Physical Analysis of the chromosome 9q34 region containing the gene for Tuberous Sclerosis (TSC1)

by

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Abstract

Tuberous Sclerosis (TSC) is an autosomal dominant disorder with a prevalence of 1 in 10,000 births. The disorder is genetically heterogeneous and is characterised by a combination of neurological and cutaneous abnormalities. One of the genes responsible for the disease, designated TSC1 (for Tuberous Sclerosis Complex 1) has been mapped to chromosome 9q34 by linkage analysis.

This thesis describes the attempts made to identify the TSC1 gene using the positional cloning approach. A panel of radiation reduced hybrid lines was constructed from a somatic cell hybrid line retaining a chromosome 9q as its only human material. Several hybrids retained fragments from the TSC1 region and a subset of these were used to construct a deletion map across the region using previously localised genetic markers. This deletion panel was used to map the Retinoid X Receptor (RXR) alpha locus to chromosome 9q34 and the chromosome position of other members of the RXR family of nuclear receptors were considered as potential candidate genes for the other TSC loci.

Genomic libraries were constructed from two irradiation hybrid lines to generate cloned material from the TSC1 region. Genomic clones were also isolated for a number of mapped genetic loci while further cloned material was identified through the use of Alu-PCR probes derived from 9q34 containing hybrids.
CHAPTER 1: INTRODUCTION

1.1 HISTORICAL AND CLINICAL ASPECTS OF TUBEROUS SCLEROSIS

1.2 THE POSITIONAL CLONING APPROACH

1.2.1 Chromosomal localisation of the gene

1.2.1.1 Linkage analysis

1.2.1.2 Chromosomal changes

1.2.2 Selective cloning of the disease interval

1.2.2.1 Chromosome microdissection

1.2.2.2 The irradiation fusion strategy

1.2.2.3 Human chromosomal retention in irradiation hybrids

1.2.2.4 Radiation hybrid mapping
1.2.3 Isolation of candidate genes from a region 29
1.2.4 Scanning for mutations 30

1.3 FLUORESCENT IN_SITU HYBRIDISATION 32

1.4 THE CANDIDATE GENE APPROACH(S) 33

1.5 DEVELOPMENT OF THE DISTAL 9q MAP 34

1.6 CHROMOSOMAL ASSIGNMENT OF THE GENES INVOLVED IN THE TSC PHENOTYPE 38

1.7 REFINEMENT OF THE GENETIC POSITION OF TSC1 39

1.8 PHYSICAL MAPPING OF THE 9q34 REGION 40

1.9 AIMS OF THE PROJECT 43

CHAPTER 2: MATERIALS AND METHODS 44-61

2.1 MATERIALS

2.1.1 Standard media and buffers 45
2.1.2 Suppliers of materials 46

2.2 GENERAL TECHNIQUES 47

2.2.1 Growth and expansion of cell lines 48
2.2.2 Isolation of DNA 48
2.2.2.1 DNA preparation of DNA from cell lines 48
2.2.2.2 Large scale preparation of plasmid and cosmid DNA 49

2.2.3 Preparation and transformation of competent E.coli 50

2.3 DNA ANALYSIS 51

2.2.1 Agarose gel electrophoresis 51
2.2.2 Precipitation of DNA 51
2.2.3 Recovery of DNA fragment from agarose gels 51
2.2.4 Concentration of DNA 52

2.4 DNA MODIFICATION REACTIONS 52

2.3.1 Restriction digestion of DNA 52
2.3.2 Dephosphorylation of DNA 52
2.3.3 Ligation of DNA 53

2.5 RADIOLABELLING OF DNA PROBES 53

2.6 SOUTHERN ANALYSIS 53

2.7 PREPARATION OF COSMID LIBRARIES FROM INDIVIDUAL HYBRID LINES 54

2.7.1 DNA preparation 54
2.7.2 Packaging reaction 55
2.7.3 The recipient bacterial strain 55
2.7.4 Screening of cosmids retaining human DNA sequences 55
CHAPTER 3: RESULTS

3.1 Identification of hybrids retaining the TSC1 region of chromosome 9q

3.1.1 Summary

3.1.2 Generation of a panel of irradiation hybrids retaining fragments derived from chromosome 9q

3.1.3 Characterisation of the chromosome 9q content retained in each hybrid through the segregation of specific markers

3.1.4 Characterisation of the human material retained in the hybrid panel by FISH

3.1.5 Correlation of the results from the 2 characterisation strategies

3.1.6 Selection of hybrids enriched for the region near the TSC1 locus

3.1.7 Deletion map

3.2 Experiments involving Alu-PCR: To characterise the chromosome 9q content of the irradiation hybrid panel and to generate region specific cloned DNA material from the TSC1 gene interval
3.2.1 Introduction 87
3.2.2 *Alu*-PCR fingerprinting of the irradiation hybrid panel 87
3.2.3 Reverse painting of *Alu*-IV products derived from irradiation hybrids 90
3.2.4 Isolation of human cosmid clones from the TSC1 interval using *Alu*-PCR products the hybrid 17B 92
3.2.5 Cloning of *Alu*-IV products from the irradiation hybrid lines 99

3.3 The generation of radiation reduced hybrid libraries to isolate cloned material from the TSC1 region 103-107

3.3.1 Introduction 104
3.3.2 Generation of cosmid libraries from the radiation hybrid lines 17B and 20A 104

3.4 The localisation of the gene for RXRA to the TSC1 interval and the assignment and localisation of the RXRB gene to chromosome 6p21.3 108-117

3.4.1 Introduction 109
3.4.2 Localisation of the RXRA gene on chromosome 9q 110
3.4.3 Localisation of the gene for RXRA to 9q34 by FISH 113
3.4.4 Assignment of the RXRB gene to chromosome 6 and its localisation to 6p21.3 113

CHAPTER 4: DISCUSSION 118-132
4.1 Summary of the genetic and physical data of the TSC1 region

4.2 The irradiation fusion panel

4.3 Generation of cloned material from the TSC1 region

4.4 Comparison of this irradiation panel with previous irradiation experiments

4.5 Candidate genes and TSC

4.6 Trinucleotide expansion and its involvement in TSC

4.7 Future studies

CHAPTER 5: REFERENCES 133-162
LISTS OF FIGURES

CHAPTER 1

1.1 The irradiation hybrid approach to generate radiation reduced hybrid lines 24
1.2 Genetic maps of chromosome 9q34 37
1.3 Physical map of chromosome 9q34 42

CHAPTER 3

3.1.1 Irradiation fusion approach for the generation of radiation reduced hybrid lines retaining unselected fragments from human chromosome 9q. 65

3.1.2a Southern analysis of the DBH locus for the irradiation hybrid lines 1A-8B 68

3.1.2b Locus specific PCR amplification of the D9S66 locus from the complete irradiation hybrid panel 70

3.1.3 Cytogenetic analysis of hybrid 64063a12 by FISH using biotinylated DNA as probe 72

3.1.4 Cytogenetic analysis of hybrid 6A by FISH using biotinylated DNA as probe 73

3.1.5 Cytogenetic analysis of hybrid 12C by FISH using biotinylated DNA as probe 73

3.1.6 Cytogenetic analysis of hybrid 17A by FISH using biotinylated DNA as probe 74

3.1.7 Cytogenetic analysis of hybrids 3C, 8B and 15B by FISH using biotinylated DNA as probe 76
3.1.8 Cytogenetic analysis of hybrid 1A by FISH using biotinylated DNA as probe

3.1.9 Cytogenetic analysis of hybrid 14A (a) and a 14A subclone 14A4 by FISH using biotinylated DNA as probe

3.1.10 Cytogenetic analysis of hybrid 20A by FISH using biotinylated DNA as probe

3.1.11 Cytogenetic analysis of hybrid 21A by FISH using biotinylated DNA as probe

3.1.12 Cytogenetic analysis of hybrid 17B by FISH using biotinylated DNA as probe

3.1.13 Cytogenetic analysis of hybrid 19B by FISH using biotinylated DNA as probe

3.1.14 Deletion map of chromosome 9q34

3.2.1 Gel electrophoresis of Alu-IV PCR fingerprint of the entire irradiation hybrid panel, hybrids 1A-22B with suitable controls DNAs

3.2.2 Alu-IV amplification of a subset of the irradiation hybrids, 17A, 14A4, 14A, 19B, 17B and 20A

3.2.3 Localisation of human fragments present in irradiation hybrids 12C and 17B on human chromosome 9 using Alu element-mediated PCR products as probe for chromosome in situ painting

3.2.4 Primary screening of a human cosmid library using Alu-IV PCR products derived from hybrid 17B

3.2.5 Secondary screening of the primary colonies isolated after probing with Alu-IV PCR products from hybrid 17B onto a human cosmid library
3.2.6 Fluorescent in situ hybridisation of biotinylated human cosmid clone cl7B6 onto human metaphase chromosome spreads

3.2.7 Fluorescent in situ hybridisation of biotinylated human cosmid clone cl7B12 onto human metaphase chromosome spreads

3.2.8 Southern analysis of an Alu IV product derived from c17B7 onto an Alu fingerprint of the entire irradiation hybrid panel (fig 3.2.1)

3.2.9 Southern analysis of an Alu IV product derived from c17B7 onto an Alu fingerprint of the entire irradiation hybrid panel (fig 3.2.1)

3.2.10 EcoRI digestion of cloned Alu IV products generated from the hybrids retaining chromosome 9q34 material

3.2.11 Southern analysis of cloned Alu IV product, clone 8, onto an Alu IV fingerprint of the entire hybrid panel (fig 3.2.1)

3.3.1 Fluorescent in situ hybridisation of biotinylated human cosmid clones, c17BL1 and c20AL1 to human metaphase chromosomes

3.3.2 Fluorescent in situ hybridisation of biotinylated human cosmid clone human metaphase chromosomes

3.4.1 PCR amplification of the RXRA 3' untranslated region in the irradiation hybrids derived from the chromosome 9q34 region

3.4.2 Localisation of the RXRA locus to 9q34 using the panel of irradiation hybrids
3.4.3 Fluorescent in situ hybridisation of biotinylated human cosmid clones for RXRA (A) and RXRB10 (B) to human metaphase chromosomes spreads.

LIST OF TABLES

CHAPTER 1

1.1 Diagnostic criteria for TSC

CHAPTER 3

3.1

3.1.1 Marker retention of chromosome 9q markers in the irradiation hybrid line

3.1.2 The markers used in the retention studies of chromosome 9q34, their chromosomal position and references for primer sequences or probes used in their study

3.1.3 Stability and number of human fragments retaining three or fewer fragments by FISH

3.4

3.4.1 Segregation of the RXRB gene in a panel of rodent-human somatic cell hybrid lines

3.4.2 Segregation of RXRB in a human chromosome 6 mapping panel
ABBREVIATIONS

Mins Minutes
rpm Revolutions per minute
bp Basepairs
kb Kilobase-pairs
Mb Megabase-pairs
ml Millilitre
ul Microlitre
l Litre
DNA Deoxyribonucleic acid
EDTA ethylenediaminetetraacetic
g grams
PCR Polymerase Chain reaction
PEG Polyethylene glycol
RFLP Restriction Fragment Length Polymorphism
TSC1 Tuberous Sclerosis Complex 1
v/v Volume for volume
w/v Weight for Volume
YAC Yeast artificial chromosome
Contributers

Carol Jones, Sue Povey, Ben Carritt,
Provided the chromosome 9q only somatic cell hybrid line, 64063a12, the hamster cell line Wg3-h and DNA from the DORA cell line retaining the philadelphia chromosome as its only human material

Louise Sefton and Peter Goodfellow
The irradiation and fusion procedure was carried out in ICRF under their supervision

Andrea Bryant
Determined the retention of the human AK1 locus in the hybrids by enzyme assay

Joseph Nahmias and Nick Hornigold
They were involved in the characterisation of a small proportion of the marker loci, ABL, ABO and D9S7

Darren Griffin, Karen Woodward and Lyn West
They carried out all the fluorescent in situ hybridisation studies on the hybrid cell lines and the chromosomal assignment of cosmid clones using provided cell lines and cosmid DNA. The analysis of results was carried out by myself.

Joseph Nahmias
Amplified all the Alu-PCR products used in the analysis of the hybrid lines,

Karen Woodward
Played an equal role in the generation of the 17B hybrid cosmid library and the isolation of the four 17B library cosmids

Sue Povey
Provided the chromosome hybrid panel for localisation of the RXRB gene.
Godfrey Gillet
Did the PCR analysis for the localisation of the RXRB gene locus to chromosome 6p although the initial suggestion to localise this locus was my own idea

John Boyle
Provided the chromosome 6 translocation hybrid panel for sub-localisation of the RXRB locus on chromosome 6.
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To Joe and Nodie
Helene and Anne
INTRODUCTION

1.1 Historical and Clinical aspects of Tuberous Sclerosis

In 1880, Bourneville, a French physician drew attention to a 15 year old mentally retarded girl with epilepsy and the cutaneous abnormality of angiofibromatosis, who also had multiple sclerotic nodules in the cerebral cortex on postmortem examination (Bourneville, 1880). Reflecting the "potatolike" character of these cortical lesions, Bourneville applied the term "tuberous sclerosis" to her disease.

Symptoms of the disease usually begin in the first 3 years of life with a characteristic triad of mental deficiency, seizures and angiofibromatosis. Although better known for these neurological and cutaneous abnormalities the disease affects a large number of other organs including the kidneys, heart and lungs. It is now referred to as tuberous sclerosis complex (TSC) to reflect the multiplicity of organs affected in the disease.

TSC is present both in an autosomal dominant and sporadic form (Gunther and Penrose, 1935; Bundey and Evans, 1969) with a prevalence of approximately 1 in 10,000 live births (Hunt and Lindenbaum 1984) with a new mutation rate of almost 70% (Osborne et al., 1991). It is characterised by the presence of benign overgrowths of a tissue type normally occurring in the organ, called hamartomas, and developmental abnormalities of tissue combination termed hamartias. The presence of these lesions suggests that TSC may be a disorder of cell differentiation and proliferation (Gomez, 1991). These lesions do not take up the entirety of the organ but exist as small pockets of affected tissue (Gomez, 1991). Consequently the size, number and position of the lesions determine the severity of the disease. If these lesions are small or absent, none of the symptoms commonly associated with the disease may be present.
Such is the variability in the expression of these manifestations both within and between families, that various attempts have been made to define diagnostic criteria for the disease. These criteria are summarised in table 1.1 and have been comprehensively reviewed elsewhere (Gomez, 1991; Fryer, 1991; Osbourne and Fryer, 1990).

The introduction of new imaging techniques, the cranial CT scan and magnetic resonance imaging to identify intracranial lesions and cortical tubers, and renal ultrasound and echocardiography to detect lesions in the kidney and heart have allowed a more rigorous evaluation of people at risk from the disease. Previous cases of apparent non-penetrance in TSC (Wilson and Carter, 1978; Lowry et al.,1979; Michel et al.,1983; Baraitser and Patton, 1985; Connor et al.,1986) have not been supported following reanalysis of the parents involved in these cases. There continues to be diagnostic difficulties for some families at risk, where variability in expression of the disease is great. The recent cases of two patients, one who developed an ungual fibroma in his forties and a second individual who had cardiac rhabdomyomas as the only signs of TSC, exemplify the problems faced for genetic counselling (Al-Gazali et al.,1989; Webb and Osbourne, 1991). The rarity however for clinically normal parents to have a second affected child argues against non-penetrance in the disease although reduced or age-dependent effects of TSC or germ-line mosaicism must be considered (Fryer, 1991; Gomez, 1991).

To determine the underlying defect causing TSC, several groups have resorted to the use of positional cloning (Collins, 1992) to search for the genes involved in the disorder (Povey et al.,1992). The following section describes this approach, concentrating on the particular aspects of the strategy used in the work presented in this thesis.
<table>
<thead>
<tr>
<th>Organ</th>
<th>Definitive</th>
<th>Presumptive</th>
<th>Suspect</th>
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<tr>
<td>CNS</td>
<td>Cortical tuber</td>
<td>Subependymal nodules</td>
<td>Giant cell astrocytomas</td>
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<tr>
<td>Retina</td>
<td>Multiple phakomas</td>
<td>Single phakoma</td>
<td>Infantile spasms</td>
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<tr>
<td>Skin</td>
<td>Facial angiofibromas</td>
<td>Confetti-like spots</td>
<td>Other seizures</td>
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<td></td>
<td>Ungual fibroma</td>
<td>Shagreen patch</td>
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<td>Forehead fibrous plaques</td>
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<tr>
<td>Kidneys</td>
<td>Multiple angiofibromas</td>
<td>Single angiomyolipomas</td>
<td>Cysts</td>
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<td>Heart</td>
<td>Multiple rhabdomyomas</td>
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<td>Single rhabdomyomas</td>
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<tr>
<td>Lungs</td>
<td>Lymphangiomatosis</td>
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<td>Enamel pits/fibromas</td>
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<td>Teeth\Gingiva</td>
<td>Hamartomatous polyps</td>
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<td>Pseudocysts in phalanges</td>
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<td>Rectum</td>
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<td>Periosteal new bones</td>
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<td>Bones</td>
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Table 1.1 Diagnostic features in tuberous sclerosis (Taken directly from Fryer, 1991)

The definitive features are individually diagnostic for the disease. In contrast more than one of the presumpive or suspicious feature has to be present before a diagnosis can be made.
1.2 THE POSITIONAL CLONING APPROACH

This approach identifies the disease gene on the basis of its chromosomal position rather than from a knowledge of its functional properties. Previously termed reverse genetics (Orkin, 1988) this strategy has been used in the cloning of a large number of disease loci (Collins, 1992; Chen et al., 1992; Vulpe et al., 1993; Attree et al., 1992, Vetrie et al., 1993; Walker et al., 1993; Trofatter et al., 1993).

1.2.1 Chromosomal localisation of the Gene

1.2.1.1 Linkage analysis

The chromosomal position of the gene can be determined by looking for a genetic character which shows the same pattern of segregation as the disease in affected pedigrees. It is based on the assumption that as the distance between two loci increases the less likely it becomes that they will be co-inherited and therefore linked in future generations due to their separation by meiotic recombination. Consequently through a knowledge of the map position of a marker that is linked to a disease the chromosomal position of the disorder can be inferred to the same region.

Various models have been devised to determine the likelihood that two loci are linked (Ploughman and Boehnke, 1989). This can be calculated as a LOD score, a logarithmic function of the odds of linkage versus non-linkage. Generally this score is maximised assuming some degree of recombination between the two loci to estimate the most probable distance between them. Additional fine mapping of the region can then be applied to narrow the genetic boundaries of the disease to flanking markers. This genetic distance is measured using recombination rather than physical length and is expressed in centimorgans (cM) where 1cM represents 1% recombination. This
association will only hold for short distances as the probability of multiple cross-overs occurring between the two loci will increase with increasing distance.

On average a 1cM interval can be equated to a physical size of one megabase but this value will vary depending on the chromosomal region in question. For instance the frequency of recombination in the chromosome 9q34 region where the TSC1 gene is located is estimated to be two and a half times the frequency of this theoretical value (Ozelius et al., 1992). This genetic interval can be subsequently translated into a physical distance to determine the size of the region to be screened for the disease gene.

1.2.1.2 Chromosomal changes

The association between gross chromosomal rearrangements and the cause of genetic disease has also provided an important clue as to regions involved in genetic disease (Monaco et al., 1986; Wallace et al., 1990; Sinclair et al., 1990; Trofatter et al., 1993). Although linkage efforts (section 1.2.1.1) can refine the genetic position of a locus to as little as 1cM, the discovery of chromosomal aberrations in patients can help position the affected gene to a region within this genetic interval. In a number of disorders the identification of a deletion or a rearrangement has led to formal linkage studies to confirm the association between the chromosomal abnormality and the disease phenotype (Herrera et al., 1986; Francke et al., 1985). These observations help overcome the need for extensive linkage analyses to localise the disease gene to a particular chromosomal position.

1.2.2 Selective cloning of the disease interval

Once the gene responsible for the disorder has been localised a number of methods have been developed to enrich for DNA material from this chromosomal region. This greatly improves the
prospects for gene identification by reducing the complexity of the DNA to be isolated and screened.

In the case of diseases such as Duchenne muscular dystrophy (Monaco et al., 1986) and Wilm's tumor (Call et al., 1990; Gessler et al., 1990), the identification of deletions in some patients suffering from the disease have been exploited to map the area surrounding the affected locus. For diseases where there is no obvious chromosomal changes the techniques of chromosome microdissection and irradiation-fusion hybrids have been extensively used.

1.2.2.1 Chromosome Microdissection

The use of chromosome microdissection provides a direct method for the isolation of genomic sequences. This approach was first introduced by Scalenghe et al. in 1981 to clone regions from the Drosophila genome. The introduction of band specific microdissection and microcloning techniques for human chromosomes (Senger et al., 1990) has made it possible to target chromosomal regions that are known to contain human disease genes (Buiting et al., 1990; Davis et al., 1990; Ludecke et al., 1990; MacKinnon et al., 1990; Hampton et al., 1991; Puech et al., 1992; Seki et al., 1993). This approach allows the precise excision of single chromosomal bands which can be cloned as short DNA fragments and used to screen genomic reference libraries to identify cosmid and yeast artificial chromosomes present in that region. Consequently the saturation of a region with these markers contributes towards the generation of a contiguous physical map around the disease locus.
1.2.2.2 The irradiation fusion strategy

1.2.2.2.1 Introduction

A second approach relies on an irradiation-fusion strategy to isolate the region containing the disease gene as a small subchromosomal fragment contained in a human-rodent somatic cell hybrid. This is the approach I have used in order to selectively clone the region containing the TSC1 gene on chromosome 9. The technique was first described by Goss and Harris in 1975. It involves the fragmentation of human DNA from a somatic cell hybrid by exposure to a lethal amount of ionising radiation and the rescue of these fragments by fusion to a viable rodent cell (Fig 1.1). These new hybrid lines contain one or more human fragments which together constitute a fraction of the starting human material. The transferred chromosomal fragments are then characterised by marker retention and cytogenetic study to identify those hybrids which have been enriched for the region of interest.

Originally the technique was used for gene mapping. Goss and Harris showed that the linear order of markers could be deduced by comparing the frequency at which genes were transferred together after irradiation to the rodent cell. Although their initial experiments selected for fragments spanning the HPRT locus on the X chromosome (Goss and Harris, 1975; Goss and Harris, 1977a), their later work on chromosome 1 (Goss and Harris, 1977b) indicated that unselected fragments could also be retained by this approach.

The discovery that fragments can be retained at a high frequency without selection provided a general approach for the isolation of fragments from any chromosomal position (Cox et al., 1989; Benham et al., 1989). This technique has now been adopted using human-rodent hybrids containing single human chromosomes to generate fragment hybrids enriched for regions containing several disease loci. Only a small proportion of these hybrids will be suitable for selective cloning of DNA from the gene
Exposure to a large dose of X-irradiation

Fragmentation of the chromosomal material

Cells are non-viable

Cell fusion

Selection for retention of a particular locus present in the irradiated donor of either human or hamster origin

Hybrid cells will only survive

These hybrid cells are expanded for DNA isolation and marker retention analyses

Fig 1.1 The irradiation hybrid approach to generate radiation reduced hybrid lines retaining reduced amounts of human chromosomal DNA. The donor cell line is irradiated with a large dose of X-rays and the chromosomal fragments rescued by fusion to a viable rodent cell line. Hybrid cells are selected for the retention of a marker locus of human or hamster present in the donor cell line.
region although the availability of suitable selection systems, e.g. cell surface antigens or metabolically selectable enzymes, can improve the precision of the approach, by targeting retention of the specific chromosomal region of interest (Glaser et al., 1990; Henske et al., 1992; Brook et al., 1992). Unfortunately, the chromosomal regions around many disease genes do not contain known selectable markers although it has been possible to introduce new selection systems into these regions to increase the efficiency of the approach (Doucette-Stamme et al., 1991).

1.2.2.2.2 Integrity of transferred fragments

The integrity of the human fragments generated by the irradiation-fusion process has been considered in a number of ways. Pulsed-field studies comparing the physical structure of the human DNA retained in fragment hybrids with the parent line (Cox et al., 1989) and between fragment hybrids derived independently from the same region (Glaser et al., 1990) suggest that chromosomal changes do not occur as a result of the irradiation process. This is in marked contrast to chromosome-mediated gene transfer (McBride and Ozer, 1973), a method previously used for generating reduced somatic cell hybrid lines, which resulted in a significant level of DNA rearrangements (Bickmore et al., 1988; Glaser et al., 1990).

Indeed the pattern of marker retention in irradiation hybrids is so similar to the linear map order expected from genetic linkage studies that the irradiation hybrid strategy has been developed along the lines of the initial mapping studies of Goss and Harris into a statistical approach for the mapping of individual chromosomes (Cox et al., 1990; Warrington et al., 1991; Ceccherini et al., 1992; Tamari et al., 1992; Altherr et al., 1992; Rothschild, et al., 1992). Some studies have suggested that small deletions occur in at least some hybrid lines (Doucette-Stamm et al., 1991). However the frequency of chromosomal changes will be difficult to predict as this would require extensive analyses of individual hybrid DNAs.
1.2.2.2.3 Human chromosomal retention in irradiation hybrids

Human fragments in irradiation hybrids are usually retained as insertions or translocations to hamster chromosomes (Benham et al., 1989; Cox et al., 1990; Sinke et al., 1992). These associations are the result of random healing of fragmented chromosomal ends together after irradiation and may be visualised by in situ hybridisation of metaphase chromosome spreads from the hybrid using total human DNA as probe. It seems that these fragments are stabilised by their attachment to a rodent centromere although the increased retention frequency of human centromeric sequences found in irradiation hybrids and the presence of some fragments consisting entirely of human DNA implies that human centromeric sequences are also intrinsically stable in the new hybrid cell (Benham et al., 1989; Cox et al., 1990).

A tendency for multiple independent human fragments to be retained in irradiation hybrid lines after fusion (Benham et al., 1989; Goodfellow et al., 1990; Florian et al., 1991) is unfortunate as the amount of human DNA retained in a hybrid will determine its suitability as a resource to enrich for the region being studied. The size of these fragments is inversely related to the amount of starting irradiation. Cox et al. 1990 made use of the observation that irradiated cells undergo a final round of cell division to determine the number of human fragments generated by irradiation exposure. Using an estimate of the amount of human material in their starting somatic cell hybrid they were able to determine the frequency of chromosomal breakage as once every 8Mbs for a starting exposure of 8 Krads.

Some of the human fragments retained in a hybrid line will be completely stable while others will be progressively lost with culturing. A consequence of fragment instability is the generation of new hybrid lines which result from the progressive loss of unstable human fragments from the original cell line. These new lines may provide a more suitable cloning resource as a result of
the loss of unwanted human DNA from the cell (Benham and Rowe, 1992). An alternative strategy is to enrich for the fragment of interest in these hybrids by refusion to a new rodent cell (Altherr et al., 1992). The basis for this is the tendency for cells to segregate some chromosomal material after fusion. The chance segregation of extraneous human DNA can result in the generation of secondary hybrids enriched for the fragment containing the locus being studied. This strategy has been applied to the generation of single fragment hybrids around the Huntingtons disease locus on chromosome 4 and the Spinal Muscular Atrophy and Treacher Collins syndrome loci on chromosome 5 (Altherr et al., 1992).

1.2.2.2.4 Radiation Hybrid Mapping

The radiation hybrid (RH) mapping procedure introduced by Goss and Harris has now been developed into a statistical approach for the construction of long range maps of mammalian chromosomes (Cox et al., 1990). Although an individual radiation hybrid is an unreliable mapping tool the analysis of a large number of such hybrids has proved more valuable. The approach is analogous to meiotic mapping, in that the frequency of chromosomal breakage between loci acts as a measure of the distance between them. Although different methods of analysis have been proposed to determine marker order (Cox et al., 1990; Falk, 1991; Boehnke, 1992; Bishop and Crockford, 1992; Barrett, 1992), the results are in good agreement with each other and those obtained by other mapping methods (Cox et al., 1990). It is expected that these radiation panels will provide a valuable resource for constructing fine physical maps across the genome (Gerhard et al., 1992; Altherr et al., 1992).
1.2.2.2.5 The application of irradiation hybrids as a cloning resource

Traditionally, recovery of human DNA from rodent-human somatic cell hybrids has involved the generation of recombinant libraries and the identification of human material by probing with a human specific repetitive sequence probe. Although this approach has succeeded in the identification of human material from the regions around the Huntington's (Prichard et al., 1989) and Myotonic Dystrophy loci (Brooks et al., 1992) the approach becomes rather laborious and inefficient as the proportion of human material retained in a particular hybrid gets smaller.

An alternative strategy makes use of the large number of repetitive elements, called Alu repeats, present in the human genome. These repeats are present on average every 7kb and are in general randomly distributed throughout the genome with an underrepresentation at the centromeric heterochromatic regions of at least 50 fold (Moydis et al., 1989). The intervening DNA fragment between Alu sequences can be amplified by PCR using a Alu specific oligonucleotide primer when two flanking Alu elements are in opposite orientation to each other. Simultaneous amplification from many genomic loci result in the generation of a fingerprint pattern of Alu products characteristic of the DNA present. Consequently using human specific Alu primers this approach can provide an efficient mechanism for generating region specific DNA fragments from a somatic cell hybrid line (Nelson et al., 1989; Cotter et al., 1990; Brooks-Wilson et al., 1990; Ledbetter et al., 1990).

Alu products can then be used to identify chromosome and region specific yeast artificial chromosome and cosmid clones by direct hybridisation to genomic reference libraries (Monaco et al., 1991; Chumakov et al., 1992; Cole et al., 1992). These resources can then be used to identify transcribed sequences from the particular chromosomal region (section 1.2.3). As Alu products represent the human material present in the somatic cell panel they can also be used in conjunction with fluorescent in situ
hybridisation to localise the human DNA content present in a hybrid line to discrete regions of a chromosome (Sinke et al., 1992).

Another option is to isolate human transcripts directly from somatic cell hybrids. Human transcripts have been isolated from heteronuclear RNA by selecting for RNAs containing human repetitive sequences (Liu et al., 1989; Corbo et al., 1990). Recently a subtractive hybridisation strategy for cloning human cDNAs has also been developed which makes use of the reduced homology to rodent sequences in the non-coding region of mature mRNA (Jones et al., 1992). The human sequences derived from the non-coding region of the mature mRNA can be used to screen human cDNA libraries to identify the corresponding translated sequence.

1.3 Isolation of candidate genes from a region

There has been a concerted effort to identify transcribed sequences from cloned DNA sources using a number of different approaches. Initially the search for expressed sequences was conducted by identifying regions conserved between divergent species as gene sequences were known to be evolutionary conserved. The observation that CpG rich islands lie at the 5' end of many genes (Brown and Bird, 1986; Lindsay and Bird, 1987) provided a further clue to the position of transcribed regions. Such sequences could then be used to screen suitable expression libraries to identify corresponding cDNA clones.

Following advances in cloning long stretches of DNA in yeast artificial chromosomes (Burke et al., 1987) these have been used directly to increase the efficiency of cDNA isolation. The screening of appropriate cDNA libraries with YACs has led to the identification of the gene for Norrie disease and the isolation of new cDNAs from the HLA and Huntington's regions (Chen et al., 1992; El Kahloun et al., 1993; Snell et al., 1993).
The more recent methods of exon trapping (Duyk et al., 1990; Buckler et al., 1991) and direct cDNA selection (Parimoo et al., 1991; Lovett et al., 1991) apply a PCR based approach towards the isolation of candidate gene sequences. The use of exon trapping systems has made it possible to identify expressed regions by selecting for splice site sequences contained within the cloned genomic DNA. This approach has been used in the identification of the genes for X-linked glycerol kinase deficiency (Walker et al., 1993) and Neurofibromatosis 2 (Trofatter et al., 1993).

In comparison the cDNA selection approach enriches for cDNAs showing homology to cloned DNA. Appropriate cDNA libraries are amplified using primers specific to the vector arms and hybridised to the cloned material. After washing, to remove any non-specific hybridising cDNAs, sequences showing homology to the genomic clones are reamplified by PCR. Through successive rounds of hybridisation and PCR it has been possible to enrich for these cDNA clones. This approach has been adopted for the identification of the X-linked agammaglobulinaemia (XLA) gene (Vetrie et al., 1993). The utilisation of oligonucleotides corresponding to splice site consensus sequences (Melmer and Buchwald, 1992) may provide a method to preselect the cloned material for exon-trapping and cDNA selection studies.

1.2.4 Scanning for mutations

The final step towards the identification of the disease gene requires the evaluation of each candidate locus to identify the gene altered in the disorder. Initially candidate genes can be used to screen for deletions or rearrangements by Southern analysis of patient DNAs. Vetrie et al. 1993 for example identified chromosomal changes segregating with the disease in 8 out of 31 families having XLA using one out of the six candidate genes they had identified. The recent observations linking heritable unstable DNA sequences in the form of simple trinucleotide repeat units to inherited disorders may also provide a new approach to identify
some disease genes (La Spada et al., 1991; Verkerk et al., 1991; Buxton et al., 1992; Richards and Sutherland, 1992; Huntington's disease collaborative research group, 1993). However, it seems more likely that the identification of the vast majority of disorders will require a systematic search for mutation in each candidate clone to identify the aberrant gene.

In the past this has involved sequencing cDNA clones or exons from each patient. However several new scanning methods, amenable to the analysis of DNA and RNA, now exist to screen for mutation. These have been reviewed in detail (Cotton, 1989; Rossiter and Caskey, 1990). Three approaches in particular, Denaturing Gradient Gel Electrophoresis (DGGE) (Myers et al., 1985), the Chemical Cleavage Mismatch method (CCM) (Cotton et al., 1988; Saleeba et al., 1992) and the Single Strand Conformational Polymorphism approach (SSCP) (Orita et al., 1989) have been used extensively to detect mutation at a number of disease loci (Kogan and Gitschier, 1990; Reiss et al., 1992; Windericky et al., 1992; Flomen et al., 1992). The coupling of the PCR technique with these new strategies has provided an improved method for targeting regions of particular interest.

The SSCP approach is based on the observation that changes in the sequence of a single stranded molecule can have a profound effect on its electrophoretic mobility. This allows molecules differing in sequence by as little as a single nucleotide to be distinguished from each other. In contrast the DGGE strategy compares the denaturing profiles between DNA duplexes. Sequence variation between strands will lead to changes in the melting profile of a molecule allowing heteroduplexes between different alleles to be identified. Recently the DGGE approach has been adapted to detect variation in genomic DNA (Gray, 1992). The DNA is restriction digested in the normal way but the digestion products are separated on the basis of their base composition by DGGE, increasing the potential to identify sequence variation after blotting and hybridisation (Burmeister et al., 1991; Krolewski et al., 1992).
In the CCM method, heteroduplexes are formed between test and control DNA samples. Mutations are identified by modifying mismatches or unmatched residues making them more susceptible to chemical cleavage. Following the identification of base variation in the PCR product these can be sequenced to determine the nucleotide changes that have occurred.

1.3 Fluorescent In Situ Hybridisation

The application of fluorescent in situ hybridisation (FISH) to genome analysis has also contributed markedly towards recent mapping advances (Trask et al., 1991). The introduction of fluorescent detection schemes (Albertson et al., 1988; Bhatt et al., 1988), in conjunction with chromosomal in situ suppression hybridisation (Lichter et al., 1990), has increased the speed and precision of mapping cloned material. The physical and genetic analyses around disease loci have also been complemented by the introduction of dual labeling experiments to determine the linear order and distance between sequences (Trask et al., 1991).

The FISH technique also provides a simple method to test for chimeras in YAC clones (Ross et al., 1992) and is an efficient way to characterise the amount and stability of human material retained in irradiation hybrid lines. Since the introduction of painting techniques using Alu-PCR it has allowed the confirmation of the position of markers derived from radiation reduced hybrid and microdissection studies. Most importantly the speed and precision of the approach has helped to increase the number of genes which have been precisely localised to regions of the genome making it applicable to follow a candidate gene approach towards the identification of some disease genes.
1.4 The Candidate Gene Approach(s)

The candidate gene approach involves the fortuitous association between the chromosomal map position of a disease locus and a previously mapped gene whose structure or function suggests it to be a likely candidate for the disorder. This approach eliminates the need for extensive cloning efforts across the disease region and has led to the identification of genes for several disorders (Dryja et al., 1990; Farrar et al., 1991; Giesterfer-Lowrance, 1990; MacLennan et al., 1990; Malkin et al., 1990; Maslen et al., 1991; Gaate et al., 1991; Rosen et al., 1993). Unfortunately the general application of the approach for most diseases is limited as the density of characterised genes in the genome is still quite low.

A similar approach makes use of the growing knowledge of the genomes of other species. Although individual genomes are diverged, regions of synteny have been identified between them where gene order is maintained. This allows for comparative mapping of disease genes from one species to be evaluated as candidates for diseases mapping to the related region in another species. For example, following the discovery that the mouse mutant Splotch was caused by mutations in the Pax-3 gene (Epstein et al., 1991), its human homolog, Hup2, was assessed as a candidate gene for Waardenburg syndrome on the basis of their comparable map positions. Mutations at this locus have now been found in patients with this disease (Tassabehji et al., 1992; Baldwin et al., 1992). In a similar fashion the gene for human type II oculocutaneous albinism has been identified on the strength of its similar map position with the mouse pink-eyed dilution locus (Rinchik et al., 1993).

The expressed sequence tag strategy (EST) for randomly selecting and sequencing cDNAs may ultimately lead to the cloning of further disease genes through their sequence homology with genes of known function present in other organisms. The fortuitous sequence homology between an EST and the gene for glycerol kinase in *Bacillus subtilis* has for example led to the
identification of the gene for X-linked glycerol kinase deficiency (Sargent et al., 1993).

1.5 Development of the distal 9q map

In most cases the development of a high resolution map of the region containing a disease locus is required for the finer localisation and subsequent isolation of the corresponding gene. The localisation of TSC1 to chromosome 9q34 has focused attention on the development of the map across this region.

Initially genetic maps were restricted to a few small linkage groups. Linkage of the ABO blood group to Nail Patella syndrome in 1955 was the third autosomal linkage group to be defined in humans (Renwick and Lawler, 1955). Later in 1965 Xeroderma Pigmentosum was shown to be linked to the ABO blood-group (El Hefnawi et al., 1965) and soon after that the gene encoding the enzyme Adenylate Kinase was also placed in this linkage group (Rapley et al., 1967). Following the development of somatic cell genetics it became possible to correlate the retention of human biochemical markers in human-rodent hybrid cells with the retention of identifiable human chromosomes (Weiss and Green, 1967; Ruddle, 1973). This led to the assignment of AK1 and the associated ABO linkage group to chromosome 9 (Westerwald et al., 1976; Povey et al., 1976). The position was further refined to the distal long-arm of the chromosome by looking at the segregation of these markers in families with various chromosome 9 aberrations (Ferguson-Smith et al., 1976; Cook et al., 1978).

Although there existed extensive polymorphism at the structural loci of many human proteins (Harris and Hopkinson, 1972), genetic markers were still restricted to a small collection of the genes for red cell enzymes, plasma proteins and blood cell antigens that were scattered throughout the genome. In contrast the discovery of a method for analysing variation directly at the DNA level offered a vast potential for new genetic markers. Initially this variation was analysed by restriction endonuclease
digestion. Since these enzymes recognise specific DNA sequences, changes in genomic DNA can lead to the loss or gain of a digestion site. This variation in digestion patterns between different alleles, first observed in the beta globin locus (Kan and Dozy, 1979), were termed restriction fragment length polymorphism (RFLP) (Botstein et al., 1980). These polymorphic sites can be regionally positioned and their order and linkage distances determined by linkage analysis. However, although RFLPs are very common they generally consist of two alleles, i.e. the presence or absent of a restriction site and this limits their application for effective linkage analysis.

A few RFLPs show great variability in their length (Whyman and White, 1980; Bell et al., 1982; Capon et al., 1983; Jeffreys et al., 1985). These fragment are found to contain stretches of short tandem repeat units between 11-60bp in length, called minisatellites. Using consensus oligonucleotides to these minisatellites many new individual hypervariable loci have been isolated and developed as new marker systems (Nakamura et al., 1987). This approach allowed several new markers to be incorporated into the genetic map of chromosome 9 (Lathrop et al., 1988). Unfortunately these loci tend to cluster towards the telomeres which limit their application for mapping purposes (Royle et al., 1990).

As minisatellite regions often display extensive length variation the potential of shorter repeat elements, termed microsatellites as genetic markers has also been investigated (Litt and Luty, 1989; Weber and May, 1989). These short simple repeat sequences usually take the form of poly(C-A).poly(G-T) blocks scattered throughout the genome with between 10-60 repeat units present in each block. The number of repeat units can be highly polymorphic and the application of the polymerase chain reaction with primers flanking the repeat in conjunction with sizing by polyacrylamide gel electrophoresis has provided an efficient system for their analysis. Since such repeats occur as often as one in every 30-100kb in the genome (Stallings et al., 1991) they provide a vast new resource of highly informative
markers for the development of the human genome map. Using this approach a high resolution map of the q33-q34 region of chromosome 9 has been produced (Kwiatowski et al., 1992). This map has been incorporated into a consensus map of the region containing previously assigned loci, fig 1.2 (Povey et al., 1992). The development of this map has been instrumental in refining the position of the TSC1 gene locus to a much smaller genetic interval of chromosome 9q34 (section 1.7).
Fig 1.2 Genetic map of the chromosome 9q34 region containing the TSC1 gene

The genetic map is taken from the consensus map of chromosome 9q22-9ter Povey et al., 1992. Genetic distances are in sex averaged centimorgans. The interval containing the TSC1 gene is shown by the arrows, ————→
1.6 Chromosome Assignment of the genes involved in the TSC phenotype

The positional cloning approach initially requires that the chromosomal position of the gene or genes responsible for the disorder be defined. With the help of the Tuberous Sclerosis Association of Great Britain, linkage analysis was undertaken by Fryer et al. in a U.K. collaborative effort, to locate chromosomal regions involved in the TSC phenotype (Fryer et al., 1987). This study, using 19 families and 26 polymorphic markers scattered throughout the genome identified a TSC locus close to the ABO blood group locus on the distal long-arm of chromosome 9. Although this assignment was confirmed by later studies (Connor et al., 1987; Fahsold et al., 1987; Sampson et al., 1989), it quickly became apparent that not all family data supported linkage of TSC to this chromosome (Smith et al., 1987; Northrup et al., 1987; Kandt et al., 1989).

The first clue to the possible location for a second TSC locus came with the discovery of TSC in a chromosomally abnormal patient, trisomic for distal 11q and proximal 22q (Clark et al., 1988). The conclusion that a TSC gene may have been altered by this rearrangement stimulated renewed linkage study which positioned this second locus to 11q14-11q23 (Smith et al., 1990). The subsequent identification of another TSC patient with a reciprocal translocation, t(3;12)(p26.3;q23.3) led to chromosome 12q22-24.1 being suggested as a likely location for a third TSC locus (Fahsold et al., 1991).

Considered together, these studies increased speculation about genetic heterogeneity in TSC (Smith et al., 1990; Sampson et al., 1990; Janssen et al., 1990; Haines et al., 1991). A model for genetic heterogeneity was supported through analyses on a large international collaborative data set which confirmed the assignment of a TSC gene to chromosome 9 and the existence of at least one further TSC locus. These analyses failed to confirm the existence of this second TSC locus to either chromosome 11 or 12.
With this in mind Kandt et al. (1992) set about an extensive genome search to identify any remaining TSC loci. This study, using 5 large TSC families previously shown not to be linked to chromosome 9, and 116 polymorphic loci distributed over 21 chromosomes succeeded in identifying a new TSC locus on chromosome 16p13, close to the Polycystic Kidney disease type 1 gene (PKD1) (Germino et al., 1992; Somlo et al., 1992). It now appears that TSC is predominantly, if not exclusively caused by these loci on chromosome 9 and 16. Although the proportion of cases attributed to each locus is not yet certain one group has suggested that the 9q34 locus is responsible for 30% of familial cases of TSC with the second locus on chromosome 16 responsible for the remaining 70% (Pericak-Vance et al., 1992). A few families remain which show genetic recombination with markers in both regions. This suggests that the inheritance of TSC may involve a third locus in a small number of families (Short et al., 1992).

1.7 Refinement of the genetic position of TSC1

Extensive haplotyping of TSC families with these newly derived marker loci from the chromosome 9q34 region (section 1.5) have succeeded in refining the genetic position of the TSC1 gene locus to a much smaller region of distal 9q34. Unfortunately the inclusion of recombination data from unaffected individuals in these studies remains in doubt following a re-appraisal by Haines et al. 1992 of the linkage data in their families using an affecteds only analysis (Povey et al., 1992). Their study has suggested that the incidence of non-penetrance is an important factor in TSC pedigree analysis. It is therefore prudent to concentrate primarily on the exclusion data derived from affected individuals when determining the genetic boundaries of TSC1 while utilising the exclusion data available from unaffected individuals to identify regions of more immediate interest between these boundaries. The reported exclusion data for TSC1 (Povey et al., 1992) defines the genetic boundaries of the disease to a region between the markers...
D9S64 and D9S67 with the region around the ABO locus, between the markers D9S64-DBH as the most likely location for the gene.

1.8 Physical mapping of the 9q34 region

As linkage analysis is an indirect measurement of distance the application of a physical mapping strategy across the candidate region is important to complement these genetic linkage studies. This translation of the genetic distance into physical size is required to determine whether the length of the region is appropriate for contig assembly to begin.

Pulsed-field gel electrophoresis (PFGE) first described by Schwartz and Cantor in 1984 is the established method for constructing long range physical maps of chromosomal regions (Schwartz and Cantor, 1984). This approach allows the separation of large fragments of DNA up to several megabases in length. Fragments are generated by digesting the DNA with rare cutting restriction enzymes which cleave very infrequently in the genome. These fragments are resolved by applying alternating electric fields which size fractionate the DNA molecules by their ability to reorientate to the changing electric field position. Physical linkage of markers from a region to fragments of known size can be determined by sequential hybridisation with selected probes. By using a combination of complete and partial digests for a variety of rare cutting enzymes the physical map can be extended to cover the entire candidate region.

In addition to providing a comparison between the genetic and physical size of a region, the availability of a pulsed-field map can also be used to identify and assess candidate genes for the disorder. This is possible because of the association between stretches of undermethylated CpG dinucleotides sequences which correspond to the cleavage sites of rare cutting enzymes and the 5' upstream regions of housekeeping genes. Indeed it now appears that all CpG islands are associated with genes and almost all cover at least part of one exon (Larsen et.al., 1992) The clustering of
rare cutter sites across a region will therefore suggest the position of candidate gene sequences. Furthermore, although it may not be possible to identify any gross cytogenetic abnormalities in individuals suffering from the disorder, a direct comparison of the physical maps from affected and unaffected individuals has succeeded in identifying deletions or rearrangements in the candidate regions of several disorders (Vetrie et al., 1993). This may allow for finer localisation of the disease gene and confine cloning efforts to a small part of the genetic interval.

Physical mapping studies have been strongly influenced by the ongoing genetic efforts. The most recent physical map of the region is shown in fig 1.3. These studies suggest that the 9q34 region is much smaller than that expected from genetic linkage analysis (Ozelius et al., 1992). For instance the region encompassing the ASS-D9S10 region, a genetic distance of 8cM has been completely linked on PFG fragments totalling 2.35Mb (Harris et al., 1993).

The further development of the physical map will provide an opportunity to compare the candidate region in affecteds and unaffecteds in an attempt at identifying chromosomal changes which may be involved in causing the disease. In addition a physical map of the region will define the actual size of the TSC1 gene interval which will help direct the generation of a cloned contiguous map of the region.
Fig 1.3 Physical analysis of the TSC1 region of chromosome 9

References (1) Harris et al., 1993, (2) Handa et al., 1992, (3) Hornigold personal communication
1.9 Aims of the project

The localisation of a gene for TSC to chromosome 9q34 in 1987 by Fryer et al. focussed attention on the characterisation of this region in order to identify the gene involved.

The initial aim of the work was to generate a panel of radiation reduced hybrid lines from chromosome 9q and to use these as a mapping resource to assist in the ordering of markers from the TSC1 region. It was anticipated that in conjunction with the ongoing genetic data from TSC1 families a small proportion of these hybrids could then be used to generate new cloned resources from the region in order to construct a cloned contig map between the flanking markers for the disease.

These resources could also be used to search for gene sequences within the boundaries defined by genetic linkage studies to identify candidate genes for TSC1.
CHAPTER 2

Materials and Methods
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2.1 Materials and Methods

2.1.1 Standard media and buffers

Unless otherwise stated solutions were sterilised by autoclaving at 15 psi, 121 C for 20-25 min.

LB Medium
(per 100ml)

Bacto-tryptone 1g, Bacto-yeast extract 0.5g, NaCl 1g, Glucose 0.1g

LB Agar
Bacto-tryptone 1g, Bacto-yeast extract 0.5g, NaCl 1g, 1.5g Bacto-agar

Antibiotics

kanamycin sulphate was prepared at a concentration of 25mg/ml in distilled water and sterilised by filtration (0.22μm pore size). The working concentration was 25μg/ml.

5XTBE 0.5M Boric acid, 0.5M Tris, 10mM EDTA
1XTE 10mM Tris, 1mM EDTA pH 8.0
1XTNE 100mM NaCl, 10mM Tris, 1mM EDTA pH 8.0
20XSSC 3M NaCl, 0.3M Trisodium citrate

Gel loading buffer
0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol
Church hybridisation solution
0.5M NaPi pH 7.2, 7% SDS, 1mM EDTA

1M Sodium Phosphate solution pH7.2
Prepared by titration of 1 molar solutions of Na₂HPO₄·12H₂O and NaH₂PO₄·2H₂O

Denaturation solution
0.5M NaOH, 1.5M NaCl

Neutralising solution
1.5M NaCl, 1M Tris, 1mM EDTA pH 7.5

2.1.2 Suppliers of Materials

Restriction endonucleases

Restriction endonuclease were obtained from NBL or BRL

DNA modifying enzymes

Calf intestinal
phosphatase
Boehringer Mannheim

T4 DNA ligase
Anglian

Klenow DNA
polymerase
New England Biolabs

Polmerase Chain Reaction
Taq polymerase and buffer was supplied by Promega or NBL. Nucleotides were supplied by Boehringer Mannheim. All PCR was carried out in a Hybaid thermal reactor.

**Kits**

Random prime labelling kit Amersham International

**Chemicals, reagents and membrane**

Hybond N Amersham

[alpha-32-P] dCTP Amersham

X-Ray film Kodak X-omat AR

Agarose Sigma

Bacterial media Difco

General Chemicals and reagents BDH

**Cell lines**

The hypoxanthine phosphoribosyl transferase (HPRT) positive cell line, 64063a12 a hamster-human hybrid containing a single copy of chromosome arm 9q (Jones and Kao, 1984) was provided by Carol Jones. The HPRT- hamster cell line, Wg3-h, (Goss and Harris, 1975) was provided by Sue Povey. DNA from the DORA cell line which retains the philadelphia chromosome was provided by Ben Carritt

**2.2 General Techniques**
2.2.1 Growth and expansion of cell lines

The cell lines 64063a12, Wg3-h and the radiation reduced hybrid lines were grown as attached monolayers in Eagle's medium. This was supplemented with 10% v/v Foetal calf serum (FCS), 0.002M glutamine, non-essential amino acids, 0.1M hepes, 0.2%w/v NaHCO₃ and 100 units of Penicillin and Streptomycin in suitably sized flasks. The medium was brought to a slightly alkaline pH by the addition of a solution of NaOH/NaHCO₃. Radiation reduced lines were also grown in HMT selection medium, 100μm hypoxanthine, 10μm methotrexate and 10μm thymidine.

When the cells were confluent they were washed in HANKS and released from the flask surface by a brief incubation with a small volume of 0.25% trypsin. The trypsin was neutralised by the addition of some fresh media and the cells were divided or transferred to a larger flask as required. Cells were stored in 1ml aliquots in liquid nitrogen in 95% v/v FCS and 5% v/v DMSO.

Cell lines were subcloned after resuscitation from liquid nitrogen. Cells were plated at low density and colonies picked which were derived from a single cell. These colonies were expanded for DNA extraction. Cells were also retained for the generation of metaphase chromosome spreads to allow for fluorescent in situ analysis to test for the absence or retention of individual human fragments present in the parent irradiation hybrid line.

2.2.2 Isolation of DNA

2.2.2.1 DNA Preparation from Cell Lines.

Cells from 3 or more large flasks (180cm³) were thoroughly washed in HANKS. 7ml of lysing solution (0.5% SDS, 100g/ml proteinase K in STE) was added to each flask and incubated for at least 5 hours at 37°C or left incubating overnight at 37°C.
debris was removed by three extraction with phenol:CHCl₃:isoamylalcohol (25:24:1) followed by a further 2 extractions with CHCl₃ to remove any traces of phenol. The nucleic acid was precipitated with 2 volumes of ethanol and the spooled DNA was air dried and resuspended in TE.

2.2.2.2 Large scale preparation of plasmid and cosmid DNA

400ml of LB broth with the appropriate antibiotic selection was inoculated with a single bacterial clone and grown overnight with aeration in a 1 litre flask and harvested the following day by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellets resuspended in a total volume of 30ml Solution 1 (50mM glucose, 10mM EDTA, 25mM Tris.Cl (Ph 8.0), 4mg/ml lysozyme) and allowed to stand at room temperature for 5 minutes. 60ml of freshly prepared solution 2 (0.2 M NaOH, 1% SDS) was added and the mixture was gently mixed prior to incubation on ice for 5 minutes. 45ml of ice-cold solution 3 (3M potassium acetate) was lastly added followed by a further incubation on ice for 5 minutes. After lysis, the cell debris was pelleted by centrifugation at 10,000 rpm for 15 minutes and the supernatant retained.

0.6 volumes of propan-2-ol, approximately 70ml, was added to precipitate the DNA. After a 40 min period of incubation the precipitate was pelleted at 10000 rpm for 15 minutes at room temperature, drained and resuspended in 11.2 ml of TE buffer. To this suspension was added 0.4 ml of EDTA, 0.4 ml Tris Base, 12g of solid cesium chloride (CsCl) and 1.2 ml of 10mg ml ethidium bromide. The solution was thoroughly mixed and spun at 5000 rpm for 5 minutes to remove any remaining precipitate and pipetted into Sorvall polyallomer ultracentrifuge tubes. The tube was completely filled with paraffin oil and the tube was sealed with a Sorvall ultracrimp tube sealer. The tubes were spun in a Sorvall ultracentrifuge at 45000 rpm at 20°C for 18 hours. After centrifugation the plasmid DNA was visualised under UV light and removed using a syringe. The ethidium bromide was removed from
the plasmid DNA by repeated extraction with CsCl-saturated propan-2-ol and the CsCl was then removed by dialysis overnight in 5 litres of TE buffer.

The DNA was precipitated (section 2.2.1.3) and recovered by centrifugation. The pellet was washed in 70% ethanol, dried and resuspended in a suitable volume of TE. The concentration of the DNA was determined by spectrophotometry at 260nm (section 2.2.1.4.).

2.2.3 Preparation and transformation of competent E.coli

Competent E.coli JM101 cells, were prepared using the Calcium chloride (CaCl₂) procedure as described by Maniatis et al. (1982). JM101 were grown on a mimimal media plate to preserve the F' episome containing the genes for proline synthesis (this includes the gene required for blue/white selection of recombinants). 30ml of L-broth was inoculated with 0.5 ml of a fresh overnight culture of JM101 and incubated at 37°C with shaking until the culture reached an OD 550 of 0.4-0.6. The culture was chilled on ice for 10 min and pelleted by centrifugation at 500rpm at 4°C. The cell pellet was re-suspended in 15ml of ice-cold, sterile, 50mM CaCl₂ solution and kept on ice for 15 min. Cells were pelleted as before and resuspended in 3ml of ice-cold, sterile, 50mM CaCl₂. The re-suspended cells were stored on ice before being transformed.

For transformation, generally up to 20μl of DNA solution, approximately 50-100ng, was added to a 200μl aliquot of competent cells, incubated on ice for 40 min, heat shocked at 42°C for 90 seconds and reincubated for a further 2 min on ice, before plating out on L-agar containing the appropriate antibiotic resistance. When using Amp resistance vector systems, the cells were incubated in 0.5 ml of L-broth for 30 min prior to plating to allow for pre-expression of the Amp resistance. When using blue/white selection screening, 50μl of 2% X-Galactosidase and 20μl of 100mM Isopropylthiol-beta-D-galactoside was added to
the transformed cells before plating. The transformation efficiency of the cells was tested by transforming with 10ng of undigested vector DNA. The efficiency of the CIP reaction (section 2.2.2.2.) for preventing self religation was examined by transforming with dephosphorylated vector.

2.3 DNA Analysis

2.3.1 Agarose gel electrophoresis

DNA digestion products were resolved in 0.4-2.0% agarose gels prepared and run in 1xTBE buffer. Samples were loaded with the addition of 0.1 vol of 10x loading buffer. The DNA was stained with ethidium bromide at 0.5 µg/ml and visualised under ultraviolet transillumination.

2.3.2 Precipitation of DNA

DNA was precipitated by the addition of 0.1 x volume of 3M NaAc followed by 2 x volume of ice-cold ethanol. The DNA solution was incubated at -70°C for 5 minutes to allow the DNA to precipitate. The DNA was pelleted by centrifugation at 10000 rpm or in a bench centrifuge as appropriate for 10 minutes. The supernatant was discarded and the pellet washed in 70% ethanol before being dried and resuspended in an appropriate volume of TE, usually 1µg/µl.

2.3.3 Recovery of DNA fragment from agarose gels

DNA fragments were isolated from agarose gels using the Gene Clean method. The DNA fragment of interest was excised from the gel and the agarose melted by incubation in 3 x vol NaI and 1 x TBE modifier at 50°C for 5 min. Glassmilk was added and the suspension was incubated on ice for 5 minutes to allow binding of the DNA and Glassmilk together. The glassmilk-DNA complex
was pelleted by centrifugation for 5 seconds and washed in New Buffer x 3 before the DNA was eluted in double distilled water or TE.

2.3.4 Concentration of DNA

The concentration of the DNA was measured by spectrophotometry in a CIBA CORNING 2800 Spectrascan at 260nm or estimated by direct comparison of a small amount of the DNA with a known amount of lamda ladder after gel electrophoresis.

2.4 DNA Modification Reactions

2.4.1 Restriction digestion of DNA

Restriction enzyme digestion of plasmid and cosmid DNA was generally carried out in a reaction volume of 20μl using 0.5μg of DNA. Reaction buffer and restriction enzymes were those supplied by the manufacturer and incubation was typically for a period of 2 hours under the specified conditions. For the digestion of genomic and hybrid DNA reactions similar to that described above were set up but the reaction volume was increased to a total volume of 40-60μl and incubation continued overnight.

2.4.2 Dephosphorylation of DNA

The terminal phosphate group of linearised vector DNA was removed, using calf intestinal phosphatase (CIP), to prevent self ligation of the ends in subsequent cloning experiments. In general, 2-5μg of vector DNA was digested with the appropriate restriction enzyme in One-Phor-All Plus buffer. 0.1 units of CIP was added and incubated at 37°C for 30 min and the reaction stopped by heat inactivation of the enzyme at 85°C for 15 min. The CIP was removed by 2 phenol followed by 1 chloroform extraction and the DNA was recovered by ethanol precipitation, the pellet washed in 70% ethanol, dried and resuspended in a suitable volume of TE.
2.4.3 Ligation of DNA

Insert DNA was ligated to the appropriate cut and phosphatased vector in a solution containing 100ng of vector was used for most subcloning operations. Reactions were performed at 14°C overnight and generally contained at least a 3-fold molar excess of insert DNA to vector DNA with one unit of T4 DNA ligase.

2.5 Radioactive Labelling of DNA probes

A commercial labelling kit was used (Amersham). Usually 20-50 ng of insert DNA or Alu PCR products were labelled. The DNA was denatured by incubation at 99°C for 5 minutes in a Hybaid PCR automated heating block. Following a brief incubation on ice, 10ul of buffer 1 (dATP, dGTP, dTTP and labelling buffer) and 5ml of buffer 2 (containing random hexanucleotide primers) were added. 50 Ci radiolabelled nucleotide alpha 32P dCTP (10Ci/μl) was added to the reaction mix followed by 2.5μl (2.5 units) of DNA polymerase 1, klenow fragment to give a final volume of 50μl. Incubation was for at least 3 hours at room temperature or at 37°C for between 1-3 hours after which time the reaction was terminated by the addition of 50ml of TNE/0.1% SDS.

Unincorporated nucleotides were removed using a Sephadex G50 column. Sephadex suspended in TNE/0.1% SDS was compacted by centrifugation at 2000rpm for 2 minutes into a 1ml syringe. The labelling reaction was layered onto this and the spin repeated. The labelled DNA was contained in the eluate. Prior to hybridisation the probe was denatured by boiling generally in the presence of 1mg of sonicated herring sperm DNA and cooled on ice.

2.6 Southern analysis
After electrophoresis in 0.8% agarose gels, DNA was depurinated by submerging the gel in 0.25M HCl for 20 min. The gel was rinsed in distilled water and soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 2 x 20 min followed by a further 2 x 20 min in neutralising solution (1.5M NaCl, 0.5M Tris/HCl, pH 7.2), prior to Southern blotting of the DNA on to nylon membrane, Hybond N, in 20 x SSC for 16 hours. After gentle washing in 2 x SSC, the filters were baked at 80°C for 2 hours. Filters were pre-hybridised and hybridised in Church prehybridisation solution (0.5M NaPi, 7%SDS and 1mM EDTA). Pre-hybridisation was for >2 hours and hybridisation was for 14-16 hours following the addition of 32P-labelled DNA probe. Restriction fragments for Southern analysis were 32P-labelled by random priming (section 2.2.7). Filters were washed in 2 x SSC, 0.1% SDS for 2 x 20 min at 65°C and in 0.1 x SSC, 0.1% SDS for 30 min at 65°C when appropriate, before autoradiography at -70°C.

Before reuse filters were stripped of their radioactivity by incubation in 0.4M NaOH for 15 min followed by neutralisation in 0.2M NaPi pH 7.2, 0.1 x SSC and 0.1% SDS.

2.7 Preparation of cosmid libraries from individual hybrid lines

2.7.1 DNA Preparation

DNA was prepared from the radiation reduced hybrid lines 17B and 20A which retain a small amount of human DNA from the region surrounding the TSC1 locus. Hybrid DNA was partially digested with the restriction Mbo1 and size fractionated by sodium chloride density gradient. DNA from the fractions retaining 35-45 Kb fragment sizes were ligated into the cosmid vector Lorist B (Cross and Little 1986). Vector arms were prepared according to the conditions described by Little (1987). Approximately 500ng of hybrid DNA was ligated to 200ng of vector in an reaction volume of 10ul.
2.7.2 Packaging reaction

The linear cosmid molecules were packaged into bacteriophage lambda head particles in the following reaction: 2μl of the ligation reaction was mixed with 7ml of buffer A (20mM Tris.Cl pH 8.0, 1mM EDTA, 5mM MgCl, 0.05% v/v B-mercaptoethanol) and 1μl of buffer Q (6mM Tris.Cl pH7.5, 18mM MgCl, 60mM spermidine pH7.5, 15mM ATP pH7.6, 0.2% v/v B-mercaptoethanol). 4μl of sonicated extract and 5μl of freeze thaw lysate were added and incubated for 1 hour at 30°C. The reaction volume was made up to 200μl with phage storage medium and the phage were used to infect the recombinant deficient strain of E.coli ED8767 (Murray et al.,1977). Control packaging reactions were also carried out in tandem using 10ng of cosmid DNA that had been prepared by maxipreparation.

2.7.3 The recipient bacterial strain

The recipient bacteria were tested before use for retention of their RecA phenotype by exposure to UV light. The mutation that renders this strain unable to repair damage caused by UV also reduces the incidence of internal deletion events in cosmids which occur as a result of recombination between direct repeat elements.

A clone sensitive to UV irradiation was grown overnight in cosmid broth with 0.2% maltose in order to promote the production of maltose receptor molecules to which the phage molecules could adhere. The culture was pelleted and resuspended in half the volume 10mM MgSO4. An equal volume of cells was added to the phage packaging solution and following infection at 30°C for 20 minutes, 8 X vol of LB broth was added and incubation was continued for a further 45 minutes in order for the infected bacteria to express the antibiotic resistance genes. The bacteria were then plated onto agar plates containing kanamycin.

2.7.4 Screening for cosmids retaining human DNA sequences
Library filters, containing 5 genome equivalents (approximately 0.5 x 10^6 colonies) were prepared essentially as described in section 2.2.8 and hybridised to radiolabeled total human DNA to identify clones containing human inserts. Filters were prehybridised in Church solution (0.5M NaPi pH7.2, 7% SDS, 1mM EDTA) for 2-3 hours and were hybridised in the same solution with the addition of the DNA probe labelled alpha-^{32}P dCTP for approximately 16 hours. Filters were washed in 2 X SSC and 0.1% SDS for 1 X 5 minutes at room temperature followed by 2 X 30 minutes washes at 65°C. Autoradiography was for 1-3 days at -70°C.

Clones giving signals on duplicate filters were picked from the master and purified by subsequent rounds of screening. Human clones were identified, grown and stored at -70°C with 15% glycerol. The human nature of these clones were confirmed by FISH.

2.8 Cosmid Library Screening

Human genomic clones were isolated from a Mbo1 partialed human cosmid library constructed in the vector LoristB (Cross and Little, 1986) by Dr. Begona Cachon-Gonzalez in the laboratory of Dr. Jonathan Wolfe

The library was spread directly onto hybridisation membranes (Hybond N, Amersham) which had been laid on suitable selection media (L-agar plus kanamycin) and grown until the colonies were 0.5mm across. The master membrane was then removed and placed on kanamycin plates containing 25% glycerol and left 2-3hrs. This filter was removed and placed face up onto 3 filter papers, the top one prewetted with L-broth plus 15% glycerol. A hybridisation membrane was pre-wetted by laying onto a selective plate containing 25% glycerol and laid on the first master membrane covered with colonies. 3 filter papers were then laid on top of the second membrane. This sandwich was then
compressed to transfer the bacteria. The filter papers were peeled away and registration holes were made through both membranes using a sterile needle. The above process was repeated to give a duplicate hybridisation filter. The master and 2 replicas were then laid on kanamycin plates to regrow until the colonies were easily visible.

The replica membranes were floated colony side up as follows: 2 x 5 min denaturing solution (1.5 M NaCl, 0.5M NaOH), 2 x 5 min neutralising solution (1.5M NaCl, 0.5M Tris HCL (pH 7.5)), 5 min 2 x SSC. Any remaining colonies were removed by wiping with tissue paper soaked in 2 X SSC. Hybridisation filters were blotted dry and baked for 2 hrs at 80°C.

Following screening to detect recombinants the appropriate region of the filter was identified and colonies were scraped from an area of 0.5-1.0 cm diameter using a sterile loop. Bacteria were resuspended in L-broth and respread on petri dishes to give 50-200 colonies per plate. These were treated as above for the secondary screening stage.

2.9 The Polymerase Chain Reaction

The PCR reactions were carried out in a Hybaid automated heating block. The gilsons used to set up the reaction, gilson tips and centrifuge tubes were kept separate to those used in other experiments. Reactions were set up in a separate room to the one used for gel electrophoresis of the PCR products. These precautions minimised the possibility of extraneous DNA contamination.

The reaction mix contained 1 X PCR buffer, 200μM dNTPs, 1 ug genomic DNA, 50pMol of each primer and 2 units of Taq Polymerase in a final volume of either 50 or 100ml made up to volume with double distilled water. The reaction mixture was given an initial 5 minute denaturation step and layered with parrafin to prevent evaporation of the mix. The cycles were
usually primer dependent but generally followed the pattern: Denaturation at 94°C 30 seconds, Annealing at 12°C below the Td of primers, and an elongation step at 70-72 °C for 30-60 seconds. 30-35 cycles were used for each primer.

After completion of the amplification reactions the reaction products were analysed by agarose gel electrophoresis in a 1.5-2.0 % mini-gel and visualised under ultra-violet transillumination.

AluIV PCR was carried out as described above with the following changes. The PCR buffer conditions also contained 0.01 % gelatin, 0.1% triton X-100, 0.01% tween 20, and 0.1% nonidet NP40. The cycles used after an initial denaturation step for 5 min were 94°C for 45 seconds, 56°C for 1 min, 72°C for 3 min for 10 cycles followed by two further sets of ten cycles for elongation periods of 4 and 5 min respectively.

**PRIMER SEQUENCES**

**Retinoid X receptor alpha (RXRA)**
GCA TCG ATA AAC AGC AAC GGA  
CTT CCT TAT GGA GAT GCT GGA  
SIZE 407bp  
(Mangelsdorf et al., 1990)

**Retinoid X Receptor beta (RXRB)**
CAG AGT CTT TCT CTC AGG GGC  
GCT TTC TTA CGT AAC ACC CCA  
SIZE 142bp  
(Leid et al., 1992)

**D9S15**
TAA AGA TTG GGA GTC AAG TA  
TTC ACT TGA TGG TGG TAA TC  
SIZE approximately 200bp  
(Wallis et al., 1990)

**Aldolase B (ALDOB)**
(Abbot and Povey, 1991)
TCA TTG CTT GCT TTC TCA AGC AGG G
CAA TGC TTC TCC GTG TTG GAA AGT C
SIZE 348bp

**Orosomucoid (ORM)**
CAG CTG TTG CCA CAC TCA GTG G
GTG TCT GTG ACA CAA TCC TGC C
SIZE 430bp

(Dente et al., 1987)

**Gelsolin (GSN)**
CAG CCA GCT TTG GAG ACA AC
TCG CAA GCA TAT GAC TGT AA
SIZE approximately 143bp

(Kwiatkowski et al., 1991)

**D9S65**
CCT TGC AGA CTG ATG GAG A
GCG GAC AAT TAG GTT TCA GG
SIZE approximately 155bp

(Kwiatkowski et al., 1991)

**Argininosuccinate synthetase (ASS)**
GGG AGC TAT AAA AAT GAC CA
TTA GGT CCG AAA ACA CAA AG
SIZE approximately 145bp

(Yuille et al., 1990)

**Abelson oncogene (ABL)**
GGA GGG TGA AGG GCT TGA AAG GC
AGT CCG CCT GCA CCA AGA CTC CT
SIZE 278BP

(Florian et al., 1991)

**D9S64**
GAA GGG CTC TTT ATT AAC TGA T
AAC CTG GGC GAC ACA GCA A
SIZE approximately 110bp

(Kwiatkowski et al., 1991)

**ABO Blood-group (ABO)**
CAC TCG CCA CTG CCT GGG TCT C
CAT GTG GGT GGC ACC CTG CCA

(Johnson et al., 1992)
The irradiation-fusion process was carried out essentially as described by Goodfellow et al. 1988. 5 x 10^6 cells of the parental donor cell line 64063a12 were harvested by trypsinisation and resuspended in 20 ml of serum containing medium. The cells were given a 45,000 rad irradiation exposure at room temperature using an industrial X-ray unit (HF320 SR, pantak) at a rate of 1000 rads per minute. After irradiation the cells were washed in HANKS and combined with 5 x 10^6 recipient hamster cells, W-g3H. The cells were pelleted by centrifugation at 1500 rpm for 5 min at room temperature. The supernatant was removed and the pellet loosened by tapping the centrifuge tube. 1 ml of prewarmed 50%v/v PEG (37°C) was added whilst gently disturbing the pellet with the tip of a pipette. The cell suspension was incubated at 37°C for 90 sec. 1 ml of serum free medium was added drop by drop over a period of 1 min with gentle stirring using a gilson tip followed by the addition of 5 ml of serum free medium over approximately 2 min with again constant stirring. 10 ml of serum containing medium was then added and the contents of the tube gently mixed as outlined. The cells were pelleted by centrifugation at 1500 rpm for 5 min. The cells were gently
resuspended in growth medium and plated out at a density of 2 x $10^6$ cells in 25 cm$^3$ tissue culture flasks. After 24 hrs incubation at 37°C the growth medium was changed to selective growth medium, medium with the addition of HAT. Colonies were visible after 14 days, picked and developed independently. Cell lines were named 1-22 depending on the flask they were picked from with a different letter suffix a, b or c to refer to independent colonies chosen from the same flask.

2.11 Fluorescence in situ hybridisation

The procedures for fluorescence in situ hybridisation (FISH) were adapted from the original description by Pinkel et al., 1986. Cosmid DNA were prepared from 10ml bacterial cultures, grown overnight at 37°C. Biotin labelled probes of cosmid DNA were hybridised, with competition to remove repetitive sequences, to human chromosome spreads, prepared from normal lymphocyte cultures, at 37°C overnight, followed by signal detection with avidin-fluorescein isothiocyanate (avidin-FITC). For R-bandning, chromosomes were counterstained with propidium iodide and diamidophenyldiiodole (DAPI) and visualised under u.v. light with an FITC filter for simultaneous viewing of signals and chromosomes. Approximately 20 metaphase chromosome spreads were analysed for accurate assessment of signal localisation. To determine the number and arrangement of human fragments in the hybrid lines biotin labelled total human DNA were hybridised to hybrid chromosome spreads followed by signal detection as described above. Approximately 20 metaphase hybrid line spreads were analysed for accurate assessment of the number of fragments retained in each hybrid.
CHAPTER 3

RESULTS 3.1: Identification of hybrids retaining the TSC1 region of chromosome 9q34
RESULTS CHAPTER 3

3.1 Identification of Hybrids Retaining the TSC1 Region of Chromosome 9q

3.1.1 Summary

The irradiation fusion approach of Goss and Harris was used to generate hybrid lines retaining small fragments of human chromosome 9q in an effort to enrich for sequences near the TSC1 gene on 9q34. The source of chromosome 9q material was an HPRT+ Chinese Hamster-Human somatic cell hybrid line, 64063a12, which retains the long-arm of human chromosome 9 as its only human material. 39 independent radiation reduced hybrid lines were analysed for the retention of 24 defined marker loci previously assigned to the chromosome arm. Fragment hybrids were also characterised by FISH, using total human DNA as probe, to determine the number, distribution and stability of the fragments retained in each cell line.

Several hybrids which retained markers near the TSC1 gene, but little or no human material from elsewhere on the chromosome arm were identified. These provided a source of human DNA, enriched for the TSC1 region which could be subsequently used to clone DNA from the interval around the disease locus. The choice of markers and the extent to which individual hybrids were considered for further analysis were strongly influenced throughout by the ongoing genetic studies of TSC.

3.1.2 Generation of a panel of irradiation hybrids retaining fragments derived from chromosome 9q
A series of irradiation-reduced hybrids were derived from 64063a12 as shown in fig 3.1.1. The chromosomal DNA from this HPRT+ donor cell line, was fragmented by exposure to a 45,000 rad dose of X-rays (section 2.10) and the chromosomal fragments generated by the process were rescued by fusion of the irradiated donor cell mixture to the unirradiated HPRT- hamster recipient cell line, Wg3-h. There was no direct selection for the retention of human chromosomal fragments as hybrid cells were selected for the rescue of the hamster HPRT+ gene, present in the irradiated donor cell line, by growing in HAT selection media.

In total, 39 independent colonies were isolated. 36 of these 39 lines were assayed at an early stage of growth for the expression of the human AK1 gene, to confirm that at least some of the irradiation lines had retained human chromosomal material. 5 of the lines were found to be positive for this human enzyme (Table 3.1.1), indicating that fragments of the human chromosome containing the AK1 gene had been rescued and retained by the process.

3.1.3 Characterisation of the chromosome 9q content retained in each hybrid through the segregation of specific markers

The panel of hybrids were tested for the retention of 24 loci which had been previously mapped to chromosome 9q (Table 3.1.2). 14 loci were investigated by southern analysis (section 2.6) for their retention in the irradiation hybrid panel. Fig 3.1.2a gives as example the retention study for the DBH locus in the irradiation hybrid lines 1A to 8B (lanes 5-19). Suitable controls of human (lanes 3 and 4), 64063a12 (lane 2) and hamster (lane 1) DNAs were included on each blot. A constant hamster band, absent in the human lanes, was present in all the hybrid lanes and the hamster control. A larger human specific band of 12 kb was present in the two human controls and in 64063a12 as expected. Two hybrid
Fig 3.1.1 Irradiation fusion approach for the generation of radiation reduced hybrid lines retaining unselected fragments from human chromosome 9q. 64063a12 cells were irradiated and fused to Wg3-h cells and hybrid cells selected for the retention of the HPRT+ locus from the irradiated donor.
Key to Table

<table>
<thead>
<tr>
<th>Key</th>
<th>Description</th>
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<td>Dark</td>
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<td>Lighter</td>
<td>hybrids testing negative for a particular marker</td>
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<td>Dots and lines</td>
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<tr>
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<td>Black and white dots</td>
<td>hybrid not tested by enzyme assay</td>
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table_3_1_1 Marker retention analysis of chromosome 9q markers in the irradiation hybrid panel. The marker loci used are shown along the horizontal axis and hybrid lines along the vertical axis. The various shadings indicate the presence or absence of a marker in a hybrid line. 25 hybrids tested positive for at least one of the markers tested (Table 3.1.2) using Southern blotting, PCR or enzyme assay studies. The remaining 14 hybrids, 3B, 3C, 4B, 8A, 8C, 9A, 9C, 11A, 11B, 12A, 15B, 16B, 16C and 22B failed to retain any of the 24 markers scored.
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<th>LOcus</th>
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<th>REFERENCE</th>
<th>PHYSICAL LOCATION</th>
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<td>D9S48</td>
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<td>Daiger et al., 1984</td>
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Table 3.1.2 The markers used in the retention studies of chromosome 9q34, their chromosomal position and references for primer sequences or probes used in their study. (* Primer sequences are presented in section 2.2.9) The loci include the genes for arginosuccinate synthetase pseudogene 12 (Assp12), arginosuccinate synthetase pseudogene 3 (Assp3), aldolase B fructose bisphosphatase (ALDOB), Orosomucoid (ORM) aminolevulinate delta dehydratase (ALAD), tenacin (HBX), gelsolin (GSN), adenylate kinase 1 (AK1), arginosuccinate synthetase (ASS), Abelson oncogene (ABL), ABO blood-group (ABO) and Dopamine beta-hydroxylase (DBH)
Fig 3.1.2a  Southern analysis of the DBH locus for the irradiation hybrid lines, 1A-8B

An EcoRI digest of 10μg of hybrid DNAs 1A-8B with suitable controls of human, hamster and parent hybrid DNA were run in a 0.8% agarose gel prior to blotting and probing with a DBH cDNA fragment. A single human band of 12Kb was found in the two human controls (lane 3 and 4), the 64063a12 hybrid line (lane 2) and the two irradiation hybrid lines 5A (lane 12) and 6C (lane 9). A constant hamster band was present in the hamster control (lane 1) and the hybrid lines.
lines, 5A (lane 12) and 6C (lane 9), also retained this human specific band. The stronger human signal in hybrid 5A probably reflects a greater stability of the human fragment containing the DBH locus in this hybrid.

This suggests that the human fragments contained in these irradiation hybrids are not always stably retained. The 5 hybrids, 2A, 5A, 9B, 12C and 14A however, which tested positive for the AK1 gene by enzyme assay of the hybrid lines were also positive by southern analyses. This implies that the human fragments containing this locus in each of these lines are stable as the initial analysis of the hybrid lines for AK1 retention was carried out at a very early stage of growth.

The pattern of retention for the remaining 10 loci studied were determined by human locus specific PCR amplification (section 2.9). Fig 3.1.2b gives as example the PCR amplification of the marker locus D9S66 for the complete irradiation hybrid panel. A human specific product of approximately 100bp was observed for the irradiation hybrid lines 5A (lane 8), 6C (lane 11), 12C (lane 23), 17B (lane 31), 20A (lane 35) and 20B (lane 36). All PCR studies included human and hamster controls.

These marker retention studies were biased towards the chromosome 9q34 region, distal to the AK1 locus, to selectively identify those hybrids retaining fragments from the region surrounding the TSC1 gene. 25 out of the 39 hybrids tested positive for at least one marker (Table 3.1.1). 16 out of these 25 hybrids retained loci from the 9q34 region.

3.1.4 Characterisation of the human material retained in the hybrid panel by FISH

The human chromosome 9q content of the hybrids was also investigated by FISH. Cell lines were resuscitated from liquid nitrogen and expanded for the preparation of metaphase
Fig 3.1.2b  PCR analysis of the D9S66 locus

Example of locus specific amplification using the polymerase chain reaction in order to characterise the chromosome 9q regions retained in each individual hybrid line. The primers used in the reaction generate a human specific 100bp PCR product for the D9S66 marker locus. Human specific amplification was observed for hybrids 5A, 6C, 12C, 17B, 20A and 20B (lanes 8, 11, 23, 31, 35 and 36). Positive controls of the donor hybrid line 64063a12 (lane 40), DORA hybrid (lane 41) and human DNA (lane 42) tested positive while the recipient hamster hybrid line, Wg3-h (lane 39), tested negative. All other hybrids failed to amplify the 100bp product.
chromosome spreads (section 2.2.1). Biotinylated total human DNA was used as probe to metaphase chromosome spreads from each cell line to estimate the approximate number of discrete human fragments retained, their stability and arrangement in each hybrid line.

The donor cell line, 64063a12, was used as a positive control in these studies. FISH using total human DNA revealed a single fluorescent chromosome consistent with the sole retention of a single copy of chromosome 9q (Fig 3.1.3). The radiation reduced lines retained several small chromosomes not present in either the irradiated donor or recipient hamster cell lines consistent with the fragmentation of the chromosomal material by the irradiation treatment. Human chromosomal fragments were retained as translocations (Fig 3.1.4) or insertions (Fig 3.1.5) onto hamster chromosomes or as fragments apparently consisting entirely of human chromosomal material (Fig 3.1.6).

Of the 37 hybrids studied by FISH (Table 3.1.3), 26 hybrids gave visible signals when probed with biotin labelled total human DNA. 14 of these retained between 1-3 human fragments per cell, while the remainder retained multiple human chromosomal segments. In general, hybrids retaining multiple human fragments were not studied in further detail.

FISH studies identified 4 hybrids which retained human chromosomal material but were negative for the 24 marker loci tested on the hybrid panel. Three of these hybrids 3C, 8B, and 15B all retained a single human fragment when visualised by FISH (Fig 3.1.7). In contrast hybrid 9A retained multiple human fragments.

The stability of the human fragments in the 14 hybrid lines retaining a small number of human segments was investigated by looking at the retention of human material in 20 metaphase spreads from each cell line (Table 3.1.3). The stability of the human component varied between hybrids. Hybrids 13B and 21A for instance retain their human fragment in approximately 30% of cells, in contrast to hybrids 1A and 17B where the human
NOTE: fig 3.1.3- 3.1.13  Cytogenetic analysis of the donor hybrid 64063a12 and the radiation reduced hybrid lines by fluorescent in situ hybridisation (FISH).

Biotinylated human DNA was hybridised to metaphase chromosome spreads from each hybrid line. Human DNA was detected using fluorescein conjugated avidin (green) and the chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanning laser microscopy. The stability of the human DNA was determined by looking at the presence or absence of individual human fragments in 20 metaphases from each hybrid line.

Fig 3.1.3  Cytogenetic analysis of hybrid 64063a12 by FISH.

The fluorescently labelled chromosome corresponds to the intact human chromosome 9q present in the hybrid. This chromosome is retained in 100% of the metaphases analysed.
Fig 3.1.4  Cytogenetic analysis of hybrid 6A by FISH.

The hybrid retains a single human fragment integrated into the telomere of a small hamster chromosome. This fragment is retained in 90% of the metaphases analysed.

Fig 3.1.5  Cytogenetic analysis of hybrid 12C by FISH.

The hybrid retains 2 small human fragments integrated close together into a metacentric hamster chromosome. Both fragments are retained in 100% of the metaphases analysed.
Fig 3.1.6  Cytogenetic analysis of hybrid 17A by FISH.

The hybrid retains a single human fragment consisting entirely of human DNA. This fragment is retained in 80% of the metaphases analysed.
<table>
<thead>
<tr>
<th>Hybrid Name</th>
<th>Number of Human Fragments</th>
<th>Stability of Human Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>2A</td>
<td>1</td>
<td>80%</td>
</tr>
<tr>
<td>3C</td>
<td>1</td>
<td>60%</td>
</tr>
<tr>
<td>6A</td>
<td>1</td>
<td>90%</td>
</tr>
<tr>
<td>8B</td>
<td>1</td>
<td>50%</td>
</tr>
<tr>
<td>12C</td>
<td>2: a, b</td>
<td>a-100%, b-100%</td>
</tr>
<tr>
<td>13B</td>
<td>1</td>
<td>30%</td>
</tr>
<tr>
<td>14A</td>
<td>3: a, b, c.</td>
<td>a-90%, b-20%, c-nt</td>
</tr>
<tr>
<td>15A</td>
<td>1</td>
<td>80%</td>
</tr>
<tr>
<td>17A</td>
<td>1</td>
<td>80%</td>
</tr>
<tr>
<td>17B</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td>20A</td>
<td>1</td>
<td>60%</td>
</tr>
<tr>
<td>20B</td>
<td>1</td>
<td>60%</td>
</tr>
<tr>
<td>21A</td>
<td>1</td>
<td>25%</td>
</tr>
</tbody>
</table>

Table 3.1.3 Stability and number of the human fragments retained in the hybrids retaining three or fewer fragments by FISH.

Hybrids retaining greater than 3 human fragments by FISH, 2B, 3A, 5A, 6B, 6C, 7A, 7B, 9A, 9B, 18A, 18B and 19B were not included. Of the remaining thirteen hybrids, eleven, hybrids 3B, 4B, 8A, 8C, 9C, 11A, 11B, 12A, 13A, 16A, 16B failed to retain any human material by FISH and two, hybrids 13C and 22B were not tested. (nt—not determined; a,b and c refer to different fragments present in the same hybrid line)
Fig 3.1.7 (1) (2) (3) Cytogenetic analyses of hybrids 3C, 8B and 15B by FISH.

Hybrid 3C retains a single human fragment integrated into a hamster chromosome. This fragment is retained in 60% of the metaphases analysed. Hybrid 8B retains a single human fragment consisting entirely of human DNA. This fragment is retained in 50% of the metaphases analysed. Hybrid 15B retains a single human fragment integrated into the telomere of an acrocentric hamster chromosome. This fragment is retained in 80% of the metaphases analysed.
fragments are completely stable. There is also variability in the stability of human fragments within the same cell line. Hybrid 14A retains one fragment which is almost completely stable while the remaining 2 fragments are retained in a much reduced number of the cells studied (Table 3.1.3).

3.1.5 Correlation of the results from the 2 characterisation strategies

There was good agreement between the chromosome 9q content of the hybrids as assessed by Southern blot/PCR analyses and by the FISH studies using total human DNA as probe. For instance:

Hybrid 1A retains a single human fragment attached to a small hamster chromosome (Fig 3.1.8). It was positive for a single chromosomal marker, D9S48.

Hybrid 14A retains 3 separate regions of the long-arm of the chromosome as defined by the 3 non-contiguous markers ASSp12, AK1 and D9S7. It also retained 3 discrete human chromosomal fragments when tested by FISH (Fig 3.1.9). A subclone of 14A, 14A4 (section 2.2.1) retaining a single human fragment has also been generated.

Hybrids 20A and 20B both retain identical human fragments by FISH (Fig 3.1.10), consisting almost entirely of human DNA. They also exhibit the same pattern of marker retention from chromosome 9q.

Hybrid 21A retains only the D9S7 locus out of the 24 markers tested. It retains a small single human fragment when studied by FISH (Fig 3.1.11).

There are at least two exception. Hybrid 13A which retains 2 discrete regions of chromosome 9q from marker retention studies failed to give any evidence by FISH to suggest that it
Fig 3.1.8  Cytogenetic analysis of hybrid 1A by FISH.

Hybrid 1A retains a single human fragment consisting entirely of human DNA. This fragment is retained in 100% of the metaphases analysed.

Fig 3.1.9  Cytogenetic analysis of hybrid 14A (a) and a 14A subclone 14A4 (b).

Hybrid 14A retains 3 separate human fragments. Fragment a is integrated into the telomere of an acrocentric hamster chromosome and is retained in 90% of the metaphases analysed. Fragment b is also integrated into an acrocentric hamster chromosome. It is retained in 20% of the metaphases analysed. Fragment c is integrated into a small hamster chromosome. This fragment's stability has not been determined. A subclone of 14A, 14A4 retains fragment a and neither of the other fragments.
Fig 3.1.10 Cytogenetic analysis of hybrid 20A by FISH.

Hybrid 20A retains a single human fragment attached to a small hamster fragment. This fragment is retained in 60% of the metaphases analysed.
Fig 3.1.11 Cytogenetic analysis of hybrid 21A by FISH.

Hybrid 21A retains a single human fragment integrated into the telomere of a small hamster chromosome. This fragment is retained in 25% of the metaphases analysed.

Fig 3.1.12 Cytogenetic analysis of hybrid 17B by FISH.

Hybrid 17B retains a single human fragment integrated into a small hamster chromosome. This fragment is retained in 100% of the metaphases analysed.
retained human material. Similarly FISH of hybrid 8C which tested positive for the ALAD gene failed to identify any human fragment in any of the metaphases analysed. It is possible that the human DNA present in these hybrid lines has been lost following their resuscitation from liquid nitrogen and re-expansion of the cell line for FISH analysis. It could also reflect the small size of the human fragments present in the cell line or its retention in only a small percentage of cells as this would reduce the likelihood of detection. The identification of the 4 hybrids, 3C, 8B, 9A and 15B retaining human material by FISH but not retaining any of the tested markers could be attributed to the paucity of markers tested from the proximal regions of the chromosome 9q arm.

3.1.6 Selection of Hybrids enriched for the region near the TSC1 locus

At the time of these studies the most conservative estimate of the genetic position of the TSC1 gene was between the loci ASS and D9S14. Cell lines which retained markers from this interval included: 6C, 12C, 17A, 17B, 19B and 20A.

Hybrid line 6C retains multiple human fragments by FISH. The stability of and pattern of fragment retention in the hybrid were not determined by FISH. The hybrid tested positive for a contiguous set of markers, distal to D9S64 and proximal to D9S67.

Hybrid line 12C retains only 2 human fragments by FISH (Fig 3.1.5) both inserted into the same hamster chromosome. It retains many markers present in the 9q33-34 region, distal to ORM, although their retention is not consistent with the rescue of 2 contiguous fragments. It seems likely that a number of deletions have occurred during the irradiation-fusion process. Both human fragments are stable, present in 100% of the metaphases analysed.
Hybrid 17A retains 2 discrete regions of chromosome 9q, the centromeric region as defined by the marker D9S48 and the region around the ASS-ABL loci on 9q34. The hybrid contains a single human chromosomal fragment as defined by FISH (Fig 3.1.6), consisting entirely of human DNA and maintained in 80% of metaphases studied. It seems likely that this fragment has arisen through the healing of 2 independent segments of the chromosome arm after irradiation and has been stabilised by the presence of a human centromere.

Hybrid line 17B retains a contiguous set of markers from DBH to D9S14 from the 9q34 region. It retains a single human chromosomal fragment (Fig 3.1.12) as determined by FISH. The human fragment is present as a translocation onto a small hamster chromosome and is completely stable.

Hybrid line 19B retains 4 discrete regions of chromosome 9q, including 3 separate segments of the 9q34 region. Multiple human fragments were observed (Fig 3.1.13) which were very unstable. These were present in less than 40% of the cells analysed.

Hybrid line 20A maintains at least 2 discrete regions of band 9q34, one segment containing 4 tightly linked markers, ABO-DBH-D9S10-D9S66 and a more distal region around the D9S17 locus. Surprisingly FISH results suggests that this hybrid contains a single chromosomal fragment attached to a small hamster fragment (Fig 3.1.10). This is retained in 60% of the metaphases analysed. Further studies indicated that it also contains some centromeric sequences (section 3.3). Consequently, it seemed probable that the single human fragment observed by FISH is composed of 3 human fragments from separate regions of the chromosome arm including a considerable amount of DNA from the TSC1 interval.
Fig 3.1.13a and b Cytogenetic analysis of hybrid 19B by FISH.

Hybrid 19B retains multiple human fragments whose retention varies between metaphases. (a) A human fragment is integrated into a small hamster chromosome and is retained in 40% of the metaphases analysed. (b) The other fragments show more reduced retention frequencies. Not all fragments present in hybrid 19B are represented in these two metaphase spreads.
3.1.7 Deletion Map

Using the 6 hybrids, 6C, 12C, 17A, 17B, 19B and 20A, a deletion map of the region has been constructed (Fig 3.1.14). The pattern of marker retention observed for the hybrids in conjunction with FISH studies has allowed the order of markers to be inferred from the TSC1 gene region.

The close proximity of the ASS and ABL loci are suggested by their co-retention in hybrid 17A which retains a single human fragment by FISH (fig 3.1.6). Hybrid 17B retains the DBH, D9S10, D9S66, D9S14 and D9S67 loci from chromosome 9q34. The apparent absence of other human material from the distal region of the chromosome suggests that these markers form a contiguous set. Given the genetic information placing D9S66 proximal to D9S67 (Kwiatkowski et al., 1992), the retention of ABO, DBH, D9S10 and D9S66 in both hybrid 6C and 20A supports a proximal location for the ABO blood group locus with respect to these loci. This subdivides hybrid 17B into two discrete regions, containing the DBH-D9S10-D9S66 set of markers and D9S14 and D9S67. Hybrid 17A does not show any overlap with hybrids 6C and 20A as all 3 are negative for the marker D9S64 which has been genetically positioned between D9S66 and ASS-ABL (Kwiatkowski et al., 1992).

The interval into which the TSC1 gene must map is defined by recombination events in affected individuals for the marker loci D9S64 and D9S67 (section 1.7). A combination of the human DNA retained in the hybrid lines 17B and 20A covers the ABO-D9S67 part of this region. However the most proximal part of the interval containing the D9S64 locus, with the possible exception of hybrid 19B, is not contained within these hybrid lines.
Fig 3.1.14 Deletion map of chromosome 9q34

The pattern of marker retention in the six irradiation hybrid lines, 6C, 12C, 17A, 17B, 19B and 20A were used to construct a deletion map of the 9q34 region containing the TSC1 interval D9S64-D9S67. The ABO-D9S67 region is covered by the 2 hybrid lines 17B and 20A (shown in bold lines). The more proximal part of the interval, D9S64-ABO, with the possible exception of the D9S64 fragment present in hybrid 19B is not contained in any of the irradiation hybrids.
CHAPTER 3

RESULTS 3.2: Experiments involving Alu-PCR: To characterise the chromosome 9q content of the irradiation hybrid panel and to generate region specific cloned DNA material from the TSC1 gene interval.
3.2 Experiments involving Alu-PCR: To characterise the chromosome 9q content of the irradiation hybrid panel and to generate region specific cloned DNA material from the TSC1 gene interval.

3.2.1 Introduction

The use of Alu primed PCR has provided a new method for the rapid isolation of human specific probes from complex sources of DNA material (Nelson et al., 1989; Cotter et al., 1990; Brooks-Wilson et al., 1990; Ledbetter et al., 1990). The method amplifies the sequences between Alu repeats giving a PCR fingerprint pattern representative of the human DNA content present in a particular DNA mixture.

Alu products have been used to identify chromosome and region specific YAC and cosmid clones by direct hybridisation of Alu PCR products derived from somatic cell hybrids to appropriate reference libraries (Monaco et al., 1991; Chumakov et al., 1992). FISH using Alu-PCR products as probes has also been used to localise the human DNA content present in a hybrid line to discrete regions of the genome (Pinkel et al., 1988; Lichter et al., 1990; Sinke et al., 1992).

In an effort to characterise the radiation reduced panel in more detail and to isolate new cloned resources near the TSC1 region, these various applications of the Alu-PCR technique have been utilised.

3.2.2 ALU-PCR fingerprinting of the irradiation hybrid panel

Alu-PCR using a human specific Alu primer, Alu-IV (Cotter et al., 1990), was used to amplify human DNA specifically from the irradiation reduced chromosome 9q hybrid lines (section 2.9). Agarose gel electrophoresis of the Alu IV PCR products gave rise
to a fingerprint of fragments representative of the human content in each hybrid line, in the size range 0.3-3Kb (fig 3.2.1). 26 hybrids gave visible Alu IV PCR products. There was no observed amplification of DNA from the recipient hamster genome, Wg3-h (fig 3.2.1, lane 42). The donor hybrid, 64063a12 generates a greater number of Alu-PCR products (fig 3.2.1, lane 41) than any of the radiation reduced hybrid lines, consistent with its retention of the largest amount of human DNA.

Hybrids 3B, 4B, 8A, 9C, 11A, 11B, 12A, 16B, 16C and 22B which failed to retain human material by marker retention or FISH analyses did not generate any visible Alu-PCR products. Three of the four hybrids, 8B, 9A and 15B (fig 3.2.1, lanes 15, 17 and 28) which tested negative for marker retention studies but retained some human DNA by FISH gave discrete patterns of Alu-PCR products. The fourth hybrid 3C (fig 3.2.1, lane 6) failed to give any amplification products. This may reflect the small size of the chromosome 9q fragment retained in this hybrid.

Hybrid 8C also failed to give any visible Alu-PCR products (fig 3.2.1, lane 16). Although it tested positive for the ALAD locus, FISH analysis of this hybrid also failed to identify any human material. It is possible that a small human fragment may have gone undetected in this hybrid as this would also explain the absence of Alu products from the hybrid. Hybrid 9B, positive for human DNA by marker retention and FISH studies, also failed to give any Alu amplification pattern (fig 3.2.1, lane 18). This was not unexpected as the hybrid DNA had previously failed to PCR any of the non-specific hamster bands which appeared in some of the locus specific PCR reactions, suggesting that the DNA generated from this hybrid could not be amplified.

Hybrid 13A gives a number of Alu products after amplification (fig 3.2.1, lane 24) although no human fragments were detected by FISH. This could be explained however by the loss of the human component from this hybrid following resuscitation of the cell line for FISH analysis. The presence of identical ALU-IV patterns for hybrids 20A and 20B (fig 3.2.1, lanes
Fig 3.2.1 Gel electrophoresis of Alu-IV PCR fingerprint of the irradiation hybrid panel, hybrids 1A-22B, with suitable control DNAs.

Alu-IV PCR products from the irradiation hybrid panel (lanes 1-40), the donor hybrid cell line 64063a12 (640) (lane 41), the recipient hamster Wg3-h (Ham) (lane 42) and human DNA (Hum) (lane 42) were electrophoresed on a 2% agarose gel. Visible amplification was observed in 26 of the 39 radiation reduced hybrid lines. Hybrids failing to generate a visible Alu amplification are shown plain text.
37 and 38) is consistent with the marker retention studies and FISH analysis which suggest that they retain the same human chromosome 9q component.

Hybrids with overlapping regions of chromosome 9q on the basis of marker retention analyses are expected to have sets of \textit{Alu} PCR products in common. Fig 3.2.2 shows the \textit{Alu} IV fingerprint of the hybrids 17A, 14A4, 14A, 19B, 17B and 20A. Hybrid 14A4 is a subclone product of hybrid 14A retaining 1 of the 3 human fragments present in the parent irradiation hybrid 14A (fig 3.1.9). The \textit{Alu}-IV fingerprint pattern of both these hybrids (fig 3.2.2, lanes 3 and 4) are similar, consistent with their retention of an identical human fragment. Hybrids having much smaller regions of overlap can be compared in the same manner. Hybrid 17B (fig 3.2.2, lane 6) shares a number of visible co-migrating \textit{Alu}-PCR products in common with hybrids 19B (fig 3.2.2, lane 5) and 20A (fig 3.2.2, lane 7) consistent with their co-retention of the D9S67 and DBH regions on chromosome 9q34 respectively. Hybrid 17A (fig 3.2.2, lane 2) has no obvious \textit{Alu}-PCR products in common with any other hybrid lines.

A comparison of the marker retention analyses (section 3.1) and physical map of the 9q34 region (section 1.8) suggests that the amount of human DNA retained in the hybrids 17A, 17B and 20A are less-than than 2Mb of DNA. As the \textit{Alu}-IV fingerprint for these hybrids generate a pattern of a least ten discrete products (fig 3.2.2) it is likely that this primer will generate a product approximately every 100-200Kb from the TSC1 containing region.

3.2.3 Reverse painting of \textit{Alu}-IV products derived from irradiation hybrids

The human content of 2 radiation reduced hybrids, 17B and 12C, were precisely localised by \textit{in-situ} hybridisation of the \textit{ALU}-IV PCR products derived from each hybrid to human metaphase
The hybrids generate a discrete set of Alu products ranging in size from 0.3-3 kb. Hybrid 14A4 is a subclone of hybrid 14A. It retains only the most stable of the three human fragments retained in the parental irradiation hybrid line. The Alu PCR fingerprints of these two hybrids have a large number of visible amplification products in common (lane 3 and 4) as expected from their co-retention of an identical fragment generated from chromosome 9q. Hybrids 17B and 19B retain 2 co-migrating visible products in common of between 1.6 and 2.0 kb. Hybrids 17B and 20A retain 3 visible products in common of approximately 1.3, 1.0 and 0.6 kb. Visible Alu products in common between hybrids are arrowed.
chromosome spreads (fig 3.2.3). Both hybrids gave signals solely on the distal long-arm of chromosome 9. This is consistent with the marker retention studies of both hybrids (Table 3.1.1). The larger signal obtained using 12C \textit{Alu}-PCR products reflects the retention of a larger segment of the distal region of chromosome 9q in this hybrid in comparison to hybrid 17B.

3.2.4 Isolation of human cosmid clones from the TSC1 interval using \textit{Alu}-PCR products from the hybrid 17B

Radiolabelled \textit{Alu}-PCR products generated from hybrid 17B were hybridised to lysed colony filters of a human cosmid DNA library (sections 2.7.4, 2.8) in duplicate (fig 3.2.4) to identify cosmid clones contained within the limits of the human DNA present in the hybrid line 17B. Several positive hybridising signals were identified and subsequently purified (fig 3.2.5).

In total 4 cosmids, c17B2, c17B6, c17B7 and c17B12 were isolated and mapped to chromosome 9q34 by FISH of total cosmid (section 2.2.2.2) DNA to human metaphase chromosome spreads. Fig 3.2.6 and fig 3.2.7 shows the FISH analysis of cosmids c17B6 and c17B12. The three cosmids c17B6, c17B7, and c17B12 were sublocalised to discrete regions of the chromosome 9q region contained in the 17B hybrid by probing with the \textit{Alu} products derived from these cosmids onto the \textit{Alu}-PCR fingerprint of the entire hybrid panel (fig 3.2.1).

The \textit{Alu} products derived from the cosmids c17B6 and c17B7 tested positive for the hybrids 6C, 12C, 17B, and 19B and were weakly positive for hybrid 14A and the parent line 64063a12 (fig 3.2.8). This suggests from the pattern of marker retention in these hybrids that these 2 cosmids map to the region proximal to D9S67 and D9S14 and distal to D9S66 (see fig 3.1.14). The \textit{Alu} product derived from cosmid c17B12 tested positive for hybrids 6C, 17B, 20A and 20B and was weakly positive for hybrids 12C, 13A, 13B
Fig 3.2.3 Localisation of human fragments present in irradiation hybrids 12C and 17B on human chromosome 9 using Alu element-mediated PCR products as probe for chromosome in situ painting.

Biotinylated Alu-PCR products were hybridised under conditions that suppress signals from repetitive DNA sequences to human metaphase chromosome spreads. Hybridisation signals were detected using fluorescein conjugated avidin (green). Chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanner laser microscopy. Arrows indicate the fluorescent signals on chromosome 9 (A) Hybridisation of 12C Alu-products to the distal long-arm of chromosome 9, 9q33-34; (B) Hybridisation of 17B Alu-products to the distal long-arm of chromosome 9, 9q34.
Fig 3.2.4 Primary screening of a human cosmid library using Alu-IV PCR products derived from the irradiation hybrid line 17B.

Lysed colony filters were probed in duplicate, replica 1 and 2 with radiolabelled Alu-IV PCR products. Colony areas giving duplicate positive signals (arrowed) were picked and secondary screens carried out (fig 3.2.5).
Fig 3.2.5 Secondary screening of the primary colonies isolated after probing with Alu-IV PCR products from hybrid 17B onto a human cosmid library.

Between 50 and 200 colonies were rescreened in duplicate with the Alu-IV PCR products. The number of positives identified in secondary screenings varied considerably eg secondary 1 and 2. These were independent of the actual number of rescreened colonies and most likely reflected the size of the region picked to isolate the primary signals. A number of positively hybridising colonies were picked, DNA isolated and their 9q34 localisation confirmed by FISH.
3.2.6 Fluorescent in situ hybridisation of biotinylated human cosmid clone c17B6 onto human metaphase chromosome spreads

The whole cosmid was used as probe under conditions that suppress signals from repetitive DNA sequences. Hybridisation signals were detected using fluorescein conjugated avidin (green). Chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanning laser microscopy. Arrows indicate the signals on chromosome 9.

3.2.7 Fluorescent in situ hybridisation of biotinylated human cosmid clone c17B12 onto human metaphase chromosome spreads

The whole cosmid was used as probe under conditions that suppress signals from repetitive DNA sequences. Hybridisation signals were detected using fluorescein conjugated avidin (green). Chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanning laser microscopy. Arrows indicate the signals on chromosome 9.
Fig 3.2.8 Southern analysis of an Alu IV product derived from c17B7 onto an Alu fingerprint of the entire irradiation hybrid panel (fig 3.2.1).

Hybrids 6C, 12C, 14A, 17B, 19B and 64063a12 gave positive signals. All remaining hybrids gave no visible hybridising signal.
Fig 3.2.9 Southern analysis of an Alu IV product derived from c17B12 was used as probe for southern analysis onto an Alu IV fingerprint of the entire hybrid panel (fig 3.2.1).

and 64063a12 (fig 3.2.9). This places c17B12 distal to the ABO locus and proximal to the D9S14-D9S67 loci. The identification of overlapping regions between c17B12 and a cosmid clone retaining the D9S66 locus, placing c17B12 adjacent to the D9S66 (Hornigold, personal communication) is consistent with the mapping information derived from southern analysis of the hybrid lines (fig 3.2.9, fig 3.1.14).

In general the intensity of the hybridisation signal seems to be inversely proportional to the number of Alu products generated by a given irradiation hybrid line, for example the intensity of the hybridisation signal in hybrid 17B is greater than that observed for hybrid 12C although the human fragments retained in both hybrid lines are completely stable (fig 3.2.8; fig 3.2.9). This is consistent with the retention of a smaller amount of human DNA in the former. The signal strength will also decline with fragment instability as this reduces the amount of starting template available for amplification. The weaker hybridisation signal observed for some hybrids, for example hybrid 13A and 13B in fig 3.2.9 in contrast to the parent hybrid 64063a12 which retains a larger proportion of human material supports this suggestion (Table 3.1.3). No hybridisation signal was ever observed in the human lane which reflects the large number of Alu products generated from the human genome as a whole and the obvious reduction in the amount of product generated for any particular Alu amplification.

3.2.5 Cloning of Alu-IV products from the irradiation hybrid lines

There is preferential amplification of some Alu products over others as observed by the differential ethidium bromide staining intensities of products under transillumination (fig 3.2.2). This suggests that the use of pooled Alu amplification products from any hybrid will invariably consist of a large proportion of the
better amplified sequences. The presence of an EcoRI site in the Alu IV primer allows the efficient cloning of Alu-PCR products generated from any particular hybrid (section 2.9). Using this approach Alu IV PCR products from a pool of Alu IV products derived from the hybrid lines 17A, 17B, 19B and 20A have been cloned into the vector bluescript (fig 3.2.2, fig 3.2.10). Vector DNA was digested with EcoRI (section 2.4.1), dephosphorylated (section 2.4.2) and ligated to EcoRI digested Alu IV products. Recombinant clones were identified after transformation of JM101s (section 2.2.3).

Preliminary examination of a small number of these clones have suggested that certain Alu products are preferentially cloned, for example clones 1, 2 and 5 and 6, 9, 12 and 15 are identical in size (3.2.10) suggesting that they are the same clone. Other clones, for example clones 1 and 10 generate a number of individual EcoR1 products after digestion. This most probably reflect co-ligation events during the ligation procedure.

The pattern of retention of a number of these products has been determined by southern analysis of these Alu products to the Alu IV fingerprint of the entire hybrid panel (fig 3.2.1). The segregation of these Alu IV products in the hybrid panel was consistent with their localisation to the chromosome 9q34 region. One exception, clone 8, was found to hybridise to the Alu fingerprint of hybrid 17A and more weakly to hybrids 19B, 20A and 20B (fig 3.2.11). This was not consistent with marker retention analyses which failed to detect overlap between the human material retained in hybrids 17A and 20A (fig 3.1.14). The finding that hybrid 20A has retained some human material from the centromeric region (section 3.3) and the retention of the D9S48 marker in hybrids 17A and 19B suggests that this Alu product is probably derived from the centromeric region. This suggests that hybrid 20A retains more human material than determined by marker retention analysis.
Fig 3.2.10  EcoRI digestion of cloned Alu IV products generated from the hybrids retaining chromosome 9q34 material

1 μg of plasmid DNA from 15 cloned Alu PCR products in the vector bluescript and a vector control were digested with EcoRI and electrophoresed on a 0.8% agarose gel (lanes 1-16). Several Alu products were preferentially cloned while other products may reflect co-ligation events during the cloning process, see text for more details.
Fig 3.2.11 Southern analysis of a cloned *Alu* IV product, clone 8, onto an *Alu* IV fingerprint of the entire hybrid panel (fig 3.2.1).

Hybrids 5A, 6C, 12C, 13A, 17A, 19B, 20A and 64063a12 gave positive hybridising signals. The hybridisation signals show great variation in their intensities. All other hybrids failed to give any visible hybridisation signal.
CHAPTER 3

RESULTS 3.3: The generation of radiation hybrid libraries to isolate cloned material from the TSC1 region.
3.3 The generation of radiation hybrid libraries to isolate cloned material from the TSC1 region

3.3.1 Introduction

Radiation reduced hybrid lines have previously been used to construct genomic libraries to saturate the regions around the Huntington's Chorea (Pritchard et al., 1989; Doucette-Stamm et al., 1991) and Myotonic Dystrophy loci (Brook et al., 1992) with new cloned material. In the same way the identification of 2 hybrid lines, hybrids 17B and 20A, which together retain a large proportion of the DNA from the TSC1 interval (Table 3.1.1, fig 3.1.14) have provided a suitable resource to focus cloning efforts to this region.

3.3.2 Generation of cosmid libraries from the radiation hybrid lines 17B and 20A

The irradiation hybrid lines 17B and 20A were regrown from frozen cell aliquots (section 2.2.1) and DNA was extracted (section 2.2.2.1). The retention of marker loci from the TSC1 region and the stability of these human fragment were confirmed by Southern blotting analysis (section 2.6) of the DNA and FISH studies of metaphase spreads from each of the regrown lines. The Alu-IV PCR fingerprints for both hybrid DNAs were identical to the PCR patterns generated from their primary DNA aliquots.

The DNA from each hybrid was partially digested using the enzyme Mbo1 and digestion products in the size range 35-45Kb were purified by NaCl density gradient centrifugation (section 2.7.1). These fragments were ligated into the vector Lorist B and packaged into bacteriophage lamda head particles prior to infection of the bacterial strain ED8767 (section 2.7.2; 2.7.3). The libraries generated for each hybrid were small. Approximately
20,000 clones were screened from each library for the retention of human DNA inserts by probing with total human DNA. Positively hybridising clones were picked and purified. 8 human cosmids, 4 from each hybrid line were identified. The chromosome 9q origin of the positively hybridising clones 17BL1, 17BL2, 17BL3 and 17BL4 and 20AL1, 20AL3, 20AL5 and 20AL7 was confirmed by FISH of total cosmid DNA (section 2.2.2.2) to human metaphase chromosome spreads.

6 out of these 8 cosmids mapped to 9q34 by FISH. Fig 3.3.1 shows the FISH studies of cosmids c17BL1 and c20AL1 to human metaphase chromosome spreads. The remaining 2 cosmids 20AL5 and 20AL7 localised close to the centromere (fig 3.3.2). The varying intensities of the signals observed for these cosmids by FISH for the different chromosome 9 homologues suggest that these cosmids retain a polymorphic repeat from the heterochromatic region of the chromosome. Although 50% of the cosmids isolated from the 20A library were derived from this region the small number of actual clones isolated in total make it difficult to estimate the relative proportion of human DNA in the 20A line from outside the TSC1 region.

These result suggest that the hybrid 20A in addition to retaining markers from the 9q34 region also retains sequences derived from the centromere of chromosome 9. This is consistent with identification of a common Alu product with the hybrid 17A, which is known to retain a chromosome 9 centromeric component but which fails to retain any of the defined marker loci present in hybrid 20A (section 3.2, fig 3.2.11).
Fig 3.3.1 Fluorescent in situ hybridisation of biotinylated human cosmid clones, c17BL1 and 20AL1, to human metaphase chromosomes

The whole cosmids were used as probes under conditions that suppress signals from repetitive DNA sequences. Hybridisation signals were detected using fluorescein conjugated avidin (green). Chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanning laser microscopy. Arrows indicate the fluorescent signals on chromosome 9. (A) Hybridisation of cosmid 17B1 to the 9q34 region of chromosome 9. (B) Hybridisation of cosmid 20A1 to the 9q34 region of chromosome 9.
Fig 3.3.2 Fluorescent in situ hybridisation of biotinylated cosmid clone c20A5L to human metaphase chromosomes

The whole cosmids were used as probes under conditions that suppress signals from repetitive DNA sequences. Hybridisation signals were detected using fluorescein conjugated avidin (green). Chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanner laser microscopy. Arrows indicate the fluorescent signals on chromosome 9. Cosmid 20A5L hybridises to the centromeric region of chromosome 9.
CHAPTER 3

RESULTS 3.4: The localisation of the gene for RXRA to the TSC1 interval and the assignment and localisation of the RXRB gene to chromosome 6p21.3.
3.4 The localisation of the gene for RXRA to the TSC1 interval and the assignment and localisation of the RXRB gene to chromosome 6p21.3.

3.4.1 Introduction

The gene for the Retinoid X Receptor, RXR alpha (RXRA), was recently assigned to the long-arm of chromosome 9 (Karen Jones, personal communication). This gene is a member of the retinoid X receptor (RXR) family of nuclear receptors (Mangelsdorf et al., 1992), which also includes the genes for retinoid X receptor beta (RXRB) and retinoid X receptor gamma (RXRG). These RXRs together with the retinoic acid receptor family (RAR), mediate the effect of retinoic acid (RA) (Mangelsdorf et al., 1990), a derivative of vitamin A, which is important for growth, development and differentiation (Sporn et al., 1984). Previously it had been suggested that TSC may be a disorder of cell differentiation and proliferation (Gomez, 1991).

These nuclear receptor proteins, upon ligand activation, control the expression of their target genes by binding to specific DNA sequences, called hormone response elements. The receptor proteins consist of a highly conserved 68 amino acid domain that confers sequence specific DNA-binding capabilities to each receptor and a less well conserved C-terminal region of approximately 220 amino acid that functions as the ligand-binding site. RXRs bind to response elements that are distinct from those that are regulated by RARs and the 2 receptor systems are also sensitive to different isoforms of RA, RXRs responding specifically to 9-cis-RA with RARs respond to all-trans-RA (Heyman et al., 1992; Levin et al., 1992).

The study of RXRB (Yu et al., 1991), which shows 95% homology to RXRA in the DNA-binding domain and greater than 90% homology in their ligand-binding domain suggests that RXRs also function as nuclear receptor co-regulators which enhance the binding of RARs and other nuclear receptors to their respective
response elements. This implies that the RXR class of nuclear receptors are involved in a distinct pathway of retinoid metabolism (Mangelsdorf et al., 1991) but are also interactive with the RAR response system.

The RXRA gene was regionally localised to chromosome 9q34 between the markers D9S66-D9S67 by the use of somatic cell and fragment hybrid lines specific to chromosome 9q. This regional localisation was confirmed by FISH studies of genomic RXRA clones. In light of these results the chromosomal position of a second member of the RXR family, RXRB, was also determined to consider the potential involvement of the RXR family of nuclear receptors as candidate loci for the TSC disorder.

3.4.2 Localisation of the RXRA gene on chromosome 9q

DNA from the DORA cell line and the panel of radiation reduced hybrids retaining chromosomal fragments from 9q (section 3.1), were typed for retention of the RXRA gene, using primers based on the published sequence of the RXRA 3' untranslated region (section 2.9). The amplification of this 407bp human specific PCR fragment in the hybrids 6C, 12C, 17B and 19B and in the DORA cell line (fig 3.4.1) was consistent with the localisation of the gene to the long-arm of chromosome 9, band q34, distal to the marker D9S66 and proximal to D9S67 (Fig 3.4.2). RXRA could also be positioned distal to D9S67 if hybrid 6C, which retains a large number of human fragments (Table 3.1.3), also retains a human fragment from this region. The other 3 hybrids which tested positive for RXRA retain human DNA from this region.
Fig 3.4.1  PCR amplification of the RXRA 3' untranslated region in the irradiation hybrids derived from the chromosomal 9q34 region.

Hybrids 6C, 12C, 17B, 19B and the DORA (Dor) cell line tested positive for the 407 bp PCR RXRA product. Hybrids 17A and 20A tested negative. Suitable controls of hamster (Ham) and Human (Hum) were also included.
Fig 3.4.2 Localisation of the RXRA gene to chromosome 9q34 distal to D9S66

The 4 hybrids 6C, 12C, 17B and 19B retain the RXRA gene which places the RXRA gene locus between the loci D9S66-D9S14. The likely position of the Retinoid X Receptor alpha gene is indicated in heavy dotted lines.
3.4.3 Localisation of the gene for RXRA to 9q34 by FISH

A cosmid library (section 2.2.8.) was screened with the 407-bp PCR fragment from the 3'-untranslated region, of the human RXRA cDNA sequence. Two positive clones, RXRA7 and RXRA14 were isolated and used for FISH studies to human metaphase chromosome spreads. Hybridisation signals were observed near the telomere on the long-arm of chromosome 9 (fig 3.4.3 A), confirming the localisation of the RXRA gene to the 9q34 region.

3.4.4 Assignment of the RXRB gene to chromosome 6 and its regional localisation to 6p21.3

DNA was amplified from a panel of hamster-human somatic cell hybrids which retains various human chromosomes (Table 3.4.1) using primers (section 2.9) based on the published 5'-untranslated RXRB cDNA sequence. Complete concordance was observed between the amplification or non-amplification of the human specific 142bp RXRB sequence from the hybrid cell line DNA and the presence or absence of human chromosome 6 (Table 3.4.1).

For regional localisation of the RXRB gene the human cosmid library (section 2.2.8.) was rescreened using the 142bp RXRB PCR products to isolate a genomic clone. 3 cosmids RXRB10, RXRB11, RXRB12 were identified. FISH of these cosmids to human metaphase chromosome spreads localised the RXRB gene to 6p21.3 (fig 3.4.3 B). This assignment is consistent with the segregation of the RXRB locus in a human chromosome 6 mapping panel (Table 3.4.2) which localises RXRB to 6p21.3-p21.1.
Fig 3.4.3 Fluorescent in situ hybridisation of biotinylated human cosmid clones for RXRA (A) and RXRB10 (B) to human metaphase chromosome spreads.

The whole cosmids were used as probes under conditions that suppress signals from repetitive DNA sequences. Hybridisation signals were detected using fluorescein conjugated avidin (green). Chromosomes were counterstained with propidium iodide (red). The two fluorochromes were simultaneously visualised using confocal scanner laser microscopy. Arrows indicate the fluorescent signals on chromosome 6 and 9. (A) Hybridisation of cosmid RXRA to the 9q34 region of chromosome 9; (B) Hybridisation of cosmid RXRB to the 6p21.3 region of chromosome 6.
Table 3.4.1 Segregation of the RXRB gene in a panel of rodent-human somatic cell hybrid lines provided by Sue Povey. The cell lines are described in the references indicated. nt in the table indicates not determined and f that a hybrid contains only a fragment of a particular human chromosome.
<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Chromosome 6 Component</th>
<th>RXRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDAG 3R</td>
<td>6pter- 6q21</td>
<td>+</td>
</tr>
<tr>
<td>RAGMH 9.4.8</td>
<td>6pter- 6q15(q21)</td>
<td>+</td>
</tr>
<tr>
<td>RAGSU 3.1.2.3</td>
<td>6pter- 6q14</td>
<td>+</td>
</tr>
<tr>
<td>56.47</td>
<td>6pter- 6p21.1</td>
<td>+</td>
</tr>
<tr>
<td>CALLA9 1.9.9</td>
<td>6pter- 6p23</td>
<td>-</td>
</tr>
<tr>
<td>EDAG 2.9.8</td>
<td>6q21- 6qter</td>
<td>-</td>
</tr>
<tr>
<td>IJA9 2.21.14</td>
<td>6q12- 6qter</td>
<td>-</td>
</tr>
<tr>
<td>MCP-6</td>
<td>6p21.3-6qter</td>
<td>+</td>
</tr>
<tr>
<td>CALLA9 1.13.10</td>
<td>6p23- 6qter</td>
<td>+</td>
</tr>
<tr>
<td>2068RAG 22.2</td>
<td>6pter- 6q27</td>
<td>+</td>
</tr>
<tr>
<td>ROHRAG 9.32</td>
<td>6q27</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.4.2 Segregation of RXRB in a human chromosome 6 mapping panel as described Boyle et al., 1992. The pattern of retention is consistent with a localisation of RXRB to 6p21.3.
CHAPTER 4

DISCUSSION
CHAPTER 4 DISCUSSION

4.1 Summary of the genetic and physical data of the TSC1 region.

An absence of associated chromosomal aberrations or biochemical abnormalities has restricted efforts to refine the position of the TSC1 gene on chromosome 9q34 to the use of genetic linkage analyses. This has led to the development of new highly polymorphic marker loci from the region (Kwiatkowski et al., 1992), the improvement in the informativeness of existing markers (Johnson and Hopkinson, 1992; Nahmias et al., 1992; Porter et al., 1992) and the incorporation of these markers into a high resolution genetic map (Povey et al., 1992). In the search for the causative gene extensive haplotyping of TSC families with these new loci has succeeded in refining the genetic position of TSC1 to a 20cM interval flanked by the microsatellite markers D9S64 and D9S67 with the most likely location for the gene within this region close to the ABO blood-group locus (section 1.7).

Current estimates of the physical size of this region is limited to the 6cM proximal portion of the interval, between the markers D9S64 and D9S10. Harris et al. (1993) using a combination of PFGE and FISH studies have constructed a physical map across this region linking the ABL oncogene and D9S10 markers to common PFG fragments totaling 2.35 Mb. The D9S10 marker and the genetic marker D9S66 have been mapped within 60 Kb of each other and so can be considered the same for physical analyses (N. Hornigold personal communication). Handa et al. (1992) have physically mapped the ABO blood-group and D9S10 locus to the same 550 Kb Nru1 fragment within this interval. These results suggest that the physical distance between the markers D9S64 and D9S66 range from no greater than 0.55 Mb to 2.35 Mb suggesting that a recombination event may occur as often as once every 100-400Kb in this region.

119
There are no physical data available to compare with the 13cM genetic distance of the D9S66-D9S67 subregion (Kwiatkowski et al., 1992). However, considering the increased recombination frequency observed in the D9S64-D9S66 region and the tendency for further increases as one approaches the telomere of chromosomes (Buetow et al., 1991; Tanzi et al., 1992) it could be expected that the size of this distal section (Kwiatkowski et al., 1992) may be as small as 1.3Mb. This would place the closest flanking markers for TSC1 within approximately 2 Mb of each other.

4.2 The irradiation fusion panel

In conjunction with the development of the genetic and physical maps of chromosome 9q34 and the progressive refinement of the genetic position of the TSC1 region the irradiation fusion approach was used in this study to generate hybrids retaining small chromosomal fragments from chromosome 9q to focus in on the TSC1 gene. The hybrid cell line 64063a12 (Jones and Kao, 1984), which contains an intact chromosome arm 9q as its only human material was used as a source of human material from the region. A dose of 45,000 rads of X-rays were used to fragment the chromosomal DNA and after fusion 39 radiation reduced hybrids were isolated and expanded for further analysis. A comparable amount of irradiation had previously been shown to fragment the chromosomal DNA into fragments of between 1-10Mb of human DNA (Benham et al., 1989). The size of fragments generated by irradiation exposure has been more accurately quantified by Cox et al. (1990) suggesting that a 45,000 rad dose is expected to generate fragment sizes of approximately 1.5Mb of DNA. This is fortunately in the same size range as the physical size one might expect for the TSC1 gene region in view of the recent physical and genetic studies.

It was anticipated that by biasing the marker retention studies to loci originating from the 9q33-34 region all the hybrids
retaining human fragments from the TSC1 region would be identified. 16 of the 25 hybrids which tested positive for at least one marker retained some human material from the 9q34 region. The paucity of markers tested for the more proximal regions of the chromosome is reflected in the identification of an additional 4 hybrids retaining human DNA by FISH. Alu-PCR analyses of the entire hybrid panel was in general consistent with the retention of human DNA in these 29 hybrids and the apparent lack of human DNA in the remaining 12 lines (section 3.2).

A subset of the 16 hybrids retaining DNA from the 9q34 region were used to construct a deletion map of the interval between the markers ASS and D9S67 (Fig 3.1.14). This mapping panel supported the map position of previously mapped loci present in the region and allowed the localisation of new genes to this interval. The hybrids 17A, 17B and 20A were particularly useful in this regard.

Hybrid 17A retains the ASS and ABL loci from 9q34 and the centromeric marker D9S48. Its retention of a small amount of human DNA is consistent with the amplification of a small number of Alu-PCR products from the hybrid. The presence of ASS and ABL in this hybrid suggests that these genes are close together on chromosome 9. This supports the PFGE information which places these genes on the same 800Kb Mlu1 fragment (Harris et al.,1993) and the genetic data which has failed to identify any recombination between the 2 genes (Ozelius et al.,1992). ASS is known to be proximal to the ABL gene by the mapping of ASS using translocated chromosomes from a chronic myeloid leukemia patient (Leversha et al.,1991; Harris et al.,1993).

Hybrid 17B also retains a small amount of human DNA. FISH analyses using biotin labeled total human DNA and reverse painting of Alu-PCR products are consistent with its retention of a single human fragment from the TSC1 region (fig 3.1.10, fig 3.2.3). The retention of the marker loci DBH, D9S10, D9S66, RXRA, D9S67 and D9S14, in the absence of the ABO blood-group gene, supports a localisation for the ABO blood-group either proximal to this group
of markers or distal to D9S67. The co-retention of ABO with DBH, D9S10 and D9S66 in hybrids 6C and 20A supports a proximal position for the ABO blood-group in relation to these markers. This is in agreement with the genetic order proposed by Lathrop et al. in 1988 which places D9S10 distal to ABO by linkage analysis.

However, subsequent genetic studies have failed to orientate the ABO locus with respect to DBH and D9S10. The recent isolation of a dinucleotide repeat polymorphism for the DBH gene (Nahmias et al. 1992; Porter et al., 1992) and the use of DGGE to increase the heterozygosity at the ABO locus (Johnson and Hopkinson, 1992) has led to the identification of a number of new recombination events (Sampson personal communication, Povey et al., 1992, Ozelius et al., 1992) which support the initial linkage observation by Lathrop et al. and the irradiation hybrid information presented here. The close proximity of these genes has now been confirmed by PFGE studies which place these markers on a common 550Kb NruI fragment (Handa et al., 1992). The precise order of markers in this interval is of particular importance as no recombinants have been observed between the ABO blood-group and the disease in TSC1 pedigrees.

The deletion panel has also allowed the localisation of the RXRA gene to a region between the microsatellite repeat sequences D9S66 and D9S67. This regional assignment has been confirmed by FISH of a genomic clone isolated for this locus and from comparative mapping data in the mouse (Hoopes et al., 1992). The localisation of the RXRA locus distal to D9S66 also agrees with the retention studies by Zhou et al. 1992 using flow sorted chromosomes derived from individuals containing translocations involving the chromosome 9q34 region.
4.3 Generation of cloned material from the TSC1 region

The identification of 2 hybrid lines, 17B and 20A, which covered a large part of the TSC1 interval and which seemingly retained little DNA from elsewhere on the chromosome arm (section 3.1, 3.2) provided a good starting resource to generate new markers from the region.

The Alu-PCR products from hybrid 17B were used to screen a total human genomic library in an effort to isolate cosmid clones from the region. Although 4 cosmid clones were subsequently isolated from 9q34 (section 3.2) the lack of a gridded flow sorted chromosome 9 or YAC library to increase the efficiency of this approach, seriously diminished the potential of the strategy as a method for region specific DNA isolation. This was disappointing in view of the success that this approach has had in the isolation of cloned material from defined regions of various chromosomal sub-regions with the availability of more suitable reference libraries (Monaco et al. 1991; Chumakov et al.,1992; Zuchman et al.,1992).

The more traditional approach involving the construction of recombinant libraries was used as an alternative strategy. A further 6 cosmids, 4 from hybrid 17B and 2 from hybrid 20A were isolated from 9q34 (section 3.3). The difficulty encountered in the generation of a representative library for each hybrid was compounded by the small proportion of human DNA present in each hybrid line, making this a rather laborious and inefficient method for retrieving human DNA from these rodent-human resources.

The identification of 2 further cosmid clones from hybrid 20A which originate from the centromere (section 3.3) suggests that the human fragment present in hybrid 20A consists of three separate human segments, including two from the 9q34 region (Table 3.1.2). This hybrid is therefore not as useful as previously anticipated as it retains a far greater amount of extraneous DNA from outside the TSC1 region than expected. The amount of DNA
retained from the centromere has not been determined. However, as the 9q34 segments are likely to be stabilised by their attachment to these centromeric sequences, this fragment could be quite large.

The application of the Alu-PCR based strategy is still appropriate for hybrid 20A since Alu sequences are under represented at the centromere by at least 50-fold (Moyzis et al., 1989). The identification of an Alu product common to both 17A and 20A (fig 3.2.11) suggest that some Alu products are derived from the centromeric region of chromosome 9. However, the correlation of Alu products between hybrid lines sharing marker loci from chromosome 9q34 (section 3.2) suggests that differential screening of gridded libraries with Alu sequences derived from these hybrids would allow clones to be assigned to discrete intervals of the TSC1 region.

In view of the increased frequency of recombination observed across the TSC1 interval (Ozelius et al., 1992; section 4.1), the 20cM genetic distance between the flanking markers D9S64 and D9S67 (Kwiatkowski et al., 1992) may have an actual physical size of approximately 2Mb. The use of the irradiation hybrid lines, 17B and 20A, have succeeded in the identification of 10 cosmid clones from this interval (section 3.2, 3.3). Additional clones have also been isolated for the RXRA locus (section 3.4) and the loci D9S10 and DBH which map within this region (J. Wolfe, personal communication). These are being used in conjunction with other cloned resources provided by D. Kwiatkowski and S. Graw to construct a cloned cosmid map of this region (Hornigold personal communication). The exon trapping approach (section 1.2.3) is being pursued to identify gene sequences from the cloned material generated in this study (Woodward, personal communication). At present with the apparent difficulties encountered in further refining the location of the TSC1 gene (section 1.7) the development of the physical map would appear more appropriate than concentrating on the identification of new genes.
It is disappointing, given the success of the irradiation-hybrid experiment to generate hybrids retaining DNA almost exclusively from the TSC1 region, that the number of cosmid clones isolated from this region is still relatively few. Fortunately the recent availability of a flow sorted gridded chromosome 9 library (J. Wolfe personal communication) has provided a better resource for the utilisation of these hybrids. The cosmid clones identified so far will provide a starting point for the generation of a cloned map of the entire TSC1 interval. These clones can then be used to identify candidate genes for the disease as outlined previously (section 1.3)

4.4 Comparison of this irradiation panel with previous irradiation experiments

Previous irradiation hybrid studies have shown using in-situ hybridisation that human fragments are retained as translocations or insertions onto hamster chromosomes or as fragments consisting entirely of human DNA (Cox et al., 1990). An identical pattern of fragment retention has been observed in this panel (section 3.1). Glaser et al. (1990) have suggested that this approach will give only a minimum estimate of the number of human fragments in each cell, since it is possible that some fragments will consist of noncontiguous human fragments healed together after irradiation. The observation that hybrid 17A gives a single human signal by FISH but consists of 2 noncontiguous fragments from marker retention studies supports this suggestion.

Fragment sizes of between 1-2Mb are expected from using an irradiation dose of 45,000 rads. The 9q34 region encompassing the ASS-D9S10 region allows for a direct comparison between the observed fragment size and the size one expects from this irradiation exposure. Hybrid 17A for instance is limited by the ASS and ABL loci which have been shown to reside on the same 800 Mlu1 PFGE fragment (Harris et al., 1993). The hybrid tested negative for the D9S65 and D9S64 markers which flank the ASS-
ABI region. Hybrid 20A retains a fragment containing the ABO-D9S66 region which is present on a common 550Kb Nru1 fragment (Handa et al., 1992). It is also likely that the human fragments in hybrids 6C and 17B from 9q34 contain between 1-2Mb of human DNA from a comparison of the genetic distance between the loci present in each hybrid and the frequency of recombination observed in the region (section 4.1).

Although the integrity of human fragments present in radiation reduced lines is supported by PFGE and marker retention studies, chromosomal deletions have also been reported (section 1.2.2.2.2). From this study there is little evidence to suggest that deletions are a common phenomenon in radiation reduced lines. This may reflect the small number of hybrids in the panel and the selection of only a subset of these for extensive characterisation. There is evidence to suppose that a number of small deletions have occurred in hybrid 12C. Reverse painting of 12C Alu-PCR products (section 3.2) together with marker retention studies suggest that the hybrid has retained DNA solely from the 9q33-34 region. The pattern of the markers retained in the hybrid divide this region into 5 discrete intervals (Table 3.1.2, Fig 3.1.14) which is inconsistent with FISH studies using human DNA as probe and the identification of 2 human fragment in the cell line (Fig 3.1.5). The observation that all the DNA retained in the hybrid line originated from the same region argues against the healing together of fragments after irradiation as an explanation for the reduced fragment number observed by FISH. It is probable that the inconsistencies between the marker retention and FISH data result from small deletions in the human material in the hybrid line.

The frequency of human retention in the hybrid panel is approximately 76% (29 of the 38 independent hybrids retain some DNA material. Hybrids 20A and 20B can be considered identical from a combination of FISH, marker retention and Alu-PCR analyses). For previous irradiation hybrid studies using a similar dose of irradiation retention frequencies of 100%, 75% and 30% have been observed in panels generated from donor somatic cell hybrid lines retaining chromosomes X, 10, and 12p respectively.
(Benham et al., 1989; Goodfellow et al., 1990; Sinke et al., 1992). The observed retention frequency of 76% in this experiment supports the suggestion by Sinke et al. (1992) who proposed that the frequency of retention of human DNA may depend on the amount of human chromosomal material present in the starting donor somatic cell line. This suggestion is supported by the observation that increasing irradiation dose also leads to increases in the frequency of hybrids retaining human DNA (Benham et al., 1989; Doucette-Stamm et al., 1991). Together these results suggest that the number of human fragments generated by the irradiation exposure will influence the retention of human DNA in the new hybrid cell.

However, the number of human fragments retained varies considerably between hybrids after fusion (Siden et al., 1992). Greater than 3 human fragments were visualised in 12 out of the 26 hybrids which were shown to retain human DNA by FISH. The remaining 14 hybrids retained far fewer human fragments, between 1-3 fragments per cell. This variation in the number of fragments retained between hybrids may depend on the amount of fragmentation caused by the irradiation exposure, in so much as donor cells retaining the greater number of human fragments after exposure will have an increased likelihood of retaining a larger number of human fragments after fusion than donor cells retaining fewer fragments.

The retention of the heterochromatic marker D9S48 in 24% of hybrids supports the previous suggestion that human centromeres are intrinsically stable in irradiation hybrid lines. The lack of an alphoid probe to test for the retention of the centromere may suggest why the frequency of retention of the D9S48 marker is less than the 65% retention observed in a previous study using a comparable amount of irradiation (Benham et al., 1989). This would also support the suggestion by Benham et al. (1989) that the centromeric region may be more susceptible to breakage after irradiation than other chromosomal regions and would explain the identification of centromeric sequences in the
human fragment present in hybrid 20A, although this hybrid had not retained the D9S48 locus (section 3.3).

In summary the distribution and size of the fragments generated in this panel is similar to that which has previously been observed. The integrity of the fragments transferred from the donor cells is supported by marker retention studies of the panel although there is some evidence to suggest that deletions have occurred in at least one of the irradiation hybrid lines. The frequency of human DNA retention in the panel is consistent with previously reported retention frequencies with a preferential retention of centromeric sequences.

4.5 Candidate Genes and TSC

Several genes have been proposed as likely candidates for the TSC disorder. The suggestion of the neural cell adhesion molecule (Clark et al., 1988), the dopamine receptor D2 (Janssen et al., 1990) and the gene for tyrosinase (Fahsold et al., 1991) on chromosome 11 and the genes for phenylalanine hydroxylase (Fahsold et al., 1991) and insulin like growth factor 1 (Fahsold et al., 1991) on chromosome 12 reflect an earlier interest in these chromosomes as possible locations for TSC genes. The genes for HBX, ABL and DBH have also been put forward as likely candidates on chromosome 9 (Gulcher et al., 1990; Sampson et al., 1989; Fahsold et al., 1991). HBX and ABL have been excluded as candidate loci for TSC1 following the identification of recombination events in affected individual in chromosome 9 linked families. The localisation of the RXRA gene to chromosome 9q34 in this study led to the RXR receptor family of nuclear receptors being considered for their possible involvement as a cause of the TSC phenotype.

The chromosomal position of the RXRA gene was initially determined in light of the involvement of the RARA gene in acute promyelocytic leukemia (Borrow et al., 1990: The et al., 1990) and
its role as a candidate gene for ovarian and breast cancer (Black and Solomon, 1993). The RXRA gene was subsequently assigned to chromosome 9q by somatic cell hybrid analysis (Karen Jones, personal communication). The panel of irradiation hybrids derived from chromosome 9q (Table 3.1.2) was used to localise the gene to the interval between the microsatellite markers D9S66 and D9S67 on distal 9q34 placing it within the maximum region in which TSC1 must map. This regional assignment has been subsequently confirmed in a number of ways (section 4.2).

The involvement of RXRA in mediating the dermatological effects of retinoids (Mangeldorf et al., 1990) and its expression in a number of other organs affected by TSC (Mangelsdorf et al., 1992) provided additional evidence in support of its role as a candidate gene for TSC1. In light of the genetic heterogeneity present in TSC the chromosome position of the remaining 2 members of this nuclear receptor family, RXRB and RXRG, were investigated to consider their possible involvement's as candidate genes for other TSC loci.

It was predicted that these genes would reside either close to the PKD1 disease locus on chromosome 16p13 or at the chromosomal breakpoints which had previously been identified in TSC patients. The RXRB gene was mapped to chromosome 6p21.3 (section 3.4) and this was subsequently confirmed by comparative mapping studies in the mouse (Hoopes et al. 1992). In addition Hoopes et al. have mapped the rxrg gene to mouse chromosome 1 which from comparative mapping analyses places the RXRG gene on human chromosome 1. The mapping of these genes to regions outside of those previously implicated in the disorder excludes from serious consideration their involvement as the cause of the disease.

Indeed the most recent genetic data for TSC1, based on the presence of recombination events with the DBH gene in 2 unaffected individuals (section 1.7) also excludes the region containing the RXRA gene. Although serious doubts have been raised over the inclusion of recombination data from unaffected
individuals in defining the position of the TSC1 gene (Povey et al., 1992) there is a general acceptance that the interval between the markers D9S64 and DBH represents the most likely position for the gene (Povey, personal communication). Although it is still difficult to argue against RXRA remaining a candidate gene for TSC1, it is accepted that the region around the ABO gene is the more likely position of the TSC1 interval to consider in detail at the present time.

4.6 Trinucleotide expansion and its involvement in TSC?

The observation that amplification of a simple trinucleotide repeat sequence is the basis for the disorders, spinal and bulbar muscular atrophy (SBMA) (La Spada et al., 1991), fragile X (FX) (Yu et al., 1992) and myotonic dystrophy (DM) (Mahadevan et al., 1992) has led to DNA instability being considered as the possible mutational mechanism involved in a number of other diseases (Li et al., 1992; Riggins et al., 1992). Clinically FX and DM exhibit a phenomenon called anticipation where one observes increasing severity of the disease in subsequent generations of the affected family. Molecular analysis of the trinucleotide repeat sequences in these pedigrees suggest a correlation between the size of the amplified element in a patient and the severity of the phenotype observed in these individuals (Yu et al., 1992; Tsilfidis et al., 1992; La Spada et al., 1992). Sutherland and colleagues (Sutherland et al., 1991) have proposed that this form of DNA instability might also explain other phenomenon including incomplete penetrance, variable expression and variegation which have been observed in TSC.

Although TSC does not show anticipation in its expression a similar mechanism of trinucleotide expansion could explain the diverse and heterogeneous phenotypes observed both within and between TSC families. However, the disorders identified so far involving simple expansion of a trinucleotide repeat result from
mutations in a few founder chromosomes (Tsiltidus et al., 1992; Caskey et al., 1992: Richards et al., 1992). It has been proposed that a founder effect may be characteristic of other disorders resulting from a similar mechanism of mutation (Tsiltidus et al., 1992). It seems reasonable that a search for linkage disequilibrium between flanking microsatellites and the disease would be the appropriate starting point prior to any protracted search for trinucleotide involvement in any disease. The high mutation rate observed in TSC suggests that the disorder is not the result of a mutation in a few founder chromosomes which argues against the involvement of DNA instability as the probable mutational mechanism.

4.7 Further study

The breakpoints of the hybrids 6C, 17B, 19B and 20A in the D9S64-DBH region should be refined with the availability of additional repeat polymorphisms from within this interval. These could provide a small deletion panel to map candidate cDNAs to discrete parts of the TSC1 region around the ABO blood-group locus. The availability in time of a gridded chromosome 9 or gridded human YAC libraries will provide more suitable reference resources for the efficient screening with Alu-PCR products derived from these radiation reduced hybrid lines. This should provide additional cloned material for the construction of a contig map of this 9q34 region and the isolation of genes from the region.

In an effort to direct the construction of a contiguous map of the entire region it will be useful to extend the physical map to include the markers D9S67 and D9S14. Previously overlapping radiation reduced hybrid lines have been used in conjunction with PFGE to generate a physical map of the WAGR region of chromosome 11p13 (Rose et al., 1990). The retention of small amounts of human DNA in the hybrid lines 17B and 20A suggest that a similar approach could be used to develop the physical map of the distal 9q34 region in tandem with the generation of a cloned contig from the region using this approach.
These hybrids could also be directly used to isolate transcripts from the region (section 1.2.2.2.4). For instance the subtractive hybridisation strategy of Jones et al. (1992) has succeeded in identifying nine independent human transcripts from a hybrid line retaining a human fragment of between 10-12Mb of DNA. Hybrid 17B and 20A should also be amenable to this approach. This would in part provide an independent method for the isolation of genes from the 9q34 region in contrast to the more conventional routes outlined in the introduction.

A large number of apparently sporadic patients have been collected since the interest in isolating the genes involved in TSC has gathered momentum. Studies of sporadic cases of TSC have not led to the discovery of any chromosomal alterations or long range restriction fragment changes associated with the disease which would assist in the refinement of the position of the TSC1 gene. It is possible that extensive haplotyping of these individuals using available highly informative marker loci from the TSC1 region could efficiently identify hemizygous patients for loci within the region which have occurred as a result of small submicroscopic deletions in these individuals. This would provide an efficient method to sub-divide sporadic cases into a smaller group most likely to contain deletions and these could then be evaluated by PFGE to consider the potential involvement of a deletion in these individuals as a cause of TSC1.
CHAPTER 5

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142


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