PHYSICAL MAPPING OF A CHROMOSOMAL REGION DELETED IN SMALL CELL LUNG CANCER

by

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Abstract

Characteristic deletions have been associated with a number of solid tumours, which represent the sites of tumour suppressor genes. The deletion of 3p21 sequences in small cell lung carcinoma has been demonstrated. To identify and isolate this tumour suppressor gene by positional cloning, a precise knowledge of the boundaries of the deletion is required. A detailed physical map of this region is important in the identification of genes with potential tumour suppressing properties.

The thesis describes the physical mapping of a region of the human genome, 3p21, which is consistently deleted in small cell lung carcinoma, SCLC. These deletions are thought to indicate the presence of a tumour suppressor gene. Somatic cell hybrids and pulsed field gel electrophoresis were used to map within the deleted region.

Somatic cell hybrids were established to provide a mapping resource for the fine mapping of markers on 3p21. Each panel of hybrids were characterised with available 3p markers and genes.

One hybrid containing a portion of 3p21 representing a part of the deletion was used further in physical mapping studies. A gene was isolated from this hybrid and it's genomic structure determined. A cosmid encompassing this gene was used to initiate a chromosomal walk and construction of a contig in this region.

Using pulsed field gel electrophoresis, a long range map around this gene was generated and a physical link to another marker in 3p21 shown. This defined region was analysed in SCLC tumours using similar techniques.

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To my Father

Jai. K. Gulati

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
cos	cosmid
DEAK	Deakin hybrid
DEPC	Diethylpyrocarbonate
ddH_2O	deionised distilled water
dH_2O	distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diaminetetraacetic acid
GPS	Greig cephalopolysyndactyly syndrome
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobases
µl	microlitres
ml	millilitre
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PMSF	Phenylmethylsulfonylfluoride
RCC	renal cell carcinoma
RFLP	Restriction fragment length polymorphism
RHF	relative heterozygous frequencies
RNA	Ribonucleic acid
SCLC	small cell lung carcinoma
SDS	Sodiumdodecyl (lauryl) sulphate

T_4	Tetraiodthyroine (thyroxine)
Tris	2-Amino-2-(hydroxymethl)-1.3-propanediol
VHL	Von-Hippel Lindau
VRC	Vanadyl ribonucleosidase complex
XGal	5-Bromo-4-chloro-3-indolyl-β-Dgalatopyranoside

1. INTRODUCTION

1. INTRODUCTION

This thesis is concerned with the extension of the current cytogenetic and molecular characteristics of small cell lung cancer. It is specifically concerned with the physical mapping of a region of the human genome, 3p21, which is consistently deleted in small cell lung carcinoma.

Lung cancer accounts for 25% of all cancer deaths. About one third of these is thought to be attributable to smoking. Tobacco smoke is a mixture of carcinogens and clastogenic agents which play an interactive role in the genetic events occurring during the development and progression of the tumour. An inherited chromosomal weakness acting in concert with carcinogens has been proposed as a possible mechanism leading to the malignancy.

The WHO classification of lung cancer categorises the tumours into two major classes; small cell lung carcinoma (SCLC) and non small cell lung carcinoma (non-SCLC). Non small cell lung carcinoma is subdivided into squamous cell carcinoma, adenocarcinoma and large cell carcinomas. Studies of the biology of lung tumours have demonstrated the unique morphology and neuroendocrine properties of the small cell lung carcinomas.

The molecular genetic studies of small cell lung carcinoma have revealed the activation of oncogenes and chromosomal deletions at several identified loci. However deletions on the short arm of chromosome 3 are the most consistent chromosomal abnormality associated with SCLC.

A brief overview of oncogenes and tumour suppressor genes identifed in cancer genetic studies is presented. The review focuses on the chromosome rearrangements of SCLC and specifically concentrates on the cytogenetic and mapping studies on the short arm of chromosome 3. Finally, the importance of chromosome 3 in other related and unrelated malignancies is discussed.

1.1.CHROMOSOMAL REARRANGEMENTS IN CANCER

The use of chromosome banding in oncology genetics has revealed consistent chromosomal rearrangements in a number of cancers. These abnormalities include chromosomal translocations, homogeneously staining regions (HSR), double minutes (DM's) and deletions of a chromosomal band or segment. At the molecular level these chromosomal abnormalities are thought to represent the mechanisms of oncogene activation and loss of tumour suppressor gene function.

1.1.1. ONCOGENES

Oncogenes have been identified at or near the junction of chromosomal translocations in the studies of leukaemias and lymphomas (Collins et al. 1982, Dalla-Favera et al. 1982a&1982b, Yunis et al 1983). For example, in malignant cells from Burkitt's lymphoma patients a consistent translocation involving chromosomes 8 and 14 has been characterised. The *c-myc* protooncogene on chromosome 8 is translocated to a location adjacent to the immunoglobulin constant region on chromosome 14. This results in the activation of the oncogene through the transcriptional influence of the immunglobulin promoter. The translocation breakpoints and potential oncogene activation have been characterised in chronic myelogenous leukaemia and acute nonlymphocytic leukaemia.

1.1.2 TUMOUR SUPPRESSOR GENES

1.1.2.1. Retinoblastoma

Knudson's hypothesis for the origins of retinoblastoma (1971) proposed that at least two independent events are necessary for the expression of the malignant phenotype. These events are mutations of the two alleles of a single gene. In familial cases the first mutation is inherited in the germline and a further mutation in a cell of the retinal tissue is required for tumour development. However, in sporadic cases

two mutations must occur in the same somatic cell. Familial cases of retinoblastoma are characterised by an earlier age of onset and the high frequency of bilateral tumours whereas sporadic tumours are very often unilateral and arise at a later age (Knudson et al. 1971). The differences between sporadic and familial retinoblastoma can be explained by the existence of a tumour suppressing factor acting in a mode postulated by Knudson. Together these observations provide strong evidence for a tumour suppressor gene whose loss of function through the mutation of both alleles leads to tumour development. The retinoblastoma gene, RB1, at 13q14 was the first tumour suppressor gene to be cloned. (Dryja et al. 1986, Friend et al 1986 &1987) . Gross alterations of the gene are limited to 30% of the tumours. Abnormal sized and mutated transcripts have been characterised in a number of retinoblastoma patients. In all cases no Rb tumour expresses the "wild type" form of the protein (Lee et al.1987, Dunn et al. 1988). The RB1 gene encodes a phosphoprotein with DNA binding activity regulated through a phosphorylation/dephosphorylation mechanism (Lee et al. 1987, DeCaprio et al. 1988).

1.1.2.2. Wilm's Tumour

The two hit mutational model for Wilm's tumour proposed by Knudson and Strong (1972) was based on early epidemiological studies which suggested that retinoblastoma and Wilm's tumour behave in similiar genetic fashion. However, no significant differences of age of onset and proportion of bilateral tumours are seen in Wilm's tumour patients.

A consistent deletion at the chromosomal locus 11p13 has been identified in the Wilm's tumour aniridia genitourinary abnormalities and mental retardation,WAGR (Ricardi et al.1980, Koufos et al. 1984, Orkin et al. 1984, Fearon et al 1984). The region harbours at least two genes, AN2, aniridia and WT, Wilms tumour.

The Wilm's tumour gene is a zinc finger polypeptide containing four zinc fingers. In addition, its extensive homology with the human epidermal growth factor gene

and its high proline and glutamine content suggests the gene may be a transcription factor (Call et al. 1990). The AN2 gene has been mapped to 11p13 using cell lines from aniridia individuals with and without Wilm's tumour (Mannens et al. 1989).

However, the RFLP studies of markers on 11p have revealed the loss of an allele in band 11p15 to be a consistent event in about one third of Wilm's tumour patients. In addition chromosomal rearrangements and losses in a subgroup of patients with Beckwith-Wiedemann syndrome consistently include the 11p15 region (Koufos et al. 1989, Ping et al. 1989, Orkin 1984, Reeve et al 1984). These results provide convincing evidence for a Wilm's tumour associated Beckwith-Wiedemann locus at 11p15. (Raizis et al. 1986).

Less than 1% of Wilm's tumour cases are familial, compared with the 30-40% of retinoblastoma cases. By analogy with retinoblastoma, inherited predisposition to Wilm's would involve a locus on 11p. However linkage studies of familial Wilm's tumour have shown the exclusion of 11p, suggesting a third locus outside this region (Grundy et al. 1988, Huff et al. 1988). These observations reflect the heterogeneity and genetic complexity of Wilm's tumour.

1.1.2.3. Colorectal cancer

Two genes mapping to chromosome 5 , MCC (mutated in colorectal cancer) and APC (adenomatous polyposis coli), have been identified in colon cancer development (Kinzler et al. 1991, Groden et al. 1991, Nishisho et al 1991) . The APC locus is thought to be the site of mutations that causes the hereditary susceptibility to colon cancer known as familial polyposis coli (Nishisho et al. 1991). The identification of chromosome 18 allele loss in about 70% of colorectal tumours has assisted in the localisation and subsequent isolation of the DCC gene (deleted in colorectal cancer). DCC is a cell surface interaction protein. Its inactivation could result in a loss of cell growth inhibition which is thought to be mediated by cell to cell interaction (Fearon et al. 1990)

1.1.2.4. P53

Following the identification of the tumour suppressor gene p53, at 17p13, mutations in a wide of range of human cancers were reported (Isobe et al. 1986, Nigro et al.1986, Cattoretti et al. 1988) .P53 encodes a nuclear phosphoprotein controlling cell proliferation. It is present in minute amounts in the cell as the half life is limited to 20-30 minutes. Mutational hotspots have been identified coinciding with the highly conserved regions of the gene (Nishisho et al. 1991).

1.2.CHROMOSOMAL CHANGES IN SMALL CELL LUNG CANCER

1.2.1 BIOLOGY OF SMALL CELL LUNG CANCER.

The notion of a unique cellular origin for SCLC is based on the biochemical and morphological differences observed in comparison to other lung cancer types. The Kulchitsky cell or K cell, present in the basal lining of the bronchial mucosa has been proposed as the originating stem cell (Gazdar et al. 1981, Iannuzzi & Scoggin1986) in the light of the shared morphological and neuroendocrine properties of the K cell and SCLC cells.

In contrast, a common origin for all lung cancer types has been proposed. Under this model, the pulmonary endodermal stem cell would be a likely candidate for a common originating cell. The demonstration of a transition from the SCLC morphology to that of the non SCLC, together with the detection of low levels of neuroendocrine expression in some non SCLC, would support this view (Leij et al. 1985, Reeve et al. 1986). This model would require an extensive differentiation pathway to account for the range of histological types observed in lung cancer.

1.2.1.1 Biochemical properties of small cell lung cancer

Well characterised, clonable small cell cultured lines have greatly advanced the knowledge of the biology of the tumour. The lines are easily distinguishable from those of the other lung cancer types by the presence of neurosecretory granules and the secretion of neuropeptides, predominantly gastrin releasing peptides, (GRP) and neuron specific enolase, (NSE) (Cuttitta et al. 1985, Erisman et al.1982, Reeve et al. 1986).

SCLC also shares properties with the amine precursor and uptake decarboxylyation (APUD) cells, possessing high levels of two specific enzymes, DDC(L-Dopa decarboxylase) and CK (creatine kinase) (Gazdar et al.1980, 1981&1985 , Baylin et al. 1978&1980).

Creatine kinase catalyses the transfer of high energy phosphates from creatine phosphate to ADP. Abundant levels of the brain isozyme CK-BB can be detected in striated muscle, brain, bladder and gastrointestinal tract, most other organs having much lower amounts (Saks et al. 1978, Shatton et al. 1979).

Increasing levels of CK have been found during the growth of classic and variant SCLC cultures. Long term cell lines established from the extensive staged disease often adopt a large cell morphology and simultaneously display increasing CK expression (Gazdar et al.1981).

L-Dopa decarboxylase is a key enzyme in the synthesis of biogenic amines. It is present in surgical and autopsy tissues of all lung cancer types, with the highest levels in SCLC and a proportion of adencarcinomas (Baylin et al.1978 &1980).

Loss of DDC activity but increasing levels of CK-BB have been shown in a number of SCLC long term cultures indicating a discordance in the regulation of the enzymes. This suggests that CK-BB may be a more consistent biochemical feature

of SCLC.

Neuron Specific Enolase, NSE, is normally present in the peripheral neuroendocrine cells of APUD and is highly localised to the gut secretory cells (Facer et al. 1980) .Studies of NSE in lung cancer have included the use of a monoclonal antibody to quantitate its expression. NSE immunoreactivity could be detected in all SCLC with varying staining intensities. In contrast however, immunostaining of nonSCLC tissue revealed patchy areas of expression (Marangos et al. 1982, Bergh et al. 1985, Reeve et al. 1986)

Bombesin is a tetradecapeptide found in amphibians. The mammalian equivalents are gastrin releasing peptides GRPs (Spindel et al. 1984). These are naturally abundant in the mammalian brain, stomach, intestine and foetal lung. Immunocytochemical studies of the lung show GRP expression confined to the respiratory epithelial cells of the foetal and neonatal lung. The strong mitogenic properties of these peptides has been shown by the addition of GRPs to SCLC culture, causing a 100 to 150 fold increase in growth . Similarly, a decrease in the growth of SCLC cultures is observed by the blocking of the GRP receptors (Sausville et al. 1986. Cuttitta et al. 1985).

Monoclonal antibodies to gastrin releasing peptides and the use of co-chromatography were used to illustrate their increased levels in SCLC. Significant peaks of immunoreactivity were observed in all cell lines analysed. (Moody et al. 1981&1983). Further experimental support for increased GRP levels in SCLC came from a study of tumours grown in nude mice. Expression of the peptide was immunohistochemically localised, quantified and analysed using chromotography (Erisman et al. 1982) .

These data illustrate the importance of GRP expression to the continued growth of these cancer cells .

1.2.1.2. Characterisation of Established Small Cell Lines

A considerable number of SCLC lines have been established and characterised with respect to their biochemical, morphological and growth properties. There is some controversy as to whether this characterisation would apply to the original tumour since changes do occur during the establishment of the line. However, it is the general view that these changes do not make a significant difference to the results obtained (Carney et al 1985). The subclassification of SCLC lines is based on the morphological and biochemical variation. 'Classic lines ' are those that retain the small cell morphology during culture with expression of all neuropeptides. 'Variant lines' have an altered morphology and biochemical profil e. They are not artefacts of the tumour in culture as the morphological differences are seen in a number of small cell tumours at autopsy. The variant class is subdivided into biochemical variants, SCLC-BV and the morphological variants, SCLC- MV. In the former class the lines are morphologically simil ar to the classic type wih limited expression of neuropeptides. The SCLC-MV comprise loosely adhering floating cells with a different biochemical profile (Carney et al. 1985 Gazdar et al. 1985). All SCLC lines can be unambiguously classified into these subtypes and many studies have illustrated the universal and unambiguous application of this system. (Ballie-Johnson et al. 1985). Studies of SCLC-MV lines have demonstrated the particular virulence and resistance to chemotherapeutic agents in these subtypes; indeed suggestions have been that SCLC-MV is the end point of the SCLC differentiation pathway and may in fact represent the transition from SCLC to non SCLC morphology (Leij et al. 1985).

1.2.2. ONCOGENE ACTIVATION IN SMALL CELL LUNG CANCER.

The activation of cellular oncogenes can occur through a variety of mechanisms including rearrangements by chromosomal translocation, gene amplification, and

point mutations. Molecular studies support the view that the function of protooncogenes is to transduce part of the complex information involved in regulating cellular growth, and that the inactivation leads to a deregulation of the system thus contributing to the neoplastic transformation of tumour cells.

1.2.2.1. *Myc* oncogene

Combined cytogenetic and molecular studies of cellular oncogenes in SCLC has illustrated the frequent activation of the *myc* family of oncogenes. These are nuclear oncogenes and include *N-myc, C-myc,* and *L-myc.* and the latter was identified as an expressed sequence in lung tumour (Nau et al. 1985).

Amplification and over expression of *C-myc* in classic SCLC lines has been identified by the presence of unique additional multiple *C-myc* hybridising bands and a fifteen to 35 fold increase in the 27 kb transcript. Karyotype analysis of these lines revealed double minutes, (DM's), and homogeneously staining regions, (HSR's), on and close to the *C-myc* locus on chromosome 8 (Little et al. 1983). A study of *N-myc* and *C-myc* in SCLC DNA samples and cell lines illustrated the frequent amplification of one of these genes, (Wong et al. 1986, Van der Hout et al. 1989) and in support of this, HSR's and DM'S were observed in all corresponding karyotypes.

Oncogene activation is believed to have an important role in the development of the small cell tumour. For example, following the the isolation and cloning of the *L-myc* gene, 10-20 fold increases in expression of abnormal sized transcripts were observed in SCLC lines and fresh tumours, (Nau et al. 1985). A large study of the three *myc* oncogenes in 31 SCLC including both variant and classic types shortly followed (Taya et al. 1986): Twenty-one lines showed amplification of one oncogene but there was no association between the tumour type and gene amplification. Additional studies of *myc* amplification have confirmed these observations (Keifer et al. 1987, Ibson et al. 1987). The introduction of a tumour

with *myc* amplification into nude mice results in significant increases its tumourgenic properties. Transfection studies have illustrated that cells with the amplified oncogene have a greater colony forming efficiency which confers a selective tumorigenic advantage on the cell (Gemma et al. 1988).

However the amplification of *myc* is not universal and several explanations for the absence of *myc* amplification in some lines have been offered (Wong et al. 1986). These include (i) the possibility that tumours were in fact expressing one of these *myc* genes at high levels as a result of an undetectable cytogenetic, molecular or epigenetic event, (ii) the effect of an elevated *myc* was replaced by the activation of another oncogene of similar function oncogenes and (iii) *myc* amplification is advantageous for only certain tumour types.

1.2.2.2. *Ras* Oncogene

The three *ras* genes encode a 21 kilodalton GTP binding protein and are believed to play a part in the growth signal transduction pathway. Point mutations are a common mode of *ras* gene activation and are commonly found in lung cancer cell lines of the non small cell type (Bos 1989). However, a three fold increase of *k-ras* expression in a small proportion of SCLC lines has been reported, (Taya et al. 1986).

1.2.2.3. Other Oncogenes

Following the isolation of the *c-myb* oncogene, thirteen lung cancer cell lines were studied of which eight were of small cell origin (Griffin et al. 1985). *C-myb* activation was seen in twelve tumours in which there was simultaneous expression of *myc* . A number of other oncogenes were studied in lung tumours. These included *c-fms, c-fes,c-fos c-src* all of which were shown to be weakly expressed in all tumour types (Keifer et al. 1987).

The group of proto-oncogenes encoding cellular growth factors or receptors include

c-erb-1, c-erb-2, c-sis, PDGF (β chain)and *c-fms. C-erb-2* encodes a thyroid hormone receptor, THRB, assigned to the short arm of chromosome 3 and has been used in the RFLP analysis of SCLC (Doborovic et al. 1988). Expression analysis of THRB in lung cancer reveals minimal levels in SCLC as compared to tumours of the non-small cell type (Schneider et al. 1989).

1.2.3 MUTATIONS OF THE RETINOBLASTOMA GENE.

Mutations of the retinoblastoma gene have been shown in a number of unrelated cancers principally SCLC, breast and bladder carcinomas (Devilee et al. 1989, Mori et al. 1989, Yokota et al. 1988, T'ang et al. 1988). These observations have highlighted the pleiotrophic effects of the Rb gene, suggest ing a functional role in several types of unrelated tumour.

A number of investigations of the Rb gene in lung cancer have been reported (Harbour et al. 1988, Yokota et al. 1989 and Lee et al. 1990). The former study uses polymorphic markers from the 13q12-q33 region in RFLP studies of twenty-three SCLC tumours. In eighteen, reduction to homozygosity at one or more informative loci was observed including a genomic rearrangement of the Rb gene in one sample. Four SCLC cell lines and one lung carcinoid line illustrated one of two types of structural abnormalities; homozygous or hemizygous loss of the Rb gene. Of the twenty-six lines studied, twenty had no expression of the Rb transcript. In contrast, in fifteen of the nineteen NSCLC lines analysed, the Rb gene expression was apparently normal.

The investigations of the Rb gene in both NSCLC and SCLC by Yokota et al (1989) originated from his earlier observations showing 100% loss of heterozygosity at loci in the 13q12-22 region. The later study included eighteen lung tumour lines, nine SCLC and nine NSCLC. Although no gross abnormalities were found, several differing patterns of Rb expression were observed including a few cases with no detectable transcript .

Further involvement of the Rb gene in twenty-six SCLC lines was reported by Lee et al (1990). Genomic rearrangements were limited but only three lines expressed greater than trace levels of Rb RNA. Multiple forms of the Rb protein including the unphosphorylated form were shown. These three studies illustrate the frequent involvement of the Rb gene in SCLC , with a limited role in tumours of the non small cell type. Unlike retinoblastoma, SCLC does not have a clear hereditary pattern. The mutations are therefore somatic events in the lung tumour cell. SCLC phenotypically resembles Rb in that both possess neuroendocrine properties and can have deregulated N-myc expression. The involvement of the Rb gene in SCLC and other tumours suggests that mutation of this gene contributes to the malignancy. However the overlapping biochemical properties of the two tumours may indicate the Rb gene to show similar properties to the SCLC tumour suppressor and quite possibly interact with it. The cloning of the latter gene will allow studies in Rb tumours which may prove interesting. These observations together with the involvement of Rb in other tumours supports the notion that the loss of the gene function is important in the pathogenesis of other cancers by contributing to the malignancy of tissues other than that of the retina.

1.2.4. MUTATIONS OF THE P53 GENE.

Detailed mutational analysis of p53 in SCLC has been reported, (Takahashi et al. 1989, Iggo et al. 1990, Sameshima et al. 1990). Takahashi found the homozygous deletions or subtle mutations of the gene which results in over expression of mutant p53 in eleven of thirteen cell lines analysed. Immunohistochemical techniques have also illustrated high steady state levels of mutant p53 in SCLC which are likely to occur through a stabilisation mechanism by the formation of complexes with viral oncoproteins (Iggo et al. 1990). In one SCLC line analysed by Sameshima et al., (1990) an abnormal p53 transcript caused by a splicing inhibition mechanism has been shown.

Vogelstein (1990) has speculated that the p53 gene acts through a dominant negative mechanism where mutant p53, although having oncogenic potential, can also act as an inhibitory protein competing with and blocking the wild type protein, creating an inactive but stable complex.

Lung, colon and breast carcinoma together represent the majority of cancer types. Mutations of the p53 gene is the common denominator, occurring in at least 75% of these tumours illustrating that this gene is not only a widely implicated tumour suppressor but also the commonest mutation found in all cancers.

1.3. CHROMOSOME 3 AND SMALL CELL LUNG CANCER

1.3.1 CYTOGENETIC STUDIES OF SCLC

The most consistent and significant chromosomal defect in SCLC is deletion of part of the short arm of chromosome 3. This section reviews the results of karyotypes analysis from fresh tumours , established cell lines and bone marrow samples of SCLC patients. The data has provided the basis for RFLP and expression studies of genes and markers on 3p in these tumours .

The difficulties in establishing chromosomes from fresh tumours and established cell lines continued to be a problem through to the early 1980's. However, where karyotypes were obtainable, chromosomes could only be analysed and categorised on the basis of size. High modal numbers were observed and attempts were made to identify markers common to all cell lines (Oboshi et al. 1971, Ohara et al. 1977). In an extensive study of Giemsa banded bone marrow metephases from 26 patients, many numerical and structural aberrations were identified, but no consistent marker across all the patients was apparent (Wurster-Hill et al. 1978).

A breakthrough in the cytogenetics of SCLC came from the analysis of chromosomes from established cell lines, short term cultures and fresh tumours from 16 SCLC patients (Whang-Peng et al. 1982a&b). In addition to conventional Giemsa staining, trypsin- giemsa (G-banding) and C-banding were used to assist in the karyotyping of the large number of chromosomes derived from each sample. A consistent deletion on the short arm of chromosome 3 in at least one homologue in meteaphase spreads was identified. The deletions from each cell line were charted and the shortest region of overlap identified as 3p14-23.

The presentation of karyotypes from three SCLC lines at the 8th international chromosome conference (Buys et al. 1983) and four lines from Yunis' (1983) laboratrory confirmed the 3p deletion. The shortest region of overlap was identified as 3p21-23. Direct prepararations from 3 SCLC's further illustrated the 3p- marker as a consistent feature in this tumour (Falor et al. 1985)

However, only a minority of the 19 SCLC lines characterised by Wurster-Hill et al. (1984) included this 3p- marker. This study revealed high modal numbers in these tumours and several structural aberrations in each line. A further discrepency regarding a 3p deletion arose from the report of the karyotypes of six SCLC lines which failed to reveal any clonal abnormality on the short arm of chromosome 3 (Zech et al. 1985).

1.3.2. MAPPING DATA ON CHROMOSOME 3.

Polymorphic DNA markers have been used in the analysis of both lung and renal cell carcinomas. The loss of heterozygosity in tumour samples as compared to the constitutional genotype has enabled the identification of a chromosomal deletion in the tumour . The precise order of these markers used in these analysis is important in both quantifying and mapping the deleted region. Markers shown to flank or lie close to the deletion can be used as landmarks in the finer mapping of the region.

Several approaches to generate a detailed, high resolution map of the short arm of chromosome 3 have been undertaken. Conventional techniques using genetic linkage analysis, somatic cell hybrids and radioactive in situ hybridisation have been used in the assignment of several genes and markers to the short arm of chromosome 3. With the additional application of irradiation fragmented hybrid panels (Naylor et al. 1991), physical linkage studies (Smith et al. 1991) and the simultaneous hybridisation of two markers to metaphase chromsome using FISH (fluorescent in situ hybridisation), (Albertson et al. 1989, Heppell-Parton et al. 1991) the further localisation of DNA markers used in the loss of heterozygosity studies of renal and lung carcinomas have been determined.

Table 1 illustrates the methods used in assigning 8 DNA markers and 5 genes used in these analysis to the short arm of chromosome 3. The mapping data published prior to and presented at Human Gene Mapping 11, HGM11, have been used to derive an unambiguous order for a majority of these markers and genes. The exceptions include the D3F15S2 and the APEH which are separated by a distance of 4kb, (Carritt et al. 1986, Naylor et al.1989) where the relative order with respect to the centromere remains unknown.

MARKER	PROBE	LOCATION	MODE	SRO	REFERENCE
RAF	p627	3p25	ISH\SCH	**3p25**	Bonner 1984
THRB	pHeA4	3p21-p25	SCH		Drabkin 1987
	pHeA4	3p24.2	SCH	**3p24.2-24.3**	Dobrovic 1988
	pBHeA2	3p21-pter	ISH		Rider 1987
	pHeA4/A2	3p24.2-24.3	FISH		Albertson 1989
D3S11	E41	3p14-p21	ISH		Dietzsch 1986
		3p22-p23	FISH	**3p22-23**	Heppell-Parton 1991
ALAS 1	pGEM1	3p21	SCH		Sutherland 1988
		3p21.2-pter	SCH	**3p21.2-21.3**	Naylor 1991
ALAS 3		3p21	SCH/ISH		Bishop 1990
APEH	pH3E4	3p21	SCH		Davis1986
		3p21	SCH		Carritt 1986
		3p21	ISH	**3p21.2-21.3**	Kok 1987
		3p21.3-p23.	FISH		Albertson 1989
		3p21.3-p23.	FISH		Heppell-Parton 1991

Table 1 : Methods of assigning 13 markers to the short arm of chromosome 3.

MARKER	PROBE	LOCATION	MODE	SRO	REFERENCE
D3F15S2	pH3H2	3p21	SCH		Carritt 1986
		3p21	SCH	**3p21.2-21.3**	Goode 1986
		3p21.2-pter	SCH		Gemmill 1991
D3S32	pEFD145	3p21	GL		Leppert 1987
		3p21	FISH	**3p21.1**	Heppell-Parton 1991
		3p21.2-pter	SCH		Gemmill 1991

Table 1contd.: Methods of assigning 13 markers to the short atrm of chromosme 3.

MARKER	PROBE	LOCATION	MODE	SRO	REFERENCE
D3S2	pHF12-32	3p21-q21	SCH		Naylor 1984
		3p14.2-p21	FL.CYT		Harris 1986
		3p	GL		Leppert 1987
		3p14.2-pter	SCH		Gerber 1988
		3p14.2 or 3p21	SCH	**3p21.1**	Glover 1988
		3p14.2-p21.1	SCH		Drabkin 1989
		3p21-distal 3p14	ISH		Van der Hout 1990
		3p21.1	FISH		Heppell-Parton 1991
		3p21.2-pter	PL		Gemmill 1991
ACY-1	pACYC184	3p21-pter	SCH		Voss 1980
		3p21.1	SCH	**3p21.1**	Miller 1990
		3p21.1	PL		Gemmill 1991
D3S6	pMS1-37	3p14	SCH	**3p14**	Gerber 1986
		3p13-14.2	FISH		Albertson 1989

Table 1 contd: Methods of assigning 13 markers to the short arm of chromosome 3.

MARKER	PROBE	LOCATION	MODE	SRO	REFERENCE
D3S4	B67	3pter-q21	SCH		Morle 1984
		3p	GL	**3p13-p14**	Leppert 1987
		3p13-14	FISH		Heppell-Parton 1991
D3S30	pYNZ86.1	3	SCH		Nakamura 1987
		3p	GL		Leppert 1987
		3p13-p14	FISH	**3p14.1**	Heppell-Parton 1991
		3p14.1	PL		Gemmill (unpub)
D3S3	pMS1-37	3	SCH		DeMartinville 1983
				3p14.1	
		3p14	SCH		Gerber 1986
		3p13-p14.2	FISH		Albertson 1989

Table 1 contd.: Methods of assigning 13 markers to the short arm of chromosome 3.

The shortest regions of overlap for each marker are listed and have beeen agreed at HGM11 (Naylor & Carritt 1992). Different sequences for the ALAS gene were presented at HGM11 from two independent laboratories and have been assigned as

two different genes, ALAS 1 and ALAS 3.

KEY: **SRO** **shortest region of overlap**

ISH radioactive in situ hybridisation

FISH fluorescent in situ hybridisation

GL genetic linkage

PL physical linkage

FL.CYT flow cytometry

The order of these markers are listed below from 3pter-cen

RAF

THRB

D3S11

ALAS1/ALAS3

D3F15S2/APEH

D3S32

D3S2

ACY-1

D3S6

D3S4

D3S30

D3S3

A map of new markers and previously isolated genes mapping to the short arm of chromosome 3 was presented at HGM11 by Naylor et al.(1991). Of particular interest to the study of SCLC and RCC are those flanked by the reference markers

RAF, THRB and D3F15S2. Through genetic linkage analysis of CEPH families an order with estimated genetic distances has been shown.

Additional genetic maps include one showing 25 new DNA segment markers of the short arm of chromosome 3 presented by Tory et al. (1991) at HGM11 with RAF, THRB, D3F15S2 and D3S32 included as reference markers

A linkage map of 41 polymorphic markers for human chromosome 3 includes a high density of new clones in the 3p14-p24 region. Genetic distances between these markers have been defined but not relative to any of the reference loci listed above (Yamakawa et al. 1991).

An attempt to saturate chromosome 3 with cosmids derived from a somatic cell hybrid deletion mapping panel has been made (Smith et al. 1991). Many of these clones have been precisely localised to the short arm of chromosome 3 and should eventually contribute to the development of a complete physical and genetic map of the region encompassing tumour specific deletions .

1.3.3 RFLP ANALYSIS OF SMALL CELL LUNG TUMOURS.

The use of polymorphic DNA markers in the analysis of SCLC has enabled molecular characterisation of the deleted region on chromosome 3. Under the simplest possible model, the frequency with which loss of heterozygosity is observed for a marker is directly related to its proximity to genes whose loss contributes to the malignant phenotype. These gene are termed tumour suppressor genes.

Following the cytogenetic characterisation of the 3p21-23 deletion in SCLC (Yunis 1983, & Buys et al. 1983), polymorphic markers mapping in and close to this region have been used in RFLP analysis. Figure 1 presents the published data on the relative heterozygous frequencies, RHF, at six polymorphic loci in SCLC (Brauch et al.1987& 1990, Daly et al.1991, Doborovic et al.1988, Johnson et al.1988, Kok et al.1987, Mori et al.1989, Mooibroek et al.1987, Naylor et al.1986 & 1987, Rabbitts

et al.1990, Sithanadum et al.1989, Yokota et al. 1989).

The frequency of heterozygosity of these markers in the normal population ranges from 30-50%. However, most of the studies on loss of heterozygosity in SCLC include the preselection of heterozygous patients where the retention or loss of alleles will be detectable. The degree of bias that this introduces into the data is difficult to assess, but, given the relatively large number of loci studied simultaneously, and the moderately high heterozygosity at each of them, is unlikely to be significant. The matched normal and tumour pairs of SCLC included in the data in figure 1 has been extracted and plotted on a second histogram, figure 2, where the number of normal patients analysed is shown and compared to the number retaining heterozygosity in the corresponding tumour.

It is clear from the figure 1 that the relative heterozygosity frequency, RHF value of all 3p markers is depressed to less than 20% in SCLC. Figure 2 illustrates the reduced numbers of heterozygous tumours in informative patients supporting the pooled RHF data.

This is in accord with cytogenetic observations that the deletions of chromosome 3 found in SCLC are usually large but consistently include the 3p21 region (Whang-Peng et al. 1982a&b, Buys et al.1983, Falor et al.1985). The RHF of both RAF and D3F15S2 is markedly lower that that of other 3p loci. Unfortunately the single patient retaining heterozygosity at D3F15S2 was not assessed for loss of the distal markers, RAF and THRB. However, at least one patient informative at RAF in which THRB is retained had lost D3F15S2, suggesting either an interstial deletion removing D3F15S2 or a double one removing RAF as well. No single tumour has been found which supports the model of a single deletion including RAF and excluding D3F15S2.

The retention of two alleles in a patient homozygous at the D3F15S2 and THRB loci demonstrated by densitometry analysis by Daly et al. (1990) has not been included in the relative heterozygosity frequency data. This deletion maps proximally of D3S4 to

include D3S30, D3S4 and D3S3. A submicroscopic homozygous deletion at the D3S3 locus has been shown in one SCLC cell line (Rabbitts et al. 1990). The modal number is high and includes four copies of chromosome 3 all with a deletion around the D3S3 locus. These two cases illustrate uncharacteristic locations of the SCLC deletion. However, this may represent an additional region involved in the malignancy in at least a proportion of tumours.

Retention of both alleles is lowest for markers, D3S32, D3F15S2, THRB and RAF. Although the sample sizes are lower for the outer markers, D3S32 and RAF, allele loss is seen in all cases. However the considerably low RHF value for D3F15S2 and its location at 3p21 together with the cytogenetic characterisation of the SCLC deletion supports its close proximity to a tumour suppressor gene in this region.

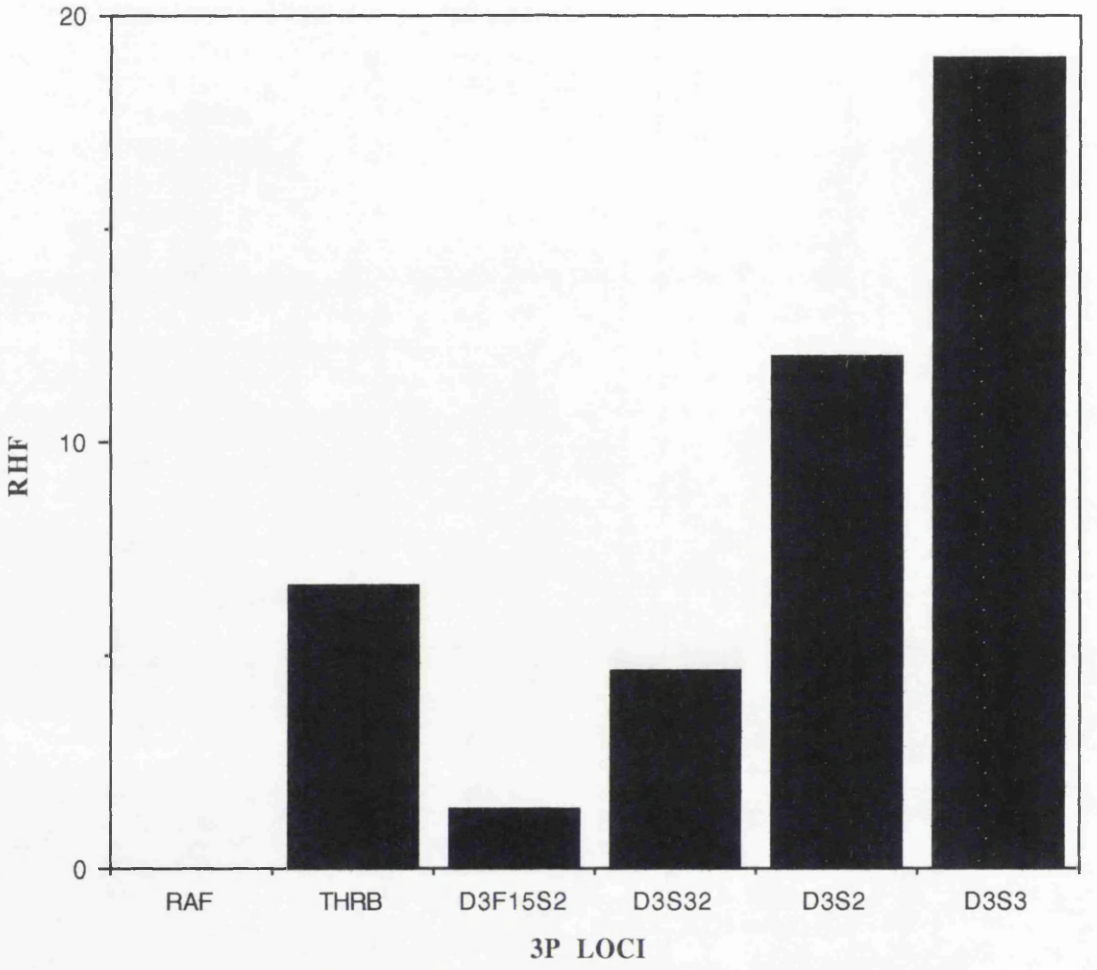

FIGURE 1 RELATIVE HETEROZYGOSITY FREQUENCIES IN SCLC AT 3P LOCI

$$\text{RHF} = \frac{\%\ \text{HETEROZYGOUS SCLC}}{\text{POPULATION FREQUENCY}} \times 100$$

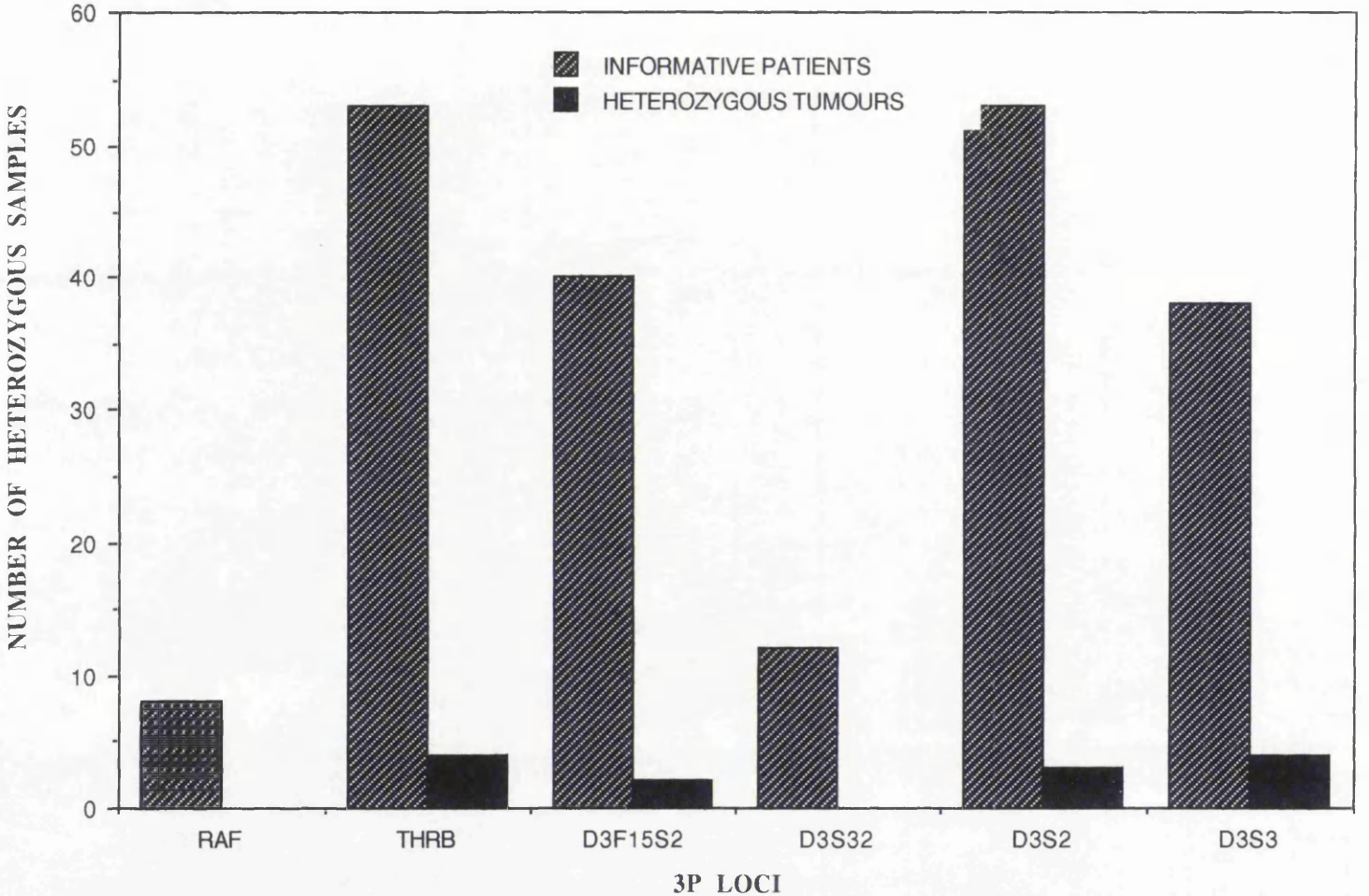

FIGURE 2 RETENTION OF HETEROZYGOSITY IN MATCHED AND INFORMATIVE SCLC

1.3.4 EXPRESSED SEQUENCES ON 3P21 IN SMALL CELL LUNG CANCER.

The use of anonymous DNA segments in RFLP studies of SCLC continues to provide valuable information on the limits of the SCLC deletion. The tumour suppressor genes cloned to date and discussed earlier illustrate the wide range of encoded functional proteins. Any prior expectations of a lung tumour suppressor gene are based on the knowledge of those already identified.

The consistent deletion at 3p21 in SCLC indicate the presence of a tumour suppressor gene whose loss of function contributes to the malignancy. Therefore any expressed sequences mapping to this region are considered potential candidates. Genes mapping to 3p21 include Aminoacylase-1, ACY-1, Aminolevulinate synthase, ALAS (ALAS 1 and ALAS 3), and a protein tyrosine phosphatase, PTPG, all of which have been assayed in a panel of SCLC samples.

1.3.4.1. Aminoacylase-1

Acy-1 is a cytosolic enzyme catalysing the hydrolysis of acylated amino acids into free amino acids and acyl groups. The study of the gene in a number of tumours has shown reduced expression in a significant number of SCLC with apparently normal expression in other neoplasms including non small cell lung cancer (Miller et al. 1989). Acy-1 was undetectable in five of the thirty-seven SCLC samples with an additional seventeen expressing less than 10% of the normal level. This finding indicates an association between Acy-1 inactivation and SCLC. No polymorphism at this locus has been shown therefore allele loss cannot be assessed. Acy-1 has been localised to the the proximal portion of 3p21 to 3p21.1 (Miller et al.1990). Long range mapping of this region using the Greig polysyndactyly translocation breakpoint in 3p21 has mapped the gene 200kb distal to the D3S2 marker (Gemmill et al.1991). If as suggested earlier, D3S2 defines the proximal boundary of the SCLC deletion then Acy-1 cannot be in the obligatory region.

The lack of expression of Acy-1 in SCLC is analogous to the esterase D enzyme in retinoblastoma. Reductions in expression of Esterse D is seen in large number of retinoblastoma patients (Sparkes et al. 1980 & 1983). Esterase D maps at least 350 kilobases from the RB gene (Ford et al. 1990). It is possible that any controlling regions, of this gene are deleted or altered in patients showing gross abnormalities of the Rb gene. Therefore it seems reasonable to propose that although Acy-1 gene is outside the SCLC deletion, it may be genetically linked to the tumour suppressor gene.

1.3.4.2. Acylpeptide Hydroylase

The expressed sequence which is closest to the polymorphic marker D3F15S2, which shows loss of heterozygosity in a majority of SCLC, is 87% homologous to acylpeptide hydroylase, APEH. This enzyme catalyses the hydroylsis of the terminal acetyl amino acid which is further processed to acetete and a free amino acid by an acylase, Acy-1. The gene lies 4 kilobases from the polymorphic D3F15S2 site and codes for a 367 kilodalton protein comprising 26 cysteine residues (Jones et al. 1991).

Genomic analysis and expression studies of 29 SCLC failed to reveal any changes in the APEH gene in all but one tumour, (Naylor et al.1989). Similarly, apparently abnormal APEH expression in sporadic renal cell carcinoma has been reported (Erlandsson et al.,1991). This supports the view of APEH is unlikely to be a key enzyme in the initiation of lung or renal tumourogenesis.

1.3.4.3. Aminolevulinate Synthase

Aminoleuvinate synthase, ALAS, catalyses the condensation of succinylcoenzyme A and glycine to form 5-aminoleuvlinate. This is the first step in the biosynthesis of haem, (Kappas et al.1983). Highest levels are seen in the liver in the hepatic and erthyroid cells. Three ALAS gene have been isolated, two of which, ALAS 1 and ALAS 3 have been shown to be housekeeping genes and map to 3p21, (Bishop et

al.1990, Sutherland et al.1988). ALAS 2 is erythroid specific and maps to the X chromosome and its deficiency is believed to contribute to some forms of hereditary anaemia (Bishop et al. 1990).

1.3.4.4. Protein-Tyrosine Phosphatase

The gene for protein-tyrosine phosphatase, PTPG, has been proposed as a candidate tumour suppressor gene in renal and small cell lung carcinomas, (LaForgia et al.1991). This suggestion originates from its functional role, allele loss in 8 of 15 tumours analysed and its map location. The latter is based on data from a panel of somatic cell hybrids, including those derived from the well characterised familial t(3;8) renal cell patient (Cohen et al. 1979). However, the data presented (LaForgia et al. 1991) is confusing and it is not clear whether the gene maps to 3p14 or 3p21.

1.4 3p AND OTHER CANCERS

1.4.1. NON-SMALL CELL LUNG CANCER

Cytogenetic studies of NSCLC have been limited over the last decade because of the difficulty in establishing cell lines and obtaining analysable karyotypes. The reports of the cytogenetic abnormalities in these tumours do not reveal a consistently deleted region. However, frequent interstitial or terminal deletions on the short arm of chromosome 3 have often been observed.

Following the identification of a interstitial deletion on chromosome 3 in SCLC ,(Whang-Peng et al.1982a&b), studies of this region were stimulated in NSCLC. 3p deletions were identified in three tumours, one from each histological type, SQC, LCC and ADC. The precise location of this deletion could not be characterised but evidence for the inclusion of the 3p14-23 region was demonstrated (Zech et al.1985). Two lung adenocarcinomas were karyotyped and deletions of observed in both cases, (Rey et

al.1987).

The frequency of allele loss at a number of 3p loci have been reported in NSCLC.(Brauch et al. 1987, Kok et al. 1987, Rabbitts et al. 1989, Becker at al. 1987, Weston et al. 1989). Much of this molecular data has been presented as part of larger studies which have included small cell type tumours. Reduction to homozygosity in at least one 3p marker has been shown in approximately 25-50% of NSCLC, (Becker et al.1989, Weston et al.1989, Leduc et al.1989). However, the 3p markers showing allele loss in five of six adenocarcinomas studied suggests a similar sized deletion to that in the small cell type (Yokota et al.1987). In one study allele loss at the D3F15S2 locus was reported in all NSCLC studied. However, this study did not include other 3p markers and therefore provides no evidence about the magnitude of the deletion (Kok et al.1987). An extensive study of 44 informative patients with nine polymorphic probes has revealed a high frequency of allele loss on 3p, (32/44). Here retention of both alleles at the D3S3 locus in two patients is reported suggesting a potential proximal boundary for the deletion. The data indicates the frequent loss of alleles distal to 3p13-14 and defines the minimal deleted region as 3p13-14-3pter (Rabbitts et al.1989).

The interpretation of this data is difficult as allele loss is not consistent for any one marker. This may reflect the greater chromosomal abnormalities observed in these tumours making the identification of a deleted region on chromosome 3 difficult. The evidence for a proximal boundary is convincing but the abundance of terminal deletions does not allow for a distal end to be identified.

Similar deletions on the short arm of chromosome 3 would support the idea of a common origin for the two major lung types. The higher number of terminal deletions and the illustration of larger interstitial deletions in the NSCLC type, would suggest an instability mechanism during the development of this malignancy causing breakage and subsequent loss of a larger proportion of 3p material.

Malignant mesothelioma represent a rare neoplasm with convincing evidence for its association with asbestos exposure. Cytogenetic deletions have been identified in a number of tumours including 3p14-21. Molecular analysis has defined the deletion further to the 3p21 region (Popescu et al.1988, Van den et al.Berg 1989, Fletjer et al.1989).

1.4.2 RENAL CELL CARCINOMA

Renal cell carcinoma, RCC, is a common adult malignancy of the kidney occurring in familial and sporadic forms at a combined frequency of 3%. Cohen et al.(1979) first described a three generation family with mortality from metastatic renal cell carcinoma in which members with an inherited chromosome translocation t(3;8) (p21;q24), were predisposed to the disease. Speculations were made that damage to genes had occurred at the rearranging loci which were responsible for the tumour development. Using high resolution banding the breakpoint was redefined as 3p14.2.(Whang-Peng et al.1984). Following this, Pathak et al. (1982) reported a 3;11 chromosomal translocation in one patient with familial renal cell carcinoma, with a breakpoint at 3p13-14. The translocaton was limited to the tumour cells, the individual being constitutionally normal.

The emergence of a consistent breakpoint with the possibility of a deletion of DNA sequences in 3p13-14 region in RCC in familial and some sporadic cases of renal cell carcinoma, stimulated detailed karyotypic studies. Cytogenetic analysis of eight sporadic cancers detected clonal rearrangements of chromosome 3 including gross deletions and translocations with breakpoints in the 3p12-14 region (Van der Hout et al.1988). An isochromosome 3p was observed in one tumour but no apparent involvement of this region was detectable in two of the samples. (Yoshida et al.1986). Further illustrations of the importance of this region came from a study of nine patients with clear cell RCC , six of which showed clonal alterations at 3p12-14. However it was speculated that alterations of the region were confined to the clear cell type.

(Carroll et al.1987). Cytogenetic studies of an apparently constitutionally normal patient with sporadic RCC identified a pericentric inversion with material deleted from 3p11-pter in addition to inversions on other chromosomes (Szucs et al.1987). An interesting study of karyotypes in 26 sporadic cases suggested that the common cytogenetic event as trisomy 7 with the limited involvement of chromosome 3.

The analysis of breakpoints in a number of renal cell tumours revealed the consistent involvement of the 3p12-3p14 region. (Kovacs et a1.987). Cytogenetic studies of sporadic renal cell carcinoma have demonstrated terminal deletions of the short arm of chromosome 3 (3p13-pter) in 95% of the samples studied (Kovacs et al.1989).

Investigations of allele loss in sporadic RCC using polymorphic markers from 3p have been used to define the limits of the deletion. Two markers mapping to 3p21, D3F15S2 and D3S2 were particularly informative for 18 tumours analysed. Loss of heterozygosity was observed in all 11 informative tumours for the D3F15S2 marker, with the frequent retention of both alleles of the D3S2 marker. However, a reduction of intensity of one D3S2 allele was evident and interpreted as being due to contaminating normal tissue; loss of heterozygosity at this locus was therefore suggested (Zbar et al.1987). Further reports of allele loss at the D3F15S2 in three sporadic tumours supported the inclusion of this locus (Kovacs et al.1988, Van der Hout et al.1988, Bergerheim et al.1989). Bergerheim showed retention of both D3F15S2 alleles in only two of the thirteen informative tumours studied.

Additional markers on 3p have been used to study allele loss in RCC including D3S4,D3S3, D3S32, THRB and RAF (Bergerheim et al.1989, Kovacs et al.1988, Anglard et al.1991 Ogawa et al.1991). The results of RFLP studies of 41 RCC patients have demonstrated potential proximal and distal boundaries of the deletion, D3S2 and THRB respectively (Van der Hout et al.1991a). Combining these data with those from two earlier studies (Zbar et al.1987, Kovacs et al.1988), the smallest deleted region appears to be in the interval between D3S2 and THRB. The additional

use of the D3S32 polymorphic marker, within this interval, in a more recent study has further defined the limits of the deletion with D3S32 and THRB as the flanking markers (Van der Hout et al.1991b).

This is in contrast to familial RCC where molecular analysis has illustrated the translocation t(3;8) breakpoint to be flanked by the D3S3 and D3S2 markers (Gerber et al.1986, Van der Hout et al.1991a). If, as is suggested this translocation breakpoint on chromosome 3 is responsible for the inherited predisposition to RCC, it would appear that distinct genetic loci at 3p21 and 3p14.2 are involved in the sporadic and familial forms respectively.

1.4.3 VON HIPPEL-LINDAU SYNDROME

Von Hippel Lindau syndrome (VHL) is an hereditary precancerous disorder with complex manifestations in a number of organs. It is an autosomal dominant trait highly variable and pleiotrophic in expression. The lesions have an earlier of age of onset than corresponding non familial tumours with an increased susceptibility for malignancy in a number of tissues. Individuals with VHL may develop tumours in the eye, brain, spinal cord, kidney, pancreas, adrenal gland or epididymis. Eye lesions occur in 60% of patients at any age from infancy through to eighty or ninety years, half of which progress to retinal detachment and blindness. Lesions of the central nervous system are common affecting the cerebellum, spinal cord, brainstem and cerebrum. Pancreatic cysts, phaeochromocytomas, epididymal cysts and associated malignancies arise in 40% of VHL patients (Lamielle et al. 1989).

The VHL disease was first mapped to a region of chromosome 3 by Seizinger. et al (1988). Linkage to the *c-raf* oncogene at 3p25 was reported with a positive lod score of 4.38 through the analysis of nine large affected pedigrees. Crossovers were observed between VHL and *c-raf* illustrating that VHL is not a mutation in *c-raf* itself. Confirmation of linkage was obtained by Vance et al. (1990) when THRB was used in addition to *c-raf* and VHL in multipoint analysis.

Renal cell carcinoma constitutes a frequent cause of death in VHL patients, presenting at an earlier age than in sporadic cases of this tumour type. This stimulated a number of studies using markers close to the *c-raf* gene in addition to those from the proximal region 3p14-21.

Tory et al (1989) examined seventeen renal cell tumours from seven VHL patients using the available RFLP markers on 3p. Loss of heterozygosity was observed at several loci mapping from 3cen-3p26 including the *c-raf* region. Patients with more then one tumour were shown to lose the same 3p allele suggesting that the same chromosome was deleted in the different tumours.

Cytogenetic analysis of the constituitional karyotypes of VHL patients have been documented by King et al.(1987) and Decker et al.(1989). The earlier study reports a deletion at 3p14 as the only visible change. Decker describes a VHL/RCC patient with a balanced translocation, t(X;3), with loss of 3p material. The RFLP analysis illustrated loss at the D3F15S2 locus .

Further localisation of the VHL locus by genetic mapping around two markers, D3S18 and D3S191 showed them to be closely linked and distal to the *c-raf* locus at 3p25 (Seizinger et al.1991). The data supports the presence of the VHL gene in the *c-raf* and D3S18 interval (Maher et al. 1991). The close linkage to the recently isolated polymorphic marker, D3S601 within this interval, maps the VHL to a 3.5 megabase interval (Lerman et al. 1991).

Controversy has arisen over the locus heterozygosity of the VHL gene. Evidence for a complex genetic locus for VHL comes from two studies; the first of 92 patients from 29 kindreds (Neuman et al1991) illustrating a clustering of specific lesions in individual families. An additional report of 41 families provides further confirmation and suggests that mutant alleles exist for each tissue abnormality. However, tests for locus heterogeneity in 36 families has proved negative (Maher et al. 1991).

1.4.4. 3P AND OTHER MALIGNANCIES

The karyotypic studies of solid tumours have illustrated the frequent involvement of chromosomes 5,11,13,17 and 18. Tumour suppressor genes on each of these chromosomes have been cloned on the basis of tumour specific alterations. Molecular studies support the concept of their inactivation in specific cancers and their extended role in unrelated malignancies. The specificity of tumour suppressor genes on the short arm of chromosome 3 is unknown. However, RFLP analysis of 3p markers of malignancies outside the lung indicate at the very least that of this region contributes to the malignant phenotype.

Cytogenetic studies of ovarian tumours has revealed deletions of the short arm of chromosome 3 (Whang-Peng et al.1984, Pannani et al.1985). Other genetic losses have been identified at 6q and 11p (Trent et al.1981, Lee et al.1989). The frequent loss of heterozygosity at D3S2, D3F15S2 and *c-raf* in ovarian tumours has been shown (Yokota et al. 1989, Ehlen et al.1990). This indicate the loss of DNA sequences at loci which may contribute to the development of this tumour but may not represent the primary event.

RFLP analysis at the D3S2 locus of carcinoma of the uterine cervix supports the loss of DNA sequences on the short arm of chromosome 3. However with the limited cytogenetic knowledge and the number of uninformative markers in this study , the significance of this loss is unclear (Yokota et al.1989).

Molecular analysis of breast carcinoma has illustrated the non random involvement of at least four chromosomal loci (3p, 11p 17p, and 13q) in the progression of this tumour. Allele loss of markers on 3p is confined to the 3p14-21 region and reflects the instability of the tumour which additional changes in the loss of allele at loci harbouring tumour suppressor genes (Devilee et al.1989).

1.5. AIMS OF STUDY

The overall objectives of this project is to generate physical mapping data of the 3p21 region. The aims of this thesis can be briefly summarised as follows:

1) To characterise a panel of somatic cell hybrids established from material containing deletions, translocation breakpoints and small fragments of the 3p21 region.

2) Genomic analysis around a gene isolated from 3p21

3) Long range physical mapping around the D3F15S2 locus and other genes in this region.

4)The genomic analysis of SCLC DNA in the 3p21 region by pulsed field gel electrophoresis.

Each of these topics is presented in a separate chapter of this thesis, following the material and methods section.

2.MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. CHEMICALS

Unless other stated general laboratory chemicals were obtained from Merck Ltd., Poole, Dorset. All buffers comprised "Analar" or biochemical grade reagents and, where appropriate, were sterilised by autoclaving at 15lbs psi 121°C for 30 minutes.

2.1.2. ENZYMES

Restriction endonucleases were obtained from NBL UK or BRL Ltd. UK

T4 ligase was supplied by BRL UK.

RNase Free DNase (10 units/µl) supplied by Stratagene (California)

2.1.3. POLYMERASE CHAIN REACTION

Taq polymerase and buffer was supplied by Promega or NBL. Nucleotides were obtained in solution from Pharmacia, UK. For all PCR reactions a Hybaid thermocytometer was used.

2.1.4 HOST CELLS

XLI-Blue (Epicurian Coli) supplied by Stratagene (California)

JM109/JM101/DH1 were available as glycerol stocks in the laboratory

2.1.4. PULSED FIELD GEL ELECTROPHORESIS

All pulsed field runs were carried out on LKB Pharmacia-"Pulsaphor" using the manufacturer's hexagonal electrode array.

Lambda-PFGE marker was obtained from LKB Pharmacia, UK.

2.1.5. TISSUE CULTURE

RPMI medium and other reagents were obtained from Gibco Ltd.

2.1.6 SOURCE OF PROBES

pACY-1 was a kind gift from York Miller.

ZnFP16 was a kind gift from Dr Peter Little, Imperial College London.

2.1.7 SOURCE OF LYMPHOCYTES FOR ESTABLISHING HYBRIDS.

GPS cell line was obtained from M.Farrall.

Blood lymphocytes from the Deakin patient were obtained from I.Glass. The patient is referred to as 'Deak'.

2.1.8. SCLC LUNG LINES

Lines prefixed "GLC" were a kind gift from Pr. C.H.C.M. Buys. The remaining lines were a kindly donated by Dr. P.H. Rabbitts

2.1.9. CHROMOSOME 3 HYBRID.

The somatic cell hybrid containing a single copy of chromosome 3 as the only human component was obtained from Eric Stanbridge and is referred to as hybrid, 429.

2.2 METHODS

2.2.1 CULTURING OF CELL LINES

2.2.1.1 Small Cell Lung Lines

2.2.1.1.1 Culturing from liquid nitrogen stocks

The Groningen small cell lung lines, prefixed GLC (appendix) and those obtained from P.H. Rabbitts, were grown from stocks frozen in liquid nitrogen in SCLC medium 1 (appendix). The cells were thawed immediately at 37°C and poured into a vial containing 8-10ml medium. The cells were pelleted at 1K for 5 minutes. The medium was aspirated and replaced with 5ml of fresh medium. The cells were resuspended gently to avoid the disaggregation of cell lumps. The suspension was then poured into a $25cm^3$ tissue culture flask. The caps were released slightly once placed in a 5% CO_2 incubator.

The medium was changed every second day by aspirating and replacing with 5ml of medium for surface growing cells. However, cell lines growing in suspension were pelleted as described previously and resuspended in 5ml fresh medium prior to returning the cells to the flask. For slow growing lines, 3ml of medium was added each time to increase the concentration of growth factors available to the cells.

2.2.1.1.2 Dividing Cells.

Cells were divided into two flasks when at approximately two- thirds confluent. For surface growing lines, the medium was aspirated and 1ml of 1% Trypsin/0.5 M EDTA was added and incubated at room temperature for 5-10 minutes. Once cells were detached from base of flask, half the suspension was pipetted off into a fresh flask of the same cells. 5ml of media was then added to each half. Once confluent, lines could be established in larger volumed flasks.

2.2.1.1.3 Freezing cells

Once two $75cm^3$ flasks were established, a frozen stock was made from one flask as follows.: Cells were pelleted as described previously and 1.5ml of freezing media was added and immediately transferred to a 2.0ml ampule. The ampule was sealed with an open flame and kept on ice during transfer to liquid nitrogen. The freezing media, dimethylsulphonoxide, DMSO, is harmful to growing cells and therefore as soon as it is added to the cells the suspension was kept on ice.

2.2.1 2. Establishment of hybrid cell cultures

The method used for hybrid cell fusion is that described in 'Cell & Tissue: Laboratory Procedures'. In brief, the mixed cell pellet, human and chinese hamster, a23, is suspended in 50% w/v PEG 6000. The fusion is plated and maintained in HAT medium for DNA isolation.

2.2.2 EXTRACTION OF DNA FROM HYBRID AND SCLC LINES

DNA was extracted from cell lines by removing two-thirds of the cells from a confluent $75cm^2$ flasks. 10ml of SE buffer was added and the cells gently resuspended. To lyse the cells SDS at a final concentration of 1% and proteinase K at 0.2mg/ml was added and the suspension incubated at 55°C overnight. The following day sodium chloride was added to final concentration of 1.5M and gently mixed. An equal volume of chloroform was added and rotated on a 'Daisy Wheel' for 30-60 minutes. To split the two phases the suspension was centrifuged at 2.5K for 10 minutes. The top layer was removed and precipitated with an equal volume of isopropanol. The DNA was hooked out and washed with 70% ethanol and finally resuspended in TE.

2.2.3 PROBE PREPARATION

2.2.3.1 Preparation of Competent Cells

A fresh overnight culture of an appropriate E.coli strain (JM109, 831 or DH1) was grown in 10ml L-Broth (appendix). The cells were diluted in 200ml of fresh sterile L-Broth and grown at 37°C with agitation to ensure thorough aeration until an absorbance reading of 0.4-0.5 at 550nm was obtained. The suspension was immediately chilled on ice and then centrifuged at 5K at 4°C for 10 minutes. The supernatent was discarded, the pellets resuspended in 100ml fresh ice-cold sterile 100mM $CaCl_2$ and the mixture was homogenised by sucking gently up and down with a wide bore, cold pipette. The solution was then incubated on ice for 30 minutes with occasional swirling and then centrifuged as before. The cells were gently resuspended in 10ml ice-cold 100mM $CaCl_2$ to which was added 15% w/v glycerol. 0.2ml aliquots were made in sterile microcentrifuge tubes and stored at 4°C for 12-24 hours for high transformation efficiency. The alliquots were then stored at -70°C. Using this method competency was maintained for several months.

2.2.3.2. Plasmid Transformation

A 0.2ml alliquot of frozen competent E. coli was thawed slowly on ice. To this was added 10-50ng plasmid DNA and the mixture gently agitated and incubated on ice for 45 minutes. The cells were then heat shocked for 2 minutes at 42°C. 0.4ml of L-Broth (non-selective media) was added and incubated shaking at 37° C for 1 hour. 200µl was plated out using the appropriate antibiotic and IPTG and X-gal (appendix) for blue/white selection. White recombinants were picked and grown in 3ml of L-Broth with the appropriate antibiotic overnight. A small scale "mini prep" DNA isolation was then carried out.

2.2.3.3. Small Scale Plasmid/ Cosmid Preparation (Alkali Lysis Method, Birnhoim & Doly 1979)

A 3ml overnight culture was divided into two microcentrifuge tubes and spun for 1 minute in a microcentrifuge. The supernatent was aspirated and 100µl of lysozyme buffer was added (appendix). The tube was vortexed to resuspend the cell pellet in the buffer. 200 µl of freshly prepared alkaline SDS was added and the tube gently inverted to mix. The mixture was incubated at room temperature for 5 minutes after which 150µl of ice-cold acid KOAc was added and the tube inverted. After 5 minutes at room temperature the mixture was centrifuged for 3 minutes. The DNA containing supernatent was transferred to a fresh tube and 250µl of buffer-saturated phenol was added. The tube was vortexed, centrifuged for 1 minute and the supernatent transferred to fresh tube. Two volumes of absolute ethanol was added and the tube vortexed. After 5 minutes at room temperature the tube was spun for 10 minutes. The supernatent was aspirated and the DNA dried in a vacuum. The pellet was dissolved in 30µl of TE (appendix) and digested as follows:

15µl DNA in TE

4µl 100mM spermidine

4µl restriction buffer (supplied by manufacture with each enzyme)

20-30 units restriction enzyme

0.5µl RNase (10mg/ml)

12.5µl ddH_2O

The mixture was incubated at 37°C for 90 minutes and electrophoresed as described in 2.2.4.1.

2.2.3.4. Large Scale Plasmid/Cosmid Preparation (Alkali lysis method)

A 10ml aliquot of L-broth (appendix) containing the appropriate antibiotic (appendix) was inoculated and grown up overnight . This culture was used to prepare 1ml glycerol stocks (15% v/v glycerol) for long term storage at -70°C and to seed (1/100) a 250ml L-broth culture with antibiotic. The culture was grown overnight at 37° with aeration and harvested the following day at 6000rpm for 10 minutes at 4 °C. The supernatent was discarded and the pellets resuspended in 10ml of lysozyme buffer (appendix). A wooden applicator was used to break down the pellet to a thick slurry and the suspension transferred to a 250 ml conical flask . To this was added 20 ml of freshly prepared alkaline SDS solution (appendix) and left to incubate on ice for 10 minutes. 15 ml of acid KOAc (appendix) was added and again the left to stand on ice for 10-30 minutes. Following lysis, centrifugation was carried out at 10000rpm for 15 minutes at 4°C. The plasmid containing supernatent was retained and the pellets containing the cell debris discarded.

The volume of supernatant was measured and 0.6 volumes of propan-2-ol was added to precipitate the DNA. Following incubation at room temperature for 10 minutes, the precipitate was pelleted at 10000rpm for 15 minutes at room temperature. The pelleted DNA was washed with 20ml of 70% ethanol and centrifuged at 10000rpm for 5 minutes. The pellet was drained thoroughly and resuspended in 11ml of 10x TE (appendix).

2.2.3.5. Plasmid Purification by CsCl Gradient Centrifugation

As explained above the plasmid pellets were suspended in 11ml TE buffer. To this was added:

0.44ml 0.2M K_2HPO_4 pH 7.5

11.44g solid Caesium Chloride

and when dissolved

1.14ml 10mg/ml ethidium bromide

The solution was mixed thoroughly and pipetted into Sorvall polyallomer ultracentrifuge tubes. A sufficient amount of paraffin oil was added to prevent leakage and the tubes sealed with a Sorvall "ultracrimp" tube sealer. The tubes were spun in a Sorvall ultracentrifuge at 45000rpm at room temperature for 18 hours.

On removal the tubes were examined under UV light and the plasmid band removed using a syringe. The ethidium bromide was removed by repeated extraction with CsCl saturated iso-amyl alcohol and the CsCl then removed by dialysis in Sartorius collodian bags (presoaked in ddH_2O) against six changes of distilled water. The plasmid DNA was precipitated by addition of 1/10 volume of 4M sodium chloride and 2 volumes of absolute ethanol at -20°C overnight. The DNA was pelleted by centrifugation at 10000rpm at 4°C, resuspended in 0.5ml TE and transferred to a microcentrifuge tube. A further precipitation using 1/2 volume ammonium acetate and 2 volumes of absolute ethanol was carried out at -20°C overnight or -70°C for 1-2 hours. Using a microcentrifuge the DNA was pelleted and finally resuspended in an appropriate amount of TE. Plasmid DNA was routinely stored at -20°C.

2.2.3.6. Preparation of Inserts

The concentration of plasmid DNA prepared as above was calculated by measuring its optical density at 260nm. 10µg was digested with 40 units of the appropriate restriction enzyme (appendix) in a 40µl volume.

(i) Oligolabelling: the sample was electrophoresed overnight at 25 volts on 0.8% low melting point agarose containing 100µg/ml ethidium bromide to separate vector and insert fragments. A 1Kb ladder (appendix) was loaded in a neighbouring lane. The

gel was viewed under UV light at 354nm and the insert cut out from the gel. The gel slice was transferred to a microcentrifuge tube, weighed and 3 volumes of ddH_2O added. This was then boiled for seven minutes and alliquoted into 30µl volumes and stored at -20°C.

(ii) Subcloning: the sample was electrophoresed overnight at 25 volts on 0.8% agarose containing 100µg/ml ethidium bromide to separate vector and insert fragments. A 1Kb ladder was loaded in a neighbouring lane The gel was viewed under UV light at 354nm and a slit cut in the gel under the insert fragment required. A small piece of NA45 DEAE membrane (Schleicher & Schull) was placed through the slit and under the the gel. The gel was electrophoresed further to allow the insert to run onto the membrane. The DNA was recovered from the membrane by placing it into a microcentrifuge tube and incubating it at 70°C in 400µl of 2M sodium chloride for 30 minutes. The tube was then centrifuged and the membrane discarded. The solution containing the DNA was dialysed to remove the salt using presoaked Sartorius collodian bags against 3 changes of distilled water. The volume was reduced to 50-100µl by attaching the Sartorius apparatus to a vacuum. To check the concentration of the recovered DNA, 5µl was electrophoresed on 1% agarose mini-gel with known concentrations of standard lambda DNA.

2.2.4 ANALYTICAL GELS: SOUTHERN BLOTTING AND HYBRIDISATION

2.2.4.1. Restriction Digestion of Genomic and Cosmid DNA samples

In general restriction digest mixtures were set up as follows:

(i) for genomic DNA

8µg genomic DNA

4µl 100mM spermidine

4µl reaction buffer supplied with each enzyme

40 units restriction enzyme

sterile ddH_2O to 40µl

(ii) for cosmid DNA

550ng cosmid DNA

2µl 100mM spermidine

2µl appropriate restriction buffer

2µl restriction enzyme

sterile ddH_2O to 20µl

The mixtures were spun for a few seconds in a microcentrifuge and incubated at 37°C (65°C- Taq 1) for 4 hours.

Following digestion, 4µl sucrose blue loading buffer was added to stop the reaction and the digests run at 20-25V for 8-20 hours on 0.7-1.0% agarose gels containing ethidium bromide at a concentration of 100µg/ml in TBE running buffer (appendix). A 1Kb ladder was loaded on cosmid analytical gels only The gel was visualised under short wave UV and photographed using an orange filter.

2.2.4.2. DNA Transfer (Southern Blotting)

The gel was treated in 0.25N HCL for 15 minutes to partially depurinate the DNA. 0.4N Sodium hydroxide was used to denature the gel for 30 minutes. The gel was capillary blotted onto Hybond N+ (Amersham) according to manufacture specifications using the alkali blotting procedure. Blotting times were as follows:

(i) cosmid DNA- up to three blots were taken from one gel the first after 15 minutes and the second and third after 25 and 60 minutes.

(ii) genomic DNA- one overnight blot per gel .

Following blotting the membrane was treated in 2x SSC (appendix) for a few minutes and then wrapped wet in cling film and stored at 4°C to prevent drying out.

2.2.4.3. Radiolabelling of DNA probes for Southern Hybridisation

Probes for Southern Hybridisation consisted of low melting point agarose slices (described in 2.2.3) labelled to high specificity by the random hexanucleotide priming method (Feinberg and Vogelstein 1983) using the multiprime DNA labelling system (Amersham). 30µl DNA gel slice containing approximately 25ng DNA boiled for 1 minute to melt the agarose and denature the DNA duplex. To prevent the agarose setting the gel slice was incubated at 37°C for 10 minutes and then the following reagents added:

10µl buffer (solution 1 multiprime kit)

5µl primer (solution 2 multiprime kit)

3µl α^{32}P dCTP (3000Ci mmol)

2µl enzyme (solution 3 multiprime kit)

The mixture was incubated at 37°C for 3-6 hours. To remove the unincorporated labelled nucleotides, the reaction mixture was expanded to 100µl with TE (appendix) and spun through a G-50 sephadex column at 1500rpm for 3 minutes.

2.2.4.4. Southern Hybridisation

Filters were prehybridised in Church buffer (Church & Gilbert 1984) for at least two hours at 65°C before addition of the radiolabelled probe DNA. The probe was

denatured by boiling for 5 minutes and quenched on ice for a further 5 minutes and then added to the prehybridisation mixture. Hybridisation was carried out at the same temperature for 12-24 hours after which the probe solution was then poured off and retained for future use.

2.2.4.5. Filter Wash

Filters were washed in 100mm phosphate buffer (appendix), 2% SDS and 2mm EDTA at 65°C for 15 minutes and then for a further 15 minutes in 50mm phosphate buffer, 1%SDS and 1mm EDTA. If required, an additional wash at this stringency was carried out 65°C. The filter was wrapped wet in cling film and autoradiographed at -70°C using Fuji RX-L X-ray for between one and seven days.

2.2.4.6. Removal of Probe for Rehybridisation

Filters were incubated at 65°C for 15 minutes in 500ml of 2.0mM Tris pH7.5, 1.0mM EDTA 0.1% SDS. Removal of probe was checked by autoradiography.

2.2.5 COSMID LIBRARY SCREENING

2.2.5.1 Cosmid Library Screening (first round)

For screening the cosmid library 1-2 x 10^5 cosmid/bacteria were plated on Sartorius nitrocellulose filters as follows:

Two plates of an area 24cm^2 were filled with 200ml of L-Agar each containing kanamycin at a concentration of 3µg/ml The nitrocellulose filters were placed gently over the agar plates to avoid air bubbles between the filter and agar. 1-2 x 10^5 cosmid bacteria were added to 2ml L-Broth + kanamycin and poured down one side of the filter. A sealed Pasteur pipette was used to spread the suspension evenly over the filter. The plate were inverted and incubated at 37°C overnight. The following day replica filters were made by prewetting the nitrocellulose on fresh agar megaplates and then placing them over the master filters. Double thickness Whatman 3mm filter was placed over the

two filters and firm pressure was applied. Landmarks were made to enable orientation after screening. The master plates were stored wrapped at 4°C and the replicas allowed to grow for 2-6 hours at 37°C or alternatively overnight at room temperature.

To lyse the cells and bind the DNA to the filters the replicas were treated as follows:

	time (minutes)
Double thickness 3mm filter paper soaked in 10%SDS	3
Double thickness 3mm filter paper soaked in (0.5N NaOH, 1.5M NaCl)	5
Neutralising Solution (0.5M Tris, pH7.4, 3M NaCl) -floating initially	2
-submerged	3
2x SSC, 0.1% SDS bath - firmly wiped with Kimwipe tissue	1
- final rinse	2

The filters were then blotted dry and baked at 80°C for 2 hours to bind the DNA to the filters. Prior to hybridisation the filters were rinsed in 2x SSC. Hybridisation was carried out at 65°C overnight using Denhardt's solution (appendix).

Filters were washed in 2xSSC at room temperature for 20 minutes and then at 65°C in 2xSSC, 0.1% SDS 15-25 minutes.The filters were blotted dry and autoradiographed using Fuji RX-L X-ray film at -70°C.

2.2.5.2. Second and Third Round Cosmid Screening.

Positive clones were picked into 1ml L-Broth+kanamycin. A 1:100 and 1:1000 dilution in L-Broth was made and the following volumes plated on 80mm diameter petri dishes containing L-Agar and kanamycin:

Dilution	Volume
1:100	2µl
1:100	20µl
1:1000	2µl

The plates were incubated, replica plated and hybridised as described 2.3.1. A third round was sometimes necessary in order to pick single positive colonies. Single positive clones were grown in 3ml of L-Broth and kanamycin overnight at 37°C. Small scale DNA isolations were made as described in section 2.2.3.1. Analytical gels were performed and Southern blotted and probed with the original screening probe to ensure genuinity of cosmid clones (appendix 2.2.). Glycerol stocks were made as described in 2.2.3.4. from which large scale DNA isolations were prepared

2.2.5.3.Riboprobe synthesis from Lorist B cosmids

The T7 promoter in Lorist B was used to synthesize short riboprobes from the T7 end of the cosmid insert . Owing to the poor riboprobe synthesis achieved with SP6 promoter ,where probes were required from the opposite end of the insert the cosmids were reorientated by cutting with the restriction enzyme Hind III and religated as described below.

Hind III restriction digest of cosmid DNA

2µg of cosmid DNA

4µl of restriction buffer

40 units of Hind II enzyme

4µl of 100mM spermidine

sterile water to 40µl volume

After incubation at 37°C for 3 hours the mixture was extracted with an equal volume of phenol. The supernatent was them extracted with chloroform. Two volumes of absolute ethanol was added o the supernatent to precipitate the DNA AT -70°C for 20-30 minutes. The mixture was then spun in a microcentrifuge for 10 minutes at 4C. The supernatent was aspirated and the pellet redissolved in 10µl of TE. The ligation reaction was set up as follows:

5µl of HindIII cut DNA

4µl 5x ligase buffer (BRL)

1.5 unit T4 ligase (BRL)

sterile water to 20µl volume

The reaction was incubated overnight in a water bath at 12°C. The DNA was transformed using competent 831 cells as described in 2.2.3.1 and 2.2.3.2. Small scale isolations of DNA and analytical gels were carried to ensure authenticity of clone prior to large scale DNA isolation and riboprobe synthesis.

Riboprobes were synthesised using tips, microcentifuge tubes and water pretreated with diethylpyrocarbonate. Rsa I was used to cut the 5µg of cosmid DNA for each synthesis (as described above except with Rsa I and not HindII).

Following incubation at 37°C for 3 hours the DNA was purified by phenol and chloroform extractions as described above. Following ethanol precipitation the pelleted DNA was resuspended in 7.5µl TE and 7.5µl DEPC treated water. The riboprobe synthesis reaction was set up as follows:

7.5µl DNA

1.5 units T7 polymerase (BRL)

6µl T7 buffer (BRL)

1µl 100mM dithiroretiol

2 units RNasin (Stratagene)

1µl CTP mM(Pharmacia)

1µl CTP mM(Pharmacia)

1µl CTP mM(Pharmacia)

1µl α^{32}P UTP

sterile water to 30µl volume

Transcription was allowed to proceed for 2-5 hours at 37°C. The reaction was then ethanol precipitated with two volumes of absolute ethanol overnight at -20°C.

The following day the DNA was pelleted by spinning in a microcentrifuge for 10 minutes. The pellet was resuspended in 40µl TE, 50µl 3xSSC and 10µl 200mM vanadyl ribonucleosidase complex, (VRC). VRC is added to inhibit ribonuclease activity.

The synthesised probe was used immediately in hybridisation on Hybond N+ filters with Church Buffer or nitrocellulose filters with Denhardt's as described in 2.2.4.4. with the addition of VRC to a final concentration of 10mM.

2.2.6 PHAGE LIBRARY SCREENING

2.2.6.1 Mouse cDNA Library

A mouse cDNA 'lambda ZAP' library (Stratagene) at a concentration of 2x 10^{10} plaque forming units per ml was plated on eight plates, (area 24cm^{2}). Host XL-1 Blue (see 2.1.4) were grown overnight with 0.2% maltose in order to induce the maltose operon which includes the phage attachment site. 400µl of XL-IBlue were added to 400µl Mg-Ca solution followed by an aliquot of the library to give a final number of plaques per plate of approximately 40,000 to 50,000. The mixture was gently agitated and incubated for exactly 10 minutes for adsorption of phage. The time was limited to 10 minutes to prevent premature lysis which would inflate the titre. 20ml of Top-Agar at 45-50°C was added, mixed and quickly poured over the L-Agar plate. Once set the plates were incubated overnight at 37°C .

The following day the plates were placed at 4°C for 2 or more hours to harden the agar before taking phage lifts. Nitrocellulose filters (Schleicher &Schull) were prewetted in 3xSSSC and then placed over the phage plates for 1.5 minutes Landmarks were made before removal of filter. The filter was then treated as follows:

Pad of denaturing solution (0.5N NaOH, 1.5N NaCl)	3 minutes
Tray of Neutralising solution (0.5 Tris pH7.4, 3M NaCl)	3 minutes

The filters were baked at 80°C, hybridised and washed as described in 2.2.5 for cosmid replicas.The filters were blotted dry and autoradiographed as described in 2.2.5.1.

Positive clones were picked and into 400µl phage buffer (appendix). A 10,000 dilution was made using phage buffer and 10µl and 50µl alliquots were plated out on petri dishes

as described above except using 100µl of fresh overnight XL-1 Blues and 100µl of Mg-Ca solution.

2.2.6.2 Preparation of Plate Lysate

After a third round of screening single colonies were picked into 100µl of phage buffer. For a confluent 80mm radius plate, 1/10th of a plaque was required. As described above host XL1-Blue were used to plate out 10µl of the plaque. Top agarose was used in this instance to facilitate satisfactory digestible DNA preparations.

Following incubation at 37°C overnight the phage were eluted in phage buffer by adding 5mls to the phage plate and placed it on a rocker for 2 hours. This was poured into a glass bijou and 500µl of chloroform added. This was referred to as the plate lysate and stored for several months at 4°C.

2.2.6.3. Preparation of Liquid Lysates

For a liquid lysate host XL1-Blue were grown overnight in NZYDT (appendix) with 0.2% maltose and 10mM $MgSO_4$. 2.5ml of the host cells were used to seed 100ml of NZYDT with 10mM $MgSO_4$. The culture was vigorously shaken (> 250rpm) at 37°C until an optical density reading of 0.2 (absorbance 600nm) was reached (approximately 90 minutes). An excess of plate lysate, 2.5ml, was added and further incubated at 37°C until lysis occurred (up to 4 hours). Chloroform was then added to a final concentration of 1% and left overnight at 4°C.

2.2.6.4. Preparation of Phage DNA.

The liquid lysate described above was pelleted at 7.5K at 4°C for 30 minutes.The DNA containing supernatent was poured into a conical flask and the cell debris discarded. 6g of sodium chloride was gently mixed in until dissolved. Polyethelyne Glycol (PEG 6000) was added to precipitate the phage DNA. 7.5g of PEG was then added and stirred gently in the cold room (4°C) for 30 minutes and then left to stand for at 1 hour

The mixture was then centrifuged at 7.5k for 15 minutes at 4°C to pellet the DNA. The supernatent was discarded and the pellet dissolved in 5ml of phage buffer. RNase was added to 3µg /ml and DNase to 5µg /ml and incubated for 30 minutes at 37°C. The DNase was then inactivated at 65°C for 10 minutes prior to adding SDS for cell lysis. This prevented any exposure of phage DNA to active DNase. 0.1ml of 10%SDS and 0.25ml 100mM EDTA was added and the mixture incubated at 65°C for 15 minutes.To remove proteins and other cell matter, two phenol extractions and one chloroform extractions were carried out.

The DNA was precipitated by the addition of 2 volumes of absolute ethanol and 1/10 volume of 4M NaCl at -20°C overnight. A further precipitation was carried out the next day using 0.5 volumes of 7.5M ammonium acetate and two volumes of absolute ethanol. The DNA was then dissolved in an appropriate volume of TE buffer.

2.2.6.5 Subcloning of cDNA clones

Clones from "Lambda ZAP" were excised by digesting with EcoRI and Hind III and inserts prepared as described in 2.2.3.6. The cloning vector pUC18 was used to subclone these inserts. The vector was digested at 37°C for 2 hours as follows:

2µg vector DNA

40 units EcoRI

40 units Hind III

4µl restriction buffer

4µl 100mM spermidine

sterile water to 40µl volume

The vector DNA was purified by phenol/ chloroform extraction. The supernatent

removed and ethanol precipitated with two volumes of absolute ethanol at -20°C overnight. The DNA was resuspended in 30µl TE

The ligation mix was set up as follows:

5µl vector DNA (approximately 100ng)

500ng insert DNA

5µl 5x ligase buffer (BRL)

1 unit T4 ligase (BRL)

TE up to 25µl volume

The reaction mix was incubated at 12°C overnight in a polystyrene water bath. 10µl of ligated mixture was transformed using competent JM109 or DH1 cells as described in 2.2.3.1 and 2.2.3.2 Small scale DNA isolations were made to check inserts of recombinants (2.2.3.3).

2.2.7. POLYMERASE CHAIN REACTION

2.2.7.1 Oligonucleotide Synthesis

Oligonucleotide were synthesised on a 'PCR-mate' (Applied Biosystems) and purified using ammonium hydroxide. 2ml of ammonium hydroxide were taken up in a 10ml syringe and attached to the oligo column. To the opposite orifice of the column an empty 10ml syringe was attached The oligo was gradually saturated by passing ammonium hydroxide back and forth through he column. This was left at room temperature for 2-3 hours with intermittent flushing of ammonium hydroxide through the column.

The eluate was placed in 7ml sterile bijou and the column and syringes rinsed through with more ammonium hydroxide which was also added to the bijou. The

oligo was incubated at 55°C overnight. Prior to drying of the oligo the oligo was cooled to 4°C . A Gyrovap was used to dry the oligo to a gel-like form.

A G25 sephadex column was used to purify the oligo gel by washing it though with sterile TE. Three fractions were collected, the second containing most of the oligo. The absorbance at 260nm of each fraction was assayed and the concentrations calculated based on the following standardisation:

For each oligo:

50pmol. of a 20mer =320 ng

50pmol. of a 25mer =400ng

2.2.7.2 Polymerase Chain Reaction Conditions -General Method (Saiki et al., 1988)

PCR was carried out using *Taq* polymerase from Promega or NBL. Reactions were carried out in 50µl volumes except for the D3F15S2 set of primers where a 20µl volume was used. Primers were titrated for optimum magnesium concentrations and recorded in table 2. Negative controls were set up on each occasion. Table 2 presents the primer sequences used for each marker.

Each reaction contained: 500ng-1000ng of genomic DNA

0.2µ m of each primer

200µm each dNTP (Pharmacia)

2µl 10x PCR buffer (Promega/ NBL) containing:

15mM $MgCl_2$

500mM KCl

10% Triton X 100

additional $MgCl_2$ if necessary (1M stock)

0.2 units Taq polymerase

MARKER	LOCATION	PRIMER SEQUENCE	ANNEALING TEMP C	Mg++ (mm)	AMPLICON (base pairs)	REFERENCE
THRB	3p24.2-p24.3	5'-TTCCAGGTGGCTGAGAGATAT-3' 5'-CCACTCCTGATATTCTAAAGGGG-3'	6O	2.0	226	Naylor 1991
D3S11	3p22-p23	5'-AAGGCAGATCCAAGTACTCA-3' 5'-CATAAGCAACTGATTAGAACC-3'	5O	2.0	265	Naylor 1991
D3S1100	3p	5'-GGTTTCATATACCATCAATCCCAC-3' 5'-GTACACCATCATGAGGAGTCTGG-3'	55	1.5	179	Naylor 1991
D3F15S2	3p21.2-p21.3	5'TAGATCTGAGCCCGGTGCCTGGGCCA CGAA-3' 5'GAGTCCATGGCAGCAGTTGGCAACCA TGGC-3'	65	1.5	535	Ganly 1991
ALAS 1	3p21.2-p21.3	5'-TGCAACTTCTGCAGGAGG-3' 5'CAACTTGAAGACCATCTGG-3'	55	1.5	162	Naylor 1991

Table 2: Primer sequences and conditions for markers used for PCR.

MARKER	LOCATION	PRIMER SEQUENCE	ANNEALING TEMP C	Mg++ (mm)	AMPLICON (base pairs)	REFERENCE
ALAS 3	3p21.2-p21.3	5'-CTGAGCATGACCTCAATTATT-3'	40	1.5	135	Bishop 1990
		5'-AAGCAGATTTATTTCCAGGAC-3'				
ARH12	3p21	5'-TTAATCTTAGTGTATGATTACTGGCCT-3'	65	2.0	209	Naylor 1991
		5'-TCCTTGAATTAGCGCCTGGTGTGTC-3'				
ZNF35	3p21	5'-CTCTGATTGCTGTCCTCTCTTGTC-3'	60	1.5	196	Naylor 1991
		5'-CTCTGCATTAATCTCSGCTGCTGA-3'				
D3S2	3p21.1	5'-CCCTAGAGCAATGTTAATTCTTCT-3'	55	2.0	139	Naylor 1991
		5'-TCCTGCAAGTAGTTGCACTTC-3'				
SI	3q25-q26	5'-GATTGGATCTCTTCCTTTTGATGAC-3'	50	1.5	170	Abbott 1991
		5'-CTGGGGGAGGAAAACTCAATAAAAT-3'				

Table 2 continued: Primer sequences and conditions for markers used for PCR.

The reaction mix was denatured at 95°C for 5 minutes prior to the addition of the *Taq* polymerase. This was followed by 30 cycles of amplification each cycle consisting of 92°C for 30 seconds, an annealing temperature and time specific for each primer set (see table 2) and 72°C for 1 minute.

The products were run on 3% Nusieve 3:1 gel with a 1kb ladder and a ϕX marker (appendix) stained with ethidium bromide.

2.2.8 PULSED FIELD GEL ELCTROPHORESIS (PFGE)

2.2.8.1 Preparation of Samples

2.2.8.1.1 Human DNA Samples

Analysis of human DNA using PFGE requires high molecular weight DNA. The method of DNA preparation must eliminate shear breakage and enzymatic degradation. The simplest method consists of embedding living cells in blocks of low melting point agarose (0.5%) followed by detergent lysis and proteinase K treatment. The method described below is based on the Schwatz and Cantor method (1984).

10ml of freshly taken blood was incubated on ice for 5-10 minutes. Three volumes of ice cold lysis buffer (appendix) was added and the gently inverted to mix. The mixture was incubated on ice for 30-45 minutes. The lymphocytes were then pelleted by centrifuging at 1K for 10 minutes at 4°C. The supernatant was discarded and the pellet gently redissolved in 5mls of lysis buffer. The samples were centrifuged as described above and the cells washed and pelleted several times in phosphate buffered saline, PBS. Prior to the last spin the exact volume was noted and a small volume removed for counting.

A calibrated haemocytometer is used in which a specific number of squares is equal to an exact volume. Twenty-five squares of the haemocytometer used in this instance was equal

to 10^{-5} of a ml. A final concentration of 2-3x 10^6 cells per agarose block (100l ie 20-30 x 10^6 per ml) is ideal.The calculations are made as follows:

Number of cells in 25 squares = A

therefore concentration per ml = A x 10^5

Total number cells = Ax 10^5 x number of mls of cell suspension (B)

therefore total number of cells = A x B x 10^5

number of mls of PBS/1% LMP (1:1) to add to final cell pellet for required concentration:

$$\frac{A \times B \times 10^5}{25 \times 10^6} \text{ mls}$$

The cells were initially resuspended in half this volume of PBS and warmed to 37°C. An equal volume of molten LMP agarose (appendix) was added and gently mixed at 37°C.The moulds used for making the blocks were cleaned in 0.1N HCl and then rinsed in several changes of sterile distilled water before allowing to dry. The mixture was casted into 0.1ml blocks, and placed into 2-3 volumes of ESP solution (appendix) at 50°C for 24-48 hours.

The blocks were then dialysed against TE containing 0.04mg/ml phenylmethylsulfonylfluoride, PMSF, to inactivate proteinase K and sarcosyl activity. The agarose blocks were stored in 0.5M EDTA at 4°C. This high concentration of EDTA allowed storage for several months without apparent degradation.

2.2.8.1.2. Lymphoblastoid and Hybrid Cell lines

Cell lines were grown to an optical density of 1 x 10^6 cells per ml. For surface attached cell lines, cultures were trypsinised using trypsin/versene at 37°C for 5-10 minutes

(appendix). Cells remaining attached after this period were manually removed using an 'L' shaped glass rod. The cell suspension were poured into capped tubes and the flask rinsed through with PBS and added to the flask. The cells were pelleted by centrifuging at 1K for 10 minutes. Several washing were carried out using PBS to remove traces of media and trypsin/versene. The cells were counted and blocks prepared and treated as described previously.

2.2.8.2 Restriction Endonuclease Digestion of DNA in Agarose Blocks

Analysis of large DNA fragments suitable for PFGE analysis requires restriction endonucleases which cut human DNA infrequently. The cleavage sites are rare because they are 8 base pairs long (Not I and Sfi I) and/or contain the dinucleotide CpG , which is under represented in the mammalian genome. The enzymes and cleavage sites used in the experiments are listed below:

Rare Cutting Enzyme	Cleavage Sites
BssHII	GCGCGC
EagI	CGGCCG
MluI	ACGCGT
NaeI	GCCGGC
NarI	GGCGCC
NotI	GCGGCCGC
Nru	TCGCGA
PvuI	CGATCG
SacII	CCGCGG

SalI	GTCGAC
SfiI	GGCC(N)5GGCC
XhoI	CTCGAG

Prior to digestion of DNA the agarose blocks were washed several times in sterile distilled water to remove EDTA which at high concentrations inhibits enzyme activity. The 10x restriction enzyme buffers recommended by the manufacturer (NBL, UK) were modified by the addition of 40µg/ml of BSA, Bovine Serum Albumin) and 40mM spermidine.

One half of an agarose block (50µl) was incubated for 15 minutes on ice with 15µl 10 x restriction buffer, 85 l sterile distilled water and up to 10 units of enzyme, permitting the diffusion of enzyme into the agarose The reaction was then incubated at 37°C for 4 hours except when using BssHII or SfiI which require higher temperatures of 55°C.

For double digestions, reaction components from the first digestion were removed by dialysis with sterile distilled water for 15-30 minutes. The second digestion was then set up using the same conditions as described previously.

2.2.8.3 Electrophoresis of DNA Fragments

All gels were 1%/1.2% agarose in 0.5x Tris-borate-EDTA as electrophoresis buffer. Agarose blocks were sealed in using LMP agarose to prevent them floating out of the well during electrophoresis. Separation of DNA fragments were carried out using the LKB "Pulsaphor" (Pharmacia, Houston).The size range of molecules is strongly affected by switch times. With increasing switch times more molecules enter the resolved area of the gel and the zone of no resolution, the compression zone gradually disappears. The more favoured method of resolving fragments was achieved through the use of ramped programmes which allows two or more switch times to be used over a given period. The programme conditions used and the size separations achieved are shown below. A set

pulse time was used for a few runs which are listed below. However, the majority involved a ramping or stepping of pulse times which are described on the following page.

Set Pulse Time Programmes:

Prog. no.	N/S (secs)	E/W (secs)	Time (hours)	Voltage (volts)	Separation
1	50	50	40	170	50kb-850kb
2	90	90	40	170	100kb-1200kb

Ramping Programmes:

Prog. no.	N/s (secs)	E/W (secs)	Time (Hours)	Voltage (Watts)	Separation
3	15	15	11		
	45	45	11	200	50kb-700kb

	15	15	0		
Prog.	N/s	E/W	Time	Voltage	Separation
no.	(secs)	(secs)	(Hours)	(Watts)	
4	20	20	20		
	90	90	20	200	100kb-1600kb
	20	20	0		
5	20	20	18		
	120	120	30	120	100kb-1600kb
	120	120	18		
	400	400	0		
6	20	20	18		
	120	120	40	120	200kb-1800kb
	120	120	18		
	400	400	0		

2.2.8.4 Pulsed field Gel Blotting and Hybridisation

After electrophoresis gels were stained for 20 minutes in 0.5 x TBE and ethidium bromide at a final concentration of 3µg/ml. The gel was then destained prior to photography to remove non specific staining. Prior to blotting the gel was treated as described in 2.2.4.4 with an additional 30 minute treatment with 0.4N NaOH. Filters were hybridised, washed and stripped as described in 2.2.4.3-6.

2.2.8.5 Preparation of Saccharomyces Cerevisae Chromosomal DNA

This method is based on a protocol presented by Overhauser and Radic to BRL and published in "Focus 9.3". Yeast cells were grown from frozen stock in 200ml of YPD medium to an optical density of 1.0 at an absorbance of 600nm. The cells were recovered by centrifugation at 4000rpm for 5 minutes. The supernatent was resuspended in 10ml of SE buffer and centrifuged as before. The cells were washed twice more with SE before being resuspended in 4ml of SE. The cell suspension was placed in a plastic flask and warmed to 45°C before adding an equal volume of LMP agarose and blocks made as described in 2.2.8.1.1. Approximately 40-50 blocks were made with this volume of yeast culture.

To lyse the cells, 0.5ml 2-mercaptoethanol, 5mg zymolase was added with the final volume adjusted to 10ml with SE and incubated at 37°C for 2 hours. The blocks then rinsed in sterile distilled water and incubated in 20ml ESP solution overnight.

2.2.9 COSMID DNA FINGERPRINTING

2.2.9.1. DNA micropreps

Cosmid clones were grown from glycerol stocks in 96- well microtitre plates. Each well was filled with 0.5ml of L-Broth/kanamycin and inoculated. The plate was

incubated overnight at 37°C. The following day 0.2mls of the culture was removed and placed into a fresh microtitre plate and pelleted by spinning at 2500 rpm for 2 minutes. The supernatent was discarded and the pellet drained. 25µl of lysis buffer was added to each well and the pellets fully resuspended by tapping or using a tooth pick. 25µl of alkaline/SDS solution was added to each well and incubated at room temperature for 5 minutes. This was followed by the addition of 25µl of 3M sodium acetate solution, which was gently mixed and incubated at room temperature for a further 5 minutes. Prior to pelletting the cell debris the suspension was vortexed. After centrifuging at 3500 rpm for 9 minutes, 60µl of the supernatent was transferred from each well to a fresh microtitre plate containing 100µl of isopropanol in each well. The plate was recapped and incubated at -20°C for 30-60 minutes to precipitate the DNA.

The DNA was pelleted at 3500 rpm for 9 minutes and the supernatent discarded. The pellets resuspended in 25µl of sterile distilled water using a toothpick on stubborn pellets. An equal volume of 4.4M Lithium Chloride was added and the suspension incubated at 4°C for 1 hour.

Following centrifugation at 3500rpm for 9 minutes, 50µl of the DNA-containing supernatant is transferred to fresh plate containing 100µl of isopropanol. To precipitate the DNA, the suspension was left overnight at -20°C and centrifuged at 3500 rpm for 9 minutes. The pellet was washed in 200µl of 95% ethanol and pelleted and drained as described previously. Once dry, the pellets were resuspended in 10 l of TE and stored at -20°C until ready for use.

2.2.9.2 Fingerprinting DNA with Sau 3A1 and Hind III

2µl of the DNA samples were placed in a fresh microtitre plate. The following reagents were added to a microcentrifuge tube in the order listed :for 96 wells:

160µl ddH_2O

40µl 10 x M buffer (supplied by enzyme manufacturer)

5µl ddGTP (1mM)

8µl α-^{32}PATP

80units AMV reverse transcriptase

4µl Hind III (11 units/µl)

Using a 2µl dispenser, 2µl were added to the side of each well. The plate was covered with cling film and the reactions incubated at 37°C for 45 minutes, the optimum temperature for HindIII cutting and 'filling in' with reverse transcriptase. To inactivate both enzymes the reactions were incubated at 65°C for 25 minutes.

A second mix was prepared in a microcentrifuge tube in the following order:

for 96 wells: 197µl ddH_2O

22µl 10 x M buffer

5.7µlSau 3AI (50 units/µl)

Two microlitres of the mix was added to the side of each well as described above and incubated at 37°C for 2 hours. Following digestion, 4µl of formamide dye was added to each well and stored at -20°C until ready to load

2.2.9.3 Preparation of Lambda DNA marker

Five micrograms of lambda DNA are cut with Sau 3AI in the following reaction mix:

33µl ddH_2O

5µl restriction enzyme buffer (supplied by enzyme maunufacturer)

5µl lambda DNA (1mg/ml)

1µl Sau3AI (10units/µl)

The mixture was incubated at 37°C for 1 hour. The Sau3AI fragments were then labelled by the addition of the reagents:

4µl γ^{32}P dATP

2µl 10mM dGTP

3µl 10mM ddTTP

11units AMV-reverse transcriptase

The labelling reaction was incubated at 37°C for 30 minutes and 25µl of formamide dye added before storing at -20°C.

2.2.9.4 Sequencing Gel Preparation

For one gel the following mix was incubated at 37°C with occasional stirring to dissolve the urea:

42.0 g Analar Urea

10.0mls 10 x TBE

45.0 mls sterile distilled water

Once dissolved 10mls of 40% acrylamide:bisacryl was added to the solution. The setting agents were then added; 0.8ml of 10% ammonium persulphate and 80µl of Temed. The solution was swirled briefly to mix and quickly poured between two sequencing plates (30cm x 43cm) using a 50ml syringe avoiding air bubbles.The comb was placed onto the top of the gel and allowed to set for about one hour. The gels were run in a 0.5 x TBE buffer chamber.

2.2.9.5 Loading and Running of Fingerprinting Gels

Prior to loading of samples the wells were flushed through to remove dried urea and other small loose gel particles. The samples and marker were denatured at 80°C for 10 minutes and 4µl of each sample and 1µl of marker loaded.

The gel was run at 74 watts for 90 minutes or until the bottom dye band was half an inch from the bottom.

2.2.9.6. Fixing gels

The apparatus was disassembled and the small plate, with gel facing uppermost fixed in 10% acetic acid.The acid was then drained out and placed in distilled water and left for 35 minutes. The gel was dried using a gel dryer at 80°C for 45 minutes. The gel was covered with cling film, avoiding air bubbles on the surface and autoradiographed overnight at -70°C using Fuji X-ray film.

3. RESULTS

3. RESULTS

The results section is presented in six chapters. The first part, 3.1, is concerned with the characterisation of a panel of somatic cell hybrids for the preliminary analysis of the short arm of chromosome 3. The second section, 3.2, summarises the collaborative project with Pr. C.H.C.M. Buys on the isolation and characterisation of a coding sequence, D8, from 3p21 and its implications in SCLC. The subsequent chapters are related to the detailed studies of this region. The genomic cloning around D8 is presented in section 3.3. and the long range mapping of this region presented in 3.4. The fifth section, 3.5, is concerned with the genomic analysis of SCLC using the techniques and resources described in the 3.3 and 3.4. The final section presents the isolation of a possible homologue of the D8 sequence.

3.1 CHARACTERISATION OF A PANEL OF SOMATIC CELL HYBRIDS: mapping of 3p21 markers.

The characterisation of the 3p21 deletion in SCLC requires a fine resolution map of the region. The locating and ordering of genes and markers within 3p21 will provide landmarks to assist in defining the limits of the SCLC deletion.

Somatic cell hybrids were established to provide a mapping resource for the fine characterisation of markers on 3p21. Hybrids were specially constructed from material containing deletions and translocation breakpoints of the 3p21 region. In addition an already available somatic cell hybrid shown to contain a fragment of 3p21 as the only representation of chromosome 3 was fully characterised. Each panel of somatic cell hybrids was characterised with available 3p markers and genes. In combination with existing mapping data these hybrids have enabled the ordering of markers and genes in the 3p21 region. An additional resource was a somatic cell hybrid, 429, already prepared and fully characterised, containing a single copy of chromosome 3 as its sole human componentwas used as an important control.

3.1.1 CHARACTERISATION OF THE 3P COMPONENT IN THE HYBRID Dis2.6.

The hybrid, Dis2.6 contains a fragment of chromosome 3p21(Carritt et al. 1986 &1992), which includes the marker D3F15S2 and the closely mapping gene, APEH. The absence of the markers, D3S2 and THRB in this hybrid suggest its representation of chromosome 3 to be no more than 3p21-p24 (Carritt et al. 1992). Since this hybrid contains the most frequently lost marker in SCLC, D3F15S2, its further characterisation with respect to other 3p assigned markers may contribute to the identification of a defined region of 3p which is consistently involved in the SCLC deletion.

Primer sequences from nine 3p21 markers (section 2.6) were used to screen the

Dis2.6 hybrid by the polymerase chain reaction. Primers designed from the mouse p53 pseudogene which cross hybridised to hamster DNA were used as a positive control to illustrate satisfactory amplification of the hybrid in all polymerase chain reactions (C.M. Abbott pers. communication). In addition, DNA from the single chromosome 3 hybrid, 429, in conjunction with a rodent control was used to confirm the human and locus specificity of the primers used. The data is presented in the summary section (3.1.4)

A genomic clone which contains a region of homology to a zinc-binding finger motif has been mapped to distal 3p21 by in situ hybridisation (Hoovers et al. 1992) The shared map location with the marker, D3F15S2 suggested the possibility that ZnFP16 may be included in the hybrid. A cDNA homologous to the cosmid clone Znf 16BamHI4.5, was used as a probe in Southern analysis of the Dis2.6 hybrid and the results are presented in figure 3. The results obtained with each marker in the Dis 2.6 hybrid are recorded in table 3. As indicated in the table three additional markers were identified in the hybrid, AlAS 3, ALAS 1, ARH12 and ZnFP16. ARH12 encodes the ras related related gene (Madaule 1985).

3.1.1.2. Physical mapping around the D3F15S2 locus in Dis 2.6

A physical map around the D3F15S2 region in the Dis 2.6 hybrid has been constructed. Since this marker is consistently lost in SCLC, characterisation of its flanking region may assist in the isolation of neighbouring DNA sequences which contribute to the development of the lung tumour. In an attempt to assess and quantify the 3p21 human component in Dis 2.6, pulsed field gels of the hybrid cell lines were blotted and hybridised to the probe H3H2 which detects the D3F15S2 locus. Figure 4 presents the results obtained following digestion with the rare cutters, MluI and NotI. As illustrated in the figure (figure 4), H3H2 recognises a human-hamster boundary approximately 400kb from the D3F15S2 locus in the Dis 2.6 with the enzyme MluI. This data is illustrated in the physical map in figure.5.

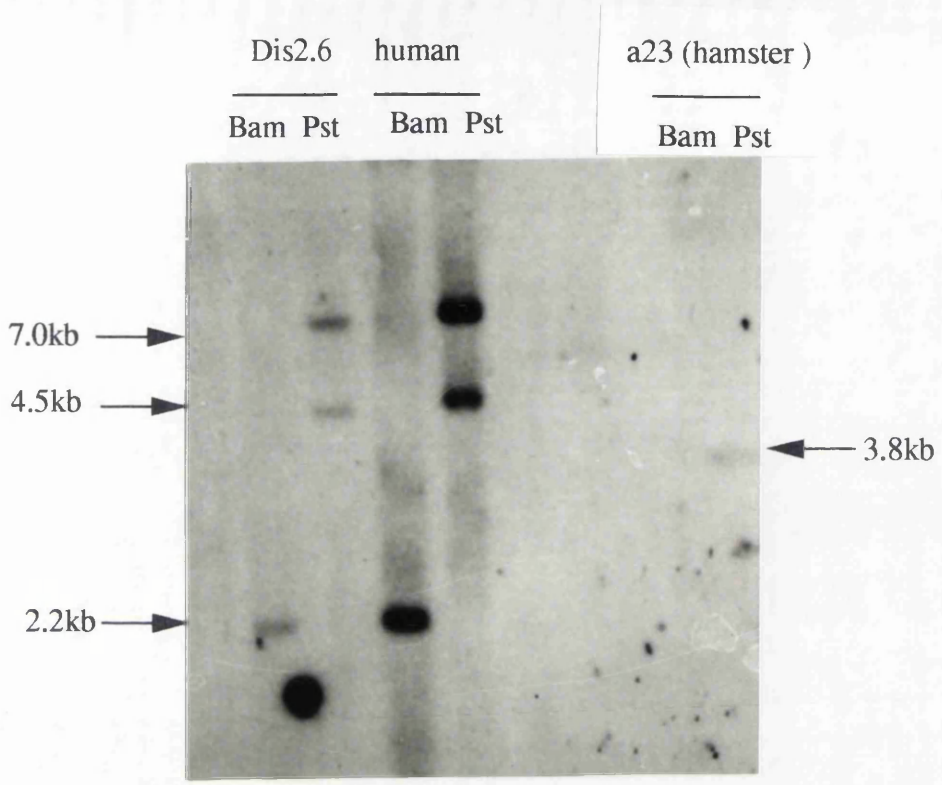

Figure 3: Mapping of ZnFP16 in Dis 2.6.

Genomic DNA from the Dis 2.6 cell line and the control samples, a23 and total human were digested with PstI and BamHI, blotted and hybridised to the ZnFP16 cDNA clone. Two BamHI fragments, 7.0kb and 4.5kb and a single 2.2kb PstI fragment are observed in the total human and Dis2.6 samples. A weak cross-hybridising 3.8kb BamHI fragment is observed in a23 DNA. This indicates that the fragments observed in Dis2.6 following digestion with BamHIand PstI are human specific, confirming the presence of the ZnFP16 clone in the hybrid.

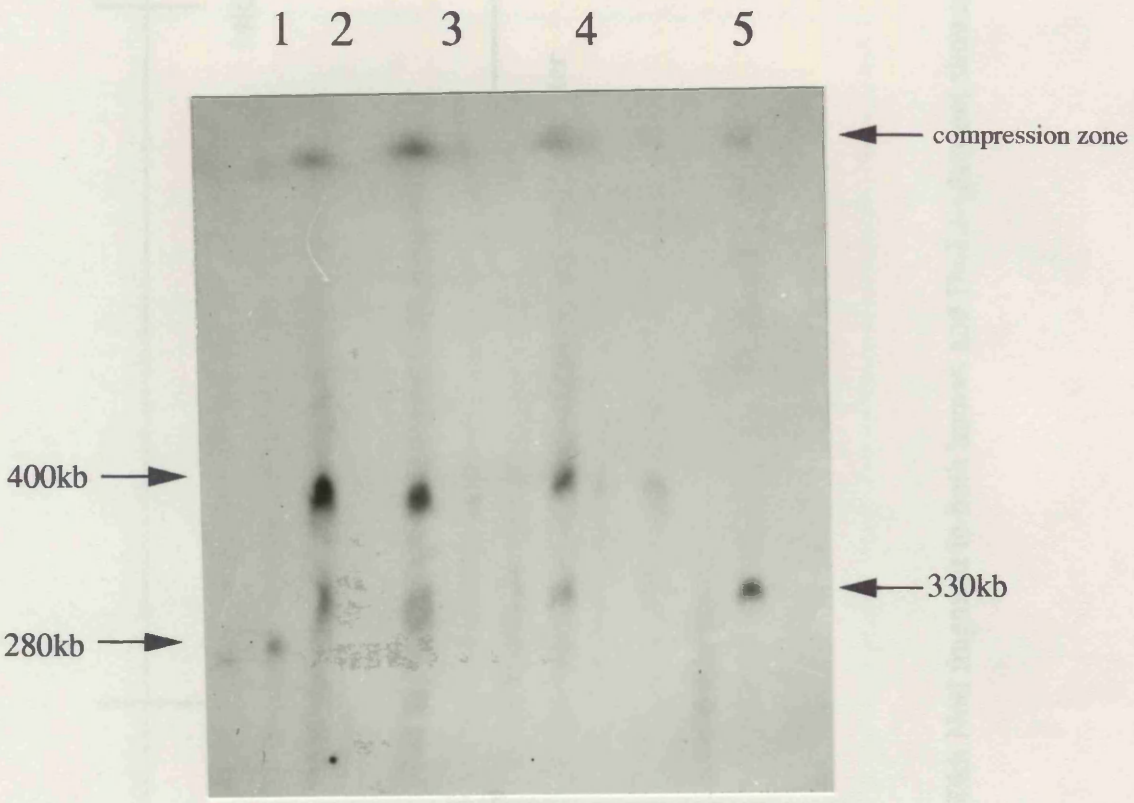

Figure 4: Long range mapping of Dis2.6 around APEH.

DNA agarose blocks were digested with MluI and run on pulsed field gels using ae 66 hour ramping programme. The blotted DNA was then hybridised to the probe, APEH.

The figure illustrates the following hybridising bands; human genomic DNA x MluI- 280kb (lane1), Dis2.6 x MluI-400kb+330kb (lanes 2-4 are different agarose blocks prepared from the Dis2.6 cell line) and hamster, a23 x MluI-330kb (lane 5).

APEH recognises a 280kb human genomic MluI fragment and a 330kb hamster MluI fragment. However, in addition to the hamster specific band in Dis2.6, an abnormal sized MluI fragment of 400kb is observed. This represents the human component of the hybrid, indicating a potential human-hamster or human-human boundary.

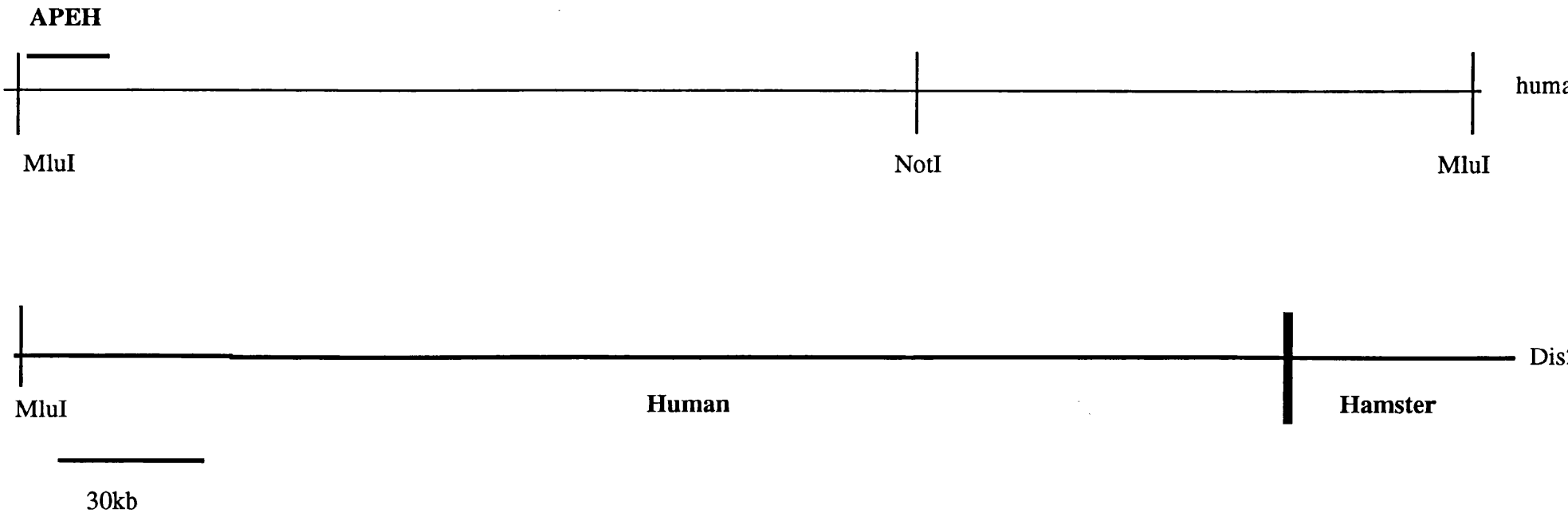

Figure 5: Long range map of Dis2.6 around the APEH locus.

The hamster-human boundary lies in the 270kb MluI fragment. The shared 650kb NotI fragment in both human and Dis2.6 (data not shown) positions the boundary in the non APEH hybridising MluI-NotI fragment.

MARKER	Dis2.6
THRB	-
D3S1100	-
DS11	-
ZnF35	-
ALAS 3	+
D3F15S2	+
ARH12	+
ZnFP16	+
ALAS 1	+

Table 3: Mapping of 3p markers in Dis2.6 by PCR and Southern analysis.

3p markers screened for in Dis 2.6 hybrid. '+' denotes presence of marker, '-'denotes absence of marker .

3.1.2. GREIG CEPHALOPOLYSYNDACTYLY 3:7 (p21.1:p13) TRANSLOCATION.

The Greig cephalopolysyndactyly-craniofacial abnormalities syndrome is an autosomal dominant disorder involving a gene located in band 7p13. A reciprocal translocation involving this locus and the 3p21 region has been characterised in one patient. Using markers assigned to 3p21 the breakpoint on chromosome 3 has been shown to involve band 3p21.1 (Drabkin et al. 1989, Gemmill et al. 1991). The marker D3S2 (3p21.1) and the gene ACY-1 (3p21.1) segregate with the proximal half of the translocation and the the markers D3F15S2 (3p21.2-p21.3) and D3S32 (3p21) were shown to segregate with the distal portion of the translocation (Gemmill et al. 1991).

The distance from the D3S2 marker to the translocation breakpoint has been illustrated by pulsed field gel electrophoresis as 1 megabase. The Acy-1 gene has been mapped to the same pulsed field gel fragment at a distance of 200kb proximal of D3S2. The frequent loss of the markers D3S2 and D3F15S2 in SCLC illustrates the need for additional markers around the interval.

Through the establishment of somatic cell hybrids, designated as GPS hybrids, containing each translocation half in individual hybrids, 3p21 assigned markers and genes were mapped with respect to the 3p21.1 breakpoint.

3.1.2.1 Segregation of translocation in GPS Hybrids

Hybrids were made from the lymphocytes of the Greig polysyndactyly patient by PEG-induced fusion with the tk^- chinese hamster line, a23. To ascertain hybrids containing either the proximal or distal halves of the translocation, each hybrid was assayed for the presence of the SI gene (3q) and the two genes, ACY-1 and THRB, which have previously been mapped proximal and distal respectively to the breakpoint (Gemmill et al.1991). Hybrids positive for only one of these markers

would therefore contain one half of the translocation.

Human-specific primers representing coding sequence of the SI gene mapping to the long arm of chromosome 3, 3q35-q26 (Abbott et al 1990) were used in the PCR analysis of the GPS hybrids and control DNA's. Figure 6 illustrates an ethidium bromide stained agarose gel of the PCR products from each hybrid cell line. GPS 15 and GPS17 are positive for 3q and therefore contain at least one copy of chromosome 3 and or the distal portion of the translocation. One or more of the the remaining hybrids, negative for 3q, may contain the distal portion of the translocation in their complement. The ACY-1 gene (3p21.1), previously shown to segregate with the proximal portion of the translocation, was used in southern analysis of the GPS hybrids (Drabkin et al. 1989, Gemmill et al.1991). Figure 7 presents a Southern blot of the GPS hybrids following hybridisation to the ACY-1 genomic clone. The results demonstrates the co-segregation of the 3q marker, SI and the ACY-1 gene.

THRB was used as a marker to assay for the presence of 3p in the hybrids. Figure 8 illustrates an ethidium bromide stained gel of negative and positive PCR products detected in each of the hybrids. GPS1, GPS13 and GPS15 are shown to be positive for the THRB gene. The data obtained in this primary analysis of the GPS hybrids is presented in table 4.

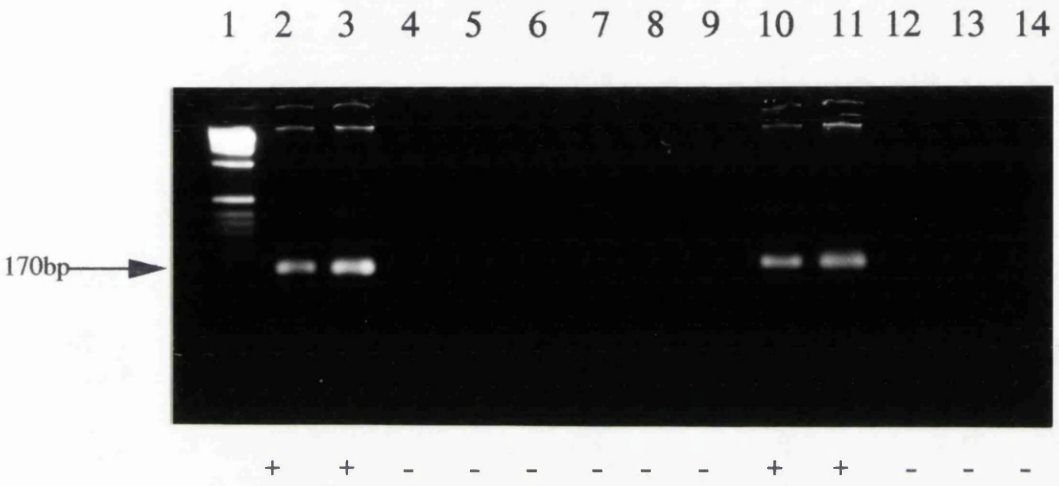

Figure 6: PCR analysis of 3q marker in GPS hybrids.

Lane 1: 1kb ladder. Lanes 2 and 3 illustrate the 170 base pair amplicon following amplification of total genomic DNA and the control hybrid DNA, 429 respectively. Lanes 4-12 show the results obtained for each GPS hybrid; Lane4:GPS1, Lane 5:GPS7, Lane 6:GPS8, Lane7:GPS11, Lane8:GPS12, Lane9:GPS13, Lane10:GPS15, Lane11:GPS17, Lane 12:GPS19.Lane 13-hamster, a23 Lane 14-negative control.

The hybrids GPS 15 and 17 are positive for the 3q marker.

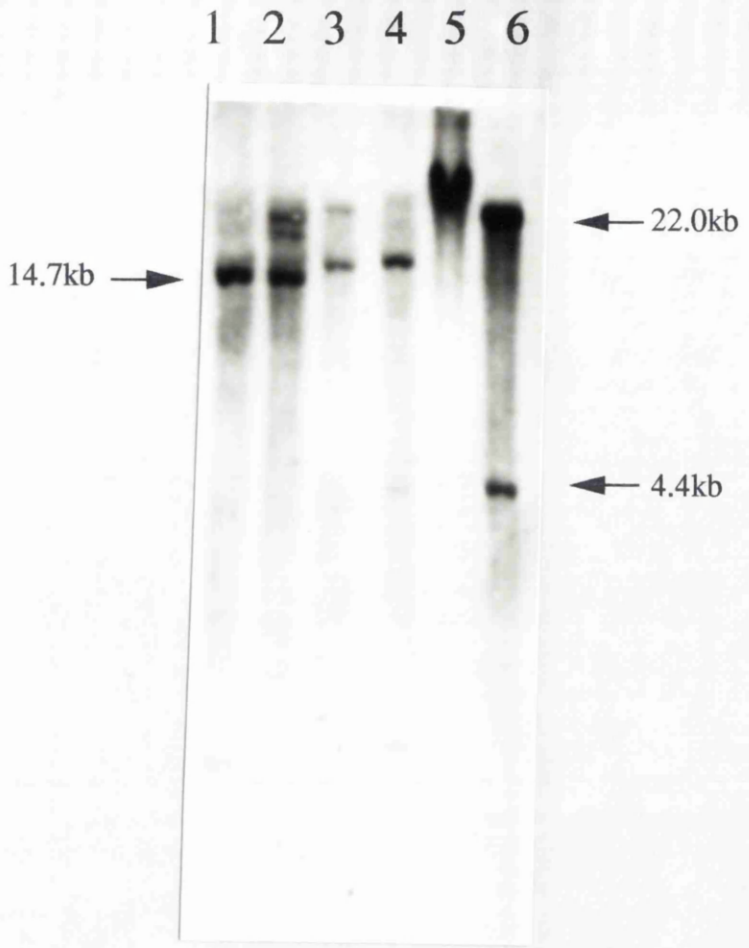

Figure 7: Mapping of ACY-1 in GPS hybrids .

The GPS hybrids and control DNA samples were digested with EcoRI, blotted and hybridised to the ACY-1genomic clone. Lane 1: a23, hamster DNA, Lane 2: GPS17, Lane 3:GPS15, Lane 4: GPS13, Lane 5: total genomic DNA(undigested), Lane 6: total genomic DNA. A single ACY-1 cross hybridising 14.7kb band is seen in the a23 chinese hamster DNA.

The two hybridising bands, 22.0kb and 4.4kb in total genomic DNA (lane 6), represent the ACY-1 locus on chromosome 3 and chromosome 8 respectively (Miller et al 1990). A single ACY-1 cross-hybridising 14.7kb is observed in the hamster and hybrid DNA samples.

The 22.0kb band representing the ACY-1 chromosome 3 locus is observed in GPS 15 and not in GPS 13.

In lane 1 (a23) two faint bands are observed around the 22.0kb region. A similar banding pattern is also observed in lane 2 (GPS17) and therefore difficult to decide whether one of these bands represent the human ACY-1 locus as observed in lane 3 (GPS13) or a partial hamster band as indicated in lane1. Using this result alone, it is not possible to confirm the segrgation of the ACY-1 locus with GPS17.

However, the results presented in figure 6 and those presented further on in this chapter together with studies using the GPS hybrids by Gemmill et al. (1991) positions the ACY-1 locus proximal of the GPS breakpoint and therefore in GPS17.

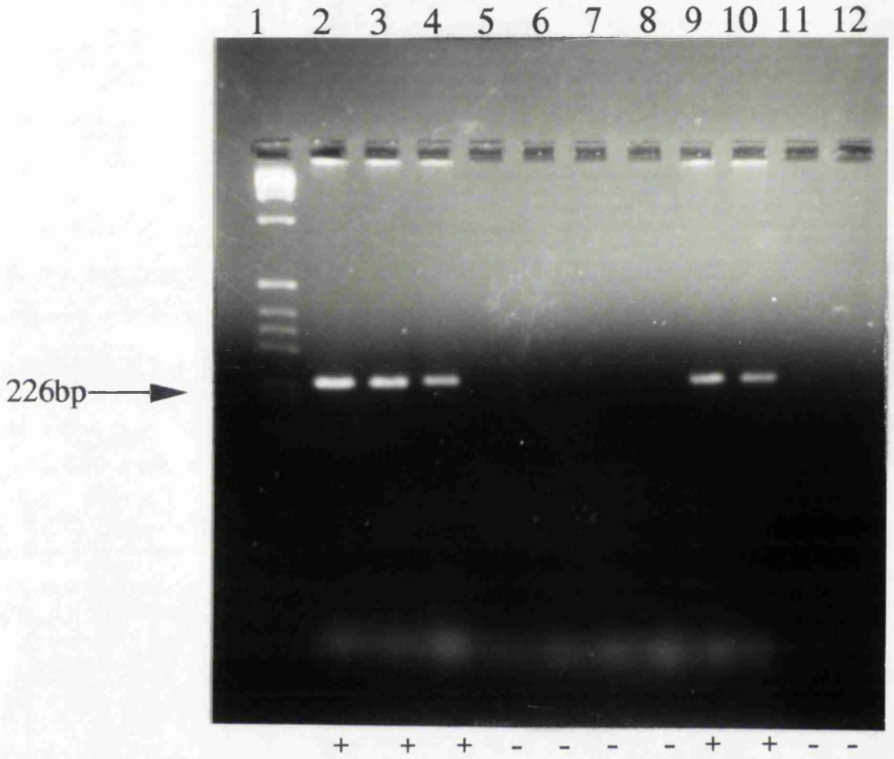

Figure 8: Screening of GPS hybrids using THRB.

Lanes 1: 1kb ladder, Lane 2 and 3 illustrate the 226 base pair amplicon following amplification of total genomic DNA and the control hybrid DNA, 429 respectively.
Lane 4: GPS1, Lane 5: GPS 7, Lane 6: GPS8, Lane 7: GPS10, Lane8: GPS17, Lane9: GPS13, Lane10: GPS15, Lane11: a23, hamster DNA. Lane 12: negative control.
The hybrids GPS1, GPS13 and GPS15 are positive for the THRB marker.

The following table (table 4) presents the results of screening the GPS hybrids for the segregation of the two halves of the translocation. On the basis of this data, GPS13 and GPS 17, containing complementary translocation fragments were used in the PCR and Southern analysis of markers and gene assigned to 3p.

GPS hybrid	SI 3q25-q26	ACY-1 3p21.1	THRB 3p24.2
1	-	ND	+
7	-	ND	-
8	-	ND	-
11	-	ND	-
12	-	ND	-
13	-	-	+
15	+	+	+
17	+	+	-
19	-	ND	ND

Table 4: Segregation of translocation in GPS hybrids

Hybrids are listed in the left hand column.and the markers used in PCR and Southern analysis in the latter 3 columns. Positive (+) and negative (-) signs are used to denote presence and absence of marker respectively in PCR analysis. 'ND' denotes not determined.

As described in section 3.1.1, primer sequences available for 3p markers were used in the analysis of the GPS hybrids to determine their map position relative to the GPS breakpoint. The data obtained in the PCR and Southern analysis is presented in table 5 and the raw data presented in the summary section, 3.14.

As indicated in table 5, the ALAS1 gene segregates with the proximal half of the translocation whereas the ALAS3 gene segregates with the distal half of the translocation. This implies that aminolevulinate synthase, is encoded by 2 genes which map to different loci in 3p21. In addition, ARH12, ZnFP16 and D3S1100 map distal to the GPS 3p21.1 breakpoint.

MARKER	GPS13	GPS17
THRB	+	-
D3S1100	+	-
D3S11	+	-
ZnF35	+	-
ALAS3	+	-
D3F15S2	+	-
APEH	+	-
ARH12	+	-
ALAS1	-	+
ACY-1	-	+
3q(SI)	-	+

Table 5: Mapping of 3p markers in GPS hybrids

The table presents the results of assaying four of the GPS hybrids with 3p markers using the polymerase chain reaction and Southern analysis. Symbols used: '+' denotes amplification product , '-' denotes no amplfication product

3.1.3. CHARACTERISATION OF 3P DELETION IN DEAK PATIENT USING SOMATIC CELL HYBRIDS.

Peripheral blood lymphocytes were obtained from a mentally handicapped young male with a cytogenetically detected interstitial deletion of one homologue of chromosme 3 and were used to establish somatic cell hybrids. The patient suffered from impaired speech, weight and height in addition to facial abnormalities. G-Banded karyotypes of a lymphocyte culture revealed an interstitial deletion of the short arm of chromosome 3, del (3) (p14.2;p21.3) as the only visible karyotypic abnormality. To characterise the deletion at the molecular level and its possible value as a 3p21 mapping resource, somatic cell hybrids were established to segregate the deleted chromosme 3 from the normal homologue.

Nine hybrid cell lines were established from lymphocytes of the patient. The hybrid cell lines were screened for 3q using primers from the SI gene (3q25-q26), with the appropriate controls as described in 3.1.1. Figure 9 presents an ethidium bromide stained agarose gel of PCR products from each hybrid cell line with hamster and human controls. The five hybrids, IB2, IIA2, IIC5, IB1 and IIA4 are shown to contain at least one copy of human chromosome 3.

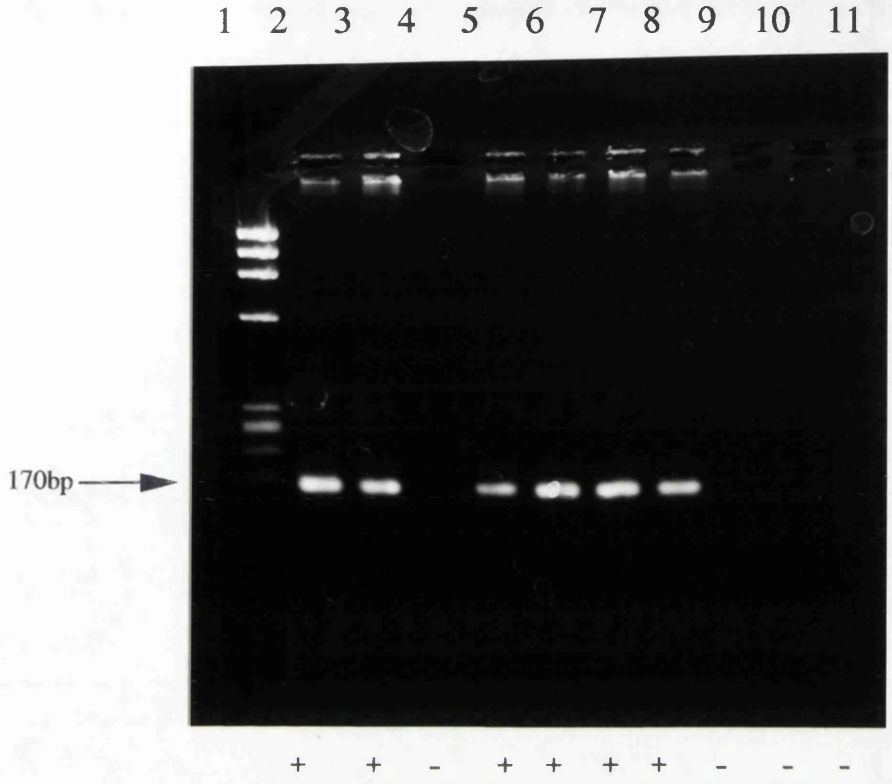

Figure 9: PCR analysis of 3q marker (SI) in the Deak hybrids.

Lane 1: 1kb ladder. Lanes 2 and 3 illustrate the 170 base pair amplicon following amplification of total genomic DNA and the control hybrid, 429, DNA respectively. Lanes 4-9 show the results obtained for each Deak hybrid; Lane 4:IA2, Lane 5:IIA2, Lane 6:IIA4, Lane 7:IB1, Lane 8:IB2, Lane9:IB5. Lane10 confirms the human specificity by using a23, hamster DNA as the template. Lane 11: negative control.

The hybrids IIA2, IIA4, IB1and IB2 therefore contain the 3q marker, SI.

In the case of a heterozygous interstitial deletion, the determination of which hybrids contain the deleted homologue and which contain the normal chromosome 3 is not straightforward. An attempt was made to do this using a highly polymorphic 3q minisatellite marker (D3S621) in the hope that the patient, who was no longer available for testing directly, might have been heterozygous. However, all hybrids in which the locus could be detected appeared to be identical, indicating that either the patient was homozygous or that all hybrids contained the same homologue of chromosome 3, or both (data not shown).

In a further attempt, therefore, to determine which hybrids contain the deleted chromosome 3, a marker highly likely to map into the deletion was required. It was believed that on the basis of the cytogenetic characterisation of the deletion, the marker, D3F15S2 assigned to 3p21.3 would map into this interval if the deletion included the whole of this band. However, both PCR and Southern analysis revealed positive signals in all hybrids, which either indicated that the marker mapped outside the deletion or a normal chromosome 3 was present in all the hybrids. Nevertheless it is possible that the cytogenetic characterisation was inexact and it was decided therefore to continue to screen for all markers on 3p where primer sets were available for PCR and to use APEH in Southern analysis. The results are presented in table 6 and indicate that the three markers, D3S11, ALAS 3 and ZNF35 map into the deletion, the remaining markers showing positive signals. In combination with existing data, the proximal boundary of the deletion must lie distal of D3F15S2 and the distal boundary flanked by the markers D3S11 and D3S1100. The raw data obtained with the hybrid IIA4 containing the deleted chromosome 3 homologue is presented in the summary section 3.1.4, figures 10-18.

	Hybrids					
MARKER	IIA2	IIA4	.IB1	IB2	IIC5	IB5
THRB	+	+	+	+	+	-
D3S1100	+	+	+	+	+	-
D3S11	+	-	+	-	+	-
ZnF35	+	-	+	-	+	-
ALAS3	+	-	+	-	+	-
D3F15S2	+	+	+	+	+	-
APEH	ND	+	ND	ND	ND	ND
ARH12	+	+	+	+	+	-
ALAS1	+	+	+	+	+	-
3q (SI)	+	+	+	+	+	-

Table 6: Characterisation of 3p component in Deak hybrids.

The table presents the results of assaying six of the Deak hybrids with 3p markers using the polymerase chain reaction and Southern blotting. The symbols are as follows: '+' denotes amplfication product , '-' denotes no amplfication product.and 'ND' denotes not determined.

3.1.4. SUMMMARY OF HYBRID MAPPING DATA

Figures 10-18 present the results of the hybrid screening by the polymerase chain reaction and Southern analysis. Table 7 presents the data obtained for each of the informative hybrids with each of the 3p markers. The data can be pooled by scoring for the presence of markers in the Dis2.6 and GPS hybrids and the absence of markers in the Deak deletion hybrid. As indicated in the table 7, the Dis2.6 hybrid shares markers with each of the GPS hybrids, locating the 3p21 fragment of Dis2.6 across the GPS breakpoint. An overlap of one marker is shown between the Deakdeletion and the Dis2.6 hybrid.

Five markers, ALAS 3, ZnF35, D3F15S2, ZnFP16 and ALAS 1 are contained in Dis2.6. Only one of these, ALAS 1 maps proximal of the GPS breakpoint, the remaining map distal to the breakpoint. This immediately indicates that the Dis2.6 represents a 3p21 fragment which crosses the GPS boundary.

Of particular interest is the distance of ALAS 1 from the GPS breakpoint. On the basis of the physical mapping data of D3S2, which indicates a distance of 800kb from the breakpoint to the marker (Gemmill et al. 1991), and the absence of D3S2 in Dis 2.6, the ALAS 1 gene must map in the 800kb interval flanked by D3S2 and the GPS breakpoint.

The human-hamster or human-human boundary in Dis2.6 observed using the D3F15S2 marker, distal of the GPS breakpoint, therefore represents the distal end of the Dis 2.6 fragment and that ALAS 1, mapping proximal of the GPS breakpoint, is the closeset marker to the proximal boundary of the Dis2.6 fragment.

Markers absent in the Deak hybrid but present in Dis 2.6 hybrid would represent an overlap of the deletion and the 3p21 fragment contained in Dis 2.6 ALAS 3, is the only marker where this is the case and therefore of the loci tested, the deletion and the 3p21 fragment only overlap at this locus. On the basis of the ALAS 3 gene

mapping distal of the GPS breakpoint, its inclusion in Dis 2.6 and in the Deak deletion, the gene must map to the distal overlapping end of the 3p21 fragment of Dis2.6. Since D3F15S2 isolated from Dis 2.6, is outside the Deak deletion ALAS 3 must map distal of D3F15S2 and be contained within the pulsed field fragment which harbours the human-hamster boundary in Dis 2.6.

The zinc finger gene, ZnF35, ALAS 3 and D3S11 map into the Deak deletion. On the basis of the mapping of D3S11to 3p22 (Heppell-Parton et al. 1991), and the presence of the ALAS 3 gene in Dis2.6, the order of markers in this region is:

cen-D3F15S2-ALAS3-ZnF35-D3S11-D3S1100-tel.

----------[Deak deletion]----------

The relative position of D3S11 and ZnF35 is based on the previously published mapping of ZnF35 to 3p21 (Donti et al. 1990). Of particular interest is the anonymous marker, D3S1100, isolated from an irradiation fragment hybrid, mapping in the interval D3F15S2 and THRB (Naylor 1991). D3S1100 which is not contained in Dis2.6, maps distal of the GPS breakpoint and outside the Deakin deletion. Therefore, the marker, D3S1100 must lie in the interval between D3S11and THRB. On the basis of the data presented here combined with existing data, the Deakin deletion can therefore be mapped as 3p21.3-p22. This is smaller and more distal than the original cytogenetic characterisation.

The zinc finger clone shown to lie distal of the GPS breakpoint and contained in Dis2.6 but mapping outside the Deakin deletion positions it in the interval flanked distally by ALAS 3 and proximally by the GPS breakpoint.

On the basis of the results obtained and the reasons discussed the following map presented in figure 19 was constructed.

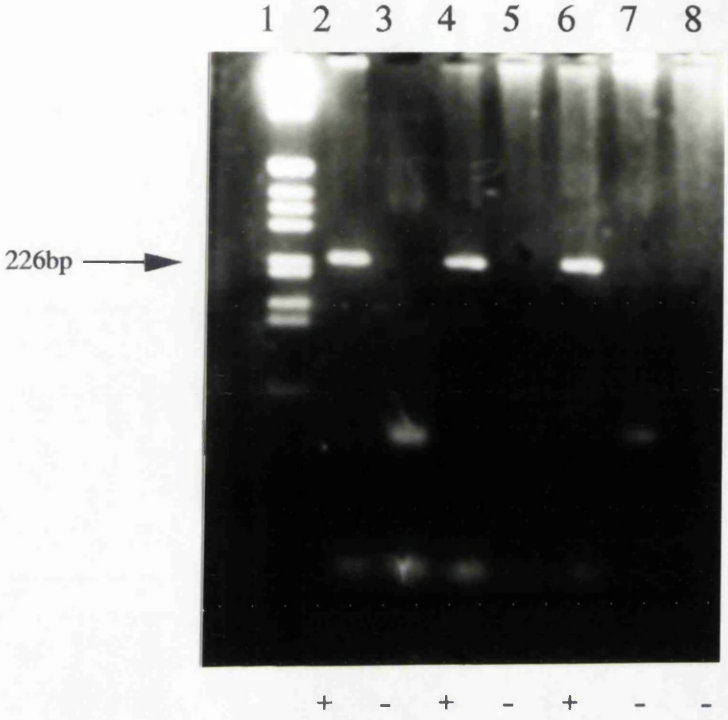

Figure 10: Screening of hybrid panel with THRB

Lane 1:1kb ladder. Lane 2 illustrates the 226 base pair amplicon following amplification of total geneomic DNA with the THRB primers. Lane 3: negative control, Lanes 4-7 are the hybrids screened with THRB. Lane 4: GPS 13, Lane 5: GPS17, Lane 6: : IIA4, Lane 7: Dis2.6, Lane 8: a23, hamster DNA.
The hybrids, GPS13 and IIA4 contain THRB.

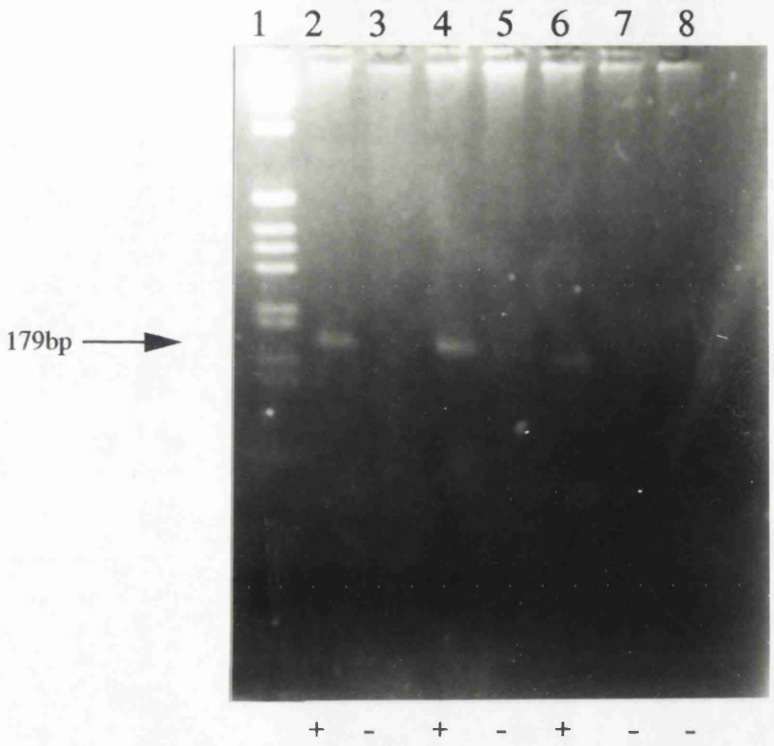

Figure 11: Screening of hybrid panel with D3S1100

Lane 1:1kb ladder. Lane 2: illustrates the 179 base pair amplicon following amplification of total genomic DNA with the D3S1100 primers. Lanes 3-6 are the following hybrids: Lane 3: Dis2.6, Lane 4: GPS 13, Lane 5: GPS17, Lane 6: IIA4, Lane 7: a23, hamster DNA, Lane 8: negative control.
The hybrids, GPS13 and IIA4 contain themarker D3S1100.

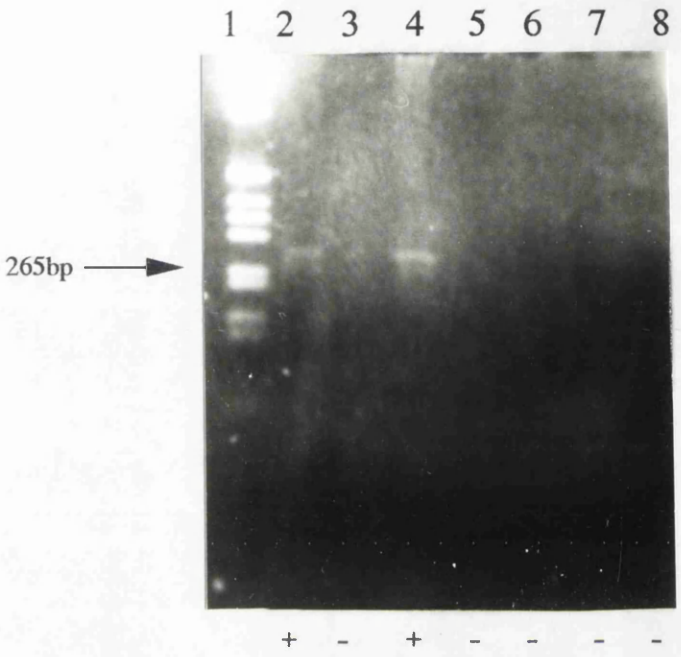

Figure 12: Screening of hybrid panel with D3S11

Lane 1:1kb ladder. Lane 2: illustrates the 265 base pair amplicon following amplification of total genomic DNA with the D3S11 primers. Lane 3-6 are the hybrids with this marker. Lane 3: Dis2.6, Lane 4: GPS 13, Lane 5: GPS17, Lane 6 : IIA4, Lane 7: a23, hamster DNA, Lane 8: negative control.
The hybrid GPS13 contains the marker D3S11.

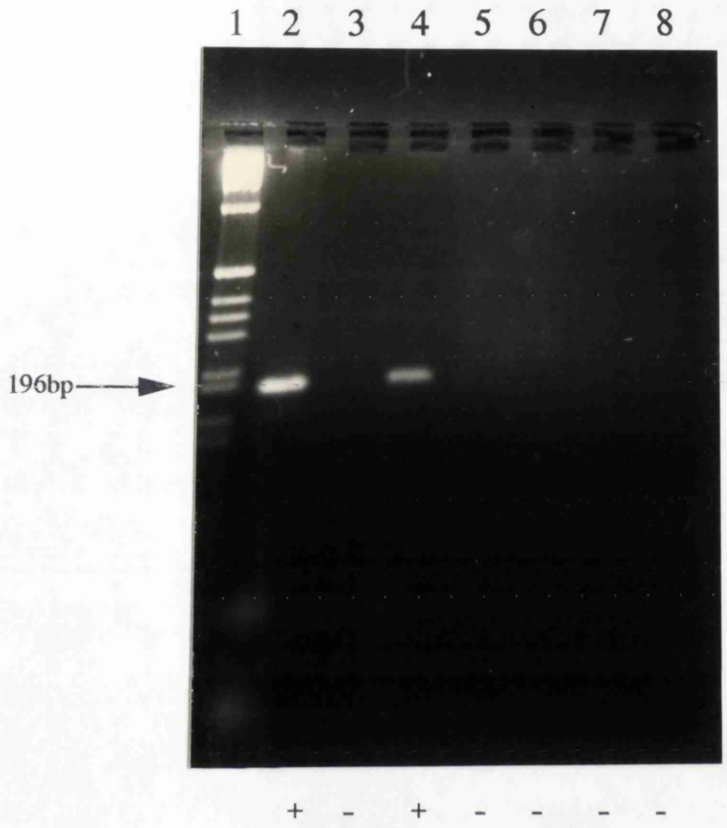

Figure 13: Screening of hybrid panel with ZNF35.

Lane 1:1kb ladder. Lane 2: illustrates the 196 base pair amplicon following amplification of total genomic DNA with the ZNF35 primers. Lane 3-6 are the hybrids screened with ZNF35. Lane 3: Dis2.6, Lane 4: GPS13, Lane 5: GPS17, Lane 6: IIA4, Lane 7: a23, hamster DNA, Lane 8: negative control.
Only the hybrid GPS13 is positive for ZNF35.

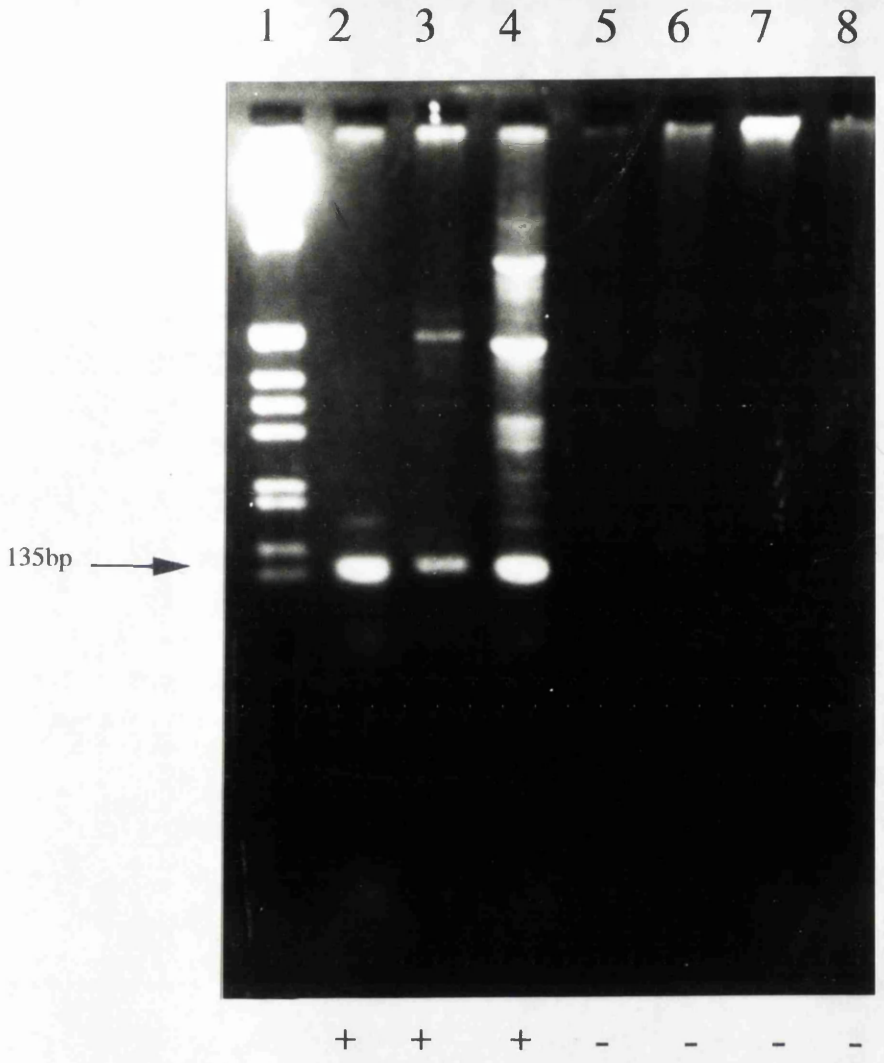

Figure 14: Screening of hybrid panel with ALAS 3

Lane 1:1kb ladder. Lane 2: illustrates the 135 base pair amplicon following amplification of total genomic DNA with the ALAS 3 primers. Lane 3-6 are the following hybrids with this marker: Lane 3: Dis2.6, Lane 4: GPS 13, Lane 5: GPS17, Lane 6: : IIA4, Lane 7: a23, hamster DNA, Lane 8: negative control.
The hybrids, Dis2.6 and GPS13 contain ALAS 3.

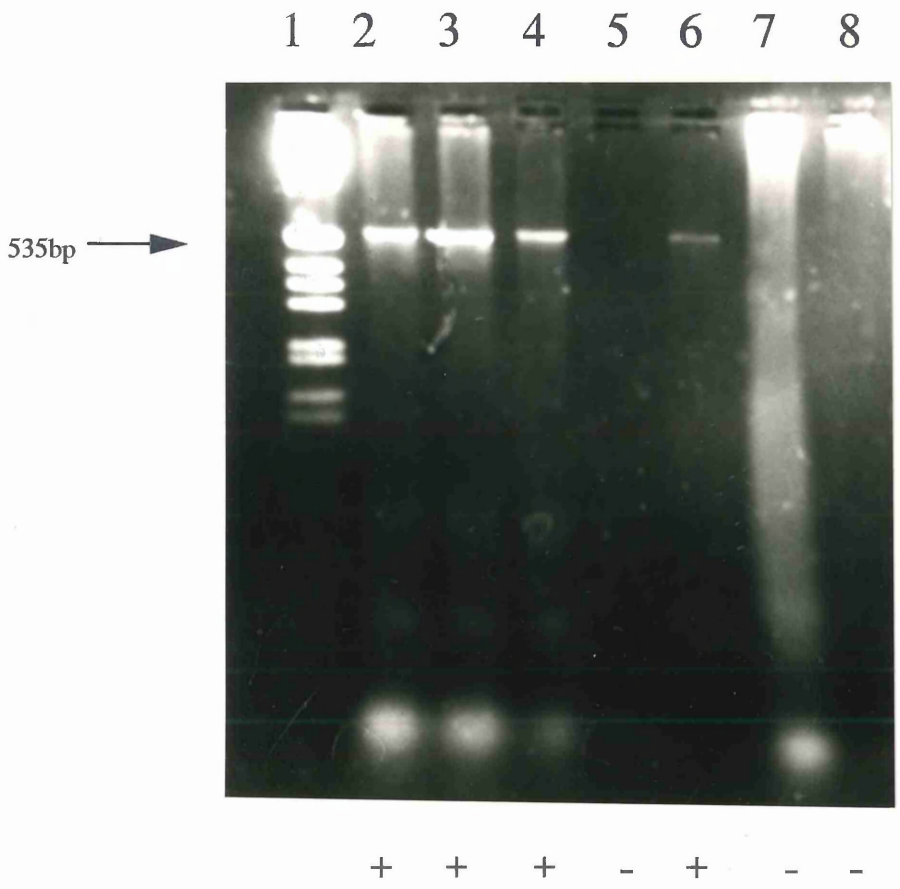

Figure 15: Screening of hybrid panel with D3F15S2.

Lane 1: 1kb ladder. Lane 2: illustrates the 535 base pair amplicon following amplification of total genomic DNA with the D3F15S2 primers. Lane 3-6 are the hybrids screened with D3F15S2. Lane 3: Dis2.6, Lane 4: GPS13, Lane 5: negative control, Lane 6: IIA4, Lane 7: GPS17, Lane 8: hamster DNA.
The hybrids Dis2.6, GPS13 and IIA4 are positive for D3F15S2.

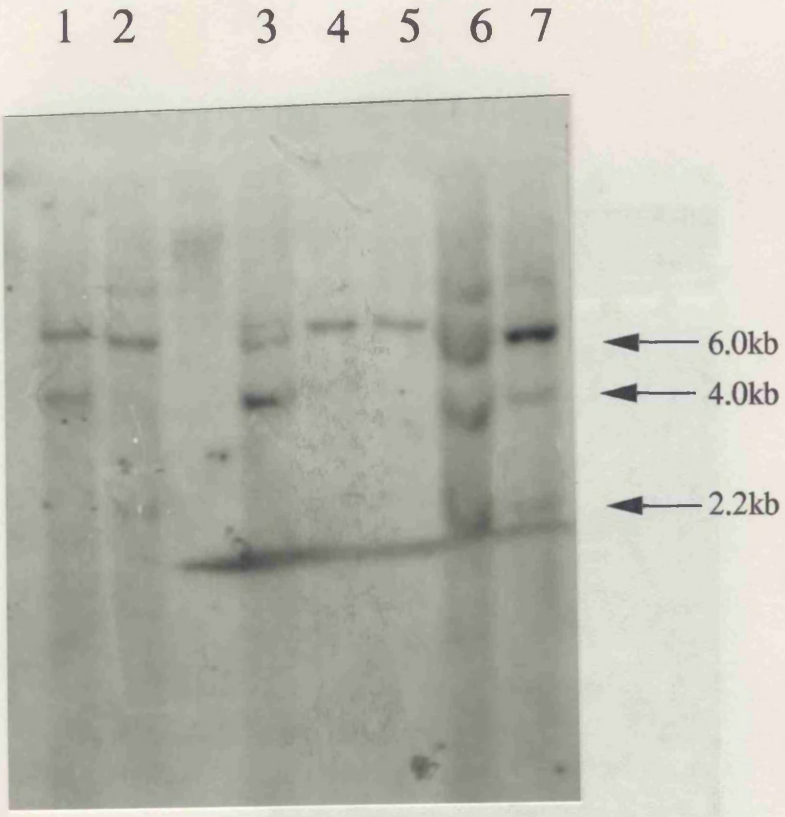

Figure 16: Mapping of APEH in hybrid panel.

DNA from the hybrids, IIA4, GPS13, GPS 15 and GPS17 were digested with EcoRI. Following Southern blotting, the DNA's were hybridised to the probe, APEH .

Lane 1: total genomic DNA, Lane 2: a23, hamster DNA, Lane 3: single 3 micro-cell hybrid, Lane 4: IIA4, Lane 5: GPS17, Lane 6: GPS15, Lane 7: GPS17. A 6.0kb EcoRI hamster specific fragment is observed in the a23 hamster DNA (lane 2) and the five hybrid DNA samples (lanes 3-7).

The human chromosome 3 specific 4.0kb EcoRI fragment is observed with total human genomic DNA (lane1) and the control hybrid, 429, DNA. The 4.0kb EcoRI fragment is observed in the hybrids, IIA4, GPS15 and GPS 13 and are therefore positive for APEH. The extra 2.2kb fragment observed in GPS13 and GPS15 may represent a non specific hybridising band.

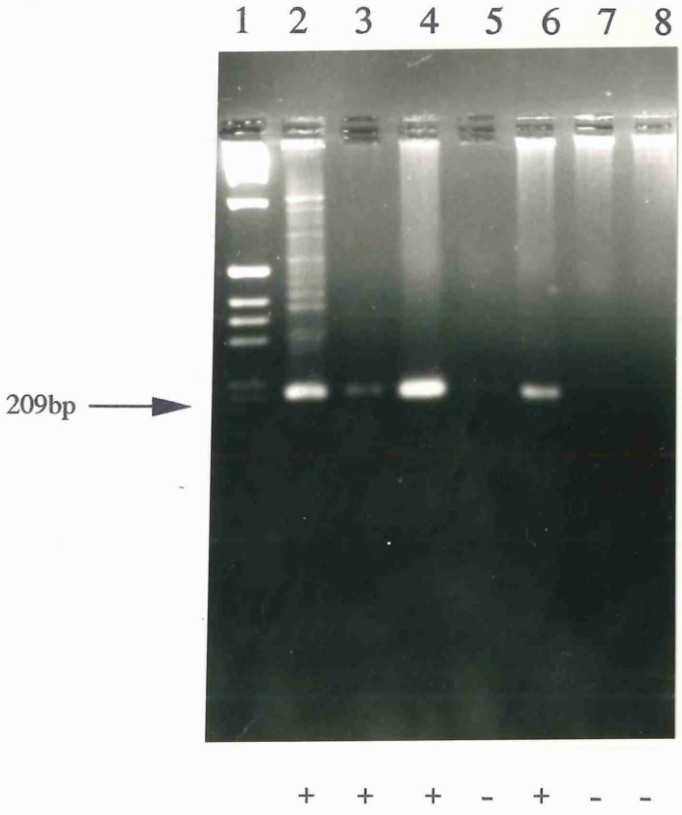

Figure 17: Screening of hybrid panel with ARH12.

Lane 1:1kb ladder. Lane 2: illustrates the 209 base pair amplicon following amplification of total genomic DNA with the ARH12 primers. Lane 3: Dis2.6, Lane 4: IIA4, Lane 5: negative control, Lane 6: GPS13, Lane 7: GPS17, Lane 8: a23, hamster DNA. The hybrids Dis2.6, GPS13 and IIA4 are positive for ARH12.

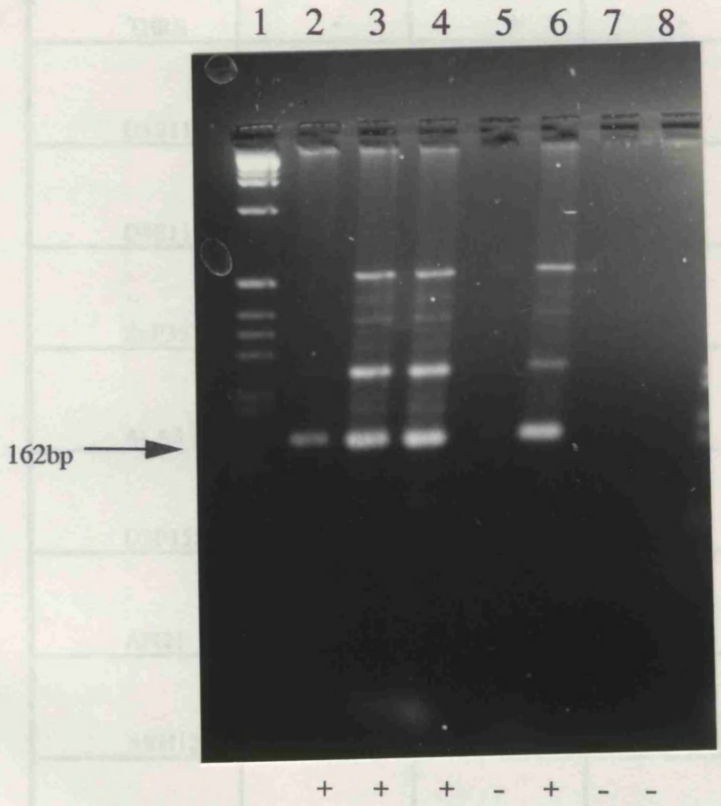

Figure 18: Screening of hybrid panel with ALAS 1.

Lane 1:1kb ladder. Lane 2: illustrates the 162 base pair amplicon following amplification of total genomic DNA with the ALAS 1 primers. Lanes 3-6 are the hybrids screened with D3F15S2. Lane 3: Dis2.6, Lane 4: GPS17, Lane 5: GPS13, Lane 6: IIA4, Lane 7: a23, hamster DNA. Lane 8: negative control.
The hybrids Dis2.6, GPS17 and IIA4 are positive for ALAS 1 .

	Hybrids			
MARKER	Dis2.6	GPS13	GPS17	IIA4
THRB	-	+	-	+
D3S1100	-	+	-	+
D3S11	-	+	-	-
ZnF35	-	+	-	-
ALAS3	+	+	-	-
D3F15S2	+	+	-	+
APEH	+	+	-	+
ARH12	+	+	-	+
ZnFP16	+	ND	ND	ND
ALAS1	+	-	+	+
ACY-1	ND	-	+	ND
3q(SI)	-	-	+	+

Table 7: Characterisation of hybrid mapping panel using 3p markers.

Dis2.6 is the 3p21 fragment hybrid. GPS13 and GPS 17 represent the Greig cephalopolysyndactyly hybrids containing the different halves of the translocation. IIA4 is the Deak hybrid containing the deleted chromosome 3 homologue. '+/-' denote presence and absence of marker respectively and 'ND' not determined.

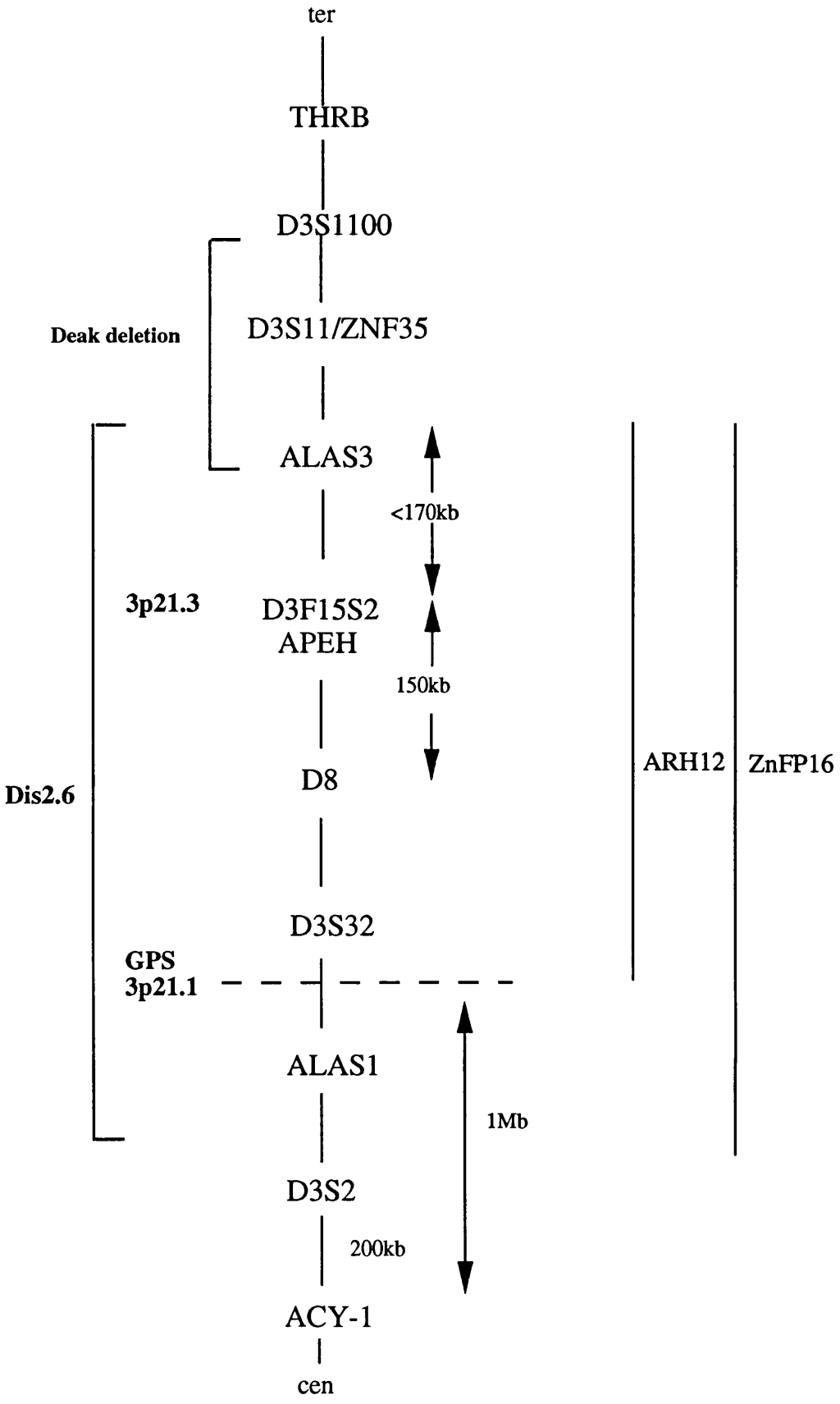

Figure 19: Physical map of markers and genes on 3p .

The map illustrates the ordering of markers through the characterisation of a panel of somatic cell hybrids. The positions of the Deak deletion and the 3p21 fragment of Dis2.6 are illustrated. The dashed line symbolises the GPS translocation breakpoint at 3p21.1. The ARH12 gene could only be assigned to the region contained in Dis2.6, distal of the GPS breakpoint. The ZnFP16 gene was only analysed in the Dis2.6 hybrid and therefore localised to the interval 3p21.1-p21.3.

3.2 Dis2.6 AND THE ISOLATION OF D8

The isolation and characterisation of the gene D8 was part of a collaborative project with Pr C.H.C.M.Buys at the Department of Medical Genetics, State University of Groningen. The entire collaborative programme which led to the isolation of D8 and the studies of its expression by analysis of mRNA in adult and foetal lung and cultured small cell lung lines is summarised here. The work is fully described in the publication, "*Cancer Research* " (in press), by Carritt et al. My specific role in the project was to isolate single copy clones around D8 for the isolation of coding sequence and to contribute he physical mapping of the D8 region on 3p21. This is an important component of the overrall thesis and is presented in subsequent chapters.

As discussed earlier, the marker D3F15S2 is consistently reduced to hemizygosity in SCLC. The hybrid, Dis2.6 previously shown to contain this locus was used in this study (Carritt et al. 1986). The 3p component of the hybrid, Dis2.6 is a fragment of 3p21 which maps in the interval D3S2-THRB (Carritt et al. 1992).

A previously identified neighbouring gene, acylpeptide hydroylase, APEH, mapping 4kb from the D3F15S2 locus has been shown to be expressed at convincingly normal levels in SCLC (Naylor et al.1989).

Therefore, in an attempt to isolate other coding sequences in the proximity of the D3F15S2 locus, a genomic library was constructed from the Dis2.6 hybrid. A random human genomic clone, pD8, was isolated from the library and localised to 3p21 by in situ hybridisation. A single copy probe D8A1 was isolated and used to examine DNA from a wide variety number of species. Extensive cross hybridisation was observed suggesting that D8 contained conserved sequences which may include coding DNA. The same clone was therefore used to screen a lung cDNA library.

Following the isolation of a partial cDNA clone, cD8, a number of different cDNA libraries were used in an attempt to isolate a full length cDNA. To date the longest

cDNA identified is a 3.3kb clone, PB3.3, which detects a 3.4kb transcript in normal lung mRNA. Sequence analysis has illustrated that all the coding region is contained within the clone PB3.3.

A wide variety of cell types were shown to express the D8 transcript including adult and foetal lung . . However, no detectable transcript could be observed in all 23 SCLC cell lines analysed. Further analysis of the D8 transcript in SCLC included the use of quantitative PCR. This method allowed the detection of some endogenous D8 RNA in SCLC lines but only 0.5-3% of the normal levels of D8 mRNA were detectable.

The reduced expression of D8 in SCLC cell lined illustrates its possible involvement in the origin or progression of the tumour. The search for point mutations and genomic rearrangements involving this gene, in at least a subset of tumours may assist in defining of the role of this gene.

3.3 GENOMIC CLONING AROUND D8

3.3.1 ISOLATION OF COSMID D8A1.4

The original genomic clone isolated from the Dis2.6 hybrid and assigned to 3p21 was named pD8, and a single copy sequence derived from it, D8A1, was used to isolate the D8 cDNA (see section 3.2). A Lorist B cosmid library of normal genomic DNA was screened with D8A1 and two positive clone, cos D8A1.4.and cos4.13 were isolated. The restriction enzymes BamH1, EcoRI, and Bgl II were used in single and double digests of the cosmids, illustrated on ethidium stained agarose gels in figure 20a. DNA was transferred by Southern blotting and the filters hybridised to the original screening probe and Lorist B vector DNA to identify vector containing fragments. Following hybridisation with the D8 cDNA only the cosmid D8A1.4 was shown to contain coding regions of the gene with no signals in the 4.13 cosmid (figure 20b).

3.3.2 ISOLATION AND FINE MAPPING OF SINGLE COPY REGIONS.

Southern blots of restriction digests of cos D8A1.4 with the enzymes described above were screened with sonicated total genomic DNA in order to identify potential single copy sequences. Fragments failing to hybridise signified single copy regions in the cloned DNA. With the double digest, BamH1/EcoRI, two possible single copy fragments were identified. The first, a 4.1 kb fragment which overlapped with the original clone pD8. and the second a 0.6kb BamH1 fragment (see figure 22). The 4.1kb BamH1 fragment was isolated and subcloned into pUC18 and referred to as pD8B4.1 hereafter

3.3.3 RESTRICTION MAPPING OF pD8B4.1 AND pD8

The two overlapping clones pD8B4.1 and the phage clone pD8 were mapped using the enzymes, BamHI, EcoRI, BstEII and Sst I Figures 21a illustrates an analytical ethidium bromide stained gel of pD8 and pD8B4.1. As shown in figure 21b.the partial cDNA of D8, E1.6, isolated at this time, was shown to map outside the D8A1 region extending into the pD8B4.1 fragment.

This data was used in the construction of a restriction map of the cosmid D8A1.4 which is presented in figure 22.

3.3.4 MAPPING OF THE D8 cDNA IN COSMID D8A1.4 AND GENOMIC DNA

The longest D8 cDNA isolated to date is 3.3kb and is believed to contain all the coding sequence of the D8 gene (Carritt et al. 1992). In order to assess the amount of coding sequence contained within the cosmid D8A1.4, the cDNA was used in hybridisation experiments of genomic and cosmid DNA.

The PB3.3 was hybridised to the BamHI restriction fragments of the D8A1.4.cosmid. As illustrated in figure 20b, four fragments were were shown to hybridise to the cDNA. According to the relative position of these fragments (figure 22), the data suggests that the cDNA maps to a contiguous 9.4 kb region in the cosmid D8A1.4. The largest hybridising fragment, greater than 12kb, includes the vector containing 5.5kb fragment, and therefore does not represent the actual size of the BamHI fragment in genomic DNA.

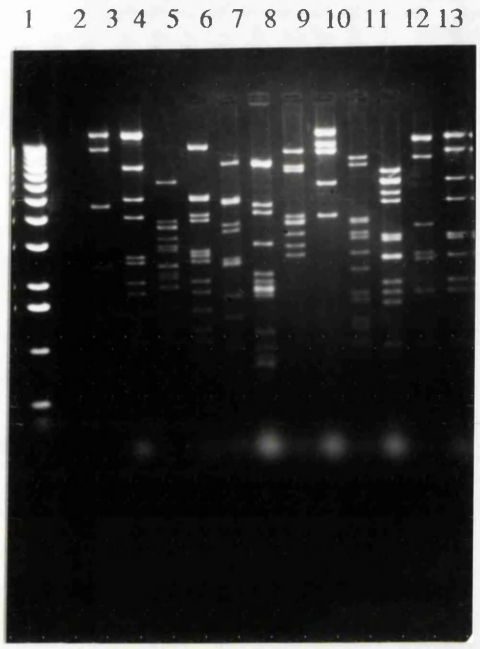

Fig. 20a

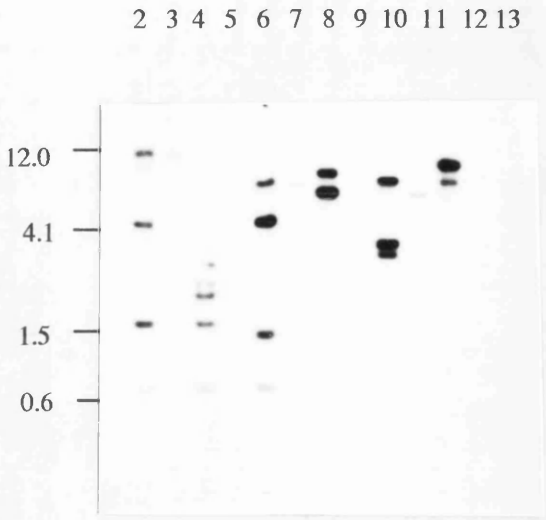

Fig. 20b

Figure 20: Analytical studies of cosmds D8A1.4 and 4.13.

Figure 20a: Analytical ethidium bromide gel of cosmids D8A1.4 and 4.13.
Lane 1: 1 kilobase size marker. Lane 2: D8A1.4 x BamHI, LAne 3: 4.13 x BamHI, Lane 4: D8A1.4 x BamHI/EcoRI, Lane 5: 4.13 x BamHI/EcoRI, Lane 6: D8A1.4 x BamHI/BglII, Lane 7: 4.13 x BamHI/BglII, Lane 8: D8A1.4 x EcoRI, Lane 9: 4.13 x EcoRI, Lane 10: D8A1.4 x EcoRI/BglII, Lane 11: 4.13 x EcoRI/BglII, Lane 12 : D8A1.4 x BglII, Lane 13: 4.13 x BglII.

Figure 20b: Autoradiogram of analytical gel following hybridisation to the D8 cDNA (PB3.3).
The figure presents an autoradiogram of the cosmid D8A1.4 analytical gel (fig 20a) following hybridsation to D8 cDNA, PB3.3. Four BamHI fragments ,12kb, 4.1kb, 1.5kb and 0.6kb, (lane 2) and two EcoRI fragments, 8.8kb and 6.4kb, (lane 8) of the D8A1.4 cosmid hybridise to the cDNA. However, no signals are observed with the 4.13 cosmid.

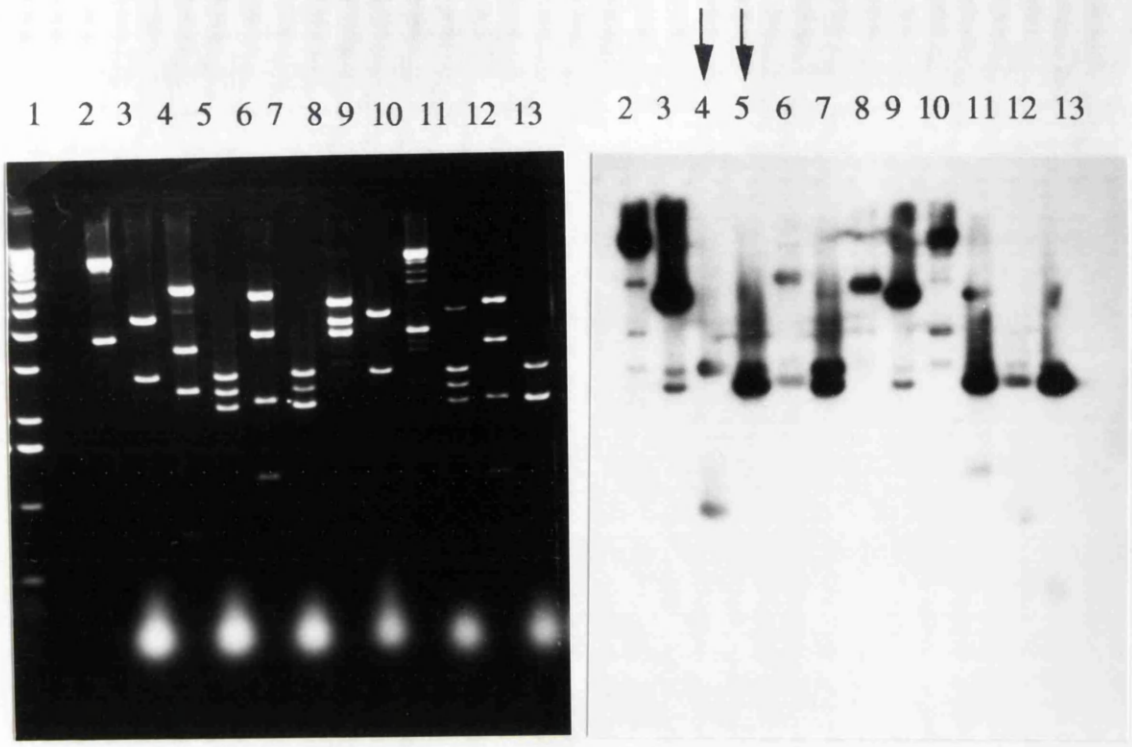

fig21a

fig21a

Figure 21: Analytical studies of pD8 and pD8B4.1.

Figure 21a: Analytical restriction enzyme analysis of the two subclones pD8 and pD8B4.1.

Lane 1 is the 1kilobase size marker . Lanes 2 and 3 are single digest of pD8 and pD8B4.1 respectively to release each. Lane 4-13 are double and triple digets of the subclones, which includes the enzyme required to cleave the inserts. Lane 4:pD8 x EcoRI/BamHI, Lane 5: pD8B4.1 x BamHI/EcoRI, Lane 6: pD8 x EcoRI/BstEII, Lane 7: pD8B4.1 x BamHI/BstEII, Lane 8: pD8 x EcoRI/MluI. Lane 9: pD8B4.1 x BamHI/ MluI, Lane 10: pD8 x EcoRI/SstI, Lane 11: pD8B4.1 x BamHI x SstI, Lane 12: pD8 x EcoRI/BstEII;/BamHI, Lane 13: pD8B4.1 x BamHI/BstEII/EcoRI.

Figure 21b: Hybridisation of the partial cDNA D8 clone, E1.6 to pD8 and pD8B4.1.

The figure presents the autoradiograph of the analytical gel (figure 21a) following hybrisation to the partial cDNA clone, E1.6. The cDNA hybridises to the same 2.3kb BamHI/EcoRI in pD8B4.1 and pD8 suggesting an overlap of the two clones. The 0.6kb BamHI/EcoRI fragment in the pD8 and a 2.0kb fragment in pD8B4.1 (lanes 4 &5 respectively (arrows) represent the non-overlapping ends of each clone as illustrated in figure 20.

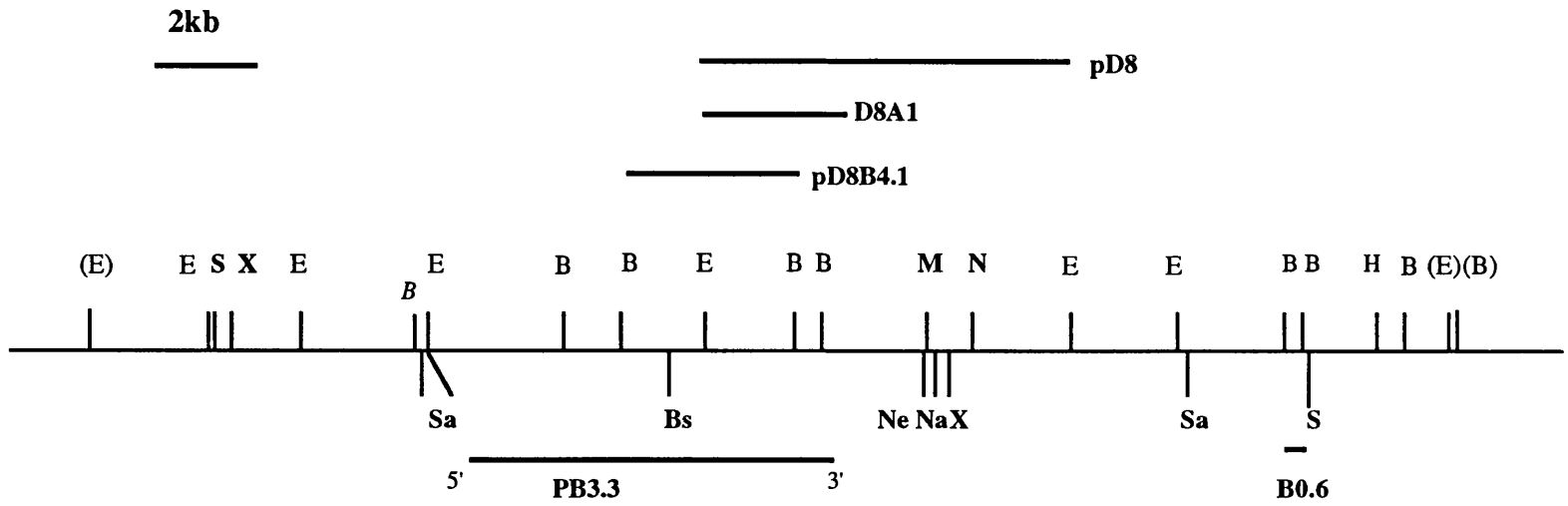

Figure 22: Restriction map of the cosmid D8A1.4.

The position of the D8 coding sequence, PB3.3, and the relative position of the subclones B0.6,D8A1, pD8B4.1 and pD8 illustrated.

'B ' denotes BamHI site lost in the cloned DNA but present in genomic DNA. (B) and (E) denote restriction sites in Lorist vector.

(Rare cutting enzymes are shown in bold. B:BamHI, B:BssHII, E:EcoRI, H:HindIII, S:SacII, Sa:SalI, M:MluI, N:NruI, Na:NarI, Ne:NaeI, X:XhoI)

In order to determine the actual size and number of BamHI fragments containing D8 coding sequence, genomic DNA was restriction digested and hybridised to the PB3.3 cDNA. As indicated in figure 23, five BamHI fragments are shown to hybridise to total genomic DNA. The largest fragment of approximately 10-12kb may represent the 10-12kb vector-containing fragment in the cloned DNA. However, the additional 3kb BamHI fragment, not observed in the D8A1.4 cosmid, must either represents a fragment outside the cloned region, a restriction fragment length polymorphism or a lost BamHI site in the cosmid (see figure 22).

To test for the possibility of an RFLP, the cDNA was used in hybridisation experiments of 50 random genomic samples. As illustrated in figure 24 the 3kb BamHI hybridising fragment can be observed in all genomic samples and in 429, the somatic cell hybrid containing a single chromosome 3. This suggests that the 3kb BamHI fragment does not represent an RFLP but may represent a cross hybridising locus, not 3p21. The PB3.3 cDNA was therefore used as a hybridising probe of BamHI digests of the hybrid 429. However, as illustrated in figure 24b , the 3kb BamHI fragment is observed in the hybrids, suggesting that this 3kb BamHI fragment maps to chromosome 3p.

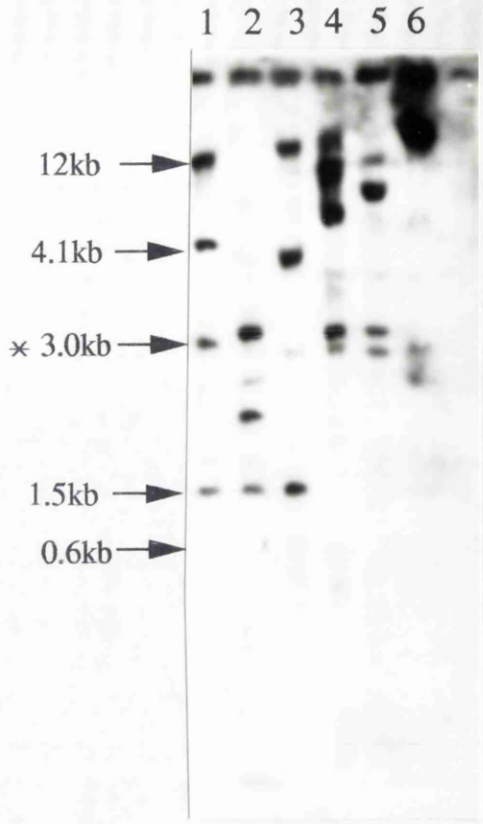

Figure 23: Hybridisation of D8 cDNA to total genomic DNA.

The figure presents an autoradiogram of genomic DNA following restriction enzyme digest, blotting and hybridsation to the D8 cDNA, PB3.3.

Lane 1:BamHI, Lane 2: BamHI/EcoRI, Lane 3: BamHI/HindIII, Lane 4: EcoRI, Lane 5: EcoRI/HindIII, Lane 6: HindIII. Five BamHI fragments, 12kb, 4.1kb, 3.0kb, 1.5kb and 0.6kb, (lane1) and two EcoRI fragments, 8.8kb and 6.4kb, (lane 4) hybridise to genomic DNA. The 3kb fragment not observed in the cosmid D8A1.4 (figure20) is shown by the asterix.

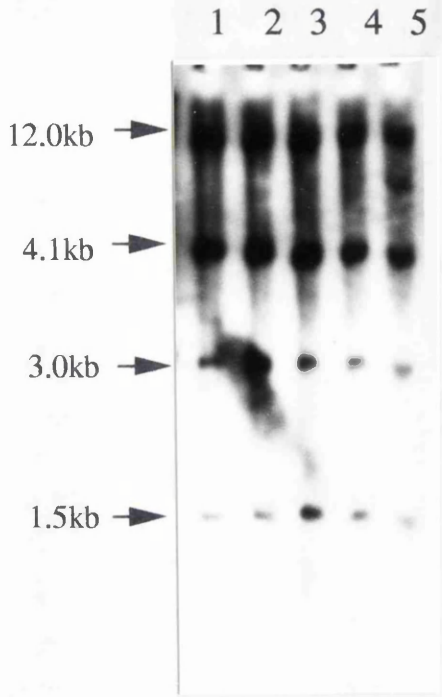

Fig 24a

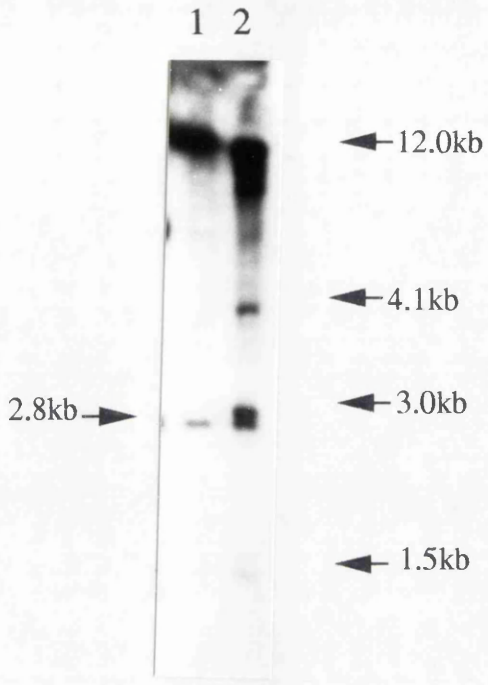

Fig 24b

Figure 24:Genomic analysis of the D8 cDNA (PB3.3)

Figure 24a: RFLP search with BamHI restriction enzyme.

Lanes 1-5 each contain 10 pooled BamHI genomic DNA samples following Southern blotting and hybridsation to PB3.3. Four BamHI bands, 12kb, 4.1kb, 3.0kb and 1.5kb are seen in the pooled genomic samples. The expected 0.6kb BamHI genomic fragment is not visible here.

Figure 24b: Analysis of 3kb BamHI fragment.
Genomic DNA from the control hybrid, 429 and the hamster cell line, a23, were digested with BamHI, Southern blotted and hybridised to PB3.3. Lane 1: 429, control hybrid DNA, Lane 2: a23 DNA (chinese hamster). In the 429 hybrid, four BamHI fragments, 12kb, 4.1kb, 3.0kb and1.5kb are observed. A hamster specific 2.8kb fragment is present in the 429 hybrid, (lane 2) and the hamster DNA, (lane 1).

3.3.5. BIDIRECTIONAL WALK FROM D8

Bidirectional cosmid walks from the D8 cosmid were carried out to extend the cloned region around D8

3.3.5.1.Right hand walk-Isolation of cos 6.1

The 0.6kb BamHI single-copy fragment, B0.6, was used to screen the cosmid library in an attempt to isolate cosmid clones extending outside the cloned region. A low melting point agarose slice of the BamHI fragment was used as a probe in library screening. After the second round positive clones were rescreened with the subclone, pD8B4.1, isolated from cos D8A1.4. Positive clones were discarded, to reduce the possibility of isolating clones with extensive overlaps to original cosmid, D8A1.4. One cosmid clone, cos 6.1 was chosen for further study.. Figure 25 illustrates the restriction fragments observed with this and the "parental" cosmid D8A1.4. The 0.6kb BamHI fragments can be observed in both clones. Following hybridisation with the BamHI 0.6kb single copy fragment, both cosmids were positive for the 0.6kb fragment. The absence of any hybridisation of the cDNA, PB3.3, to the cosmid 6.1 suggests the an extension of the cosmid D8A1.4 in the 3' direction of the D8 gene (figure 26). The comparison of the EcoRI fragments of the cosmids 6.1 and D8A1.4 suggests that the overlap is limited to 6-7kb. Figure 27 presents a map of the two clones, D8A1.4 and 6.1 to illustrate the overlapping regions.

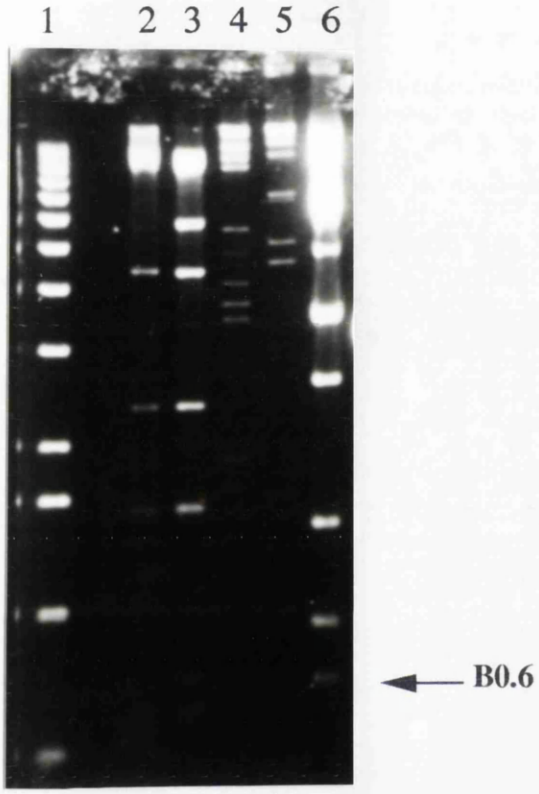

Figure 25: Comparative analytical restriction digests of cosmids D8A1.4 and 6.1.

The figure illustrates an ethidium analytical bromide stained gel of the cosmids D8A1.4 and 6.1 following digestion with BamHI. The arrow indicates the BamHI 0.6kb fragment used to isolate the cosmid clone, 6.1. Lane 1: 1 kilobase size marker, Lane 2 : cos D8A1.4, Lane 3: D8A1.4.2 (a D8A1.4 related cosmid), Lanes 4 + 5: cos 6.2 and 6.3 respectively, (isolated during the bidirectional walk but not related to the cosmids D8A1.4 or 6.1), Lane 6: cos 6.1. The 0.6kb BamHI fragment is seen in the two cosmids D8A1.4 and 6.1.

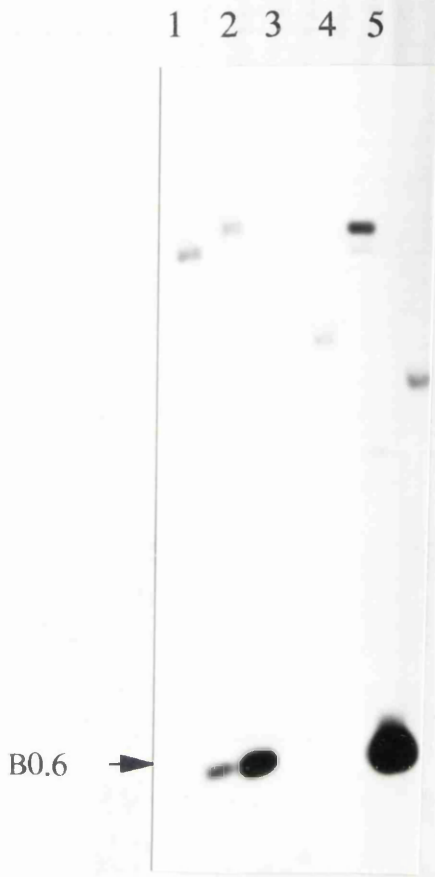

fig26a

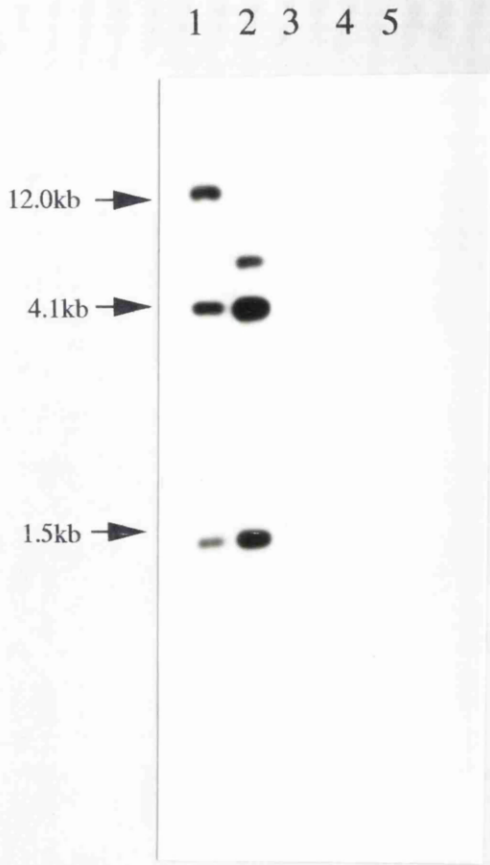

fig26b

Figure 26: Analysis of the cosmids D8A1.4 and 6.1.

Figure 26a: Hybridisation of D8A1.4 and 6.1 with the subclone B0.6kb.

The analytical gel in figure 25 was blotted and hybridised with the BamHI 0.6kb fragment, B0.6. The same BamHI 0.6kb fragment is observed in both cosmids, D8A1.4 (lane 1) and 6.1(lane 5), in addition to the D8A1.4 related clone (lane 2).

Figure 26b: Hybridisation of D8A1.4 and 6.1 with the D8 cDNA.

The analytical gel presented in figure 25 was blotted and hybridised to the the D8 cDNA, PB3.3. Only the original cosmid, D8A1.4 and its related clone hybridises to the cDNA, PB3.3. Three of the four BamHI fragments, 12.0kb, 4.1kb and 1.5kb can be observed in the cosmid D8A1.4 (lane1) and three fragments in the D8A1.4 related clone. No hybridisation to the cosmid 6.1 (lane 5) can be observed.

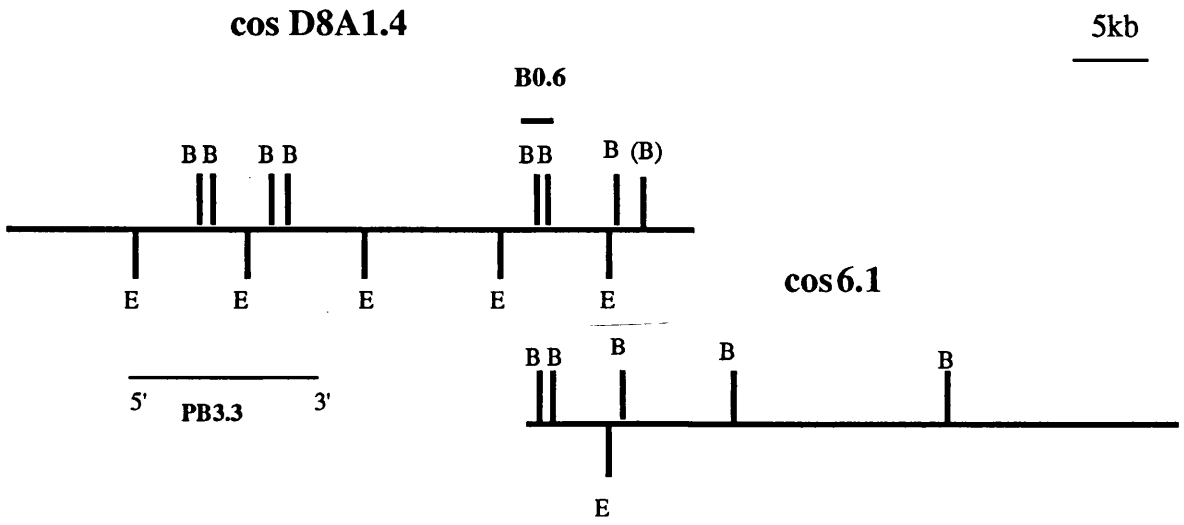

Figure 27: Overlap of cosmids D8A1.4 and 6.1.

Cosmid 6.1 was isolated using the B0.6kb single copy fragments as a screening probe. The overlap is limited to the end 5-6 kb of cosmid D8A1.4 with one BamHI fragment, 0.6kb, shared with the cosmid 6.1. (B) denotes the BamHI site in Lorist B.

3.3.5.2 Left hand walk-Isolation of Cos D8.3 and D8.10

The Lorist B vector contains SP6 and T7 RNA polymerase promoters flanking the BamHI cloning site which provide a convenient way of synthesizing RNA probes ("riboprobes") specific for each end of the cloned insert DNA (Cross & Little 1986). However, although this worked well using the T7 polymerase, very poor results were repeatedly obtained using SP6 polymerase. The following protocol for performing bidirectional walks using T7 polymerase was devised. Cosmid clones were cleaved with the restriction enzyme HindIII which is at a single site in the vector between the T7 promoter and the cloning site. Recircularisation of Hind III cleaved cosmid DNA thus has the effect of positioning the T7 promoter near the other end of the original insert; recircularised Hind III cut cosmid DNA was introduced into DHI cell and recombinants selected in kanamycin. This was applied to the cosmid D8A1.4.

The riboprobe synthesised from cos D8A1.4 and hybridised to a BamHI restriction digest of cos D8A1.4. The expected single BamHI hybridising band was observed, thus confirming the origin of the riboprobe and its low number of repeat sequences. The riboprobe could therefore be used to screen the cosmid library. Following the second round of screening, positive clones were screened out with the MluI-NruI single copy fragment isolated from cosmid D8A1.4, to prevent extensive overlaps of the new cosmids.

The two new clones, D8.6 and D8.10, together with the original cosmid, D8A1.4, were digested with BamHI and analysed on an agarose gel shown in figure 28. The riboprobe hybridised to the same BamHI fragments in the cosmid clones, D8A1.4and D8.10 suggesting an overlap between the three cosmids, (figure 29).

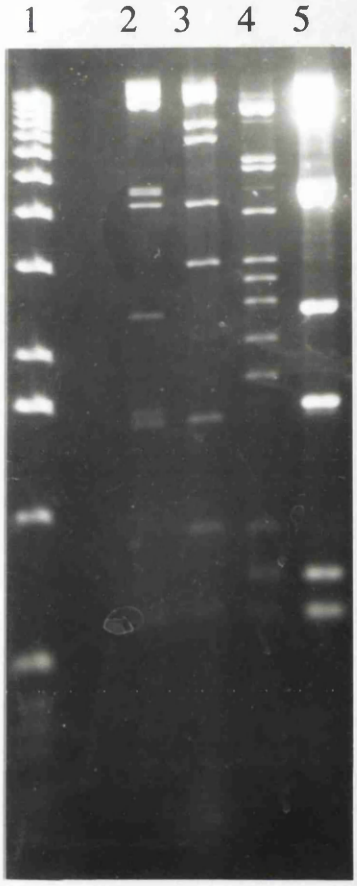

Figure 28: Analytical gel of the cosmid clones D8A1.4 , D8.3, D8.6 D8.10.

The figure illustrates an analytical gel of the cosmid clones isolated during the walk from the parental cosmid D8A1.4. Lane 1: 1kb ladder, Lanes 2-4 are BamHI restriction digests of cosmids D8.3, D8.10, D8.6 and D8A1.4.

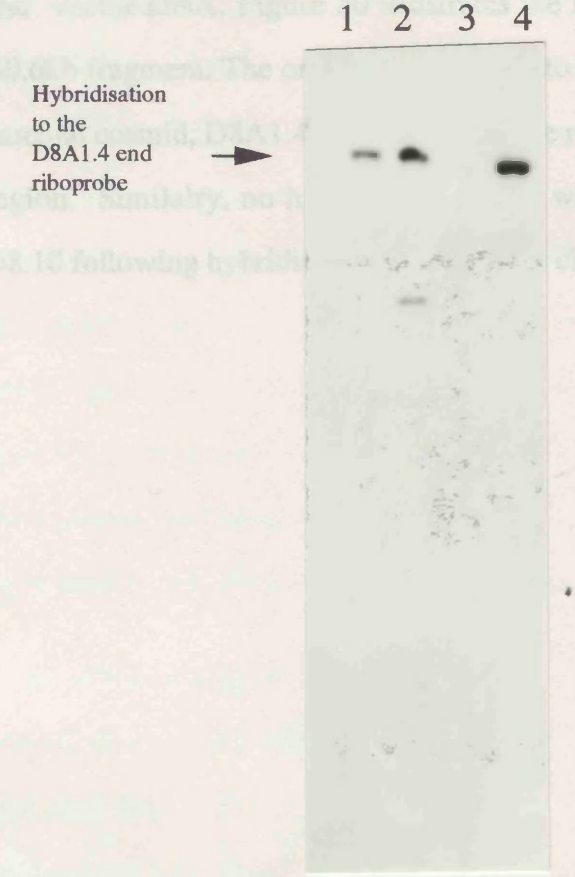

Figure 29: Hybridisation of cosmids D8A1.4, D8.3, D8.6 and D8.10 to the D8A1.4 end riboprobe.

The analytical gel presented in figure 28 was blotted and hybridised to the riboprobe synthesised from D8A1.4.
Lane 1: D8.3, Lane 2: D8.10, Lane 3: D8.6, Lane 4: D8A1.4. The riboprobe hybridised to all cosmids except D8.6.

The cosmids were also hybridised to the subclone, B 0.6, the D8 cDNA, PB3.3 and vector DNA. Figure 30 illustrates the results following hybridisation to the B0.6kb fragment. The only cosmid shown to contain this B0.6 kb subclone was the parental cosmid, D8A1.4, indicating that the remaining clones did not overlap at this region. Similalry, no hybridising signals were observed in the cosmids, D8.3 or D8.10 following hybridisation to the PB3.3 cDNA (data not shown).

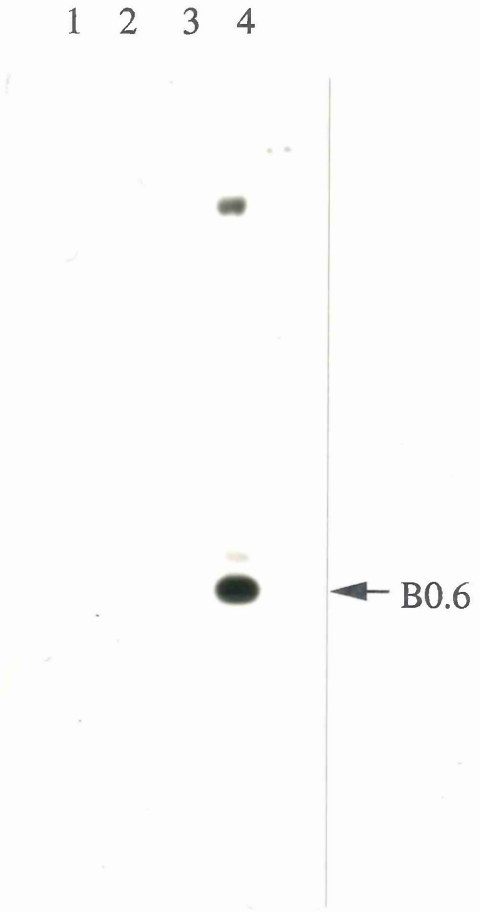

Figure 30: Hybridisation cosmids D8A1.4 , D8.3, D8.6 D8.10 to the BamHI 0.6kb subclone.

The analytical gel presented in figure 28 was blotted and hybridised to the B0.6 subclone. Lanes 1-4 are BamHI restriction digests of cosmids D8.3, D8.10, D8.6 and D8A1.4 respectively following hybridsation to the BamHI 0.6kb subclone. Only the parental cosmid, D8A1.4 contains the B0.6kb fragment.

3.3.6 DNA FINGERPRINTING OF D8A1.4 AND RELATED COSMIDS

As part of a collaborative project with Dr P.F.R. Little, a majority of the cosmid clones isolated during this study were fingerprinted. This enabled the confirmation of overlapping cosmid clones previously illustrated through hybridisation studies. The DNA fingerprinting technique used here is based on the method of Coulson et al. (1986). It involves the stereotyped restriction digest and radioactive labelling of DNA fragments. The banding pattern of each of these cosmids is resolved on polyacrylamide gels with standard markers and autoradiographed. Scanning densitometer and image processing software described by Sulston et al (1988&1989) were used for the analysis of cosmid clones and cosmid contig construction.

One of the contigs identified through this method included cos D8A1.4 and D8.10 (RP10) described above. Figure 31 illustrates the cosmids contained in the contig and their fingerprints. The absence of D8.3 and D8.6 from the contig confirms the early suspicion that they are unrelated and that their isolation was based on homologous sequences contained in the screening riboprobe.

Of particular interest here is the presence of the cosmid 4.13 discussed in section 3.3.1.This cosmid is buried in the contig suggesting that on the basis of DNA fingerprints, 4.13 is homologous to a region covered by D8.10 and D8A1.4. As described earlier, this cosmid was isolated using a genomic clone D8A1, which contains at least 700bp of the D8 gene. However, the cosmid showed no hybridisation to the D8 cDNA(D8). On the basis of the fingerprinting data, which demonstrates convincing homology of cos4.13 to D8A1.4 and D8.10, a homologous region must exist at another distinct locus. In situ chromosome hybridisation of the cosmid 4.13 would at least indicate if this region is on chromosome 3 or elsewhere in the genome.

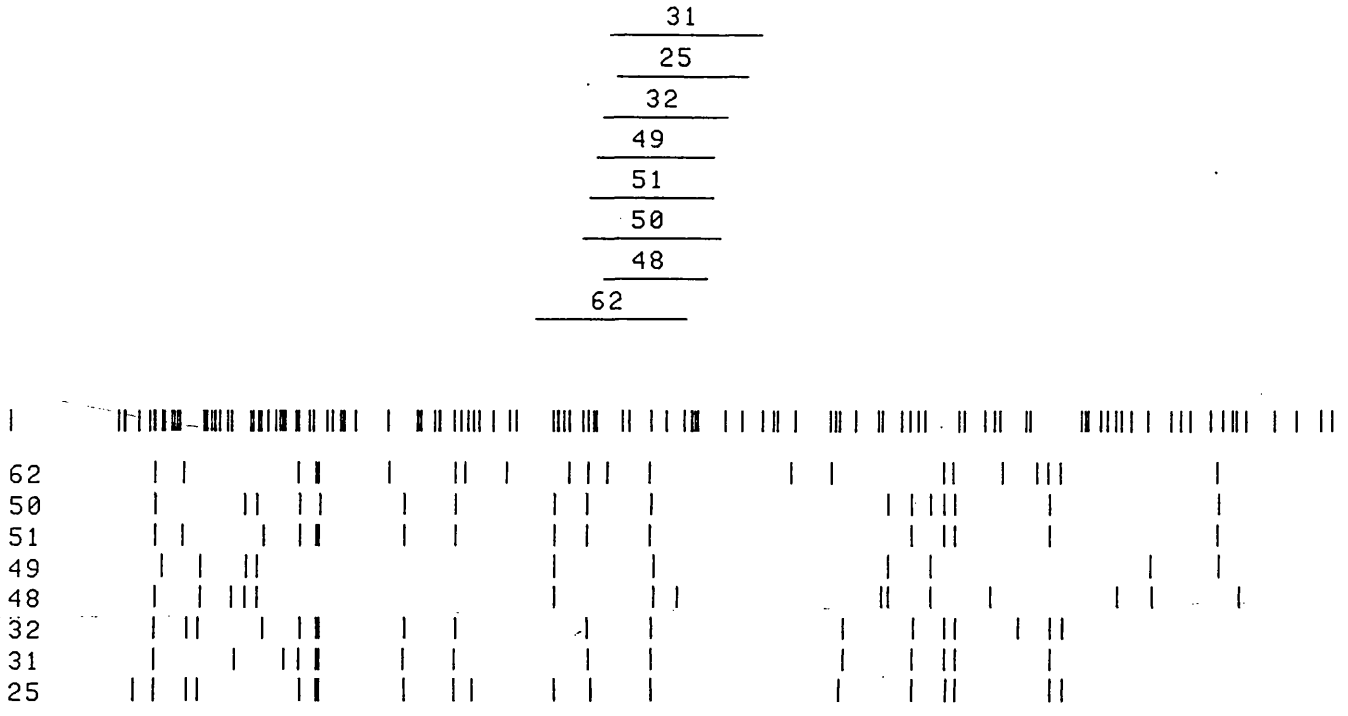

Figure 31: Cosmid contig and fingerprints of D8A1.4, D8.10 and 4.13.

The figure above illustrates the assembled contig following DNA fingerprinting of the cosmids isolated during the walk from the parental clone D8A1.4.The clones are as follows: 31:D8.10, 25:D8.9, 32:D8.11, 49:4.13, 51:D8A1.2, 50:D8A1.1, 48:D8.7, 62:D8A1.4. The figure below illustrates the fingerprints of each cosmid, the first row is the marker lane.

The contig demonstrates the overlap between the D8A1.4 (62) and D8.10 (31). In addition the cosmid clone 4.13 (49) which does not contain any D8 coding region is embedded in the contig above.

3.3.7 IDENTIFICATION OF POTENTIAL CpG ISLANDS IN COSMIDS D8A1.4 and D8.10

CpG islands are considered to mark many genes and are likely to contain control regions and 5' ends of genes Bird (1987). It is therefore valuable to identify potential islands in cloned DNA. Restriction endonucleases with recognition sites that are G+C rich have been shown to show a strong preference for island over inter-island DNA (Bird 1987) In genomic DNA inter-island CpGs are not only rare but are methylated and therefore immune to cutting by C-G enzymes. The absence of methylation in cloned DNA prevents this screening out of non-island DNA as all C-G recognition sites are cut. However, certain C-G enzymes still cut preferentially at islands in cloned DNA. In addition a high proportion of clusters containing restriction sites for the rare cutting enzymes, MluI, NotI, PvuI and NruI are believed to represent a CpG island in genomic DNA.

In order to determine any potential CpG islands in the three cosmids identified,one containing the D8 gene, each was cleaved with BamHI or EcoRI together with a panel of rare cutting enzymes. DNA fragments flanked by rare cutting sites in cloned DNA can be used as potential linking markers for long range mapping by pulsed field gel electrophoresis (section 3.4).

3.3.7.1 Rare cutting sites in cosmid D8A1.4

Figure 32 shows the changes in restriction fragment lengths with the EcoRI enzyme plus a series of rare cutters.in the cosmid D8A1.4 With the exception of SalI and BssHII, six rare cutting enzymes,(MluI, NruI, SacII, NarI) are present in 8.8kb EcoRI fragment. BssHII cleaves the 6.4kb EcoRI fragment. As indicated in figure 22, the 6.4kb and 8.8kb EcoRI fragments are adjacent to each other in the cosmid.

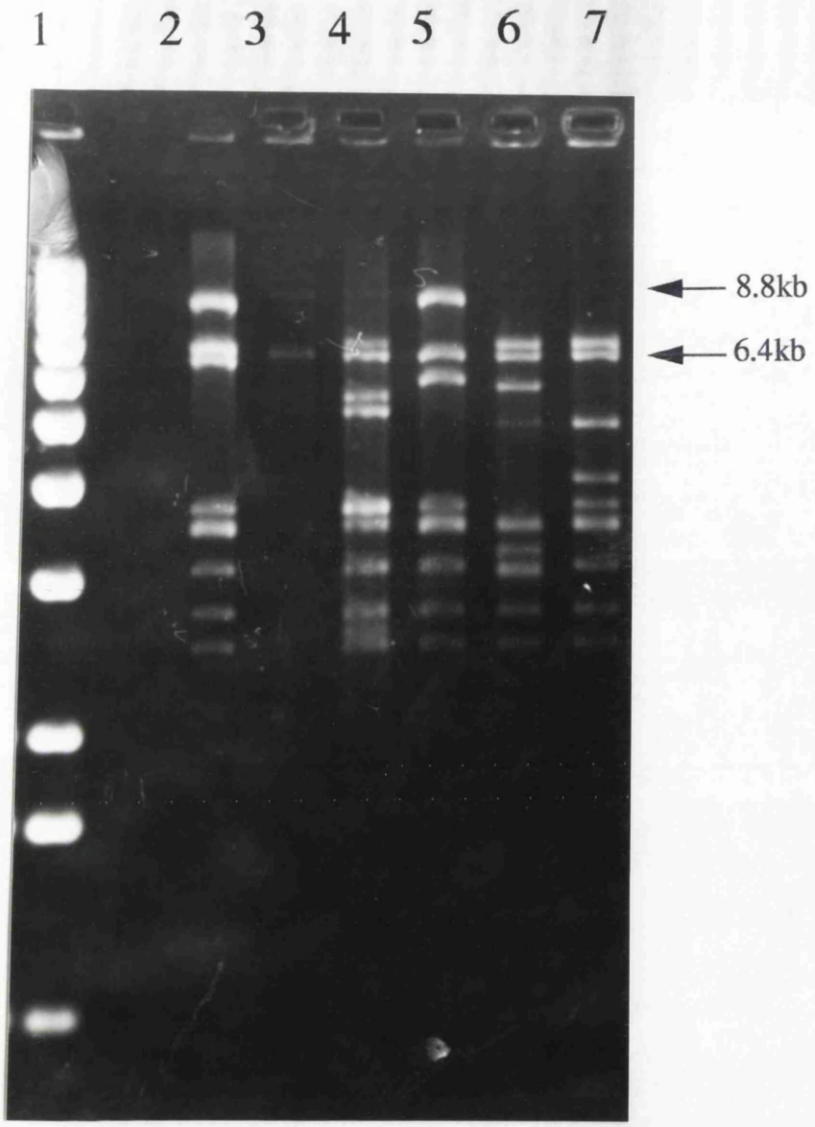

Figure 32: Analytical gel of cosmid D8A1.4: Idenitifying rare cutting sites.

Lane 1: 1kb size marker, Lane 2: EcoRI only, Lane 3: EcoRI+BssHII, Lane 4: EcoRI + MluI, Lane 5: EcoRI+ SalI, Lane 6: EcoRI+NruI and Lane 7: EcoRI+NarI.

All rare cutting enzymes cleave the 8.8kb EcoR1 fragment except BssHII and SalI which cleave the adjacent 6.4kb EcoRI fragment.

3.3.7.2 Rare cutting sites in cosmid D8.10

Figure 33. presents an analytical gel of rare cutting enzymes. Three rare restriction sites, SacII and NaeI were contained within the same EcoRI fragment indicating a locally high C+G content and raised incidence of the CpG dinucleotide.

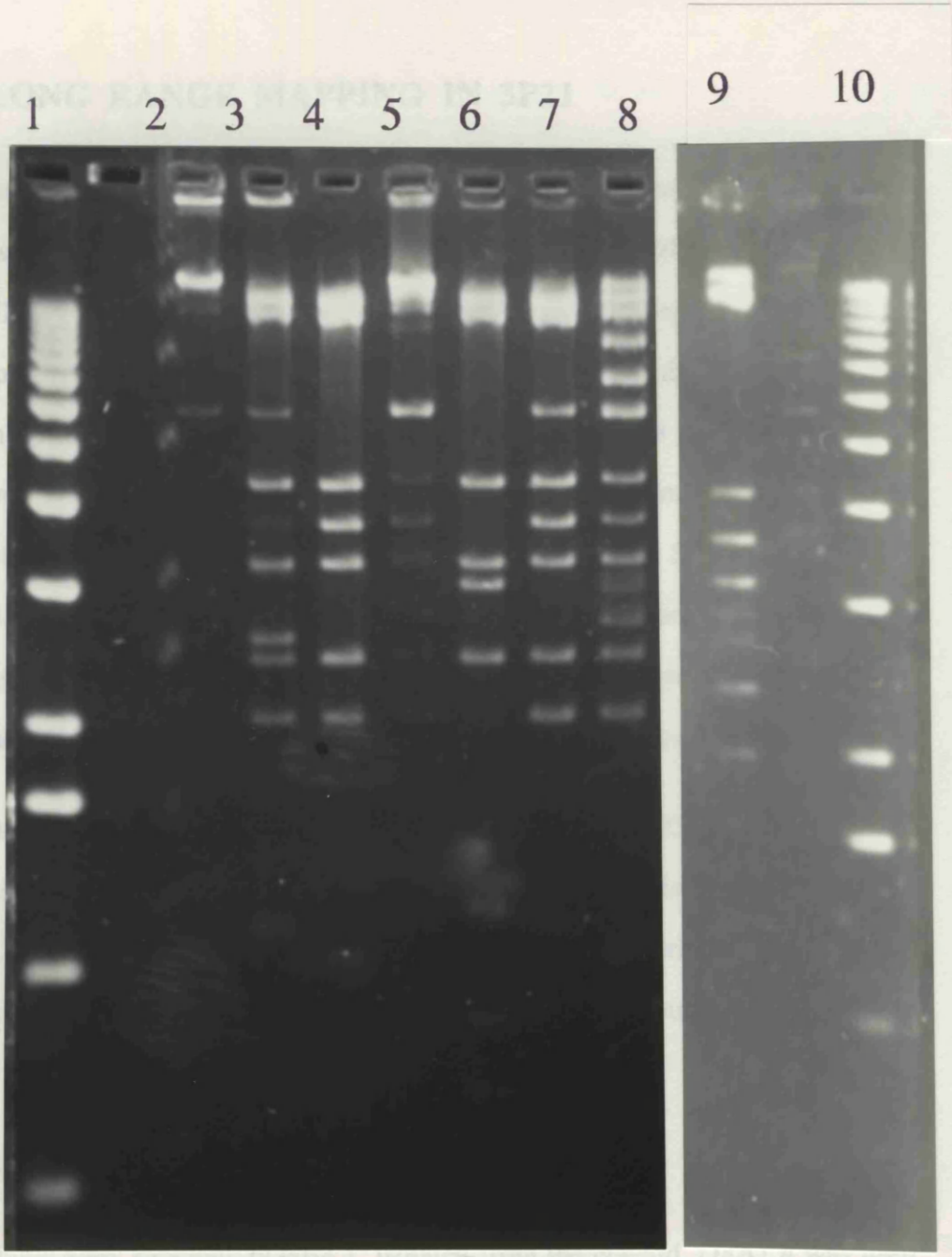

Figure 33: Analytical gel of cosmid D8.10: Idenitifying rare cutting sites.

Lanes 1: 1kb size marker, Lane 2: EcoRI only , (repeated in lane 10), Lane 3: EcoRI + SalI, Lane 4: EcoRI + MluI, Lane5: EcoRI+ NaeI , Lane6: EcoRI+NruI, Lane 7: EcoR1+ NaeI, Lane8: EcoRI+SacII, Lane 9: 1 kilobase ladder and Lane 10 EcoRI only.

The rare cutting enzymes, SacII and NaeI cleave the same 6.0kb EcoRI fragment .

3.4 LONG RANGE MAPPING IN 3P21

Long range physical maps can be generated from DNA separated on pulsed fields by the use of standard southern blot techniques. Mammalian DNA can be analysed by PFGE after cleavage by rare cutting enzymes. As described in section 2.2.8.2 these contain one or more CpG dinucleotides in their recognition sites. Some of these enzymes cut very rarely, every 500-1500kb and therefore generate very large fragments. These include PvuI, NotI NruI and MluI. Other enzymes cleave more frequently generating fragments from 50-500kb, for example SacII, SalI, BssHII, NarI, NaeI, and SfiI. (Barlow & Lehrach 1987). Depending on the enzymes used and fragment sizes expected, different pulsing times, voltages and agarose gel percentage have been used to adjust the size resolution to the required range.

To extend the physical maps around the three markers, D8, D3F15S2 and ZnF16, contained in the Dis2.6 hybrid, pulsed field gels were hybridised to each of these probes. In an attempt to quantify the 3p21 material in the hybrid and to assess the relative position of these clones, comparisons betweeen the pulsed field data was made.

3.4.1 LONG RANGE MAP AROUND D8

The single copy subclone pD8B4.1, isolated from the cosmid, D8A1.4 was used as a probe on pulsed field gels of digested lymphocyte DNA. BssHII, NaeI, Nar I, SacII, SalI and SfiI were used in both single, and in double digests with NruI and MluI of lymphocyte DNA. These enzymes, NruI and MluI were used in double digests since their sites had been identified in the D8A1.4 cosmid which could be used as landmarks. In an attempt to extend the mapping region, partial restriction digests of some of these enzymes were achieved.

Following digestion of the samples, the gel was electrophoresed using a 22 or 40 hour ramping programme as described in 2.2.8.3. These conditions allowed smaller

fragments of 50-100kb to be retained on the gel. Figures 34 and 35 present pulse field blots of the rare cutting restriction enzymes, NarI and SalI following hybridisation with the D8 probe, pD8B4.1. The data combined with data not shown is presented in table 8 and used to construct the long range map illustrated in figure 36.

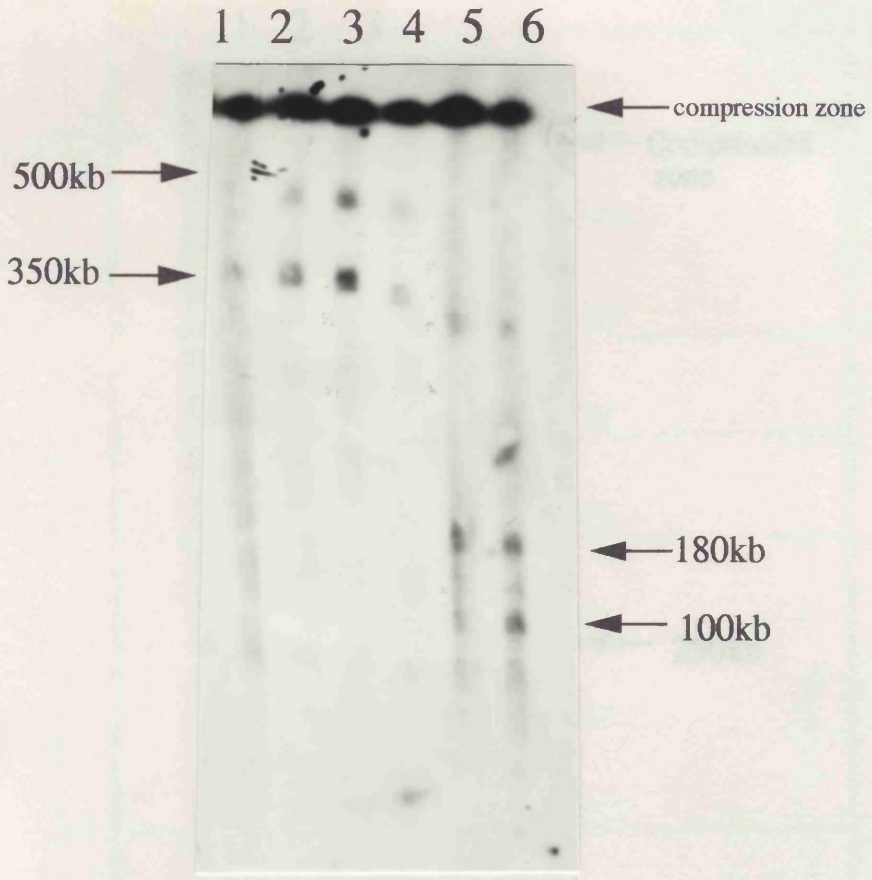

Figure 34: Mapping of NarI restriction sites around the D8 locus.

DNA agarose blocks were digseted with NruI and NarI and run on a pulsed field gel using a 22 hour ramping programme. The blotted DNA was hybridised to the D8 probe, pD8B4.1. The figure illustrates the fragment sizes observed using NarI in partial restriction enzyme analysis with NruI.

The D8 probe, pD8B4.1, hybridises to a partial NruI fragment of 500kb and a 350kb complete NruI fragment (lane 1). Lanes 2-5 are NruI digests wtih increasing amounts of NarI enzyme concentration as follows: Lane 2: 0.5units, Lane 3: 1 unit, Lane 4: 2 units, Lane 5: 5 units.
Two fragments of 180kb and 100kb are observed with NruI+ NarI and NarI alone, the 180kb band representing a partial fragment (lanes 4 and 5 respectively).
Since the NruI and NarI sites map to the same postion in the cosmid D8A1.4, the NarI restriction site therefore maps 100kb from D8 with a second site a further 80kb awav.

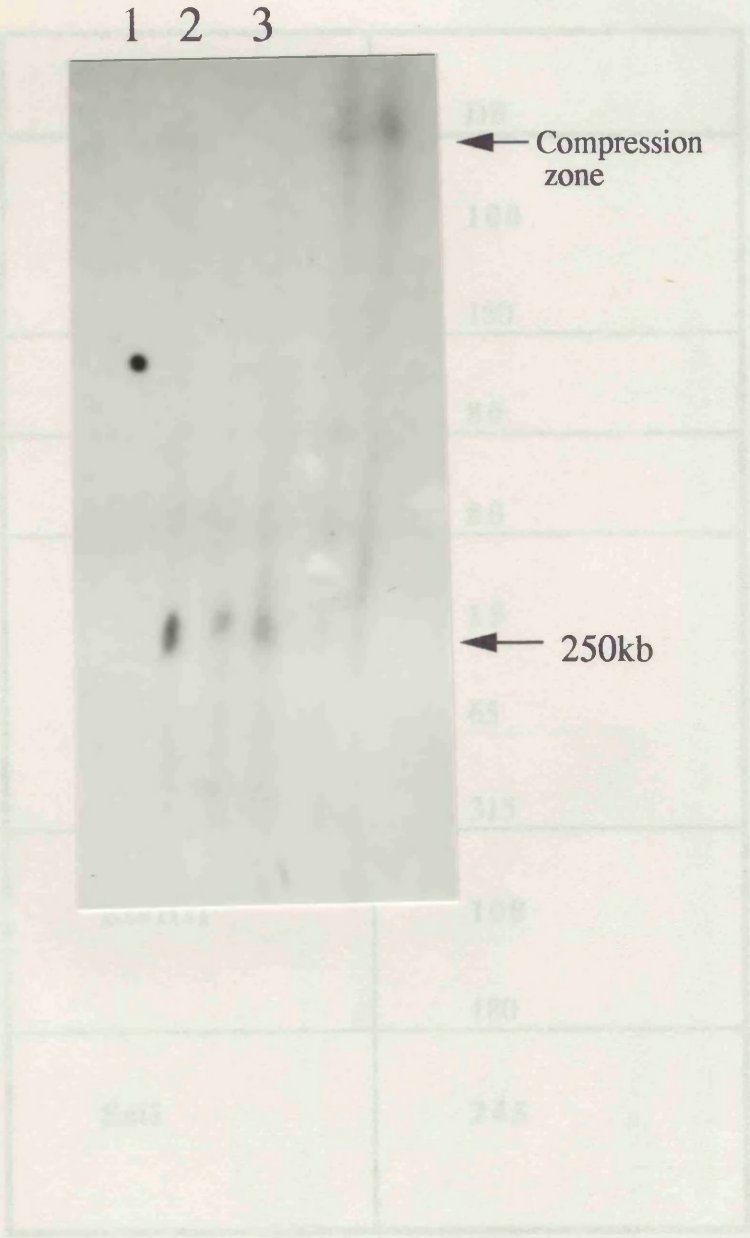

Figure 35: Mapping of SalI restriction site around the D8 locus.

DNA agarose blocks were digested with MluI+SalI and run on a pulsed field gel using a 40 hour ramping programme. The blotted DNA was hybridised to the D8 probe, pD8B4.1. pD8B4.1 hybridises to a MluI+SalI fragment of 250kb (lane 1).The same fragments is observed with SalI alone (lane2). Lane 3 is a complete MluI digest with a SalI partial digest. No second hybridising band representing a partial SalI fragment can be observed. A SalI restriction site therefore maps 250kb from the MluI site.

The previously mapped SalI site in the cosmid D8A1.4 postions the restrisction site 8kb from the MluI site. Owing to the resolution limits of PFGE, a MluI-SalI double digest would reveal the same sized band as a single SalI digest when probed with pD8B4.1.

Enzyme	D8
NarI	**100** 180
NaeI	**80**
SfiI	**80**
SacII	**15** 65 315
BssHII	**100** 180
SalI	**245**

Table 8: Pulsed field data around the D8 gene using relatively frequent enzymes in partial and complete digestion.

Fragment sizes observed following complete digestion are presented in bold leterring the remainder as partial. (Sizes are in kb).

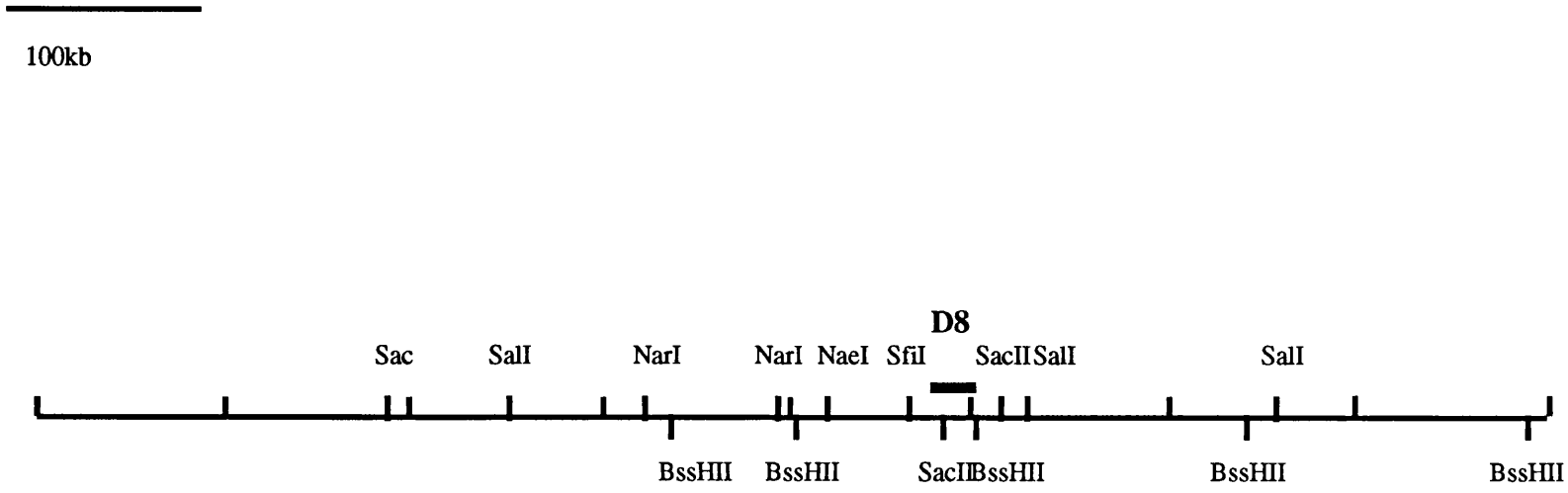

Figure 36: Physical map around the D8 locus.

Physical map constructed using pulsed field gel electrophoresis of the enzymes listed above in partial digestion with the D8 hybridisation probe, pD8B4.1.

The enzymes MluI, NotI, NruI and PvuI were used in single and double digestions of lymphocyte DNA. The 66 and 76 hour ramping programmes (see 2.6.3) were used for the electrophorese of the DNA.The blots was hybridised to pD8B4.1and are presented in figure 37-39. Partial digest were achieved with NruI (illustrated in figure 34). In addition to a 375kb ragment representing the NruI fragment encompassing D8, a second partial hybridising band of 500kb suggests additional NruI site at a distance 125kb from the first. The pulsed field mapping data.is presented in the table below (table 9) and used to construct the long range map in figure 40.

ENZYMES	PvuI	NruI	NotI	MluI
MluI	280	375	350	400
NotI	580	340	650	
NruI	280	375		
PvuI	1000			

Table 9: Pulsed field gel fragments with four rare cutting enzymes in single and double digestion using the D8 probe.

The table presents the sizes of pulsed field gel fragments obtained with the above listed rare cutting enzymes with the D8 probe, pD8B4.1. This data was used to construct a long range map around D8 presented in figure 40. (Sizes are in kb)

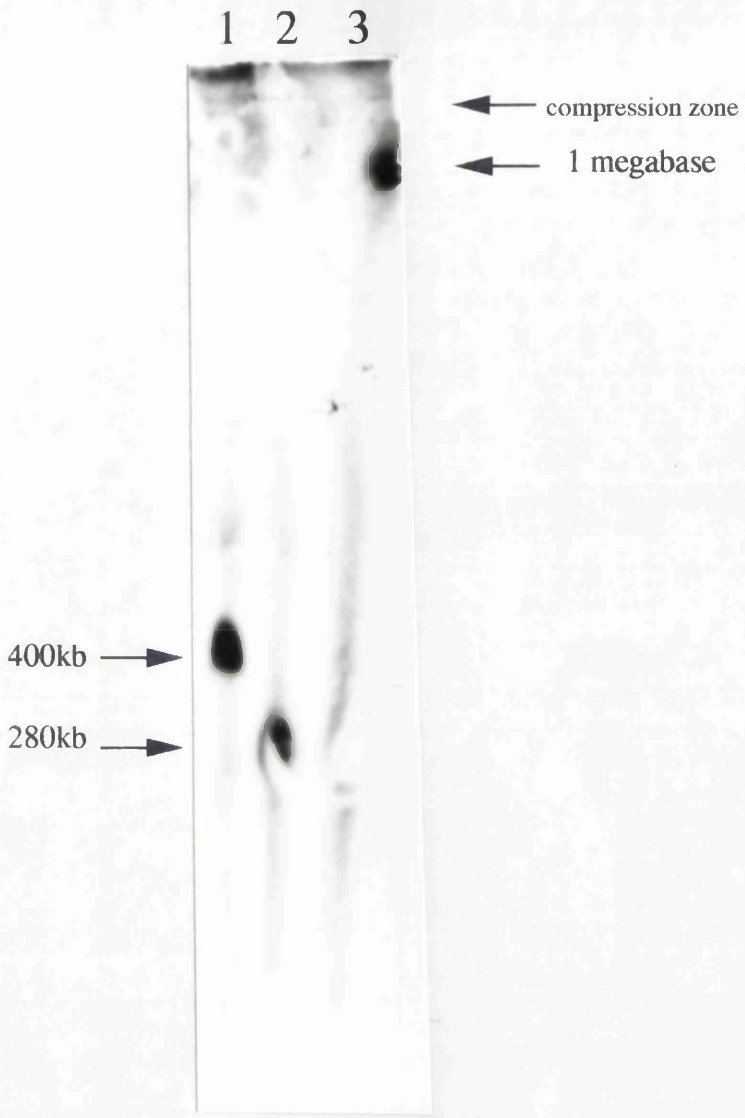

Figure 37: Physical mapping of PvuI and MluI restriction sites around the D8 locus.

DNA agarose blocks were digested with the rare cutting enzymes, PvuI and MluI and run on pulsed field gels using the 76 hour ramping programme. The blotted DNA was then hybridised to the D8 probe, pD8B4.1.

The figure illustrates the following hybridising bands; PvuI- 1megabase (lane1), PvuI+MluI-280kb (lane2) and MluI-400kb (lane3). The hybridisation of a smaller PvuI-MluI fragment in comparison to the single digests indicates that one MluI site maps internal to the 1megabase PvuI fragment and that one PvuI site maps internal to the 400kb MluI fragment.

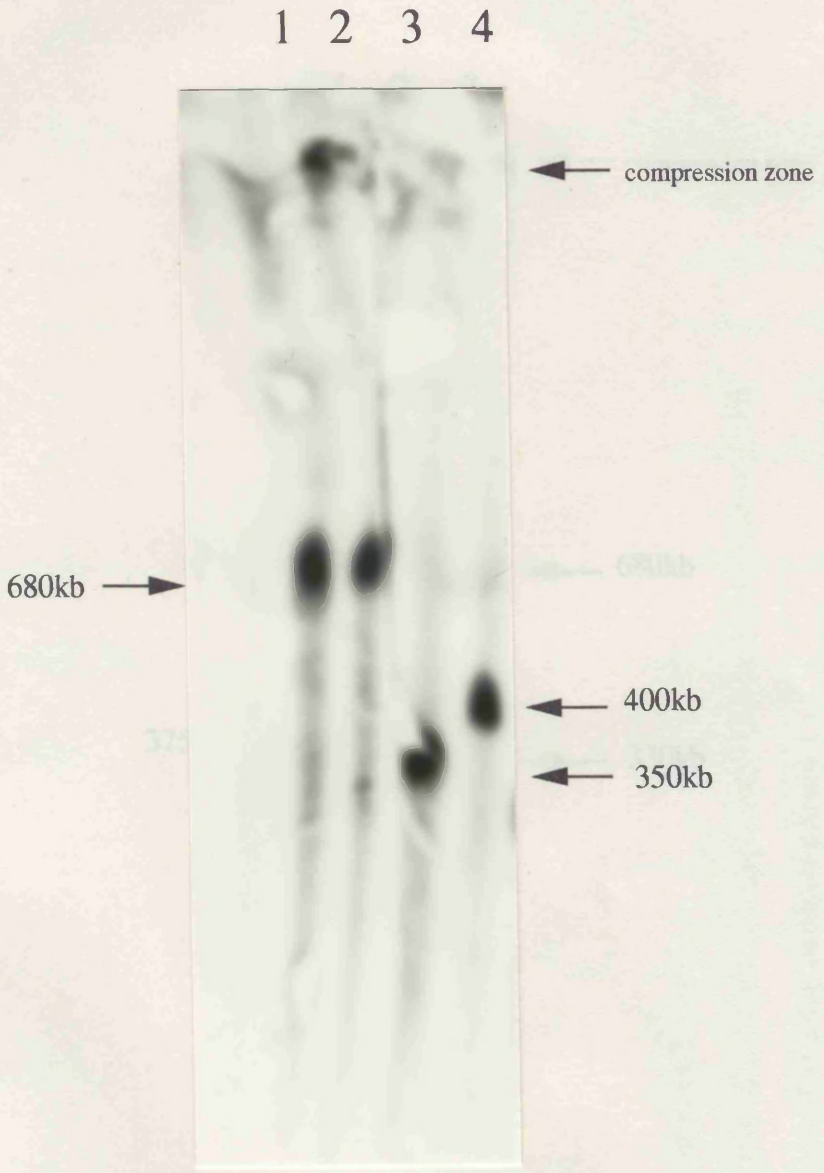

Figure 38: Physical mappin g of MluI and NotI restriction sites around the D8 locus.

DNA agarose blocks were digested with the rare cutting restriction enzymes, MluI and NotI and run on pulsed field gels using a 66 hour ramping programme. The blotted DNA was then hybridised to the D8 probe, pD8B4.1.
The figure illustrates the following sized hybridising bands; NotI- 650kb (lanes1+2), MluI+NotI-350kb (lane 3) and MluII-400kb (lane 4).
The hybridisation of a smaller MluI-NotI fragment in comparison to the single digests indicates that one MluI site maps internal to the 650kb NotI fragment and that one NotI site maps internal to the 400kb MluI fragment.

Figure 39: Physical mappin g of NruI and NotI restriction sites around the D8 locus.

DNA agarose blocks were digested with the rare cutting restriction enzymes, MluI and NotI and run on pulsed field gels using a 66 hour ramping programme. The blotted DNA was then hybridised to the D8 probe, pD8B4.1.

The figure illustrates the following sized hybridising bands; NruI- 375kb (lane1), NotI-650kb (lane2) and NruI+NotII-330kb+650kb. The larger 650kb band observed in the double digest NruI-NotI represents incomplete NruI digestion of the 650kb NotI fragment The reduction in size of the NruI fragment with NotI-NruI suggests that one NotII site maps internal to the 375kb NruI fragment.

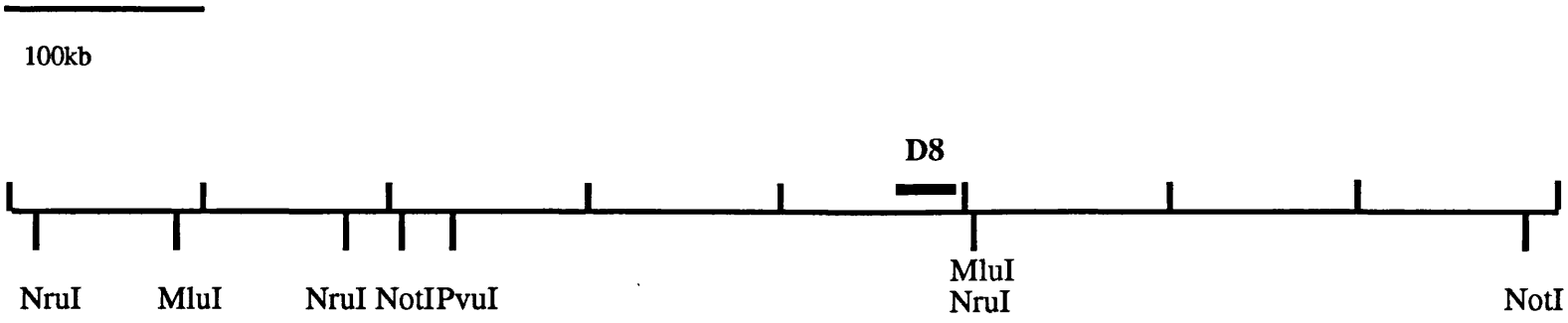

Figure 40: Long Range map around the D8 locus.

Long range map constructed using pulsed field gel electrophoresis and the hybridisation probe, pD8B4.1. The two NruI sites were identified using partial digestion.

3.4.2 LONG RANGE MAP AROUND D3F15S2

The acylpeptide hydroylase gene, APEH, mapping 4kb from the D3F15S2 locus, was used as a hybridisation probe in pulsed field gel electrophoresis. The previously identified MluI site close to the gene allowed its use as a landmark in single and double digestions with rare cutting enzymes. Figure 41 presents the pulsed field fragments with the rare cutting enzymes, MluI, PvuI and NotI using APEH as a probe. All the pulsed field data obtained with this probe is presented in table 10.

ENZYMES	PvuI	NruI	NotI	MluI
MluI	280	280	175	280
NotI	580	300	650	
NruI	700	700		
PvuI	1000			

Table 10: Pulsed field data obtained around the D3F15S2/APEH locus.

The table presents the restriction fragment sizes obtained with the four listedrare cutting enzymes in single and double digests.(Sizes are given in kilobases).

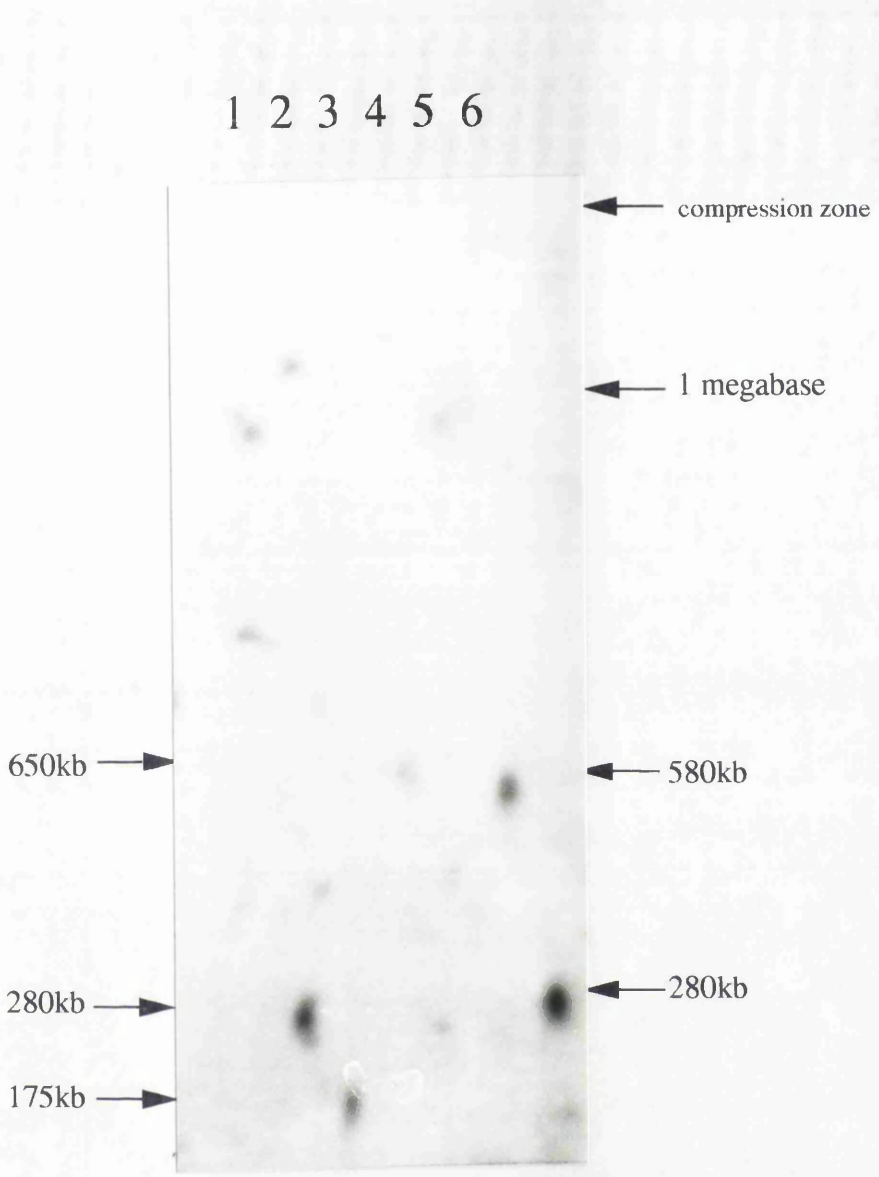

Figure 41: Physical mappimg of PvuI, MluI and NotI restriction sites around the D3F15S2/ APEH locus.

DNA agarose blocks were digested with the rare cutting restriction enzymes, PvuI, MluI and NotI and run on pulsed field gels using a 66 hour ramping programme. The blotted DNA was then hybridised to the probe, APEH.

The figure illustrates the following sized hybridising bands; Lane 1: MluI-280kb, Lane 2: MluI+NotI-175kb, Lane 3: NotI-650kb, Lane 4: PvuI-1megabase, Lane 5: PvuI+NotI -580kb and Lane 6: PvuI+MluI-280kb.

The NotI 680kb fragment is reduced in the double digests with PvuI and MluI. The same sized fragment with MluI alone and MluI+PvuI of 280kb indicates that both MluI sites around APEH lie internal to the PvuI fragments.

3.4.3. LINKAGE OF D3F15S2 AND D8 BY PULSED FIELD GEL ELECTROPHORESIS

The same sized PvuI and NotI fragments of 1megabase and 650kb respectively were identified with D8 and APEH. This suggested the possibility of the two markers sharing the same pulsed field fragments. In an attempt to link the two markers, the same pulsed field gels were hybridised to the two clones, D8 (probe:pD8B4.1) and APEH (figure 42). The NotI and PvuI enzymes in single and double digests were the same for each of the two markers The same sized 280kb PvuI/MluI fragment with D8 and APEH is also shown. However, a different sized MluI hybridising band with each marker excluded the possibility of the 280kb fragment being the same PvuI/MluI fragment. The shared NotI 650kb fragment is reduced to 175kb with APEH and 350kb with D8 in the double digest, NotI/MluI.

The combined data obtained for the two clones is presented in table 11. The identification of 700kb NruI fragment with the APEH probe (data not shown) enabled the positioning of the partial NruI fragment detected by D8. The second NruI site thus maps away from APEH. The previously identified human-hamster boundary characterised with the APEH gene in Dis2.6, and the presence of the D8 gene in this hybrid suggests that APEH is flanked by the hamster boundary on one side and the D8 gene on the other. A pulsed field map of the D8 and APEH region is presented in figure 43.

fig.42a: APEH

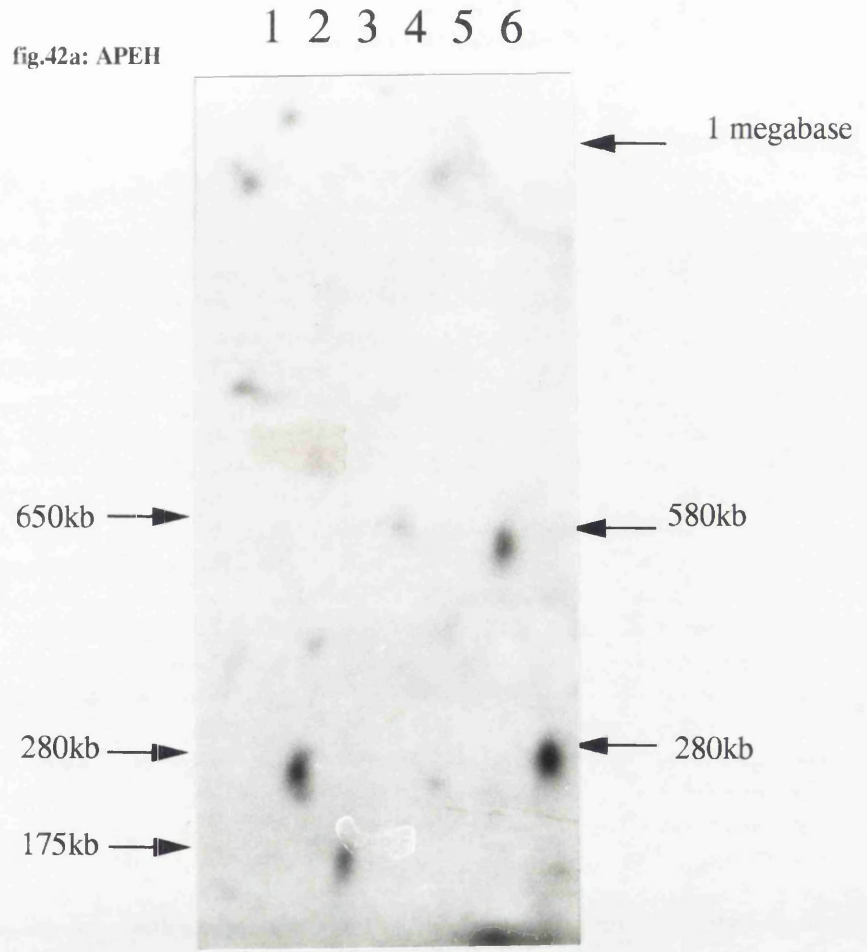

fig.42b: D8

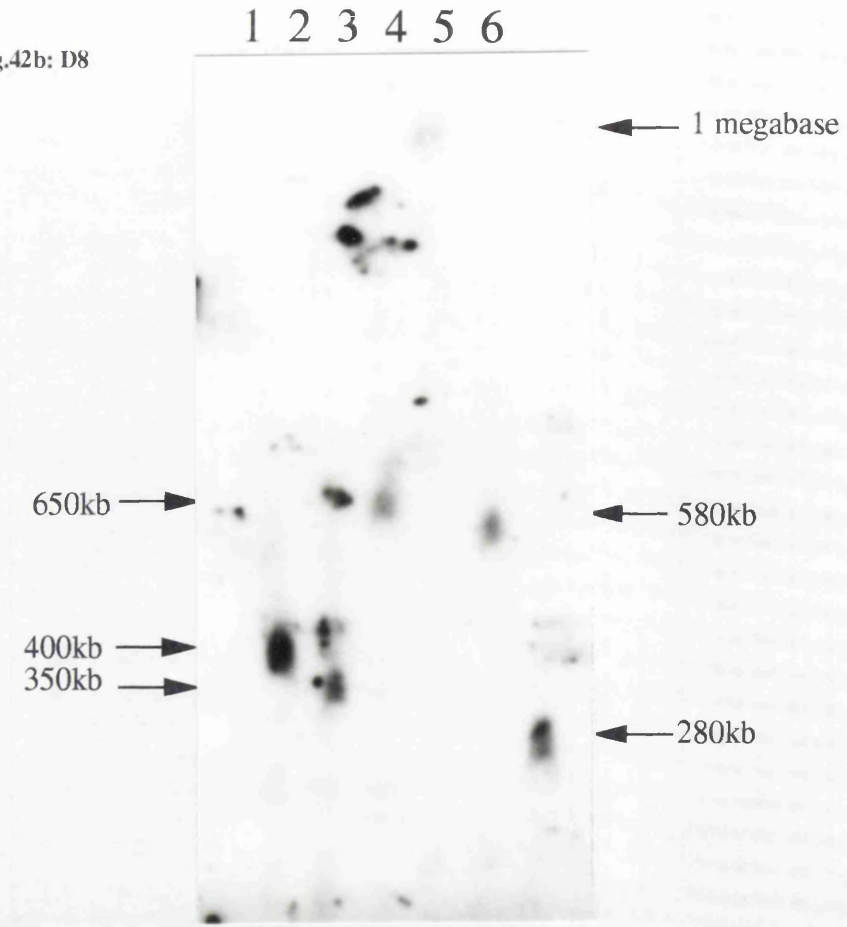

Figure 42: Linkage of D3F15S2 and D8 by pulsed field gel electrophoresis.

DNA agarose blocks were cleaved with PvuI, MluI and NotI in single and double digests and run on pulsed field gels using a 66 hour ramping programme. The blotted DNA was hybridised to APEH (fig42a) and then strippid and rehybridised to the D8 probe (fig42b), pD8B4.1. Lane 1: MluI- 280kb, Lane 2: MluI+NotI-175kb, Lane 3: NotII-650kb, Lane 4: PvuI-1megabase, Lane 5: PvuI+NotI -580kb and Lane 6: PvuI+MluI-280kb.

As illustrated above, the same PvuI , NotI and PvuI+NotI fragments are identified with APEH and D8. However, a different sized hybridising band is observed with MluI and MluI+NotI using the APEH and D8 probes. Although the same sized PvuI+MluI fragment is observed each probe, they cannot represent the same double digest fragment, since APEH and D8 recognise differnt sized MluI fragments.

	Marker	Marker
Enzymes	**D8**	**APEH**
BssHII	**80**	**140**
MluI	**400**	**280**
NotI	**650**	**650**
NruI	**375** (500)	**700**
PvuI	**1000**	**1000**
SalI	**25** 245 450	**125** 300

Table 11: Pulsed field mapping data around the two markers, D8 and APEH.

Open boxes indicates the same pulsed field gel fragment hybridisng to both clones.Complete digests are given in bold leterring and partial fragments in standard lettering. (Sizes are given in kilobases).

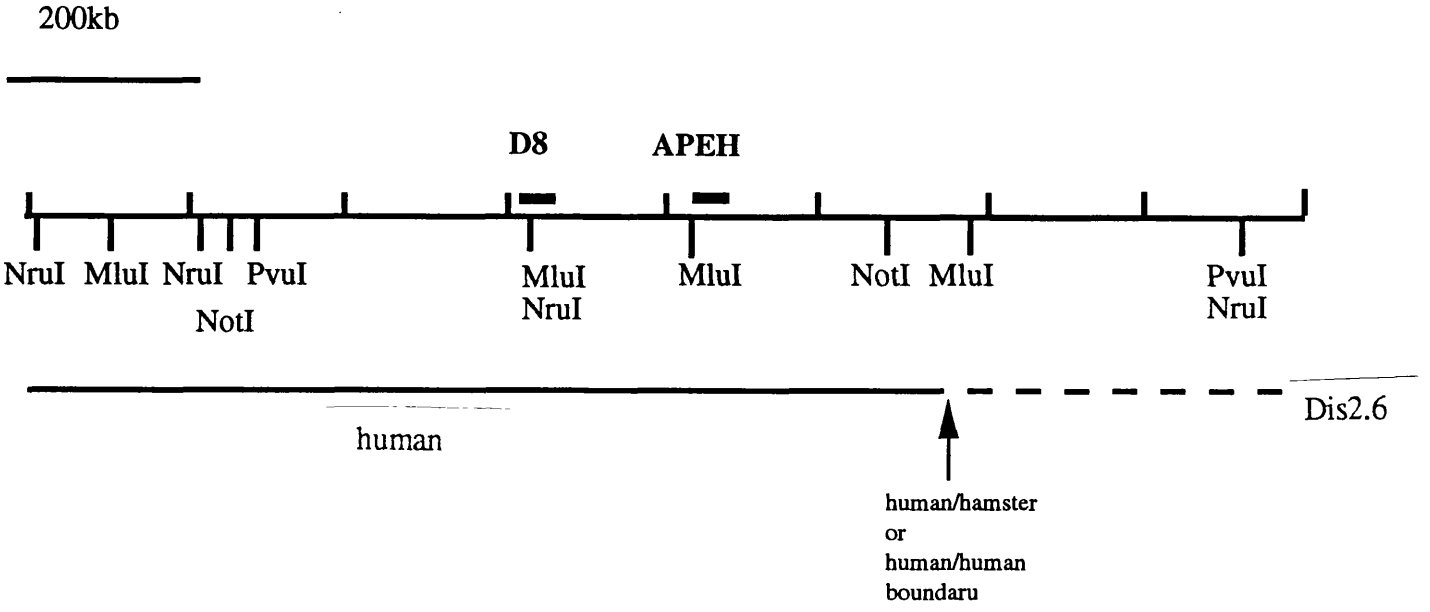

Figure 43: Long range mapping around the D8 and APEH loci.

Long range map constructed using pulsed field gel electrophoresis and the probes pD8B4.1 and APEH to illustrate the linking of the D8 and APEH/D3F15S2 markers respectively. The human-human or hamster boundary identifeid in 3.1.1 is illustrated here with the arrow indicating the approximate position of the boundary.

3.4.4 LONG RANGE MAP AROUND ZnFP16

Following the identification of the zinc finger clone, ZnFP16, in the hybrid Dis2.6, the correspondng genomic clone, 16B4.5 was used as a probe in hybridisation experiments of pulsed field gel elctrophoresis. Figure 44 presents the results obtained with four rare cutting enzymes, PvuI, MluI, NruI, EagI and NotI. The data is presented in table 12.below. and used in combination with short range mapping data available (K. Hadjantonakis & P.Little, personal communication) used to construct a long range map around the ZnFP16 gene. It was not possible to link any of the pulsed field fragments with those previously identified with the D8 or APEH probes.

Enzyme	ZnF16
PvuI	>2000
PvuI NruI	345
PvuINotI	450
MluI	1800
NotI	450
NruI	345

Table 12: Pulsed Field Gel Fragments observed using the zinc finger clone, ZnPF16.(sizes in kilobases)

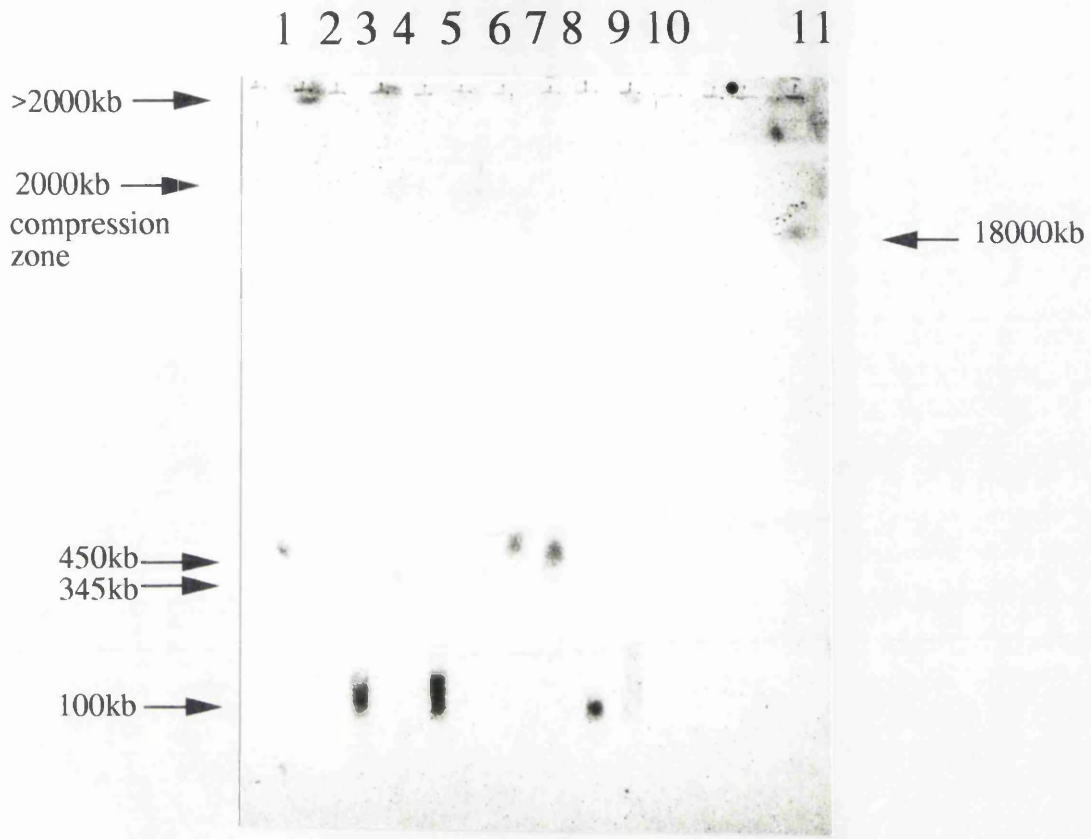

Figure 44: Physical mapping around ZnFP16 locus.

DNA agarose blocks were digested with PvuI, NotI, NruI and EagI and run on a pulsed field gel using a 76 hour ramping programme. The blotted DNA was hybridised to the ZnFP16 genomic clone, 16B4.5.

The figure illustrates the fragment sizes observed using EagI, PvuI, NruI, NotI and MluI as follows:

Lane1: NotI-450kb, Lane 2: PvuI: >2000kb, Lane 3: EagI,-90kb, Lane 4:NruI,-345kb, Lane 5:PvuI/EagI,-90kb Lane 6: PvuI/NruI-345kb, Lane 7: PvuI/NotI-450kb, Lane 8: NruI/NotI-380kb, Lane 9: NotI/EagI-60kb, Lane 10: NruI/EagI-90kb, Lane11: MluI-1800kb.

The compression zone represents fragment sizes of approximately 2000kb (according to yeast size marker). Therefore, the PvuI fragment is estimated as >2000kb (lane 2).

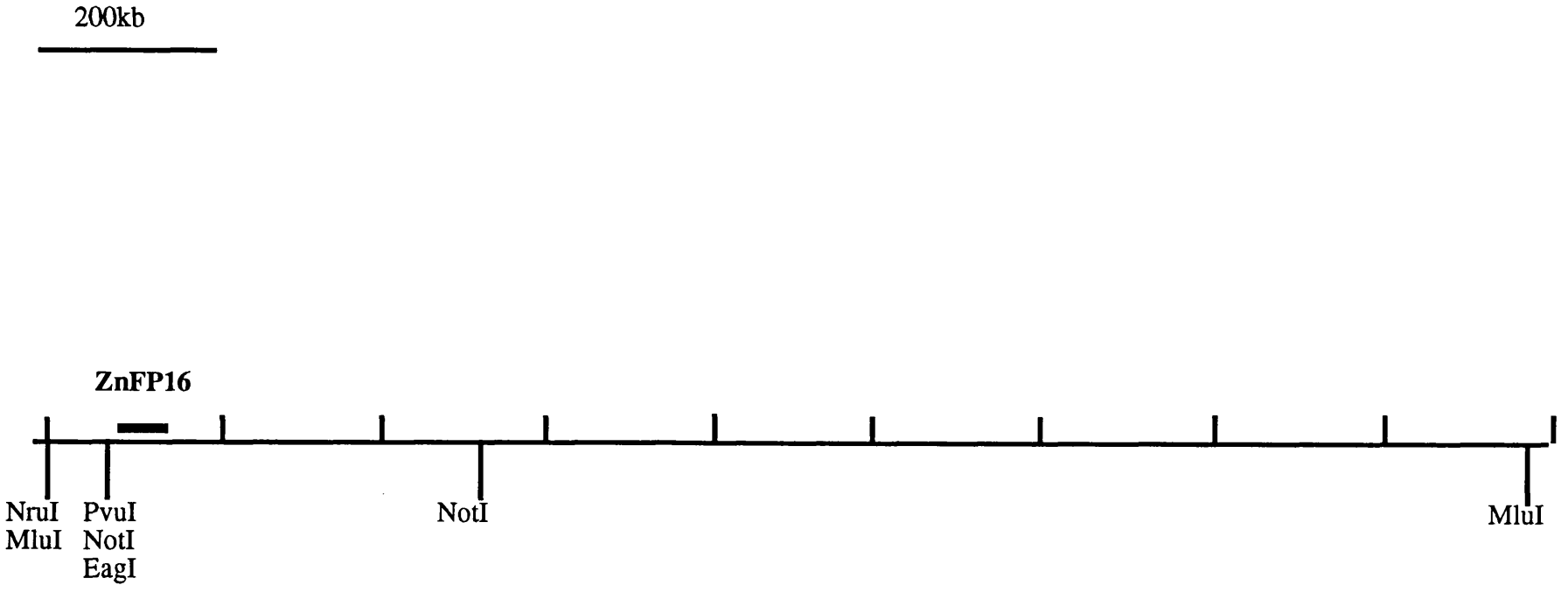

Figure 45:Long range map around the ZnFP16 locus.

Physical map constructed using pulsed field gel electrophoresis of the enzymes listed above in partial digestion with the ZnFP16, hybridisation probe, 16B4.5.

3.5 ANALYSIS OF THE D8 REGION IN SCLC

3.5.1 SEARCH FOR GENOMIC REARRANGEMENTS AROUND D8

On the basis of the marker D3F15S2 being included in the 3p deletion in a significant proportion of SCLC, any gene mapping in close proximity is likely to be included in the deletion and may be viewed upon as a candidate gene involved in small cell lung tumourogeneis. In an attempt to detect genomic rearrangements of the D8 gene, the cDNA was used in hybridisation experiments of a panel of SCLC DNA. Using the BamHI digests of genomic DNA and the longest cDNA, PB3.3, five fragments spanning a total region of over 20kb could be assayed. The analysis included EcoRI in single and double digestion with BamHI to assay for smaller fragments contained within this region. Fifteen SCLC DNA samples were used in the analysis. Figure 46 presents the results of one SCLC DNA samples, GLC42, following hybridisation with the 3.3kb cDNA. As illustrated in the figure no aberrant genomic fragments were detectable in GLC42 using the PB3.3 D8 cDNA. An additional eight SCLC lines were analysed by this method (GLC42, N417, L88, H69, FRE, GLC20, GLC8, GLC16.) but no aberrant could be detected.

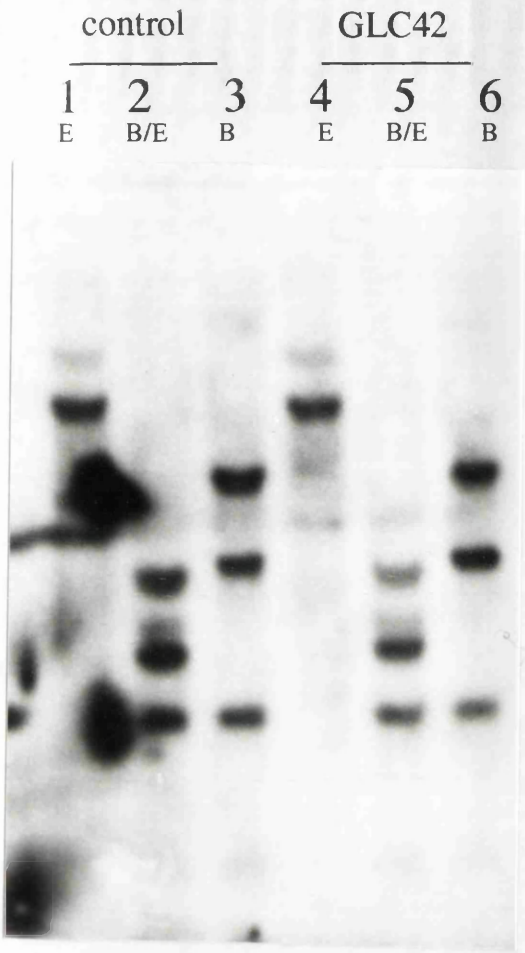

Figure 46: Genomic analysis of D8 in SCLC.

DNA isolated from the lung line, GLC42 and a control human sample were digested with enzymes listed above (E-EcoRI, B/E-BamHI/EcoRI, B-BamHI). The blotted DNA was hybridised to the D8 cDNA, PB3.3. Lanes1-3 are control DNA digests and lanes 4-6 are GLC42 samples. As indicated in the figure the lung line, GLC42, does not show any aberrant bands in comparison to the control DNA.

3.5.2 LONG RANGE MAPPING AROUND THE D8 GENE IN SCLC

The loss of one allele at the D3F15S2 locus is the most consistent event in SCLC. The characterisation and isolation of markers from the genomic region encompassing the D3F15S2 locus has enabled the genomic analysis of this region in small cell tumour DNA samples. The two markers, D8 and APEH were used as hybridisation probes in pulsed field gel electrophoresis of SCLC DNA, permitting the study of a 1 megabase region in 3p21. Any aberrant pulsed field gel fragments may be attributable to mutations around the D8, APEH or any other gene unidentified in the region.

Using the data from section 3.4 rare cutting enzymes were preselected to generate pulsed field fragments of differing sizes which in combination would cover the 1 megabase region. PvuI, MluI, NotI and NruI were used in single and double digestion of eight SCLC lines. To examine smaller pulsed field gel fragments, BssHII and SacII were used in pulsed field gel analysis (figures 47 and 48).

An appropriate control cell line for the analysis of SCLC remains unknown. An EBV transformed lymphoid line established from unaffected lung tissue was available from P.H. Rabbitts and used as a control lung cell line (AGLCL).

The methylation of vertebrate genes is not uncommon. It is believed that 55%-90% of CpGs are methylated (Bird1987). Often methylation differences are dependent on the type of cells used for DNA isolation. For example DNA isolated from blood lymphocytes and established cell lines have been known to demonstrate different methylation patterns of the same gene (K.Kok, pers. communication). The use of a lymphoblastoid cell line and blood lymphocytes as controls in the analysis of established SCLC lines provide adequate controls to prevent the misinterpretation of aberrant fragments sizes as mutations but as methylation differences .

Figures 47&48 present the pulsed field results of a subset of SCLC DNA samples

following hybridisation to the D8 gene. As mentioned above, enzymes were selected to provide an efficient method of covering the 1 megabase region. PvuI and MluII were used to assay the shared fragments whereas BssHII, NruI and SacII because of the smaller average fragment sizes they produce provided analysis around the D8 and APEH genes. As illustrated in figures 47&48 no aberrant pulsed field fragment could be detectable.

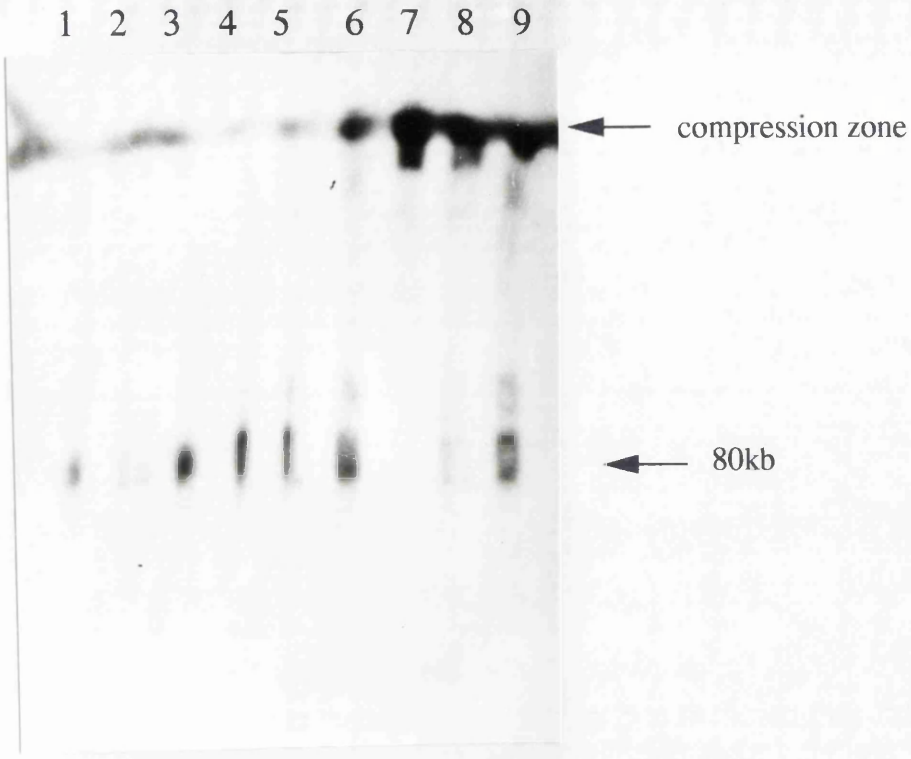

Figure 47: Short range by PFGE around D8 in SCLC.

DNA agarose blocks were digested with BssHII and run on a pulsed field gel using a 22 hour ramped programme. Lane1: GLC42, Lane2: GLC 44, Lane 3: GLC20. Lane4: N417, Lane 5: L88, Lane 6: GLC 8, Lane7: control lymphoblastoid cell line, Lanes 8+9: random control lymphocyte DNA.
In all control samples and small cell lines a 80kb BssHII fragment is observed.

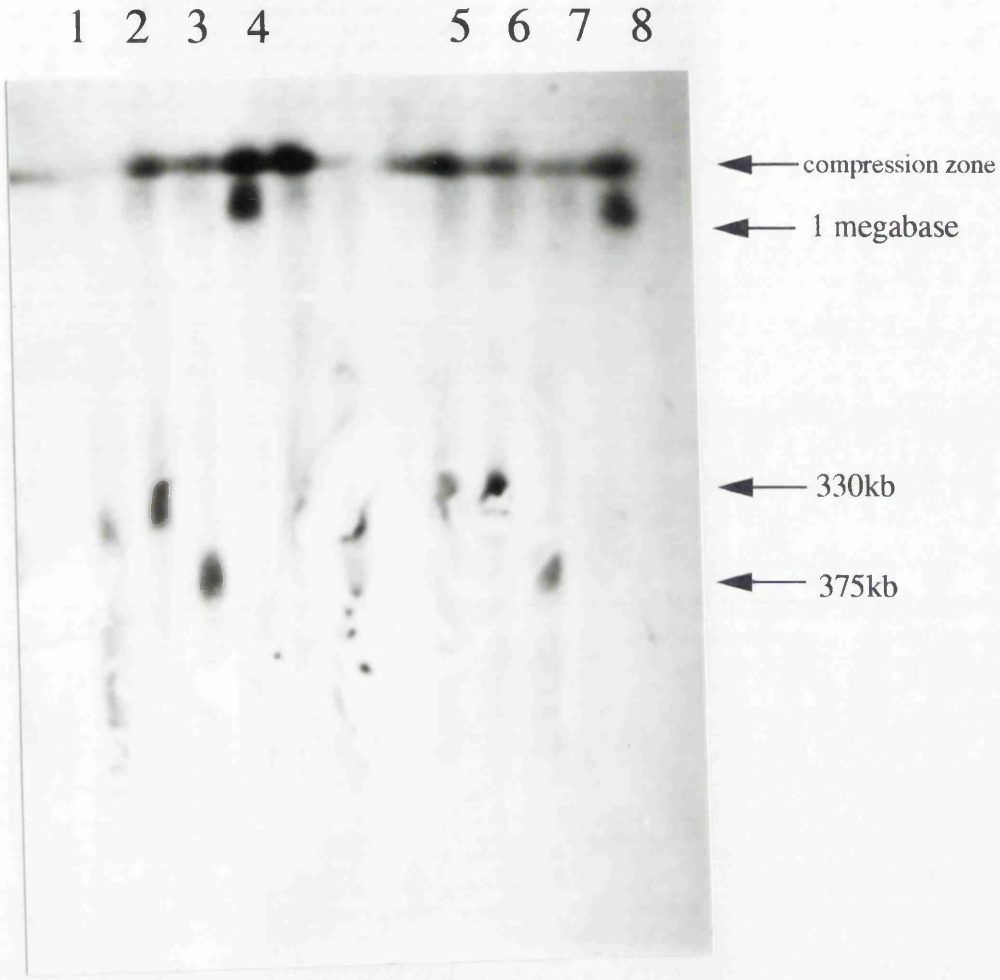

Figure 48: Long range mapping by PFGE around D8 in SCLC

DNA agarose blocks were digested with PvuI, MluI and PvuI+MluI and run on a pulsed field gel using a 66 hour ramping programme. Blotted DNA was hybridised to the D8 probe, pD8B4.1. Lane1 +2:Lymphoblastoid cell line x MluI, Lane 3:Lymphoblastoid cell line x MluI+PvuI, Lane 4:Lymphoblastoid cell line x PvuI, Lane 5+6:GLC44 x MluI, Lane 7:GLC44 x MluI+PvuI, Lane 8:GLC44 x PvuI. As illustrated in the figure, fragment sizes were the same in the control and SCLC line, GLC44.

3.6 MOUSE HOMOLOGUE OF D8 GENE

This section presents the isolation of the mouse homologue of the D8 gene. At an early stage in the collaborative project, difficulties arose over the isolation of a full length cDNA clone of D8. A 1.6kb partial cDNA, E1.6, had been isolated which included the 3' untranslated sequence and poly A+ tail. A number of cDNA libraries available to the laboratory were screened using 5' end subclones of E1.6 but failed to identify a longer cDNA of the D8 clone.

A mouse cDNA lung library was available to the laboratory and thus it was decided to isolate mouse homologue of D8. It was hoped that that any positive clones would include new 5' sequences which could then be used to screen a human cDNA library. In addition the clone would be available to the collaborating group to study the expression profile of the gene in a relatively easily available panel of mouse RNA.

3.6.1 SCREENING MOUSE cDNA LIBRARY

A random primed lambda mouse cDNA library was screened with the partial human cDNA of D8. As described above only the 3' 1.6 kb of the human cDNA had been isolated. Sequence analysis of this partial clone revealed anumber of repeat sequences at the 3' end and therefore it was decided to use the 1.4kb 5' BamHI-KpnI subclone to screen the mouse cDNA lung library.

Following three-rounds of screening, one positive phage clone, Mu195, was identified. Plate lysates were made from confluent platings and used for a liquid lysates. Phage DNA was prepared and the insert excised using the restriction enzymes EcoRI and HindIII. The 2.4kb insert was subcloned in pUC18.

Figure 49 is an ethidium bromide stained analytical agarose gel of the mouse clone,

Mu195. As illustrated in the figure the restriction enzyme, XbaI cuts the insert into two fragments of 1.6 and 0.8kb in size. To enable restriction mapping, these two fragments were excised from preparative gel and used in hybridisation experiments of southern blots of restriction digests of the mouse clone. Figure 50 presents the restriction map of the clone Mu195 illustrating the restriction sites of the enzymes; HincII, KpnI, PstI, PvuII and XbaI.

3.6.2 COMPARISON OF HUMAN D8 WITH MOUSE HOMOLOGUE, Mu195.

Towards the end of this work a 3.3kb human D8 cDNA had been isolated (Carritt et al. 1992) It was decided to to characterise the overlapping homologous regions of the mouse and human clones of D8.

Two subclones of PB3.3 and the two XbaI fragments of Mu195 were used as hybridisation probes of analytical digests of human cDNA,PB3.3 and the mouse cDNA, Mu195 (Figures 51 and 52). The data was used to construct comparative maps of the two clones to illustrate the overlapping regions (figure 53). As indicated in figure 53 the mouse clone is contained within the 3'Bam-KpnI 1.4kb fragment.

Mu195 was hybridsed to the cosmid D8A1.4 Figure 54 illustrates a single hybridisng bands with each of the enzymes EcoRI, BamHI and BglII in single and double restriction digests of the cosmid D8A1.4. The mouse clone is contained within the BamHI/EcoRI 2.4kb fragment. As illustrated in section 3.3 this fragment includes the 3' end of E1.6.confirming the 3' end homolgy of the mouse and human cDNA's. As expected human genomic DNA restriction digested with BamHI and probed with the Mu195 clone shows convincing hybridisation to the 4.1kb BamHI fragment (figure 55).

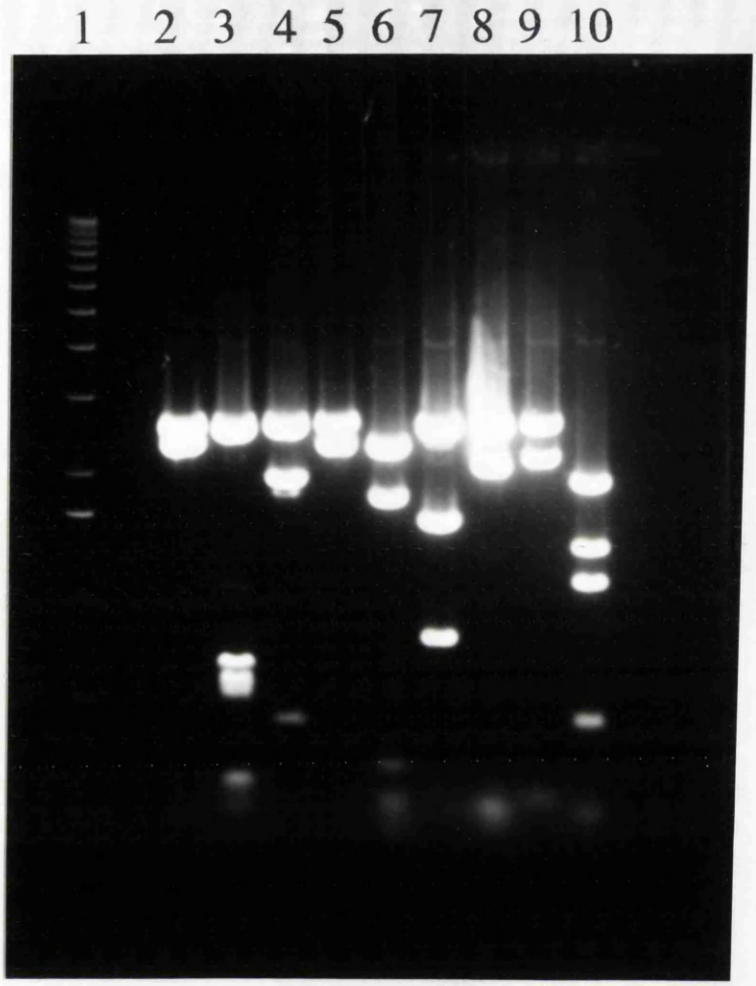

Figure 49: Analytical gel of mouse homologue of D8, Mu195 .

The figure presents an analytical gel of the mouse clone, Mu195. The insert was excised from pUC18 with the restriction enzymes EcoRI and HindIII (lane2).

Lane 1: 1kb ladder, Lane 2: EcoRI+ HindIII, Lanes 3-10 are all EcoRI+HindIII digests of the clone plus the following enzymes: Lane 3: + PstI, Lane 4: +StuI, Lane 5: +BamHI, Lane 6: +PvuII, Lane 7:+XbaI, Lane 8: +HincII, Lane 9: +KpnI, Lane 10: +BglI. The two XbaI fragments of 1.6kb and 0.8kb observed in lane 7 were excised from preparative gels for hybridisation studies.

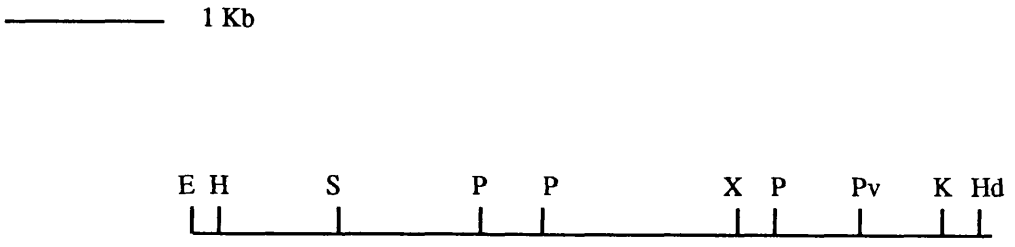

Figure 50: Restriction map of D8 mouse homologue, Mu195.

(B:BamHI, E:EcorI, H:HincII, Hd:HindIII, K:KpnI, P:PstI, Pv:PvuII, S:StuI, X:XbaI)

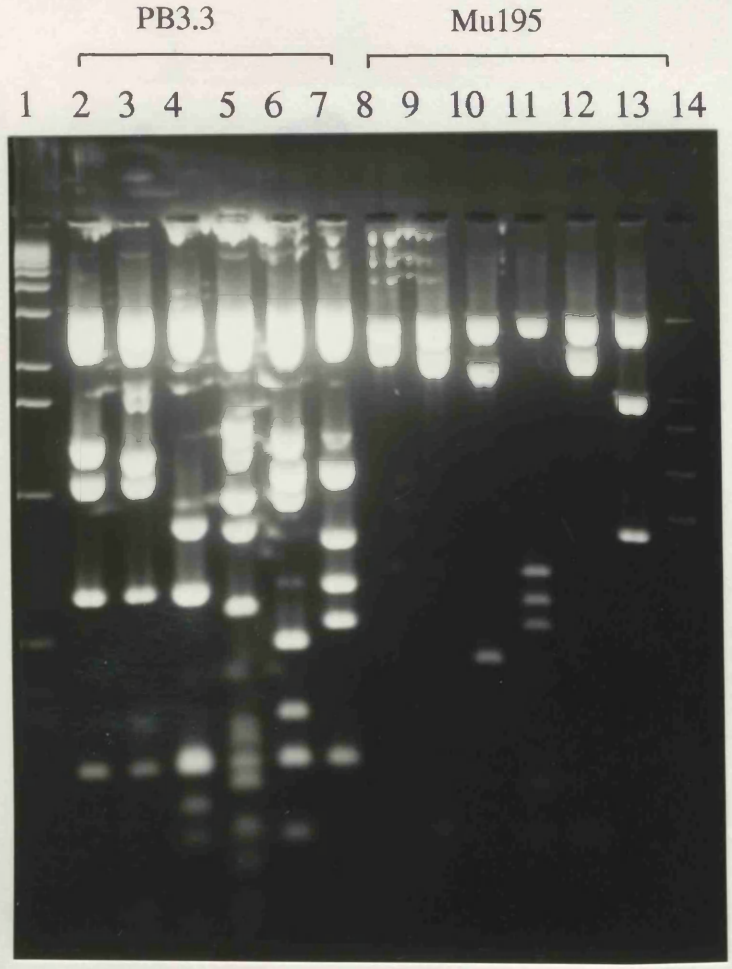

Figure 51: Comparative analytical gel of PB3.3 and mouse homologue of D8, Mu195 .

The figure presents an analytical gel of the human D8 cDNA clone, PB3.3 and the mouse clone, Mu195. Lanes 2-7 are restriction digests of PB3.3 and lanes 8-13 are restriction digests of Mu195.
Lane1: 1kb size marker. Lane 2: PB3.3 x BamHI/EcoRI, Lane 3-7: PB3.3 x BamHI/EcoRI plus the following enzymes: Lane 3:+KpnI, Lane 4: StuI, Lane 5: +PstI, Lane 6: +HincII, Lane7: +XbaI.
Lane 8: Mu195 x EcoRI/HindIII, Lanes 9-13: Mu195 x EcoRI/HindIII plus the following enzymes: Lane 9:+KpnI, Lane 10: +StuI, Lane 11: +PstI, Lane 12: +HincII, Lane 13:+XbaI.
Lane14: Øx size marker.

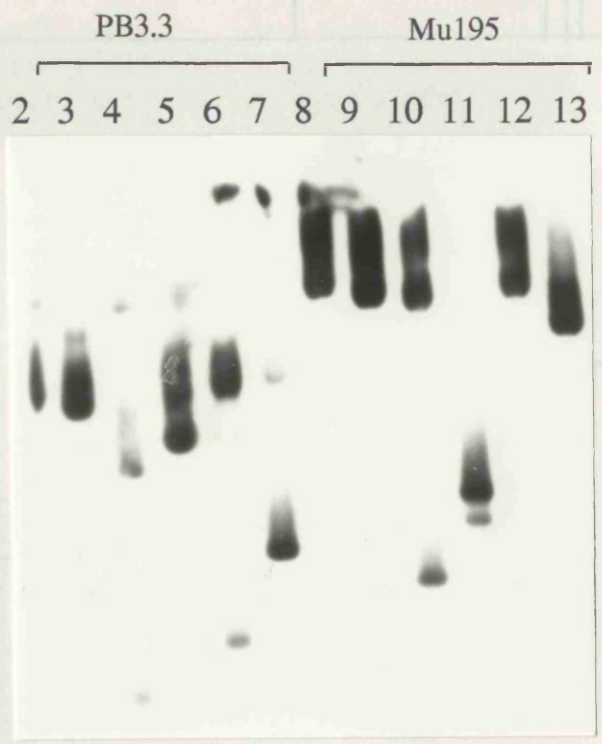

Fig52a

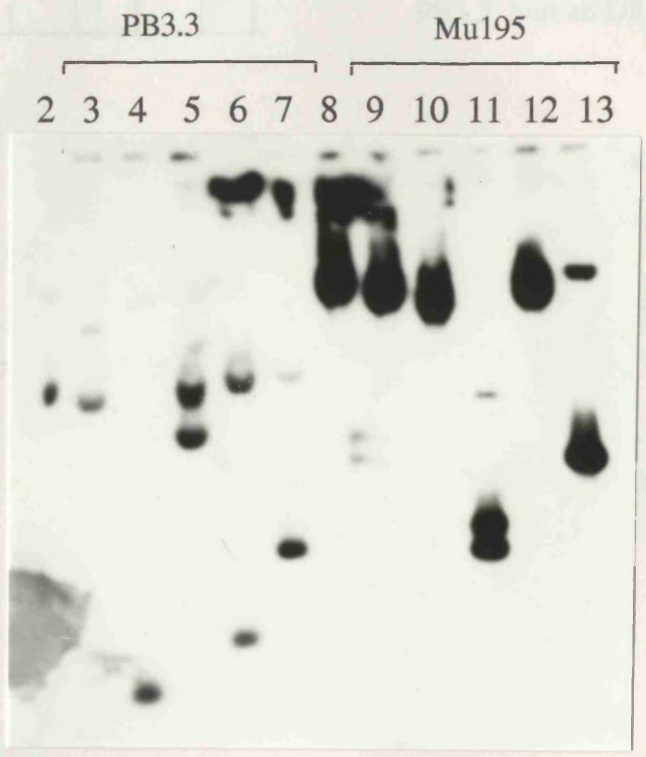

Fig52b

Figure 52: Cross hybridisation of Mu195 with the human D8 clone PB3.3.

The analytical gel presented in figure 51 was blotted and hybidsed to (52a): 1.6kb XbaI fragment and (52b) 0.8kb XbaI fragment of Mu195.
Lane 2: PB3.3 x BamHI/EcoRI, Lane 3-7: PB3.3 x BamHI/EcoRI plus the following enzymes: Lane 3:+KpnI, Lane 4: StuI, Lane 5: +PstI, Lane 6: +HincII, Lane7: +XbaI. Lane 8: Mu195 x EcoRI/HindIII, Lanes 9-13: Mu195 x EcoRI/HindIII plus the following enzymes: Lane 9:+KpnI, Lane 10: +StuI, Lane 11: +PstI, Lane 12: +HincII, Lane 13:+XbaI.
The two halves of the Mu195 clone hybridise to the same BamHI, KpnI, PstI and XbaI fragments of PB3.3 However, the 1.6kb XbaI fragment hybridises to two StuI fragme (lane 4, fig 52a) therefore crossing a StuI site. The 0.8kb XbaI fragment only hybridise to one StuI fragment (lane 4 ,fig52b).

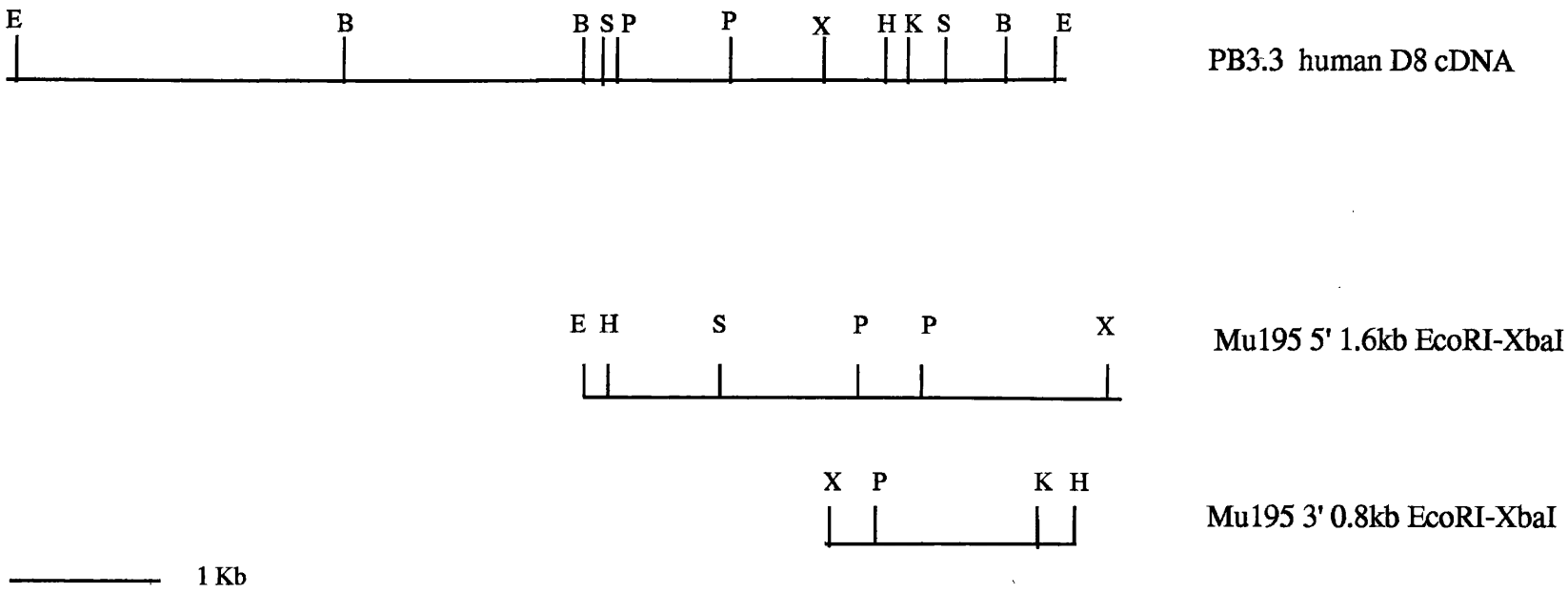

Figure 53: Comparitive restriction analysis of PB3.3 and Mu195 .

The two XbaI fragments of Mu195 are contained within the 3' BamHI-KpnI 1.4kb.

(B:BamHI, E:EcorI, H:HincII, Hd:HindIII, K:KpnI, P:PstI, S:StuI, X:XbaI)

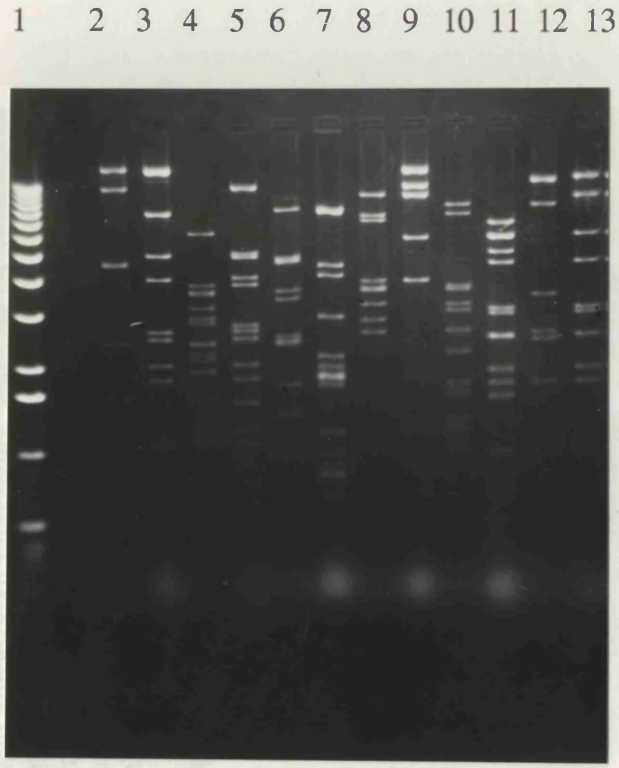

fig 54a

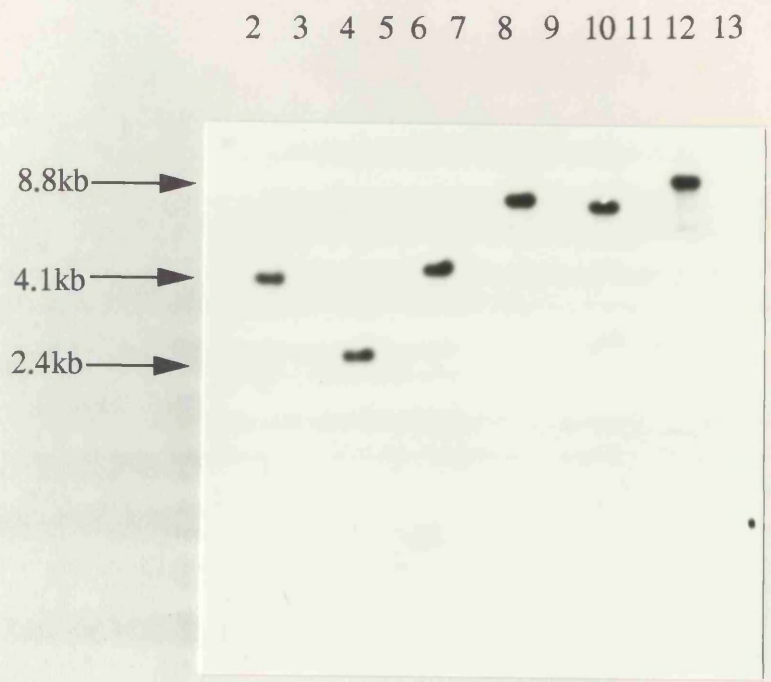

fig 54b

Figure 54: Mapping of Mu195 in the cosmid clones, D8A1.4 and 4.13.

Figure 54a: Analytical ethidium bromide gel of cosmids D8A1.4 and 4.13.

Lane 1: 1 kiolbase size marker. Lane 2: D8A1.4 x BamHI, Lane 3: 4.13 x BamHI, Lane 4: D8A1.4 x BamHI/EcoRI, Lane 5: 4.13 x BamHI/EcoRI, Lane 6: D8A1.4 x BamHI/BglII, Lane 7: 4.13 x BamHI/BglII, Lane 8: D8A1.4 x EcoRI, Lane 9: 4.13 x EcoRI, Lane 10: D8A1.4 x EcoRI/BglII, Lane 11: 4.13 x EcoRI/BglII, Lane 12 : D8A1.4 x BglII, Lane 13: 4.13 x BglII.

Figure 54b: Autoradiogram of analytical gel following hybridisation to the mouse D8 homologue, Mu195.

The analytical gel (fig 54a) was blotted and hybridsedto the mouse homologue, Mu195.

The mouse clone hybridises to a 4.1kb BamHI fragment (lane2) and a 8.8kb EcoRI fragment (lane 8). In the double digest, BamHI-EcoRI, a 2.4kb fragment hybridises to the mouse clone, indicating that all the mouse cDNA is contained within this 2.4kb region (lane 4).

No hybridisation to the cosmid 4.13 is observed suggesting that the cosmid does not contain any regions of homology the mouse genomic DNA.

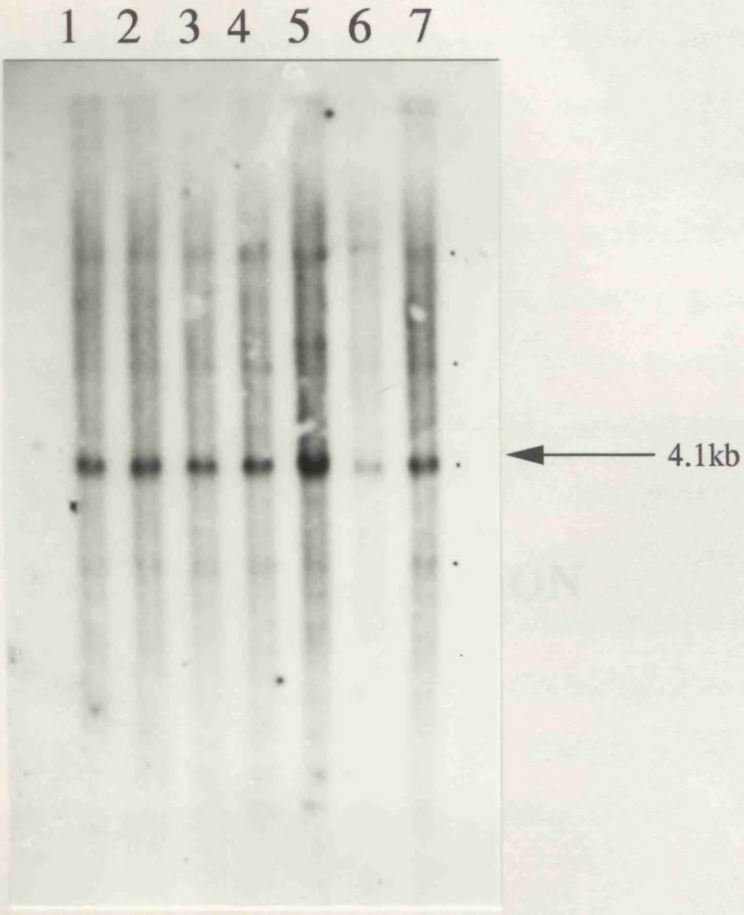

Figure 55: Mapping of Mu195 in human genomic DNA.

The figure presents an autroradiograpm of seven randon genomic DNA samples digested with BamHI restriction enzyme, Southern blotted and hybridised to the mouse clone, Mu195. The mouse clone identifies a 4.1kb band in each of the seven genomic DNA samples. Three faint hybridising bands are shown which may represent cross hybridisng loci or extension of the cDNA clone into other BamHI fragments.

4. DISCUSSION

4. DISCUSSION

Any locus which is believed to harbour a gene involved in a genetically defined disease will be a focus for intensive mapping studies. Efforts are being directed towards the saturation of regions of human DNA markers which can act as landmarks or anchor points in the mapping of a disease locus. Polymorphic DNA markers mapping into defined regions have been widely used in linkage analysis to study the segregation of a diseased phenotype as a preliminary to gene cloning. The successful implementation of this "reverse genetic" technique is exemplified by the isolation and characterisation of the Cystic Fibrosis gene (Kerem et al.1989). Similarly,where the loss of a specific allele or alleles appears to initiate or facilitate the progression of a disease, the polymorphic DNA markers can lead directly to the candidate genes. This is illustrated in the RFLP studies of several chromosome 5 markers used to identify the deleted region implicated in adenomatous polyposis coli, APC (Bodmer et al. 1987, Leppert et al. 1987). The precise location of such polymorphic markers and their flanking markers are thus essential in refining disease associated regions.

Characteristic deletions are often associated with a number of cancers. The deleted region is believed to harbour a tumour suppressor gene whose loss of function contributes to the development of the tumour. To define the limits of a deletion and thus localise potential tumour suppressor genes, polymorphic DNA markers have been used in the comparative analysis of tumour and normal patient material. The loss of heterozygosity of DNA markers in a particular tumour type, enables the sublocalisation of a region which may include the gene or genes of interest. Familial cancers enable linkage analysis of families with affected and unaffected members, to provide information on the segregation of a chromosomal region with the diseased phenotype.

In the case of lung cancer, there is little evidence for familial predisposition and as yet no clear pathological state representing the early stages of the tumour phenotype has been characterised (Iannuzzi 1986). However, cytogenetic analysis of primary tumours and lung cancer lines has led to the discovery of deletions on the short arm of chromosome 3; by far the most consistent event being the deletion of 3p14-23 in small cell lung cancer (Whang-Peng et al. 1982a&b). Additional SCLC karyotype analysis has further localised the deletion to 3p21-p23 (Yunis 1983, Buys et al. 1983). Other forms of lung cancer, although including the 3p14-p23 region, involve much larger deletions extending to the telomere (Zech et al. 1985, Rey et al. 1985).

In an attempt to define the smallest overlapping deleted region in SCLC, polymorphic DNA markers mapping in the 3p14-p23 interval have been used in the RFLP analysis of SCLC patients (Brauch et al.1987&1990, Daly et al.1991, Doborovic et al.1988, Johnson et al.1988, Kok et al.1987, Mori et al.1989, Mooibroek et al.1987, Naylor et al.1986&1987, Rabbitts et al.1990, Sithanadum et al.1989, Yokota et al.1987). This approach has been the most widely used strategy in cancer genetics in defining the molecular aberrations associated with a particular cancer phenotype.

Typically, DNA markers which are constitutionally heterozygous in SCLC patients have been assayed for allele loss in the corresponding tumour DNA. In some instances, patients uninformative for a particular marker have been assayed for allele loss using densitometric comparisons of normal and tumour DNA. The polymorphic markers, D3S2 and D3F15S2 mapping to 3p21.1 and 3p21.3 respectively have been used extensively in the RFLP analysis of SCLC patients. As described earlier, allele loss at the D3F15S2 locus represents the most consistent event in SCLC. Although loss of heterozygosity at the D3S2 locus has been demonstrated, a significant percentage of tumours retain two alleles of this marker. Additional markers mapping and flanking the 3p21-p23 region have been used in loss of heterozygosity studies.

These include RAF, THRB, D3S11, D3S4 and D3S3, (data presented in figure 1&2), each of which retains both alleles in a proportion of cases. On the basis of the pooled data, D3F15S2 may represent the closest marker to a locus implicated in small cell lung cancer.

Consistent rearrangements and deletions of chromosome 3 have been demonstrated in other cancers (Devilee et al.1989, Zbar et al.1987). Familial and sporadic renal cell cancers have been shown to include deletions of 3p, with the sporadic form illustrating consistent loss of heterozygosity of 3p21 DNA markers, including the marker D3F15S2 (Zbar et al. 1987, Kovacs et al.1988, Van der Hout et al. 1991a). These observations provide additional evidence for the existence of a tumour suppressor gene mapping at or close to 3p21.

To enable further characterisation of the 3p deletion in SCLC, additional markers are required from the 3p14-p23 region. Recent studies have been focused on the isolation of DNA markers in an attempt to construct high resolution physical and genetic maps of the 3p14-p23 interval (Yamakawa et al. 1991, Naylor et al.1991, Tory et al. 1991).

4.1 MAPPING STRATEGIES USED ON 3p21

A number of strategies have been used to isolate and map new DNA markers from chromosome 3. The availability of human chromosome 3-specific clone libraries permits the easy isolation of DNA sequences from chromosome 3. Early studies have demonstrated the approaches used in isolating and mapping chromosome 3 specific DNA fragments (Gerber et al. 1988, Atchison et al. 1988) using panels of deletion hybrids and conventional in situ hybridisation techniques. However, the mapping and assignment of DNA markers in these early studies was limited by the available hybrid panel, and simply permitted the assignment of markers to one of

four large physical regions on the chromosome.

During the last few years, an increasing number of DNA markers have been assigned to subregions of chromosome 3. The recent isolation of 75 markers, 52 of which contain RFLP's, from chromosome 3, were regionally assigned using fluorescent in situ hybridisation, FISH (Tory et al. 1991). A genetic linkage map of 41 new RFLP markers for chromosome 3 presented recently (Yamakawa et al. 1991) included a subset of 24 which were physically assigned by in situ hybridisation and in agreement to a genetic map.

These physical and genetic maps have great potential in identifying tumour suppressor genes responsible for the 3p associated malignancies. However, it is an essential requirement that new DNA markers are mapped with respect to pre-exisistng markers, in particular those used in the RFLP analysis of SCLC. This would enable the construction of a high resolution physical and genetic map spanning the SCLC deleted region, 3p14-p23.

The relative ordering and mapping of DNA markers has been markedly improved by the application of fluorescent in situ hybridisation, FISH, and by the availability of more sophisticated somatic cell hybrid panels. FISH permits the direct determination of the chromosomal location of DNA markers. The technique often enables the simultaneous mapping of two markers. Probe order has successfully been derived using interphase nuclei and multicolour labelling (Trask 1991). Markers separated by up to at least 1 megabase can be mapped using FISH in combination with metaphase or prometaphse nuclei. In one instance, double FISH has enabled the ordering and mapping of DNA markers in the 3p14-p25 interval (Heppell-Parton et al. 1991).

Somatic cell hybrid panels continue to provide a valuable resource for the isolation and mapping of DNA markers. Single chromosome micro-cell hybrids, deletion hybrids and irradiation fragment hybrids together facilitate the sublocalisation of

markers to relatively small chromosomal regions. This technique offers an excellent approach to study chromosomal subregions encompassing a few megabases. Irradiation fragment hybrids can be characterised with pre-existing markers to quantify the amount of chromosomal material and to assess their potential in mapping and isolating new fragments from the region. In combination, these hybrids can be used in deriving physical maps of a defined chromosomal region.

In this study a panel of somatic cell hybrids containing deletions, fragments and translocation halves of 3p have been characterised to assess their potential as a 3p mapping resource: The Dis2.6 hybrid contains a fragment of 3p21 which includes the D3F15S2 locus as its only representation of chromosome 3 (Carritt et al. 1992). Alu-PCR clones generated from the hybrid were used in fluorescent in situ hybridisation of metaphase chromosomes (Pr.C.H.C.M. Buys, pers. communication). A tight localised fluorescence band was observed in the distal region of 3p21. Since D3F15S2 represents the closest known marker to a tumour suppressor gene on 3p21, the characterisation of Dis2.6 was used to establish markers mapped in close proximity to the D3F15S2 locus. Pulsed field gel electrophoresis of the Dis2.6 hybrid followed by Southern blotting and hybridisation with APEH, identified a change in size of the MluI fragment. This has been interpreted as a human-hamster boundary in the hybrid. However, in view of the possibility of more than one 3p21 fragment contained in Dis2.6, it is feasible that this may represent a human-human boundary.

The potential of the deleted chromosome 3 homologue in the Deak hybrid as a mapping resource for the specific region, 3p21, was not evident at the outset. The deletion was characterised cytogenetically as 3p14-p21.3 (I.Glass, pers communication) but this proved to be an over estimation since preliminary analysis showed that markers which map in this interval were outside the deletion. It was soon apparent that the deletion was significantly smaller and more distal than originally suggested.

Hybrid cell lines established from a t (3;7)(p21.1;p13) cell line derived from a patient with the Greig cephalopolysyndactyly syndrome have previously been characterised with respect to the markers, D3F15S2, D3S32, D3S11, THRB, D3S2 and ACY-1 (Gemmill et al. 1991). In addition, physical mapping of the breakpoint demonstrated that the translocation interrupts a 1.8 megabase MluI fragment recognised by the genomic clone, MW-Not153. D3S2 recognises the adjacent but proximal 300kb MluI fragment. A maximum distance of 1 megabase has been suggested between the D3S2 marker and the translocation breakpoint (Gemmill et al. 1991). The potential of the independently isolated GPS hybrids as a mapping reagent was known and the two translocation halves separated in independent hybrids complemented the Dis2.6 and Deak hybrid panel.

The four hybrids, Dis2.6, Deak (IIA4), GPS-proximal and GPS distal were used successfully in deriving the map of 3p21 as illustrated in figure 19. The chromosome 3 component of Dis2.6 overlaps with the deletion in the Deak hybrid and extends across the GPS translocation breakpoint. Hence, in combination these four hybrids permitted the unambiguous mapping and ordering of 3p markers, therefore providing a valuable resource for additional mapping studies in this region.

The polymerase chain reaction has been shown to be very successful in the characterisation of the hybrids. The human specificity of primer sequences from a proportion of the DNA markers were assessed prior to the screening process. Primers from gene sequences used in the PCR analysis were taken from those presented by Naylor et al. (Human Genome Mapping Workshop 11,1991) except however for ALAS 3. Primer sequences from ALAS3 were designed from the published 3' untranslated sequence (Bishop et al. 1990), believed to demonstrate the maximum divergence with hamster and other species. The primers were shown to be human specific and thus used in hybrid screening.

4.2 PHYSICAL MAP OF 3p21.

4.2 1. ORDERING OF 3p MARKERS

The physical mapping of 3p markers and genes is presented in figure 19 . As illustrated in the figure there are instances where the relative order of two markers could not be determined. D3S11 and ZNF35 have been mapped in the interval between D3F15S2 and D3S1100, but their relative order could not be determined.

cen-D3F15S2-ZNF35/D3S11-D3S1100-tel

The physical assignment of the zinc finger clone, ZNF35, (Donti et al.1990) is 3p21-p22 whereas D3S11 has been assigned 3p22-p23. By multiprobe labelling of ZNF35 and D3S11 in double FISH, one could map these two markers unambiguously relative to the centromere.

The potential human zinc finger gene, ZnFP16,was isolated using an oligonucleotide specific for the zinc ginger "link" region (Hoovers et al. 1992). It has previously been physically assigned using FISH on prometephase chromosomes to 3p21.31 (Hoovers et al. 1992). As this is the chromosomal region to which D3F15S2 and D8 have been assigned, ZnFP16 was included in this study. The genomic clone was shown to be contained in the hybrid Dis2.6, but has not been assayed in the Deak and GPS hybrids. Its exact position in the 3p21 fragment of Dis2.6 can only be speculated upon. The physical assignment of ZnFP16 at 3p21.3 would suggests that it maps distal of the GPS breakpoint characterised as 3p21.1. However, the relative order of ZnFP16, D8 and D3F15S2 would be of interest. If ZnFP16 maps distal of D3F15S2/APEH then it must map in the 170-300kb interval between D3F15S2 and the presumptive human-hamster boundary, which was

shown to interrupt a 280kb MluI fragment. However, the fact that ZnFP16 lies on a 1800kb MluI pulsed field gel fragment would seem to exclude this possibility. On this argument, ZnFP16 must map proximal of D3F15S2 and D8. It is unlikely that 1800kb MluI fragment recognised by ZnFP16 is the neighbouring MluI fragment to the 400kb MluI fragment of D8, since this would indicate that ZnFP16 lies on the 150kb NruI fragment identified by partial restriction analysis of D8 (figure 34). A genomic clone, MWNot153, has been shown also to map to a 1800kb MluI fragment which crosses the GPS breakpoint (Gemmill et al. 1991). On the basis of its size this may be the same MluI fragment which is identified by ZnFP16. This would indicate a more proximal location for the ZnFP16 clone as 3p21.2-p21.3 Figure 56 presents a physical map of this region and indicates the likely position of the ZnFP16 clone.

The results obtained using the primer sequences designed from the cDNA's encoding the two ALAS genes, ALAS 1 and ALAS 3 indicate a different location for each. ALAS 1 maps proximal of the GPS breakpoint and ALAS 3 maps distal of it. The ALAS gene encodes the enzyme which catalyses the first step in the biosynthesis of haem (Kappas et al. 1983). A third ALAS gene, ALAS 2 is erythroid specific and maps to the X chromosome. The mapping of ALAS 1 and ALAS 3 to different loci on chromosome 3 suggests that one may be a pseudogene and is not translated or that both are translated in the cell and either interact at the protein level or are involved in alternative pathways of the biosynthesis of haem (Bishop et al. 1990).

The presence of both these genes in the Dis 2.6 hybrid suggests that if the 3p21 component is continuous, the fragment extends from distal 3p21.3 across the breakpoint to 3p21.2. Although the chromosome painting experiment provides no evidence for more than a single fragment, one must not exclude the possibility that Dis2.6 contains relatively small fragments of 3p21 which map at defined regions in this band (Carritt et al. 1992). It is unfortunate that D3S32, flanked by D3F15S2 and

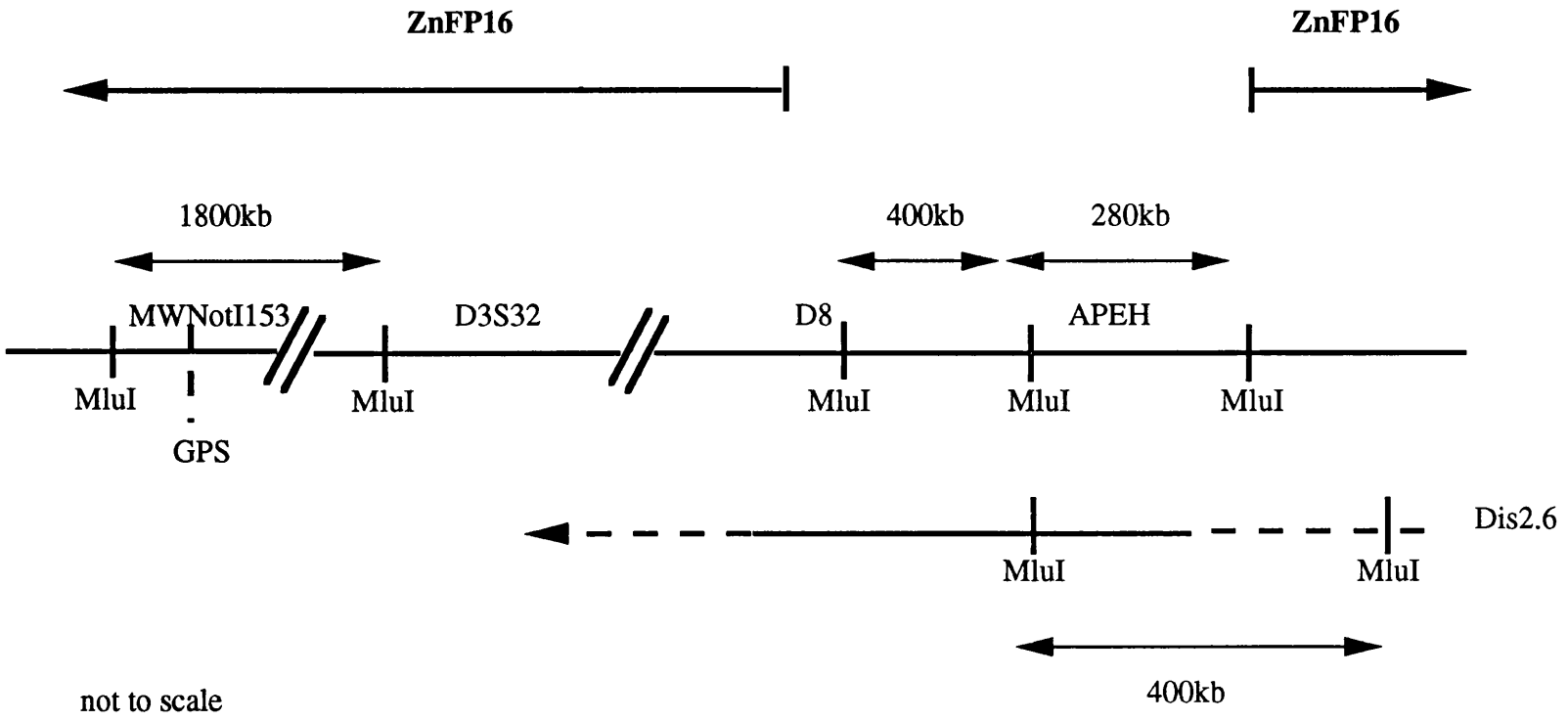

Figure 56 : Physical map to demonstrate order of 3p markers and possible positioning of ZnFP16.

The figure demonstrates the relative oreder of D3S32, D8 and APEH. MWNot153 is the genomic clone which identifies the 1800kb MluI spanning the GPS breakpoint (- - -). The positon of the 3p21 fragment in Dis 2.6, the dashed line on the right hand side symbolises the human-human or human-hamster boundary. The left hand arrowed dashed lineindicates possible inclusion of this region in Dis2.6..The two positions of the ZnFP16 gene are either side of the D8 and APEH genes and are indicated as arrowed lines.

the GPS breakpoint (Gemmill et al. 1991) was not assigned in the present study as the map presented in figure 19 predicts its presence in Dis2.6. The proximal boundary of the Dis2.6 3p21 fragment maps in the 800kb interval between D3S2 and the GPS breakpoint (figure 19).

The screening of hybrids positions the ALAS 3 gene between D3F15S2 and D3S1100. However, the distal boundary of Dis2.6 is 170kb-300kb from D3F15S2 which would indicate that ALAS 3 maps in this relatively small region. The availability of a cDNA or genomic clone would permit pulsed field gel analysis around this gene. Shared pulsed field fragments would confirm the relative position of ALAS 3 with respect to D3F15S2.

The map illustrated in figure 19 positions the *ras* related gene, ARH12, to 3p21.2-p22. In contrast, the previous assignment of this gene by conventional in situ hybridisation using tritium labelling techniques maps the gene in the interval 3p14-p22, with a significant clustering of silver grains at 3p21 indicating its most likely position (Cannizzarro et al. 1990). The mapping of the ARH12 gene using the somatic cell hybrids described in this study locates the gene to 3p21.2 - 3p22.

4.2.2. ISOLATION OF GENOMIC CLONES AROUND D8.

Cosmid clones encompassing the D8 gene were generated to provide a further insight into the genomic structure of the gene. Of particular value in deriving cosmid maps is the identification of rare cutting sites and potential CpG islands. CpG islands are unmethylated whereas inter-island CpGs are methylated (Bird 1987). Therefore, islands are detectable by cutting with enzymes with a high G+C content in their recognition site. Although methylation of inter-island CpGs is lost in cloned DNA, certain C-G enzymes still cut preferentially at islands, and can be used to select them from cosmid clones (Lindsay & Bird 1987). In addition, single copy sequences flanking rare cutting sites in island or inter-island DNA can be used in deriving long range maps by pulsed field gel electrophoresis.

In this study a cosmid clone was isolated from the phage subclone D8A1. As described in section 3.3, the cosmid was used in the mapping of the D8 cDNA. In addition a cluster of rare cutting sites including MluI, NruI, NarI and NaeI were characterised which may indicate a CpG island. These sites were subsequently shown to be unmethylated in genomic DNA. Unmethylated CpG islands are associated with the start site of transcription of housekeeping genes and some tissue specific genes (Lindsay & Bird 1987). However, this potential island maps at the 3' end of the D8 gene (figure 22) and it may therefore mark the 5' end of another gene, mapping in this region.

Following the isolation of the full length cDNA, pB3.3, it was apparent that not all the coding region of the gene was contained in the cosmid, D8A1.4 and that the further screening of a cosmid library was necessary. The D8 cDNA, pB3.3, was used to screen a Lorist B cosmid library. However following analysis of several positive clones only cosmids identical to the original cosmid, D8A1.4 were isolated. Many clones were found to contain vector sequences only as if the insert had been deleted. This may, as suggested by Harrison-Lavoie et al. (1989) reflect recombinant cosmids that are unstable and thus prone to deletion or rearrangement of sequences.

To extend the cloned region around D8, bidirectional walks were initiated from cosmid D8A1.4. The Lorist B vector has been constructed to facilitate the isolation of RNA probes from the extreme ends of the cosmid (Cross & Little 1986). To determine the presence of repeat sequences in theses RNA probes, they were initially used in hybridisation of the originating cosmid. In the cases of the right-hand walk, although the cosmid 6.1 was shown to overlap with the parental cosmid, D8A1.4 , several unrelated clones were isolated. Thus, although the use of end-fragment RNA probes provides a rapid method of chromosome walking by eliminating the need for intensive restriction mapping (Cross & Little 1986), it is important to be alert to the

possibility that where several clones are positive, they may not all be related except that they contain homologous sequences.

Similar difficulties arose during the isolation of D8.10 in the left hand walk. As described in section 3.3.6 this cosmid, D8A1.4 and other cosmids isolated during the study were analysed using DNA fingerprinting. This method has been successfully used in the construction of cosmid contigs on chromosome 11 (Harrison-Lavoie et al. 1989). The contig derived in this study although confirms the relationship of the two cosmids, D8A1.4 and D8.10. includes the clone 4.13 (figure 31). As described earlier (section 3.3.1), according to hybridisation studies, cosmid 4.13 contains no D8 coding sequence. However, since it was isolated with the same genomic clone, D8A1, it must have some region of homology with D8A1.4, and this is illustrated by its inclusion in the D8A1.4 contig. This may indicate a difficulty in the DNA fingerprinting and contig construction procedure which is based on the number of fragments shared between two clones and does not take into account homologous regions or repeat sequences shared at other distinct loci.

Perhaps a more refined approach in deriving cosmid contigs of this region is the use of cosmid libraries constructed from a single chromosome 3 hybrid or alternatively, a derived from the hybrid Dis2.6 containing a fragment of 3p21.

4.2.3. LONG RANGE MAPPING AROUND D8.

Long range mapping by pulsed field gel electrophoresis, PFGE, allows measurement of large physical distance between two markers. In this study, PFGE was used to illustrate the linking of the D8 gene isolated in this laboratory and D3F15S2. The use of the D8 gene and D3F15S2 as probes on PFGE enabled the characterisation of a 1 megabase region of 3p21. As described previously, this technique was used in the analysis of the hybrid Dis2.6, characterising a human-hamster or human-human boundary.

The isolation of YAC clones and appropriate jumping and linking libraries would facilitate an extension of the physical map around D8 and D3F15S2. This may enable the linking of additional markers and genes mapped in this study.

4.3 TUMOUR SUPPRESSOR GENES

Genes encoding growth factors or growth factor-like proteins interact with specific membrane receptors to transduce an intracellular biochemical signal in the cell growth pathway. This signal can activate or repress a cellular growth pathway. It is not surprising therefore that any genetic aberration of a growth factor gene results in a growth abnormality (Aaronson et al. 1991).

Malignant cells arise as a result of a progression of genetic events which lead to the unregulated expression of growth factors or components of the cell cycle pathway. Many oncogenes encode growth factor receptors which participate in the mitogenic signals of cellular growth (Aaronson et al. 1991). Activation and over expression of these genes have been shown to contribute to uncontrolled cellular proliferation. For example, members of the *myc* family of oncogenes are frequently activated in lung cancer (Little et al. 1983, Wong et al. 1986).

Tumour suppressor genes may encode proteins whose normal cellular role is to constrain cellular proliferation. They are components of the normal cell growth pathways which enable a cell to receive and process growth inhibitory signals (Weinberg 1991). Through their own specific mechanisms they act to suppress the neoplastic phenotype. Any functional loss of a tumour suppressor gene would initiate or contribute to tumourogenesis. These properties are shared amongst all the tumour suppressor genes identified. However, their resemblance ends here, each exercises its own function through specific interactions with other components of the cell.

There is considerable evidence for at least one tumour suppressor gene on chromosome 3. The consistent inclusion of 3p21 in the deleted region in SCLC and in other related and unrelated cancers suggests that one is located in this region. It is very difficult to speculate what properties to expect from a lung or lung associated tumour suppressor gene. The following section reviews the genes recently mapped to 3p21 and assesses their possible role in the cell growth pathway and their potential as tumour suppressor candidates.

4.4 GENES ASSIGNED TO 3p21

The mapping studies presented here have enabled the positioning of five genes in 3p21, ALAS 1, ALAS 3, ZNF35, ARH12 and ZnFP16. By virtue of their assignment in 3p21, it is of interest to learn more about the biology of the proteins they encode in order to assess them as candidate tumour suppressor genes.

4.4.1 ZINC FINGER GENES.

Since the first description of a zinc finger DNA binding domain in TFIIIA (Miller et al 1985), an increasing number of similar protein structures have been identified. TFIIIA is required for the transcription of *Xenopus laevis* 5S rRNA genes (Rosenberg et al. 1986). Similar domains have been identified in other genes controlling developmental processes as illustrated in *Drosophila*, where the Kruppel gene product is involved in the control of morphogenesis and embryogenesis (Preiss et al. 1985). Through their ability to bind DNA and RNA directly they have great potential in controlling fundamental cellular processes.

Zinc finger motifs have a consensus unit with a protein sequence of $CX_2CX_3FX_5LX_2HX_3$ where the the cysteine residues co-ordinate a zinc ion (Ruiz i Altaba et al. 1987). Each unit is separated by a stretch of seven amino acids which

act as a link enabling the independent interaction of each finger with nucleic acids via hydrophilic residues (Johnston et al. 1987).

Zinc finger genes have been implicated in the control of cellular proliferation and differentiation. Examples include the retroviral activation of a zinc finger protein, Evi-1, in the transformation of myeloid leukemia cell lines (Chowdhury et al. 1988) and the co-regulation of a zinc finger gene, Egr-1, with the oncogene *c-fos* during growth and differentiation of fibroblasts, epithelial cells and lymphocytes. In the latter case, the proteins specified by this zinc finger gene serve as transcriptional regulators (Sukhatme et al. 1988).

Of particular significance to cancer genetics is the characterisation of the Wilm's tumour gene, WT-1, as a zinc finger gene. The four zinc finger domains encoded by the gene and its proline-glutamine rich content indicate its potential role as a transcription factor (Call et al. 1990). In addition, the early growth response gene, EGR-1 and the WT-1 gene products have been shown to bind strongly to a recognition site commonly found in promoter regions (Rauschner et al. 1990). This results in the transcriptional activation of the promoter and associated gene. EGR-1 has a clearly defined role in the progression of cellular proliferation. The binding of the WT-1 gene product to the same sequence indicates that it may act to suppress transcriptional activation by EGR-1 (Madden et al. 1990).

These examples demonstrate the potential of zinc finger genes to play a role in the initiation or progression of a cancer phenotype. It is not surprising therefore, that studies involving the isolation of potential zinc finger genes are focused around disease associated regions (Donti et al. 1990, Hoovers et al. 1992). The finger gene (HF.10), ZNF35, was the first zinc finger gene to be mapped to 3p. By in situ hybridization and analysis of somatic cell hybrids the gene was mapped to 3p21-p22 (Donti et al. 1990).

The mapping of ZNF35 in this study does not contradict its previous localisation

but refines its position relative to other markers. The data from this study maps ZNF35 distal of D3F15S2 but proximal to D3S1100/THRB. As described previously, THRB is believed to lie distal of the SCLC deletion. If, as is suspected, the distal boundary of the deletion is close to the D3F15S2 locus, it is possible that ZNF35 is included in the deleted region.

The potential zinc finger gene, ZnFP16 mapped to 3p21.3, is one of several identified in this region (Hoovers et al. 1992). It will be of particular interest to determine the gene product of these zinc finger genes and whether they interact with one another. In addition their interaction with other non-zinc finger protein products may assist in determining their specific role in cellular function. Whether one or more zinc finger genes on chromosome 3 plays a significant role in lung tumourogenesis through the interaction with a tumour suppressor gene is an interesting and likely possibility.

4.4.2 ARH12: A *RAS*-RELATED GENE

ARH12 is a member of the ras-related gene family. Sequence analysis reveals up to 35% homology with the *ras* gene family (Madaule et al. 1985). Several common properties have been described between ARH genes and the *ras* family including the protein size and their ability to attach to the cell membrane (Madaule et al. 1985). However, the significant regions of diversity between the two families indicate possible functional differences. Speculations have been made suggesting the possibility of the ARH family encoding different components of the G-protein complex (Madaule et al. 1985). Like the *ras* family of oncogenes, ARH may potentially interact with anti-oncogenes, ie. tumour suppressor genes.

The mapping of ARH to 3p21.2-p21.3 indicates its close proximity to the D3F15S2 locus which may represent the closest marker to the tumour suppressor gene. It is feasible that at the protein level, a *ras* related gene may directly or indirectly interact with the SCLC tumour suppressor gene product at the cell

membrane to contribute to the development of the malignancy.

4.4.3. HEPATOCYTE GROWTH FACTOR LIKE GENE AT THE D3F15S2 LOCUS

A recent study of the D3F15S2 locus on chromosome 3 has identified it as a gene encoding kringle domains with homology to the hepatocyte growth factor, HGF (Lorie et al. 1991). A kringle domain is a protein binding domain originally identified in the plasma protein, prothrombin, which regulates the prothrombin-thrombin conversion through an interactive binding process with other proteins (Magnusson et al. 1975). The function of other genes encoding kringle domains depends on the conformational differences resulting from amino acid substitutions in the variable region of the domain. However, their direct binding capability with other proteins demonstrates a potential regulatory role in the cell (Lorie et al. 1991).

In an attempt to isolate the human prothrombin gene, a bovine cDNA probe containing the kringle domains was used to screen cDNA libraries under reduced stringency conditions. In addition to the isolation of the human prothrombin cDNA, a strongly hybridising genomic clone with an open reading frame was identified (Lorie et al. 1991). Sequence analysis revealed four kringle domains and a serine protease-like domain in the encoded gene product. In addition a 50% homology with the hepatocyte growth factor was demonstrated, which included the kringle domains. The gene was referred to as the HGF-like gene.

Hepatocyte growth factor is the most potent mitogen for mature hepatocytes (Rubin et al. 1989). The molecular cloning and expression of human hepatocyte growth factor has highlighted its function as a growth factor (Nakamura et al. 1989). HGF induces the autophosphorylation of the *c-met* tyrosine kinase. *C-met* binds HGF as its cell surface receptor to mediate the mitogenic signal of the growth factor.

A homology has been observed with the HGF-like protein and sequences

previously identified at the D3F15S1, D3F15S2 and rat acyl-peptide hydroylase loci. Further analysis demonstrated the HGF-like protein to be located at the D3F15S2 locus on chromosome 3 (Lorie et al. 1991). Three regions of homology in the genomic 3'-flanking region of the complementary strand were shown to be homologous to human lung cDNA, APEH. Therefore, in addition to the APEH gene , a second overlapping gene encoding a HGF-like protein exists downstream of APEH, transcribed in the opposite direction.

The presence of a growth factor-like encoding gene at a locus shown to be consistently involved in SCLC is of great significance. Its potential role in the control of cell growth makes it an attractive candidate for a tumour suppressor gene. Lorie et al (1991) have speculated that the gene product may be a competitive inhibitor for a growth factor receptor which if absent would permit any growth factor to bind to its receptor and uncontrolled growth may occur.

The ability of a tumour suppressor gene to interact with an oncogene product is not uncommon. Since the mitogenic signal of HGF is mediated by the *c-met* oncogene it is possible that the recently identified HGF-like gene possesses similar if not the same interactive abilities with an oncogene product (Nakamura et al. 1989). This is demonstrated further by the four Kringle domains of the HGF-like gene illustrating its ability to directly bind and quite possibly regulate other proteins in the cell (Nakamura et al. 1989). The abnormal expression of any growth factor or growth factor related protein can not only influence normal cell differentiation but activate growth-promoting pathways in cancer cells.

4.4.4 D8 AND ITS HOMOLOGY WITH THE UBIQUITIN ACTIVATING ENZYME, E1

The gene isolated in this laboratory, D8, at 3p21 showing reduced expression in SCLC has been used in sequence databases analysis, to identify homologies with other proteins which may assist in defining a functional role for the gene (K.Kok

pers. communication). Of particular significance was the extensive homology found between D8 and the human X linked A1S9 gene cDNA.

The A1S9 gene complements a temperature-sensitive cell cycle mutation in mouse cells. The temperature inactivation of this gene has the most significant effect on nuclear DNA replication (Zackenhaus et al. 1990). The gene encodes the ubiquitin-activating enzyme, E1, a 90kd protein (Handley et al. 1991). The protein is unable to enter the nucleus by passive diffusion on the basis of its large size. Two nuclear organising sites have been identified which may assist in the translocation through the nuclear membrane.

A candidate spermatogenesis gene on the mouse Y chromosome has recently been been reported and is homologous to the ubiquitin-activating enzyme, E1(Mitchell et al. 1991, Kay et al. 1991). The Y chromosome has a sex-reversed region which encodes the *Tdy* primary sex determining gene in addition to other male determining genes. Since the candidate spermatogenesis gene, *Sby* is homologous to the ubiquitin activating enzyme, E1 it indicates a potential role in the control of a specific ubiquitin-dependent pathway in spermatogenesis.

Therefore the ubiquitin-activating enzyme is encoded by the A1S9 gene and the candidate spermatogenic gene from mouse Y (Handley et al. 1991). The enzyme catalyses the first step in ubiquitin conjugation with a key role in any process affected by ubiquitin modification (Handley et al. 1991). These have been identified as ubiquitin protein degradation and specific ubiquination of histones involved in transcriptional control. In addition and of particular interest here is the fact that several cell-surface molecules like the platelet-derived growth factor, PDGF, and growth hormone receptors have been found to be modified by ubiquitin (Yarden et al. 1986, Spencer et al. 1988). Ubiquitin covalently binds to the PDGF receptor exercising some form of post translational modification which maybe significant in the signal transduction process of the cell growth pathway (Yarden et al. 1986).

If D8 encodes a ubiquitin activating enzyme or an enzyme with very similar properties then it is feasible that it may play a role in regulating growth factors in a similar mode as PDGF. Obviously, if D8 is a ubiquitin activating enzyme then its mouse homologue should be able to complement the temperature sensitive mutation in mouse cells.

4.5 3p AND CANCER

Critical loci on the short arm of chromosome 3 have been implicated in the cancerous phenotype. The repeated reduction to homozygosity at the D3F15S2 locus in SCLC and its high frequency of involvement in non-SCLC, renal cell carcinoma and other forms of cancer provide firm evidence for a tumour suppressor gene close to this locus. There are now several cases of both SCLC and non-SCLC which provide evidence for a tumour suppressor gene located proximal to the D3F15S2 locus. Of particular significance are the two cases of SCLC which show no evidence for allele loss at the D3F15S2 locus. In one SCLC, U2020, a homozygous deletion at the D3S3 locus was detected (Daly et al.1991) The second cases is from a SCLC patient biopsy which demonstrates retention of heterozygosity at THRB and D3F15S2. Loss of heterozygosity is observed at the D3S30 and D3S32 loci (Ganly et al.1992). In addition, a preferential involvement of the markers, D3S32 and D3S2 (3p21.1), both of which have been shown to be proximal of D3F15S2, in non-SCLC has been observed Brauch et al. (1990). Their published data on loss of heterozygosity studies of SCLC have been widely taken to support the idea that there is a tumour suppressor gene mapping close to D3F15S2 and possibly in the interval flanked by this marker and the proximal locus, D3S2.

The 3p13-p14 region has been implicated in both familial and sporadic renal cell carcinoma. In addition reduction to hemizygosity of markers mapping in this interval

(D3S3, D3S4 and D3S30) has been reported in SCLC and non-SCLC indicating a second 3p locus inmplicated in renal carcinoma with a secondary role in lung cancer (Brauch et al. 1990, Daly et al.1991).

Therefore, in the interval 3p13-p25 there are at least two genes whose loss of function would initiate or contribute to a malignant phenotype. On the basis of some of the studies reported on SCLC, non-SCLC and renal cell carcinoma one might be tempted to offer the easy explanation that mutation of one gene is the primary event for the kidney malignancy at 3p13-p14, and an other for the lung malignancy at 3p21. However, there are many instances of renal cell and lung tumours which exclude this idea, and clearly indicates a more complicated model (Brauch et al. 1987, Rabbitts et al. 1987, Daly et al. 1991, Van der Hout et al.1991, Ganly et al 1992). It is difficult to explain the observations of non-SCLC which on the basis of molecular analysis, include the frequent loss of alleles distal of 3p13-p14. However, no consistent loss of one allele has been satisfactorily demonstrated in non-SCLC, making the characterisation of a 3p deletion difficult.

The idea of a common origin for all lung cancers is based on the observations of a transition of the SCLC phenotype to the non-SCLC phenotype and the shared biological properties of these two tumour types (Leij et al. 1985, Reeve et al. 1986). It is possible that the two lung tumour phenotypes share an early genetic event, which may include the deletion of sequences on the short arm of chromosome 3. This may be followed by an accumulation of genetic and biological events unique to SCLC and to non-SCLC. If loss of one allele at the D3F15S2 locus represents a consistent event in SCLC, one might expect all non-SCLC tumours to show reduction to hemizygosity. However, there are some albeit a few cases, which show retention of heterozygosity at this locus and this may indicate a different genetic mechanisms in at least some non-SCLC cases (Brauch et al. 1990) .

The minimally deleted region in sporadic renal cell carcinoma has recently been identified as 3p21-p25. This is clearly distinct from the the 3p13-p14 region which was characterised both in familial and sporadic RCC (Van der Hout et al. 1991a&b, Cohen et al. 1979, Kovacs et al.1987&1989). These observations have several implications. First, there is more than one tumour suppressor gene involved in the renal cell tumour phenotype, and second could one of these genes be the same as that involved in the lung cancer phenotype? It is possible that there is an additional gene implicated in RCC, distinct from the first which is involved in sporadic forms of the cancer. It may be that this is the same gene implicated in SCLC and non-SCLC since they are also non-familial cancers.

Therefore, it seems very likely that a number of related or unrelated tumour suppressor genes important in a broad spectrum of malignancies must co-exist in the interval 3p14-p25. It must be borne in mind that the mode of action of the tumour suppressor genes implicated in SCLC, non-SCLC and RCC are unlikely to be as simple as the retinoblastoma model.

5. APPENDIX

5.APPENDIX

5.1 STANDARD BUFFERS

TE	1mM Tris pH8
	0.1mM EDTA (disodium salt)
10xTE	10mM Tris pH8
	1mM EDTA (disodium salt)
STE	150mM NaCl
	10mM Tris pH8
	10mM EDTA (disodium salt)
20xSSC	3M NaCl
	0.3M Na_3 citrate
	pH to 7.0
10x TBE	0.89M Tris.HCl pH 7.9-8.1
	0.89M Boric acid
	0.02M EDTA

Loading buffer	40% sucrose
(10x)	0.025%w/v bromophenol blue
	0.025%w/v xylene cyanol

5.2.TISSUE CULTURE AND DNA EXTRACTION

5.2.1 SCLC MEDIUM

To 85ml RPM1the following reagents are added:

10ml foetal caf serum

1ml fungicon (1mM)

1ml pencillin/streptomycin (10,000 Gibco)

1ml glutamine (10mM)

1ml sodium pyrivate (100mM)

1ml B-mercaptoethanol (0.0.5%)

The antibiotics are made up in sterile distilled water, filtered and stored at -20°C until ready for use. The sodium pyruvate is obtained in powder form and dissolved in sterile distilled water, filtered through a 0.45 m filter and stored at 4°C. B-mercaptoethanol is obtainedn as a 40m stock and diluted in sterile water, filtered and stored at 4°C.

The media is filter sterilised, stored at 4°C and a small aliquot tested by incubation at 37°C for 48 hours prior to use.

5.2.2 FREEZING MEDIUM

To fresh SCLC medium 1, 10% dimethylsulphonoxide is added and the mix cooled on ice prior to use.

5.3 BACTERIAL AND YEAST MEDIA, BUFFERS AND ANTIBIOTIC CONCENTRATION

All media and buffers were prepared with deionised idstilled water and immediately autoclaved at 15lbs psi 121°C for 30 minutes.

L-Broth	g per 100ml
Bacto tryptone	1.0
Bacto Yeast Extract	0.5
NaCl	0.5
Glucose	0.1

L-Agar

Bacto tryptone	1.0
Bacto Yeast Extract	0.5
NaCl	0.5
Bacto Agar Noble	1.5

Top Agar/ Agarose

Bacto tryptone	1.0
NaCl	0.5
Bacto Agar Noble/Agarose	1.5

NZYDT

pH to 7.4	2.0

YPD medium

Yeast Extract	1.0
Peptone	2.0
Glucose	2.0

Phage Buffer

Tris.HCl,pH 7.6	10mM
$MgCl_2$	10mM

Mg-Ca solution

$MgCl_2$	10mM

$CaCl_2$	10mM

Antibiotic	working conc.	stock conc.	solvent
	g/ml	mg/ml	
Ampicilin	50	5	water
Kanamycin	30	3	water

X-Gal	25µl	4mg/ml	dimethylformamide
IPTG	10µl	100mM	water

5.4 SOLUTIONS FOR LARGE AND SMALL SCALE PLASMID PREPARATION

Solution I

50mM Glucose

25mM Tris HCl pH8

10mM EDTA

Filtered through 0.45 m filter and stored at 4°C

Solution II

2 pellets BDH NaOH (0.2N)

18ml ddH_2O when dissolved

2ml 10% SDS

Solution III 60ml 5M KOAc

11.5ml glacial acetic acid

28.5 ml H_2O

Phenol Preparation: 1 litre of 0.1M EDTA was added to 1 kg of solid phenol and melted at 42°C. The resulting solution was shaken vigorously to form an emilsion and the top layer removed. The remainder was extracted once with 1 litre 0.1M Tris; 10mM EDTA and once with 1 litre 10mM Tris; 1mM EDTA Both times the top layer was syphoned off and discarded. Finally the top layer was replaced with 100ml TE buffer.

5.5 SOLUTIONS FOR HYBRIDISATIONS

Hybridisation mix for Hybond N+ (Church Buffer):

0.5m phosphate buffer (0.5M NaH_2PO_4 + phophoric acid to pH7.4)

7.0% SDS

1mM EDTA

Hybridisation for nitrocellulose:

5x SSC

5x Denhardt's solution

0.5% w/v SDS

Denhardt's solution:

	g/100ml
Fraction V, Sigma BSA	0.4
Pharmacia Polyvinyl pyrrolidone	0.4
Pharmacia Polyethylene Glycol 400	0.4

5.6 STANDARD MARKERS

1Kb and ΦX ladders were obtained from BRL,U.K.The sizes are listed below (in Kb):

1Kb ladder	ΦX 174 RF DNA/Hae III
12.2	1.35
11.2	1.08
10.2	0.87
9.2	0.60
8.1	0.31
7.1	0.28
6.1	0.27
5.1	0.23
4.1	0.19
3.1	0.12
2.0	0.07
1.6	
1.02	
0.51	
0.39	
0.34	
0.3	
0.22	
0.20	
0.154	
0.134	
0.075	

YEAST MARKER SIZES: (in kb)1900,1640,1120, 1100,945,915,815,785,745,680,585,450,375,295,225.

5.7 REAGENTS FOR PULSED FIELD GEL ELECTROPHORESIS.

5.7.1 PREPARATION OF DNA AGAROSE BLOCKS

5.7.1.1 Lysis Buffer (working solution):

155mM Ammonium Chloride

10mM Potassium Hydrogen Carbonate

10mM EDTA

5.7.1.2 Phosphate Buffered Saline (PBS):

10 x PBS (Gibco) diluted to working strength with sterile deionised water and neutralised wtih sterile 1M NaOH.

5.7.1.3 Low Melting Point Agarose (LMP) for Agarose Blocks

1% LMP dissolved in SE buffer

5.7.1.4. SE buffer:

75mM Sodium Chloride

25mM EDTA

5.7.1.4 ESP Solution

0.5M EDTA

1% N-lauroylsarcosine

0.5mg/ml proteinase K

5.8 REAGENTS FOR DNA FINGERPRINTING

5.8.1 MICROPREP SOLUTIONS

5.8.1.1 Lysis solution (500ml)

4.504g glucose

10ml 0.5 EDTA

12.5ml 1M Tris.HCl pH8

filtered to sterilised and stored at 4°C

5.8.1.2 Alkaline/SDS

9.1ml sterile distilled water

0.5ml 20% SDS

0.4ml 5M NaOH

freshly prepared before use

5.8.1.3. Sodium Acetate

3m NaoAc pH 5.5

autoclaved to sterilised

stored at 4°C

5.8.1.4. Lithium Chloride

9.328g in 50ml (4.4M) sterile distilled water

stored at 4°C

5.8.2 FINGERPRINTING REACTION

Formamide Dye: 98% deionised formamide

10mM EDTA (pH8.0)

0.3% bromophenol blue

0.3% xylene cyanol

stored at -20°C

5.8.3 SEQUENCING GEL PREPARATIONS

5.8.3.1 Preparation of plates

5.8.3.1.1 Large back plate

The plate was washed with lipsol detergent, dried and then washed with 70 % ethanol. The plate is siliconoised periodically to prevent the gel sticking. Liberal amounts of BDH "Repelcote" (ie 2% solution of Dimethyldichlorosilane in 1,1,1-trichloroethane).are wiped over the back plate and allowed to dry for at least 10 minutes.

5.8.3.1.2 Small front plate

The plate was washed with lipsol detergent, dried and then washed with 70 % ethanol. One side was applied with freshly prepared bondng solution:

3ml ethanol

50µl 10% acetic acid

5µl Wakker soluiton

A small amount was placed on a tissue and wiped evenly over the plate. It is important not to apply too much as this results in the gel bonding to both plates. The plate is the left to dry and ethanol washed and dried once again.

5.8.3.2. Sequencing gel mix

Acrylamide: 40% stock solution of 19:1 acrylamide:bis-acrylamide (Sigma) made up in sterile deionised water and stored at 4°C

Ammonium Persulphate (AMPS): 10% w/v solution of AMPS in sterile distilled water.

Temed: stock from Biorad

5.8.4. DIETHYLPYROCARBONATE TREATMENT

Tips and microcentrifuge tubes were soaked in 0.1% DEPC/ water overnight and sterilisesd by autoclaving before use.

6. REFERENCES

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