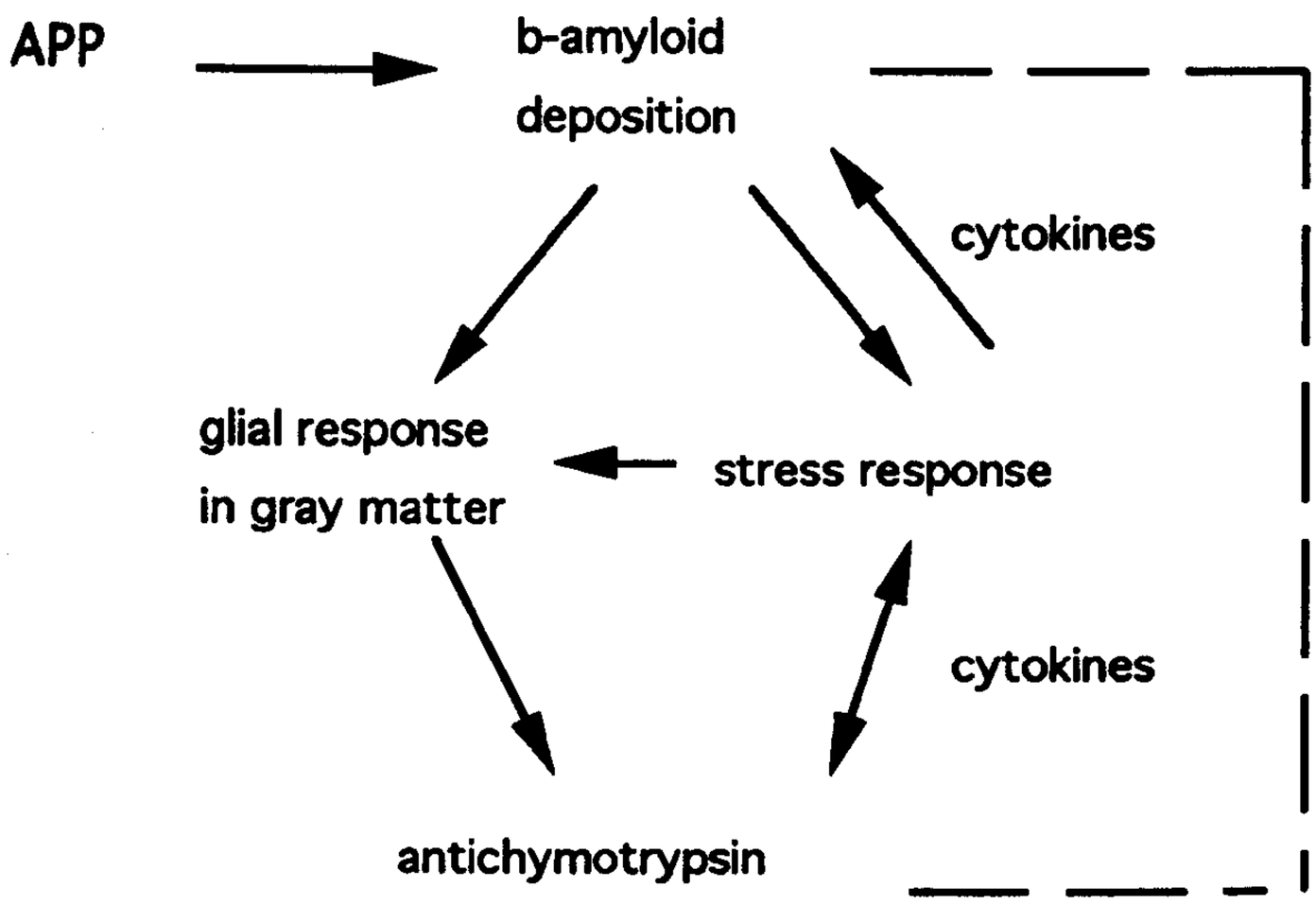
The existence of genetic heterogeneity in familial early onset Alzheimer's disease (St George Hyslop *et al*.1990) makes genetic studies much more difficult since there may be several other loci involved in the pathogenesis of Alzheimer's disease in non-APP cases of the disease. With this in mind we can only be certain of the exclusion of the AACT locus in those two families which have frank recombinants between the locus and the disease. However, since the data in 6 out of the 7 remaining families is also negative, we regard it as highly unlikely that AACT is the defective gene in the other pedigrees. These data, therefore,

suggest that AACT is not a pathogenic locus for Alzheimer's disease.

Thus, the deposition of AACT must be a secondary effect in the disease pathogenesis, most likely initiated by ß-amyloid deposition and possibly consequent upon a stress response reaction and a glial reaction (figure 5.14). AACT is specifically associated with ß-amyloidoses (Abraham *et al*.1990). Both APP and AACT, share a consensus sequence in their promoters (Tsuchiya *et al*.1987) supporting the idea that they may be upregulated by the same stimuli. One likely such stimulus is the cytokines. The cerebral acute phase response involves the cytokines, which stimulate both the production of stress proteins (Vandenabeele and Fiers, 1991), and an increase in the number of astrocytes and microglial cells producing them (Rothwell, 1991; Pasternack *et al*.1989). It is known that antichymotrypsin is an inhibitor of several inflammation associated proteases and this could account for its specific increase in response to stress. A complex of AACT with cathepsin G is known to stimulate interleukin 6 and thus to signal for acute phase protein synthesis (Kurdowska and Travis, 1990).

Since AACT is always present in mature senile and diffuse plaques in Alzheimer's disease (Shoji *et al*.1991), in diffuse plaques found in Hereditary Cerebral Haemorrhage with Amyloidosis (Dutch type) (Rozemuller *et al*.1992) and in brain amyloid deposits in normal aging (Abraham and Potter, 1989) it may be involved in stabilising the ß-amyloid deposits through a specific

Figure 5.14


APP
b-amyloid
deposition
cytokines
glial response
in gray matter
stress response
senile plaques
(AD pathology)
cytokines
antichymotrypsin

interaction. The fact that neither AACT or ß-amyloid are found in the amyloid lesions of other neurological diseases, for example, prion diseases (Abraham *et al*.1988) further suggests its tight association with ß-amyloid in the formation of the amyloid deposits of Alzheimer's disease.

In conclusion, AACT, although apparently essential for the formation of the amyloid aggregates in Alzheimer's disease, is not found at an initiating point in the pathogenic process resulting in Alzheimer's disease and further loci involved with the Alzheimer's disease pathogenic pathway remain to be tested.

Table 5.1

(Modified from Table 4.1)

Family No	Age of onset	Affected (Path. confirmed)
F53	43-60	5 (1)
F74	39-50	7 (2)
F84	59-64	3
F105	36-39	14
F121	35-39	6
F126	48-63	4
F134	41-50	6 (2)
F148	42-46	5 (1)
F206	37-42	7

Table 5.2

a) Two-point linkage analysis between the PI locus and disease

	Thetas						
Pedigree	0	0.01	0.03	0.05	0.07	0.09	0.1
53	-0.044	-0.041	-0.037	-0.033	-0.030	-0.026	-0.025
74	0.381	0.373	0.356	0.339	0.321	0.302	0.293
84	0.008	0.008	0.008	0.008	0.008	0.008	0.008
105	0.013	0.011	0.009	0.008	0.006	0.005	0.004
121	-0.055	-0.056	-0.057	-0.057	-0.057	-0.056	-0.055
126	-0.243	-0.234	-0.216	-0.199	-0.181	-0.165	-0.157
134	-0.062	-0.058	-0.055	-0.052	-0.041	-0.036	-0.034
148	-1.56	-1.25	-0.953	-0.774	-0.647	-0.548	-0.506
206	-4.2	-1.18	-0.72	-0.515	-0.386	-0.294	-0.294
TOTALS	-5.813	-2.481	-1.17	-1.314	-1.050	-0.852	-0.770

b.Two-point linkage analysis between AACT marker and disease

	Thetas						
Pedigree	0	0.01	0.03	0.05	0.07	0.09	0.1
53	-0.122	-0.115	-0.102	-0.091	-0.08	-0.071	-0.066
74	-3.00	-1.37	-0.905	-0.692	-0.553	-0.453	-0.412

84	-0.282	-0.246	-0.187	-0.141	-0.106	-0.079	-0.067
105	-0.036	-0.035	-0.032	-0.029	-0.027	-0.024	-0.023
121	0.406	0.397	0.377	0.357	0.336	0.314	0.303
126	-0.555	-0.495	-0.402	-0.332	-0.277	-0.233	-0.214
134	-0.068	-0.066	-0.061	-0.057	-0.052	-0.048	-0.046
148	-0.038	-0.038	-0.040	-0.041	-0.042	-0.043	-0.043
206	-0.774	-0.758	-0.726	-0.692	-0.656	-0.619	-0.600
TOTALS	-4.469	-3.725	-2.381	-1.709	-1.269	-0.952	-0.826

c. Cumulative *Lod* scores for AACT/PI haplotype and disease

Thetas

Pedigree	0	0.01	0.03	0.05	0.07	0.09	0.1
53	-0.166	-0.156	-0.138	-0.122	-0.107	-0.094	-0.09
74	-3.668	-1.016	-0.571	-0.381	-0.266	-0.189	-0.16
84	-0.282	-0.248	-0.191	-0.147	-0.113	-0.086	-0.07
105	-0.089	-0.085	-0.077	-0.070	-0.063	-0.057	-0.05
121	0.771	0.753	0.715	0.677	0.637	0.597	0.57
126	-1.342	-1.039	-0.749	-0.586	-0.476	-0.394	-0.36
134	-0.005	-0.004	-0.003	-0.001	-0.000	0.001	0.001

148	-1.282	-0.985	-0.691	-0.524	-0.409	-0.325	0.290
206	-7.199	-2.312	-1.405	-1.008	-0.762	-0.589	-0.52
TOTALS	-20.464	-7.723	-4.970	-3.633	-2.768	-2.148	-1.89

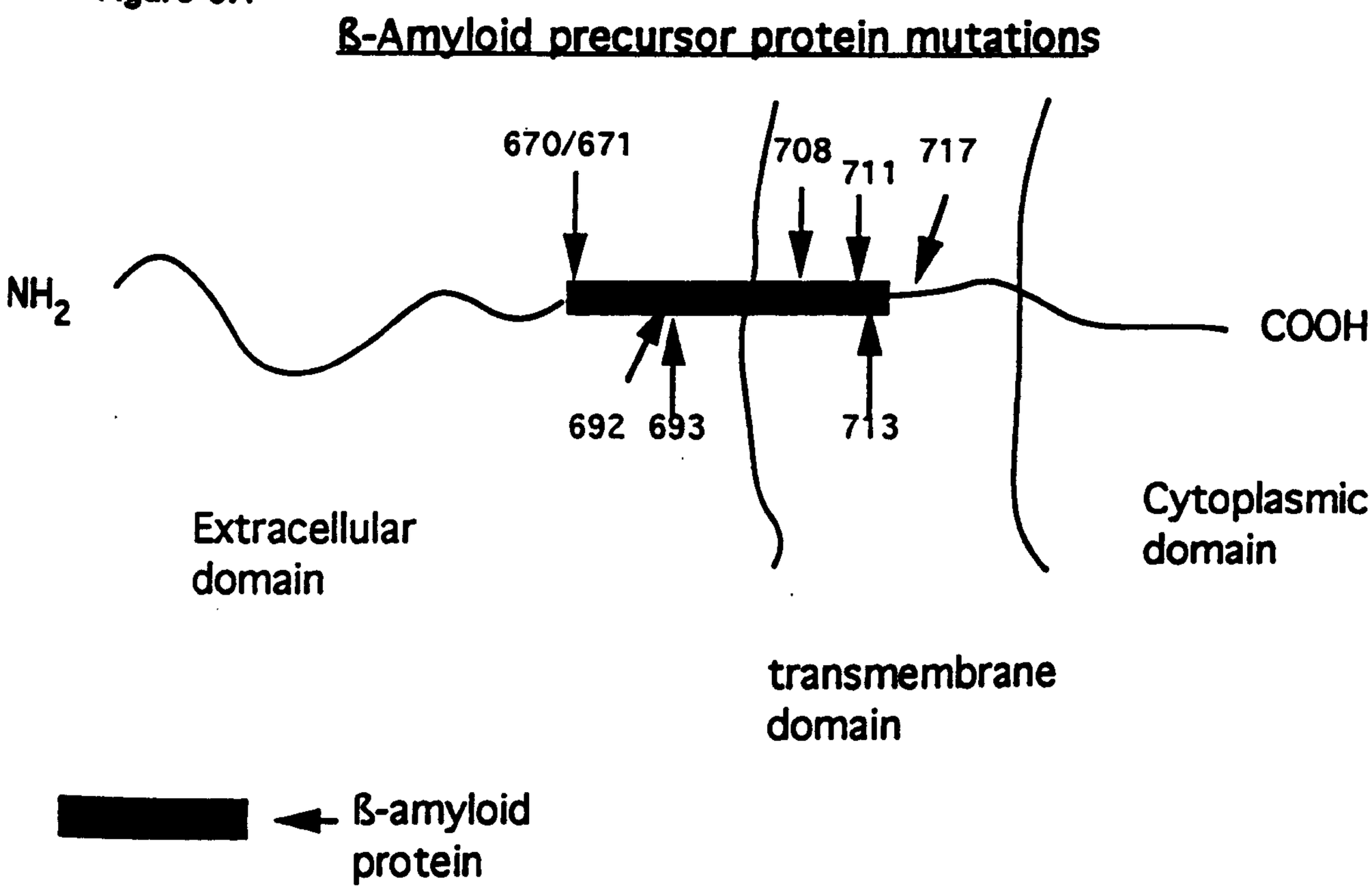
CHAPTER 6

DISCUSSION

The discovery of the pathogenic mutations in the ß-amyloid precursor gene has given a new insight in the pathogenesis of Alzheimer's disease. It is now evident that the complex pathology of the disease may result from different causes.

APP mutations (figure 6.1), play a significant, although not yet clear role, in ß-amyloid deposition. The first mutation described causes Hereditary Cerebral Haemorrhage with Amyloidosis-Dutch type disease, which is characterised by ß-amyloid deposits in cerebral vessels. This mutation changes a glutamate to a glutamine at codon 693 of the APP770 transcript (Levy *et al*.1990) . Recently another mutation was identified, which gives rise to a phenotype which sometimes resembles HCHWA, this time at codon 692, changing an alanine for a glycine (van Duijn *et al*.1991). The phenotype caused by this mutation in some individuals is slightly different than straight HCHWA-D, involving neuritic plaques and dementia. Both those mutations are close to the normal site of cleavage of APP (Esch *et al*.1990) suggesting that those mutations may change the structure of the molecule in this region so that the normal cleavage enzyme is less efficient.

Figure 6.1


ß-Amyloid precursor protein mutations
670/671
708
711
717
NH2
COOH
692
693
713
Extracellular domain
Cytoplasmic domain
transmembrane domain
ß-amyloid protein

In Alzheimer's disease the three pathogenic mutations at codon 717 of the APP770 transcript change the native valine to an isoleucine (Goate *et al*.1991; Naruse *et al*.1991; Yoshioka *et al*.1991; Hardy *et al*.1991), a phenylalanine (Murrell *et al*.1991), and a glycine (Chartier Harlin *et al*.1991). The identification of three mutations in the same codon of the gene accentuates their significance making this codon particularly important for the abnormal processing of the amyloid protein, leading to the development of Alzheimer's disease pathology.

It has been suggested that the mutations disrupt a regulatory stem loop in APP mRNA which alters translational regulation leading to the production of abnormally high amounts of protein (Tanzi and Hyman, 1991) (figure 6.2). Several observations make it unlikely that this hypothesis is correct:

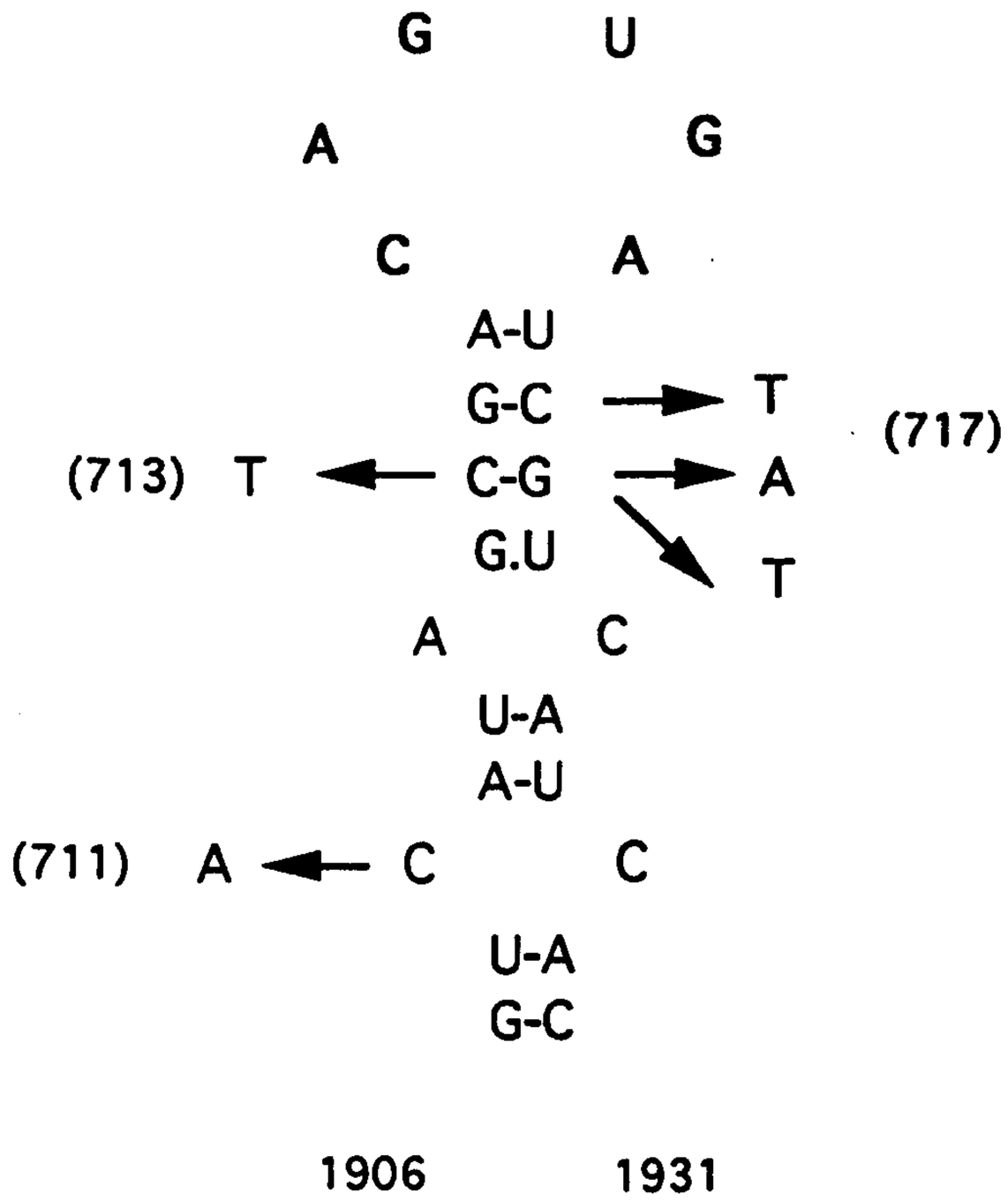
a) levels of APP mRNAs do not appear to be altered significantly by the val->ile mutation (Harrison *et al*.1991) as expected if the mRNA stability was altered (Klausner AND Harford, 1989) and

b) silent mutations, not altering the amino acid sequence but disrupting the stem loop structure would be predicted to cause Alzheimer's disease.

A number of silent variants have been identified (Balbin *et al*.1992; Adroer *et al*.1992) but they do not in any way disrupt the loop structure, and are probably rare polymorphisms having no effect in Alzheimer's disease pathogenesis.

Figure 6.2

RNA stem loop structure and APP mutations



In bold is the consensus sequence for the iron responsive elements (IRE), which in the genes of ferritin and transferrin, interacts with a binding protein, which regulates protein production at a translational level, in response to iron concentration.

Figure modified from Tanzi & Hyman,1991.

Recently a double mutation has been identified in two Swedish pedigrees (Mullan *et al*.1992), at the N-terminus of ß-amyloid very close to the endosomal/lysosomal cleavage site for APP (Estus *et al*.1992; Golde *et al*.1992), at codons 670 and 671 changing an asparagine to a lysine (Asn->Lys) and a leucine to a methionine (Leu->Met) respectively.

Another variant, associated with familial chronic schizophrenia at codon 713 changing an alanine to a valine, is puzzling and further studies will determine whether it is a rare polymorphism or a pathogenic mutation with no relevance to either Alzheimer's disease or HCHWA-D.

It is now clear that there are several causes of Alzheimer's disease although the relationship between precise phenotype and underlying genotype is not straightforward. Allelic heterogeneity in APP appears to account for diverse phenotypes, (HCHWA-D and Alzheimer's disease) which in some cases overlap as for APP692. At present, families with each APP mutation, are known to have a consistent family specific age of onset, but it is not possible yet to determine whether this is specific for each mutation, since families with no APP mutations have similar ages of onset as well (Fidani *et al*.1992).

As mutations and variations are frequently now being identified in the APP gene it is interesting to define the proportion of genetic cases of Alzheimer's disease they cause. Taking into consideration the data discussed in previous chapters it is evident that they account for a very small proportion of

genetic cases, indeed. No alterations were observed in the whole coding sequence of APP gene in 11 families with early onset Alzheimer's disease, and no mutations were identified in (40) separate genetic cases of early onset where only exon 16 and 17 were tested. It is very unlikely that there are mutations in the APP gene in the families tested, which are not identified, since apart from the SSCP analysis data, linkage studies between the disease and APP markers show clear recombinants in most cases.

Our negative data in the screening of sporadic cases (>100) for exon 16 and exon 17 further supports the rarity of these mutations, suggesting also that the alterations identified are only found in genetic cases where the disease is inherited through generations and that sporadic cases have a different etiology (environmental effects) resulting in the same pathology. A tissue specific somatic mutation may though account for some of these cases as well. The clinical features of the sporadic cases are undistinguishable from those of the genetic cases.

As yet one can only hypothesize as to how ß-amyloid deposition is caused by these mutations:-

a) Recent research concentrating on -COOH-terminal derivatives of APP suggests that these fragments are processed via the endosomal-lysosomal pathway potentially generating amyloidogenic fragments as well as non amyloidogenic fragments (Estus *et al*.1992; Golde *et al*.1992). The mutations may just help "shift" the processing from the "secretase" pathway, which cleaves the ß-amyloid at amino-acid 16 on the cell surface (Esch *et al*.1990; Anderson *et al*.1991), to the endosomal/lysosomal

system where amyloidogenic fragments may be generated and subsequently secreted and form extracellular aggregates.

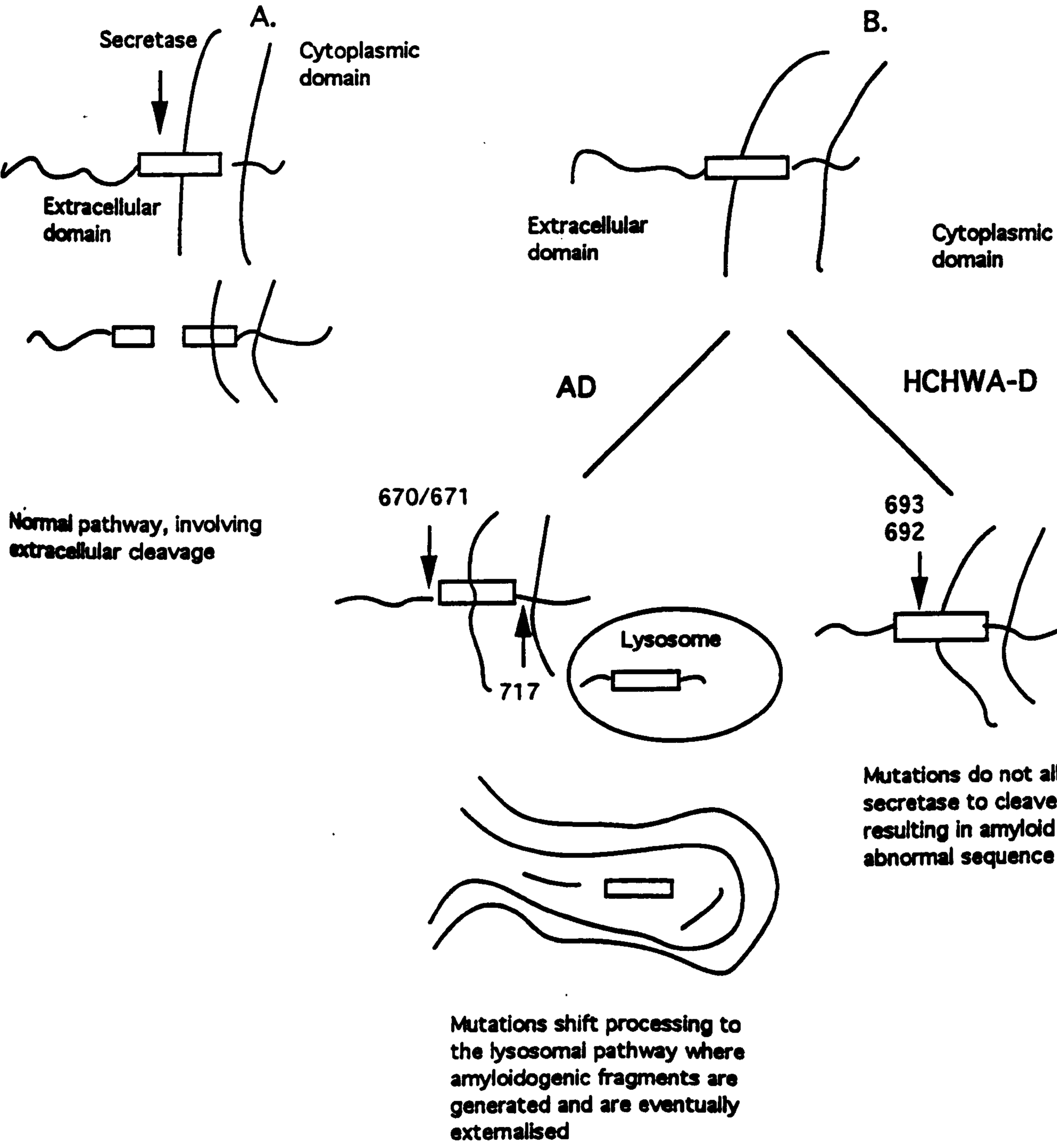
b) The amino acid changes in the membrane segment may open the way to membrane damage, so cleavage in the membrane becomes possible.

c) The mutations somehow change the conformation of the protein making recognition by the "secretase" difficult and thus result in the shift to the alternative pathway. "Secretase" is quite independent of the sequence of the substrate but recognises alpha-helical conformation at a distance from the membrane (Sisodia, 1992). This hypothesis may overlap with the first.

With the recent discovery of ß-amyloid peptide of normal sequence secreted in normal and Alzheimer's disease individuals (Haass *et al*.1992; Seubert *et al*.1992; Shoji *et al*.1992) a slight modification of the first hypothesis seems to gain credit. The Alzheimer's disease mutations in both ends of the peptide do not alter its sequence but somehow guide it follow an alternative metabolic pathway in the lysosomes. There, amyloidogenic fragments are generated, which subsequently are externalised and thus form the extracellular ß-amyloid. When this pathway is favoured as a result of the mutations, abnormal levels of ß-amyloid are generated which initiate the pathogenic process resulting in Alzheimer's disease phenotype (figure 6.3). This is believed to be a normal alternative processing pathway, existing possibly for conditions of stress, so that ß-amyloid can be produced in greater amounts if necessary. If this is correct then other factors may have the same effect as the mutations, and

Figure 6.3

A. Normal pathway of APP metabolism
B. Amyloidogenic pathways of APP metabolism



Normal pathway, involving extracellular cleavage

Mutations shift processing to the lysosomal pathway where amyloidogenic fragments are generated and are eventually externalised

Mutations do not allow secretase to cleave, resulting in amyloid of abnormal sequence

those remain to be discovered.

The mutations in the coding sequence of the ß-amyloid peptide though have a different effect, by possibly inhibiting normal cleavage, thus resulting in the generation of amyloidogenic fragments (figure 6.3).

Irrespective of the exact mechanism of pathogenesis, the mutant variants represent one identified cause of the disease, making aetiologic heterogeneity of Alzheimer's disease evident (figure 6.4). Nevertheless, ß-amyloid deposition appears to be at the initiating position in the chain of events (Hardy and Allsop, 1991; Hardy and Higgins, 1992) (figure 6.5), marking the first time that we begin to have an insight on the sequence of events which result in the complex pathology of Alzheimer's disease. Supporting evidence comes from research with Down's syndrome patients showing diffuse ß-amyloid deposits as early as their mid-teens whereas the later brain lesions they develop are no different from those in Alzheimer's disease individuals (Mann AND Esiri, 1989). In Down's syndrome ß-amyloid deposition may result from increased APP transcription (3 copies of chromosome 21), also causing APP to be processed by an alternative pathway generating amyloidogenic fragments (Rumble *et al*.1989).

Both work on Alzheimer's disease cases, staged for severity, and on Down's syndrome cases at different ages (Mann AND Esiri, 1989), suggests that the disease process starts with neuritic plaque formation in the amygdala.

Figure 6.4

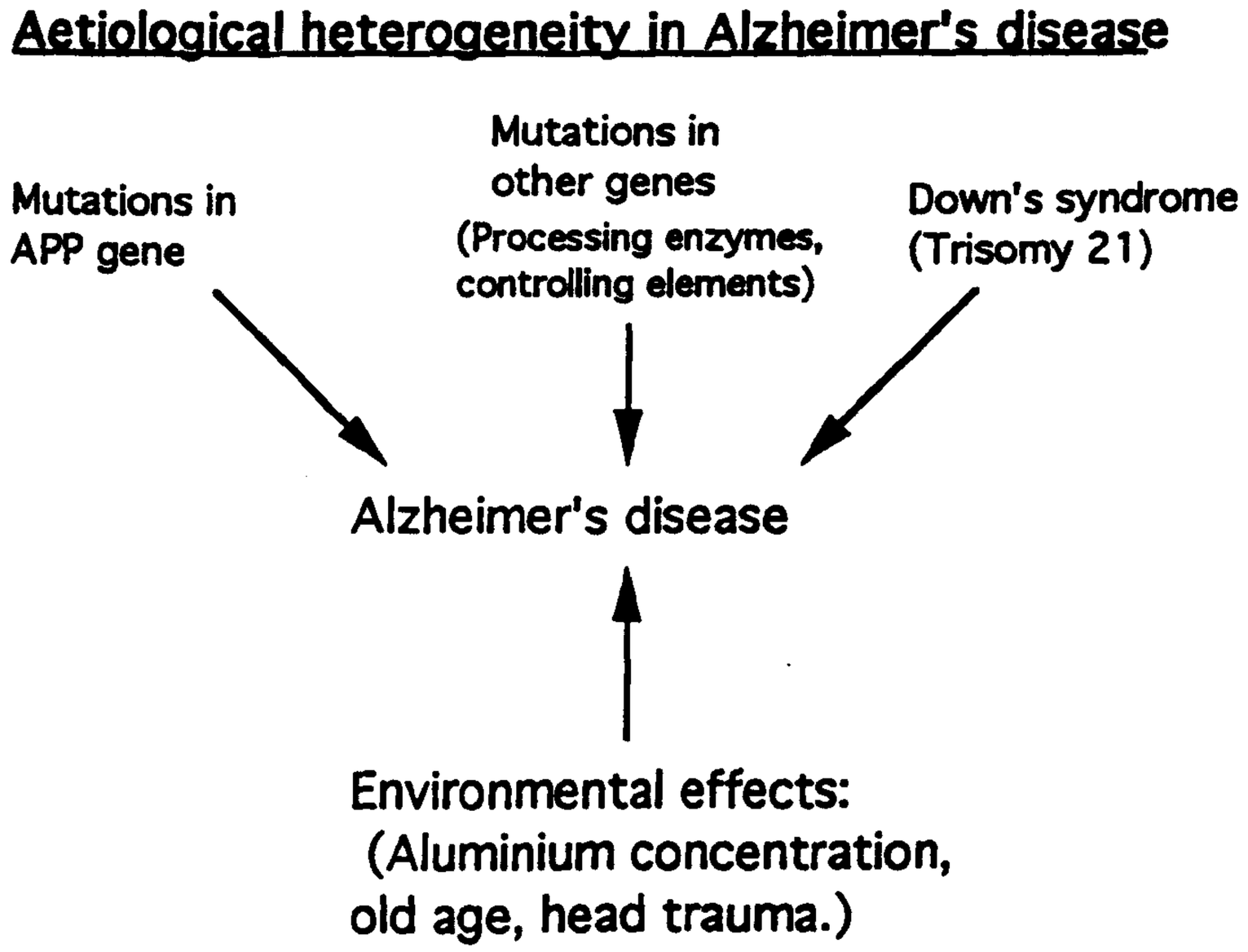
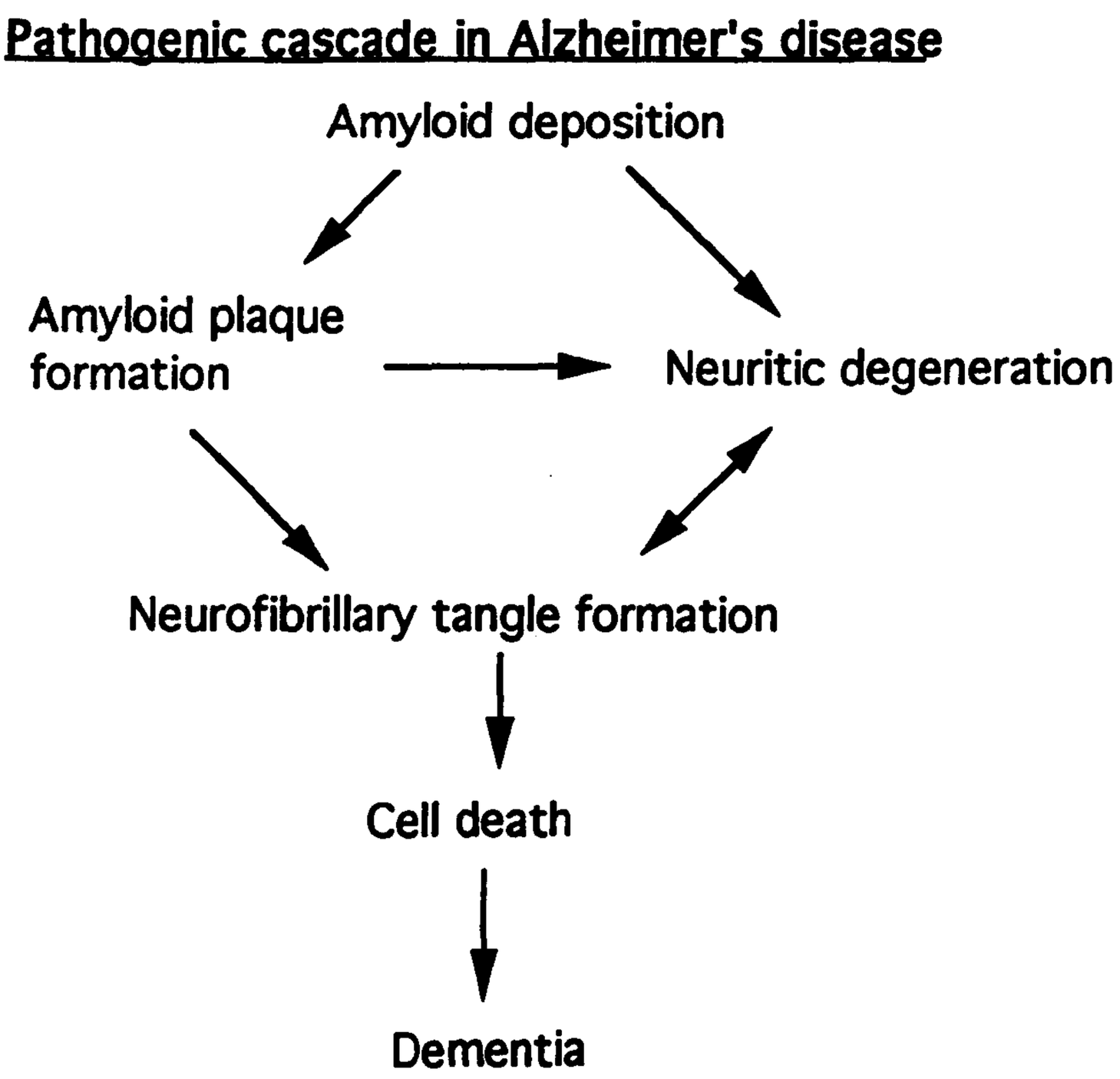

Aetiological heterogeneity in Alzheimer's disease
Mutations in APP gene
Mutations in other genes (Processing enzymes, controlling elements)
Down's syndrome (Trisomy 21)
Alzheimer's disease
Environmental effects: (Aluminium concentration, old age, head trauma.)

Figure 6.5


Pathogenic cascade in Alzheimer's disease
Amyloid deposition
Amyloid plaque formation
Neuritic degeneration
Neurofibrillary tangle formation
Cell death
Dementia

When focal ß-amyloid deposition is initiated, it provokes a neuronal response: the nature of this neuronal response is not yet clear. However, it seems that ß-amyloid is either directly or indirectly neurotoxic (Yankner *et al*.1990; Yankner *et al*.1989). It has also been suggested that ß-amyloid destabilises neuronal calcium homeostasis and thus makes neurons more vulnerable to environmental effects (Mattson *et al*.1992). The neurotoxicity is likely to induce an upregulation of stress response proteins, either via heat shock or interleukin mediated events. In this respect it is of interest that several components of plaques are stress response proteins (Vandenabeele and Fiers, 1991). Consistent with this hypothesis are the recent reports of abnormal ß-amyloid staining in both humans and animals after neuronal injury induced in a variety of ways (Roberts *et al*.1991; Kawarabayashi *et al*.1991; Abe *et al*.1991). These data, together with evidence that APP is axonally transported (Koo *et al*.1990), form the basis of the following notion:-

a) Initial deposition of ß-amyloid in the amygdala, for unknown reasons,

b) Stimulation of stress response in the surrounding neuropil,

c) Up regulation of APP and other proteins in the vicinity of the plaque and thus increased ß-amyloid deposition. Because ß-amyloid is axonally transported (Koo *et al*.1990), it also leads to increased APP turnover at the terminals of those neurons which are being damaged and thus to initiation of the same process at their terminals.

It is clear that our results do not represent the majority of cases of Alzheimer's disease. However, by pointing to the initiating point of the disease, they predict that the other causes will involve APP processing or upregulation. A better understanding of the APP metabolism will help in the identification of new putative candidate genes. Environmental factors, as head trauma, which interfere with the amyloid cascade pathway are also likely to be candidates for the development of the disease in sporadic cases. The loci identified as pathogenic in the early onset cases will be good candidate loci whose genetic contribution to late onset Alzheimer's disease should be assessed. This will be difficult given the problems associated with this subgroup of the disorder (Ott, 1990). However there is hope that by delineating the genetics of early onset Alzheimer's disease, identifying all the loci responsible, our understanding of the pathogenesis will be such, as to allow therapeutic intervention.

At the moment and after the test of α1-antichymotrypsin there is still a large number of genes which could be considered as reasonable candidates to Alzheimer's disease pathogenesis. The difficulties in their precise identification supplement the difficulties in the genetic studies in a complex disease with relatively late onset such as Alzheimer's. However, the candidate gene approach presents many advantages over a genome search at the moment, while there are not many large well characterised pedigrees in which Alzheimer's disease affects individuals in more than two generations. Among a collection of putative candidate loci, a few, appear to be stronger, taking

into account our knowledge for the disease's pathogenesis.

For example, interleukins are good candidates because of the fact that amyloid deposition, as an acute response, may happen via increased interleukin-1 (IL-1) production induced by injury or a defect in its expression, which in turn induces an acute phase response within the brain leading to increased synthesis of both α1-antichymotrypsin and APP (Royston *et al*.1992). Excessive APP production may have the same effect as an extra dosage of the gene seen in Down syndrome.

Other genes could involve transcription factors binding to the APP promoter (Salbaum *et al*.1988) and regulating APP expression like the heat schock protein HSP70, or genes coding for enzymes involved in APP processing like the lysosomal enzymes cathepsins.

One putative way of developing the same pathology as with the mutations would be a defect in the enzyme "secretase" itself, weakening its activity in certain individuals.

Identification of the other causes will help in the understanding of the exact mechanism of the pathogenic process, which will undoubtedly lead to treatments for Alzheimer's disease in the future. Animal models of the disease will be important not only in the dissection of these biochemical processes but also in the testing of drugs to alter them. If the above cascade of events is correct then drugs interfering with the stress

response and the accumulation of acute phase proteins, which upregulate APP, might prove to be helpful in the delay of the symptoms. Although treatment is still a long time away, we can now be more optimistic that with the help of the techniques of molecular genetics and biology, what was an almost impossible dream may now be considered as a realistic hope for the future.

Addendum: chromosome 14 linkage

As this thesis was being finally submitted, our group continuing to use the 9 families in which we knew there was no APP lesion, identified genetic linkage, close to the AACT locus on ch14. From our data it is clear that the pathogenic locus is not AACT, nor is it cathepsin G, which is also on this chromosome. A likely candidate gene, which is now being investigated by sequence analysis, is the heat shock protein HSP70. This is found at approximately the correct genetic location and is a good candidate gene (by the amyloid cascade hypothesis) because it is involved in the upregulation of APP (Abe *et al*.1991). The preliminary data indicate that whatever the nature of this second locus, it is indeed the major locus as all the families in which I have helped exclude APP as the pathogenic locus, show positive evidence for genetic linkage to chromosome 14 markers.

ADDITIONAL REFERENCES

Farrer, L.A., Myers, R.H., Cupples, L.A., St.George-Hyslop, P.H., Bird, T.D., Rossor, M.N., Mullan, M.J., Polinsky, R., Nee, L., Heston, L., et al., (1990). Transmission and age-at-onset patterns in familial Alzheimer's disease: evidence for heterogeneity. Neurology 40, 395-403

Farrer, L.A., Myers, R.H., Connor, L., Cupples, L.A. and Growdon, J.H. (1991). Segregation analysis reveals evidence of a major gene for Alzheimer's disease. Am.J.Hum.Genet. 48, 1026-1033.

Hardy, J.A., Goate, A.M., Owen, M.J., Mullan, M.J., Rossor, M.N., and Pearson, R.C. (1989). Modelling the occurence and pathology of Alzheimer's disease. Neurobiol. Aging 10, 429-431

Mohs, R.C., Breitner, J.C., Silverman, J.M., and Davis, K.L. (1987). Alzheimer's disease. Morbid risk among first degree relatives approximate 50% by 90 years of age. Arch. Gen. Psychiatry 44, 405-408.

REFERENCES

1. ABE, K. , ST.GEORGE HYSLOP, P. H. , TANZI, R. E. , AND KOGURE, K. (1991). Induction of amyloid precursor protein mRNA after heat shock in cultured human lymphoblastoid cells. *Neurosci.Letts.* **125**: 169-171.

2. ABE, K. , TANZI, R. E. , AND KOGURE, K. (1991). Selective induction of Kunitz-type protease inhibitor domain-containing amyloid precursor protein mRNA after persistent focal ischemia in rat cerebral cortex. *Neurosci.Letts.* **125**: 172-174.

3. ABRAHAM, C. R. , DRISCOLL, J. , POTTER, H. , VAN NOSTRAND, W. E. , AND TEMPST, P. (1991). A calcium-activated protease from Alzheimer's disease brain cleaves at the N-terminus of the amyloid beta-protein. *Biochem.Biophys.Res.Commun.* **174**: 790-796.

4. ABRAHAM, C. R. AND POTTER, H. (1989). The protease inhibitor, alpha 1-antichymotrypsin, is a component of the brain amyloid deposits in normal aging and Alzheimer's disease. *Ann.Med.* **21**: 77-81.

5. ABRAHAM, C. R. , SELKOE, D. J. , AND POTTER, H. (1988). Immunochemical identification of the serine protease inhibitor alpha 1-antichymotrypsin in the brain amyloid deposits of Alzheimer's disease. *Cell* **52**: 487-501.

6. ABRAHAM, C. R. , SELKOE, D. J. , POTTER, H. , PRICE, D. L. , AND CORK, L. C. (1989). Alpha 1-antichymotrypsin is present together with the beta- protein in monkey brain amyloid deposits. *Neuroscience* **32**: 715-720.

7. ABRAHAM, C. R. , SHIRAHAMA, T. , AND POTTER, H. (1990). The protease inhibitor α1-antichymotrypsin is associated solely with amyloid deposits containing the ß-protein and is localized in specific cells of both normal and diseased brain. *Neurobiology of Aging* **11**: 123-129.

8. ABRAMS, E. Z. , MURDAUGH, S. E. , AND LERMAN, L. S. (1990). Comprehensive detection of single base changes in human genomic DNA using denaturing gradient gel electrophoresis and a GC clamp. *Genomics* **7**: 463-475.

9. ADROER, R. , CHARTIER-HARLIN, M. C. , CRAWFORD, F. , AND OLIVA, R. (1992). Improved direct sequencing of Alzheimer's amyloid precursor protein (APP) exons 16 and 17. *Neurosci.Letts.* **141**: 69-71.

10. ADROER, R. , LOPEZ-ACEDO, C. , OLIVA, R. , HARDY, J. , AND FIDANI, L. (1992). A novel silent variant at codon 711 and a variant at codon 708 of the APP sequence detected in Spanish Alzheimer and control cases. *Neurosci.Letts.* **in press**:

11. ALTSCHUL, S. F. , GISH, W. , MILLER, W. , MYERS, E. W. , AND LIPMAN, D. J. (1990). Basic local alignment search tool. *J.Mol.Biol.* **215**: 403-410.

12. ANDERSON, J. P. , ESCH, F. S. , KEIM, P. S. , SAMBAMURTI, K. , LIEBERBURG, I. , AND ROBAKIS, N. K. (1991). Exact cleavage site of Alzheimer amyloid precursor in neuronal PC-12 cells. *Neurosci.Lett.* 128: 126-128.

13. BAKKER, E. , VAN BROECKHOVEN, C. , HAAN, J. , VOORHOEVE, E. , VAN HUL, W. , LEVY, E. , LIEBERBURG, I. , CARMAN, M. D. , VAN OMMEN, G. J. B. , FRANGIONE, B. , AND ROOS, R. A. C. (1991). DNA diagnosis for hereditary cerebral hemorrhage with amyloidosis (Dutch type). *Am.J.Hum.Genet.* 49: 518-521.

14. BALBIN, M. , ABRAHAMSON, M. , GUSTAFSON, L. , NILSSON, K. , BRUN, A. , AND GRUBB, A. (1992). A novel mutation in the ß-protein coding region of the amyloid ß-protein precursor (APP) gene. *Hum.Genet.* 89: 580-582.

15. BAO, J. , SIFERS, R. N. , KIDD, V. J. , LEDLEY, F. D. , AND WOO, S. L. C. (1987). Molecular evolution of serpins: homologous structure of the human α1-antichymotrypsin and α1-antitrypsin genes. *Biochemistry* 26: 7755-7759.

16. BATES, G. P. , MACDONALD, M. E. , BAXENDALE, S. , YOUNGMAN, S. , LIN, C. , WHALEY, W. L. , WASMUTH, J. J. , GUSELLA, J. F. , AND LEHRAH, H. (1991). Defined physical limits of the Huntington disease gene candidate region. *Am.J.Hum.Genet.* 49: 7-16.

17. BEAUDET, A. , BOWCOCK, A. , BUCHWALD, M. , CAVALLI-SFORZA, L. , FARRALL, M. , KING, M. C. , KLINGER, K. , LALOUEL, J. M. , LATHROP, G. , NAYLOR, S. , OTT, J. , TSUI, L. C. , WAINWRIGHT, B. , WATKINS, P. , WHITE, R. , AND WILLIAMSON, R. (1986). Linkage of cystic fibrosis to two tightly linked markers: joint report from a collaborative study. *Am.J.Hum.Genet.* 39: 681-693.

18. BENHAM, F. AND ROWE, P. (1992). Use of Alu-PCR to characterize hybrids containing multiple fragments and to generate new Xp21.3-p22.2 markers. *Genomics* 12: 368-376.

19. BERKNER, K. L. AND FOLK, W. R. (1977). Polynucleotide kinase exchange reaction EcoR1 cleavage and methylation of DNAs containing modified pyrimidines in the recognition sequence. *J.Biol.Chem.* 252: 3176.

20. BOTSTEIN, D. , WHITE, R. L. , SKOLNICK, M. , AND DAVIES, R. W. (1980). Construction of a genetic linkage map in Man using restriction fragment length polymorphisms. *Am.J.Hum.Genet.* 32: 314-331.

21. BREITNER, J. C. AND FOLSTEIN, M. F. (1984). Familial Alzheimer Dementia: a prevalent disorder with specific clinical features. *Psychol.Med.* 14: 63-80.

22. BRITTEN, R. J. , BARON, W. F. , STOUT, D. B. , AND DAVIDSON, E. H. (1988). Sources and evolution of human Alu repeated sequences. *Proc.Natl.Acad.Sci.* 85: 4770-4774.

23. BROOKS-WILSON, A. R. , GOODFELLOW, P. N. , POVEY, S. , NEVANLINNA, H. A. , DE JONG, P. J. , AND GOODFELLOW, P. J. (1990). Rapid cloning and characterization of new chromosome 10 DNA markers by Alu element-mediated PCR. *Genomics* 7: 614-620.

24. CAWTHON, R. M. , WEISS, R. , XU, G. , VISKOCHIL, D. , CULVER, M. , STEVENS, J. , ROBERTSON, M. , DUNN, D. , GESTELAND, R. , O'CONNELL, P. , AND WHITE, R. (1990). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* 62: 193-201.

25. CHADEFAUX, B. , ALLARD, O. , RETHORE, M. O. , RAOUL, O. , POISSONNIER, M. , GILGENKRANTZ, S. , CHERUY, C. , AND JEROME, H. (1984). Assignment of human phosphoribosylglycinamide synthetase locus to region 21q22.1. *Hum.Genet.* 66: 190-192.

26. CHARTIER HARLIN, M. C. , CRAWFORD, F. , HAMANDI, K. , MULLAN, M. , GOATE, A. , BACKHOVENS, H. , MARTIN, J. J. , AND VAN BROECKHOVEN, C. (1991). Screening for the ß-amyloid precursor protein mutation (APP:Val->Ile) in extended pedigrees with early onset Alzheimer's disease. *Neurosci.Letts.* 129: 134-135.

27. CHARTIER HARLIN, M. C. , CRAWFORD, F. , HOULDEN, H. , WARREN, A. , HUGHES, D. , FIDANI, L. , GOATE, A. , ROSSOR, M. , ROQUES, P. , HARDY, J. , AND MULLAN, M. (1991). Early onset Alzheimer's disease caused by mutations at codon 717 of the ß-amyloid precursor protein gene. *Nature* 353: 844-846.

28. CHARTIER-HARLIN, M. -C. (1991). Amyloid protein and early onset familial Alzheimer's disease. *Bull.Inst.Pasteur* 89: 159-186.

29. COTTER, F. E. , HAMPTON, G. M. , NASIPURI, S. , BODMER, W. F. , AND YOUNG, B. D. (1990). Rapid isolation of human chromosome-specific DNA probes from a somatic cell hybrid. *Genomics* 7: 257-263.

30. CRAWFORD, F. , HARDY, J. , MULLAN, M. , GOATE, A. , HUGHES, D. , FIDANI, L. , ROQUES, P. , ROSSOR, M. , AND CHARTIER-HARLIN, M. C. (1991). Sequencing of exons 16 and 17 of the ß-amyloid precursor protein gene in families with early onset Alzheimer's disease fails to reveal mutations in the ß-amyloid sequence. *Neurosci.Letts.* 133: 1-2.

31. DE SAUVAGE, F. AND OCTAVE, J. N. (1989). A novel mRNA of the A4 amyloid precursor gene coding for a possibly secreted protein. *Science* 245: 651-653.

32. DEAN, M. , WHITE, M. B. , AMOS, J. , GERRARD, B. , STEWART, C. , KHAW, K-T. , AND LEPPERT, M. (1990). Multiple mutations in highly conserved residues are found in mildly affected cystic fibrosis patients. *Cell* 61: 863-870.

33. DEININGER, P. L. , JOLLY, D. J. , RUBIX, C. M. , FRIEDMAN, T. , AND SCHMID, C. W. (1981). Base sequence studies of 300 nucleotide renatured repeated human DNA clones. *Journal of Molecular Biology* 151: 17-33.

34. DELABAR, J. M. , GOLDGABER, D. , LAMOUR, Y. , NICOLE, A. , HURET, J. L. , DE GROUCHY, J. , BROWN, P. , GAJDUSEK, D. C. , AND SINET, P. M. (1987). Beta amyloid gene duplication in Alzheimer's disease and karyotypically normal Down syndrome. *Science* 235: 1390-1392.

35. DIETZ, H. C. , CUTTING, G. R. , PYERITZ, R. E. , MASLEN, C. L. , SAKAI, L. Y. , CORSON, G. M. , PUFFENBERGER, E. G. , HAMOSH, A. , NANTHAKUMAR, E. J. , CURRISTIN, S. M. , STETTEN, G. , MEYERS, D. A. , AND FRANCOMANO, C. A. (1991). Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature* 352: 337-339.

36. ECONOMOU, E. P. , BERGEN, A. W. , WARREN, A. C. , AND ANTONARAKIS, S. E. (1990). The polydeoxyadenylate tract of Alu repetitive elements is polymorphic in the human genome. *Proc.Natl.Acad.Sci* 87: 2951-2954.

37. EIKELENBOOM, P. , HACK, C. E. , ROZEMULLER, J. M. , AND STAM, F. C. (1989). Complement activation in amyloid plaques in Alzheimer's dementia. *Virchows Arch.B.* 56: 259-262.

38. ESCH, F. S. , KEIM, P. S. , BEATTIE, E. C. , BLACHER, R. W. , CULWELL, A. R. , OLTERSDORF, T. , MCCLURE, D. , AND WARD, P. J. (1990). Cleavage of amyloid beta peptide during constitutive processing of its precursor. *Science* 248: 1122-1124.

39. ESTUS, S. , GOLDE, T. E. , KUNISHITA, T. , BLADES, D. , LOWERY, D. , EISEN, M. , USIAK, M. , QU, X. , TABIRA, T. , GREENBERG, B. D. , AND YOUNKIN, S. G. (1992). Potentially amyloidogenic, carboxyl terminal derivatives of the amyloid protein precursor. *Science* 255: 726-728.

40. FABER, J-P. , POLLER, W. , WEIDINGER, S. , LINDMARK, B. , ERIKSSON, S. , AND OLEK, K. (1991). Identification of point mutations in the Alpha-1-antichymotrypsin gene. *The American Journal of Human Genetics* Supplement, 49: 134.(Abstract)

41. FEINBERG, A. P. AND VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal.Biochem.* 132: 6-13.

42. FIDANI, L. , ROOKE, K. , CHARTIER-HARLIN, M. C. , HUGHES, D. , TANZI, R. , MULLAN, M. , ROQUES, P. , ROSSOR, M. , HARDY, J. , AND GOATE, A. M. (1992). Screening for mutations in the open reading frame and promoter of the ß-amyloid precursor protein gene in familial Alzheimer's disease: identification of a further family with APP717 Val-> Ile. *Hum.Mol.Genet.* 1,3: 165-168.

43. FRASER, B. M. , POON, M-C. , AND HOAR, D. I. (1992). Identification of factor IX mutations in haemophilia B: application of polymerase chain reaction and single strand conformation analysis. *Hum.Genet.* 88: 426-430.

44. GARDINER, K. , HORISBERGER, M. , KRAUS, J. , TANTRAVAHI, U. , KORENBERG, J. , RAO, V. , REDDY, S. , AND PATTERSON, D. (1990). Analysis of human chromosome 21: correlation of physical and cytogenetic maps; gene and CpG island distributions. *EMBO* 9,1: 25-34.

45. GLENNER, G. G. AND WONG, C. W. (1984). Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem.Biophys.Res.Commun.* 122: 1131-1135.

46. GLENNER, G. G. AND WONG, C. W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem.Biophys.Res.Commun.* 120: 885-890.

47. GOATE, A. , CHARTIER HARLIN, M. C. , MULLAN, M. , BROWN, J. , CRAWFORD, F. , FIDANI, L. , GIUFFRA, L. , HAYNES, A. , IRVING, N. , JAMES, L. ,MANT, R. ,NEWTON, P. ,ROOKE, K. ,ROQUES, P., TALBOT, C. ,PERICAK-VANCE, M. ,ROSES, A. ,WILLIAMSON, R. ,ROSSOR, M. ,OWEN, M. ,HARDY, J. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349: 704-706.

48. GOATE, A. M. , HAYNES, A. R. , OWEN, M. J. , FARRALL, M. , JAMES, L. A. , LAI, L. Y. , MULLAN, M. J. , ROQUES, P. , ROSSOR, M. N. , WILLIAMSON, R. ,HARDY, J. (1989). Predisposing locus for Alzheimer's disease on chromosome 21. *Lancet* 1: 352-355.

49. GOLDE, T. E. , ESTUS, S. , USIAK, M. , YOUNKIN, L. H. , AND YOUNKIN, S. G. (1990). Expression of beta amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR. *Neuron* 4: 253-267.

50. GOLDE, T. E. , ESTUS, S. , YOUNKIN, L. H. , SELKOE, D. J. , AND YOUNKIN, S. G. (1992). Processing of the amyloid protein precursor to potentially amyloidogenic fragments. *Science* 255: 728-730.

51. GOLDFARB, L. G. , KORCZYN, A. D. , BROWN, P. , CHAPMAN, J. , AND GAJDUSEK, D. C. (1990). Mutation in codon 200 of scrapie amyloid precursor protein gene linked to Creutzfeldt-Jakob disease in Sephardic Jews of Libyan and non-Libyan origin. *Lancet* 336: 637-638.

52. GOLDGABER, D. , LERMAN, M. I. , MCBRIDE, O. W. , SAFFIOTTI, U. , AND GAJDUSEK, D. C. (1987). Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science* 235: 877-880.

53. GOLDGABER, D. , LERMAN, M. I. , MCBRIDE, W. O. , SAFFIOTTI, U. , AND GAJDUSEK, D. C. (1987). Isolation, characterization, and chromosomal localization of human brain cDNA clones coding for the precursor of the amyloid of brain in Alzheimer's disease, Down's syndrome and aging. *J.Neural Transm.Suppl.* 24: 23-28.

54. GRIMALDI, G. , SKOWRONSKI, J. , AND SINGER, M. F. (1984). Defining the beginning and end of KpnI family segments. *EMBO* 3,8: 1753-1759.

55. GUSELLA, J. , VARSANYI-BREINER, A. , KAO, F-T. , JONES, C. , PUCK, T. T. , KEYS, C. , ORKIN, S. , AND HOUSMAN, D. (1979). Precise localization of human ß-globin gene complex on chromosome 11. *Proc.Natl.Acad.Sci.* 76,10: 5239-5243.

56. HAASS, C. , SCHLOSSMACHER, M. G. , HUNG, A. Y. , VIGO-PELFREY, C. , MELLON, A. , OSTASZEWSKI, B. L. , LIEBERBURG, I. , KOO, E. H. , SCHENK, D. , TEPLOW, D. B. , AND SELKOE, D. J. (1992). Amyloid ß-peptide is produced by cultured cells during normal metabolism. *Nature* 359: 322-325.

57. HARDY, J. AND ALLSOP, D. (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol*. 12: 383-388.

58. HARDY, J. , MULLAN, M. , CHARTIER HARLIN, M. C. , BROWN, J. , GOATE, A. , ROSSOR, M. , COLLINGE, J. , ROBERTS, G. , LUTHERT, P. , LANTOS, P. , NARUSE, S. , KANEKO, K. , TSUJI, S. , MIYATAKE, T. , SHIMIZU, T. , KOJIMA, T. , NAKANO, I. , YOSHIOKA, K. , SAKAKI, Y. , MIKI, T. , KATSUYA, T. , OGIHARA, T. , ROSES, A.,, PERICAK-VANCE, M. , HAAN, J. , ROOS, R. , LUCOTTE, G. , AND FAVID, F. (1991). Molecular Classification of Alzheimer's disease. *Lancet* 337: 1342-1343.

59. HARDY, J. A. AND HIGGINS, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 286: 184-185.

60. HARDY, J. A. , MANN, D. M. , WESTER, P. , AND WINBLAD, B. (1986). An integrative hypothesis concerning the pathogenesis and progression of Alzheimer's disease. *Neurobiol.Aging* 7: 489-502.

61. HARRISON, P. J. , BARTON, A. J. L. , AND PEARSON, R. C. A. (1991). Expression of amyloid ß-protein precursor mRNAs in familial Alzheimer's disease. *Neurorep*. 2: 152-154.

62. HAYASHI, K. (1991). Single Strand Conformation Analysis. *PCR Methods and Applications* 1: 34-38.

63. HESTON, L. , MASTRI, A. , ANDERSON, V. , AND WHITE, J. (1981). Dementia of the Alzheimer type: clinical genetics, natural history and associated conditions. *Arch.Gen.Psychiatry* 38: 1085-1090.

64. HESTON, L. L. , ORR, H. T. , RICH, S. R. , AND WHITE, J. A. (1991). Linkage of an Alzheimer disease susceptibility locus to markers on human chromosome 21. *Am.J.Med.Genet*. 40: 449-453.

65. HOUCK, C. M. , RINEHART, F. P. , AND SCHMID, C. W. (1979). A ubiquitous family of repeated DNA sequences in the human genome. *J.Mol.Biol*. 132: 289-306.

66. HSIAO, K. , BAKER, H. F. , CROW, T. J. , POULTER, M. , OWEN, F. , TERWILLIGER, J. D. , WESTAWAY, D. , OTT, J. , AND PRUSINER, S. B. (1989). Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. *Nature* 338: 342-345.

67. HUBBARD, B. M. AND ANDERSON, J. M. (1985). Age-related variations in the neuron content of the cerebral cortex in senile dementia of Alzheimer type. *Neuropath.Appl.Neurobiol*. 11: 369-382.

68. HULTMAN, T. , STAHL, S. , HORNES, E. , AND UHLEN, M. (1989). Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucl.Acids Res*. 17: 4937-4946.

69. JELINEK, W. R. AND SCHMID, C. W. (1982). Repetitive sequences in eukaryotic DNA and their expression. *Ann.Rev.Biochem*. 51: 813-844.

70. JOHNSON, P. H. , CADIOU, H. , AND HOPKINSON, D. A. (1991). Detection of the common alpha-1-antitrypsin variants by denaturing gradient gel electrophoresis. *Ann.Hum.Genet*. 55: 183-198.

71. JUNG, V. , PESTKA, S. B. , AND PESTKA, S. (1990). Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites. *Nucl.Acids Res.* 18: 6156.

72. KANG, J. , LEMAIRE, H. G. , UNTERBECK, A. , SALBAUM, J. M. , MASTERS, C. L. , GRZESCHIK, K. H. , MULTHAUP, G. , BEYREUTHER, K. , AND MULLER HILL, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325: 733-736.

73. KANG, J. AND MULLER HILL, B. (1990). Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4(695) mRNA is predominantly produced in rat and human brain. *Biochem.Biophys.Res.Commun.* 166: 1192-1200.

74. KARLINSKY, H. , VAULA, G. , HAINES, J. L. , RIDGLEY, J. , BARGERON, C. , MORTILLA, M. , TUPLER, R. G. , PERCY, M. E. , ROBITAILLE, Y. , NOLDY, N. E. , YIP, T. C. K. , TANZI, R. E. , GUSELLA, J. F. , BOOKER, R. , BORG, J. M. , CRAPPER MCLACHLAN, D. R. , AND ST.GEORGE-HYSLOP, P. H. (1992). Molecular and prospective phenotypic characterization of a pedigree with familial Alzheimer's disease and a missense mutation in codon 717 of the ß-amyloid precursor protein gene. *Neurology* 42: 1445-1452.

75. KAWARABAYASHI, T. , SHOJI, M. , HARIGAYA, Y. , YAMAGUCHI, H. , AND HIRAI, S. (1991). Expression of APP in the early stage of brain damage. *Brain Res.* 563: 334-338.

76. KAZAZIAN JR, H. H. , WONG, C. , YOUSSOUFIAN, H. , SCOTT, A. F. , PHILLIPS, D. G. , AND ANTONARAKIS, S. E. (1988). Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. *Nature* 332: 164-166.

77. KITAGUCHI, N. , TAKAHASHI, Y. , TOKUSHIMA, Y. , SHIOJIRI, S. , AND ITO, H. (1988). Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature* 331: 530-532.

78. KLAUSNER, R. D. AND HARFORD, J. B. (1989). Cis-Trans models for post transcriptional gene regulation. *Science* 246: 870-872.

79. KOO, E. H. , SISODIA, S. S. , ARCHER, D. R. , MARTIN, L. J. , WEIDEMANN, A. , BEYREUTHER, K. , FISCHER, P. , MASTERS, C. L. , AND PRICE, D. L. (1990). Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. *Proc.Natl.Acad.Sci.U.S.A.* 87: 1561-1565.

80. KORENBERG, J. R. AND RYKOWSKI, M. C. (1988). Human genome organisation: Alu, Lines, and the molecular structure of metaphase chromosome bands. *Cell* 53: 391-400.

81. KURDOWSKA, A. AND TRAVIS, J. (1990). Acute phase protein stimulation by α1-antichymotrypsin-cathepsin G complexes. Evidence for the involvement of interleukin-6. *J.Biol.Chem* 265,34: 21023-21026.

82. LABRUNE, P. , MELLE, D. , REY, F. , BERTHELON, M. , CAILLAUD, C. , REY, J. , MUNNICH, A. , AND LYONNET, S. (1991). Single strand conformation polymorphism for detection of mutations and base substitutions in phenylketonuria. *Am.J.Hum.Genet.* 48: 1115-1120.

83. LATHROP, G. , LALOUEL, J. M. , JULIER, C. , AND OTT, J. (1984). Strategies for multilocus linkage analysis in humans. *Proc.Natl.Acad.Sci.U.S.A.* **81**: 3443-3446.

84. LATHROP, G. M. , LALOUEL, J. M. , JULIER, C. , AND OTT, J. (1984). Strategies for multilocus linkage analysis in humans. *Proc.Natl.Acad.Sci.* **81**: 3443-3446.

85. LEDBETTER, S. A. , NELSON, D. L. , WARREN, S. T. , AND LEDBETTER, D. H. (1990). Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence polymerase reaction. *Genomics* **6**: 475-481.

86. LEE, V. M. , BALIN, B. J. , OTVOS, L. J. , AND TROJANOWSKI, J. Q. (1991). A68: a major subunit of paired helical filaments and derivatized forms of normal Tau. *Science* **251**: 675-678.

87. LEHESJOKI, A-E. , KOSKINIEMI, M. , SISTONEN, P. , MIAO, J. , HASTBACKA, J. , NORIO, R. , AND DE LA CHAPELLE, A. (1991). Localisation of a gene for progressive myoclonus epilepsy to chromosome 21q22. *Proc.Natl.Acad.Sci.* **88**: 3696-3699.

88. LEVY, E. , CARMAN, M. D. , FERNANDEZ MADRID, I. J. , POWER, M. D. , LIEBERBURG, I. , VAN DUINEN, S. G. , BOTS, G. T. , LUYENDIJK, W. , AND FRANGIONE, B. (1990). Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* **248**: 1124-1126.

89. LONG, G. L. , CHANDRA, T. , WOO, S. L. C. , DAVIE, E. W. , AND KURACHI, K. (1984). Complete sequence of the cDNA for human α1-antitrypsin and the gene for the S variant. *Biochemistry* **23**: 4828-4837.

90. LOWE, T. , SHAREFKIN, J. , YANG, S. Q. , AND DIEFFENBACH, C. W. (1990). A computer program for selection of oligonucleotide primers for polymerase chain reactions. *Nucl.Acids Res.* **18,7**: 1757-1761.

91. MANIATIS, T., E. F. FRITSCH, AND J. SAMBROOK. 1982. *Molecular Cloning : A Laboratory Manual*, Cold Spring Harbor,

92. MANN, D. M. AND ESIRI, M. M. (1989). The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down's syndrome. *J.Neurol.Sci.* **89**: 169-179.

93. MANN, D. M. , YATES, P. O. , AND MARCYNIUK, B. (1984). Alzheimer's presenile dementia, senile dementia of Alzheimer type and Down's syndrome in middle age form an age related continuum of pathological changes. *Neuropathol.Appl.Neurobiol.* **10**: 185-207.

94. MASTERS, C. L. , SIMMS, G. , WEINMAN, N. A. , MULTHAUP, G. , MCDONALD, B. L. , AND BEYREUTHER, K. (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc.Natl.Acad.Sci.U.S.A.* **82**: 4245-4249.

95. MATTSON, M. P. , CHENG, B. , DAVIS, D. , BRYANT, K. , LIEBERBURG, I. , AND RYDEL, R. E. (1992). ß-amyloid peptides destabilise Calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J.Neurosci.* **12**: 376-389.

96. MCCONLOGUE, L . , BROW, M. D. , AND INNIS, M. A. (1988). Structure-independent DNA amplification by PCR using 7-deaza-2'-deoxyguanosine. *Nucl.Acids Res.* 16,20: 9869.

97. MOYZIS, R. K. , TORNEY, D. C. , MEYNE, J. , BUCKINGHAM, J. M. , WU, J-R. , BURKS, C. , SIROTKIN, K. M. , AND GOAD, W. B. (1989). The distribution of interspersed repetitive DNA sequences in the human genome. *Genomics* 4: 273-289.

98. MULLAN, M. , CRAWFORD, F. , AXELMAN, K. , HOULDEN, H. , LILIUS, L. , WINBLAD, B. , AND LANNFELT, L. (1992). A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of ß-amyloid. *nature genet.* 1: 345-347.

99. MURRELL, J. , FARLOW, M. , GHETTI, B. , AND BENSON, M. (1991). A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254: 97-99.

100. MYERS, R.M., V. C. SHEFFIELD, AND D. R. COX. 1988. Detection of single base changes in DNA: ribonuclease cleavage and denaturing gradient gel electrophoresis. Pages 95-139 in K.E. DAVIES, Ed., *Genome analysis*. IRL, Oxford.

101. NARUSE, S. , IGARASHI, S. , KOBAYASHI, H. , AOKI, K. , INUZUKA, T. , KANEKO, K. , SHIMIZU, T. , IIHARA, K. , KOJIMA, T. , MIYATAKE, T. , AND TSUJI, S. (1991). Mis-sense mutation Val->Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. *Lancet* 337: 978-979.

102. NELSON, D. L. , LEDBETTER, S. A. , CORBO, L. , VICTORIA, M. F. , RAMIREZ-SOLIS, R. , WEBSTER, T. D. , LEDBETTER, D. H. , AND CASKEY, C. T. (1989). Alu polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources. *Proc.Natl.Acad.Sci.* 86: 6686-6690.

103. NEVE, R. L. , ROGERS, J. , AND HIGGINS, G. A. (1990). The Alzheimer amyloid precursor-related transcript lacking the beta/A4 sequence is specifically increased in Alzheimer's disease brain. *Neuron* 5: 329-338.

104. OLIVER, C. AND HOLLAND, A. J. (1986). Down's syndrome and Alzheimer's disease: a review. *Psychol.Med.* 16: 307-322.

105. ORITA, M. , SEKIYA, T. , AND HAYASHI, K. (1990). DNA sequence polymorphisms in Alu repeats. *Genomics* 8: 271-278.

106. ORITA, M. , SUZUKI, Y. , SEKIYA, T. , AND HAYASHI, K. (1989). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5: 874-879.

107. OTT, J. (1990). Invited editorial: Cutting a Gordian knot in the linkage analysis of complex human traits. *Am.J.Hum.Genet.* 46: 219-221.

108. OWEN, F. , POULTER, M. , COLLINGE, J. , AND CROW, T. J. (1990). Codon 129 changes in the prion gene in Caucasians. *Am.J.Hum.Genet.* 46: 1215-1216.

109. PASTERNACK, J. M. , ABRAHAM, C. R. , VAN DYKE, B. J. , POTTER, H. , AND YOUNKIN, S. G. (1989). Astrocytes in Alzheimer's disease gray matter express alpha 1- antichymotrypsin mRNA. *Am.J.Pathol.* 135: 827-834.

110. PATTERSON, D. , GRAW, S. , AND JONES, C. (1981). Demonstration, by somatic cell genetics, of coordinate regulation of genes for two enzymes of purine synthesis assigned to human chromosome 21. *Proc.Natl.Acad.Sci.* 78,1: 405-409.

111. PERRY, G. , FRIEDMAN, R. , SHAW, G. , AND CHAU, V. (1987). Ubiquitin is detected in neurofibrillary tangles and senile plaque neurites of Alzheimer disease brains. *Proc.Natl.Acad.Sci.U.S.A.* 84: 3033-3036.

112. PETERSEN, M. B. , SLAUGENHAUPT, S. A. , LEWIS, J. G. , WARREN, A. C. , CHAKRAVARTI, A. , AND ANTONARAKIS, S. E. (1991). A genetic linkage map of 27 markers on human chromosome 21. *Genomics* 9: 407-419.

113. PONTE, P. , GONZALEZ-DE WHITT, P. , SCHILLING, J. , MILLER, J. , HSU, D. , GREENBERG, B. , DAVIS, K. , WALLACE, W. , LIEBERBURG, I. , FULLER, F. , AND CORDELL, B. (1988). A new A4 anyloid mRNA contains a domain homologous to serine protease inhibitors. *Nature* 331: 525-527.

114. PRITCHARD, C. , COX, D. R. , AND MYERS, R. M. (1991). Invited editorial: The end in sight for Huntington disease. *Am.J.Hum.Genet.* 49: 1-6.

115. PRUSINER, S. B. (1991). Molecular biology of prion diseases. *Science* 252: 1515-1522.

116. RABIN, M. , WATSON, M. , KIDD, V. , WOO, S. L. C. , BREG, W. R. , AND RUDDLE, F. H. (1986). Regional location of alpha-1-antichymotrypsin and alpha-1-antitrypsin genes on human chromosome 14. *Somat.Cell Molec.Genet.* 12: 209-214.

117. ROBAKIS, N. K. , WISNIEWSKI, H. M. , JENKINS, E. C. , DEVINE GAGE, E. A. , HOUCK, G. E. , YAO, X. L. , RAMAKRISHNA, N. , WOLFE, G. , SILVERMAN, W. P. , AND BROWN, W. T. (1987). Chromosome 21q21 sublocalisation of gene encoding beta-amyloid peptide in cerebral vessels and neuritic (senile) plaques of people with Alzheimer disease and Down syndrome. *Lancet* 1: 384-385.

118. ROBERTS, G. W. , GENTLEMAN, S. M. , LYNCH, A. , AND GRAHAM, D. I. (1991). ß/A4 amyloid protein deposition in brain after head trauma. *Lancet* 338: 1422-1423.

119. ROMMENS, J. M. , IANUZZI, M. C. , KEREM, B. , DRUMM, M. L. , MELMER, G. , DEAN, M. , ROZMAHEL, R. , COLE, J. L. , KENNEDY, D. , HIDAKA, N. , ZSIGA, M. , BUCHWALD, M. , RIORDAN, J. R. , TSUI, L. C. , AND COLLINS, F. C. (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 245: 1059-1065.

120. ROTHWELL, N. J. (1991). Functions and mechanisms of interleukin 1 in the brain. *Trends Pharmacol.* 12: 430-436.

121. ROUYER, F. , SIMMLER, M-C. , PAGE, D. C. , AND WEISSENBACH, J. (1987). A sex chromosome rearrangement in a human XX male caused by Alu-Alu recombination. *Cell* 51: 417-425.

122. ROYSTON, M. C. , ROTHWELL, N. J. , AND ROBERTS, G. W. (1992). Alzheimer's disease: pathology to potential treatments. *Trends Pharmacol*. 13: 131-133.

123. ROZEMULLER, J. M. , BOTS, G. T. A. M. , ROOS, R. A. C. , AND EIKELENBOOM, P. (1992). Acute phase proteins but not activated microglial cells are present in parenchymal b/A4 deposits in the brains of patients with hereditary cerebral hemorrhage with amyloidosis-Dutch type. *Neurosci.Letts*. 140: 137-140.

124. RUMBLE, B. , RETALLACK, R. , HILBICH, C. , SIMMS, G. , MULTHAUP, G. , MARTINS, R. , HOCKEY, A. , MONTGOMERY, P. , BEYREUTHER, K. , AND MASTERS, C. L. (1989). Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. *N.Engl.J.Med*. 320: 1446-1452.

125. SAIKI, R. K. , GELFAND, D. H. , STOFFEL, S. , SCHARF, S. J. , HIGUCHI, R. , HORN, G. T. , MULLIS, K. B. , AND ERLICH, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

126. SAINZ, J. , PEVNY, L. , WU, Y. , CANTOR, C. R. , AND SMITH, C. L. (1992). Distribution of interspersed repeats (Alu and Kpn) on Not I restriction fragments of human chromosome 21. *Proc.Natl.Acad.Sci*. 89: 1080-1084.

127. SALBAUM, J. M. , WEIDEMANN, A. , LEMAIRE, H. G. , MASTERS, C. L. , AND BEYREUTHER, K. (1988). The promoter of Alzheimer's disease amyloid A4 precursor gene. *EMBO J*. 7: 2807-2813.

128. SANGER, F. , NICKLEN, S. , AND COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc.Natl.Acad.Sci*. 74: 5463-5467.

129. SARKAR, G. , KAPELNER, S. , AND SOMMER, S. S. (1990). Formamide can dramatically improve the specificity of PCR. *Nucl.Acids Res*. 18,24: 7465.

130. SCHELLENBERG, G. D. , ANDERSON, L. , O'DAHL, S. , WIJSMAN, E. , SADOVNICK, A. D. , BALL, M. J. , LARSON, E. B. , KUKULL, W. A. , MARTIN, G. M. , ROSES, A. D. , AND BIRD, T. D. (1991). APP717, APP693, and PRIP gene mutations are rare in Alzheimer's disease. *Am.J.Hum.Genet*. 49: 511-517.

131. SCHELLENBERG, G. D. , BIRD, T. D. , WIJSMAN, E. M. , MOORE, D. K. , BOEHNKE, M. , BRYANT, E. M. , LAMPE, T. H. , NOCHLIN, D. , SUMI, S. M. , DEEB, S. S. , BEYREUTHER, K. , AND MARTIN, G. M. (1988). Absence of linkage of chromosome 21q21 markers to familial Alzheimer's disease. *Science* 241: 1507-1510.

132. SCHMID, C. W. AND JELINEK, W. R. (1982). The Alu family of dispersed repetitive sequences. *Science* 216: 1065-1070.

133. SCHROEDER, W. T. , MILLER, M. F. , WOO, S. L. C. , AND SAUNDERS, G. F. (1985). Chromosomal localization of the human α-1-Antitrypsin gene (PI) to 14q31-32. *The American Journal of Human Genetics* 37: 868-872.

134. SCOTT, A. F. , SCHMECKPEPER, B. J. , ABDELRAZIK, M. , THEISEN COMEY, C. , O'HARA, B. , PRATT ROSITTER, J. , COOLEY, T. , HEATH, P. , SMITH, K. D. , AND MARGOLET, L. (1987). Origin of the human L1 elements: Proposed progenitor genes deduced from a consensus DNA sequence. *Genomics* 1: 113-125.

135. SEALEY, P. G. , WHITTAKER, P. A. , AND SOUTHERN, E. M. (1985). Removal of repeated sequences from hybridisation probes. *Nucl.Acids Res.* 13: 1905-1922.

136. SEFTON, L. , KELSEY, G. , KEARNEY, P. , POVEY, S. , AND WOLFE, J. (1990). A physical map of the human PI and AACT genes. *Genomics* 7: 382-388.

137. SEUBERT, P. , VIGO-PELFREY, C. , ESCH, F. , LEE, M. , DOVEY, H. , DAVIS, D. , SINHA, S. , SCHLOSSMACHER, M. G. , WHALEY, J. , SWINDLEHURST, C. , MCCORMACK, R. , WOLFERT, R. , SELKOE, D. , LIEBERBURG, I. , AND SCHENK, D. (1992). Isolation and quantification of soluble Alzheimer's ß-peptide from biological fluids. *Nature* 359: 325-327.

138. SGARAMELLA, V. AND KHORANA, H. G. (1972). Studies on polynucleotides. A further study of the T4 ligase-catalyzed joining of DNA at base-paired ends. *Journal of Molecular Biology* 72: 493-502.

139. SHOJI, M. , GOLDE, T. E. , CHEUNG, T. T. , GHIAO, J. , SHAFFER, L. M. , CAI, X-D. , TINTNER, R. , FRANGIONE, B. , AND YOUNKIN, S. G. (1992). Normal processing produces the Alzheimer amyloid ß protein. *Science* in press:

140. SHOJI, M. , HIRAI, S. , YAMAGUCHI, H. , HARIGAYA, Y. , ISHIGURO, K. , AND MATSUBARA, E. (1991). Alpha 1-antichymotrypsin is present in diffuse senile plaques. A comparative study of beta-protein and alpha 1-antichymotrypsin immunostaining in the Alzheimer brain. *Am.J.Pathol.* 138: 247-257.

141. SHULDINER, A. R. , SCOTT, L. A. , AND ROTH, J. (1990). PCR-induced (ligase-free) subcloning: a rapid reliable method to subclone polymerase chain reaction (PCR) products. *Nucl.Acids Res.* 18,7: 1920.

142. SIDDIQUE, T. , FIGLEWICZ, D. A. , PERICAK-VANCE, M. , HAINES, J. L. , ROULEAU, G. , JEFFERS, A. J. , SAPP, P. , HUNG, W. Y. , BEBOUT, J. , MCKENNA-YASEK, D. , DENG, G. , HORVITZ, H. R. , GUSELLA, J. F. , BROWN, R. H. , AND ROSES, A. D. (1991). Linkage of a gene causing familial amyotrophic lateral sclerosis to chromosome 21 and evidence of genetic-locus heterogeneity. *N.Engl.J.Med.* 324: 1381-1384.

143. SISODIA, S. S. (1992). ß-amyloid precursor protein cleavage by a membrane-bound protease. *Proc.Natl.Acad.Sci.* 89: 6075-6079.

144. SMITH, L. M. , SANDERS, J. Z. , KAISER, R. J. , HUGHES, P. , DODD, C. , CONNELL, C. R. , HEINER, C. , KENT, S. B. H. , AND HOOD, L. E. (1986). Fluorescence detection in automated DNA sequence analysis. *Nature* 321: 674-679.

145. SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments transferred to nitrocellulose. *J.Mol.Biol.* 98: 503-517.

146. SPINARDI, L. , MAZARS, R. , AND THEILLET, C. (1991). Protocols for an improved detection of point mutations by SSCP. *Nucl.Acids Res.* 19,14: 4009.

147. ST GEORGE HYSLOP, P. H. , HAINES, J. L. , FARRER, L. A. , POLINSKY, R. , VAN BROECKHOVEN, C. , A. , MCLACHLAN, D. R. , ORR, H. , BRUNI, A. C. , SORBI, S. , RAINERO, I. ,FONCIN, JF. ,POLLEN, D. ,CANTU, JM. ,TUPLER, R. ,VOSKRESENSKAYA, N. ,MAYEX, R. ,GROWDON, J. ,FRIED, VA. ,MYERS, RH. ,NEE, L. ,BACHOVENS, H. ,MARTIN, JJ. ,ROSSOR, M. ,OWEN, M. ,MULLAN, M. ,PERCY, ME. ,KARLINSKY, H. ,RICH, S. ,HESTON, L. ,MONTESI, M. ,MORTILLA, M. ,NACMIAS, N. ,GUSELLA, JF. ,HARDY, J. & other members of the FAD collaborative study group (1990). Genetic linkage studies suggest that Alzheimer's disease is not a single homogeneous disorder. FAD Collaborative Study Group. *Nature* 347: 194-197.

148. ST GEORGE HYSLOP, P. H. , TANZI, R. E. , POLINSKY, R. J. , HAINES, J. L. , NEE, L. , WATKINS, P. C. , MYERS, R. H. , FELDMAN, R. G. , POLLEN, D. , DRACHMAN, D. , GROWDON, J. ,BRUNI, A. ,FONCIN, JF. ,SALMON, D. ,FROMMELT, P. ,AMADUCCI, L. ,SORBI, S. ,PIACENTINI, S. ,STEWART, GD. ,HOBBS, WJ. ,CONNEALLY, PM. ,GUSELLA, JF. (1987). The genetic defect causing familial Alzheimer's disease maps on chromosome 21. *Science* 235: 885-890.

149. ST GEORGE HYSLOP, P. H. , TANZI, R. E. , POLINSKY, R. J. , NEVE, R. L. , POLLEN, D. , DRACHMAN, D. , GROWDON, J. , CUPPLES, L. A. , NEE, L. , MYERS, R. H. , O'SULLIVAN,D. ,WATKINS, PC. ,AMOS, J. ,DEUTCH, CK. ,BODFISH, JW. ,KINSBOURNE, M. ,FELDMAN, RG. ,BRUNI, A. ,AMADUCCI, L. ,FONCIN, JF. ,GUSELLA, JF. (1987). Absence of duplication of chromosome 21 genes in familial and sporadic Alzheimer's disease. *Science* 238: 664-666.

150. ST.GEORGE HYSLOP, P. H. , MYERS, R. H. , HAINES, J. L. , FARRER, L. A. , TANZI, R. E. , ABE, K. , JAMES, M. F. , CONNEALLY, P. M. , POLINSKY, R. J. , AND GUSELLA, J. F. (1989). Familial Alzheimer's disease: progress and problems. *Neurobiol.Aging* 10: 417-427.

151. SUMPTER, P. Q. , MANN, D. M. , DAVIES, C. A. , YATES, P. O. , SNOWDEN, J. S. , AND NEARY, D. (1986). An ultrastructural analysis of the effects of accumulation of neurofibrillary tangle in pyramidal neurons of the cerebral cortex in Alzheimer's disease. *Neuropathol.Appl.Neurobiol*. 12: 305-319.

152. TANAKA, S. , SHIOJIRI, S. , TAKAHASHI, Y. , KITAGUCHI, N. , ITO, H. , KAMEYAMA, M. , KIMURA, J. , NAKAMURA, S. , AND UEDA, K. (1989). Tissue-specific expression of three types of beta-protein precursor mRNA: enhancement of protease inhibitor-harboring types in Alzheimer's disease brain. *Biochem.Biophys.Res.Commun*. 165: 1406-1414.

153. TANZI, R. E. , GUSELLA, J. F. , WATKINS, P. C. , BRUNS, G. A. , ST GEORGE HYSLOP, P. , VAN KEUREN, M. L. , PATTERSON, D. , PAGAN, S. , KURNIT, D. M. , AND NEVE, R. L. (1987). Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. *Science* 235: 880-884.

154. TANZI, R. E. AND HYMAN, B. T. (1991). Alzheimer's mutation. *Nature* 350: 564.

155. TANZI, R. E. , MCCLATCHEY, A. I. , LAMBERTI, E. D. , VILLA KOMAROFF, L. , GUSELLA, J. F. , AND NEVE, R. L. (1988). Protease inhibitor domain encoded by an amyloid precursor mRNA associated with Alzheimer's disease. *Nature* 331: 528-530.

156. TANZI, R. E. , ST GEORGE HYSLOP, P. H. , HAINES, J. L. , POLINSKY, R. J. , NEE, L. , FONCIN, J. F. , NEVE, R. L. , MCCLATCHEY, A. I. , CONNEALLY, P. M. , AND GUSELLA, J. F. (1987). The genetic defect in familial Alzheimer's disease is not tightly linked to the amyloid beta-protein gene. *Nature* 329: 156-157.

157. TANZI, R. E. , VAULA, G. , ROMANO, D. M. , MORTILLA, M. , HUANG, T. L. , TUPLER, R. G. , WASCO, W. , HYMAN, B. T. , HAINES, J. L. , JENKINS, B. J. , KALAITSIDAKI, M. , WARREN, A. C. , MCINNIS, M. C. , ANTONARAKIS, S. E. , KARLINSKY, H. , PERCY, M. E. , CONNOR, L. , GROWDON, J. , CRAPPER-MCLACHLAN, D. R. , GUSELLA, J. F. , AND ST GEORGE-HYSLOP, P. (1992). Assessment of amyloid ß-protein precursor gene mutations in a large set of familial and sporadic Alzheimer disease cases. *Am.J.Hum.Genet.* 51: 273-282.

158. TERRY, R. D. AND KATZMAN, R. (1983). Senile dementia of the Alzheimer type. *Ann.Neurol.* 14: 497-506.

159. TIERNEY, M. C. , FISHER, R. H. , LEWIS, A. J. , ZORZITTO, M. L. , SNOW, W. G. , REID, D. W. , AND NIEUWSTRATEN, P. (1988). The NINCDS-ADRDA work group criteria for the clinical diagnosis of probable Alzheimer's disease: a clinicopathologic study of 57 cases. *Neurology* 38: 359-364.

160. TRAVIS, J. , GARNER, D. , AND BOWEN, J. (1978). Human α-1-antichymotrypsin: Purification and Properties. *Biochemistry* 17,26: 5647-5656.

161. TSUCHIYA, Y. , HATTORI, M. , HAYASHIDA, K. , ISHIBASHI, H. , OKUBO, H. , AND SAKAKI, Y. (1987). Sequence analysis of the putative regulatory region of rat alpha 2-macroglobulin gene. *Gene* 57: 73-80.

162. VAN BROECKHOVEN, C. , GENTHE, A. M. , VANDENBERGHE, A. , HORSTHEMKE, B. , BACKHOVENS, H. , RAEYMAEKERS, P. , VAN HUL, W. , WEHNERT, A. , GHEUENS, J. , CRAS, P. , BRUYLAND, M./ ,MARTIN, JJ. ,SALBAUM, M. ,MULTHAUP, G. ,MASTERS, CL. ,BEYREUTHER, K. ,GURLING, HMD. ,MULLAN, MJ. ,HOLLAND, A. ,BARTON, A. ,IRVING, N. ,WILLIAMSON, R. ,RICHARDS, SJ. ,HARDY, J. (1987). Failure of familial Alzheimer's disease to segregate with the A4- amyloid gene in several European families. *Nature* 329: 153-155.

163. VAN BROECKHOVEN, C. , HAAN, J. , BAKKER, E. , HARDY, J. A. , VAN HUL, W. , WEHNERT, A. , VEGTER VAN DER VLIS, M. , AND ROOS, R. A. (1990). Amyloid beta protein precursor gene and hereditary cerebral hemorrhage with amyloidosis (Dutch). *Science* 248: 1120-1122.

164. VAN DUIJN, C. M. , CLAYTON, D. , CHANDRA, V. , FRATIGLIONI, L. , GRAVES, A. B. , HEYMAN, A. , JORM, A. F. , KOKMEN, E. , KONDO, K. , MORTIMER, J. A. , ROCCA, W. A. , SHALAT, S. L. , SOININEN, H. , AND HOFMAN, A. (1991). Familial aggregation of Alzheimer's disease and related disorders: A collaborative re-analysis of case-control studies. *Int.J.Epidemiol.* 20 Suppl. 2: S13-S20.

165. VAN DUIJN, C. M. , HENDRIKS, L. , CRUTS, M. , HARDY, J. A. , HOFMAN, A. , AND VAN BROECKHOVEN, C. M. (1991). Amyloid precursor protein gene mutation in early-onset Alzheimer's disease. *Lancet* 337: 978.

166. VAN DUIJN, C. M. , STIJNEN, T. , AND HOFMAN, A. (1991). Risk factors for Alzheimer's disease: Overview of the EURODEM collaborative re-analysis of case-control studies. *Int.J.Epidemiol.* 20 Suppl. 2: S4-S12.

167. VAN DUIJN, C. M. , VAN BROECKHOVEN, C. , HARDY, J. A. , GOATE, A. M. , ROSSOR, M. N. , VANDENBERGHE, A. , MARTIN, J. J. , HOFMAN, A. , AND MULLAN, M. J. (1991). Evidence for allelic heterogeneity in familial early onset Alzheimer's disease. *Brit.J.Psych.* 158: 471-474.

168. VAN DUINEN, S. G. , CASTANO, E. M. , PRELLI, F. , BOTS, G. T. , LUYENDIJK, W. , AND FRANGIONE, B. (1987). Hereditary cerebral hemorrhage with amyloidosis in patients of Dutch origin is related to Alzheimer disease. *Proc.Natl.Acad.Sci.U.S.A.* 84: 5991-5994.

169. VAN KEUREN, M. L. , STEWART, G. D. , BRADLEY, C. M. , KURNIT, D. M. , NEVE, R. L. , WATKINS, P. C. , TANZI, R. E. , GUSELLA, J. F. , AND PATTERSON, D. (1989). Characterization of an unusual and complex chromosome 21 rearrangement using somatic cell genetics and cloned DNA probes. *American Journal of Medical Genetics* 33: 369-375.

170. VAN KEUREN, M. L. , WATKINS, P. C. , DRABKIN, H. A. , JABS, E. W. , GUSELLA, J. F. , AND PATTERSON, D. (1986). Regional localization of DNA sequences on chromosome 21 using somatic cell hybrids. *Am.J.Hum.Genet.* 38: 793-804.

171. VANDENABEELE, P. AND FIERS, W. (1991). Is amyloidogenesis during Alzheimer's disease due to an IL-1-/IL-6-mediated `acute phase response' in the brain. *Immunol.Today* 12: 217-219.

172. VITEK, M. P. , RASOOL, C. G. , DE SAUVAGE, F. , VITEK, S. M. , BARTUS, R. T. , BEER, B. , ASHTON, R. A. , MACQ, A. F. , MALOTEAUX, J. M. , BLUME, A. J. , OCTAVE, JN. (1988). Absence of mutation in the beta-amyloid cDNAs cloned from the brains of three patients with sporadic Alzheimer's disease. *Brain Res.* 464: 121-131.

173. WEBER, J. L. AND MAY, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am.J.Hum.Genet.* 44: 388-396.

174. WEINER, A. M. , DEININGER, P. L. , AND EFSTRATIADES, A. (1986). Nonviral retroposons: Genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. *Ann.Rev.Biochem.* 55: 631-661.

175. WEITKAMP, L. R. , NEE, L. , KEATS, B. , POLINSKY, R. J. , AND GUTTORMSEN, S. (1983). Alzheimer disease: evidence for susceptibility loci on chromosomes 6 and 14. *Am.J.Hum.Genet.* 35: 443-453.

176. WHITE, R. , LEPPERT, M. , BISHOP, D. T. , BARKER, D. , BERKOWITZ, J. , BROWN, C. , CALLAHAN, P. , HOLM, T. , AND JEROMINSKI, L. (1985). Construction of linkage maps with DNA markers for human chromosomes. *Nature* 313: 101-105.

177. YAMAGUCHI, H. , HIRAI, S. , MORIMATSU, M. , SHOJI, M. , AND HARIGAYA, Y. (1988). Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathol.Berl.* 77: 113-119.

178. YANKNER, B. A. , DAWES, L. R. , FISHER, S. , VILLA KOMAROFF, L. , OSTER GRANITE, M. L. , AND NEVE, R. L. (1989). Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science* 245: 417-420.

179. YANKNER, B. A. , DUFFY, L. K. , AND KIRSCHNER, D. A. (1990). Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* 250: 279-282.

180. YOSHIKAI, S. , SASAKI, H. , DOH URA, K. , FURUYA, H. , AND SAKAKI, Y. (1990). Genomic organization of the human amyloid beta-protein precursor gene. *Gene* 87: 257-263.

181. YOSHIOKA, K. , MIKI, T. , KATSUYA, T. , OGIHARA, T. , AND SAKAKI, Y. (1991). The ^{717}Val-->Ile substitution in amyloid precursor protein is associated with familial Alzheimer's disease regardless of ethnic groups. *Biochem.Biophys.Res.Commun.* 178: 1141-1146.

182. ZULIANI, G. AND HOBBS, H. H. (1990). A high frequency of length polymorphisms in repeated sequences adjacent to Alu sequences. *Am.J.Hum.Genet.* 46: 963-969.

Reprinted from Nature, Vol. 349, No. 6311, pp. 704–706, 21st February, 1991
© Macmillan Magazines Ltd., 1991

Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease

Alison Goate*, Marie-Christine Chartier-Harlin*, Mike Mullan*, Jeremy Brown*, Fiona Crawford*, Liana Fidani*, Luis Giuffra†, Andrew Haynes‡, Nick Irving*, Louise James‡, Rebecca Mant‖, Phillippa Newton*, Karen Rooke*, Penelope Roques*, Chris Talbot*, Margaret Pericak-Vance§, Allen Roses§, Robert Williamson*, Martin Rossor*, Mike Owen‖ & John Hardy*¶

* Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St Mary's Hospital Medical School, London W2 1PG, UK
† Department of Human Genetics, Yale University Medical School, 333 Cedar Street, New Haven, Connecticut 06150, USA
§ Duke University Medical Center, Durham, North Carolina NC 27710, USA
‖ Departments of Psychological Medicine and Medical Genetics, University of Wales College of Medicine, Cardiff CF4 4XN, UK

A LOCUS segregating with familial Alzheimer's disease (AD) has been mapped to chromosome 21 (ref. 1), close to the amyloid precursor protein (APP) gene[2–5]. Recombinants between the APP gene and the AD locus have been reported[6–8] which seemed to exclude it as the site of the mutation causing familial AD. But recent genetic analysis of a large number of AD families has demonstrated that the disease is heterogeneous[9]. Families with late-onset AD do not show linkage to chromosome 21 markers[9,10]. Some families with early-onset AD show linkage to chromosome 21 markers, but some do not[8,9,11]. This has led to the suggestion that there is non-allelic genetic heterogeneity even within early onset familial AD[8,9]. To avoid the problems that heterogeneity poses for genetic analysis, we have examined the cosegregation of AD and markers along the long arm of chromosome 21 in a single family with AD confirmed by autopsy. Here we demonstrate that in this kindred, which shows linkage to chromosome 21 markers, there is a point mutation in the APP gene. This mutation causes an amino-acid substitution (Val→Ile) close to the carboxy terminus of the β-amyloid peptide. Screening other cases of familial AD revealed a second unrelated family in which this variant occurs. This suggests that some cases of AD could be caused by mutations in the APP gene.

Segregation of polymorphic DNA markers spanning 55% of the physical and genetic length of the long arm of chromosome 21 was analysed in family F23 in which early-onset AD has been confirmed after autopsy (Fig. 1). These markers, represented by the loci (from centromere to telomere) D21S16, D21S13, D21S1, APP, D21S17, D21S156 and D21S167 (refs 12–16), cover the genetic distance in which the AD locus has been proposed to occur, with the exception of the pericentromere. Two-point linkage analyses (MLINK; ref. 17) were carried out between each of the polymorphic loci and AD (Table 1).

Two individuals with recombinant chromosomes provide evidence for the position of the AD locus (Fig. 1). The unaffected individual in the second sibship who shares the same allele at D21S17 as the affected individuals is 15 years over the mean age of onset of the disease in this family. This recombinant excludes the disease locus from being telomeric to this marker. The individual with a recombinant chromosome in the first sibship is unaffected but is only two years above the mean age of onset within the family (giving odds of two to one against developing the disease based upon the family's risk curve). This person shares the same alleles as the affected individuals between D21S13 and D21S1/S11, but not at APP. If this person were to develop AD in future, then the disease locus must be centromeric of APP and the APP gene is excluded as the disease locus; but if the person remains healthy, then the AD locus must be telomeric of D21S1/S11 and APP is not excluded.

TABLE 1 Two-point linkage analyses between Alzheimer's disease and polymorphic DNA markers on the long arm of chromosome 21

	Recombination fraction (θ)				
Locus	0.00	0.01	0.05	0.10	0.20
D21S16	1.36	1.35	1.28	1.18	0.92
D21S13	2.21	2.17	2.06	1.89	1.44
D21S1	2.65	2.61	2.45	2.22	1.67
APP(*Eco*RI)	2.93	2.88	2.82	2.36	1.72
D21S17	0.45	0.51	0.61	0.63	0.54
D21S156	−6.31	−4.29	−2.73	−1.76	−0.77
D21S167	−8.02	−3.44	−1.88	−1.17	−0.49
APP(*Bcl*I)	3.37	3.31	3.07	2.76	2.09

Allele frequencies used for the loci D21S16, D21S13, and D21S1 have been calculated from unrelated individuals in a British population (M.M. *et al.*, manuscript in preparation). Allele frequencies used for the other polymorphisms are as previously reported[12–16]. The allele frequency used for the *Bcl*I polymorphism was 0.01, which was the highest frequency predicted from non-detection of the polymorphism in 200 normal chromosomes (99% confidence, $[1-(1-\text{confidence interval})^{1/200}]$). The following loci were not informative within the pedigree:D21S80, D21S52, D21S11, D21S8, D21S111 and D21S82.

Because we could not exclude the APP gene as the site of the mutation leading to AD in this family, we analysed the APP gene in an affected family member by PCR direct sequencing using intronic primers[18,19]. Exon 17 was sequenced first because it encodes part of the β-amyloid peptide and is the site of the mutation leading to hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D)[20,21].

Sequencing of exon 17 revealed a C to T transition at base pair 2,149, causing a valine to isoleucine change at amino acid 717 (transcript APP_{770}; ref. 19) (Fig. 2*a*). This valine residue is conserved in rodents. The base substitution may have involved deamination of a methylated cytosine situated 5′ to guanine, a fairly common mutation in human DNA[22]. The substitution creates a *Bcl*I restriction site which allows detection of the corresponding polymorphism within the PCR product (Fig. 2*b*). Linkage analysis shows that the polymorphism cosegregates with AD (Table 1).

Screening by PCR of 100 unrelated, normal individuals from the same population as the AD pedigree, and 14 cases (9 families) of familial late-onset disease, failed to demonstrate this substitution. Screening of 18 cases (16 families) of early-onset familial disease revealed the *Bcl*I restriction site in two affected individuals from a second family (family 372) in which linkage between AD and chromosome 21 markers shows small positive lod scores (log of likelihood ratio; D21S16, $z=0.6$, $\theta=0$; D21S1, $z=0.9$, $\theta=0$)[10].

Affected individuals from both families with the mutation were also genotyped for the rare *Eco*RI polymorphism detected by the 3′ end of the APP complementary DNA[12]; this probe detects a polymorphic site in the intron between exons 17 and 18. For family F23, the disease (and the mutation) cosegregates with the rare allele, which is present in only 5% of the normal population. In contrast, affected individuals in family 372 do not share this allele, but are homozygous for the common allele. This shows that the two pedigrees are very unlikely to be related to one another, as the markers are separated by less than 20 kilobases.

The genetic data show that the disease locus is linked to the missense mutation in F23 ($z=3.37$, $\theta=0$), but does not prove causation. However, failure to detect this polymorphism in 200 normal chromosomes supports the contention that it is a pathogenic mutation (the frequency of this polymorphism in

‡ Present addresses: Department of Respiratory Medicine, St Mary's Hospital Medical School, London W2 1PG, UK (A.H.); ICI Biotechnology Division, Alderley Park, Macclesfield, Cheshire, UK (L.J.).
¶ To whom correspondence should be addressed.

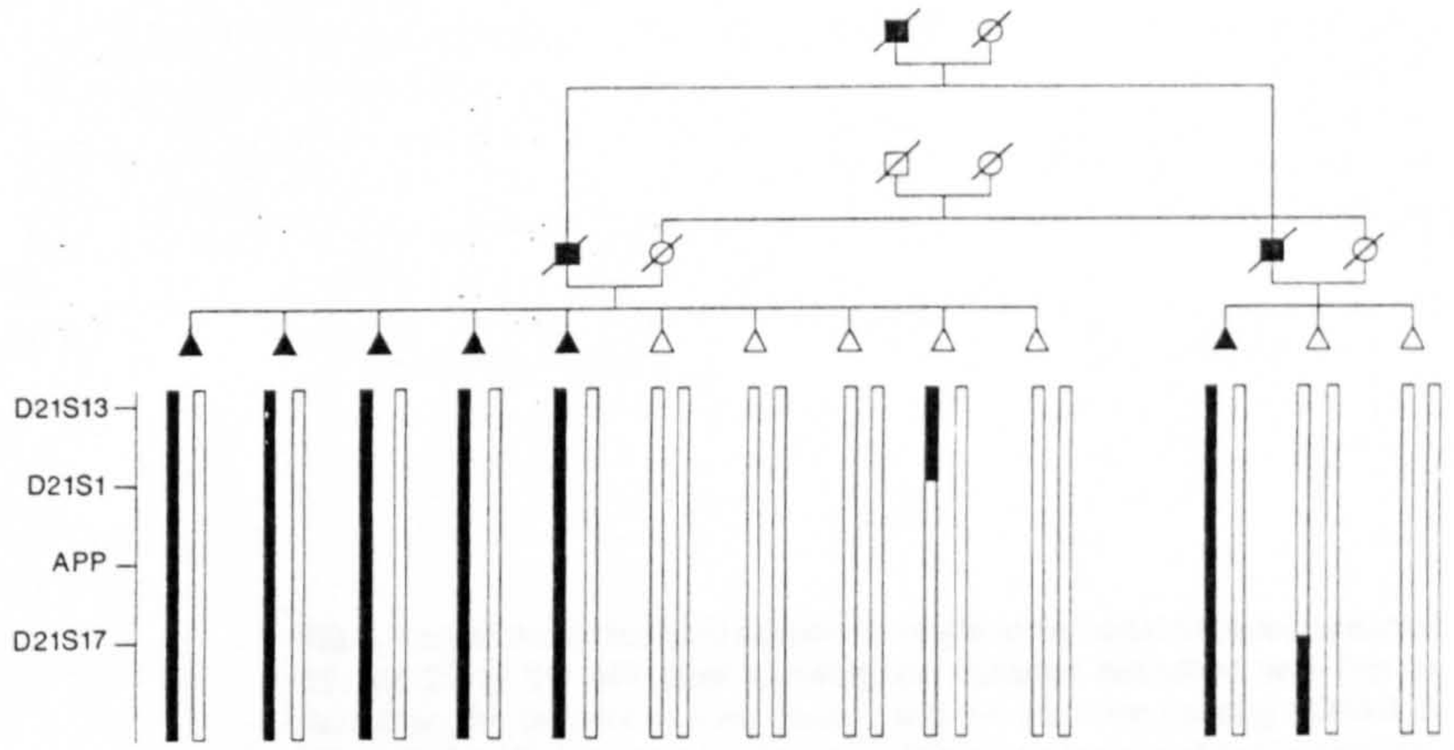


FIG. 1 Pedigree in which early-onset AD is apparently inherited as an autosomal dominant disorder. The average age of onset in this family is 57 ± 5 yr. Black symbols denote affected individuals and oblique lines indicate individuals who are deceased. Females are denoted by circles and males by squares. Triangles are used in the present generation to preserve anonymity. In generation II the spouses of the two affected brothers were sisters. Samples were available from the 13 individuals whose haplotypes are illustrated, from a further 19 children and spouses of these individuals and from 7 more distantly related unaffected individuals. Beneath the pedigree are ideograms of the two chromosomes 21 in each individual of the third generation at four loci on the long arm of the chromosome. The linkage data suggest that the chromosomes filled in in black were inherited from the affected fathers.

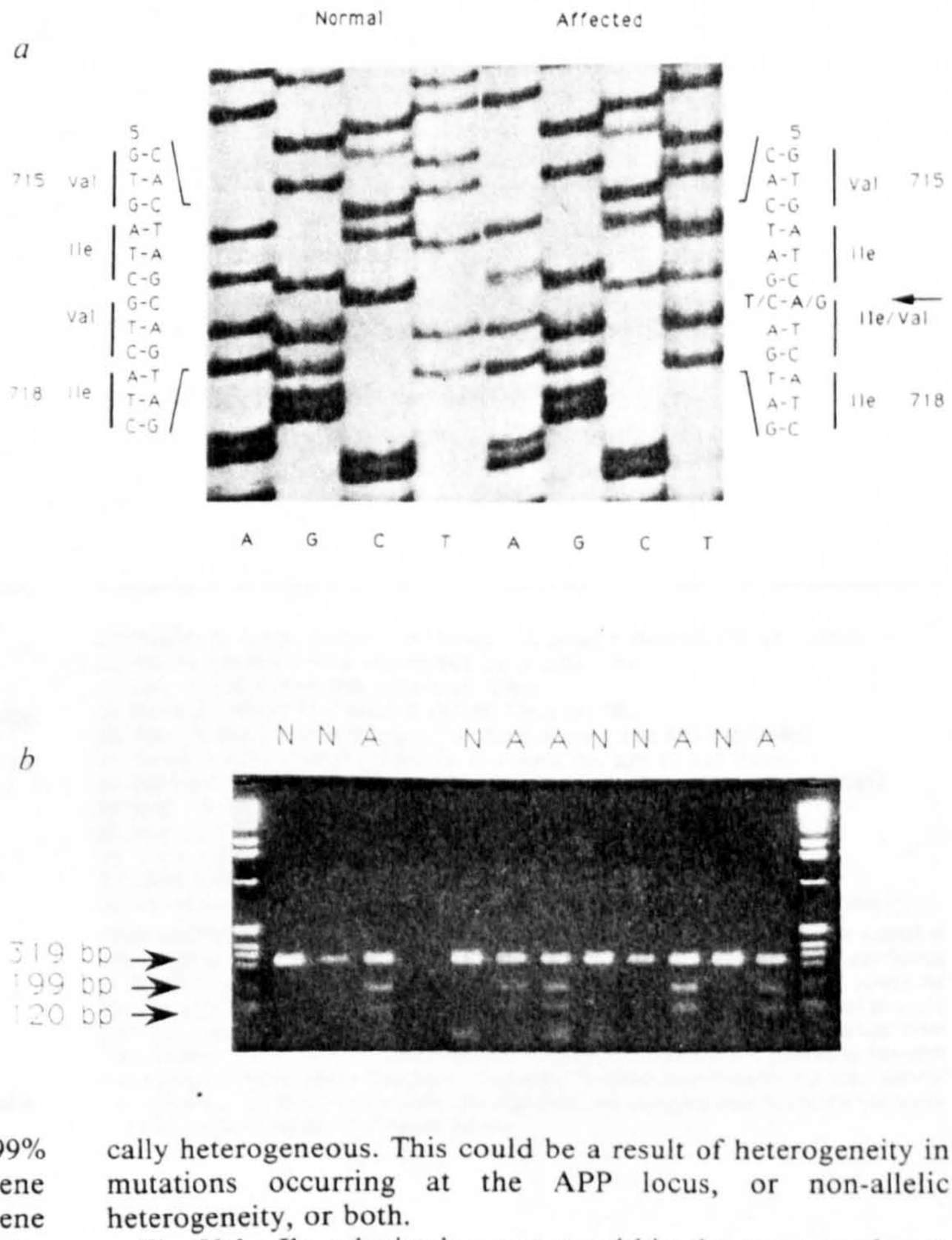


FIG. 2 *a*, Autoradiograph of a sequencing gel from part of exon 17 of the APP gene in a normal and an affected individual showing a single base-pair change in the affected individual. This C to T transition leads to an amino-acid substitution of a valine by an isoleucine at codon 717. PCR was carried out using the following intronic primers in order to amplify exon 17 of the APP gene: (1) 5′-CCTCATCCAAATGTCCCCGTCATT-3′, and (2) 5′-GCCTAATTCT-CTCATAGTCTTAATTCCCAC-3′. PCR conditions were 94 °C for 10 min to denature, then 35 cycles of 60 °C for 1 min, 72 °C for 3 min, 94 °C for 1.5 min; and a single cycle of 72 °C for 10 min. The reaction volume of 25 μl contained 50 pmol of primers, dNTPs at 200 μM, and $MgCl_2$ at 1.5 mM. A second PCR was performed which contained 50 pmol primer (1) and 0.5 pmol primer (2). The PCR product was purified on a Centricon 100 microconcentrator (Amicon) and used directly for sequencing with the Sequenase kit (version 2.0) (USB) following the manufacturer's protocol. Direct sequencing of exon 15 has revealed that this exon sequence is normal in family F23. Single-strand conformational analysis has failed to detect any sequence alterations in exons 7, 15 or 16 (our unpublished data). *b*, *Bcl*I digests of the exon 17 PCR product from unaffected (N) and affected (A) individuals in an early-onset AD family showing cosegregation of the restriction site and the disease. *Bcl*I digests were carried out at 50 °C for 2–4 h then electrophoresed in 3% agarose and stained with ethidium bromide. Size markers (indicated in base pairs) are shown in the outside tracks of the gel.

the general population has an upper limit of 0.01, with 99% confidence). As we have not yet sequenced the entire APP gene in an affected individual it remains possible that the APP gene is the disease locus but that the variant observed is not the causative mutation.

If this variant is pathogenic, previous reports of recombinants between AD and the APP gene have to be explained[6–8]. The demonstration of heterogeneity in familial AD[9] suggests that many apparent recombinants will have resulted from analysis of families without a mutation on chromosome 21. Reports of recombination in families showing evidence of chromosome 21 linkage may have been in error for several reasons, including misdiagnosis, mistyping, non-paternity and the occurrence of phenocopies. Furthermore, the APP gene is large enough for intragenic recombination to occur[19]. Alternatively, there may be two loci on chromosome 21 capable of causing AD, APP and a more proximal locus. If the mutation we describe is pathogenic, then the failure to detect the resulting *Bcl*I site in all cases of familial early-onset AD proves that this disease must be genetically heterogeneous. This could be a result of heterogeneity in mutations occurring at the APP locus, or non-allelic heterogeneity, or both.

The Val → Ile substitution occurs within the transmembrane domain two residues from the carboxy terminus of the β-amyloid peptide (Fig. 3). Computer analysis predicts that the substitution would make the transmembrane domain more hydrophobic and so might anchor the protein more firmly in the membrane[23–29], which could influence the stability of the deposited peptide.

A clear prediction of the hypothesis that mutations in the APP gene can predispose to AD is that examination of the APP gene in other families should lead to the identification of other mutations. The occurrence of AD pathology in trisomy 21 suggests that these mutations need not be in the coding region but may also be in controlling elements, leading to overexpression of APP. Whatever the aetiology of individual cases of AD, the results reported here suggest that β-amyloid peptide deposition is the central event in the pathogenesis of the disorder[30]. □

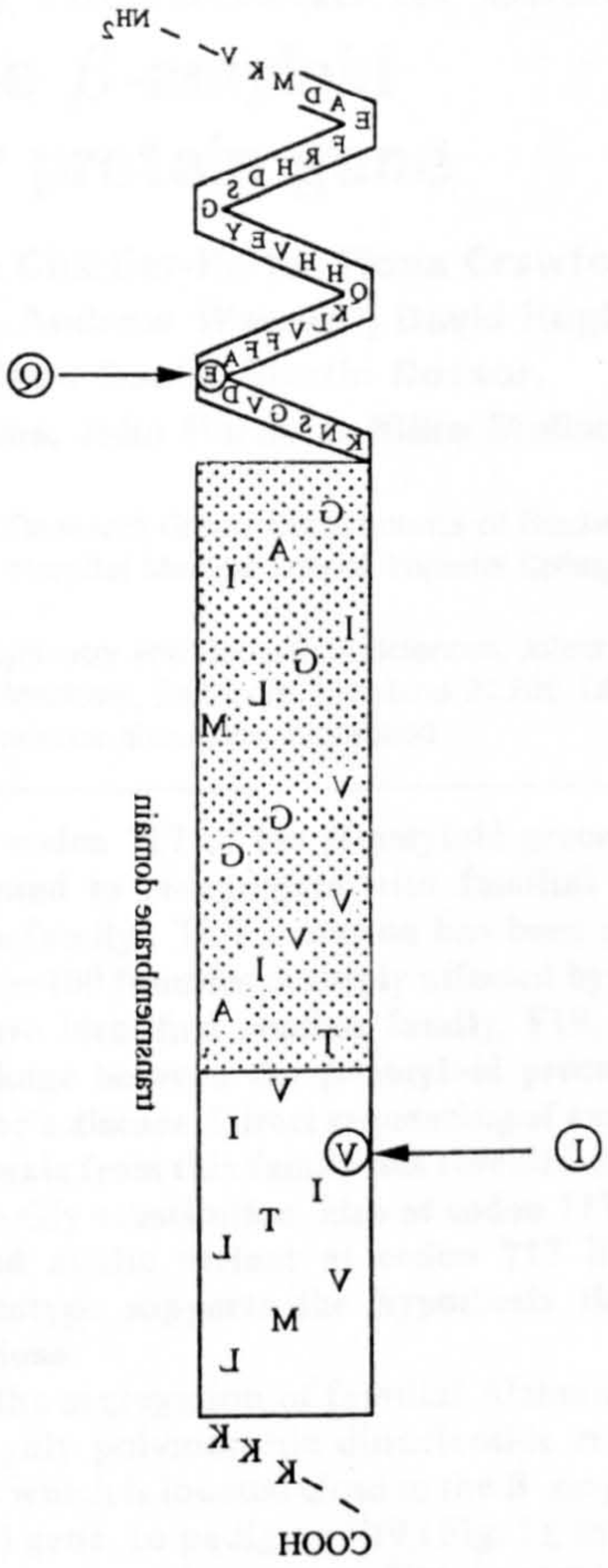


FIG. 3 Part of the amino-acid sequence (single-letter code) encoded by exons 16 and 17 of the APP gene showing the mutation described here (Val to Ile) within the transmembrane domain and the mutation causing HCHWA-D (Glu to Gln) in the extracellular domain[21]. The shaded region of the transmembrane domain and the boxed amino acids of the extracellular domain represent the sequence of the deposited β-amyloid peptide. Adapted from ref. 2.

Received 11 December 1990; accepted 7 January 1991.

1. St George-Hyslop, P. *et al. Science* **235,** 885–890 (1987).
2. Kang, J. *et al. Nature* **325,** 733–736 (1987).
3. Goldgaber, D., Lerman, M. I., McBride, O. W., Sappiotti, U. & Gajdusek, D. C. *Science* **235,** 877–880 (1987).
4. Robakis, N. K., Ramakrishna, N., Wolfe, G. & Wisniewski, H. M. *Proc. natn. Acad. Sci. U.S.A.* **84,** 4190–4194 (1987).
5. Tanzi, R. *et al. Science* **235,** 880–884 (1987).
6. Van Broeckhoven, C. *et al. Nature* **329,** 153–155 (1987).
7. Tanzi, R. *et al. Nature* **329,** 156–157 (1987).
8. Schellenberg, G. *et al. Science* **241,** 1507–1510 (1988).
9. St George-Hyslop, P. *et al. Nature* **347,** 194–197 (1990).
10. Pericak-Vance, M. *et al. Expl Neurol.* **102,** 271–279 (1988).
11. Goate, A. *et al. Lancet* **1,** 352–355 (1989).
12. Tanzi, R. *et al. Genomics* **3,** 129–136 (1988).
13. Warren, A. *et al. Genomics* **4,** 579–591 (1989).
14. Lewis, G. *et al. Genomics* **8,** 400–402 (1990).
15. Guo, Z., Sharma, V., Patterson, D. & Litt, M. *Nucleic Acids Res.* **18,** 4967 (1990).
16. Kidd, K. *et al. Cytogenet. Cell Genet.* **51,** 622–947 (1989).
17. Lathrop, G. M., Lalouel, J. M., Julier, C. & Ott, J. *Proc. natn. Acad. Sci. U.S.A.* **81,** 3443–3446 (1984).
18. Gyllensten, U. in *PCR Technology* (ed. Erlich, H. A.) 45–60 (Stockton, London, 1989).
19. Yoshikai, S., Sasaki, H., Doh-ura, K., Furnya, H. & Sakaki, Y. *Gene* **87,** 257–263 (1990).
20. Van Broeckhoven, C. *et al. Science* **248,** 1120–1123 (1990).
21. Levy, E. *et al. Science* **248,** 1124–1126 (1990).
22. Barker, D., Schafer, M. & White, R. *Cell* **36,** 131–138 (1984).
23. Argos, P., Rao, J. K. M. & Hargrave, P. A. *Eur. J. Biochem.* **128,** 565–575 (1982).
24. Garnier, J., Osguthorpe, D. J. & Robson, B. *J. molec. Biol.* **120,** 97–120 (1978).
25. Gaboriaud, C., Bissery, V., Benchetrit, T. & Mornon, J. P. *FEBS Lett.* **224,** 149–155 (1987).
26. Kyte, J. & Doolittle R. F. *J. molec. Biol.* **157,** 105–132 (1982).
27. Levin, J., M., Robson, B. & Garnier, J. *FEBS Lett.* **205,** 303–308 (1986).
28. Lim, V. *J. molec. Biol.* **88,** 857–872 (1974).
29. Gibrat, J., Garnier, J. & Robson, B. *J. molec. Biol.* **198,** 425–443 (1987).
30. Glenner, G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **120,** 1131–1135 (1984).

ACKNOWLEDGEMENTS. We thank the family and their clinician Irene Ovenstone for their support in this project; C. Van Broeckhoven, B. Bakker, J. Haan and R. Roos for discussion of the significance of HCHWA-D to AD; Y. Sakaki for intron sequence data; C. Van Broeckhoven and P. Hyslop for discussing unpublished genetic data with us; F. Chartier who ran and interpreted the protein structure prediction programmes; M. Fagan and H. Beswick for oligonucleotide synthesis; and the MRC Brain Bank (Institute of Psychiatry) for confirmation of diagnosis. This work was supported by Research into Ageing, the Mental Health Foundation, the Medical Research Council, NATO, The Royal Society, the Wellcome Trust, Shin-Etsu Bio (Japan), National Institutes on Ageing (AG-05128), the MacArthur Foundation and the Alzheimer's Disease Society.

Early-onset Alzheimer's disease caused by mutations at codon 717 of the β-amyloid precursor protein gene

Marie-Christine Chartier-Harlin, Fiona Crawford, Henry Houlden, Andrew Warren*, David Hughes, Liana Fidani, Alison Goate, Martin Rossor, Penelope Roques, John Hardy & Mike Mullan†

Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St Mary's Hospital Medical School, Imperial College, London W2 1PG, UK
* Departments of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA
† To whom correspondence should be addressed

A MUTATION at codon 717 of the β-amyloid precursor protein gene has been found to cosegregate with familial Alzheimer's disease in a single family[1]. This mutation has been reported in a further five out of ~100 families multiply affected by Alzheimer's disease[1-4]. We have identified another family, F19, in which we have detected linkage between the β-amyloid precursor protein gene and Alzheimer's disease. Direct sequencing of exon 17 (ref. 5) in affected individuals from this family has revealed a base change producing a Val→Gly substitution, also at codon 717. The occurrence of a second allelic variant at codon 717 linked to the Alzheimer's phenotype supports the hypothesis that they are pathogenic mutations.

We examined the segregation of familial Alzheimer's disease (FAD) with a highly polymorphic dinucleotide repeat marker GT12 (*D21S210*) which is located close to the β-amyloid precursor protein (APP) gene. In pedigree F19 (Fig. 1), in which FAD was confirmed by autopsy (onset age, 59±4 years), an allele of *D21S210* cosegregates with the disease. Linkage analysis gave a peak lod score between the marker and the disease of 3.02 at a recombination fraction, θ, of zero (Table 1).

We therefore sequenced the APP gene in this family starting with exon 17. Direct sequencing (Fig. 2) revealed a T→G transversion at base pair 2,150 changing valine to glycine at codon 717 of the APP transcript 770. Direct sequencing and single-strand conformation analysis (SSCA)[6,7] show that the mutation cosegregates with the linked marker GT12 and therefore the disease (lod score, 3.66; $\theta = 0$; three affected and 10 unaffected family members). It does not occur in any of the other 24 pedigrees with FAD that we have sequenced, nor does it occur in 52 normal individuals. The occurrence of this mutation is remarkable because it is a change at the same codon as that previously reported[1]. Direct sequencing or SSCA of other exons has not revealed any other changes in the APP gene in this family (Fig. 2 legend), but it is known that SSCA does not detect all single base-pair changes[7]. It remains a possibility that other sequence differences in the APP gene exist between affected and unaffected individuals either in exons not yet examined or sequenced or in the promoter.

However, the occurrence of this mutation in a family with histologically confirmed FAD, together with the multiple occurrence of the APP717 Val→Ile mutation, strongly support the hypothesis that these mutations are pathogenic[1]. The main features of FAD in F19 and families with the APP717 Val→Ile mutation clinically fulfill NINCDS[8] criteria for probable Alzheimer's disease and neuropathologically fulfill NINCDS criteria for definite Alzheimer's disease. Clinico-pathological features discriminating between these allelic variants have so far not been detected (M.M., unpublished data). The fact that both mutations are at the same codon may be a coincidence, but this is unlikely. There are two possibilities that may explain our data. First, substitution of the valine residue with Ile or Gly at codon 717 results in increased β-amyloid deposition because of changes in APP processing. Second, variation in the sequence around this position may result in increased translation of APP messenger RNAs, causing Alzheimer's disease by a route analogous to that believed to occur in Down's syndrome[9,10].

The mutation described here, like the one reported previously[1], could destabilize a putative stem-loop structure and destroy a possible iron-responsive element between base pairs 2,131 and 2,156[9]. There are several other possible mutations that could also disrupt this structure, many of which would be silent at the protein level. Both the Val→Ile and the Val→Gly mutations involve changes in amino acid 717; no silent changes or changes to other amino acids have yet been reported. Examination of sequence data from 10 other mammalian species[11] shows that although the valine residue at codon 717 is conserved in all species, the putative stem-loop structure postulated from the human sequence[9] would not be predicted to occur in either cattle or sheep; and in pig and mouse the consensus sequence for iron-responsive elements[12] is not present. Finally, such stem-loop structures are believed to modulate gene translation by altering mRNA stability[12]. However, *in situ* hybridization suggests that amounts of APP mRNAs are not grossly altered in the brain of an individual with APP717 Val→Ile[13]. For these reasons, we suggest that alterations in the rate of APP translation caused by these mutations are not likely to be the key to their pathogenicity. The fact that the mutations can convert Val to either Gly or Ile suggests that neither side-chain hydrophobicity nor bulk are responsible for the pathogenicity of these mutations.

The principal fate of APP involves cleavage within the β-amyloid fragment[14]. However, deposition of β-amyloid occurs to a small extent under normal conditions in the absence of any mutations. Therefore an alternative pathway of APP processing must occur that involves cleavage on either side of the β-amyloid region of APP.

TABLE 1 Linkage analysis between GT12 and Alzheimer's disease

Recombination fraction	0	0.01	0.05	0.1	0.2	0.3	0.4
Lod score	3.02	2.97	2.75	2.47	1.86	1.22	0.6

Lod scores were calculated with seven liability classes modelling age-dependent penetrances from 0.01 to 0.95 with a phenocopy rate of 0.001 and a gene frequency of 0.001 using MLINK from the LINKAGE package[17]. (GT12 allele frequencies available from A.W.)

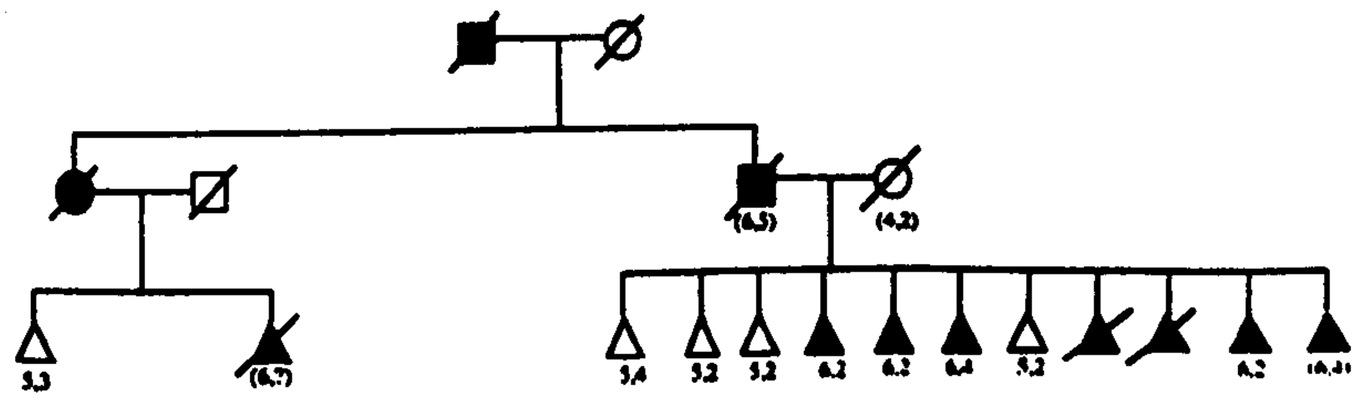


FIG. 1 Diagram of the pedigree together with the GT12 data. Filled symbols represent affected individuals. Numbers designate six alleles of this marker segregating in the pedigree. The pedigree is disguised for reasons of confidentiality. Brackets indicate data derived from reconstruction.

Amyloid molecules isolated from the brain of a patient with Alzheimer's disease show N- and C-terminal heterogeneity[15,16], suggesting that this alternative pathway involves either sequence-specific proteolysis followed by exopeptidase activity (creating end-heterogeneity), or that it is not sequence-specific. We suggest that proteolysis on either side of the β-amyloid region of APP is enhanced by these mutations, increasing the rate of β-amyloid deposition. Such mutations might increase β-amyloid formation by providing a poorer substrate for the main intra-amyloid proteolytic pathway or a better substrate for the alternative pathway. The identification and characterization of further mutations in familial Alzheimer's disease, together with work aimed at elucidating APP processing, should help to resolve the mechanisms by which these mutations are pathogenic and may suggest other causes of β-amyloid deposition[16].

Note added in proof: A third mutation at codon 717 of the APP770 transcript associated with Alzheimer's disease has been reported. The predicted amino-acid change is valine to phenylalanine[20]. □

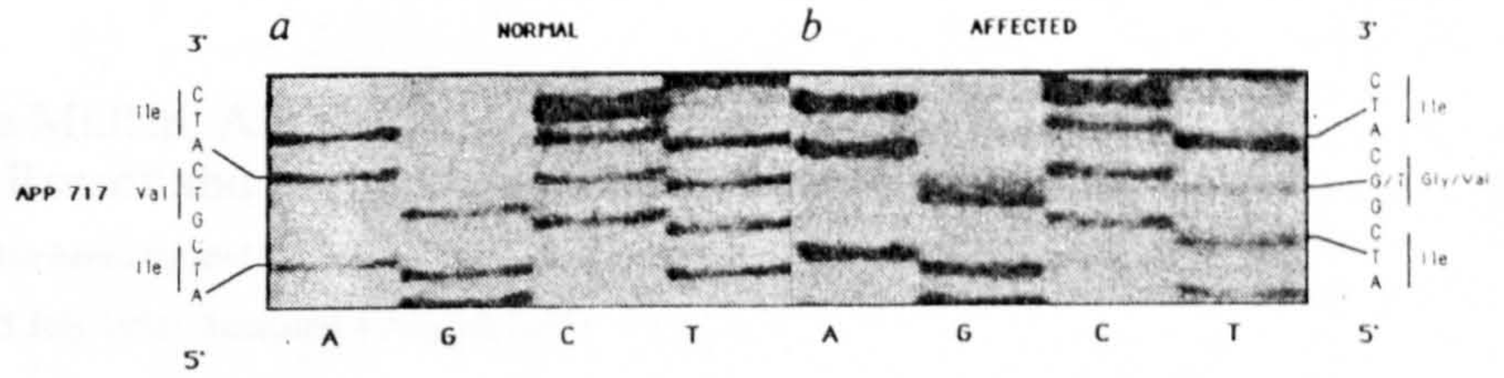


FIG. 2 APP exon-17 sequences in an affected and an unaffected member of F19. In the affected member, there is a T→G transition at nucleotide position 2,150.

METHODS. Amplification of exon 17 was as described[18], with the following modifications: (1) the amplification primer sequences were 5'-ATAACCTCATCCAAATGTCCCC−3' and 5'-GTAACCCAAGCATCATGGAAG−3'; (2) PCR conditions were 94°C for 10 min, then 35 cycles of 60 °C for 1 min; 72 °C, 1 min; 94 °C, 1 min, followed by 72 °C for 5 min. The second primer (50 pmol) was used to generate single-stranded product which was then purified and sequenced with the Sequenase kit (2.0) (USB) using a primer of sequence 5'-AAATGAAATTCT-TCTAATTGCG-3'. The presence of the T→G transversion creates artefacts on gels which are resolved by inclusion of inosine (Sequenase kit) in the sequencing reaction. Direct sequencing of exons 7 and 16 from affected individuals from F19 showed that these were of normal sequence and SSCA failed to identify changes in exons 2, 3, 7, 9, 12, 13, 15 or 18. SSCA of exon 17 detects APP693 (ref. 19), and both APP717 Val→Ile and Val→Gly (conditions available from the authors).

Received 16 August; accepted 4 October 1991.

1. Goate, A. *et al. Nature* **349,** 704-706 (1991).
2. Hardy, J. *et al. Lancet* **337,** 1342-1343 (1991).
3. van Duijn, C. M. *et al. Lancet* **337,** 978 (1991).
4. Naruse, S. *et al. Lancet* **337,** 978-979 (1991).
5. Yoshikai, S., Sasaki, H., Dohura, K., Furuya, H. & Sakaki, Y. *Gene* **87,** 257-263 (1990).
6. Orita, M., Suzuki, Y., Sekiya, T. & Hayashi, K. *Genomics* **5,** 874-879 (1989).
7. Orita, M., Sekiya, T. & Hayashi, K. *Genomics* **8,** 271-278 (1990).
8. McKhann, G. *et al. Neurology* **34,** 939-944 (1984).
9. Tanzi, R. E. & Hyman, B. T. *Nature* **350,** 564 (1991).
10. Rumble, B. *et al. New Engl. J. Med.* **320,** 1446-1452 (1989).
11. Johnstone, E. M., Chaney, M. O., Norris, F. H., Pascual, R. & Little, S. P. *Molec. Brain Res.* **10,** 299-305 (1991).
12. Klausner, R. D. & Harford, J. B. *Science* **246,** 870-872 (1989).
13. Harrison, P. J., Barton, A. J. L. & Pearson, R. C. A. *Neuro Rep.* **2,** 152-154 (1991).
14. Esch, F. S. *et al. Science* **248,** 1122-1124 (1990).
15. Master, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **82,** 4245-4249 (1985).
16. Glenner, G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **120,** 885-890 (1984).
17. Lathrop, G., Lalouel, J. M., Julier, C. & Ott, J. *Proc. natn. Acad. Sci. U.S.A.* **81,** 3443-3446 1984).
18. Chartier Harlin, M. C. *et al. Neurosci. Lett.* **129,** 134-135 (1991).
19. Levy, E. *et al. Science* **248,** 1124-1126 (1990).
20. Murrell, J., Farlow, M., Ghetti, B. & Benson, M. *Science* **253,** 97-98 (1991).

ACKNOWLEDGEMENTS. We thank R. Harris for referring the family to us, D. Bowen and D. Mann for samples of brain tissue from an affected individual and D. Mann for confirmation of diagnosis, S. Antonarakis and M. McInnis for sharing data with us before publication, and M. Palmer, F. Chartier and F. Ashall for discussion. This work was supported by the Mental Health Foundation, Research into Ageing, the Alzheimer's Disease Society, the Royal Society, the IPSEN Foundation, the Wellcome Trust and the Medical Research Council.

Neuroscience Letters, 133 (1991) 1–2
© 1991 Elsevier Scientific Publishers Ireland Ltd. All rights reserved 0304-3940/91/$ 03.50

NSL 08175

Sequencing of exons 16 and 17 of the β-amyloid precursor protein gene in 14 families with early onset Alzheimer's disease fails to reveal mutations in the β-amyloid sequence

Fiona Crawford, John Hardy, Mike Mullan, Alison Goate, David Hughes, Liana Fidani, Penelope Roques, Martin Rossor and Marie-Christine Chartier-Harlin

Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St. Mary's Hospital Medical School, London (U.K.)

(Received 25 July 1991; Accepted 4 August 1991)

Key words: Alzheimer's disease; Chromosome 21; Genetics, β-Amyloid precursor protein

A mutation within exon 17 at codon 717 of the β-amyloid protein precursor (APP) gene is one cause of early onset familial Alzheimer's disease. Direct sequencing of exons 16 and 17 of the β-amyloid precursor protein gene in 14 families with familial early onset Alzheimer's disease without the known pathogenic mutation (APP717) failed to reveal other mutations within the β-amyloid sequence in this form of the disorder.

The valine to isoleucine mutation at residue 717 of the β-amyloid precursor protein (APP) gene [2] has been found in several families with early onset familial Alzheimer's disease (AD) [2, 3]. These data, together with the fact that this variant has not been found in the general population [2, 3] strongly suggests that this mutation is a rare cause of early onset familial AD in Caucasian population [2, 3], though it may be a relatively common cause of this form of AD in Japanese populations [3, 4]. While there is strong genetic evidence that not all cases of the early onset familial AD are encoded within the APP locus [7, 10, 11], mutations within the β-amyloid section of the APP gene (exons 16 and 17 [13]) other than APP717 are likely causes of some the other cases. For this reason we decided to sequence these exons in affected individuals from 11 other families in our collection with familial AD (Table I) 1, and three families (FAD1, FAD2, FAD4) from the Coriell cell repository [9].

PCR direct sequencing was carried out exactly as we have previously described [1, 7]. PCR amplification of exon 17 was carried out using primers of sequence: (A) 5′-CCT CAT CCA AAT GTC CCC GTC ATT-3′ and (B) 5′-GCC TAA TTC TCT CAT AGT CTT AAT TCC CAC-3′. to amplify the exon. PCR conditions were 94°C, 10 min to denature; then 35 cycles of 60°C, 1 min; 72°C, 3 min; 94°C, 1.5 min and a single cycle of 72°C, 10 min. The reaction volume used was 25 μl with a final concentration of primers at 50 pmol, dNTPs of 200 μM and a magnesium chloride concentration of 1.5 mM. A second PCR reaction as then performed with a final concentration of 50 pmol of primer A and 0.5 pmol of primer B. The PCR product was purified on a Centricon 100 microconcentrator (Amicon) and used directly for sequencing with the Sequenase kit (version 2.0) (USB) following the manufacturer's protocol. PCR amplification of exon 16 was initially carried out using primers of sequence: - GGG TAG GCT TTG TCT TAC AG and GGC AAG ACA AAC AGT AGT GG. Direct sequencing of this exon used an internal primer of sequence: ACA AAC AGT AGT GGA AAG AGG TAA ATT AT. The conditions used for the amplification and sequencing reactions were initially the same as for exon 17. Sequencing exon 16 by this method, while it generated some data, proved troublesome. We, therefore, switched to using biotinylated primers with Dynabeads (M280 Streptavidin, Dynal, Merseyside, U.K.) (sequence GGG TAG GCT TTG TCT TAC AG) to produce single stranded DNA for sequencing of this exon. Single strand conformation analysis [5, 6] of these exons using these PCR primers failed to reveal any mutations in either exon 16 or exon 17 although it does show band shifts in exon 17 with families with both APP693 and APP717 mutations (unpublished data).

Correspondence: M.C. Chartier-Harlin, Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St. Mary's Hospital Medical School, London W2 1PG, U.K.

TABLE I

Brief clinical information from the FAD series of families have been previously reported (see ref. 9). We have previously reported the sequence data for FAD4 [1]. Many of these families are also discussed in ref. 12.

Families	Age of onset (range)	No. of affected	No. path. confirmed
F53	52 (43–60)	5	1
F74	43 (39–50)	7	2
F75	51 (44–58)	11	0
F84	61 (59–64)	3	0
F97	62 (62–63)	2	0
F105	38 (36–39)	14	0
F121	37 (35–39)	6	0
F126	54 (48–63)	4	0
F134	45 (41–50)	6	2
F148	44 (42–46)	5	1
F160	37 (36–38)	4	2

Examination of the sequence data from these families identified no other mutations within the β-amyloid containing exons. There are two possible explanations for these finding which are not mutually exclusive. The first is that there are mutations elsewhere in the APP gene (either within the open reading frame, or within controlling elements) which are pathogenic. The second is that these families have mutations in genes other than the APP gene. While the precedence of other diseases suggests that other mutations within the APP locus are likely causes of AD (for example, there are many mutations within the prion gene which are pathogenic), it is notable that the linkage data suggesting a locus on chromosome 21 are largely contributed by those families with the APP717 mutation (these pedigrees are F23 (pedigree 26 [8]) and F372 [8]). Thus, one does not need to postulate the occurrence of other mutations elsewhere on chromosome 21 to explain the data relating to chromosome 21 linkage for AD.

This work was supported by grants from Research into Ageing, IPSEN, the Mental Health Foundation, the Royal Society, and the Medical Research Council. The samples from the FAD series of pedigrees which were used in this study were AG06849 and AG07647 (FAD1), AG08554 and AG08526 (FAD2) and AG08554 and AG08109A (FAD4) from the Coriell Cell repository.

1 Chartier Harlin, M.C., Crawford, F., Hamandi, K., Mullan, M., Goate, A., Backhovens, H., Martin, J.J. and Van Broeckhoven, C., Screening for the β-amyloid precursor protein mutation (APP:Val->Ile) in extended pedigrees with early onset Alzheimer's disease, Neurosci. Lett., 129 (1991) 134–135.

2 Goate, A., Chartier Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. et al, , Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease, Nature, 349 (1991) 704–706.

3 Hardy, J., Mullan, M., Chartier Harlin, M.C., Brown, J., Goate, A., Rossor, M., Collinge, J., Roberts, G., Luthert, P., Lantos, P., Naruse, S., Kaneko, K., Tsuji, S., Miyatake, T., Shimizu, T., Kojima, T., Nakano, I., Yoshioka, K., Sakaki, Y., Miki, T., Katsuya, T., Ogihara, T., Roses, A., Pericak-Vance, M., Haan, J., Roos, R., Lucotte, G. and Favid, F., Molecular classification of Alzheimer's disease, Lancet, 337 (1991) 1342–1343.

4 Naruse, S., Igarashi, S., Kobayashi, H., Aoki, K., Inuzuka, T., Kaneko, K., Shimizu, T., Iihara, K., Kojima, T., Miyatake, T. and Tsuji, S., Mis-sense mutation Val->Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease, Lancet, 337 (1991) 978–979.

5 Orita, M., Sekiya, T. and Hayashi, K., DNA sequence polymorphisms in Alu repeats, Genomics, 8 (1990) 271–278.

6 Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K., Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction, Genomics, 5 (1989) 874–879.

7 Schellenberg, G.D., Bird, T.D., Wijsman, E.M., Moore, D.K., Boehnke, M., Bryant, E.M., Lampe, T.H., Nochlin, D., Sumi, S.M., Deeb, S.S., Beyreuther, K. and Martin, G.M., Absence of linkage of chromosome 21q21 markers to familial Alzheimer's disease, Science, 241 (1988) 1507–1510.

8 St George Hyslop, P.H., Haines, J.L., Farrer, L.A., Polinsky, R., Van Broeckhoven, C., Goate, A., McLachlan, D.R., Orr, H., Bruni, A.C., Sorbi, S. et al, , Genetic linkage studies suggest that Alzheimer's disease is not a single homogeneous disorder. FAD Collaborative Study Group, Nature, 347 (1990) 194–197.

9 St George Hyslop, P.H., Tanzi, R.E., Polinsky, R.J., Haines, J.L., Nee, L., Watkins, P.C., Myers, R.H., Feldman, R.G., Pollen, D., Drachman, D. et al, , The genetic defect causing familial Alzheimer's disease maps on chromosome 21, Science, 235 (1987) 885–890.

10 Tanzi, R.E., St George Hyslop, P.H., Haines, J.L., Polinsky, R.J., Nee, L., Foncin, J.F., Neve, R.L., McClatchey, A.I., Conneally, P.M. and Gusella, J.F., The genetic defect in familial Alzheimer's disease is not tightly linked to the amyloid beta-protein gene, Nature, 329 (1987) 156–157.

11 Van Broeckhoven, C., Genthe, A.M., Vandenberghe, A., Horsthemke, B., Backhovens, H., Raeymaekers, P., Van Hul, W., Wehnert, A., Gheuens, J., Cras, P. et al, , Failure of familial Alzheimer's disease to segregate with the A4-amyloid gene in several European families, Nature, 329 (1987) 153–155.

12 van Duijn, C.M., Van Broeckhoven, C., Hardy, J.A., Goate, A.M., Rossor, M.N., Vandenberghe, A., Martin, J.J., Hofman, A. and Mullan, M.J., Evidence for allelic heterogeneity in familial early onset Alzheimer's disease, Br. J. Psychol., 158 (1991) 471–474.

13 Yoshikai, S., Sasaki, H., Doh ura, K., Furuya, H. and Sakaki, Y., Genomic organization of the human amyloid beta-protein precursor gene, Gene, 87 (1990) 257–263.

Human Molecular Genetics, Vol. 1, No. 3 165–168

Screening for mutations in the open reading frame and promoter of the β-amyloid precursor protein gene in familial Alzheimer's disease: identification of a further family with APP717 Val→Ile

Liana Fidani[1], Karen Rooke[1], Marie-Christine Chartier-Harlin[1], David Hughes[1], Rudi Tanzi[2], Mike Mullan[1,3], Penelope Roques[1], Martin Rossor[1], John Hardy[1,3]* and Alison Goate[1]

[1]Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St Mary's Hospital Medical School, London W2 1PG, UK, [2]Molecular Neurogenetics Laboratory, Massachusetts General Hospital, East Bldg. 13th St, Charlestown, MA 02129 and [3]Suncoast Alzheimer's Disease Research Laboratories, Department of Psychiatry, University of South Florida, Tampa, FL 33612, USA

Received March 27, 1992; Revised and Accepted May 8, 1992

ABSTRACT

Following the identification of mutations in the β-amyloid precursor protein (APP) gene in familial, early onset Alzheimer's disease (AD), we have developed a screening protocol using single strand conformation analysis (SSCA) to screen exon 17 for the known mutations within APP. In addition, we used this protocol to screen the other seventeen exons of APP and a three hundred and thirty base pair regulatory region of the promoter for new mutations in 9 families with early onset AD. Exons 16 and 17, which encode the deposited beta-amyloid peptide, were screened in a further 10 families. Our screening procedure identifies all the reported mutations within APP. While we have identified a further family with APP717 Val→Ile, we did not find any previously undescribed mutations. Screening of other exons of APP in 2 families in which we have previously reported mutations at APP717, failed to reveal other sequence abnormalities supporting the hypothesis that the mutations at APP717 cause the disease in these families. These data suggest that mutations in APP are a rare cause of familial early onset AD (3/21 families tested) and that within APP most, possibly all, mutations which cause AD are in exon 17.

INTRODUCTION

The identification of mutations in the APP gene in families with early onset AD has led to the hypothesis that mutations in this gene are pathogenic (1–3). However, genetic data have suggested that the disease is heterogeneous (4) and that the APP gene is not the only predisposing locus (5,6). It is often not possible to determine whether the APP locus is pathogenic in any particular family by linkage analysis because many pedigrees with early onset AD are too small to obtain significant genetic information. Since evidence for linkage between AD and the long arm of chromosome 21 is strongest in familial early onset AD, we restricted our analysis to families with an onset of less than 65 years.

Exons 16 and 17 of the APP gene encode the β-amyloid peptide (7) and have been hypothesized to be the most likely sites for mutations leading to AD (1). However, sequencing of these exons has previously failed to reveal any alterations in a number of families with early onset AD (8,9). We therefore decided to test the hypothesis that other exons of the APP gene contain pathogenic mutations by screening these areas of the gene in families with early onset, familial AD (see table 1) by SSCA (10,11) and to confirm that the exon 17 mutations we have previously reported are the only changes from the normal sequence in those families in which they occur.

SSCA is based on the tendency of single stranded DNA molecules to adopt a sequence dependent secondary structure. These structures can be separated as they migrate through a nondenaturing polyacrylamide gel. Thus SSCA can be used as a sensitive indicator of sequence variation at the level of a single base pair, and provides a fast protocol to identify new mutations and also to screen familial and sporadic cases for already characterised mutations. Data suggest that over 80% of mutations can be detected by SSCA under a single set of conditions (10,11,12).

In addition to the mutations in the APP gene, the only other known cause of AD neuropathology is trisomy 21. All trisomy 21 individuals who live beyond the age of thirty show a neuropathology indistinguishable from that seen in AD. Down syndrome individuals have an extra copy of a normal APP gene and expression analysis has shown that APP is expressed above the expected 3:2 ratio in the brain (13). This suggests that mutations in the promoter which affect the level of expression of APP might also cause AD. Several reports characterising the APP promoter have identified a region lying between −495 and +100 which is sufficient for neuronal expression of APP (14–16). Within this region, several consensus sequences (heat shock elements and AP-1 binding sites) have been identified and shown to regulate expression of APP in cell lines.

* To whom correspondence should be addressed at: Departments of Biochemistry and Neurology, St Mary's Hospital Medical School, London W2 1PG, UK

RESULTS

In all cases, PCR amplification resulted in a single product of the predicted size (see table 1). The identity of the bands was confirmed by PCR amplification of a yeast artificial chromosome containing the APP gene which gave the same size bands in all cases (Talbot, Rooke and Goate: unpublished results).

The known APP717 mutations were resolved using our protocol as was APP693 (17) (Figure 1). The only sequence variation detected during this screening effort was a single family, F172, which had an SSCA mobility consistent with the occurrence of the APP717 Val→Ile mutation. Direct sequencing (9) of exon 17 in this family confirmed the presence of this mutation (data not shown). No additional sequence variation was observed in samples from those families (F23 and F19) in which we have previously found mutations in exon 17.

Since we optimised our SSCA conditions using exon 17 mutations, we cannot use our data from this exon to gain a realistic estimate of its sensitivity. However, we tested the same protocol on PCR products derived from characterised variants (single base changes) of the prion gene. Our protocol detected 9 out of 10 point variants tested (Fidani, Collinge and Palmer unpublished data). Two further mutations in exon 17 of the APP gene have recently been identified (Drs van Broeckhoven and St. Clair unpublished). We have found our SSCA conditions also detect these mutations.

DISCUSSION

The screening system reported herein is preferable to enzyme digestion because it is generally applicable. It is preferable to direct sequencing because of its ease and efficiency and yet the procedure has a high probability of detecting new mutations (10,11,12). However, the nature of mutations need to be confirmed by direct sequencing.

The screening of the APP gene reported here confirms the impression that exon 17 is the only site of mutations causing AD in APP since the only change we have found is a new example of the original APP717 Val→Ile mutation. Genetic analysis of this family indicates it is not related to any of the Caucasian families in which this mutation has been found (Houlden, Mullan and Hardy, unpublished observations). Thus, we have found APP mutations in 2 (F23 and F19) out of 11 families in which the whole gene has been screened, and in 1 out of 10 families in which we have only examined the promoter region and exons 16 and 17 (172). While these numbers are small, they are consistent with the notion that mutations in APP are a relatively rare cause of early onset familial AD. It remains possible that there are mutations which have not been detected using the SSC-A conditions employed herein. However, we regard this as unlikely since two reports have estimated that the technique identifies 80−90% of variations in a PCR product of 100−450 bp using a single protocol (11,12). Our experience with screening known prion variants is consistent with this. We have used both the protocol described by Orita and colleagues and our modifications of this protocol to screen the families presented here. We have calculated the GC content of all exons of APP analysed and that of the two PrP PCR products. The GC content of all PCR products except those of exon 1 and the promoter (74%) are comparable with our control samples. PrP 1 and 2 have GC contents of 55% and 50% respectively whilst exons 16 and 17 have a 37% GC content. The remaining exons have GC contents between 44.5−54.8%. The efficiency of detection of mutations in exons 2−18 should therefore be comparable with our control samples. We suggest that 80% must be a minimum estimate for the sensitivity of the procedure in these exons. Since the GC content of the PCR products for exon 1 and the promoter are very much higher than any of our control samples the efficency of detection of mutations in these products is unknown and may be quite different.

However, because of this uncertainty and because of the possibility that there may be mutations in controlling elements of the gene which we have not screened, accurate estimation of the proportion of cases of early onset, familial AD caused by mutations at the APP locus will probably not be possible until the other pathogenic loci are identified.

Despite the conservative nature of the APP717 Val−Ile mutation, evidence is accrueing in support of its pathogenicity.

Table 1. Clinical Samples Used

Family No	Age of onset	Affecteds (path. confirmed)	Mutations
a) Whole gene screened			
F53	43−60	5 (1)	None
F74	39−50	7 (2)	None
F84	59−64	3	None
F105	36−39	14	None
F121	35−39	6	None
F126	48−63	4	None
F134	41−50	6 (2)	None
F148	42−46	5 (1)	None
F168	40−54	6 (1)	None
F19	48−58	10 (1)	APP717 Val−Gly
F23	53−61	11 (1)	APP717 Val−Ile
b) Mutation Controls: exon 17 tested only.			
INDIANA	41−45	5	APP717 Val−Phe
HCHWA-D	40−60	Many	APP693 Gln−Glu
c) Samples in which exons 16 and 17 and the promoter region were tested.			
F172	44−64	10	APP717 Val−Ile
F161	50−55	3 (1)	None
F165	53−60	2	None
F175	36−42	3 (1)	None
F184	50−55	5	None
F196	32−38	5	None
F206	37−42	7	None
FAD1	Mean 52	Many (Many)	None
FAD2	Mean 48	Many (Many)	None
FAD4	Mean 40	Many (Many)	None

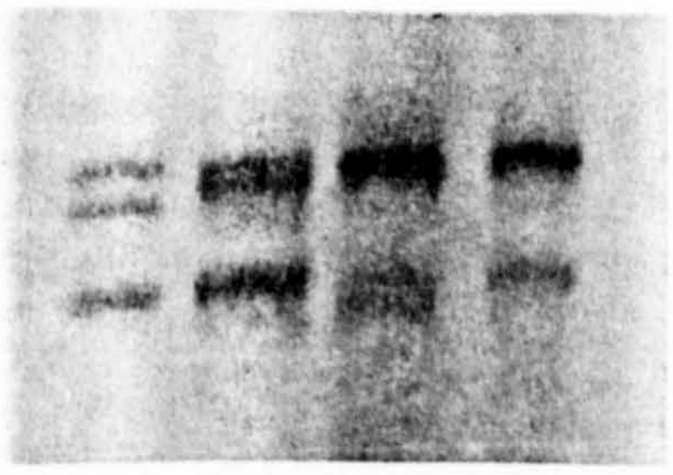


Figure 1. SSCA of variant APP exon 17 samples are shown. From left to right, these are APP717 Val−Ile, APP717 Val−Phe, APP717 Val−Gly and control.

Seven families with early onset AD have now been identified carrying this mutation but no control sample from many hundreds screened has shown the mutation(1,18−20) (this study). All exons and a promoter region of the APP gene have now been screened for other mutations in two families with APP717 mutations and no other changes have been found. For these reasons, and because of the identification of two other mutations at this amino acid (2,3), it is probable that these mutations are pathogenic rather than rare polymorphisms.

Full details of the clinical phenotype of F172 are not yet available. However, our preliminary information suggests that the onset age is in the mid 50s like the other families which have been reported with this mutation. F19 (APP717 Val→Gly) has a mean onset in the late 50s and the Indiana family (APP717 Val→Phe) has a mean onset in the mid 40s. While more families with each mutation will have to be identified before firm conclusions can be drawn, it seems as if age of onset is in part determined by the nature of the pathogenic mutation. However, clearly there is considerable overlap in the onset age, both between families with different characterised mutations and families in which the APP gene is not the site of the pathogenic change.

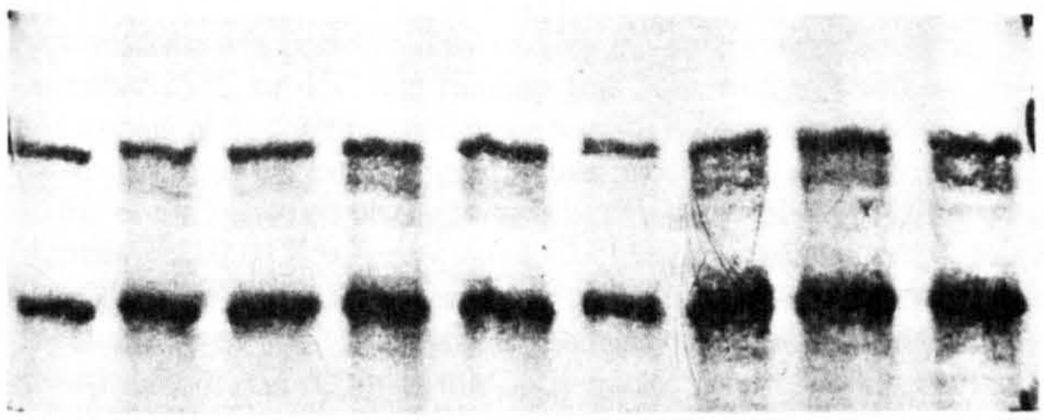


Figure 2. SSCA of exon 18 samples are shown from 8 cases of AD and (right) a control sample.

METHODS

DNA from an affected individual from each of 21 families multiply affected by early onset AD was used for the SSCA (see table 2). The age of onset was below 65 years in all families. A clinical diagnosis of AD was confirmed by histopathology in 12 families.

Specific primers for PCR amplification of the 18 exons of the APP gene and for a regulatory region within the promoter were made (7,15,16). Primers were chosen which allowed amplification between −556 and −225 of the promoter. This region contains a heat shock consensus sequence and a binding site consensus sequence for the transcription factor AP-1 (16). We screened this region of the promoter in these families by SSCA (see table 1). Intronic primers were chosen to give product sizes of 100−400bp whenever possible since SSCA is believed to be most effective at detecting sequence variability in this range (12). Conditions for the PCR reactions were optimised as shown in table 2.

Table 2. Primer Sequences for APP Gene Amplification

Sequences	(product size)	$MgCl_2$	An.Temp.
Promoter	5′-GCTTTTGACGTTGGGGGTTA-3′		
(331)	5′-AGACTCTCCCTCCCACTGTT-3′	1.5mM	60°C
Exon 1:	5′-AGCAAGGACGCGGCGGATCC-3′		
(172)	5′-GTATCGCGTCCCCACCGTGC-3′	1.5mM	58°C
Exon 2:	5′-GAATTC-TGCCTTGGAGCTATGGATAC-3′		
(262)	5′-GAATTC-ATCCAACGTGAATTGCTAGC-3′	2.0mM	58°C
Exon 3:	5′-GAATTC-ATGCTTGTAAATGCCAGCCC-3′		
(262)	5′-GAATTC-TTTCTTCCCTCAAGACCAGG-3′	1.5mM	58°C
Exon 4:	5′-CCTTGATGTCTTCTGGGGTA-3′		
(210)	5′-CAGGAAATCTTCACTTTGCA-3′	1.5mM	55°C
Exon 5:	5′-ACACATCTACTCTAGCCAC-3′		
(399)	5′-ACTCCATACAAGATGTTTCAGC-3′	1.5mM	63°C
Exon 6:	5′-GACGGCCGCTGTTTGCCTTCACAGTG-3′		
(474)	5′-GCGGCGCGCCCTTGGGATTTGCCAAGCAGC-3′	3.0mM	60°C
Exon 7:	5′-GAATTC-TCATGCTAAATGTGGTTCCC-3′		
(282)	5′-GAATTC-ACAAAAGCAGAGTCAGTGGC-3′	1.5mM	60°C
Exon 8:	5′-GAGCGCGCTTGGTTTTGTTGGAGGGACC-3′		
(250)	5′-GCCGATCGCTGGAGTTATGTGAACCCAA-3′	3.0mM	60°C
Exon 9:	5′-GAATTC-TTCTCAAACCTCCTTCTC-3′		
(211)	5′-GAATTC-ATCGTAGGGCTGAATGATGG-3′	2.0mM	58°C
Exon 10:	5′-AACATAATCATCCATCCTAT-3′		
(257)	5′-GCAGACATATTAAATGGATG-3′	1.5mM	53°C
Exon 11:	5′-GCTGCTTCATCCTACTTATTC-3′		
(266)	5′-CTTACTGTCTGTGCTGTGAC-3′	1.5mM	55°C
Exon 12:	5′-GAATTC-GCCAATGGAAGAAGCAGTGT-3′		
(262)	5′-GAATTC-CCAAGAAGCAAGAATCCTGG-3′	1.5mM	58°C
Exon 13:	5′-GAATTC-ATTCCTACCCCCAGACATGT-3′		
(196)	5′-GAATTC-ACATGTCCATGTGCAGCATC-3′	1.5mM	58°C
Exon 14:	5′-GTTACTCACCAAAGAGATGGT-3′		
(282)	5′-CCCTGCTGGCTCAGGGGACTC-3′	1.5mM	55°C
Exon 15:	5′-CGAATTC-CGACTTGTTTGGGAGCCAC-3′		
(256)	5′-CGAATTC-CTGCAGTAAGTACAATTGAG-3′	1.5mM	55°C
Exon 16:	5′-GGGTAGGCTTTGTCTTACAG-3′		
(250)	5′-GGCAAGACAAACAGTAGTGG-3	1.5mM	58°C
Exon 17:	5′-ATAACCTCATCCAAATGTCCCC-3′		
(402)	5′-GTAACCCAAGCATCATGGAAGC-3′	1.5mM	60°C
Exon 18:	5′-GAATTC-CTTCTCCATGTTCACCCTTA-3′		
(313)	5′-GTTACAGCACAGCTGTCAAAAGGG-3′	1.5mM	60°C

For exonic sequences: 10min at 94°C initial denaturation then 35 cycles of 45sec at the optimum annealing temperature (table 2), 2min at 72°C, 1.5min at 94°C, and a final cycle of 10min at 72°C. For the promoter region: 5min at 94°C, 1.5min at 60°C., then 35 cycles of 1min at 60°C, 1min at 72°C and 1min at 94°C, and a final cycle of 10min at 72°C. The reaction volume used was usually 25μl with a final concentration of primers of 0.2μM. Final concentrations of 200μM of all nucleotides were used apart from dATP which was used at a concentration of 24μM, 0.5μl of [α-^{35}S]dATP (Specific activity 600Ci/mmol). 1 unit of Taq polymerase was added. PCR of exons 1, 5, 10, 11 and 14 was performed in a total volume of 50μl and PCR of exons 6 and 8, and the promoter region was performed in a total volume of 100μl.

The resolving power of non-denaturing acrylamide gels is determined by the acrylamide concentration and the proportion of bisacrylamide added as crosslinker. We optimised our conditions to give maximal separations of the APP693 (the pathogenic mutation for Hereditary Cerebral Haemorrhage with Amyloidosis) (17) and APP717 Val→Ile and APP717 Val→Gly mutations (figure 1). This optimisation was performed by varying the ratio of acrylamide to bisacrylamide at either 25°C or 4°C and running gels both with and without 10% glycerol. As a result of this optimisation procedure we adopted the following as our standard protocol for all 18 exons (figure 2 illustrates SSCA of exon 18 as an example).

5μl of the amplified product was mixed with gel loading buffer containing 98% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue, 10mM EDTA, heated to denature at 80°C for 10min and placed om ice. The mixture was loaded and separated on 5% polyacrylamide gel (acrylamide/N',N'-methylene bisacrylamide 100:1) in 90mM Tris-borate, 2mM EDTA and 10% glycerol (pH8.0). Electrophoresis was carried out at 30W for 7–8 hours at 25°C. The gel was dried and exposed to X-ray film for 2–5 days.

In addition to analysis using our standard protocol, samples were also run using different ratios of acrylamide to bisacrylamide (19:1, 49:1) at 4°C with or without glycerol (10%) and at room temperature with and without glycerol (10%). The conditions described above gave the clearest separation and sharpest focussing of the different variants of exon 17.

SSCA of the promoter region was performed under the same standard conditions as above and also under four other conditions (1) 49:1; 0% glycerol; 4°C; 90mM Tris borate (2) 49:1; 10% glycerol; 25°C; 90mM Tris borate (3) 100:1; 0% glycerol; 4°C; 45mM Tris borate (4) 100:1; 5% glycerol; 25°C; 45mM Tris borate. Gels were run at 20W for 4 hours. It was found that for this GC rich tract of DNA, Method (4) resulted in the sharpest focussing of the DNA strands: this condition also, detects the known exon 17 mutations.

ACKNOWLEDGEMENTS

We would like to thank NATO, the MRC, the Mental Health Foundation, Athena Neuroscience, the Alzheimer's Disease Society, the IPSEN Foundation and the Royal Society for financial support and the families for their support and co-operation. We would like to thank Dr. Gerry Higgins and his colleagues for sending us the sequence of exon 6 and 8 primers through the APP Consortium and Drs. Bert Bakker, Christine Van Broeckhoven, Raymund Roos, David St. Clair and Merrill Benson for control samples with APP mutations. Samples from the FAD families came from the Coriell Cell Repository.

REFERENCES

1. Goate,A., Chartier Harlin,M.C., Mullan,M., Brown,J., Crawford,F., Fidani,L., Giuffra,L., Haynes,A., Irving,N., James,L. and et al, (1991) Nature, 349, 704–706.
2. Chartier Harlin,M.C., Crawford,F., Houlden,H., Warren,A., Hughes,D., Fidani,L., Goate,A., Rossor,M., Roques,P., Hardy,J. and Mullan,M. (1991) Nature, 353, 844–846.
3. Murrell,J., Farlow,M., Ghetti,B. and Benson,M. (1991) Science, 254, 97–99.
4. St George Hyslop,P.H., Haines,J.L., Farrer,L.A., Polinsky,R., Van Broeckhoven,C., Goate,A., McLachlan,D.R., Orr,H., Bruni,A.C., Sorbi,S. and et al, (1990) Nature, 347, 194–197.
5. Van Broeckhoven,C., Genthe,A.M., Vandenberghe,A., Horsthemke,B., Backhovens,H., Raeymaekers,P., Van Hul,W., Wehnert,A., Gheuens,J., Cras,P. and et al, (1987) Nature, 329, 153–155.
6. Tanzi,R.E., St George Hyslop,P.H., Haines,J.L., Polinsky,R.J., Nee,L., Foncin,J.F., Neve,R.L., McClatchey,A.I., Conneally,P.M. and Gusella,J.F. (1987) Nature, 329, 156–157.
7. Yoshikai,S., Sasaki,H., Doh ura,K., Furuya,H. and Sakaki,Y. (1990) Gene, 87, 257–263.
8. Chartier Harlin,M.C., Crawford,F., Hamandi,K., Mullan,M., Goate,A., Backhovens,H., Martin,J.J. and Van Broeckhoven,C. (1991) Neurosci. Letts., 129, 134–135.
9. Crawford,F., Hardy,J., Mullan,M., Goate,A., Hughes,D., Fidani,L., Roques,P., Rossor,M. and Chartier-Harlin,M.C. (1991) Neurosci. Letts., 133, 1–2.
10. Orita,M., Suzuki,Y., Sekiya,T. and Hayashi,K. (1989) Genomics, 5, 874–879.
11. Orita,M., Sekiya,T. and Hayashi,K. (1990) Genomics, 8, 271–278.
12. Hayashi, K. (1991) PCR Methods and Applications 1, 34–38.
13. Rumble,B., Retallack,R., Hilbich,C., Simms,G., Multhaup,G., Martins,R., Hockey,A., Montgomery,P., Beyreuther,K. and Masters,C.L. (1989) N. Engl. J. Med., 320, 1446–1452.
14. Wirak,D.O., Bayney,R., Kundel,C.A., Lee,A., Scangos,G.A., Trapp,B.D. and Unterbeck,A.J. (1991) EMBO J., 10, 289–296.
15. Goldgaber,D., Harris,H.W., Hla,T., Maciag,T., Donnell,R.J., Jacobsen,J.S., Vitek,M.P. and Gadjusek,D.C. (1989) Proc. Natl. Acad. Sci. USA, 86, 7606–7610.
16. Salbaum,J.M., Weidemann,A., Lemaire,H.G., Masters,C.L. and Beyreuther,K. (1988) EMBO J., 7, 2807–2813.
17. Levy,E., Carman,M.D., Fernandez Madrid,I.J., Power,M.D., Lieberburg,I., van Duinen,S.G., Bots,G.T., Luyendijk,W. and Frangione,B. (1990) Science, 248, 1124–1126.
18. Naruse,S., Igarashi,S., Kobayashi,H., Aoki,K., Inuzuka,T., Kaneko,K., Shimizu,T., Iihara,K., Kojima,T., Miyatake,T. and Tsuji,S. (1991) Lancet, 337, 978–979.
19. Yoshioka,K., Miki,T., Katsuya,T., Ogihara,T. and Sakaki,Y. (1991) Biochem. Biophys. Res. Commun., 178, 1141–1146.
20. Hardy,J., Mullan,M., Chartier Harlin,M.C., Brown,J., Goate,A., Rossor,M., Collinge,J., Roberts,G., Luthert,P., Lantos,P., Naruse,S., Kaneko,K., Tsuji,S., Miyatake,T., Shimizu,T., Kojima,T., Nakano,I., Yoshioka,K., Sakaki,Y., Miki,T., Katsuya,T., Ogihara,T., Roses,A.., Pericak-Vance,M., Haan,J., Roos,R., Lucotte,G. and Favid,F. (1991) Lancet, 337, 1342–1343.

027/1

NEURODEGENERATION, *Vol. 1, pp 000-000* (1992)

Screening of the Promoter and the β-Amyloid Sequence of the APP Gene for Polymorphism in Families with Late Onset Alzheimer's Disease

Karen Rooke*, Alison Goate, Liana Fidani, Mike Mullan, Penelope Roques, Martin Rossor, John Hardy and Marie-Christine Chartier Harlin

Alzheimer's Disease Research Group, Departments of Biochemistry and Neurology, St Mary's Hospital Medical School, London W2 1PG

Genetic studies of Alzheimer's disease have shown that mutations within the coding region of the amyloid precursor protein (APP) gene on chromosome 21 can be a cause of the disorder. Another cause of the disease is trisomy 21, which presumably acts through overexpression of this gene. These facts lead to the hypothesis that other cases of the disease might be generated through overexpression of APP. For this reason we have assessed whether there is genetic variability within the APP promoter in individuals suffering from the late onset familial disease. We also screened these samples for mutations within the β-amyloid sequence (exons 16 and 17 of APP770). No variability was detected. These results suggest that neither polymorphism within the APP promoter nor mutations within the β-amyloid sequence are major contributory factors in the aetiology of late onset, familial Alzheimer's disease.

Key words: Alzheimer's disease, genetics, APP, amyloid, promoter, SSCP

MUTATIONS in exon 17 of the β-amyloid precursor protein (APP) gene have been found in some families multiply affected by Alzheimer's disease (Goate *et al.*, 1991; Naruse *et al.*, 1991; Yoshioka *et al.*, 1991; Murrell *et al.*, 1991; Chartier Harlin *et al.*, 1991; Hardy *et al.*, 1991). All the families with a mutation at codon 717 have early-onset disease (< 65 years) and a segregation pattern consistent with fully penetrant autosomal dominant transmission. The occurrence of AD in individuals with trisomy 21 suggests that a second pathogenic mechanism for the disease is over-expression of APP (Tanzi *et al.*, 1987; Rumble *et al.*, 1989; Hardy & Allsop, 1991). These data suggest that APP mismetabolism is the common, unifying event in the pathogenesis of AD.

Mutations in the open reading frame region of the APP gene have not been detected in sporadic early-onset or in late-onset AD, whether sporadic or familial (Goate *et al.*, 1991; Chartier Harlin *et al.*, 1991; van Duijn *et al.*, 1991): indeed the entire sequence of the open reading frame in three cases of sporadic late onset disease have been reported to be normal (Vitek *et al.*, 1988). Furthermore, we have recently completed a mutation screen of the open reading frame and promoter region of the APP gene in 21 families with early onset disorder without finding novel mutations in either the promoter or the open reading frame of the APP gene (Fidani *et al.*, 1992). However, it remains possible that abnormal expression of APP is a contributory factor to the development of familial late onset AD, particularly in view of recent reports that this form of the disorder can be predisposed to by a locus on chromosome 21 (Heston *et al.*, 1991). Consistent with this hypothesis, in-situ hybridization analyses have indicated an alteration of the amount of APP mRNA in brains of individuals with AD compared with normals (Cohen *et al.*, 1988; Johnson *et al.*, 1988; Lewis *et al.*, 1988; Higgins *et al.*, 1988; Palmert *et al.*, 1989; Clark & Parhad, 1989). However, it is likely that the aetiology and genetics of late onset familial AD are complex since segregation analysis does not support the view that it is a monogenic disorder (Farrer *et al.*, 1990) and genetic predisposition at a chromosome 19 locus has also been reported (Pericak-Vance *et al.*, 1991).

The promoter of the APP gene lacks a typical TATA

*Author to whom correspondence should be addressed

Received 27 April 1991; revised and accepted for publication 30 July 1992

©1992 Academic Press Limited

1055-8330/92/000000+00 $08.00/0

box and its organization resembles promoters of housekeeping genes (see Figure 1). There is a heat shock consensus sequence at position −317: upregulation of APP in response to stress may be modulated at this element (Abe *et al.*, 1991). There is also a region with homology to the binding site consensus sequence of the transcription factor AP-1 at position −350 (Goldgaber *et al.*, 1989; Salbaum *et al.*, 1988): it has been suggested that interleukin 1 (IL-1) upregulates APP expression through a pathway mediated by protein kinase C using this site (Goldgaber *et al.*, 1989). Transgenic experiments have shown that this region of the promoter directs reporter gene expression in neuronal cells with a similar, but not identical, distribution to endogenous APP (Wirak *et al.*, 1991).

In view of the possibility that late onset familial AD could be caused by overexpression of APP determined by variability within these promoter elements, we decided to screen them for sequence variability in individuals from families with this form of the disorder. Since mutations causing early onset AD occur close to the β-amyloid sequence in exons 16 and 17, we also screened these exons for variability: samples with known mutations served as positive controls in our analyses.

We used single strand conformation polymorphism analysis (SSCP) (Orita *et al.*, 1989, 1990) as the screening technique. SSCP was chosen because it is less expensive than denaturing gradient gel electrophoresis and the procedure is quicker and easier to set up. Recent data suggest that it is between 80% and 95% effective at detecting mutations (Hayashi, 1991). SSCP is preferable to direct sequencing because of its ease and efficiency and yet the procedure has a high probability of detecting mutations (Orita *et al.*, 1989, 1990).

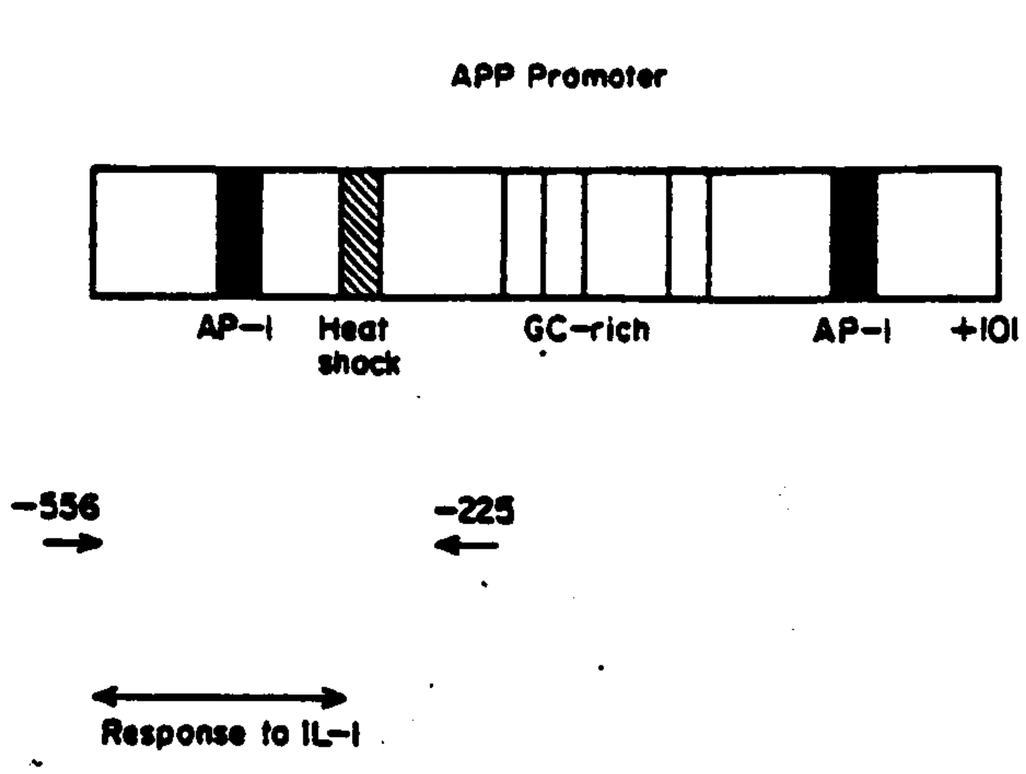


Figure 1. Diagram of the promoter of the APP gene showing regulatory elements; adapted from Goldgaber *et al.* (1989).

Methods

Families were collected as part of our ongoing AD genetics programme. In all cases, family members met standard clinical criteria for probable AD: in some families, pathological confirmation of this diagnosis was available (Table 1). Blood DNA was extracted by standard methods.

PCR of the promoter region was carried out using the following primers: [sense] (at −558) 5′-GCT-TTT-GAC-GTT-GGG-GGT-TA-3′ and [reverse antisense] (at −225) 5′-AAC-AGT-GGG-AGG-GAG-AGT-CT-3′. The PCR using 30 ng of DNA was performed in a reaction volume of 100 μl with a final concentration of primers of 0.2 μM. Final concentrations of 200 μM of all nucleotides were used apart from dATP which was used at a concentration of 24 μM, 1.0 μl of [α-^{35}S] dATP. (Specific activity 600Ci/mmol) and 1 unit of Cambio Taq polymerase]. PCR amplification was optimized by using an initial cycle of 5 min at 94°C, 1.5 min at 60°C then 35 cycles of 72°C:1 min, 94°C:1 min and 60°C:1 min and finally 10 min at 72°C. PCR of exons 16 and 17 was carried out as we have previously described (Fidani *et al.*, 1992).

The mobility of single-stranded DNA is affected by: (i) percentage of acrylamide and proportion of bisacrylamide added as cross-linker, (ii) glycerol concentration, (iii) temperature and (iv) ionic strength of electrophoretic buffer. SSCP of the promoter region was performed under five conditions by varying the above parameters (Table 2, Figure 2). SSCP of exons 16 and 17 of the APP gene was also carried out under condition 5 (Table 2) which gives the clearest separation of the variants of exon 17, detecting APP693, APP717Val→Ile, APP717Val→Gly and APP717Val→Phe (Figure 3).

The PCR amplified product (3–5 μl) was mixed with a gel-loading buffer containing 98% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue and 20 mM EDTA to a total volume of 8 μl, heat denatured at 80°C for 10 min and quenched on ice before being quickly loaded onto a non-denaturing polyacrylamide gel. DNA from a yeast artificial

Table 1. Details of families screened for variability

Family no.	Age of onset	Number of clinically affected individuals with number confirmed at autopsy in parentheses
F15	60–70	2 (1)
F38	66–77	5 (1)
F43	69–75	5
F44	70–80	5 (1)
F45	75–90	5
F46	70–80	4
F49	70–80	3 (1)
F78	67–78	5
F79	75–84	6 (1)
F80	72–80	5 (1)
F101	65–75	5 (1)
F107	65–75	2
F116	68–74	4 (1)

627/3

Table 2. SSCP Conditions used for variability screen

Condition	Acrylamide:bis	glycerol	temperature	conc.TBE
(1)	49:1	0%	4°C	1×
(2)	49:1	10%	25°C	1×
(3)	100:1	0%	4°C	0.5×
(4)	100:1	5%	25°C	0.5×
(5)	100:1	10%	25°C	1×

chromosome containing the APP promoter was used as a control. For conditions (1)–(4), electrophoresis was carried out at 20W for 4 h; for condition (5) electrophoresis was at 30W for 7 h. The gel was vacuum dried at 80°C for 1 h and exposed to X-ray film for 2–5 days.

Results

All five conditions gave clear separation of the single stranded products derived from the promoter (Figure 2). The bands observed correspond to the APP promoter as they were observed in DNA derived from a yeast strain harbouring an artificial chromosome containing the APP gene promoter. Furthermore, enzyme digests of the amplified product with *Sma*1 and *Msp*1 revealed a restriction pattern consistent with the sequence of the promoter.

No variability in the SSCP pattern was observed in any individual from any of the families we examined.

SSCP of exons 16 and 17 from the families with late onset disease failed to reveal any variation, although the conditions used did detect all the known mutations (Figure 3).

Discussion

SSCP is reported to detect between 80% and 95% of point mutations (Orita *et al.*, 1989, 1990; Hayashi, 1991). There may be mutations not detected by conditions tested here but this is unlikely as a range of conditions were used. Thus, the data we present here are strong evidence that differing DNA sequences in the AP-1 binding domain, the heat shock element, or the β-amyloid region do not contribute to the familial clustering of late onset AD.

If upregulation of APP expression is involved in the aetiopathogenesis of late onset AD, and if genetic variability in the regulation of this expression is responsible for familial clustering, this genetic variability must be at other points in the pathways regulating APP expression. There are at least two potential pathways of regulation of APP expression in which there may be genetic variation. These are the 'acute phase' response to neuronal damage mediated through the interleukin (IL-1/IL-6) pathway (Goldgaber *et al.*, 1989; Vandenabeele & Fiers, 1991) and the 'stress response' mediated by heat shock proteins (Abe *et al.*, 1991).

Figure 3. SSCP gel of variant APP exon 17 samples from left to right: APP693, control, APP717 Val→Phe, APP717 Val→Gly, APP717 Val→Ile.

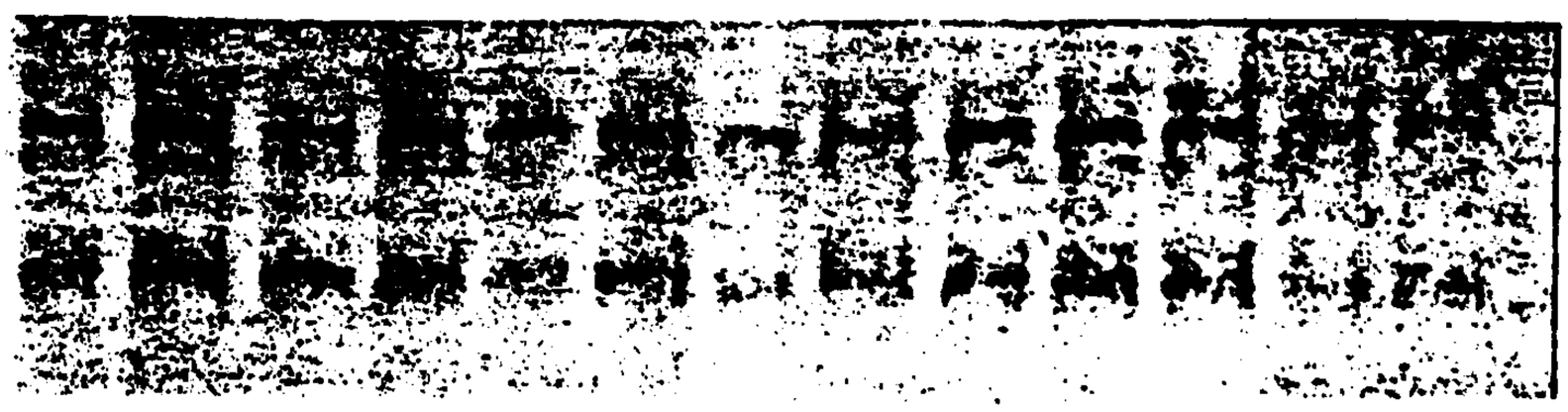


Figure 2. SSCP gel of PCR amplified promoter from 12 individuals with familial late onset AD and (right hand side) the amplified yeast artificial chromosome containing the APP gene.

Acknowledgements

We would like to thank the Alzheimer's Disease Society, MRC, the Mental Health Foundation, Athena Neuroscience, NATO, the IPSEN Foundation and the Royal Society for financial support and the families for their support and co-operation. We would like to thank Drs Bert Bakker, Christine Van Broeckhoven, Raymund Roos and Merrill Benson for samples of APP693 and APP717 mutations.

References

Abe K, St. George Hyslop PH, Tanzi RE, Kogure K (1991) Induction of amyloid precursor protein mRNA after heat shock in cultured human lymphoblastoid cells. Neurosci Letts 125:169–171

Chartier Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, Mullan M (1991) Early onset Alzheimer's disease caused by mutations at codon 717 of the β-amyloid precursor protein gene. Nature 353:844–846

Clark AW, Parhad IM (1989) Expression of neuronal mRNAs in Alzheimer type degeneration of the nervous system. Can J Neurol Sci 16:477–482

Cohen ML, Golde TE, Usiak MF, Younkin LH, Younkin SG (1988) In situ hybridisation of nucleus basilis neurons shows increased β-amyloid mRNA in Alzheimer disease. Proc Natl Acad Sci USA 85:1227–1231

Farrer LA, Myers RH, Cupples LA, St. George Hyslop PH, Bird TD, Rossor MN, Mullan MJ, Polinsky R, Nee L, Heston L et al. (1990) Transmission and age-at-onset patterns in familial Alzheimer's disease: evidence for heterogeneity. Neurology 40:395–403

Fidani L, Rooke K, Chartier Harlin MC, Hughes D, Tanzi R, Mullan M, Roques P, Rossor M, Hardy J, Goate AM (1992) Screening for mutations in the open reading frame and promoter of the β-amyloid precursor protein gene in familial Alzheimer's disease: identification of a further family with APP717 Val→Ile. Hum Mol Genet 1:165–168

Goate A, Chartier Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Hynes A, Irving N, James L et al. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704–706

Goldgaber D, Harris HW, Hla T, Maciag T, Donnell RJ, Jacobsen JS, Vitek MP, Gadjusek DC (1989) Interleukin 1 regulates synthesis of amyloid β-protein precursor mRNA in human endothelial cells. Proc Natl Acad Sci USA 86:7606–7610

Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol 12:383–388

Hardy J, Mullan M, Chartier Harlin MC, Brown J, Goate A, Rossor M, Collinge J, Roberts G, Luthert P, Lantos P, Naruse S, Kaneko K, Tsuji S, Miyatake T, Shimizu T, Kojima T, Nakano I, Yoshioka K, Sakaki Y, Miki T, Katsuya T, Ogihara T, Roses A, Pericak-Vance M, Haan J, Roos R, Lucotte G, Favid F (1991) Molecular classification of Alzheimer's disease. Lancet 337:1342–1343

Hayashi K (1991) Single strand conformation analysis. PCR Methods and Applications 1:34–38

Heston LL, Orr HT, Rich SR, White JA (1991) Linkage of an Alzheimer disease susceptibility locus to markers on human chromosome 21. Am J Med Genet 40:449–453

Higgins GA, Lewis DA, Bahmanyar S, Goldgaber D, Gajdusek DC, Young WG, Morrison JH, Wilson MC (1988) Differential regulation of amyloid-beta-protein mRNA expression within hippocampal neuronal subpopulations in Alzheimer disease. Proc Natl Acad Sci USA 85:1297–1301

Johnson SA, Pasinetti GM, May PC, Ponte PA, Cordell B, Finch CE (1988) Selective reduction of mRNA for the beta-amyloid precursor protein that lacks a Kunitz-type protease inhibitor motif in cortex from Alzheimer brains. Exp Neurol 102:264–268

Lewis DA, Higgins GA, Young WG, Goldgaber D, Gajdusek DC, Wilson MC, Morrison JH (1988) Distribution of precursor amyloid-beta-protein messenger RNA in human cerebral cortex: relationship to neurofibrillary tangles and neuritic plaques. Proc Natl Acad Sci USA 85:1691–1695

Murrell J, Farlow M, Ghetti B, Benson M (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science 254:97–99

Naruse S, Igarashi S, Kobayashi H, Aoki K, Inuzuka T, Kaneko K, Shimizu T, Iihara K, Kojima T, Miyatake T, Tsuji S (1991) Mis-sense mutation Val→Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. Lancet 337:978–979

Orita M, Sekiya T, Hayashi K (1990) DNA sequence polymorphisms in Alu repeats. Genomics 8:271–278

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874–879

Palmert MR, Podlisny MB, Golde TE, Cohen ML, Kovacs DM, Tanzi RE, Gusella JF, Whitehouse PJ, Witker DS, Oltersdorf T et al. (1989) The beta amyloid protein precursor: mRNAs, membrane-associated forms, and soluble derivatives. Prog Clin Biol Res 317:971–984

Pericak-Vance MA, Bebout JL, Gaskell PC, Yamaoka LH, Hung WY, Alberts MJ, Walker AP, Bartlett RJ, Haynes CA, Welsh KA, Earl NL, Heyman A, Clark CM, Roses AD (1991) Linkage studies in familial Alzheimer's disease: evidence for chromosome 19 linkage. Am J Hum Genet 48:1034–1050

Rumble B, Retallack R, Hilbich C, Simms G, Multhaup G, Martins R, Hockey A, Montgomery P, Beyreuther K, Masters CL (1989) Amyloid A4 protein and its precursor in Down's syndrome and Alzheimer's disease. N Engl J Med 320:1446–1452

Salbaum JM, Weidemann A, Lemaire HG, Masters CL, Beyreuther K (1988) The promoter of Alzheimer's disease amyloid A4 precursor gene. EMBO J 7:2807–2813

Tanzi RE, Gusella JF, Watkins PC, Bruns GA, St George Hyslop P, Van Keuren ML, Patterson D, Pagan S, Kurnit DM, Neve RL (1987) Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science 235:880–884

van Duijn CM, Hendriks L, Cruts M, Hardy JA, Hofman A, Van Broeckhoven CM (1991) Amyloid precursor protein gene mutation in early-onset Alzheimer's disease. Lancet 337:978

Vandenabeele P, Fiers W (1991) Is amyloidogenesis during Alzheimer's disease due to an IL-1-/IL-6-mediated 'acute phase response' in the brain. Immunol Today 12:217–219

Vitek MP, Rasool CG, de Sauvage F, Vitek SM, Bartus RT, Beer B, Ashton RA, Macq AF, Maloteaux JM, Blume AJ et al. (1988) Absence of mutation in the beta-amyloid cDNAs cloned from the brains of three patients with sporadic Alzheimer's disease. Brain Res 464:121–131

Wirak DO, Bayney R, Kundel CA, Lee A, Scangos GA, Trapp BD, Unterbeck AJ (1991) Regulatory region of human amyloid precursor protein (APP) gene promotes neuron-specific gene expression in the CNS of transgenic mice. EMBO J 10:289–296

Yoshioka K, Miki T, Katsuya T, Ogihara T, Sakaki Y (1991) The 717Val→Ile substitution in amyloid precursor protein is associated with familial Alzheimer's disease regardless of ethnic groupe. Biochem Biophys Res Commun 178:1141–1146

Down Syndrome and Alzheimer Disease, pages 195-214
© 1992 Wiley-Liss, Inc.

MUTATIONS IN APP AND THEIR ROLE IN β-AMYLOID DEPOSITION

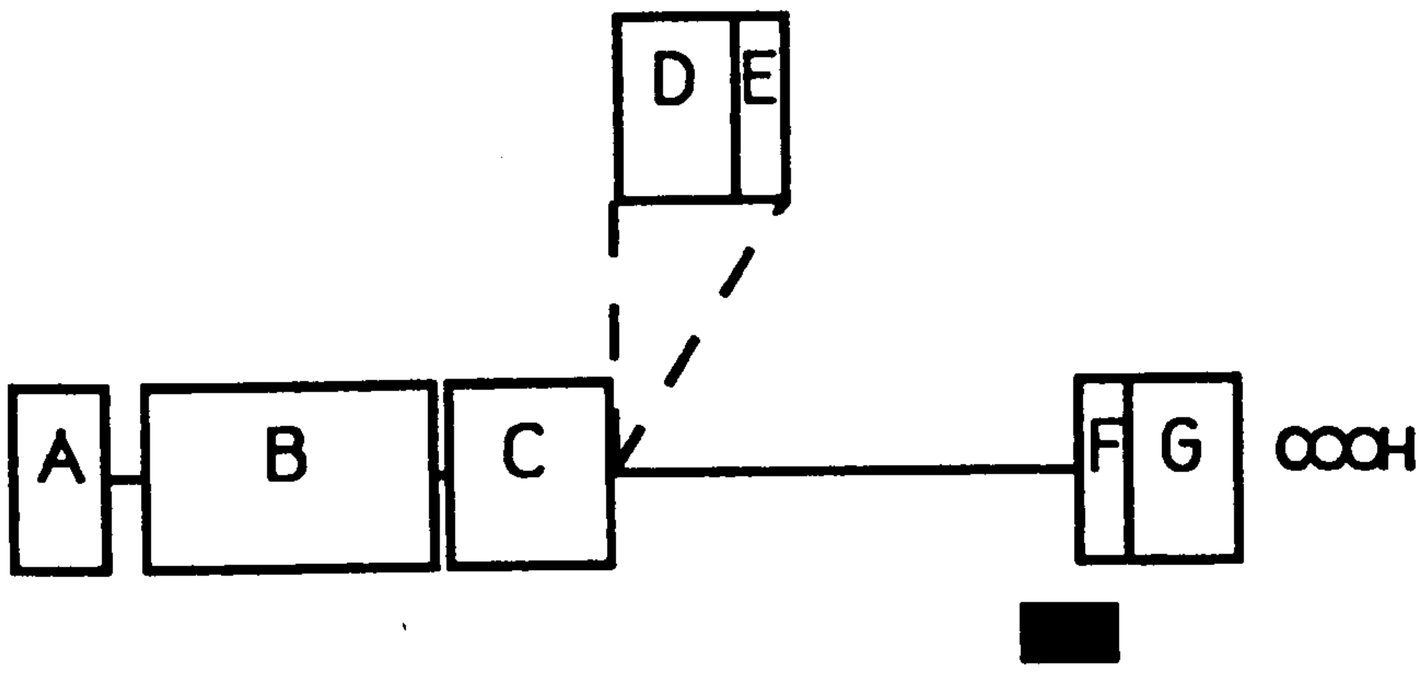
Liana Fidani & Alison Goate

Department of Biochemistry & Molecular Genetics, St. Mary's Hospital Medical School, Imperial College, London W2 1PG, UK.

FAMILIAL ALZHEIMER DISEASE

Alzheimer disease (AD) is a common neurodegenerative disorder characterised by the presence of large numbers of senile plaques and neurofibrillary tangles within the brain cortex. Senile plaques are extracellular deposits of an amyloidogenic peptide surrounded by degenerating nerve terminals whilst neurofibrillary tangles are intraneuronal bundles of an abnormally phosphorylated form of the cytoskeletal protein "tau". In addition there is massive neuronal cell loss and cerebrovascular amyloid deposits in blood vessels of the meninges, cerebral cortex and hippocampus (Katzman R.,1986). The same pathology is found in individuals with Down syndrome (trisomy 21) after the 3rd decade of life and cerebrovascular amyloid deposition is observed in hereditary cerebral haemorrhage with amyloidosis (HCHWA). In 1984 Glenner and Wong purified a protein from the fibrils of cerebrovascular amyloid deposits in AD and in 1985 Masters et al.purified the protein that forms the amyloid plaque core in AD and aged Downs individuals. In all cases the purified protein was a 4kd (39-42 amino-acids) protein with a β-pleated sheet conformation which was termed β-amyloid (β-A4). A pathogenic link between Alzheimer disease and cerebral amyloid angiopathies was also demonstrated since their amyloid deposits were shown to be antigenically related (Coria et al.,1987). Partial amino-acid sequencing of the amyloid subunit from a SCAA(Sporadic Cerebral Amyloid Angiopathy) and a

HCHWA-D (Hereditary Cerebral Hemorrhage With Amyloidosis-Dutch type) patient identified β-A4 protein (Coria et al.,1988). Subsequent cloning of the gene for β-amyloid has shown that the deposited peptide is a breakdown product of a larger precursor, the amyloid precursor protein (APP) which has features characteristic of a glycosylated cell-surface receptor. The β-A4 peptide is derived from part of the protein normally found within the transmembrane domain (Figure 1).



A=signal peptide
B=cysteine-rich region
C=negatively charged domain
D=Kunitz type protease inhibitor domain
E='ox' antigen domain
F=transmembrane domain
G=cytoplasmic domain
■ =β -amyloid peptide

Figure 1, Diagram showing the structural domains of the amyloid precursor protein.

The gene encoding the amyloid precursor protein was shown to map to the middle of the long arm of chromosome 21 in band 21q21 (Kang et al.,1987; Goldgaber et al.,1987; Tanzi et al.,1987a; Robakis et al.,1987). At about the same time that the APP gene was cloned and mapped, a genetic linkage was detected between chromosome 21 markers and the

disease in four families affected by an autosomal dominant form of early onset AD (St. George-Hyslop et al.,1987a). This linkage potentially explained the occurrence of AD in Down syndrome and directed the spotlight on the APP gene. Other research had shown at the time that sporadic AD patients have a duplication of the APP locus (Delabar et al.,1987). The excitement though, was soon over when different groups failed to confirm Delabar's findings (St.George-Hyslop et al.,1987b) and recombinants were detected between APP and AD (Van Broeckhoven et al,1987; Tanzi et al.,1987b; Schellenberg et al.,1988). Sequencing of APP cDNA from brains of sporadic AD patients was also undertaken but the sequence was found to be normal (Vitek et al.,1988).

Genetic linkage data from some studies suggested that the disease locus mapped close to the pericentromeric markers D21S16 and D21S13 (St.George-Hyslop et al.,1987a; Goate et al.,1989) although other researchers failed to detect this linkage in their families (Schellenberg et al.,1988; Pericak-Vance et al.,1988). In 1990 a collaborative linkage study in 48 AD families provided additional evidence of genetic heterogeneity in Alzheimer disease: the families with early onset AD were linked but families with late onset disease showed no evidence for linkage to chromosome 21 markers, although phenotypically the disease is similar in the two groups (St.George -Hyslop et al.,1990). Thus, genetic heterogeneity was confounding efforts to localise the disease gene.

MUTATIONS IN THE APP GENE

Studies of Down syndrome, which is caused by trisomy of chromosome 21, have suggested that deposition of β-A4 is an early event in the devlopment of AD pathology (Rumble et al., 1989). Trisomy of chromosome 21 leads to an increase in the levels of expression of APP in the brain which may lead directly to early deposition of β-A4 and AD-like pathology.

In HCHWA-D the APP gene was shown to be tightly linked to the disease locus (Van Broeckhoven et al.,1990) and a point mutation, a C to G transversion was identified which changes a glutamic acid residue to glutamine at codon 693 of the APP770

transcript (position 22 of the β-A4 protein) suggesting that this is the cause of the specific amyloid deposition in the cerebral vessel walls of HCHWA-D patients (Levy et al.,1990). Indeed subsequent research has shown that this codon 693 variant segregates with the disease in different ethnic backgrounds increasing the likelihood that the mutation is pathogenic (Fernandez-Madrid et al.,1991).

The demonstration of genetic heterogeneity in AD led us to use a single large, clinically well characterised, autopsy confirmed pedigree (F23) which showed linkage between the disease and chromosome 21 markers, to localise the disease gene. Although APP had been excluded as the disease locus previously (Van Broeckhoven et al,1987; Tanzi et al.,1987; Schellenberg et al.,1988), the demonstration of genetic heterogeneity meant that APP must be excluded in each family individually. Indeed in F23 we detected linkage between APP and the disease suggesting that it could be the site of the genetic defect in this family. Exon 17 was sequenced first since it encodes part of the β-A4 protein and is the site of the HCHWA-D mutation. A G to A transition at bp 2,149 was detected changing a valine to isoleucine at APP717, within the transmembrane segment two residues beyond the C-terminus of the β-A4 protein (Goate et al.,1991) (Figure 2). The mutation cosegregates with the disease within the family but has not been found in over 400 normal chromosomes analysed, (Chartier-Harlin et al.,1991a; Goate et al., 1991) making the occurrence of the mutation in 6 early onset AD families of different ethnic background remarkable (Goate et al.,1991; Naruse et al.,1991; Yoshioka et al.,1991) and its pathogenicity more likely. The APP717 val->ile mutation seems to be associated with a disease onset between 50 and 60 years of age with a typical clinical picture and severe AD pathology. This particular mutation accounts for only a small proportion of familial early onset Alzheimer disease cases(Van Duijn et al.,1991; Goate et al.,1991; Hardy et al.1991; Schellenberg et al.,1991) and both linkage analysis and sequencing suggests that other loci are causative for most other cases of familial AD (Chartier-Harlin et al.,1991a; Crawford et al.,1991). It remains though the first known cause of the disease.

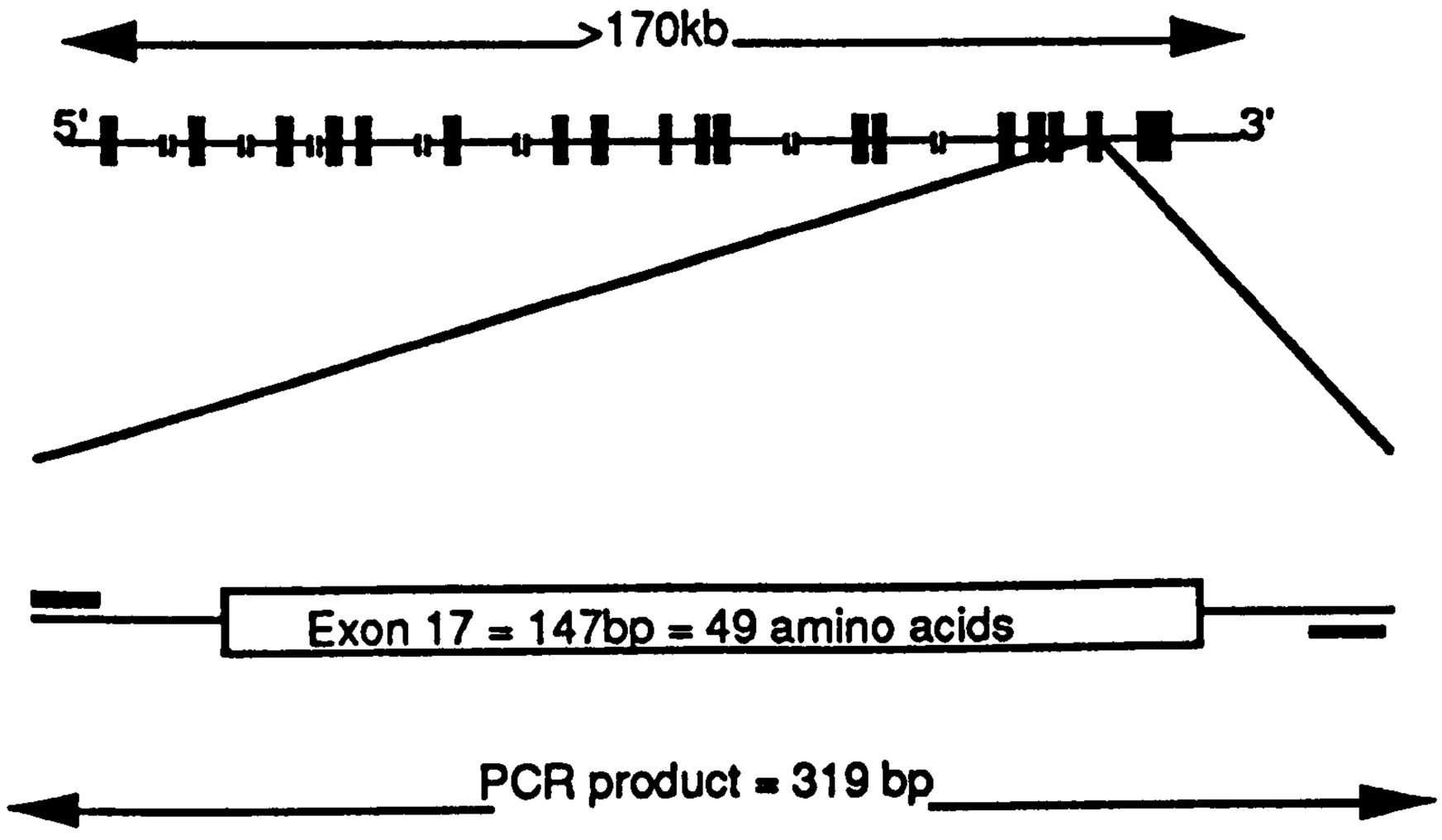


Figure 2, Genomic organisation of the human amyloid precursor protein gene and mutations in exon 17 which cause Alzheimer disease and Hereditary cerebral haemorrhage with amyloidosis-Dutch type.

Subsequent investigations have identified two further mutations within the same codon which cause familial AD. One occurs in a British family with disease onset of 59+/-4 years. Sequencing of exon 17 in this family revealed a T to G transition at bp 2150 changing valine to glycine at codon 717 (Chartier-Harlin et al.,1991b) (Figure 2). Sequencing of a US family with early onset AD (age of onset 40 years) revealed a C to T change at bp 2,148 changing the valine to a phenylalanine (Murrell et al.,1991). These newly found mutations have not yet been identified in any other cases of AD.

APP PROCESSING AND AMYLOIDOGENESIS

Delineation of the pathways by which the APP molecule can be proteolytically processed, will give us insight into possible pathogenic mechanisms. An intact amyloidogenic fragment is not generated during one pathway of normal APP processing since cleavage occurs within the β-A4 peptide (Sisodia et al.,1990). APP processing is thought to occur within the endosomal-lysosomal pathway. A processing event by a protease termed "APP secretase" results in the release of a 100 kD soluble amino-terminal fragment and a 9 kD membrane bound carboxy-terminal APP fragment (Esch et al.,1990). Several groups have tried to determine the substrate specificity of this "secretase" using site-directed mutagenesis. Preliminary evidence suggests that it has a very broad sequence specificity but cleaves APP at a defined distance from the membrane. Cathepsin B, which generally distributes in the lysosomes of mammalian cells, has been suggested as a candidate for the APP processing enzyme (Tagawa et al.,1991). Cathepsins D and B are components of senile plaques (Cataldo & Nixon et al.,1990).

Carboxyl terminal fragments containing the entire β-A4 sequence can also be derived by normal processing. It has been suggested that it is these fragments which lead to β-A4 formation and deposition (Nordstedt et al., 1991). A study to determine whether normal aging or AD are associated with alterations in the amounts of individual isoforms of APP or in their proteolytic fragments has shown that brains from aged individuals and AD pa-

tients contain the same amount of total APP protein as those of young individuals.(Nordstedt et al.,1991) but aging alters the metabolism of APP leading to a substantial increase of APP 19kDa carboxyl-terminal fragment thought to contain an intact β-A4.
Recent work suggests that processing of the amyloid precursor protein may be regulated by protein phosphorylation. Activation of protein kinase C or inhibition of protein phosphatases α-1 and α-2A diminish recovery of the mature amyloid precursor protein while enhancing recovery of a carboxy-terminal fragment of approximately 15kDa (Buxbaum et al.,1990). A combination of kinase activation and phosphatase inhibition leads to the generation of a 15kDa and a 19kDa carboxy-terminal fragment. This latter product might represent processing via an alternative and potentially amyloidogenic pathway, perhaps by cleavage at or near the β-A4 amino-terminus (Buxbaum et al.,1990; Gandy et al.,1990).

THE EFFECTS OF A POINT MUTATION ON THE PROCESSING OF APP

The identification of three mutations in the same codon of the gene accentuates their significance making this codon particularly important for processing of the amyloid protein, leading to the development of AD pathology. It has been suggested that the first mutation, val->ile, would make the transmembrane domain more hydrophobic and so anchor the protein more firmly in the membrane (Goate et al.,1991). The identification of other mutations involving different amino-acid changes which do not increase the hydrophobicity indicate that this is unlikely to contribute to the mechanism of pathogenicity.
A second hypothesis suggested that the mutations disrupt a regulatory stem loop in APP mRNA which alters translational regulation leading to the production of abnormally high amounts of protein. The effect of the mutation would therefore be identical to the effect of trisomy 21 (Tanzi & Hyman,1991). Several observations make it unlikely that this hypothesis is correct: a) although the valine residue is well conserved in mammalian species (Johnstone et al.,1991) the putative stem loop structure is not and b) levels of APP mRNAs do not appear to be

altered significantly by the val->ile mutation (Harrison et al.,1991) as expected if the mRNA stability was altered (Klausner et al.,1989). In addition mutations which do not alter the amino acid sequence but disrupt the stem loop structure would be predicted to cause AD. The most recent hypothesis is that β-A4 is generated by abnormal cleavage of APP, and that the mutations cause APP to be a worse substrate for the normal processing enzymes or a better substrate for the peptidases of the alternative pathway (Chartier-Harlin et al.,1991b). The HCHWA-D mutation is close to the normal site of cleavage of APP (Esch et al, 1990). It has been suggested that the mutation may change the structure of the molecule in this region so that the normal cleavage enzyme is less efficient (Levy et al.,1990). However, since recent evidence suggests that the enzyme has a broad sequence specificity this hypothesis is likely to be incorrect.

APP GENE - PROTEASE INHIBITION AND AMYLOIOGENESIS

The amyloid precursor protein gene is greater than 170kb and contains 18 exons (Yoshikai et al.,1990). Exons 16 and 17 code for the β-A4 protein. Screening for mutations in exons other than exon 17 has failed to reveal any further alterations (unpublished data). 5 different transcripts have been identified which can be produced from the APP gene by differential splicing (Figure 3). Two of these show homology to serine protease inhibitors (Ponte et al.,1988; Tanzi et al.,1988; Kitagushi et al.,1988). The APP714 transcript (Golde et al.,1990; Kang & Muller-Hill, 1990) has a 57 nt insert (exon 8) but lacks the protease inhibitor domain, and the APP563 transcript (Neve et al.,1990; De Sauvage et al.,1989) lacks the β-A4 region and contains an Alu domain,in the carboxyl-terminal (Figure.3). The amino terminal sequence of the largest transcripts APP751 and APP770 has been found to be identical to the sequence of a cell secreted protease inhibitor, protease nexin-II (Oltersdorf et al.,1989). The protease inhibitor domain of APP is not found in the major transcript (APP695) found in neurons. However, those transcripts containing the protease inhibitor domain have been shown to be increased in the brain tissue from AD patients (Tanaka et al.,1989) suggesting

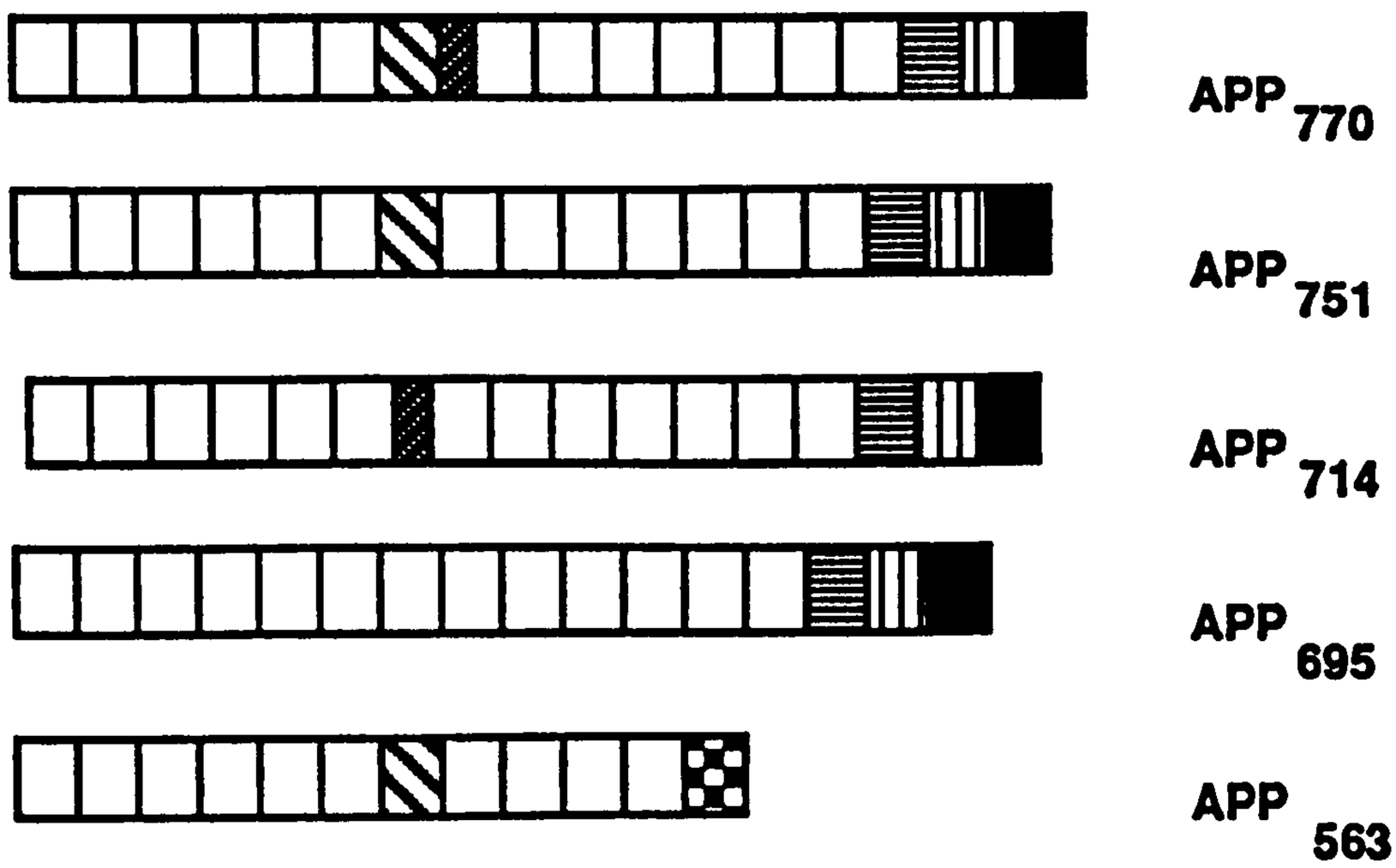


Figure 3, Alternative splicing in the amyloid precursor protein gene.

that levels of this protein may be important in the pathogenesis of the disease.

Protease nexin-II (PN-II) has growth promoting activities and is secreted at wound sites where platelets aggregate and thus influence inflammatory responses and tissue repair. As a serine protease inhibitor, APP effectively inactivates chymotrypsin (Van Nostrand et al.,1989) indicating that a physiological function of PNII/APP may be the regulation of a chymotrypsin-like protease. This protease could generate β-A4 protein or other peptides such as α1-antichymotrypsin, another serine protease inhibitor, that is also deposited in the senile plaques (Abraham et al.,1988). α1-antichymotrypsin

is a rather weak inhibitor of chymotrypsin but an effective inhibitor of several inflammation-associated proteases. The concentration of α1-antichymotrypsin doubles in serum in response to inflammation. A local excess of α1-antichymotrypsin might prevent a normal brain protease from clearing cleavage products of APP. The amino terminal of the two cleavage sites that flank the β-A4 protein within its precursor contains a methionine and could therefore result from processing by a chymotrypsin-like protease (Abraham et al.,1988).

APP: INITIATING POINT OF AETIOLOGY

The fact that patients with trisomy 21 and therefore altered expression of the APP gene show diffuse β-A4 deposits as early as their mid-teens (Rumble et al.,1989) and that the brain lesions of older Down individuals are no different than those of AD, suggests that it is likely that the β-A4 deposition precedes neuritic and glial alteration in AD as well.

In Down syndrome the increase in APP transcription may cause APP molecules to be processed by an alternative pathway, leading to β-A4 formation and early amyloid deposition. A different genetic mechanism altering β-A4 in FAD may result in the increased use of the alternative catabolic pathway.(Selkoe et al.,1990)

The mutations in the APP gene in AD and HCHWA-D suggest that APP and β-amyloid deposition are central to the pathogenic mechanism in these diseases and that neurofibrillary tangles and neuronal death occur later (Hardy & Allsop,1991). Indeed, recent evidence from a transgenic mouse model shows that overexpression of a carboxy-terminal fragment of APP in neurons can lead to the full pathology of AD (Kawabata et al., 1991). This carboxyl-terminal fragment (105 aa) has previously been shown to be neurotoxic (Yankner et al.,1989) whereas an amino-terminal peptide (28 aa) has been shown to increase the survival of hippocampal neurons.(Whitson et al.,1989). Although β-A4 is not itself neurotoxic it makes neurons more sensitive to excitotoxic damage (Whitson et al.,1989). Thus altered processing or overexpression of APP in AD may result in neurotoxicity. Finally it has been suggested that β-amyloid destabilizes neuronal calcium homeostasis

and therefore makes neurons more vulnerable to environmental effects (Mattson et al.,1992) explaining in part the toxicity of β-amyloid to neurons. This might explain the abnormal phosphorylation of tau which is controlled by calcium dependent enzymes. The occurrence of tangle formation in a number of unrelated conditions, Guam Parkinsonism dementia complex, dementia pugilistica, Heelervorden-Spatz disease, suggests that neurofibrillary tangles may represent a common pathological response to cell death.

OTHER CAUSES APART FROM APP MUTATIONS

Mutations in APP account for a small proportion of cases of AD. However, the occurrence of AD in virtually all individuals with Down syndrome over the age of thirty suggests that control of the levels of APP is very important. Other aetiologies may act by perturbing APP expression. For example Roberts et al., 1991 have recently shown that head trauma can trigger amyloid deposition as an acute response to neuronal injury and may therefore be a risk factor for AD. This could happen via increased interleukin-1 (IL-1) production induced by injury which in turn induces an acute phase response within the brain leading to increased synthesis of α1-antichymotrypsin and APP (Royston et al.,1991). Excess APP production may have the same effect as an extra dosage of the gene seen in Down syndrome.

Transcription factors could bind to the APP promoter (Salbaum et al.,1990) and regulate APP expression. Mutations in genes coding for enzymes involved in APP processing or genes for proteins which interact with the APP gene (transcription factors) may also account for other cases of familial AD.

In conclusion mutations in the gene for APP can cause both Alzheimer disease and hereditary cerebral haemorrhage with amyloidosis. Overexpression of APP in Down syndrome and in transgenic mouse models also leads to AD pathology. This suggests that processing of APP is central to the pathogenic mechanism of AD and that the other pathological features are secondary events. Understanding the pathogenic process will undoubtedly lead to treatments for AD in the future.

REFERENCES

Abraham CR, Selkoe DJ, Potter H (1988): Immunochemical Identification of the Serine Protease Inhibitor α1-Antichymotrypsin in the Brain Amyloid Deposits of Alzheimer's Disease. Cell 52:487-501

Buxbaum JD, Gandy SE, Ciccheti P, Ehrich ME, Czernik AJ, Fracasso RP, Ramabhadran TV, Unterbeck AJ, Greengard P. (1990): Processing of Alzheimer βA4 amyloid precursor protein: Modulation by agents that regulate protein phosphorylation. Proc.Natl.Acad.Sci. 87:6003-6006

Cataldo AM, Nixon RA (1990): Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain. Proc.Natl.Acad.Sci. 87:3861-3865

Chartier-Harlin MC, Crawford F, Hamandi K, Mullan M, Goate A, Hardy J, Backhovens H, Martin JJ, Van Broeckhoven C (1991a): Screening for the β-amyloid precursor protein mutation (APP717:val->ile) in extended pedigrees with early onset Alzheimer's disease. Neurosci. Letts. 129:134-135

Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, Mullan M (1991b): Early-onset Alzheimer's disease is caused by mutations in codon 717 of the β-amyloid precursor protein. Nature 353: 844-846

Coria F, Castano EM, Frangione B (1987): Brain amyloid in normal aging and cerebral amyloid angiopathy is antigenically related to Alzheimer's disease β-protein. Am.J.Path. 129: 422-428

Coria F, Prelli F, Castano EM, Larrondo-Lillo M, Fernandez-Gonzalez J, Van Duinen SG, Bots GTAM, Luyendijk W, Shelanski ML, Frangione B (1988): β-Protein deposition: a pathogenetic link between Alzheimer's disease and cerebral amyloid angiopathies. Brain Res. 463:187-191

Crawford F, Hardy J, Mullan M, Goate A, Hughes D, Fidani L, Roques P, Rossor M, Chartier-Harlin MC (1992): Sequencing of exons 16 and 17 of the β-amyloid precursor protein gene in 14 families with early onset Alzheimer's disease fails to reveal mutations in the β-amyloid sequence. Neurosci.Letts. in press

Delabar J-M, Goldgaber D, Lamour Y, Nicole A, Huret J-L, Grouchy J, Brown P, Gajdusek DC, Sinet P-M (1987): β- Amyloid gene duplication in Alzheimer's disease and karyotypically normal Down syndrome. Science 235,1390-1392

De Sauvage F, Octave JN (1989): A novel mRNA of the A4 Amyloid precursor gene coding for a possibly secreted protein. Science 245:651-653

Esch FS, Keim PS Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ (1990): Cleavage of amyloid β-peptide during constitutive processing of its precursor. Science 248:1122-1124

Fernandez-Madrid I, Levy E, Marder K, Frangione B (1991): Codon 618 Variant of Alzheimer Amyloid Gene Associated with inherited Cerebral Hemorrhage. Ann. Neurol. 30:730-733

Gandy SE, Buxbaum JD, Greengard P (1991) : Signal transduction and the pathology of Alzheimer's disease. In Iqbal K, McLachlan D, Winblad B, Wisniewski H(eds): Alzheimer's disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies.England: Wiley-Liss, Inc., pp 155-172

Glenner GG, Wong CW (1984): Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem. Bioph.Res.Comm.120,3:885-890

Goate AM, Haynes AR, Owen MJ, Farral M, James LA, Lai LYC, Mullan MJ, Roques P, Rossor MN, Williamson R, Hardy JA (1989): Predisposing locus for Alzheimer's disease on chromosome 21. Lancet 1:352-355

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving

N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J (1991):Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704-706

Golde TE, Estus S, Usiak M, Yopunkin LH, Younkin SG (1990): Expression of β-amyloid Protein Precursor mRNAs: Recognition of a novel Alternatively Spliced Form and Quantitation in Alzheimer's Disease Using PCR. Neuron 4:253-267

Goldgaber D, Lerman MI, McBride WO, Saffioti U, Gajdusek DC (1987): Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease.Science 235:877-880

Hardy J, Mullan M, Chartier-Harlin MC, Brown J, Goate A, Rossor M, Collinge J, Roberts G, Lunther P, Lantos P, Naruse S, Kaneko K, Tsuji S, Miyatake T, Shimizu T, Kojima T, Nakano I, Yoshioka K, Sakaki Y, Miki T, Katsuya T, Ogihara T, Roses A, Pericak-Vance M, Haan J, Roos R, Lucotte G, Favid F (1991):Molecular classification of Alzheimer's disease. Lancet 337:1342-1343

Hardy J, Allsop D (1991): Amyloid deposition as the central event in the aetiology of Alzheimer's disease. TiPS 12:383-388

Harrison PJ, Barton AJL, Pearson RCA (1991): Expression of amyloid beta-protein precursor mRNA in familial Alzheimer's disease. NeuroReport 2:152-154

Johnstone E, Chaney M, Norris F, Pascual R, Little S. (1991) Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis. Mol. Brain res. 10: 299-305

Kang J, Muller-Hill B (1990): Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4695 mRNA is predominantly produced in rat and human brain. Biochem.Biophys.Res.Comm. 166,3:1192-1200

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B (1987): The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325:733-736

Katzman R (1986): Alzheimer's disease. N.Engl.J.Med.314,15:964-973

Kawabata S, Higgins G, Gordon J. (1991) Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein. Nature 354, 476-478

Kitagushi N, Takahashi Y, Tokushima Y, Shiojiri S, Ito H (1988): Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. Nature 331:530-532

Klausner RD, Harford JB (1989): Cis-Trans Models for Post-transcriptional gene regulation. Science 246:870-872

Levy E, Carman MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, Van Duinen SG, Bots GTAM, Luyendijk W, Frangione B (1990): Mutation of the Alzheimer's disease Amyloid Gene in Hereditary Cerebral Hemorrhage, Dutch-type. Science, 248:1124-1126

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985): Amyloid plaque core protein in Alzheimer disease and Down's syndrome. Proc.Natl.Acad.Sci. 82:4245-4249

Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydell RE (1992): β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J. Neurosci.in press

Murrell J, Farlow M, Ghetti B, Benson MD (1991): A mutation in the Amuloid precursor protein associated with hereditary Alzheimer's disease. Science

Naruse S, Igarashi S, Kobayashi H, Aoki K, Inuzuka T, Kaneko K, Shimizu T, Iihara K, Kojima T,

Miyatake T, Tsuji S (1991):Mis-sense mutation val->ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. Lancet 337:978-979

Neve RL, Rogers J, Higgins GA (1990):The Alzheimer amyloid precursor-related transcript lacking the β/A4 sequence is specifically increased in Alzheimer's disease brain. Neuron 5:329-338

Nordstedt C, Gandy SE, Alafuzoff I, Caporaso GL, Iverfeldt K, Grebb JA, Winblad B, Greengard P (1991): Alzheimer β/A4 amyloid precursor protein in human brain: Aging-associated increases in holoprotein and in a proteolytic fragment. Proc.Natl.Acad.Sci.88:8910-8914

Oltersdorf T, Fritz LC, Schenk DB, Lieberburg I, Johnson-Wood KL, Beattie EC,Ward PJ, Blacher RW, Dovey HF, Sinha S (1989): The secreted form of the Alzheimer's amyloid precursor protein with the Kunitz domain is protease nexin-II. Nature 341:144-147

Pericak-Vance M, Yamaoka L, Haynes C, Speer M, Haines J, Gaskell P, Hung W, Clark C, Heyman A, Trofatter A, Eisenmenger J, Gilbert J, Lee J, Alberts M, Dawson D, Bartlett R, Earl N, Siddique T, Vance J, Conneally P, Roses A. (1988) Genetic linkage studies in Alzheimer's disease families. Exp. Neurol. 102: 271-279

Ponte P, Gonzalez-DeWhitt P, Schilling J, Miller J, Hsu D, Greenberg B, Davis K, Wallace W, Lieberburg I, Fuller F, Cordell B (1988): A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. Nature 331:525-527

Robakis NK, Wisniewski HM, Jenkins EC, Devine-Gage EC, Houck GE, Yao XL, Ramakrishna N, Wolfe G, Silverman WP, Brown WT (1987): Chromosome 21q21 sublocalisation of gene encoding beta-amyloid peptide in cerebral vessels and neuritic (senile) plaques of people with Alzheimer disease and Down syndrome. Lancet 384-385

Roberts GW, Gentleman SM, Lynch A, Graham DI (1991): βA4 amyloid protein deposition in brain after head trauma. Lancet 338:1422-1423

Royston MC, Rothwell NJ, Roberts GW (1992): Alzheimer's disease: from molecular biology to treatment. submitted

Rumble B, Retallack R, Hilbich C, Simms G, Multhaup g, Martins R, Hockey A, Montgomery P, Beyreuther K, Masters CL (1989):Amyloid A4 protein and its precursor in Down syndrome and Alzheimer's disease. N.Engl.J.Med. 320,22:1446-1452

Salbaum J, Weidemann A, Lemaire H-G, Masters C, Beyreuther K. (1988) The promoter of Alzheimer's disease amyloid A4 precursor gene. EMBO J. 7: 2807-2813

Schellenberg GD, Bird TD, Wijsman EM, Moore DK, Boehnke M, Bryant EM, Lampe TH, Nochlin D, Sumi SM, Deeb SS, Beyreuther K, Martin GM (1988): Absence of linkage of chromosome 21q21 markers to familial Alzheimer's disease. Science 241, 1507-1510

Schellenberg GD, Anderson L, O'Dahl S, Wisjman EM, Sadovnick AD, Ball MJ, Larson EB, Kukull WA, Martin GM, Roses AD, Bird TD (1991): APP717, APP693 and PRIP gene mutations are rare in Alzheimer disease. Am.J.Hum.Genet. 49:511-517

Selkoe DJ (1990) Deciphering Alzheimer's disease: The amyloid precursor protein yields new clues. Science 248:1058-1060

Sisodia SS, Koo EH, Beyreuther K, Unterbeck A, Price DL (1990): Evidence that β-amyloid protein in Alzheimer's disease is not derived by normal processing. Science 248:492-495

St.George-Hyslop P, Tanzi R, Polinsky R, Haines JL, Nee L, Watkins PC, Myers RH, Feldman RG, Pollen D, Drachman D, Growdon J, Bruni A, Foncin JF, Salmon D, Frommelt P, Amaducci L, Sorbi S, Piacentini S, Stewart GD, Hobbs WJ, Conneally PM, Gusella JF (1987a): The genetic defect causing familial

Alzheimer's disease maps on chromosome 21 Science 235:885-890

St.George-Hyslop P, Tanzi RE, Polinsky RJ, Neve RL, Pollen D, Drachman D, Growdon J, Cupples LA, Nee R, Myers RH, O'Sullivan D, Watkins PC, Amos JA, Deutsch CK, Bodfish JW, Kinsbourne M, Feldman RG, Bruni A, Amaducci L, Foncin J-F, Gusella JF (1987b): Absence of duplication of chromosome 21 genes in familial and sporadic Alzheimer's disease. Science, 238, 664-666

St.George-Hyslop PH, Haines JL, Farrer LA, Polinsky R, Van Broekhoven C, Goate A, Crapper McLachlan DR, Orr H, Bruni AC, Sorbi S, Rainero I, Foncin JF, Pollen D, Cantu JM, Tupler R, Voskresenskaya N, Mayeux R, Growdon J, Fried VA, Myers RH, Nee L, Backhovens H, Martin JJ, Rossor M, Owen MJ, Mullan M, Percy ME, Karlinsky H, Rich S, Heston L, Montesi M, Mortilla M, Nacmias N, Gusella JF, Hardy JA & other members of the FAD collaborative syudy group (1990): Genetic linkage studies suggest that Alzheimer's disease is not a single homogeneous disorder. Nature 347: 194-197

Tagawa K, Kunishita T, Maruyama K, Yoshikawa K, Kominami E, Tsuchiya T, Suzuki K, Tabira T, Sugita H, Ishiura S (1991): Alzheimer's disease amyloid β-clipping enzyme (APP secretase): Identification, purification, and characterization of the enzyme. Biochem.Biophys.Res.Comm.177,1:377-387

Tanaka S, Shiojiri S, Takahashi Y, Kitaguchi N, Ito H, Kameyama M, Kimura J, Nakamura S, Ueda K (1989): Tissue-specific expression of three types of β-protein precursor mRNA: enhancement of protease inhibitor-harboring types in Alzheimer's disease brain. Bioch. Bioph. Res. Comm. 165, 1406-1414

Tanzi RE, Gusella JF, Watkins PC, Bruns GAP, St.George-Hyslop P, Van Keuren ML, Patterson D, Pagan S, Kurnit DM, Neve RL (1987a): Amyloid β-Protein gene:cDNA, mRNA distribution and genetic linkage near the Alzheimer locus. Science 235:880-884

Tanzi RE, St.George-Hyslop P, Haines JL, Polinsky RJ, Nee L, Foncin JF, Neve RL, McClatchey AI, Conneally PM (1987b): The genetic defect in familial Alzheimer's disease is not tightly linked to the amyloid β-protein gene. Nature 329:156-157

Tanzi RE, McClatchey AI, Lamperti ED, Villa-Komaroff L, Gusella JF, Neve RL (1988): Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. Nature 331:528-530

Tanzi RE, Hyman BT (1991): Alzheimer's mutation. Nature 350:564

Van Broeckhoven C, Genthe AM, Vandenberghe A, Horsthemke B, Backhoven H, Raeymaekers P, Van Hul W, Wehnert A, Gheuens J, Cras P, Bruyland M, Martin JJ, Salbaum M, Multhaup G, Masters CL, Beyreuther K, Gurling HMD, Mullan MJ, Holland A, Barton A, Irving N, Williamson R, Richards SJ, Hardy JA (1987): Failure of familial Alzheimer's disease to segregate with the A4-amyloid gene in several European families. Nature 329:153-155

Van Broeckhoven C, Haan J, Bakker E, Hardy JA, Van Hul W, Wehnert A, Vegter-Van der Vlis M, Roos RAC (1990): Amyloid β-protein precursor gene and hereditary cerebral hemorrhage with Amyloidosis (Dutch). Science 248:1120-1122

Van Duijn CM, Hendriks L, Cruts M, Hardy JA, Hofman A, Van Broekhoven C (1991): Amyloid precursor protein gene mutation in early-onset Alzheimer's disease. Lancet 337:978

Van Nostrandt WE, Wagner SL, Suzuki M, Choi BH, Farrow JS, Geddes JW, Cotman CW, Cunningham DD (1989): Protease nexin-II, a potent anti-chymotrypsin, shows identity to amyloid β-protein precursor. Nature 341:546-549

Vitek MP, Rasool CG, DeSauvage F, Vitek SM, Bartus RT, Beer B, Ashton RA, Macq AF, Maloteaux JM, Blume AJ, Octave JN (1988): Absence of mutation in the β-amyloid cDNAs cloned from the brains of three patients with sporadic Alzheimer's disease. Mol.Br.Res. 4:121-131

Yankner BA, Daws LR, Fisher S, Villa-Komaroff L, Oster-Granite ML, Neve RL (1989): Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. Science 2 :417-420

Yoshikai S, Sasaki H, Doh-ura K, Furuya H, Sakaki Y (1990):Genomic organisation of the human amyloid beta-protein precursor gene. Gene 87:257-263

Yoshioka K, Miki T, Katsuya T, Ogihara T, Sakaki Y (1991): The 717 Val-> ile substitution in amyloid precursor protein is associated with familial Alzheimer's disease regardless of ethnic groups. Biochem. Biophys. Res.Comm. 178,3:1141-1146

Whitson JS, Selkoe DJ, Cotman CW (1989):Amyloid β-Protein enhances the survival of hippocampal neurons in vitro. Science 243:1488-1490

A NOVEL SILENT VARIANT AT CODON 711 AND A VARIANT AT CODON 708 OF THE APP SEQUENCE DETECTED IN SPANISH ALZHEIMER AND CONTROL CASES.

Rosa Adroer[1], Cristina Lopez-Acedo[1], Rafael Oliva [1], John Hardy[2,3] and Liana Fidani[2*]

[1] Molecular Genetics Research Group,
Faculty of Medicine,
University of Barcelona,
08028, Barcelona,
Spain

[2] Department of Biochemistry and Molecular Genetics,
St. Mary's Hospital Medical School,
London W2 1PG
UK

[3] Suncoast Alzheimer's Disease Laboratories,
Department of Psychiatry,
University of South Florida,
Tampa,
FL33612
USA

Keywords: APP, ß-amyloid, mutations, Alzheimer's disease, Genetics, Spanish population.

Pathogenic mutations have been identified in exons 16 and 17 of the ß-amyloid precursor protein (APP) gene in some cases of early onset Alzheimer's disease. Screening of these exons in a number of familial and sporadic cases of Alzheimer's disease in Spain, resulted in the identification of a novel silent variant at codon 711 whose relevance to the AD pathogenesis remains unclear. The 708 variant was also detected in one of normal controls.

It is clear that mutations in exons 16 and 17 of the ß-amyloid precursor protein (APP) gene cause some cases of familial Alzheimer's disease (AD). With this in mind, our laboratories have embarked upon a screening programme designed to identify further cases with the defined mutations, to find new sequence variants, and to determine whether "sporadic" cases of the disease can also be caused by variability in the APP gene.

In sporadic cases, or in families whose pedigree structure precludes linkage analysis, our strategy is to screen exons 16 and 17 by single strand conformation polymorphism analysis (SSCP)[12]. We have developed conditions, which detect all three APP717 mutations,[3,6,11] APP693Glu->Gln [9], APP708(silent)[2], APP692Ala->Gly[7], APP713Ala->Val[8]. These same conditions detect APP670/1Lys/Met->Asn/Leu[10] in exon 16 and the majority of prion gene variants[5]. When a variation is detected, we characterise it by direct sequencing[1,4].

We have applied this strategy to the analysis of DNA from 34 cases of AD from Spain. These individuals all met the clinical criteria for NINCDS probable Alzheimer's disease and had ages of

BIBL. LONDIN. UNIV.

onset, 9 cases 43-55, 12 cases 55-60 and 13 cases >60. Of the 34 cases, 10 had a definite family history of dementia occurring at about the same age (two or three siblings and one parent), 12 had a possible family history of late onset dementia and 12 had no documented family history.

SSCP of all but one of these samples, suggested that both exon 16 and exon 17 were of normal sequence. However, one sample derived from a man with probable AD of onset age 53, showed evidence for a sequence variant (figure 1). The mother of this man had shown evidence for cognitive decline towards the beginning of her 82nd year, but no other members of this man's large family had had any evidence for dementia, according to information we collected from the family. Direct sequencing of this variant revealed a silent change at codon 711 changing C->A in the wobble position (figure 2).

To further assess the significance of this alteration, we screened 100 cases with unrelated genetic conditions (thalassaemia, cystic fibrosis, myotonic dystrophy) from the same population. 99 of these cases had SSCP mobilities consistent with the normal sequence. One case, however showed a band shift indicative of a different variant sequence (figure 1). This case was from a girl of 11 years of age with a family history of myotonic dystrophy. Direct sequencing of exon 17 in this case revealed a C->T transversion at codon 708. This is a silent change previously reported in Swedish population [2], and it is unlikely that it represents anything other than silent genetic variability.

The position of the APP711 variant is of interest because it is within the putative "stem loop structure" proposed as of pathogenic importance in APP mutations[14]. However, this variant does not alter the bonding in the structure because it changes one mismatched base for another. The fact that this case does not have a convincing family history of the disorder strongly suggests that the variant is unrelated to the disease pathogenesis. However the mutation might have arised de novo in the proband and be pathogenic for him and for any of his offspring had they inherited the variant.

The sequence of exons 16 and 17 of the APP gene have now been examined by many laboratories in a very large number of individuals (controls, Alzheimer cases and Angiopathy cases)[4,13,15]. The variants reported here are almost certainly not pathogenic and almost certainly reflect normal DNA variation. The data presented here further demonstrate the efficacy of SSCP for detection of this variation.

Acknowledgments
We would like to thank NATO, MRC, Athena Neurosciences, Comision Interministerial de Ciencia y Tecnologia (CICYT), and Pia Gallano and Montserrat Baiget for supplying 100 random DNA samples.

BIBL. LONDIN. UNIV.

1 Adroer, R., Chartier-Harlin, M.C., Crawford, F. and Oliva, R., Improved direct sequencing of Alzheimer's amyloid precursor protein (APP) exons 16 and 17, Neurosci. Letts., 141 (1992) 69-71.

2 Balbin, M., Abrahamson, M., Gustafson, L., Nilsson, K., Brun, A. and Grubb, A., A novel mutation in the ß-protein coding region of the amyloid ß-protein precursor (APP) gene, Hum. Genet., 89 (1992) 580-582.

3 Chartier Harlin, M.C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J. and Mullan, M., Early onset Alzheimer's disease caused by mutations at codon 717 of the ß-amyloid precursor protein gene, Nature, 353 (1991) 844-846.

4 Crawford, F., Hardy, J., Mullan, M., Goate, A., Hughes, D., Fidani, L., Roques, P., Rossor, M. and Chartier-Harlin, M.C., Sequencing of exons 16 and 17 of the ß-amyloid precursor protein gene in families with early onset Alzheimer's disease fails to reveal mutations in the ß-amyloid sequence, Neurosci. Letts., 133 (1991) 1-2.

5 Fidani, L., Rooke, K., Chartier-Harlin, M.C., Hughes, D., Tanzi, R., Mullan, M., Roques, P., Rossor, M., Hardy, J. and Goate, A.M., Screening for mutations in the open reading frame and promoter of the ß-amyloid precursor protein gene in familial Alzheimer's disease: identification of a further family with APP717 Val-> Ile, Hum. Mol. Genet., 1,3 (1992) 165-168.

6 Goate, A., Chartier Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. and et al, , Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease, Nature, 349 (1991) 704-706.

7 Hendriks, L., van Duijn, C.M., Cras, P., Cruts, M., Van Hul, W., Van Harskamp, F., Warren, A., McInnis, M.G., Antonarakis, S.E., Martin, J-J., Hofman, A. and Van Broeckhoven, C., Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the ß-amyloid precursor protein gene, nature genet., 1 (1992) 218-221.

8 Jones, C.T., Morris, S., Yates, C.M., Moffoot, A., Sharpe, C., Brock, D.J.H. and St.Clair, D., Mutation in codon 713 of the ß amyloid precursor protein gene presenting with schizophrenia, nature genet., 1 (1992) 306-309.

9 Levy, E., Carman, M.D., Fernandez Madrid, I.J., Power, M.D., Lieberburg, I., van Duinen, S.G., Bots, G.T., Luyendijk, W. and Frangione, B., Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type, Science, 248 (1990) 1124-1126.

10 Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B. and Lannfelt, L., A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of ß-amyloid, nature genet., 1 (1992) 345-347.

BIBL. LONDIN. UNIV.

11 Murrell, J., Farlow, M., Ghetti, B. and Benson, M., A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease, Science, 254 (1991) 97-99.

12 Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K., Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction, Genomics, 5 (1989) 874-879.

13 Schellenberg, G.D., Anderson, L., O'dahl, S., Wijsman, E., Sadovnick, A.D., Ball, M.J., Larson, E.B., Kukull, W.A., Martin, G.M., Roses, A.D. and Bird, T.D., APP717, APP693, and PRIP gene mutations are rare in Alzheimer's disease, Am. J. Hum. Genet., 49 (1991) 511-517.

14 Tanzi, R.E. and Hyman, B.T., Alzheimer's mutation, Nature, 350 (1991) 564.

15 Tanzi, R.E., Vaula, G., Romano, D.M., Mortilla, M., Huang, T.L., Tupler, R.G., Wasco, W., Hyman, B.T., Haines, J.L., Jenkins, B.J., Kalaitsidaki, M., Warren, A.C., McInnis, M.C., Antonarakis, S.E., Karlinsky, H., Percy, M.E., Connor, L., Growdon, J., Crapper-Mclachlan, D.R., Gusella, J.F. and St George-Hyslop, P., Assessment of amyloid ß-protein precursor gene mutations in a large set of familial and sporadic Alzheimer disease cases, Am. J. Hum. Genet., 51 (1992) 273-282.

BIBL.
LONDIN.
UNIV.

A LOCUS FOR FAMILIAL EARLY ONSET ALZHEIMER'S DISEASE ON THE LONG ARM OF CHROMOSOME 14, PROXIMAL TO α1-ANTICHYMOTRYPSIN

Mike Mullan[1], Henry Houlden[2], Mike Windelspecht[1], Liana Fidani[2], Chris Lombardi[1], Pat Diaz[1], Martin Rossor[2], Richard Crook[2], John Hardy[1], Karen Duff[1] and Fiona Crawford[1]

1 Alzheimer's Disease Research Laboratories,

Psychiatry Center,

University of South Florida,

3515 E. Fletcher Avenue,

Tampa,

FL33613

USA

2 Departments of Biochemistry and Neurology,

St. Mary's Hospital Medical School,

London,

W2 1PG

UK.

Correspondence should be addressed to MM.

BIBL.
LONDIN.
UNIV.

ß-amyloid deposition in the brain parenchyma as congophilic plaques is one of the characteristic features of Alzheimer's disease (AD)[1,2]. Previous studies have shown that mutations in APP segregate with the disease in some early onset families[3-6]. To test for linkage in other early onset families (for clinical details see Table 1) we examined the segregation of the marker, GT12 at D21S210, which is tightly linked to APP. Of the nine remaining families, only one had a modestly positive lod score at D21S210. We therefore began to look at linkage to other candidate loci and chromosomes in these families. Data suggestive of linkage on chromosome 14 has previously been reported[7]. In addition, α1-antichymotrypsin (AACT) is a component of plaque cores and a protease inhibitor and has been proposed as a possible candidate gene for AD[8]. It has been localised to chromosome 14[9].

We analysed this locus by converting a protein polymorphism within the signal peptide of the AACT gene to a PCR detectable polymorphism and developing an SSCP polymorphism within the adjacent anti-trypsin (PI) locus. Overall, two point analyses of both polymorphisms were negative (unpublished data). Haplotyping the two polymorphisms (Table 2) gave exclusion (assuming genetic homogeneity) to 9 centimorgans. As there are other candidate genes (Cathepsin G and HSPA2[9]) for early onset familial AD which are located on this chromosome we examined loci adjacent to AACT.

BIBL.
LONDIN.
UNIV.

A genetic linkage map of chromosome 14 PCR-amplified micro satellites has recently been reported[10]. The accepted criterion for linkage was observed for both D14S43 and D14S53 with early onset AD (Table 2). No family typed for these markers gave any evidence for recombination except F126. This family is the only one in our data set showing any evidence of linkage to D21S210 (Table 2). HOMOG[11] analysis of the D14S43 and D14S53 was negative indicating no evidence of heterogeneity. A multipoint analysis was performed and showed that the lod score went above 7.0 with a maximum at D14S43 ($z=7.8$, $\theta=0$, Figure 1).

DISCUSSION

These data are consistent with a major early onset AD locus in the middle long arm of chromosome 14. An overview of our data set might suggests that approximately 25% of early onset familial AD are encoded at the APP locus and the remainder by a gene close to D14S43 and D14S53. At present, although we have flanking markers the multipoint map is quite flat over a relatively large genetic distance. Further recombination in other early onset families (for instance at D14S43 or D14S53) would localise the gene more precisely. Alternatively, extension of the present families may reveal recombinants as the apparent absence of recombination at D14S43 and D14S53 may partly be due to incomplete or incompletely informative data. As we had no evidence of heterogeneity we constructed a multipoint map of the region but until homogeneity is established the maximum likelihood position of the disease gene may be misleading. In

BIBL. LONDIN. UNIV.

addition, analysis for heterogeneity with a larger data set is required to examine the question of genetic heterogeneity. In previous analyses we have examined the segregation of APP in three families and have found pathogenic mutations[3-6]. In this paper we report a fourth family (F172) segregating with GT12 which was removed from the chromosome 14 analyses and subsequently shown to contain an APP mutation[12]. Similarly, F126 shows segregation with GT12 and partial recombination at D14S43 and D14S53. However, until we demonstrate an APP mutation the family remains in the data set.

The second pathogenic locus for early onset familial AD is clearly centromeric to AACT. During revison of this manuscript independent evidence of linkage to this approximate region has been published[20]. For both data sets, the most likely candidate gene in the general region, based on the amyloid cascade hypothesis[13-15], is the 70 kD heat shock protein gene, HSPA2[16], since the Cathepsin G gene is too close to the centromere. The promoter of the APP gene has a heat shock consensus sequence and APP has been shown to be upregulated by heat shock[16]. Clearly, chronic up regulation of APP as a cause of AD is consistent with this hypothesis. The genetic localisation of the HSPA2 is unknown and needs to be determined. However, it is of course possible that the gene encoding some other protein interacting with APP is encoded in this area of chromosome 14, or that the amyloid cascade hypothesis is wrong. We regard the identity of this second gene as a test of this hypothesis.

BIBL. LONDIN. UNIV.

METHODOLOGY

Clinical Studies. DNA samples from all affected individuals fulfilled NINCDS criteria[17] for probable or definite AD. Autopsy confirmation was available for some families (Table 1). All unaffected individuals for which DNA was analysed scored normally on the Mini-Mental State screening instrument[18]. Age of onset for each affected individual was estimated from a consensus history derived from the patient, primary carers, hospital and general practitioner records.

Linkage and Simulation Analysis. D21S210, D14S52, D14S43, D14S53, D14S48, AACT and AT data were analysed with MLINK from the LINKAGE package using two-point and multipoint analyses[19]. Autosomal dominance was assumed, a disease allele frequency of .001, a phenocopy rate of .0001 and seven age dependent liability classes were assigned from the distribution of the known ages of onset in each family. The age of onset distribution was assumed to be normal with no important truncation effects. Published allele frequencies were used[10]. Neutered map distances were taken from published data[9,10].

AC data was generated for D21S210 (GT12), D14S43, D14S53, D14S52 and D14S48 using published primers and conditions[10]. Primers used for the detection of the AACT polymorphism were: 5'-AGAGTTGAGAATGGAGAG-3' and 5'-GTCCACGTGTGTCCCTCG-3', and for the AT gene: 5'-GTGATTCCCCAACCTGAGGGTGACC-3' and 5'-

BIBL. LONDIN. UNIV.

CTGGGCCTTGCCACACAGGCTCTTC-3'. Conditions for both these primer sets were 10 minutes initial denaturation followed by 35 cycles of 72C for 1min, 94C for 1min and 55C for 45sec, in a 50ul reaction volume. The optimum $MgCl_2$ concentration for the AACT primers was 1.5mM and for the AT primers 1.0mM. The PCR product generated by the AACT primers is 155bp which after BstNI digestion gives three fragments of 84,33 and 32bp. The polymorphism changes an alanine to a threonine and abolishes a restriction site resulting in two fragments of 116 and 33bp. The allele frequencies estimated were 0.3 for alanine and 0.7 for threonine.

ACKNOWLEDGMENTS

We would like to thank Cecilia Figueredo for help with the manuscript and Craig Bennett, the Mental Health Foundation, Research into Aging, the MRC, NATO, private donors.

REFERENCES

1. Masters, C.L., Simms, G., Weinman, N.A., et al. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natn. Acad. Sci. USA* 82, 4245-4249 (1985).

2. Wong, C.W., Quaranta, V. & Glenner, G. Neuritic plaques and cerebrovascular amyloid in Alzheimer disease are antigenically related. *Proc. Natn. Acad. Sci. USA*, 82, 8729-8732 (1985).

BIBL. LONDIN. UNIV.

3. Goate, A., Chartier Harlin, M. C., Mullan, M., et al. *Nature* 349, 704-706 (1991).

4. Chartier Harlin, M.C., Crawford, F., Houlden, H., et al. *Nature* 353, 844-846 (1991).

5. Murrell, J., Farlow, M., Ghetti, B. & Benson, M. *Science* 254, 97-99 (1991).

6. Mullan, M., Crawford, F., Axelman, K., et al. *Nature Genet.* 1, 345-347 (1992).

7. Weitkamp, L.R., Nee, L., Keats, B., Polinsky, R.J. & Guttormsen, S. *Am. J. Hum. Genet.* 35, 443-453 (1983).

8. Abraham, C.R., Selkoe, D.J. & Potter, H. *Cell* 52, 487-501 (1988).

9. Cox, D., Walter, M.A. & Coulson, S.E., et al. *Cytogenetics and Cell Genetics* 46:600 (1991).

10. Wang, Z. & Weber, J. *Genomics* 13, 532-536, (1992).

11. Ott, J. *Am. J. Hum. Genet.* 27, 588-597 (1984).

12. Fidani, L., Rooke, K., Chartier-Harlin, M.C., et al. *Hum. Mol. Genet.* 1, 165-168 (1992).

BIBL. LONDIN. UNIV.

13. Glenner, G.G. & Murphy, M.A. *J. Neurol. Sci.* 94, 1- 28 (1989).

14. Selkoe, D.J. *Neuron* 6, 487-491 (1991).

15. Hardy, J.A. & Higgins, G.A. *Science* 256, 184-185 (1992).

16. Abe, K., St.George Hyslop, P.H., Tanzi, R.E. & Kogure, K. *Neurosci. Letts.* 125, 169-171 (1991).

17. McKhann, G., Drachman, D., Folstein, M., et al. *Neurology* 34, 939-944, (1984).

18. Folstein, M.F., Folstein, S.E. & McHugh, P.R. *J. Psychiatr. Research* 12, 189-198, (1975).

19. Lathrop, G.M. & Lalouel, J.M. *Am. J. Hum. Genet.* 36, 460-465 (1984).

20. Schellenberg, G., Bird, T., Wijsman, E., et al. *Science* 258, 668-671 (1992)

BIBL. LONDIN. UNIV.

TABLE 1. Clinical Details of Early Onset Families.

Family number	Mean age of onset	Number of generations	Affecteds (path. confirmed)
53	52	3	5 (1)
74	43	4	7 (2)
105	38	6	14
121	37	4	6
126	54	4	4
134	46	4	6 (2)
148	41	4	5 (1)
168	41	4	6 (1)
172	54	3	10
206	39	6	7

BIBL.
LONDIN.
UNIV.

TABLE 2. Lod Scores for Markers on Chromosomes 21 and 14.

Family number	D21S210 lod score at recombinant fraction (θ)					AACT/AT lod score at recombinant fraction (θ)				
	0.00	0.02	0.05	0.10	0.20	0.00	0.02	0.05	0.10	0.20
53	-0.91	-0.82	-0.70	-0.52	-0.27	-0.13	-0.12	-0.10	-0.07	-0.03
74	-3.48	-1.79	-1.25	-0.79	-0.31	-5.85	-0.66	-0.31	-0.10	0.03
105	-1.13	-0.99	-0.81	-0.59	-0.29	-0.37	-0.34	-0.30	-0.23	-0.12
121	-0.67	-0.51	-0.35	-0.20	-0.07	-0.11	0.02	0.12	0.19	0.17
126	0.72	0.69	0.64	0.55	0.37	-1.36	-0.98	-0.70	-0.45	-0.20
134	-0.25	-0.22	-0.17	-0.11	-0.05	-0.10	-0.09	-0.08	-0.06	-0.03
148	-0.19	-0.18	-0.16	-0.14	-0.08	0.06	0.07	0.08	0.09	0.07
168	-5.91	-3.34	-2.24	-1.40	-0.60	-14.36	-2.15	-1.39	-0.85	-0.36
206	-0.05	0.40	0.60	0.69	0.59	-12.21	-1.71	-0.99	-0.50	-0.14
Totals	-11.05	-6.76	-4.44	-2.51	-0.71	-34.43	-5.96	-3.67	-1.98	-0.61
172	1.76	1.69	1.58	1.40	0.99					

Family number	D14S52 lod score at recombinant fraction (θ)					D14S43 lod score at recombinant fraction (θ)				
	0.00	0.02	0.05	0.10	0.20	0.00	0.02	0.05	0.10	0.20
53	0.00	-0.01	-0.02	-0.03	-0.03	-0.03	-0.03	-0.03	-0.03	-0.02
74	-0.10	-0.09	-0.08	-0.06	-0.03	1.57	1.51	1.41	1.24	0.87
105	0.26	0.25	0.24	-0.20	0.13	-0.20	-0.19	-0.16	-0.13	-0.07
121	-0.15	-0.14	-0.12	-0.09	-0.04	0.19	0.16	0.12	0.08	0.03
126	-0.02	-0.01	-0.00	-0.00	0.00	-0.47	-0.39	-0.27	-0.19	-0.08
134	0.00	0.00	0.00	0.00	0.00	0.19	0.19	0.18	0.16	0.12
148	-5.40	-2.10	-1.34	-0.80	-0.33	1.45	1.40	1.33	1.20	0.91
168	0.41	0.39	0.36	0.31	0.21	0.07	0.07	0.05	0.03	0.03
206	0.28	0.29	0.28	0.26	0.18	0.50	0.46	0.41	0.33	0.18
Totals	-4.72	-1.42	-0.68	-0.61	0.09	3.27	3.18	3.04	2.69	1.97

Family number	D14S53 lod score at recombinant fraction (θ)					D14S48 lod score at recombinant fraction (θ)				
	0.00	0.02	0.05	0.10	0.20	0.00	0.02	0.05	0.10	0.20
53	0.06	0.05	0.03	0.01	-0.01	0.00	0.00	0.00	0.00	0.00
74	0.68	0.18	0.20	0.22	-0.09	-5.80	-0.64	-0.29	-0.08	0.05
105	1.11	0.32	0.29	0.23	0.64	-6.66	-1.18	-0.79	-0.50	-0.22
121	0.12	0.09	0.08	0.08	0.02	-0.41	-0.38	-0.34	-0.27	-0.15
126	-0.53	-0.49	-0.42	-0.32	-0.18	0.00	0.00	0.00	0.00	0.00
134	0.20	0.20	0.20	0.20	0.04	0.31	0.30	0.28	0.24	0.15
148	1.36	1.26	1.19	1.07	0.70	0.32	0.30	0.27	0.22	0.13
168	0.06	0.06	0.06	0.05	0.03	-0.12	-0.09	-0.08	-0.08	-0.06
206	0.35	0.28	0.26	0.24	0.19	0.24	0.19	0.15	0.83	0.09
Totals	3.41	1.95	1.89	1.78	1.34	-12.12	-1.50	-0.80	0.36	-0.01

BIBL. LONDIN. UNIV.

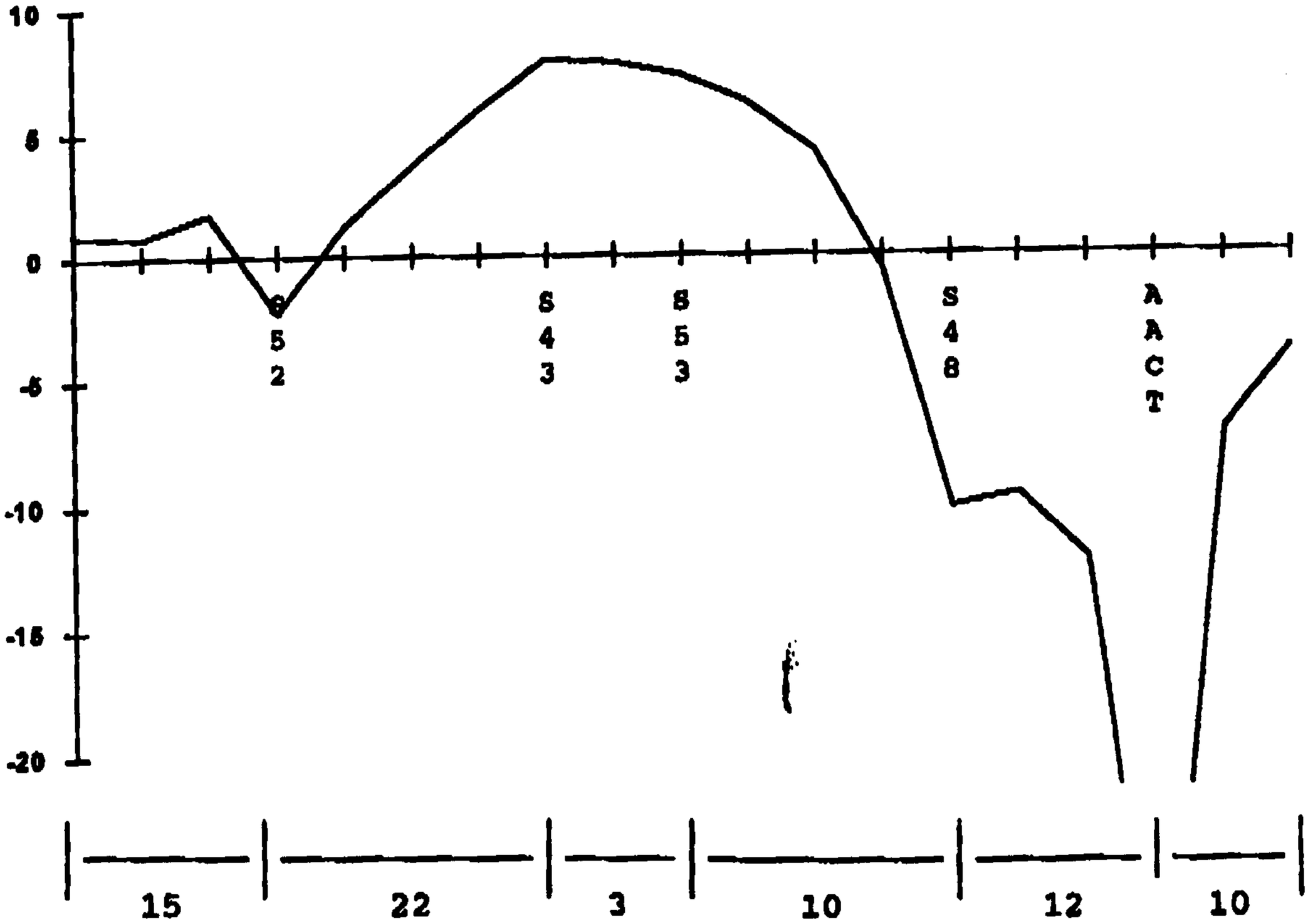


FIGURE 1. Multipoint analysis of chromosome 14 markers vs. AD in 8 early onset families (F172 not included). Indicated distances are in centimorgans.

BIBL. LONDON. UNIV.