ISOLATION OF CHROMOSOME 21 SPECIFIC DNA PROBES AND THEIR USE IN THE STUDY OF DOWN'S SYNDROME

James Galt ©

Thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow, Faculty of Medicine

Department of Medical Genetics
Duncan Guthrie Institute
Yorkhill
Glasgow

March 1988

DECLARATION

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

James Galt

To Lynne Fullarton

ACKNOWLEDGEMENTS

I would like to extend my gratitude and appreciation to the following people :

Professor M.A. Ferguson-Smith for providing me with the opportunity and the facilities to carry out this research,

Professor J.M. Connor for his help and advice in the writing of this thesis,

Dr. Elizabeth Boyd for cytogenetic analysis of Down's syndrome families,

Dr. Gordon Stewart for his assistance during the early stages of this project,

Mrs. Norma Morrison for her technical assistance with the somatic cell hybrids,

Michael Mitchell, Marianna Kalaitzidaki, Elizabeth Gillard and Doreen Jamieson for moral support.

The Medical Research Council provided financial assistance during the period October 1983 to September 1986.

I am grateful to my parents, Peter and Elizabeth Galt, for their faith in my ability to succeed and for their years of encouragement and support, both during this project and in the years before it. I would also like to thank my mother for her assistance in the typing of this thesis.

I extend my special thanks also to Dr. Lina Florentin, who helped me a great deal during the course of this research. She also taught me a great deal about life in general, for which I am most grateful.

The Northern blot experiments were carried out by Dr.Lorraine Stefani and Mrs. Anne Palmer, in the Department of Medical Genetics, Lisburn Road, Belfast.

Mr. Alan Ramsay assisted with the screening of recombinants for restriction fragment length polymorphisms, and discovered the JG90/Nde II polymorphism.

Probes E8(D21S15), H8(D21S17) and D21K9(D21S13) were supplied by Dr. Gordon Stewart; probe 10.2(D21S25) was kindly donated by Prof. Peter Pearson.

I am eternally indebted to my wife, Lynne Fullarton, without whom none of this would have been possible. I would like to express my appreciation of her emotional, moral and financial support during the seemingly endless years of this research project.

Thank you for being there when I needed you.

CONTENTS

CHAPTER 4 : DISCUSSION

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Amp	-	Ampicillin
BSA	-	Bovine Serum Albumin
CBS	-	Cystathionine Beta Synthetase
cDNA	-	Complementary DNA (Made from messenger RNA)
cm	-	centimetre
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytidine triphosphate
dGTP	-	deoxyguanosine triphosphate
dTTP	-	deoxythymidine triphosphate
DNA	-	Deoxyribonucleic Acid
DNase	-	Deoxyribonuclease
EDTA	-	Ethylene Diamine Tetra-acetic Acid
g	-	gram
GARS	-	Phosphoribosyl Glycinamide Synthetase
HEPES	-	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
IFN	-	Interferon
LMP	-	Low Melting Point
mA	-	milliamp
Ωg	-	microgram
mg	-	milligram
ml	-	microlitre
mRNA	-	messenger RNA
ng	-	nanogram
NOR	-	Nucleolar Organiser Region
o/n	-	overnight
O.D.	-	Optical Density
pfu	-	plaque forming units
PIC	-	Polymorphism Information Content
poly A^+	-	polyadenylated RNA (i.e. messenger RNA)
PRGS	-	Phosphoribosylglycinamide Synthetase
RFLP	-	Restriction Fragment Length Polymorphism
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
rpm	-	revolutions per minute
SDS	-	Sodium Dodecyl Sulphate
Tris	-	Tris (hydroxymethyl) aminomethane
tRNA	-	transfer RNA
U.V.	-	Ultraviolet
v/v	-	volume per volume
w/v	-	weight per volume
X-gal	-	5-Bromo-4-chloro-3-indoyl-beta-D-galactopyranoside

SUMMARY

Down's syndrome is caused by the presence in an individual of three copies of gene loci in a critical region in the long arm of chromosome 21. Usually a complete extra copy of chromosome 21 is present (trisomy) and at birth this has an overall frequency of 1 in 700. The most important clinical effect is severe mental retardation, and Down's syndrome represents the most commonly identified cause of human mental handicap. The phenotype also includes dysmorphic features, malformations of major organs, an increased risk of leukaemia and presenile Alzheimer-type dementia.

Older cytogenetic studies of chromosomal heteromorphisms have shown that in 80% of cases the additional chromosome in trisomy 21 is of maternal origin. Surprisingly this proportion did not seem to be increased in older mothers (over 35 years) who are known to have an increased frequency of Down's syndrome offspring. The cytogenetic studies were, however, uninformative in many cases.

The main aim of the present project was to isolate polymorphic DNA probes from chromosome 21 which could be used to study nondisjunction in Down's syndrome families and to construct a genetic linkage map of chromosome 21. Cloned sequences derived from the critical region were to be analysed for expression in an attempt to identify expressed genes of pathogenic importance.

Two chromosome 21-specific recombinant libraries were screened and of 486 bacteriophage clones initially selected, 29 recombinants were further analysed. Fourteen of these were shown to contain highly repetitive DNA sequences and were not studied further. The remaining 15 clones contained single- or low-copy number sequences and these were regionally mapped on chromosome 21 using a panel of somatic cell hybrids. Five of the single-copy sequences (JG21/D21S86, JG62/D21S90, JG72/D21S92, JG90/D21S95, JG99/D21S97) mapped in the 21q21-q22.1 region. A further six (JG12/D21S85, JG22/D21S87, JG24/D21S88, JG63/D21S91, JG81/D21S94, JG108/D21S99) were localised in the 21q22.1-qter region and one (JG77/D21S93) in the 21q22.1-q22.2 region. Probe JG373 (D21S101) was mapped in the distal part of band 21q22.3.

The remaining two DNA sequences (JG51 and JG73) are mildly repetitive and were mapped to the pericentromeric region of chromosome 21. Although these clones were isolated separately from the same recombinant library and have different human insert sizes, they appear to detect homology with the same series of sequences in the human genome, which are present on chromosome 21 and on other chromosomes. The results obtained with these two probes suggest that they are part of the alphoid repeat family, members of which are present at the centromere of every human chromosome.

Eight of the probes isolated in the present project were tested with a total of 25 restriction endonucleases and five restriction fragment length polymorphisms (RFLPs) were

discovered : JG77/D21S93 detects an Msp I polymorphism, with alleles of 6kb and 3kb, of frequencies 0.67 and 0.33, respectively; JG81/D21S94 detects two RFLPs, with the enzymes Pvu II and Eco RV - the allele sizes and frequencies (in parenthesis) for these two RFLPs are 8.5kb(0.83)/8kb(0.17) for Pvu II and 5kb(0.88)/4.5kb(0.12) for Eco RV; JG90/D21S95 detects an RFLP with the enzyme Nde II, with allele sizes/frequencies of 2.2kb(0.7) and 1.8kb(0.3); the other RFLP is detected by JG99/D21S97 with the enzyme Pst I and has 7kb(0.16) and 6.6kb(0.84) alleles.

The Msp I RFLP detected by JG77/D21S93 was used in conjunction with 4 other previously described polymorphic chromosome 21 probes to analyse the origin of nondisjunction in a total of 33 Down's syndrome families. Cytogenetic analysis by Q-banding was also used in some of these families. The parental origin of the additional chromosome was determined in 12 cases (36%) - in 9 of these (75%) the additional chromosome was of maternal origin, while in the other 3 (25%) it was derived from the father. These proportions are in agreement with those obtained previously from studies of cytogenetic heteromorphisms. The meiotic stage of the nondisjunctional event was defined in 7 families using RFLPs as follows : maternal I - 2 cases, maternal II - 3 cases, paternal II - 2 cases. Cytogenetic analysis was informative in 3 of the 5 families studied and confirmed the results obtained with DNA markers in each case. In two of the families it also allowed localisation of nondisjunction to

the first maternal meiotic division, bringing the total number of such cases to 4.

In one family results obtained with the JG77(D21S93)/Msp I polymorphism localised nondisjunction to the second maternal meiosis. Results obtained with the D21K9(D21S13) marker and Q-banding heteromorphisms, both of which are more closely linked to the centromere, showed nondisjunction to have occurred at the first maternal meiotic division. These latter results are more reliable and it can be stated that in this family crossing-over occurred between the D21K9(D21S13) and JG77(D21S93) markers in the mother, prior to nondisjunction at the first meiotic division. This is consistent with emerging data obtained from other workers and shows that reduced recombination between chromosome 21 homologues is not the major cause of trisomy in Down's syndrome, as had been suggested. The possible effects of recombination on the interpretation of RFLP data are also clearly demonstrated in this case, emphasising the importance of using pericentromeric markers to define the meiotic stage of nondisjunction.

The 13 single-copy sequences isolated during this project represent a significant contribution to the total number of arbitrary DNA sequences assigned to chromosome 21, which at present stands at 99. The recently developed technology of pulse-field electrophoresis will allow these sequences to be used in the construction of a physical map of chromosome 21. Those probes which detect RFLPs can also be

incorporated into the genetic linkage map of this chromosome. In this way the gap between the physical map (in megabases of DNA) and the genetic map (in recombination units) will be bridged.

Five of the single-copy sequences isolated during this project were also tested for expression in Northern blot experiments. Four were shown to be expressed - two (JG72/D21S92, JG108/D21S99) at a significant level only in Down's syndrome brain tissue; the other two (JG77/D21S93, JG90/D21S95) are expressed in both normal and Down's syndrome brain, and at a much higher level in the latter. It is interesting to note that JG77/D21S93 and JG90/D21S95 have been localised in the regions of chromosome 21 associated with Down's syndrome and Alzheimer's disease, respectively, and as such could represent genes or parts of genes involved in the pathogenesis of these disorders.

In the absence of an understanding of the pathogenesis of Down's syndrome, no rational therapy is available, and current emphasis must lie with prevention of the disorder. At the primary level, this would involve the prevention of nondisjunction but again this is not possible due to the lack of understanding of the mechanisms involved. As such, secondary prevention by detection and selective termination of pregnancy is the main focus of effort. To facilitate this the identification of risk factors for Down's syndrome such as increased maternal age and reduced levels of maternal serum alpha-fetoprotein is clearly important. DNA probes as

described in this study might also be of use in the definition of risk factors in families showing recurrent trisomy 21 - by comparing pericentromeric RFLP haplotypes in affected family members it could be determined whether or not all centromeres are equally predisposed to nondisjunction. Furthermore, DNA probes may also be useful for diagnosis of Down's syndrome either by in situ hybridisation to interphase nuclei or by dosage studies in fetal tissues, which might be obtained either by conventional prenatal diagnosis techniques or by isolation of fetal cells from maternal blood.

1.1 Down's syndrome

1.1.1 Historical Aspects

In 1866 an English physician, John Langdon Down, published a paper entitled "Observations on an ethnic classification of idiots". As medical superintendant of an asylum for the severely mentally retarded, he found that approximately 10% of all the inmates resembled each other and could readily be distinguished from the other inmates. He suggested that members of this group expressed some Mongolian traits : "The great Mongolian family has numerous representatives, and it is to this division I wish in this paper to call special attention. A very large number of congenital idiots are typical Mongols. So marked is this, that when placed side to side, it is difficult to believe that the specimens compared are not children of the same parents".

It is now clear that such terms as "Mongolian Idiot", "Mongol" and "Mongoloid" are not only offensive, but are also incorrect. Even Down's son, Reginald Langdon Down, who continued in his father's work with mental defectives, recognised this : "It would appear that the characteristics which at first sight strikingly suggest Mongolian features and build are accidental and superficial, being constantly associated with other features which are in no way characteristic of that race". Of the many substitute names suggested, the most commonly used is Down's syndrome.

Long before the cause of Down's syndrome was known, studies of this disorder among twins suggested a genetic role (Halbertsma 1923; Reuben & Klein 1926). Among monozygotic twins (which arise from a single egg and thus have identical genotypes) it was found that if one member had Down's syndrome, almost invariably the other was similarily afflicted. In contrast, if one member of a pair of dizygotic twins (which develop from two fertilized eggs and so are genetically equivalent to siblings) had Down's syndrome, the other was, in most cases, normal.

Because the pattern of transmission did not fit the usual models for single gene inheritance, it was suggested (Waardenburg 1923) that Down's syndrome might result from a chromosomal abnormality. Methods then available for studying human chromosomes were too primitive to test this hypothesis, but just three years after the normal human chromosome number was finally established (Tjio & Levan 1956; Ford & Hammerton 1956), came the exciting discovery that somatic cells of sporadic Down's syndrome patients contain 47 chromosomes instead of 46 (Lejeune et al 1959a,b). The extra chromosome was one of the G group, at first thought to be the next to smallest, chromosome 21. As such, Trisomy 21 was quickly accepted as an alternative name for Down's syndrome. Later, however, improved techniques showed the extra chromosome to be the smallest member of the G group, which should be called chromosome 22, but rather than try to alter the already well-established name, cytogeneticists simply learned to live

with the inconsistency. For this reason the next to smallest chromosome in the G group is designated 22, while the smallest - trisomic in Down's syndrome - is called chromosome 21 (Ridler 1971, Baikie 1971, Hsu et al. 1971).

1.1.2 Clinical Features

Of all the disabilities in Down's syndrome, the most inportant, in terms of the affected individual's ability to cope with the environment, are those connected with mental development. These patients are severely mentally retarded and generally have an I.Q. in the range of 25-50. With a frequency at birth of approximately 1 in 700 (Smith & Berg 1976), Down's syndrome thus represents the most commonly recognised cause of severe human mental handicap.

In addition to these obvious abnormalities of the central nervous system, patients are distinguished by some combination of the following traits : the face is broad with a small, flat nose, irregular teeth, and abnormally shaped ears. The eyes may be close set, with narrow, upslanting palpebral fissures; the presence of an epicanthic fold is common in Down's syndrome patients. The tongue is often large and furrowed. These dysmorphic features are very distinctive and allow the rapid clinical diagnosis of a high proportion of cases. All parts of the body are shortened, signifying poor skeletal development. Defects of the heart, intestines, kidneys, thyroid and adrenal glands are common. Males have poorly developed genitalia and are almost invariably sterile.

In females, although some have reproduced, ovarian defects and irregular menstruation are very common.

The life expectancy of Down's syndrome patients is considerably reduced. Roughly half of all Down's syndrome patients die in the first year of life, often from congenital cardiac anomalies. Another major cause of death in these patients is respiratory infection and the mortality rate is approximately 120 times greater than in the general population. Also, for unknown reasons, there is a 20-fold increased risk of dying from acute leukaemia.

The application of cardiac surgery and antibiotic therapy has, in recent years, led to an increased survival rate for Down's syndrome patients. It is unlikely, however, that their ultimate life expectancy will be in the normal range, since patients who survive longer (over 35 years) develop neurological changes typical of Alzheimer-type dementia (For further details and references on clinical features see Smith & Berg 1976 and Vogel & Motulsky 1986).

1.1.3 Cytogenetics of Down's Syndrome

The chromosome constitution most commonly associated with Down's syndrome is the presence of three complete copies of chromosome 21 in all of the cells of an individual. This occurs in about 95% of Down's syndrome individuals and is referred to as standard trisomy 21.

Another common chromosome anomaly in Down's syndrome is that of mosaicism, which results from mitotic

non-disjunction in the embryo, as opposed to meiotic non-disjunction in the parent. The degree of abnormality in such patients depends upon the proportion and tissue distribution of trisomic cells. For example, if mitotic nondisjunction occurred at an early stage of differentiation, then a high percentage of cells would be trisomic and the phenotype would show many of the characteristic Down's syndrome symptoms. If it took place later, such that cells of the nervous tissue had a normal chromosome complement, the individual may be of normal intelligence.

The true frequency of chromosome mosaicism is difficult, if not impossible, to establish, because it is theoretically impossible to rule out the presence of a second cell line in any study. In practice, however, arbitrary limits are usually imposed on the number of cells of different karyotype required for such a diagnosis. Studies of mosaicism in Down's syndrome individuals have shown that it accounts for about 1% of all cases.

In addition to standard trisomy 21 and mosaicism, two different classes of translocation can result in the Down's syndrome phenotype, and account for approximately 4% of cases. The most common is called Robertsonian translocation (Robertson 1916) or centric fusion (White 1954), and involves the translocation of the long arm of chromosome 21 to the long arm of either a large, or another small acrocentric chromosome. The complementary chromosome formed by fusion of the two short arms of the participating chromosomes is

usually lost after a few cell generations. Clinically, the result of having an additional copy of the long arm of chromosome 21 is the same whether or not it is attached to another chromosome, or isolated, as in the standard trisomic karyotype (Turpin & Lejeune 1961, Gustavson 1964, Gibson & Pozsonyi 1965, Niebuhr 1974).

Although centric fusions are the most frequent, a wide variety of reciprocal translocations involving chromosome 21 have also been reported (see deGrouchy & Turleau 1977, Smith & Berg 1976). Some of these result in the birth of a child with Down's syndrome and the study of such translocations led to the assignment of 21q22 as the chromosomal segment primarily responsible for the Down's syndrome phenotype (Aula et al. 1973, Niebuhr 1974, Williams et al. 1975, Cervenka et al. 1977, Summitt 1981). There is some disagreement regarding the involvement of the specific sub-bands of 21q22 in the determination of Down's syndrome. Some researchers have excluded band 21q22.3 from the critical region (Poissonier et al. 1976), while others have observed the Down's syndrome phenotype in patients that are trisomic only for 21q22.3 - qter (Mattei et al. 1981, Habedank & Rodewald 1982).

1.1.4 Pathogenesis of Down's syndrome

It is generally assumed that the extra 21q22 segment codes for normal products and that the abnormalities observed in Down's syndrome are produced by the excess of some of

those proteins. It has been estimated that the critical region of chromosome 21 should contain less than 100 genes (Smith & Warren 1985). Molecular cloning of genes from this area of chromosome 21 and analysis of their organisation and expression in normal and Down's syndrome individuals should lead to identification of those involved in the pathological manifestations of the syndrome.

At the time when the current project began, the only genes which had been mapped to this region of the chromosome were those for the cytoplasmic form of superoxide dismutase (SOD-1; Tan et al. 1973), phosphoribosyl glycinamide synthetase (PRGS; Moore et al. 1977) and the cell surface receptor for human interferon (IFRC; Tan et al. 1974). Although several hypotheses concerning the role of these genes have been suggested (Epstein et al. 1985), there is no compelling data linking altered expression of these genes to a specific feature of the Down's syndrome phenotype.

1.1.5 Frequency of Down's Syndrome and Parental Age Effects

Most of the published results agree that the incidence of Down's syndrome at birth is of the order of 1 in 700 (Smith & Berg 1976). The birth incidence varies greatly with the age of the mother. At about the maternal age of 20 years the incidence is 1 in 2000. This changes very little until age 35, when the risk is about 1 in 300. The exponential increase in risk continues in older mothers, such that at 45 years and over the incidence rate is approximately 1 in 45

(Smith & Warren 1985). The effect of maternal age in Down's syndrome was first described by Penrose (1933) and has subsequently been demonstrated in all populations studied, regardless of geographic location, ethnic origin or socio-economic status. One group of researchers have claimed that a proportion of Down's syndrome births are dependent on the age of the father and that the risk increases significantly after the age of 55 (Stene & Stene 1977, Stene et al. 1977). A different method of statistical analysis was used by Erickson (1978), who concluded that a paternal age effect, independent of the age of the partner, was not demonstrable in his large sample. The method used by Erickson was criticised by Stene and Stene (1978). Subsequent studies have agreed with the conclusions of Erickson, however, and failed to demonstrate the presence of an independent paternal age effect (Erickson 1981, Hook et al. 1981). The situation is the source of considerable debate and remains unresolved.

1.1.6 Recurrence Risk of Down's syndrome

The occurrence of two or more patients with Down's syndrome in the same kindred has been demonstrated (Werner et al. 1982). Occasionally this is due to a phenotypically normal parent who has a low level of mosaicism but in most cases the parents' chromosomes are normal. The observed recurrence risk for young parents in this situation is accepted to be 1% (Daniel et al. 1982).

1.2 Nondisjunction

1.2.1 Mechanisms producing standard trisomy

Standard trisomy 21, as opposed to translocations and mosaicism, is found in about 95% of Down's syndrome cases. Possible mechanisms resulting in the presence of three copies of chromosome 21 in an individual include asynapsis (failure of homologous chromosomes to pair), desynapsis* (chromosomes fall apart in the absence of chiasma formation), precocious separation (early separation of chromosomes after chiasma formation has been completed) and nondisjunction (failure of homologous chromosomes to separate during the first meiotic division or failure of chromatids to separate during the second meiotic division). Another possible mechanism leading to numeric chromosome abnormalities is loss of single chromosomes, presumably due to "anaphase lagging" : during anaphase movement, one chromosome may lag behind the others and fail to segregate properly at cell division, leading to aneuploidy.

All of these errors could result in the germ cell retaining both copies of the chromosome 21 pair, such that the gametic chromosome number is 24 instead of 23. Since it is not yet possible in man to distinguish between these different types of aberrant chromosomal behaviour by observation of the end product of meiotic division, they are all grouped, for convenience, under the general term nondisjunction.

*- It should be noted that desynapsis can be excluded as a possible mechanism in cases where recombination between nondisjoined chromosomes can be demonstrated.

1.2.2 Parental Origin of Nondisjunction

Cytogenetic Studies

Following the advent of quinacrine fluorescence chromosome banding techniques (Caspersson et al. 1970), the heritable variations in staining pattern of the short arm of chromosome 21 have been used successfully to follow the inheritance of this chromosome in Down's syndrome families and to determine the parental origin of the extra chromosome. Up until 1983, a total of 369 families had been examined using this technique. The results of these studies are summarised by Juberg & Mowrey (1983), who showed that in 80% of cases the additional chromosome 21 was of maternal origin. When maternal, nondisjunction occurred at the first meiotic division in 77% and at second meiotic division in 23% of cases. Thus in approximately two-thirds of Down's syndrome families, nondisjunction takes place at the first maternal meiotic division. The fact that in 20% of cases the additional chromosome was derived from the father helped to disprove the commonly held theory at the time that Down's syndrome was exclusively attributable to the mother. When of paternal origin, nondisjunction took place at meiosis I in 58% of cases, and at meiosis II in 42% of cases.

It should be noted, however, that determination of the meiotic stage of nondisjunction in Down's syndrome families using heteromorphisms associated with 21p is not completely reliable. Three main technical problems limit their overall usefulness. First, in the majority of cases, analysis is

based on subjective evaluation of the size and staining intensity of the chromosomal heteromorphisms. Even when the scoring of different variants is done blindly, and by independant observers, it is possible that a small proportion of parental origin assignments will be wrong. Secondly, cytogenetic analyses of Q-banding heteromorphisms are informative in only 50% of cases. When combined with the NOR-silver staining technique, this figure rises to 80% (Mikkelsen et al. 1980), but this still leaves one fifth of cases in which the origin of nondisjunction cannot be determined. Thirdly, recombination between the centromere and the short arm markers could lead to errors in the localisation of nondisjunction to a specific meiotic division.

1.2.3 Parental Origin of Nondisjunction

Use of Polymorphic DNA Markers

Polymorphic DNA markers are those which allow the detection of restriction fragment length polymorphisms (RFLPs). These are heritable variations in DNA sequence detected by differences in the band pattern shown by a particular cloned DNA probe in Southern blot experiments (Botstein et al. 1980). They represent codominant heritable markers and can be used to follow the inheritance of a particular chromosome or chromosomal region. Consequently, any polymorphic DNA probes isolated from chromosome 21 can be used to follow the inheritance of that part of the chromosome

within Down's syndrome families, thereby allowing the determination of the parental origin and in some cases the meiotic stage of nondisjunction.

The use of RFLP markers overcomes two of the problems inherent in the cytogenetic analysis of chromosome heteromorphisms mentioned previously. Firstly, the detection of allelic variation using RFLPs is objective and it is clear from the relative dosage of the bands which chromosome is present in more than one copy in the DNA of the child. Secondly, the potential availability of a large number of polymorphic DNA markers from chromosome 21 should allow the origin of the additional chromosome to be determined in most, if not all, cases.

One problem common to both cytogenetic and RFLP studies of the origin of nondisjunction is that of recombination. Crossing-over can occur between the centromere and the polymorphic DNA markers on the long arm of the chromosome, and although this would not affect determination of parental origin of nondisjunction, it may lead to errors in the localisation of nondisjunction to specific meiotic division. The use of RFLP markers close to the centromere would obviously reduce the risk of this. It should also be possible, using a sufficient number of polymorphic DNA markers dispersed along the long arm of chromosome 21, to detect the frequency and position of recombination events at any given meiosis.

1.2.4 Parental Origin of Nondisjunction

Combined Use of RFLP and Cytogenetic Methods

It seems obvious from the foregoing discussion that the origin of nondisjunction in Down's syndrome families could be analysed by a combined study of both cytogenetic heteromorphisms and polymorphic DNA markers (including at least one from the pericentromeric region). In this way the centromere could be "sandwiched" between polymorphic markers on the short and long arms. If these were concordant, then the parental origin and meiotic stage of nondisjunction could be stated with a high degree of confidence.

1.2.5 Possible Effects of Aberrant Levels of Recombination on Nondisjunction

In species with chiasmate meioses, maintenance of pairing at meiosis I is presumably dependant on the formation of at least one chiasma per bivalent (White 1973). Thus, reduction or elimination of crossing-over may lead to univalent formation, and ultimately to nondisjunction. There is some evidence from experimental animals (Henderson & Edwards 1968, Sandler 1981) to suggest that this might be an important source of trisomy. Furthermore, if multiple exchanges impede the normal separation of bivalents at anaphase I, abnormally high levels of recombination might also be associated with nondisjunction.

It is now possible to test these suggestions directly in humans using the technique of centromere mapping

(Ott et al. 1976) to compare the frequency of crossing-over in normal meioses and meioses resulting in trisomy. Chromosome heteromorphisms and/or pericentromeric RFLPs are used to determine the meiotic stage of origin of the additional chromosome. Distances from the centromere to the RFLP markers are then estimated by evaluating the level of heterozygosity among trisomic RFLPs for which the parent of origin is known to be heterozygous. In trisomy of meiosis I origin, increasing homozygosity is directly proportional to increasing centromeric distance; in trisomy of meiosis II origin the converse is true.

The combined use of pericentromeric RFLP and cytogenetic markers with more distal RFLP markers on 21q in this way should allow levels of recombination on nondisjoined chromosomes 21 to be estimated. Comparison with chromosomes involved in normal meioses should allow the possible effects of aberrant recombination on nondisjunction to be evaluated.

1.3 Aetiological Factors

Despite years of intensive study, advanced maternal age remains the only risk factor conclusively associated with nondisjunction in Down's syndrome. The failure to identify important risk factors may be due to the fact that researchers have failed to study the appropriate aetiological agents or populations at risk. Another possibility is that studies to date have been badly designed. One of the major problems associated with studies of nondisjunction is that the populations studied are intrinsically heterogeneous, and each of the four types of meiotic error (maternal/paternal, first/second division) may be caused by different environmental and stochastic factors. This situation should be resolved by the objective determination of parental origin and meiotic stage of nondisjunction in a large, coordinated study of Down's syndrome families. The risk factors for each type of meiotic error could then be analysed independantly. A discussion of the maternal age effect is presented below, and is followed by a brief summary of other proposed risk factors.

1.3.1 Maternal Age

The frequency of Down's syndrome births increases with maternal age, and as the risk shows an exponential increase in the mid 30's, prenatal diagnosis is usually recommended to all pregnant women over the age of 35. The most popular theory put forward to explain the higher

incidence of nondisjunction in older mothers is the "older egg" model. This refers to the cycle of meiosis in female mammals, which begins shortly before birth or in early postnatal life (Franchi et al.1962). Following the leptotene, zygotene, pachytene and diplotene phases, the resting stage of dictyotene is reached. The oocyte remains in this condition until its final maturation at the time of formation of the Graafian follicle. The first meiotic division is then completed with the extrusion of the first polar body. The human female reproductive capacity lasts from about the ages of 15 to 50 years and the oocytes remain in the dictyotene stage during this period. It is thought likely that harmful effects of the environment or deterioration of the nucleus may occur during this period. leading to aberrant segregation of the chromosome 21 pair and subsequent aneuploidy.

Given that the majority of nondisjunction errors occur during maternal meiosis I (see section 1.2.2), it is expected from this model that the the ratio of maternally to paternally derived cases (the M/P ratio) should increase very sharply with advancing maternal age. This is apparently not the case, however, as approximately 80% of nondisjunction errors involving chromosome 21 occur in maternal meiosis I, regardless of maternal age, and the M/P ratio changes very little with maternal age (Stein et al. 1986). These observations suggested that a mechanism other than age-related deterioration of the nucleus might be involved, and led to the proposal of the "relaxed selection" hypothesis

(Erickson 1978, Sved & Sandler 1981, Ayme & Lippman-Hand 1982). This theory states that older mothers lose the ability to reject trisomy 21 conceptuses, resulting in a higher incidence of liveborn infants with Down's syndrome.

All of the experimental evidence for an association between advanced maternal age and "relaxed selection" against trisomy 21 embryos is derived from the use of cytogenetic heteromorphisms on 21p to determine the parental origin of the additional chromosome 21. The possible errors inherent in this method (see section 1.2.2) may affect the results obtained in such studies. Indeed, other workers have expressed reservations about certain aspects of the data and methods quoted in support of the "relaxed selection" theory (Carothers 1983, Warburton 1983).

The available data do not allow a clear distinction between the "older egg" and "relaxed selection" models. It is possible that both mechanisms are involved. The resolution of this situation will involve a large, coordinated study, using both cytogenetic and polymorphic DNA markers to determine with confidence the origin of nondisjunction in Down's syndrome families (see section 1.2.4).

1.3.2 Other Candidate Risk Factors

Cytogenetic and epidemiological studies have identified many candidate risk factors including extraneous ones such as oral contraceptives (Harlap 1979), fertility drugs (Boue & Boue 1973) maternal irradiation (Uchida 1979),

cigarette smoking (Kline et al. 1983) and alcohol (Kaufman 1983). Some intrinsic risk factors have also been proposed, such as the presence of anti-thyroid autoantibodies (Fialkow et al. 1971), sharing of HLA antigens among parents (Mottironi 1983), and increased frequency of satellite associations between acrocentrics (Jacobs & Mayer 1981). Clear evidence of an association with these factors and the occurrence of Down's syndrome is still lacking in each case.

Some models have also been proposed involving specific genes which promote nondisjunction. One such theory suggests that an autosomal recessive gene which causes meiotic nondisjunction in a homozygous parent may be responsible (Holmes 1978). Another postulates the existance of a gene which induces mitotic nondisjunction in the homozygous fertilised ovum (Alfi et al. 1980). Direct evidence for both theories is currently lacking, however.

1.3.3 Recurrence Risk of Down's syndrome

Another possible factor involved in the aetiology of Down's syndrome is that there appears to be a tendency for more than one case of Down's syndrome to occur in certain families. As mentioned previously (section 1.1.5) the recurrence risk for this condition in non-mosaic couples with one affected child is approximately 1% (Daniel et al. 1982). The birth of a child with Down's syndrome raises the risk of having another trisomic child, whether or not chromosome 21 is involved.

It is also interesting that the risk to second degree relatives also seemed to be increased beyond that of the general population. A study of such individuals (Tamaren et al. 1983) suggested a risk of 0.67% of having a Down's syndrome child in the second degree relatives of such patients. Two more recent studies (Eunpu et al. 1986, Abuelo et al. 1986) dispute this finding, however, and state that the risk factor for second-degree relatives is not greater than in the general population.

These results suggest that some inherited factor may be involved in the aetiology of Down's syndrome. It may be the case that all chromosome 21 centromeres are not equal, and a predisposition to nondisjunction might then exist in certain individuals. This theory could be tested using polymorphic chromosome 21-specific DNA markers (see section 1.2.3) to determine whether a particular pericentromeric haplotype of RFLP markers might be associated more often than expected with nondisjoined chromosomes 21.

1.4 Molecular Analysis of Chromosome 21

Chromosome 21 is the smallest autosome, comprising only 1.7% of human DNA* (HGM 9, 1988). At the outset of this project, only 12 cloned genes and 9 anonymous DNA sequences had been mapped to this chromosome (HGM 7, 1984). The availability of chromosome specific recombinant DNA libraries and advances in the field of somatic cell genetics, however, would allow the rapid isolation and regional localisation of numerous cloned DNA segments for this chromosome.

Much of the interest in chromosome 21 can be attributed to its association with Down's syndrome and the fact that isolation of cloned DNA segments from the critical region of this chromosome allows the pathogenesis of the disease to be analysed at the molecular level. One of the ultimate aims of molecular genetic investigation of chromosome 21 is that it may eventually lead to the elucidation of molecular events leading to meiotic nondisjunction and consequent trisomy.

Many DNA sequences isolated from chromosome 21 detect RFLPs (almost 35% - see HGM 9, 1988) and therefore represent useful genetic markers. In addition to the analysis of nondisjunction in Down's syndrome families, these probes could be used to construct a genetic linkage map of chromosome 21, and to demonstrate linkage or non-linkage with genetic disease loci of unknown chromosomal origin.

Other possible uses for chromosome 21-specific DNA sequences, whether or not they detect RFLPs, are in (i) the isolation of genes from chromosome 21 by screening cDNA

(* - this corresponds to approximately 51 megabases of DNA)

libraries with cloned chromosome 21-specific DNA sequences (e.g Neve et al. 1986); and (ii) in chromosome "walking" and "jumping" experiments. Starting from an original cloned DNA sequence, these techniques essentially involve the isolation of either overlapping/flanking clones ("walking" - Steinmetz et al. 1984) or clones at some distance from the original one ("jumping" - Collins & Weissman 1984, Poustka et al. 1987). This means that DNA sequences not closely linked to an original clone can lead to the isolation of genes and/or sequences important in the pathogenesis of inherited disorders.

1.4 Aims of the Current Project

The general aim of this research project was the isolation of polymorphic DNA sequences from chromosome 21 which could be used to study the parental origin of nondisjunction in Down's syndrome families and in molecular genetic analysis of this chromosome. Specifically, the aims were as follows.

1. Screening of Chromosome 21-Specific Recombinant DNA Libraries

Clones containing single- or low-copy number human DNA sequences would be isolated from flow sorted chromosome 21-specific libraries.

2. Mapping of Sequences to Chromosome 21

Human DNA sequences would be mapped to chromosome 21 and regionally localised using a panel of somatic cell hybrids containing different translocation derivatives of the chromosome.

3. Testing of Cloned Probes for the Detection of Restriction Fragment Length Polymorphisms (RFLPs)

Chromosome 21-specific DNA probes would be tested for the ability to detect frequent RFLPs by hybridisation to DNA samples from different individuals digested with a range of restriction enzymes.

4. Analysis of Nondisjunction in Down's Syndrome Families

Polymorphic chromosome 21-specific DNA probes would be used in conjunction with similar probes provided by other groups and with the analysis of Q-banding heteromorphisms to determine the parental origin and, where possible, the meiotic stage of nondisjunction in Down's syndrome families. The use of RFLP markers overcomes some of the problems inherent in previous, cytogenetic studies of nondisjunction and the effectiveness of the two methods could be compared.

5. Analysis of Recombination Levels in Chromosomes 21 Undergoing Nondisjunction

Studies on animal models have indicated that aberrant levels of recombination between homologous chromosomes may be important in nondisjunction. As part of the study of Down's syndrome families, the inheritance patterns of different RFLP markers would be studied to determine whether recombination on nondisjoined chromosomes 21 could be detected.

6. Molecular Genetic Map of Chromosome 21

It was also hoped that the isolation and regional localisation of chromosome 21-specific DNA sequences during the current project would significantly augment the molecular map of chromosome 21 , which contained only 9 anonymous DNA sequences when this project began. Any probes detecting RFLPs could potentially be incorporated into a genetic linkage map of the chromosome.

7. Testing of DNA Sequences for Expression using the Northern Blot Technique

Chromosome 21-specific DNA sequences isolated during the current project would be tested in Northern blot experiments to determine whether or not they were expressed sequences. If any of the sequences localised to the Down's syndrome critical region were expressed, it is possible that they may represent genes directly involved in the pathogenesis of the disorder.

CHAPTER TWO

MATERIALS & METHODS

2.0 General

All aqueous solutions were prepared in water deionised by a Millipore "Milli-RO 15" water purification system. If required, solutions were sterilised in a pressure cooker at 15lbs/in^2 for 15-20 minutes.

Glassware, plastics and tips for micropipetting were sterilised in a Sybron/Drayton Castle industrial autoclave for 20 minutes and dried in an 80½C oven for 1-2 hours prior to use.

Plastic tubes (13ml, 30ml, 1.5ml and 0.5ml) were supplied by Sarstedt, as were all tips for micropipetting. Plastic universal (30ml) and Bijoux (7ml) bottles were Sterilin types 128B and 129C, respectively. Micropipettes were Gilson "Pipetman" type. Petri dishes were Nunc (220cm square plates, 13cm and 9cm) or Sterilin (9cm).

Centrifugation was carried out in a Sorval RC-5B refrigerated centrifuge or OTD-65 ultracentrifuge, an Eppendorf or Damon micro-centrifuge. Rotors are specified in the text.

Practical details and applications of many recombinant DNA techniques can be found in Maniatis et al. (1982).

2.1 Reagents

100x Denhardts Solution

2% Ficoll

2% Bovine serum albumin

2% Polyvinylpyrrolidone

20xSSC (Standard Saline Citrate)

for 5 litres:

441g Tris.HCl (pH7.2)

877.5 ml NaCl

TE

10mM Tris.HCl pH8.0

1mM Na EDTA pH8.0

Bacteriophage dilution buffer

0.1M Tris.HCl pH8.0

0.1M $MgSO_4$

0.1% Gelatine (w/v)

"E" buffer (Electrophoresis buffer)

40mM Tris-acetate pH8.0

20mM Sodium or potassium acetate

1mM Na EDTA

L-broth (per litre)

10g Bacto-tryptone

5g Bacto-yeast extract

10g NaCl

0.1% Maltose

10mM $MgSO_4$

Maltose : 20% stock solution, filter sterilized

Bottom agar : 15g of agar was added per litre of L-broth

Top agar : 7g of agar was added per litre of L-broth

Agar was melted by autoclaving in a pressure cooker, and cooled to approximately 45°C before use.

Ampicillin

Stock solution of 25mg/ml was prepared by dissolving the appropriate amount of powdered ampicillin in distilled water. This solution was stored at -20°C.

X-Gal (5-Bromo-4-Chloro-3-Indoyl-beta-D-Galactopyranoside)

Stock solution was prepared by dissolving 100mg X-Gal in 5ml dimethylformamide. This solution was stored at -20°C in a light-tight container.

Formamide

Formamide was deionised by stirring at 4°C for 4-5 hours with approximately 5g of Bio-Rad mixed bed resin (20-50 mesh). It was subsequently stored at -20°C.

Phenol

Phenol was distilled in this department using Quickfit distillation apparatus. It was stored at 4°C until required. 8-hydroxyquinoline was added to give a yellow colour, which, if lost, would indicate oxidisation of the phenol.

2.2 Hybrid Cell Lines used in the Regional Localisation of DNA Sequences to Chromosome 21

A brief description of each cell line is given below, together with an explanation of how they can be used to map DNA sequences on the chromosome. Schematic representations of the parts of chromosome 21 found in the various hybrids are shown in the Results section, Figures 2, 6, 8 and 11.
(Note - to facilitate interpretation of results obtained using DNA samples from the panels in Figures 6 and 8, additional, unbound copies of these Figures can be found inside the back cover of this thesis)

THYB133R is a derivative of THYB133 (Goodfellow et al. 1980) obtained from Dr.C.Bostock and contains chromosome 21 as its only detectable human chromosome against a mouse background. Any human sequences which hybridise to DNA from this cell line can therefore be assigned to chromosome 21.

AlWBf2 was constructed from fusion of human lymphocytes from a female carrying a 12;21 transloaction with Chinese Hamster A3 cells. Although a few other human chromosomes are found in this hybrid, the only chromosome 21 material present is the 21q21-qter region. Sequences from chromosome 21 which hybridise to the DNA of this cell line must therefore be derived from this part of the chromosome.

AlWC6 contains the reciprocal product of the 12;21 translocation present in AlWBf2, that is, the region 21pter-21q21. DNA clones can thus be mapped to this part of the chromosome using DNA from this hybrid cell line. Cell lines AlWBf2 and AlWC6 were obtained from Prof.P.Pearson.

AHVI-17 was constructed by fusion of mouse A9 cells with a human fibroblast line (GM1881; Camden Human Genetic Mutant Cell Repository) which carries a reciprocal balanced 1;21 translocation. This hybrid cell line contains the 21pter-21q22.1 region of chromosome 21 as part of the 1;21 translocation chromosome. Although neither the reciprocal part of the translocation nor the normal chromosome 21 are found in this hybrid, it does contain a number of additional human chromosomes. Chromosome 21 sequences hybridising to the DNA of this cell line can be assigned to the region 21pter-21q22.1; lack of hybridisation would consequently assign the sequence to the region 21q22.1-21qter. This cell line was obtained from Prof.D.Cox.

BW5147 (Hyman and Stallings 1974) is a mouse cell line, obtained from Dr.P.Goodfellow. It was included in the panel to ensure that hybridisation of probes to the hybrid cell line DNA samples was due to hybridisation to sequences on chromosome 21 and not a consequence of homology with murine DNA. Given the similarities between human trisomy 21 and mouse trisomy 16 (Epstein et al. 1985), it would also be

interesting to determine whether any of the probes isolated here did share any homology with murine DNA sequences.

Further details on the five cell lines mentioned above, including photographs showing the derivative chromosomes where appropriate, are given in Stewart (1984).

TrD$_2$

This is a human fibroblast cell line with the chromosome constitution 45XX,-21,+t(4;21)(p16.3,q21). One part of the reciprocal translocation is not present, and so this individual is essentially monosomic for the region 21pter-q21. This cell line was included to map probes by virtue of the relative signal dosage in Southern blot experiments between this hybrid and normal genomic DNA. This cell line was obtained from Dr.N.Carpenter.

72532X-6 is the result of a fusion involving Chinese Hamster ovary (CHO) and human cell lines. It contains chromosome 21 as its sole human chromosome. This hybrid and all hybrids mentioned subsequently were kindly supplied by Dr.D.Vannais/Prof.D.Patterson; further details on the construction and characterisation of these hybrids can be found in Patterson et al. (1985) and Van Keuren et al. (1986a,b).

153E7BX and 2Fu^{r}1 contain part of the long arm of chromosome 21 translocated to a Chinese Hamster chromosome. The former contains the region 21p11.1-21qter; it may also contain sequences above the p11.1 breakpoint, but analysis has so far failed to clarify this situation. The latter contains the 21q11.2-qter region. Neither hybrid contains any other human chromosomal material. DNA probes can be mapped to or excluded from these areas of the chromosome based on hybridisation or lack of hybridisation to the DNA of these cell lines.

R2-10 contains a ring 21 as its only human chromosome. It was constructed by fusion of a CHO mutant cell line deficient in GARS activity with a human cell line mosaic for a ring chromosome 21 with a breakpoint in q22.3. This hybrid contains the region 21p11.2-q22.3, and human DNA probes hybridising to the DNA of this line can be localised to this chromosomal region.

8q- contains the 8;21 translocation chromosome 8pter-q22.1::21q22.3-qter. The only part of chromosome 21 present, therefore, is the terminal part of band q22.3. Any DNA probes which hybridise to the DNA of this cell line must be derived from the terminal part of the q22.3 band.

ACEM2-90 was derived from fusion of Chinese Hamster cells with those of a Down's syndrome patient carrying a 21;21 translocation (Bradley et al. Trisomy 21, in press). This translocation chromosome is thought to have arisen from a series of complex rearrangements and contains the regions 21cen-21p11.2 and 21q22.1-qter.

Normal human and trisomy 21 DNA samples were included in the mapping panels. It was hoped that increased dosage of hybridisation signals would be evident between normal and trisomy 21 samples. As the same amount of DNA from each was added to the gel, this effect would be due to the extra copy of chromosome 21 in Down's syndrome individuals, and would support the localisation of DNA probes to chromosome 21.

HD4 contains the reciprocal product of the 8;21 translocation present in the 8q- cell line, and therefore contains all of chromosome 21 except the terminal sub-band 21q22.3. Any DNA probes failing to show hybridisation to DNA from this cell line can be mapped to the 21q22.3 region.

It should be noted that "8q-" and "HD4" are convenient abbreviations for these cell lines, their correct names being 13b1S816-10-3 and 21-8-Ab5-23a, respectively, as described by Van Keuren et al. (1986b).

2.3 Methods used in the Plating Out and Screening of the Recombinant Bacteriophage Libraries

2.3.1 Preparation of plating bacteria

The bacterial strain used was E.coli LE392. A single colony picked from an L-agar plate or 10µl taken from a glycerol stock was innoculated into a 50ml L-broth (see general section) in a 250ml conical flask. This culture was incubated with shaking overnight at 37°C in an orbital shaker (New Brunswick Scientific). To aid the absorption of bacteriophage lambda to the host bacteria, 0.2% maltose was added to the culture medium.

The cells were spun down in plastic universal containers at 2,500rpm for 15 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in sterile 10mM $MgSO_4$ to give an $O.D._{600}$ of 2.0, which represents approximately 1.5×10^9 cells per ml. The bacterial suspension was stored at 4°C and could be used for up to two weeks. The highest plating efficiency was obtained with fresh cells, however. Glycerol stocks were made following the simple procedure described in section 2.4.9.

2.3.2 Plating and titration of the libraries

In order to determine the titre of the library, ten-fold serial dilutions were prepared in phage dilution buffer in a final volume of 1ml in 1.5ml Eppendorf tubes. The suspension was thoroughly mixed before 0.1ml of each dilution

to be assayed was dispensed into sterile bijoux containing 100µl of plating bacteria. The mixture was vortexed and incubated for 20 minutes at 37°C to allow the bacteriophage particles to adsorb. Molten top agar (about 5mls) was added to each of the bijoux bottles, and immediately poured onto labelled plates containing 25-30ml of solid bottom agar. The plates were left standing half covered to allow the top agar to dry without producing condensation on the lids of the petri dishes. When the top agar had set, the plates were inverted and incubated o/n at 37°C. The number of plaques on each dilution plate was counted, and the titre of the original suspension was calculated. The libraries used in this study were found to be in the range 2 - 6 x 10^{10} pfu/ml.

2.3.3 Screening of the Recombinant Libraries

Two different recombinant libraries were used in the present study. These were screened using different strategies, as outlined below. Methods common to the screening procedures used with both libraries will be discussed first, followed by a description of the differences between the two methods.

2.3.4 Plating of the Libraries for Screening

For initial screening, the libraries were plated out at a sufficiently high density to cover chromosome 21 at least three times. Given the insert sizes of the libraries used here, this was found to correspond to approximately

10,000-20,000 plaques. These were plated out on large (220cm square) plastic culture plates. The procedure used for plating of the libraries was the same as that described previously for titration of the library, except that the number of phage used initially was calculated to give the desired number of plaques on the plates.

After o/n incubation the plates were placed at 4°C for 10-15 minutes to harden the top agar. This prevents the top agar peeling off when it comes in contact with the nylon membrane (Hybond-N, Amersham). The filter was placed carefully on the surface of the plate so that it came into direct contact with the plaques, which quickly transferred onto the filter. Both filter and plate were marked at the same locations using an ink marker (Pentel marker, black ink, types N50 or MS50 - this is the only ink not removed during the hybridisation procedure). After 30-60 seconds the filter was peeled off using blunt-end forceps and immersed, DNA side up, in a shallow tray of denaturing solution (1.5M NaCl, 0.5M NaOH) for 30-60 seconds. The filter was then transferred to neutralising solution (1.5M NaCl, 0.5M Tris.HCl pH8.0) for 5 minutes. The filter was finally rinsed twice in 2xSSC and placed on Whatman 3MM paper to dry. Subsequent impressions of the same plate were left on the plate about 30 seconds longer, or until the filter was completely wet. When dry, the filters were wrapped between sheets of Whatman 3MM paper and baked for five hours at 80°C in a vacuum oven to fix the DNA to the filter.

2.3.5 Prewashing and washing conditions

After baking, the filters were placed in heat-sealed plastic bags containing approximatley 100ml of prewashing solution (50mM Tris.HCl pH 8.0, 1M NaCl, 1mM EDTA, 0.1% SDS). The filters were incubated with gentle shaking, for 2 hours at 42°C. The prewashing solution removes from the filters any fragments of agarose or bacterial debris. The pre-hybridisation, hybridsation and posthybridisation conditions were as described below for gel filters.

2.3.6 Picking Plaques

Following the initial selection of plaques according to the screening method used, they were subjected to several purification steps. These differed for the two libraries, but the main practical points of the technique are the same, and will now be described.

Bacteriophage can diffuse condiserable distances across the layer of top agar, and so to avoid cross-contamination between neighbouring plaques, a low density of bacteriophage was plated out for such purification steps. for the same reason, this procedure was carried out as quickly as possible after plating. An effort was made to choose plaques that were well separated from each other. After the films had been exposed and developed, they were aligned with the plates, and individual plaques were picked. The plaques were picked using a glass Pasteur pipette (Bibate) with a rubber bulb. The fragments of agar were washed into 400µl of

bacteriophage dilution buffer. This was put into a 500µl Eppendorf tube and a drop or two of chloroform was added. The tubes were shaken for 30 minutes at 37°C in an orbital shaker to allow the phage particles to diffuse from the agar. These tubes were then stored at 4°C indefinitely.

2.3.7 Making High Titre Stocks

High titre stocks from those phage to be studied further were made as follows. They were plated out at a sufficiently high density to give confluent lysis, and therefore maximum yield of phage. After o/n incubation, 5ml of SM (per litre: 5.8g NaCl, 2.0g $MgSO_4$, 50ml 1M Tris.HCl pH 7.5, 5.0ml 2% gelatin) was added to each plate. The plates were then shaken gently at 4°C for several hours. As much as possible of the SM was recovered with a plastic pipette and put into a 13ml tube. A further 1ml of SM was added to the plate to rinse the surface, and the plate was stored for 15 minutes in a tilted position to allow all the fluid to drain into one area. The SM was again collected and added to the first harvest. The plates were discarded and 0.1ml of chloroform was added to each tube. These were then vortexed briefly and centrifuged for 10 minutes at 4,000rpm at 4°C. The supernatant was transferred to a fresh tube, chloroform was added to 0.3% and, after mixing the suspension very well, the stocks were stored at 4°C.

2.3.8 Selection Strategies : (i) Flow Sorted Chromosome 21 Library of Young et al. (1983)

The flow sorted chromosome 21-specific library obtained from Dr.B.D.Young was cloned into the Eco RI site of the bacteriophage vector lambda gt.WES/lambda B. The library was estimated to contain 60% chromosome 21 and 40% chromosome 22 sequences (B.D.Young, personal communication). This library was screened using the following method, which was intended to select for recombinants containing single-copy, expressed sequences.

Duplicate filter impressions from the initial, high density library plates were taken. One of these was prehybridised as normal, while the other was prehybridised in the presence of a large amount of unlabelled, sonicated human genomic DNA (final concentration 1mg/ml of prehybridisation solution). Both filters were then hybridised with a radiolabelled cDNA probe, prepared by reverse transcription (See section 2.6.4). The theory behind this was that the prehybridisation of filters with cold genomic DNA would "saturate" them, such that any clones containing repetitive DNA sequences would hybridise to this non-labelled DNA, and would therefore be unavailable for hybridisation to the labelled cDNA probe.

The mRNA from which the cDNA was prepared came from the liver of a Down's syndrome foetus. The reasons for using this were as follows: (i) any genes important in Down's syndrome would be present in more copies, due to the presence

of the additional chromosome 21, (ii) fetal tissue was used because genes important in the development of Down's syndrome may be expressed in the early stages of fetal life and "switched off" thereafter, (iii) use of liver tissue would increase the probability of isolating genes important in many biochemical pathways, since a great number of enzymes are expressed in these tissues, and (iv) cDNA was used to allow protein-coding genes to be isolated, since these are more likely to be directly involved in the pathogenesis of this disorder.

Plaques which hybridised to cDNA and which showed similar signal intensity in the autoradiographs from both filters, despite the competition with cold human DNA, were selected for further study. These were then picked as described in section 2.3.5 and then subjected to a purification procedure which involved repeating the steps of plating out, preassociation with cold genomic DNA and hybridisation with labelled cDNA. This was done four times to ensure that the plaques were pure and contained DNA from only one recombinant bacteriophage. Bacteriophage DNA was then prepared as described in Section 2.3.9.

2.3.9 Selection Strategies : (ii) Flow Sorted Chromosome 21 Library from the Lawrence Livermore Laboratory

A much simpler selection system was used with this library. Basically, impressions were taken from the initial plating out of the library. These were prehybridised as

normal (i.e. without cold human DNA) and then hybridised with labelled total genomic DNA. It was hoped that any plaques containing repetitive human DNA inserts would hybridise with the genomic DNA. Plaques which did not hybridise with genomic DNA were picked, in the hope that they would contain low- or single-copy DNA inserts. They were then purified by two rounds of plating out and picking a single plaque at random, without further hybridisation/ autoradiography. A high titre stock was then made from each candidate single-copy plaque. Bacteriophage DNA was prepared from each of these as described in the following section.

2.3.10 Preparation of Bacteriophage DNA

A liquid lysate method was used for the preparation of bacteriophage DNA. This was adapted from the method of Cameron et al. (1977). Two different versions of the same method were used, a large (500ml) and a small (5ml) scale. For the initial preparation of DNA from plaques of unknown content, the small scale method was used. The larger prep. was used for making bulk stocks of DNA for clones of known value. For convenience, only the former is described here. The protocol can be altered for larger or smaller scale DNA preparation by altering the amounts pro rata.

Large Scale Bacteriophage DNA Preparation

An aliquot of plating cells (Section 2.3.1) was incubated with the appropriate volume of bacteriophage. For a

500ml culture, 10mls of plating bacteria were used (an overnight bacterial culture typically contains 2.0×10^9 cells/ml, this volume thus represents 2.0×10^{10} cells). A ratio of 1:200 (bacteriophage : bacterial cells) was found to be optimum for this preparation, and so 10^8 bacteriophage were added to the bacterial cells in a universal. The mixture was incubated at 37°C for 15-20 minutes to allow the bacteriophage to adsorb, added to 500mls of prewarmed L-broth, and incubated at 37°C with shaking overnight.

Lysis was evident by the presence in the culture of "stringy" pieces of bacterial debris. The culture was split between two 250ml plastic tubs. (Further steps in the protocol refer to individual tubs, until the samples are pooled). DNase was added to a final concentration of 5µg/ml and the mixture incubated at 37°C for 1-2 hours. Addition of 40mls of TES (0.3M Tris.HCl pH9.0, 0.15M EDTA, 1.5% SDS) was followed by incubation at 70°C for 30 minutes. The mixture was cooled to room temperature and 50mls of 5M Potassium Acetate was added. The tubs were incubated on ice for 30 minutes and spun at 7K,4°C,20 minutes in an HS-4 rotor. The pellet was discarded and 80ml propanol added to the supernatant in a fresh tub. The mixture was spun at 7K,4°C for 30 minutes as above. The pellet was drained thoroughly and resuspended in TE buffer. At this point the pellets were pooled and transferred to 13ml plastic tubes.

Two volumes of cold (-20°C) ethanol were added and the tubes were placed at -20°C for 15-20 minutes. They were then

spun at 9K for 10 minutes in an SM24 rotor. The pellet was washed in 70% ethanol before repeating the last centrifugation step. The pellet was dried thoroughly and resuspended in 1ml of TE buffer. RNase was added to a final concentration of 100µg/µl and the mixture was incubated at 37°C for 1 hour. Proteinase K (50µl) and 10% SDS (50µl) were added, followed by incubation at 55°C for 1-2hours. The addition of 3mls of 1M Tris.HCl pH7.5 was followed by phenol extraction and ethanol precipitation. The final pellet was resuspended in approximately 1ml of TE buffer. The concentration of the DNA was measured by determining the OD_{260}, as described in section 2.5.5.

2.4 Subcloning of Recombinants into a Plasmid Vector

Once it had been established that the bacteriophage clones contained single- or low-copy number chromosome 21-specific sequences, it was decided that it would be more convenient to have them subcloned into a plasmid vector. The main reasons for this were - (i) it is easier to culture and prepare DNA from plasmid clones, and (ii) the maintenance of fresh stocks of plasmid clones is also relatively simple.

The chosen plasmid vector was pUC19, a derivative of pBR322 developed by Dr.J.Messing and co-workers (see Viera & Messing 1982). This plasmid has a useful multi-cloning site, and has ampicillin and tetracycline resistance genes. Recombinant clones can also be identified using a blue/white screening system, as outlined in section 2.4.5. Methods involved in the subcloning of human DNA inserts into the plasmid vector were as follows.

2.4.1 Production of Competent Cells

The strain of bacteria used for transformation was E.coli JM83. An aliquot of 10µl from a glycerol stock of these bacteria was inoculated into 5ml of prewarmed (37°C) L-broth in a 30ml sterile universal. The culture was incubated with shaking o/n at 37°C. From the o/n culture, 1ml was inoculated into 100ml of prewarmed L-broth and was grown to an $O.D._{600}$ of 0.6. This was reached in approximately 2 hours and represents about 6×10^8 cells/ml.

The cells were then spun down in chilled 30ml sterile plastic universals in an IEC Centra-7S benchtop centrifuge at 3,500rpm for 10 minutes at 4°C. The supernatant was poured off and each of the pellets was gently resuspended in 25ml of ice-cold 10mM $MgSO_4$ and kept on ice for 30 minutes. The cells were spun down as above and each of the pellets was resuspended in 12.5ml of ice-cold 50mM $CaCl_2$ and kept on ice for 15 minutes. The cells were finally spun down as above and each pellet was resuspended in 2ml of ice-cold 50mM $CaCl_2$. The pellets were pooled in one universal and stored at 4°C. The cells were used the day after they were made competent because the transformation efficiency increased six fold after being kept in the cold room o/n. After this the transformation efficiency decreased to the original level.

2.4.2 Preparation of Fragments for Subcloning

About 6µg of each bacteriophage DNA to be subcloned were digested in a 40µl volume (described in section 2.6.1). The reaction was stopped by addition of 5µl of 0.5M EDTA. This was followed by incubation at 70°C for 5 minutes. A 20µl aliquot of this reaction was run on a mini-gel (BRL "baby gel") to check that the DNA had been digested. If digestion was complete, the remainder of the digest mixture was diluted to 50µl with sterile distilled water. Sodium acetate was added to 0.2M. This was then extracted with 25µl of phenol/chloroform until no interphase was seen. (Note - the solution was not extracted with chloroform).

Following phenol extraction, 3 volumes of cold absolute ethanol was added to the aqueous phase and this was incubated at -30°C for 30-45 minutes and then at -70°C for another hour. The DNA was spun down in a microcentrifuge and the pellet washed with 100% of cold absolute ethanol. The pellet was finally taken up in 20µl of sterile water. The DNA concentration was usually found to be approximately 150ng/µl. If not used immediately for ligation it was stored at -20°C.

2.4.3 Ligation

Plasmid pUC19 (uncut and cut with Hind III) was commercially available from P & S Biochemicals. Ligations were performed in a 10µl volume containing 1µl 10x ligase reaction buffer, 1µl of 0.1x T4 ligase (both available from the Amersham M13 cloning kit), 1µl 10mM ATP, 1µl 50mM DTT, (both from Sigma) and a volume of vector pUC13 and digested DNA that would give approximately the same number of plasmid particles to recombinants. The reaction took place o/n at 14°C.

2.4.4 Transformation

Flat bottomed sterile bijoux bottles were chilled on ice before use. Keeping the bijoux on ice, the ligation was mixed with ice-cold DNA buffer (10mM Tris.HCl pH.7.5, 10mM $CaCl_2$, 10mM $MgCl_2$, filter sterilized) to a final volume of 100µl. The buffer was then mixed with 200µl of competent cells and the mixture was kept on ice for 25 minutes,

heat-shocked at 42°C for 2-3 minutes and kept at room temperature for 10 minutes. Prewarmed L-broth (0.7ml) was added to the above and incubated with shaking for 1 hour at 37°C in the orbital shaker. About 100µl of this mixture was spread on an Amp/X-Gal plate. The plates were left to incubate overnight and then stored at 4°C until colonies were picked.

The Amp/X-Gal plates were made as follows: 1.5% L-agar plates were made as normal. Ampicillin (Sigma, see general section) was added to the L-agar to a final concentration of 100µl/ml when its temperature was no more than 45°C. The solution was mixed well and the plates poured before the L-agar started to solidify. When the plates were set, 40µl of X-Gal (Gibco BRL, see general section) was spread on the surface of the plate. The plates were incubated at 37°C for 10 minutes until dry.

Three controls were carried out with every transformation:

(i) Competent cells were transformed with cut and religated pUC13 DNA to check the ligation procedure;

(ii) Competent cells were transformed with uncut pUC13 DNA to estimate the maximum obtainable transformation efficiency;

(iii) Competent cells were taken through the transformation procedure with no transforming DNA added to ensure that the cells were not contaminated.

2.4.5 Recombinant Selection

The bacterial host strain JM83, mentioned above, lacks part of the beta-galactosidase gene called lac Z and cannot synthesise this enzyme. This defect is complemented, however, by the presence of a lac Z' gene on the pUC series of vectors, including pUC19 used here. Cloning into such plasmids involves insertional inactivation of the laz Z' gene and recombinants are distinguished by their inability to synthesise beta-galactosidase. This is assayed during transformation by adding a lactose analogue, X-gal, which is broken down by beta-galactosidase to give a product which is deep blue in colour. If Xgal is added to the agar, along with ampicillin and an inducer of beta-galactosidase, such as isopropyl-thiogalactoside (IPTG)*, then non-recombinant colonies, the cells of which will synthesise beta-galactosidase, will be coloured blue. Recombinants with a disrupted lac Z' gene are unable to synthesise beta-galactosidase will therefore be white.

(* - N.B. E.coli stain JM83 are constitutive producers of IPTG, so there is no need to add this to the agar for this bacterial strain).

2.4.6 Picking Colonies and Small Scale Plasmid DNA Preparation

White colonies were picked using sterile toothpicks and innoculated into 3ml of L-broth/Amp in a sterile plastic universal. These were incubated at 37°C o/n in an orbital shaker. From these cultures, 1.5ml was transferred into Eppendorf tubes and spun down in a microcentrifuge for 10 minutes at room temperature. The cells were spun down and resuspended in 700µl of cold STET buffer (50mM Tris pH 8/0, 50mM EDTA, 8% sucrose, 5% Triton X-100). To this, 50µl of fresh, cold lysozyme (10mg/ml) was added and the tubes were left on ice for 5 minutes. They were then placed in boiling water for exactly 60 seconds and spun at full speed in the microcentrifuge for 20 minutes. The supernatant was transferred to a fresh Eppendorf tube and extracted with an equal volume of buffered saturated phenol/chloroform until no interphase could be seen. To the aqueous layer, sodium acetate (final concentration 0.2M) and isopropanol (0.6ml) were added. The tubes were then left for 30 minutes at -70°C, or o/n at -20°C. They were then spun down and the pellets washed in 95% cold ethanol. The tubes were inverted and left to drain on paper towels for 30 minutes. The DNA pellet was resuspended in 40µl of TE. Half of this was digested in a 40µl reaction volume using the restriction enzyme Hind III to check whether the ligation had worked and the recombinant of interest had been subcloned.

2.4.7 Large Scale Preparation of Plasmid DNA

To prepare a large amount of plasmid DNA, the method described above was simply scaled up. About 300ml of culture was routinely used. One extra step was added to this method by adding preboiled RNase to a concentration of 25µl/ml and incubating the mixture at 37°C for 60 minutes. This was followed by phenol extraction and ethanol precipitation.

2.4.8 Elution of DNA from Low Melting Point Agarose Gels

In order to isolate the insert from the plasmid DNA the following procedure was used. About 40µg of DNA was digested with *Hind* III in an 800µl reaction volume. The digestion was run on a 1% low melting point agarose (BRL) gel. The gel was stained with ethidium bromide, destained and photographed. The required band was cut out with a wide blade scalpel and placed in a preweighed 30ml plastic tube. This was reweighed and the weight of the gel slice was calculated. An equal weight of water was added and this was heated at 70°C to melt the agarose. This was incubated for at least 10 minutes at 37°C. Ice-cold phenol (saturated with 10mM Tris.HCl, pH7.5) was immediately added to it and the mixture vortexed. It was then spun in the Sorvall centrifuge using the SA 600 rotor, at 10K/4°C for 10 minutes. The supernatant was transferred to a fresh tube and the bottom layer was re-extracted after addition of an equal amount of sterile water. The samples were pooled and re-extracted until the interface was clear, to remove residual agarose. The aqueous

phase was extracted with sequential equal volumes of butan-2-ol to remove phenol and reduce the volume to approximately 100-200µl. This was transferred to an Eppendorf tube, ½ volume of 7.5M ammonium acetate and 3 volumes of cold ethanol were added. The DNA was allowed to precipitate at -20°C o/n. The precipitate was spun down at 10K/4°C for 10 minutes. The supernatant was removed and the pellet was washed with 100% cold ethanol and drained. Traces of ethanol were allowed to evaporate. The pellet was taken up in 50-150µl of water depending on the size of the pellet. A 5µl aliquot of the DNA was run on a gel to check for purity and concentration. The remainder of the DNA was stored at -20°C.

2.4.9 Storage Conditions for Plasmid Recombinants

From the 3ml o/n culture 1.5ml was transferred to a sterile plastic bijoux, 1.5ml of glycerol was added, and they were inverted several times to mix the contents. These 50% glycerol stocks were stored at -20°C and were used to inoculate new cultures. Stabs were also made, as follows. L-agar (6%) was melted and ampicillin added as described above for making L-broth/Amp plates (section 2.4.4). About 2-3ml of this was poured into sterile bijoux bottles and left to solidify. An overnight culture was grown by inoculating 10µl from the glycerol stock into 1ml of L-broth and incubating o/n at 37°C. A loopful was taken from this and stabbed into the medium in the vials, which were incubated at 37°C o/n and stored at room temperature, in the dark.

2.5 Culture and Preparation of Cells for DNA Extraction

2.5.1 Blood Samples (modified from Kunkel et.al. 1977)

Peripheral blood (5-25ml) was collected in 30ml sterile plastic universals containing 2ml of a 4.5% EDTA/ 0.7% NaCl solution. The bottles were inverted several times to ensure that the blood and EDTA solution were thouroughly mixed, as insufficient mixing could lead to coagulation of the blood, rendering the samples almost useless for the extraction of DNA. If the blood samples were not used for DNA extraction immediately, they were stored at -20°C. The samples were thawed when required by leaving them at room temperature for 2-3 hours. They were then mixed with 5 volumes of cold lysis buffer (0.32M sucrose, 10mM Tris.HCl, pH7.5, 5mM $MgCl_2$, 1% Triton X-100 (v/v)) and spun for 10 minutes at 6k,4°C in a Sorvall RC-5B centifuge, using an HS-4 rotor. The supernatant was removed and the pelleted nuclei were used for DNA extraction.

2.5.2 Lymphoblastoid Cell Lines

Established lymphoblastoid cell lines, transformed with Epstein-Barr virus, were grown in HAMS F10 (Gibco) + 10% fetal calf serum + 10% new born fetal calf serum + 1% penicillin/streptomycin (Gibco). When the culture reached about 10^8 cells, it was spun down; the cells were resuspended and washed in saline to remove any traces of serum.

2.5.3 Hybrid Cell Lines and Fibroblast Cell Lines

Fibroblast cells were grown in culture bottles in the same medium as for lymphoblastoid cell lines, until the monolayer was confluent. The cells were then trypsinised, tranferred to 30ml sterile universals and centrifuged at 10,000 rpm three times, washing with saline between spins.

2.5.4 DNA Extraction

The pelleted nuclei from the blood samples or the washed pellets from the cell lines desribed above were resuspended in 4.5ml of 0.075M NaCl/0.024M EDTA, pH8.0. Proteinase K (100µl) and 10% SDS (250µl) were added and the mixture was incubated at 37°C overnight, with gentle shaking. The sample was then transferred to a 13ml plastic tube and an equal volume of phenol/chloroform was added. This was mixed by inversion for 10 minutes and then spun at 10k for 10 minutes in a Sorvall RC-5B centrifuge, SM24 rotor at 25°C. The aqueous layer was transferred to a clean tube and the phenol extraction was repeated until the interface was clear of protein or other impurities. This usually involved 2 or 3 phenol extractions. An equal volume of chloroform was then added to the aqueous layer. The sample was again mixed and centrifuged under the same conditions. The final aqueous layer was transferred to a sterile 30ml plastic universal. Sodium acetate was added to a final concentration of 0.3M, and then 2 volumes of cold 100% ethanol were added to the mixture. Following inversion of the tube, the DNA

precipitated, and was "spooled out" using a glass Pasteur pipette with a heat-sealed end. The DNA was resuspended in TE buffer by gentle shaking over a period of 2 days at 4°C. The DNA was subsequently stored at 4°C.

2.5.5 Estimation of DNA Concentration

The concentration of the DNA samples was calculated by measuring the optical density at 260nm in a spectrophotometer (LKB or Pye/Unicam) as follows. A 10µl aliquot of the DNA was added to 990µl of TE buffer in an 1.5ml Eppendorf tube and mixed thoroughly by vortexing. The spectrophotometer was "zeroed" by measuring the $O.D._{260}$ of 1ml of TE in a quartz cuvette (Pye/Unicam). Since 1 $O.D._{260}$ unit corresponds to 50µg of DNA, and because the DNA was diluted by a factor of 100, the $O.D._{260}$ reading of the DNA sample was multiplied by 5000 to give the concentration in µg/ml.

2.5.6 DNA and mRNA Preparation from Foetal Liver

The tissue was removed and frozen immediately in liquid nitrogen, in order to prevent degradation of the nucleic acids present. The tissue was removed from liquid nitrogen and broken up into small pieces, then crushed to a powder and added to an appropriate volume depending on the size of the sample (typically 500ml) of guanidine thiocyanate solution (5M guanidine thiocyanate, 50M Tris pH7, 50mM EDTA, 5% beta-mercaptoethanol, sterilised by filtration). The mixture was shaken gently to dissolve the powdered tissue and

20% Sarcosyl was added to 0.2% final concentration. Both these chemicals prevent degradation of the tissue. The RNA/DNA was then prepared by ultra-centrifugation (Sorvall OTD-65 Ultra Centrifuge, AH627 swing out rotor) for 48-72 hours at 25K/25°C in a CsCl gradient. The correct gradient was achieved by adding 4.3ml of CsCl suspension (5.7M CsCl, 50mM EDTA, pH7) to 17ml polyallomer tubes.

After centrifugation, the DNA was collected (top layer) from the CsCl gradient, using a plastic pipette and was transferred to 30ml tubes where it was precipitated with 4-5 volumes of cold 70% ethanol. The DNA was spooled out using a heat-sealed Pasteur pipette and was resuspended in 0.075M NaCl, 0.024M EDTA, pH8.0, the volume depending on the size of the DNA pellet. Proteinase K (to 100µg/ml) and 10% SDS (to 0.5%) were added and this was incubated overnight at 37°C. The DNA was phenol and chloroform extracted (as described for blood samples) and precipitated with 0.3M sodium acetate and absolute ethanol. The pellet was washed in 70% ethanol, resuspended in TE (volume depending on size of the pellet) and finally stored at 4°C.

The RNA formed a translucent pellet at the bottom of the centrifuge tube. Approximately 100µl of filtered 8M urea was added and the pellet was resuspended using a 200µl Pipetman. Additional 8M urea was used if required. The suspension was transferred to Eppendorf tubes and 2 volumes of 4M Lithium Chloride was added to each tube. These were left at 4°C for several days to allow precipitation of the

RNA. It was then pelleted and resuspended in sterile distilled water. Sodium acetate to a final concentration of 0.3M and 2 volumes of 100% ethanol was added, the resultant suspension was then stored at 4°C.

2.5.7 Isolation of poly(A)$^+$RNA

When ready to isolate poly(A)$^+$ RNA (i.e. mRNA), the RNA was pelleted, dried, dissolved in warm (37°C) elution buffer (10mM Tris, pH7.5, 0.2%SDS). An oligo (dT) column was prepared by resuspending oligo(dT) cellulose in elution buffer and the column was poured in a Bio Rad glass column (11cm long). This was washed with 10ml of warm elution buffer and equilibrated in about 10ml of binding buffer (elution buffer + 0.4M NaCl). NaCl was also added to the RNA to a final concentration of 0.4M and the RNA was passed through the column. The effluent was collected and passed through the column twice more, to ensure that all the poly(A)$^+$ RNA was bound to the column, and finally collected in a poly A$^-$ tube. The column was washed with 3ml of binding buffer and collected into the poly A$^-$ tube. The poly(A)$^+$ RNA was eluted by passing 5 x 1ml of warm elution buffer through the column and collecting it in the poly(A)$^+$ tube. The column was washed with 3ml of binding buffer, which was also collected in the poly(A)$^+$ tube. The poly A$^-$ was again added to the column and passed over three times, so that any remaining poly A$^+$ RNA would be bound to the column. This was eluted by washing the column with warm elution buffer. A tenth volume of 4M NaCl

was added to the poly A^+ mixture and the binding and elution procedure was repeated once more. The final elution was done with 8ml of warm elution buffer. The $O.D._{260}$ of a 1ml aliquot of the poly A^+ mixture was measured in order to find the concentration. It was then lyophilised to approximately 4ml final volume. The RNA was ethanol precipitated and stored at -20°C. When required, a small aliquot was resuspended for reverse transcription, as described in Section 2.6.4.

2.6 Recombinant DNA Techniques

2.6.1 Restriction Enzyme Digestion

The basic digestion reaction was carried out in a 30µl volume which contained 4µl of 10x core buffer (BRL)*, 2µl of 0.1M spermidine, 2µl of the appropriate restriction enzyme (usually at 10 units/µl), and an appropriate volume of sterile distilled water, depending on the concentration of the DNA sample. Spermidine (Sigma) was used to improve the digestion of the DNA samples (see Bouche 1981). The amounts of DNA digested were typically 6µg from genomic samples, 10-15/g of DNA from hybrid cell lines and variable amounts for plasmids or bacteriophage clones, depending on their subsequent use. Digestions of genomic DNA samples and hybrid DNA samples were performed o/n at the required temperature for each enzyme in small Eppendorf tubes. Digests of plasmid or bacteriophage DNA were usually performed for 3-4 hours. Reactions were terminated by the addition of 5µl of "Stop" mix (50mM EDTA, 50% glycerol, 2% Ficoll, and 50µg/ml Orange G). Digested DNA samples were immediately loaded onto agarose gels, or sometimes stored at -20°C until further use. (As full listings of restriction enzyme recognition sites and reaction conditions are available elsewhere (Maniatis et al. 1982, for example), these are not presented here).

(* - Although most manufacturers supply specific buffers for use with their restriction enzymes, this buffer was found to give good results with all enzymes tested)

The choice of restriction enzymes for RFLP screening of recombinant probes (Results, Section 3.3) was largely dictated by consideration of the availability and cost of the different enzymes. Enzymes containing the CpG doublet in their recognition site have been shown to reveal RFLPs at a higher frequency than many other enzymes (Skolnick & White 1982) and as such the enzymes Taq I and Msp I were also included in the present study.

2.6.2 Gel Electrophoresis

Electrophoresis was carried out in 0.8% agarose (Sigma) gels in "E buffer" (see general section). Perspex electrophoresis tanks were prepared to our design perspex by a local supplier. LKB "Bromma/2197 constant power supply" power packs were used, typically at 200mA/3-4 hours for the 10cm x 13cm gels used. Following electrophoresis, gels were stained for 10 minutes in "E buffer" containing ethidium bromide (Sigma; final concentration 1µg/ml). The DNA was visualised on a UV transilluminator (UVP inc.) and photographed using a Polaroid CU-5 land camera fitted with a red filter, and a black and white Polaroid type 667 film.

2.6.3 Southern Blotting

After photographing the gel, it was treated with 0.25M HCl for 15 minutes to partially depurinate the DNA (see SWahl et al. 1979). This was followed by two successive 20 minute periods in denaturing solution (0.5M NaOH, 1.5M

NaCl) and two 20 minute washes in neutralising solution (0.5M Tris.HCl pH 7.4, 3M NaCl). The DNA was transferred to a nylon membrane (Hybond-N, Amersham) in 20xSSC, as described by Southern (1975). After 16-24 hours the filter was removed from the gel, washed in 2 x SSC, blotted dry and baked in an oven at 80°C for 5 hours.

2.6.4 Preparation of Radiolabelled Probes

1. Nick Translation (from Rigby et al 1977)

In some cases, DNA probes were labelled by nick translation using the Amersham nick translation kit. The reaction was performed in a 50µl volume. About 0.5µg of DNA was radiolabelled to a specific activity ranging from 15 to 85 x 10^6cpm/µg. The details of the method are as follows.

DNA was dissolved in a 5x buffer (Amersham nick translation kit) and water, when necessary. DNA polymerase (2µl) (also included in the kit) and 50µCi of [alpha-^{32}P]-dCTP (Amersham, 3000Ci/mmol) were added to each reaction. The reaction was incubated at 14-15°C for one hour. Labelled DNA was separated from unincorporated nucleotides by passage through a Sephadex G-50 column (medium type, Pharmacia; 9cm glass columns from Bio Rad), equilibrated with 1 x SSC, 0.1% SDS. The radiolabelled probe was collected as the first peak to emerge form the column. An aliquot was counted on an LKB 1215 Rackbeta liquid scintillation counter to allow the specific activity to be calculated.

2. Oligonucleotide Labelling (Feinberg & Vogelstein 1983)

The advantages of this method over nick translation are (i) it can be used to label DNA samples which are impure or at low concentration, and (ii) it can be used to label DNA cut from low melting point gels without prior elution. Details of the reagents used are as follows :

Oligo reaction mix

Firstly, the following four solutions are made up:

Solution A:	Solution B:
1.25M Tris.HCl pH8.0	1ml Solution A
0.125M $MgCl_2$	18µl beta-mercapto-ethanol
	5µl dATP,dGTP,dTTP
Solution C:	(each of these dissolved
2M Hepes, pH6.6	in TE at 100mM)

Solution D:

Hexadeoxyribonucleotides (Pharmacia, PL No. 2166) at 90 O.D. units per ml.

Solutions B, C and D are then mixed in the ratio 100:250:150 to give 10x reaction mix. Pure DNA samples were diluted to 50ng/µl for oligo-labelling, while for DNA/agarose samples, the fragment to be labelled was excised from LMP agarose, diluted with twice the weight of sterile water, and 20µl of this mixture was added to the reaction. DNA from either

source was boiled for 10 minutes, and then incubated at 37°C for at least another 10 minutes. This was mixed with 10µl of reaction mix (see below), 2µl of nuclease-free BSA (10mg/ml from Sigma), 1µl of Klenow fragment (Amersham), 2µl of [alpha-^{32}P]-dCTP (Amersham, (3000Ci/mmol) and 5µl of sterile water. The reaction was incubated at room temperature o/n, and then terminated by the additon of 50µl of stop mix (20mM NaCl, 20mM Tris.Cl pH7.5, 2mM EDTA, 0.25% SDS). As with nick translation, the DNA was separated from the unincorporated nucleotides on a G-50 Sephadex column.

3. Reverse Transcription of mRNA to cDNA

To prepare radiolabelled cDNA, 4-5µg of mRNA in ethanol was used. The RNA was spun down in an Eppendorf microcentrifuge for 5 minutes at full speed. The supernatant was discarded and the pellet was left to drain. It was then washed in 100% alcohol, spun down, again left to drain and put in an 80°C oven for 1-2 minutes to dry. The RNA was then resuspended in 32µl of water and heat-denatured for 5 minutes at 70°C. It was cooled on ice and spun down briefly before the addition of : 5µl 1M Tris.HCl pH8.3, 1.3µl 3M KCl, 10µl 0.1M $MgCl_2$, 4µl 0.25M beta-mercaptoethanol (Sigma), 4µl of 1mg/ml oligo(dT) (P-L Biochemicals, Inc.) in H_2O, 4.8µl of deoxyadenosine, deoxyguanosine and deoxythymidine (all at 20mM, from Sigma), 25µl of 1mg/ml Actinomycin D (Sigma), 8µl [alpha-^{32}P]-dCTP (3000Ci/mmol, Amersham) and 2 µl of reverse transcriptase (10 units/µl, Anglian Biotechnology). These

were mixed well and incubated for 1 hour at 37°C. The reaction was stopped by addition of 40µl of 0.5M EDTA and 30µl of 2N NaOH, was mixed well and incubated for 2 hours at 42°C. This was run through a long (18cm) G-50 Sephadex column equilibrated with 1 x SCC, 0.1% SDS. The radiolabelled cDNA was collected and its specific activity measured as described above. This was usually around 12 - 15 x 10^6 cpm/µg.

2.6.5 Hybridisation, Washing Conditions and Autoradiography

1. Prehybridisation

Filters were prehybridised in 50% formamide (deionised), 5 x Denhardts (see general section), 5 x SCC (see general section), 50mM sodium phophate pH 6.8, 380µg/ml sonicated, denatured salmon sperm DNA, 10µg/ml Poly (A), 0.2% SDS. Filters were typically incubated at 42°C o/n, but sometimes for shorter periods of time, for example 3-4 hours.

2. Hybridisation

The radiolabelled probe was denatured by boiling for 10 minutes and then added to 5-10ml of hybridisation buffer (50% Formamide, 1 x Denhardts (see general section), 5 x SSC, 20mM sodium phosphate pH6.8, 100µg/ml salmon sperm DNA, 20µg/ml Poly (A) (Boeringer), 10% dextran sulphate (Sigma). The prehybridisation buffer was discarded and the hybridsation buffer with the probe was added to the filter. The bag was re-sealed and incubated at 42°C o/n.

3. Post-hybridisation washing

After hybridisation, the filter was removed from the bag and washed at room temperature in 2 x SSC. Filters used for screening of the library with cDNA and genomic DNA were washed in 2 x SSC, 0.1% SDS twice for 10 minutes at room temperature and afterwards in 1 x SSC, 0.1% SDS for 20 minutes at 42°C. The filters were then monitored using a mini-monitor (GM Instruments) and if necessary, were washed down to 0.5 x SSC, 0.1% SDS.

Filters used for Southern blotting of genomic or hybrid DNA were washed in 2 x SSC, 0.1% SDS twice for 10 minutes at room temperature, and then in 0.5 x SSC for 20-30 minutes at 65°C. Again, if the background sounded quite radioactive after monitoring (over 10 counts per second, for example), the filter was further washed with 0.1 x SSC, 0.1% SDS at 65°C.

4. Autoradiography

Filters were placed between sheets of polythene and exposed to Kodak X-ray film between intensifying screens in Dupont Cronex X-ray cassettes. The films used were X-Omat S and X-Omat XAR-5. Exposure times varied from a few hours to weeks. The films were developed in a Fuji RG II X-Ray Film Processor, using Kodak X-Omat developer and fixer.

5. Removal of radiolabelled probes from filters

One of the advantages of using Hybond-N membrane for Southern blotting is that the radiolabelled probe can be removed efficiently and the filter can be reprobed a number of times with different probes. The procedure for removal of probes is as follows.

Filters were washed in 0.4M NaOH for 30 minutes at 45°C and for 30 minutes in neutralising solution (as used for Southern blotting). They were then rinsed in 1 x SSC, 0.1% SDS and placed in heat-sealed bags prior to further prehybridisation and hybridisation. The efficiency of the removal process was checked by exposing the stripped filters to X-ray film.

6. Pre-reassociation of Repeated Sequences from Hybridisation Probes (from Sealy et al 1985)

The method described previously (section 2.5.6) for isolating DNA from fetal tissues produces large amounts of genomic DNA (routinely, about 10 - 30mg). This was mainly used for preassociation experiments, in which the repeated sequences from radiolabelled probes were "competed out" by pre-reassociation with large amounts of unlabelled, sonicated genomic DNA. Following preparation of the DNA, it was subjected to 15 sonic bursts of 5 seconds, with 15 second intervals on ice in between, using a Soniprep 150 MSE

sonicator. The DNA was ethanol-sodium acetate precipitated as described previously and was left at -30°C o/n. The DNA was spun down to a pellet and resuspended in TE buffer to a concentration of 10mg/ml. The following were added in a 1ml reaction volume : 125µl of 30 x SSC, 5mg of the sonicated genomic DNA in a volume of 500µl and the radioactive probe. The volume was made up to 1ml with TE. The radiolabelled probe was mixed with the sonicated DNA and the salt and was denatured in boiling water for 10 minutes. It was then cooled on ice for 1-2 minutes before incubation for 20 minutes in a 68°C waterbath. The probe was then added to the prewarmed hybridisation mixture and was applied to the filter. The rest of the hybridisation and washing conditions were as described above.

2.6.6 Sizing of DNA Fragments

In order to determine the sizes of DNA fragments detected by certain probes in Southern blot experiments, the following procedure was used. Genomic DNA samples were run on 0.8% agarose gels as normal, but in one of the lanes of the gel, a set of size markers was added. The markers used were available commercially (BRL) and were roughly multiples of 1kb, and ranged in size from 1kb to 12kb. Following electrophoresis, the gel was photographed with a ruler next to it. The distances migrated by fragments of a specific length were noted, and a standard line was drawn on logarithmic graph paper. After Southern blotting,

hybridisation and autoradiography of the filter, the distances migrated by the fragments were compared with the line graph, and the fragment lengths calculated.

CHAPTER THREE

RESULTS

3.1 Isolation of Recombinants from the Flow-Sorted Chromosome 21-Specific Library of Young et al (1983)

Following extensive screening and purification in order to isolate single-copy, expressed sequences, (see Materials & Methods, Section 2.3.7), six recombinant bacteriophage were analysed : J21.1, J21.2, J21.3, J21.4, J21.5 and J21.6. DNA was prepared from each, digested with the restriction enzyme Eco RI to release any DNA inserts they might contain and separated by agarose gel electrophoresis. The results of this are shown in Figure 1 and the sizes of the human DNA inserts are given in Table 1.

3.1.1 Regional Mapping of Recombinants

The human DNA inserts contained in these clones were radioactively labelled using the random priming method (Feinberg & Vogelstein 1983) and then hybridised to Southern blots of DNA from the panel of somatic cell hybrids, normal and trisomy 21 individuals illustrated schematically in Figure 2. Although the screening procedure used was intended to select for single-copy sequences, it became obvious when these clones were hybridised to this mapping panel that each of them contained a repetitive DNA element, as might be expected for inserts of this size. All six gave an similar "smear" pattern of hybridisation; representative results are shown in Figure 3 for clones J21.1 and J21.2.

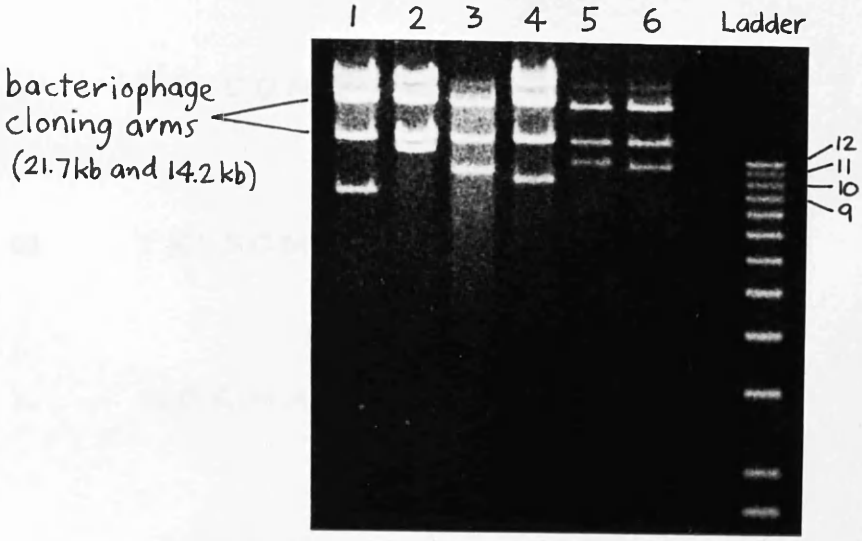


Figure 1 : Ethidium bromide-stained agarose gel showing Eco RI digestion of recombinants isolated from the flow-sorted chromosome 21 library of Young et al. (1983).

Lane numbers correspond to the J21 clone numbers (for example lane 1 contains DNA from J21.1, lane 2 from J21.2 etc.). The last lane on the right contains 1 kb ladder (BRL) as a size marker. The sizes (in kb) of the largest fragments are indicated to the right of the photograph.

Clone	Insert Size (kb)
J21.1	9.5
J21.2	13.5
J21.3	11.0
J21.4	10.0
J21.5	12.0
J21.6	11.5

Table 1 : Insert sizes of recombinants isolated from the sorted chromosome 21 library of Young et al (1983).

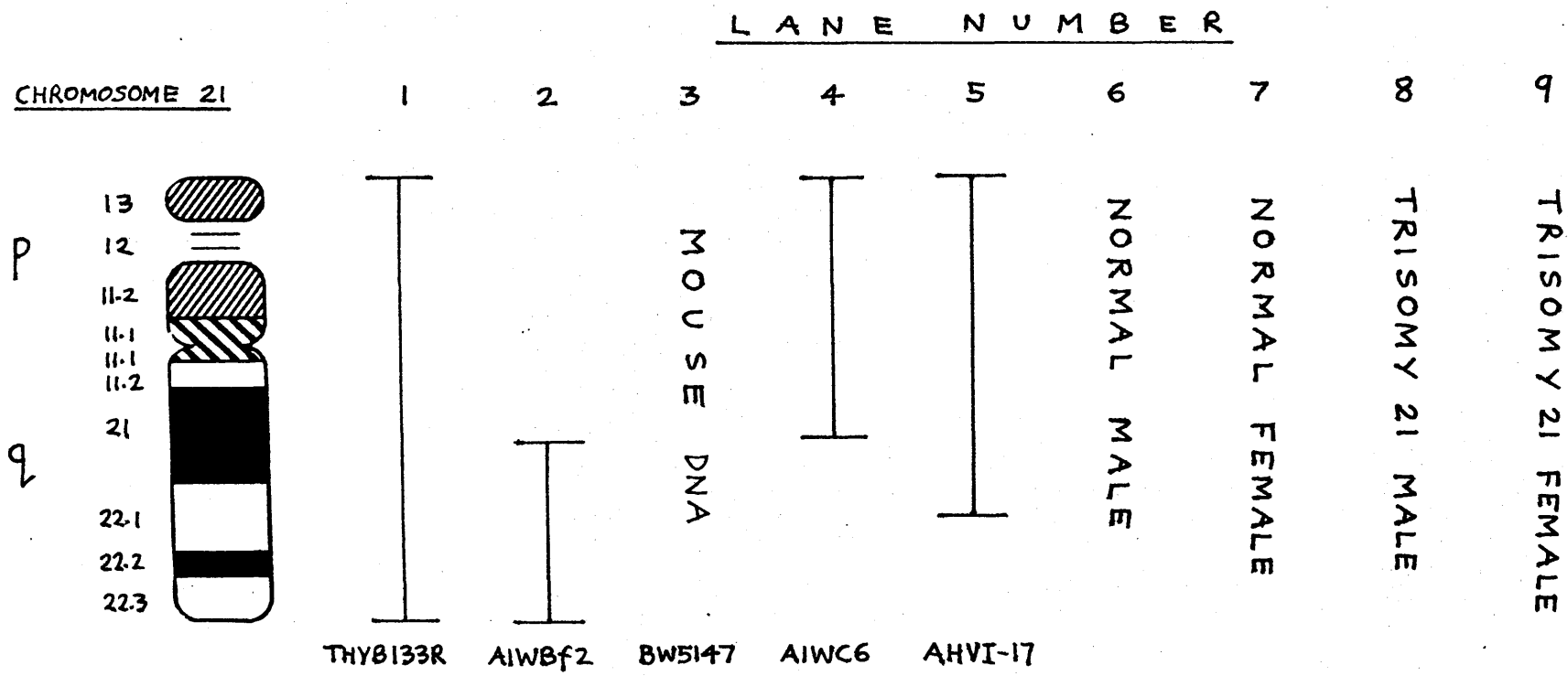
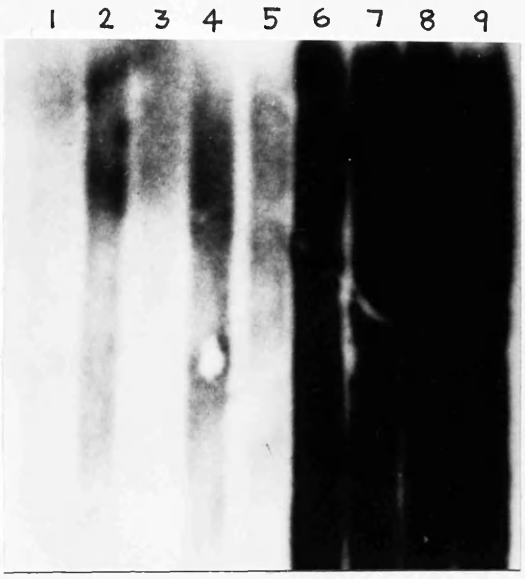


Figure 2 : Diagrammatical representation of the human chromosome 21 constitution of the original 9-member somatic cell hybrid mapping panel.

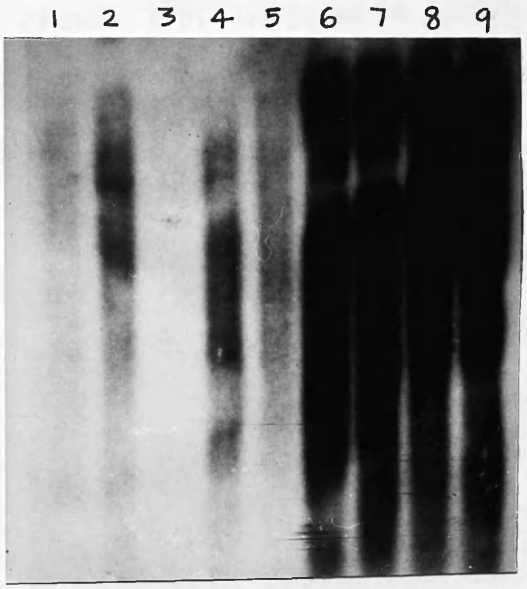
The vertical lines in lanes 1, 2, 4 and 5 indicate the parts of chromosome 21 present in each of these hybrids.

Following Page 79

Following Page 79



J21.1



J21.2

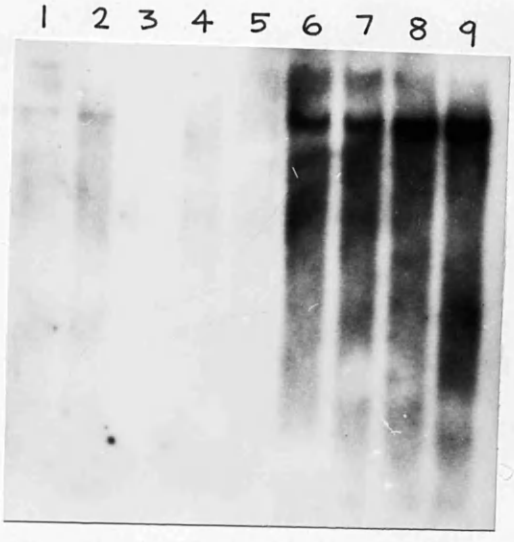
Figure 3 : Autoradiographs showing hybridisation of probes J21.1 and J21.2 to the 9-member hybrid mapping panel shown in Figure 2.

3.1.2 Identification of Clones containing Single-Copy DNA Sequences

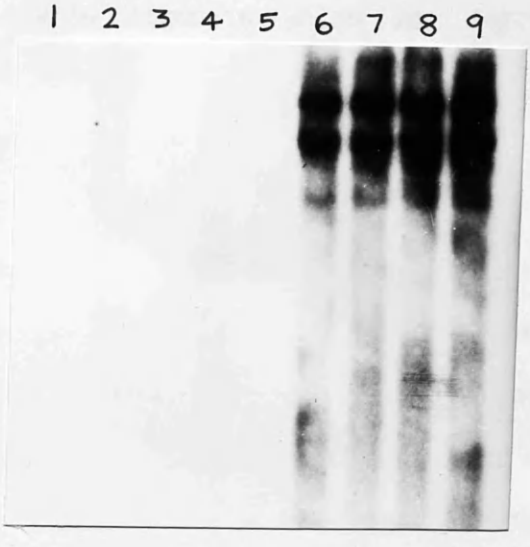
To test the possibility that these clones might also contain single-copy sequences, the pre-reassociation technique described by Sealy et al (1985) was used (details given in Materials & Methods, Section 2.6.5). This was initially attempted with clones J21.1 and J21.2 and the results are shown in Figure 4.

In Figure 4(a), a clear signal can be seen in all lanes of the panel except 3, 4 and 5. There is also a greater intensity of hybridisation to the DNA from trisomy 21 patients (lanes 8,9) compared to normal individuals (lanes 6,7). This indicates that clone J21.1 does contain a single copy sequence from chromosome 21, which maps in the region 21q22.1 - 21qter.

In Figure 4(b), two strong bands of hybridisation and two weaker signals can be seen in lanes 6 to 9 of the panel. None of these hybridisation signals appear in any of the other DNAs in the panel. There is also no difference in the intensity of hybridisation between normal and trisomy 21 individuals. This result demonstrates that there is a single- or low-copy number sequence within clone J21.2 which does not originate from chromosome 21. It should also be noted that there is no difference in hybridisation pattern or intensity between the DNAs from males and females on the panel, which suggests that these bands are not due to hybridisation to either of the sex chromosomes.



(a) J21.1



(b) J21.2

Figure 4 : Autoradiographs showing hybridisation of probes J21.1 and J21.2 to the 9-member hybrid mapping panel shown in Figure 2 following pre-reassociation of the probes with unlabelled human genomic DNA.

3.1.3 Attempted Isolation of Single-Copy Sequences from Clone J21.1

An attempt was made to separate the single-copy chromosome 21-specific sequence from the repetitive DNA also present in clone J21.1. The method used involved the following steps : 1. Digestion of clone J21.1 with a range of restriction enzymes, each used in combination with Eco RI, the enzyme originally used to construct the recombinant library; 2. Preparation of duplicate Southern blots for each digestion following separation on 0.8% agarose gels; 3. Hybridisation of one of the filters with radioactively labelled total human genomic DNA to identify weakly-hybridising fragments which may represent low- or single-copy sequences. The duplicate filter was probed with bacteriophage lambda DNA to identify fragments generated from the vector and so eliminate these from further analysis.

DNA from clone J21.1 was digested with those enzymes which generated the putative low-copy number fragments. Following electrophoresis on a 1% LMP agarose (BRL) gel, these fragments were excised from the gel, labelled by the random primer method and hybridised to the same panel of DNAs used before (Figure 2). Unfortunately all fragments gave a "smear" pattern similar to those shown in Figure 3 and typical of repetitive DNA hybridisation. Due to the lack of success in isolating any single-copy chromosome 21-specific DNA sequences from clones J21.1 and J21.2, no further analysis of clones J21.3 to J21.6 was performed.

3.2 Isolation of Recombinant Clones from the Chromosome 21-Specific Flow-Sorted Library Supplied by the National Gene Library Project (Lawrence Livermore Laboratory)

The main objective of the screening procedure used to isolate recombinant clones from this libary was to identify plaques which showed a lack of hybridisation with labelled total genomic DNA in the hope that these would contain low- or single-copy sequences. From the initial plating out of the library, involving approximately 4000 plaques, a total of 480 putative single-copy plaques were picked.

3.2.1 Restriction Enzyme Digestion of Recombinant Clones

DNA was prepared from 120 of the 480 plaques and digested with the restriction enzyme Hind III, which was used in the original construction of this recombinant library. Following separation of the resulting fragments separated on agarose gels, it was determined that 23 of the 120 purified bacteriophage plaques contained DNA inserts. The approximate lengths of these are listed in Table 2.

Following Page 82

Clone	Insert Size (kb)	Clone	Insert Size (kb)
JG12	4.1	JG81	5.0
JG21	3.2	JG83	5.0
JG22	1.7	JG87	2.7
JG24	4.3	JG88	6.0
JG51	4.7	JG90	5.0
JG61	2.9	JG93	4.0
JG62	2.0	JG95	4.2
JG63	2.3	JG99	2.7
JG68	4.2	JG101	3.2
JG72	3.2	JG108	3.2
JG73	3.0	JG373	4.1
JG77	1.8		

Table 2 - Insert sizes of recombinants isolated from the Lawrence Livermore flow-sorted chromosome 21 library, following Hind III digestion.

3.2.2 Identification of Recombinants Containing Single-Copy DNA sequences

All DNA inserts were tested to determine whether or not they were single-copy before hybridising them to the hybrid panel mapping filters. For this, "reptest" filters were prepared which contained DNA from one normal human, one trisomy 21 patient and from the hybrid cell line THYB133R, which contains chromosome 21 as its only detectable human chromosome. The results of hybridising the probes listed in Table 2 to these filters are shown in Figure 5.

It became clear that several of the cloned inserts contained repetitive sequences, as they showed typical "smear" patterns of hybridisation on all three DNA samples. Given the high degree of similarity between the hybridisation patterns of these clones, only one example of this type is shown in Figure 5, for clone JG61. The other repetitive inserts were JG68, JG83, JG87, JG88, JG93, JG95 and JG101, and no further analysis of these clones was performed.

Clones JG51 and JG73 were at first thought to contain highly repetitive sequences, but closer inspection revealed several distinct bands of hybridisation at the lower end of the filters. Although hybridisation to the 21-only hybrid was not very clear, it was decided that these probes should be investigated further.

All other probes detect a single hybridisation band in each of the three DNA samples, indicating that these are single-copy sequences derived from chromosome 21.

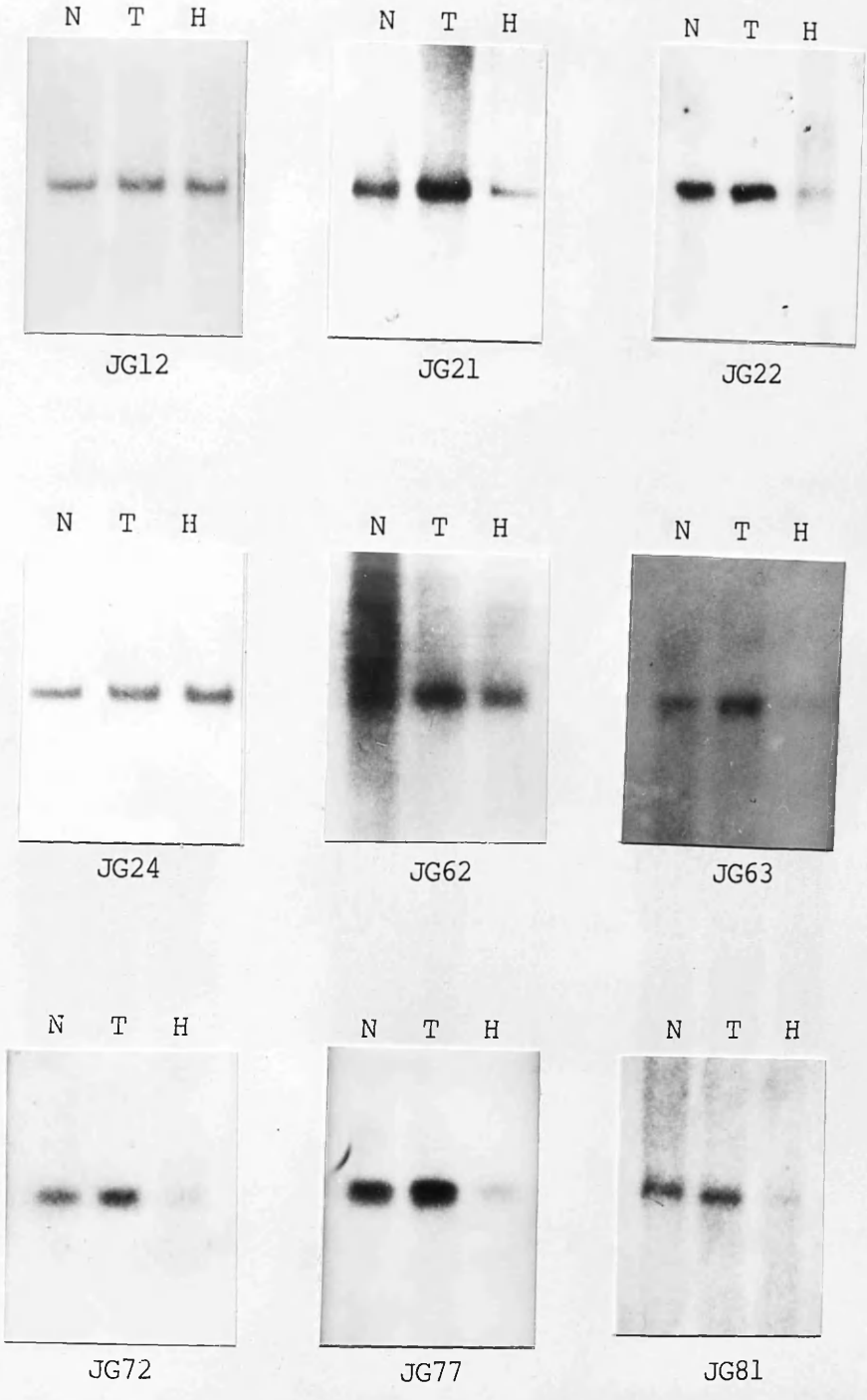


Figure 5 : Autoradiographs showing hybridisation of probes listed in Table 2 to "Reptest" filters (see text, section 3.2.2).

N : Normal Human Genomic DNA
T : Trisomy 21 Genomic DNA
H : Chromosome 21-only Hybrid Cell Line

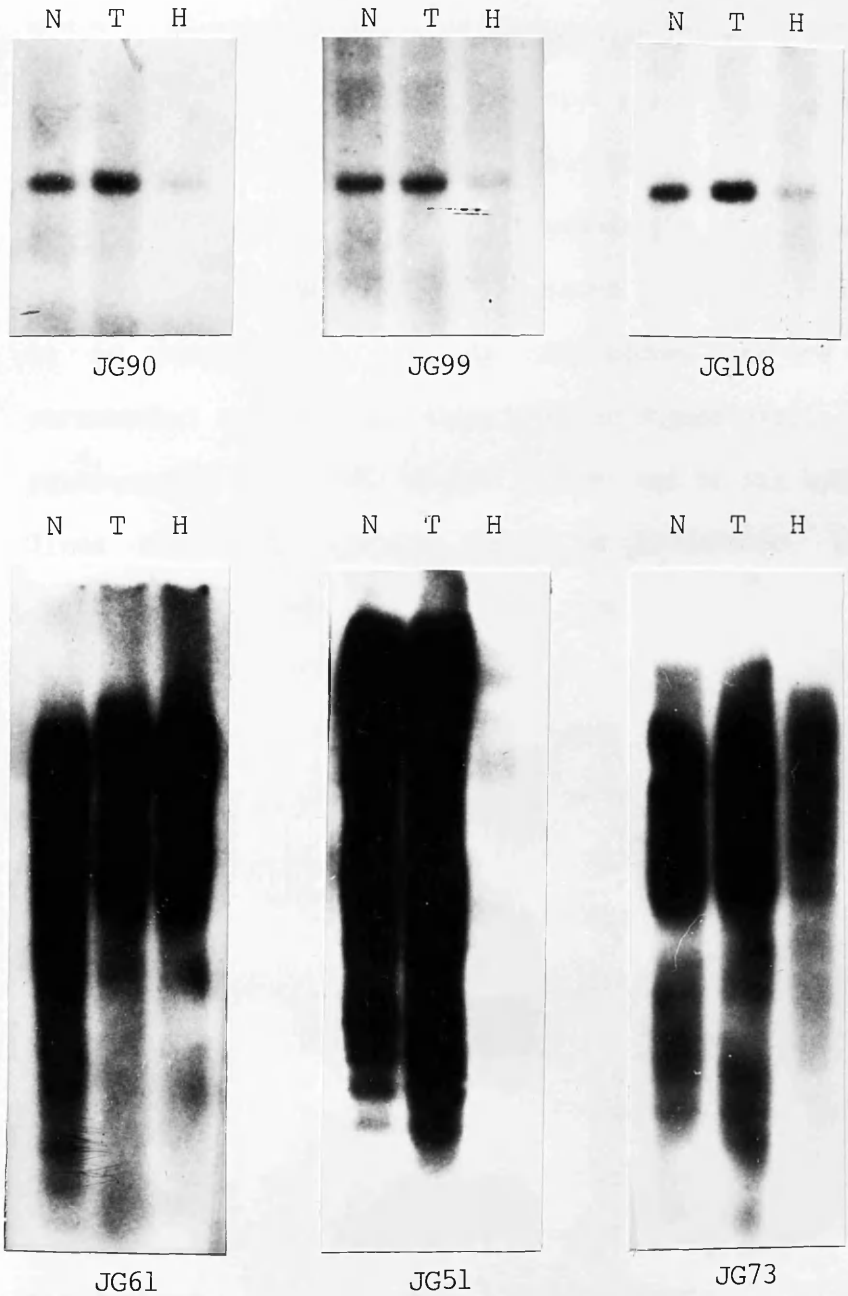


Figure 5 : Autoradiographs showing hybridisation of probes listed in Table 2 to "Reptest" filters (see text, section 3.2.2).

N : Normal Human Genomic DNA
T : Trisomy 21 Genomic DNA
H : Chromosome 21-only Hybrid

3.2.3 Regional Mapping of Recombinant Clones

All single- and low-copy number sequences were hybridised to a hybrid mapping panel simliar to that shown in Figure 2. Some changes were made in the DNA samples used, resulting in the panel shown in Figure 6. Using this panel, it is possible to localise DNA probes into one of three chromosomal regions, as illustrated in Figure 7(a). DNA was subsequently obtained from a further set of six hybrid cell lines containing various parts of chromosome 21. The inclusion of these samples in the mapping panel greatly increased the accuracy with which it was possible to localise DNA sequences, as shown in Figure 7(b). The hybrid panel incuding these additional samples is shown in Figure 8. To simplify matters the panels shown in Figures 6 and 8 will be referred to as "Panel 6" and "Panel 8", respectively. (Further details on hybrid cell lines are given in Section 2.2 of Materials & Methods).

For use in Southern blots experiments, hybrid panel DNA samples were digested with the restriction enzyme Hind III. As this was the enzyme originally used in the construction of the library, none of the cloned inserts should contain recognition sites for this enzyme and should therefore show a single hybridisation signal in each DNA sample.

Due to the multiple-band patterns observed with JG51 and JG73, results on the hybridisation of these probes to the mapping panels are considered separately from the other, single-copy probes.

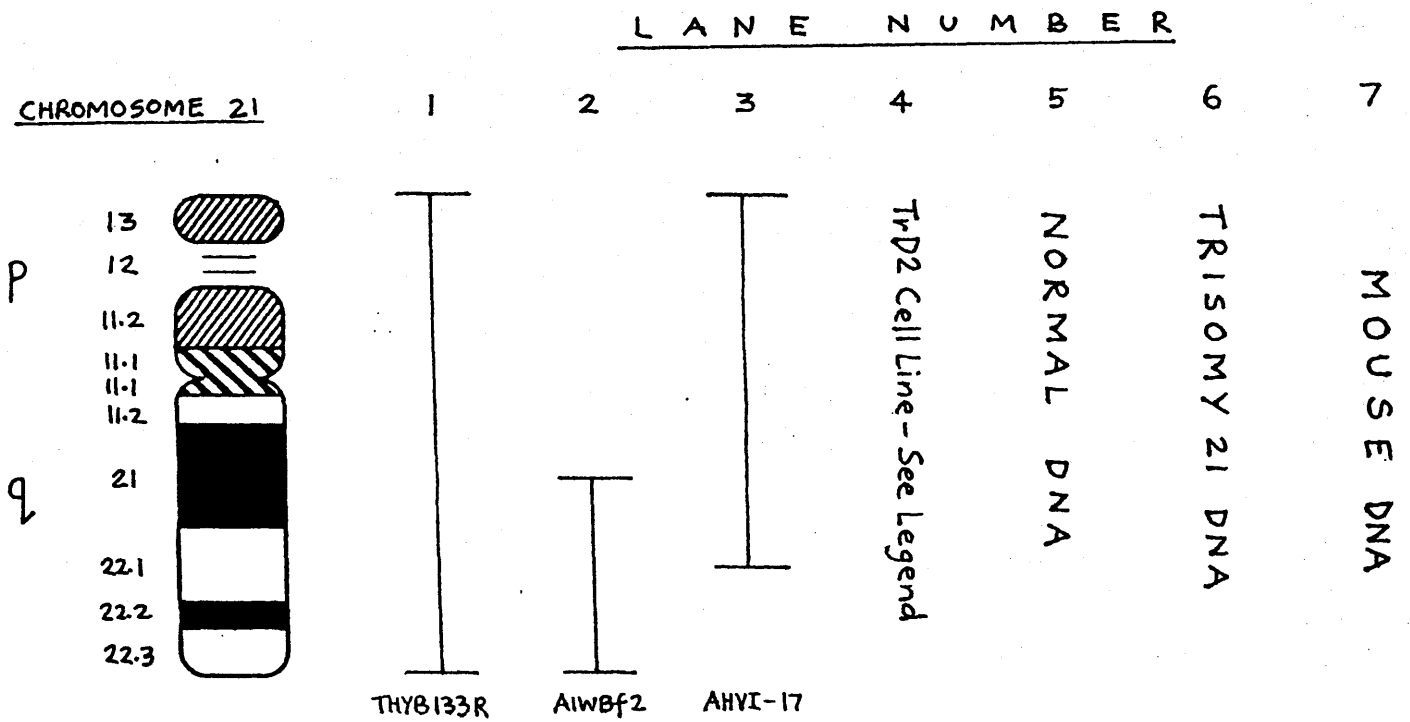


Figure 6 : Diagrammatical representation of the human chromosome 21 constitution of hybrid mapping panel 6.

The vertical lines in lanes 1, 2, and 3 indicate the parts of chromosome 21 present in each of these hybrids. TrD2 is a human fibroblast line which is monosomic for the region 21pter - 21q21 (see M & M, Section 2.2)

Following Page 84

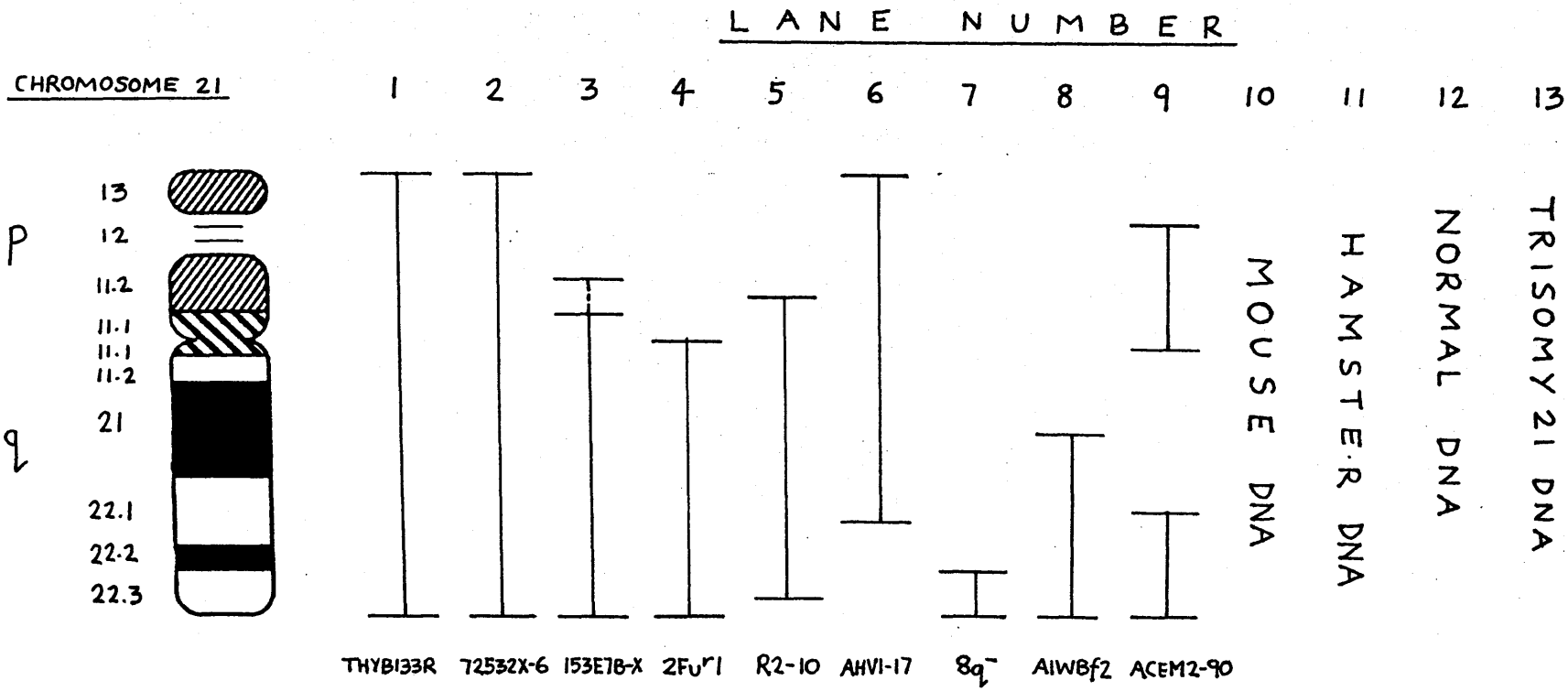


Figure 8 : Diagrammatical representation of the human chromosome 21 constitution of hybrid mapping panel 8.

The vertical lines in lanes 1 to 9 indicate the parts of chromosome 21 present in each of these hybrids.

Following Page 84

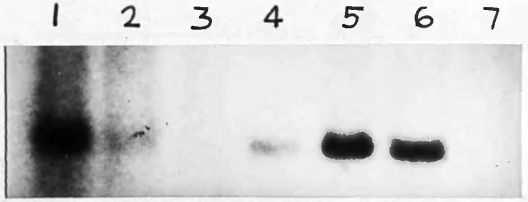
3.2.4 Single-Copy Probes - Hybridisation to Panel 6

The hybridisation patterns of the single-copy probes to this panel are shown in Figure 9 and the following information can be derived from these :

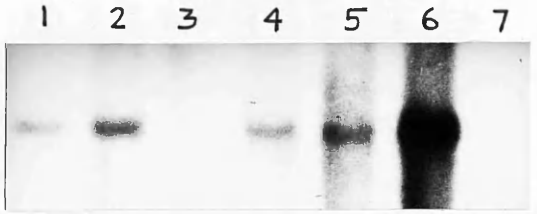
(a) JG12, JG22, JG24, JG63, JG77, JG81, JG108 and JG373 all show a similar band pattern on this panel - the only lanes to which these probes do not hybridise are tracks 3 and 7. This allows the regional localisation of these sequences to the region of chromosome 21 not present in the hybrid line AHVI-17, which is 21q22.1 - 21qter;

(b) JG21, JG62, JG72, JG90 and JG99 hybridise to all members of the panel except mouse DNA. It can be deduced from this that these probes map in the region of overlap between hybrids AIWBf2 (lane 2) and AHVI-17 (lane 3), which is defined cytogenetically as 21q21-21q22.1.

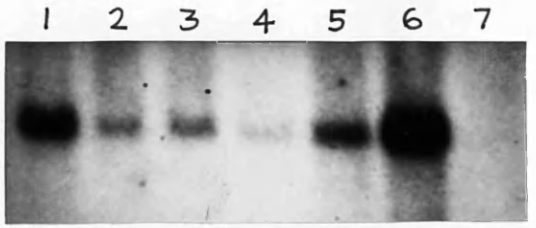
By comparing the relative intensities of hybridisation signal within each panel it is apparent that despite efforts to ensure that equal amounts of DNA were added to each track of these gels/filters, this had not been achieved. As a result of this it was decided that no conclusions on the regional localisation of DNA sequences could be drawn from differences in dosage of hybridisation signal, for example between TrD2 and normal human DNA, as had originally been hoped. It would still be possible, however, to localise probes using only positive/negative hybridisation criteria.



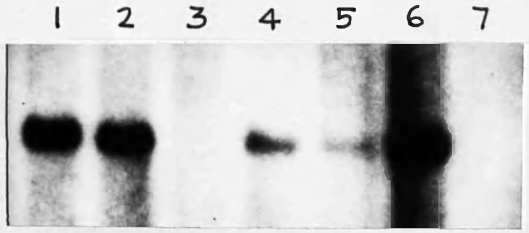
JG12



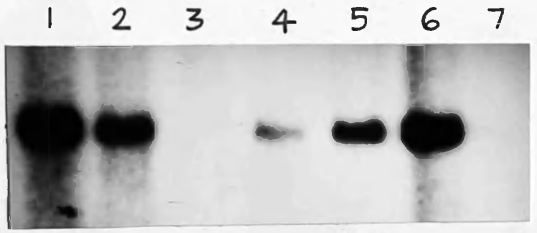
JG22



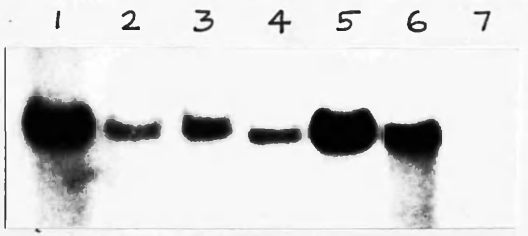
JG21



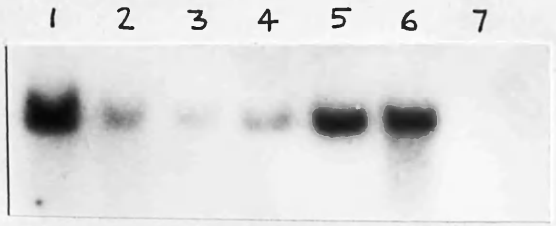
JG24



JG63



JG62



JG72

Figure 9 : Autoradiographs showing hybridisation of single-copy probes to hybrid mapping panel 6 (continued overleaf)

Following Page 85

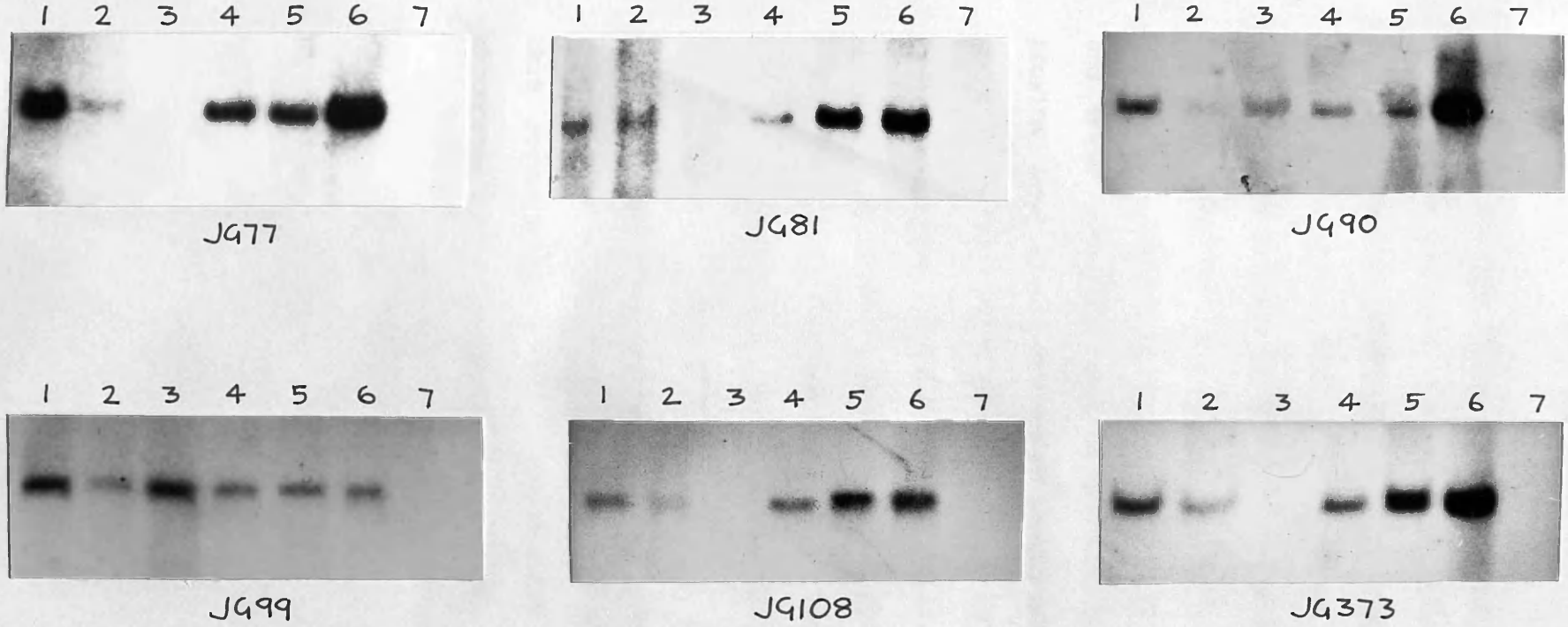


Figure 9 : Autoradiographs showing hybridisation of single-copy probes to hybrid mapping panel 6

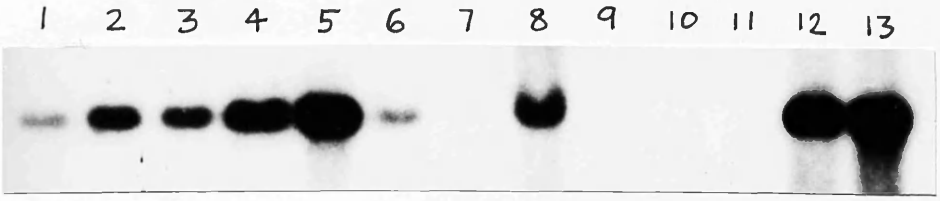
Following Page 85

3.2.5 Single-Copy Probes - Hybridisation to Panel 8

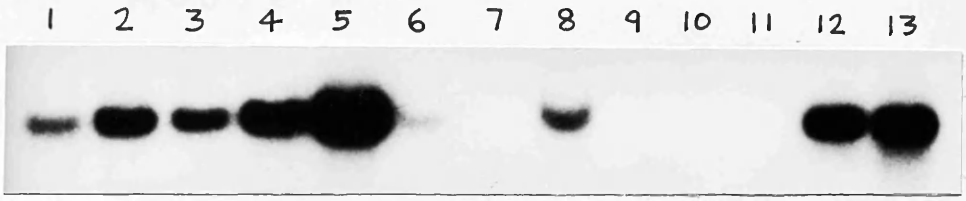
Unfortunately, time did not permit the hybridisation of all single-copy probes to this panel. Results are shown in Figure 10 for probes JG21, JG72, and JG90. These three probes show the same pattern of hybridisation on this panel - that is, they hybridise to all lanes except 7, 9, 10 and 11. This confirms the mapping of these sequences to 21q21 - 21q22.1 and moves the lower limit of this region to a slightly more proximal location, as defined by hybrid ACEM2-90 (lane 9).

Probe JG77 (also shown in Figure 10) hybridises to all lanes of the panel except 6, 7, 10 and 11. This probe can therefore be mapped in the region of chromosome 21 missing from hybrids AHVI-17 (lane 6) and 8q- (lane 7), which is defined cytogenetically as 21q22.1 - 21q22.2.

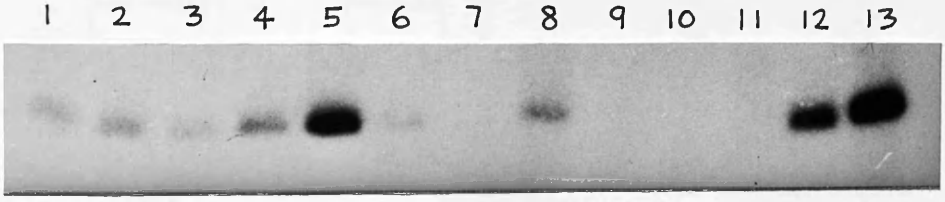
Probe JG373 was hybridised to a slightly different panel of hybrids, outlined in Figure 11. The hybridisation pattern (also shown in Figure 11) allows the localisation of this sequence to a small region close to the telomere of chromosome 21, in the distal part of band 21q22.3. This is based on its lack of hybridisation to hybrids R2-10 (lane 1) and HD-4 (lane 6).



JG21



JG72



JG90

JG77

Figure 10 : Autoradiographs showing hybridisation of single-copy probes to mappping panel 8.

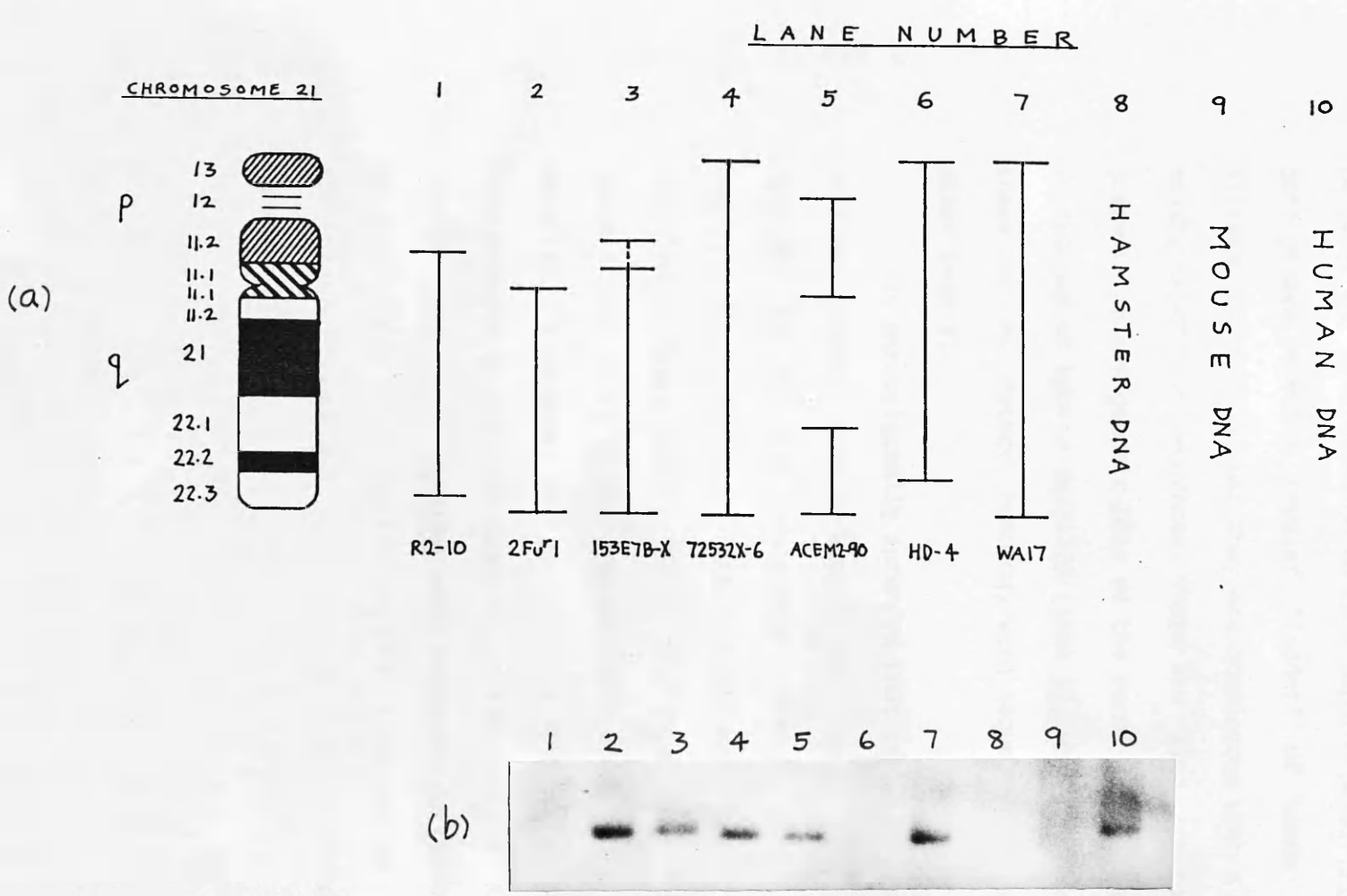


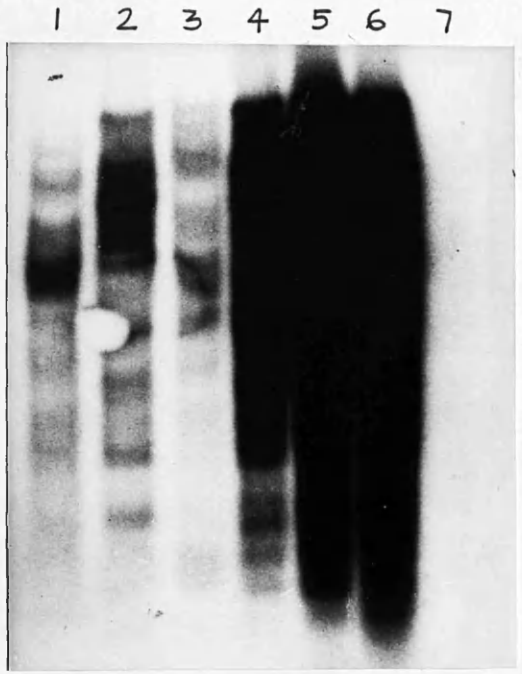
Figure 11 : (a) Diagram showing chromosome 21 constitution of hybrid mapping panel used to map probe JG373
(b) Autoradiograph showing hybridisation of probe JG373 to this mapping panel

Following Page 86

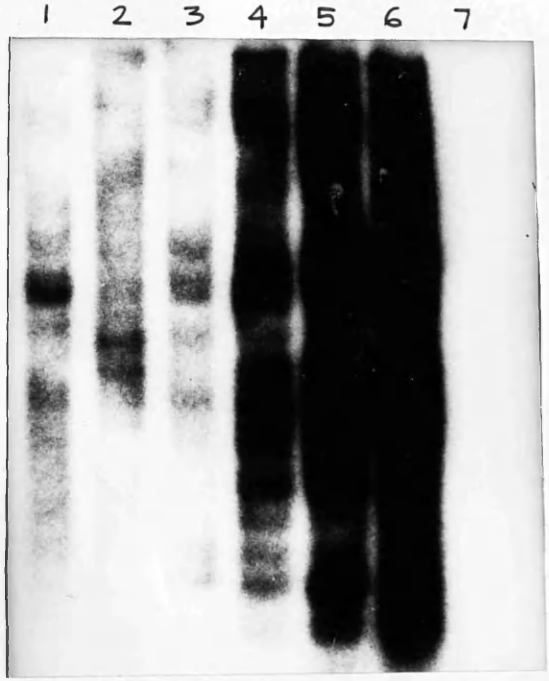
3.2.6 Mild Repeat Probes - Hybridisation to Panel 6 Using Normal (10cm long) Gels

Probes JG51 and JG73 show a similar and intriguing pattern of hybridisation on this mapping panel (Figure 12). Both probes detect a regular "ladder" of bands on these filters, suggesting that they are homologous with a series of mildly repetitive sequences. There are also numerous bands present in the genomic DNAs of the panel which do not appear in the DNA of hybrid THYB133R (lane 1), which suggests that these probes detect homology with sequences on chromosomes other than 21.

It was originally intended that regional localisation of these probes should be carried out using this panel of hybrids, as for the single-copy probes. The fact that JG51 and JG73 detect homology with non-chromosome 21 sequences complicates this, however. Since only THYB133R can be said to contain no other human chromosomes apart from 21 (see Materials & Methods, Section 2.2), it would be impossible to tell whether or not hybridisation to the DNA of the other hybrids was due to homology with sequences on chromosome 21. No conclusions regarding the regional localisation of these two probes could be drawn from the hybridisation to this panel of DNAs.



JG51



JG73

Figure 12 : Autoradiographs showing hybridisation of mild repeat probes to hybrid mapping panel 6 on normal (10cm long) agarose gels

Following Page 87

3.2.7 Mild Repeat Probes - Hybridisation to Panel 6 Using Longer Gels

Due to the multiple-band hybridisation patterns found with probes JG51 and JG73, and the difficulties in resolving these bands on the normal (10cm long) gels, further analysis of these probes was carried out using longer (20cm) gels. As mentioned above, regional localisation of sequences detected by these probes is not possible using the hybrids in panel 6. Some conclusions can still be drawn from their hybridisation patterns, however, as follows.

Probe JG51

The result of hybridising probe JG51 to mapping panel 6 on a long gel is shown in Figure 13a. The filter shown was washed at higher than normal stringency of post-hybridisation washing. Under these conditions, only one band is visible in lane 1, which contains DNA from the 21-only hybrid THYB133R. This band is approximately 4.7kb in length and is therefore likely to represent the original sequence on chromosome 21 from which JG51 was cloned.

The large number of bands present in the genomic DNAs of the panel, even at this high stringency, suggests that sequences homologous to JG51 are dispersed throughout the human genome.

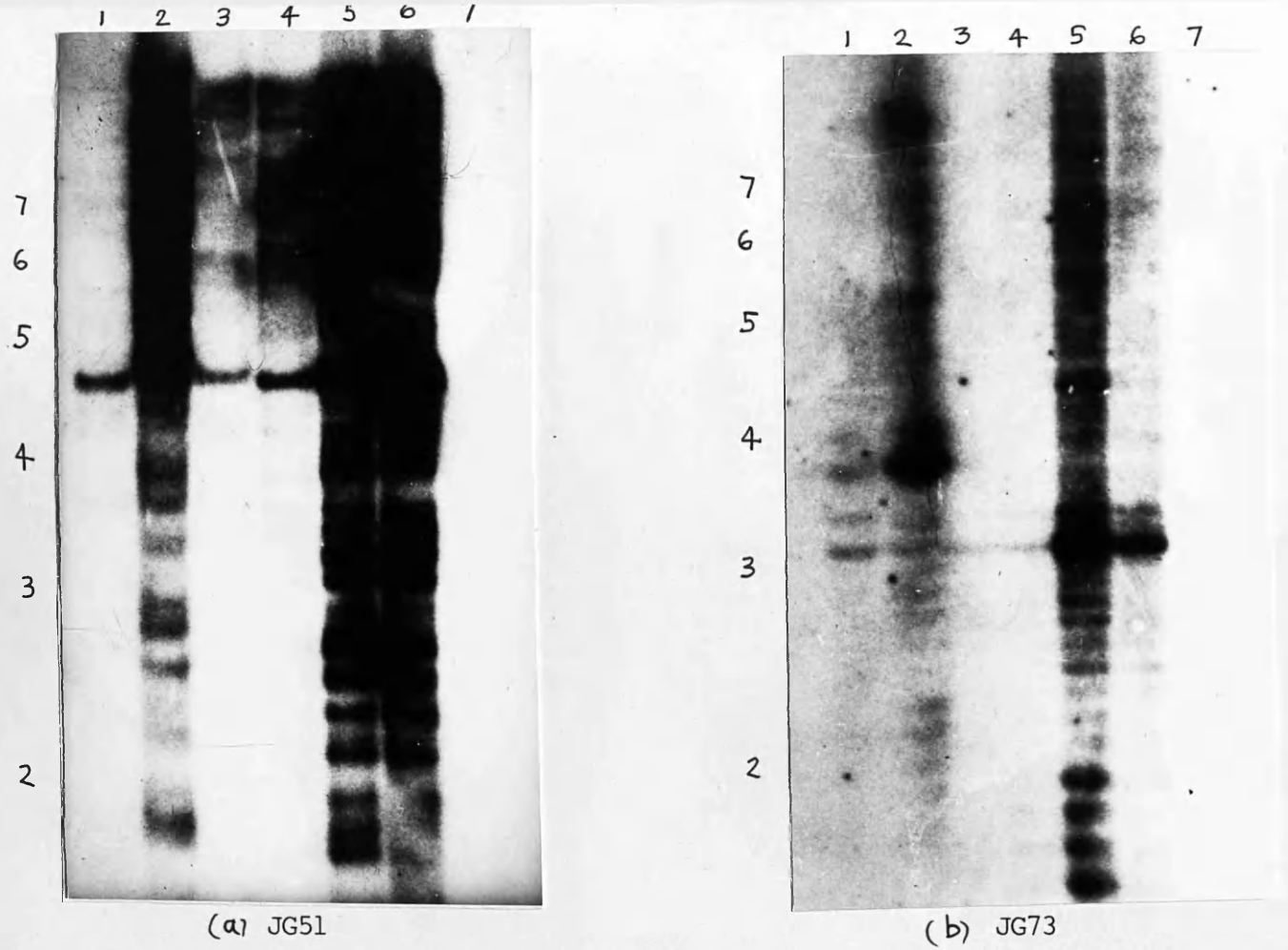


Figure 13 : Autoradiographs showing hybridisation of mild repeat probes to hybrid mapping panel 6 on long (20cm) agarose gels.

(Numbers to the left of the photographs indicate fragment sizes in kilobases)

Following Page 89

Probe JG73

Figure 13b shows the hybridisation of probe JG73 to the same DNA samples under the same conditions. It is possible to see at least 4 bands in lane 1. The lowest band is the strongest in terms of signal intensity, and was found to be 3.0kb in length. This band is therefore likely to be due to hybridisation to the original JG73 sequence on chromosome 21. Although the bands are less distinct in this autoradiograph, it is also possible to state that JG73 also detects homology with numerous non-chromosome 21 sequences in the human genome.

In summary, therefore, probes JG51 and JG73 were isolated independantly from the same chromosome 21 recombinant library and have insert sizes of 4.7 and 3.0kb, respectively. Each contains a mildly repetitive sequence which detects homology with numerous DNA sequences in the human genome, both on chromosome 21 and on other chromosomes.

3.2.8 Mild Repeat Probes - Hybridisation to Panel 8

Probes JG51 and JG73 were also hybridised to mapping panel 8, using the 20cm long gels (Figure 14). With the exception of hybrids AHVI-17 (lane 6), AIWBf2 (lane 8), and 8q- (lane 7) all of the hybrids in this panel contain no other human chromosomes apart from the region of chromosome 21 indicated in Figure 8 (Van Keuren et al. 1986a). It can be stated with confidence, therefore, that any bands visible in the DNAs of the other hybrid cell lines of the panel are due to hybridisation with sequences on chromosome 21.

The filters shown here were washed at normal stringency and under these conditions it is apparent that both probes show almost identical multiple-band hybridisation patterns. This suggests that these two probes detect homology with the same set of sequences in the human genome. With regard to the regional localisation of these sequences on chromosome 21 :

(i) there are fewer bands in lane 4 relative to lane 3. Hybrid 153E7B-X (lane 3) is known to contain the human chromosome 21 centromere, while 2Fur1 (lane 4) has only a small portion, if any, of a human centromere (Van Keuren et al. 1986a). This suggests that these probes map to the pericentromeric region of chromosome 21, and the bands visible in lane 4 are due to hybridisation to this region of the chromosome.

(ii) The only other hybrid in this panel which is known to lack the centromere of human chromosome 21 is 8q- (lane 7).

This hybrid contains the terminal part of chromosome 21 (q22.3 - qter) as part of an 8;21 translocation and therefore contains the centromere of chromosome 8. The paucity of bands found in the DNA of this hybrid supports the localisation of sequences homologous to JG51/JG73 to the pericentromeric region of chromosome 21, and suggests that the few bands visible might be due to hybridisation with homologous sequences in the pericentromeric region of chromosome 8.

(iii) The hybridisation pattern with hybrid AlWBf2 (lane 8) is also of interest. The region of chromosome 21 present in this hybrid does not include the centromere. It might be expected, therefore, that only a small number of bands would be visible in the DNA of this hybrid. This is obviously not the case, however, and multiple homologous sequences are evident in lane 8 of the panel. This result confirms that there are other human chromosomes present in this hybrid, and that probes JG51 and JG73 detect homologous sequences on these chromosomes.

(iv) It is also interesting to note that there are some differences in the intensity of bands between lanes 1 and 2. Both of these hybrids (THYB133R and 72532X6) contain chromosome 21 as their sole human chromosome. The variations in band pattern between these two must therefore represent differences in the presence, distribution or organization of sequences homologous to these probes on the different chromosome 21s present in these hybrid cell lines.

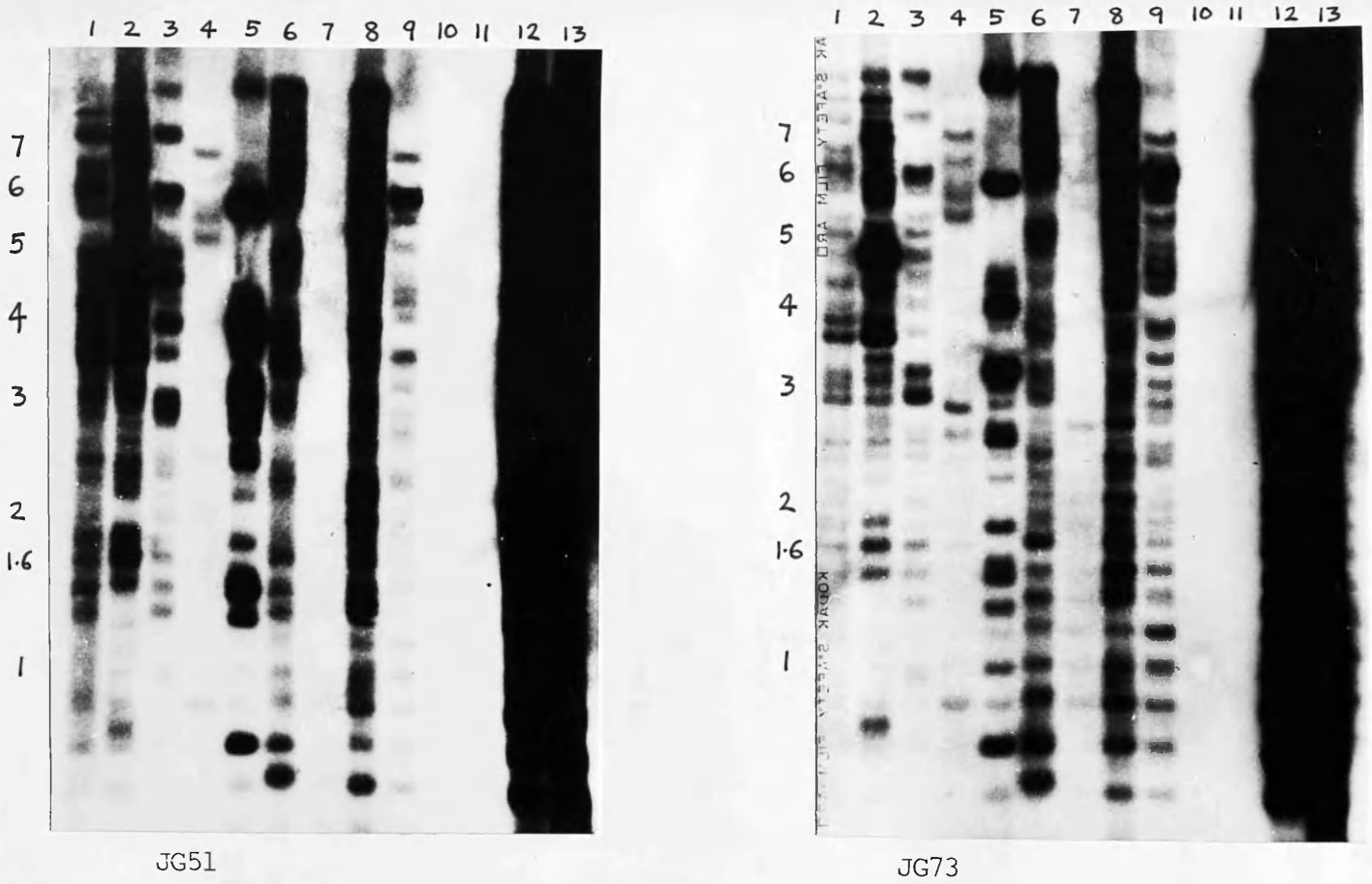


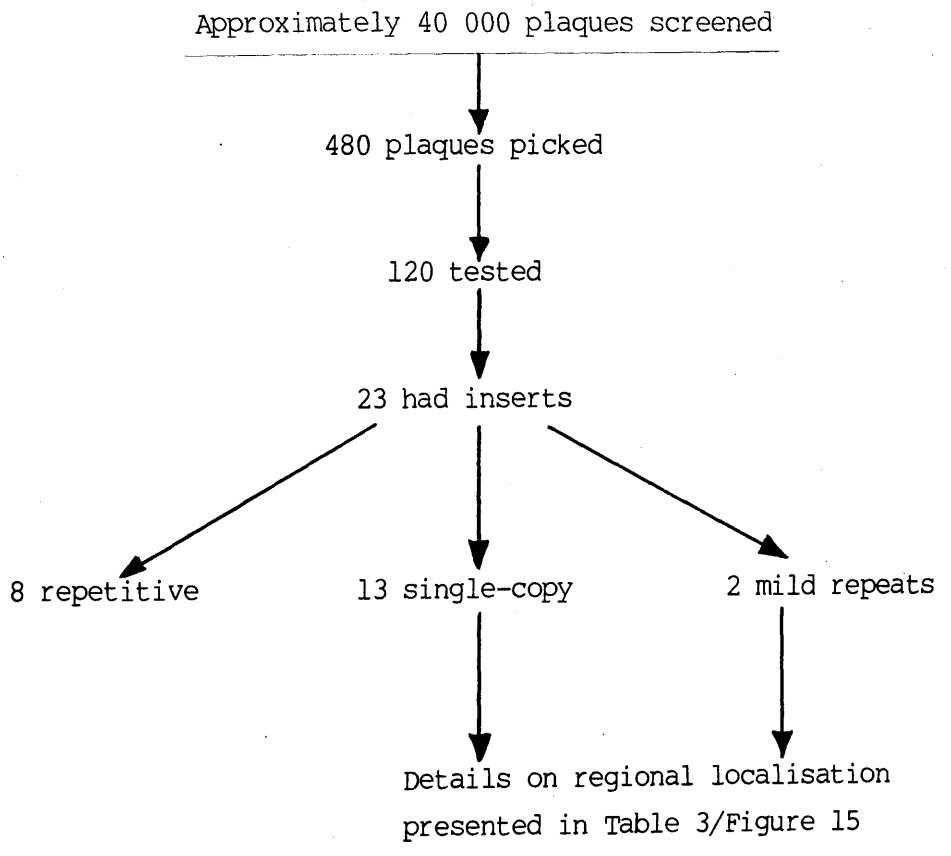
Figure 14 : Autoradiographs showing hybridisation of mild repeat probes to hybrid mapping panel 8 on long (20cm) agarose gels.

(Numbers to the left of the photographs indicate fragment sizes in kilobases)

Following Page 91

3.2.9 Summary of Characterisation of Probes Isolated from the Lawrence Livermore Sorted Library

The isolation and characterisation of recombinant clones from this library can be summarised as follows :



Following Page 92

Probe	Department Name	HGM Designation	Regional Localisation
JG12	GMG21S8	D21S85	q22.1 - qter
JG21	GMG21S9	D21S86	q2.1 - q22.1
JG22	GMG21S10	D21S87	q22.1 - qter
JG24	GMG21S11	D21S88	q22.1 - qter
JG51	GMG21S12	D21S89	pericentromeric
JG62	GMG21S13	D21S90	q2.1 - q22.1
JG63	GMG21S14	D21S91	q22.1 - qter
JG72	GMG21S15	D21S92	q2.1 - q22.1
JG73	GMG21S16		pericentromeric
JG77	GMG21S17	D21S93	q22.1 - q22.2
JG81	GMG21S18	D21S94	q22.1 - qter
JG90	GMG21S19	D21S95	q2.1 - q22.1
JG99	GMG21S20	D21S97	q2.1 - q22.1
JG108	GMG21S21	D21S99	q22.1 - qter
JG373	GMG21S22	D21S101	q22.3

Table 3 : Summary of Regional Localisation of Probes on Chromosome 21

Notes : 1. Probe JG73 has not yet been registered with the Yale Human Gene Mapping Library.

2. Although the regional localisation 21q2.1-21q22.1 is given for 5 of the 15 probes, there are slight differences in regional localisation for these probes, due to the use of different hybrid cell lines. For details see Figure 15.

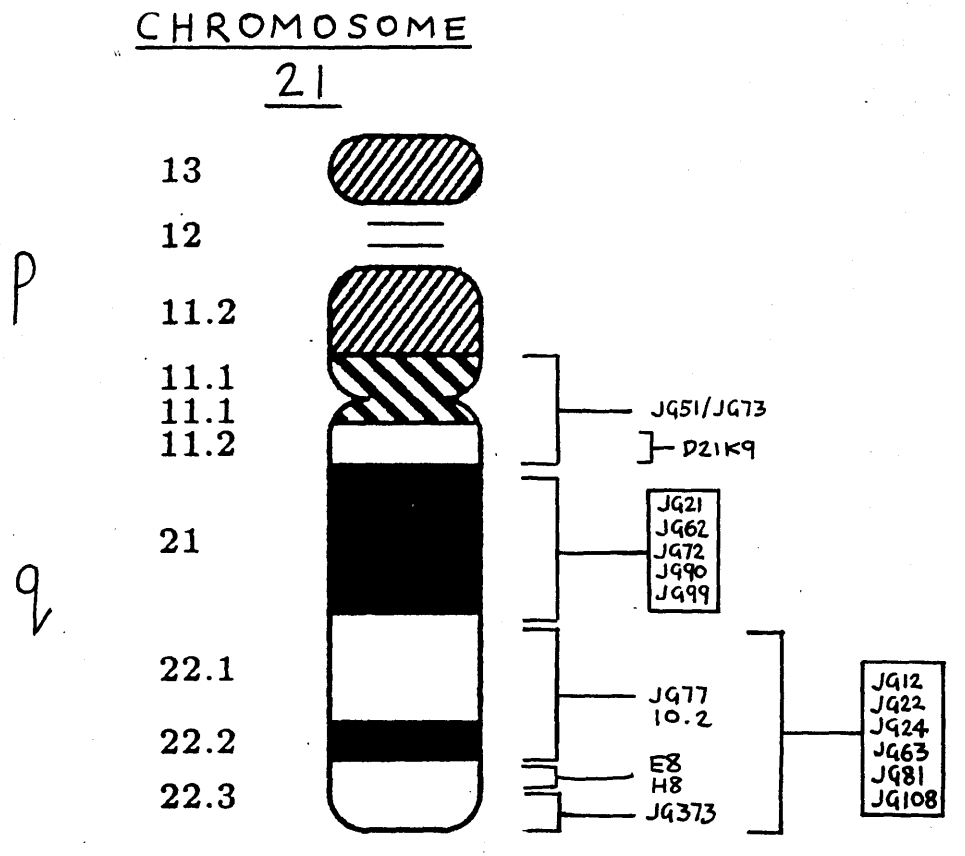


Figure 15 : Diagram summarising the regional localisation of the chromosome 21 specific DNA probes isolated during the present study.

Following Page 92

3.3 Screening of Recombinants for Restriction Fragment Length Polymorphisms (RFLPs)

DNA probes from chromosome 21 which detect RFLPs are potentially of great value in the determination of the origin of nondisjunction in Down's syndrome families. Many of the chromosome 21 probes isolated here were tested for RFLPs using a range of restriction enzymes and DNA samples from different individuals.

3.3.1 Screening for RFLPs using 5 DNA Samples

Originally, a set of DNA blots of samples from 5 different individuals was used and included digestions with 15 different restriction enzymes. As the results obtained with polymorphic sequences close to the centromere are less prone to interference caused by recombination (see Introduction, Section 1.2.3), the emphasis was placed on screening probes from this region of the chromosome. The filters were therefore hybridised with DNA probes JG21, JG62, JG72, JG77, JG81, JG90, JG99 and JG108.

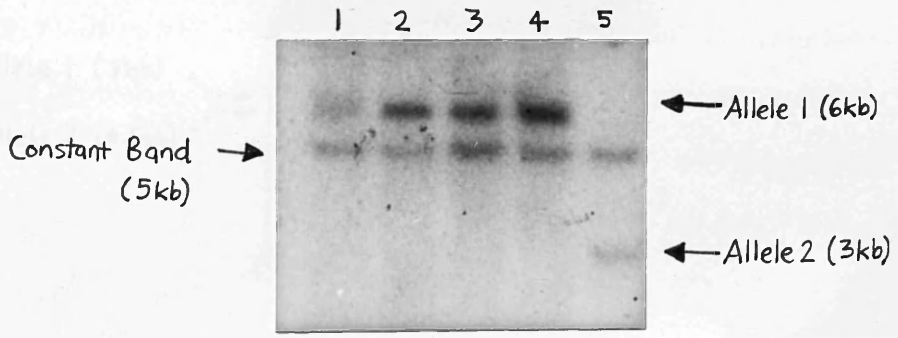
Two RFLPs were detected using this panel of filters : these were shown by probe JG77 with the enzyme Msp I and probe JG99 with the enzyme Pst I. Figure 16(a) shows the original autoradiograph illustrating the JG77/Msp I polymorphism, while Figure 16(b) demonstrates this RFLP on a further 12 individuals. The sizes of the polymorphic alleles were found to be 6kb and 3kb, respectively. There is also a constant band of 5.0kb, which appears in every genomic DNA

sample. (Details on the sizing of bands given in Materials and Methods, Section 2.6.6). The JG99/Pst I polymorphism is demonstrated in Figure 17; the alleles in this case were found to be 7.0 and 6.6kb, respectively.

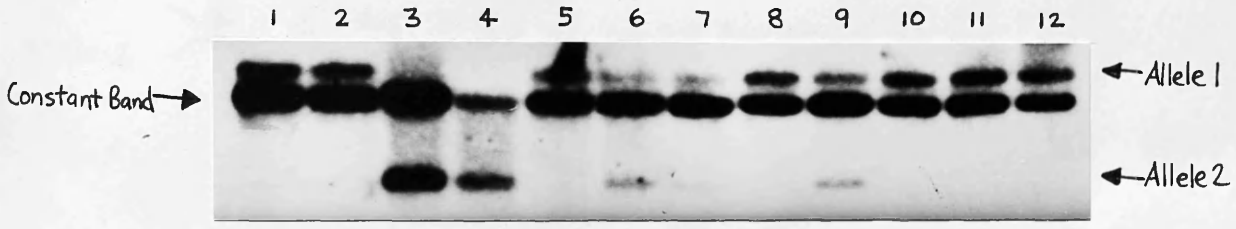
3.3.2 Screening for RFLPs using 7 DNA Samples

It was decided at this point that in order to increase the probability of detecting RFLPs, DNA from 7 different individuals should be digested with the same series of enzymes. This RFLP screening panel revealed only one additional polymorphism, however, as detected by probe JG90 with the enzyme Nde II. This probe/enzyme combination gives allele sizes of 1.8 and 2.2kb, respectively, with a constant band at 1.2kb on genomic DNA samples (autoradiographs for this RFLP not shown).

Following Page 94



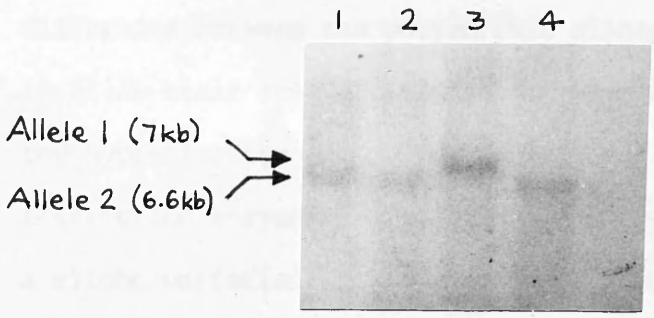
(a)



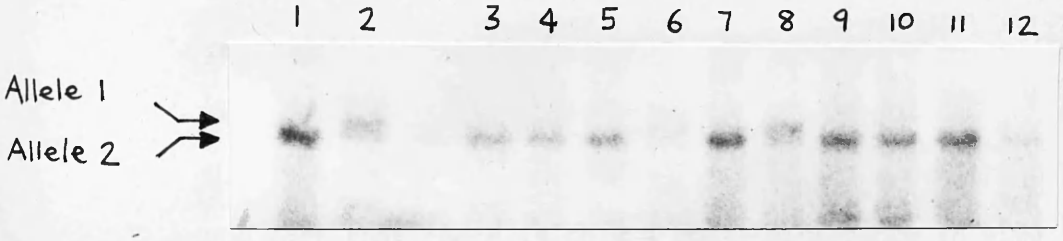
(b)

Figure 16 : Autoradiographs demonstrating the JG77/Msp I polymorphism on (a) five and (b) twelve unrelated individuals.

Following
Page 94



(a)



(b)

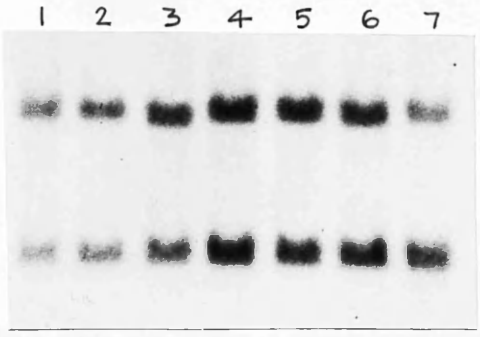
Figure 17 : Autoradiographs demonstrating the JG99/Pst I polymorphism on (a) four and (b) twelve unrelated individuals.

3.3.3 Screening for RFLPs using Longer Gels

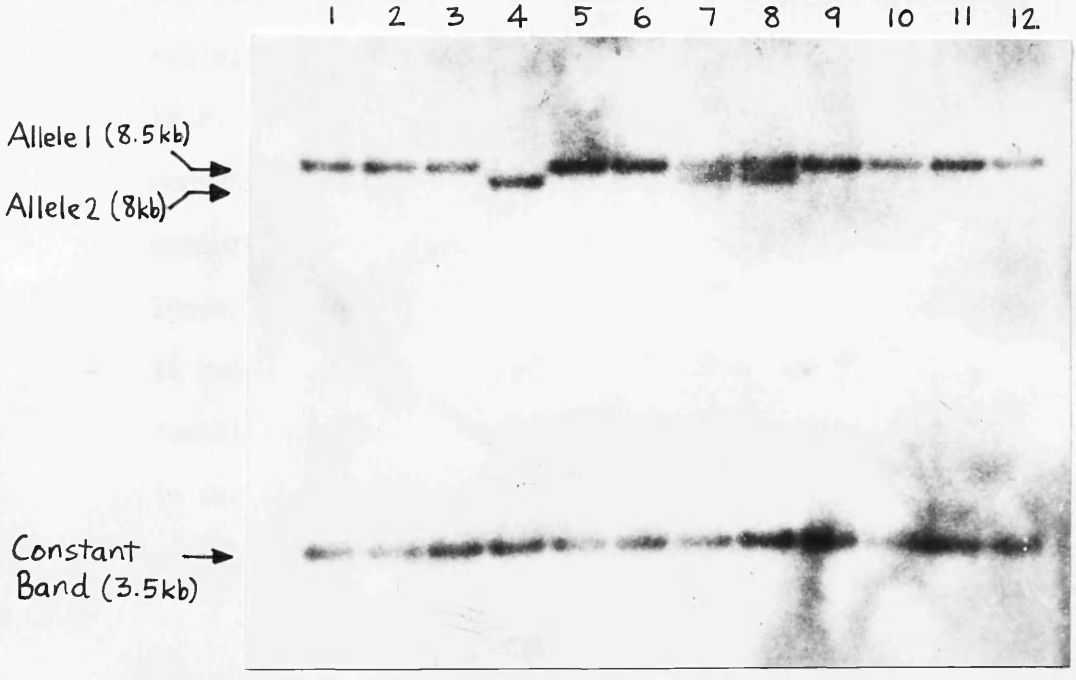
The possibility was considered that some RFLPs could perhaps remain undetected due to the fact that the size difference between the polymorphic alleles was insufficient to allow their resolution on the normal (10cm long) gels. In the hybridisation pattern shown by probe JG81 with the restriction enzyme Pvu II (Figure 18a), for example, there is a slight variation in the distance migrated by the upper band on the filter. This could be an electrophoresis artefact, but it was also considered possible that this might be a "poorly resolved" RFLP. To test this, DNA samples were run on 20cm long gels. A set of 12 genomic DNAs and a total of 20 restriction enzymes were screened in this way.

Use of longer gels for RFLP screening of probe JG81 demonstrates quite clearly that this probe does detect a polymorphism with the enzyme Pvu II (Figure 18b), confirming the above theory. The sizes of the polymorphic alleles are 8.5 and 8.0kb, respectively, with a constant band at 3.5kb. The fact that there is only a small difference in size between the alleles explains the initial difficulty in resolving this polymorphism on the shorter gels.

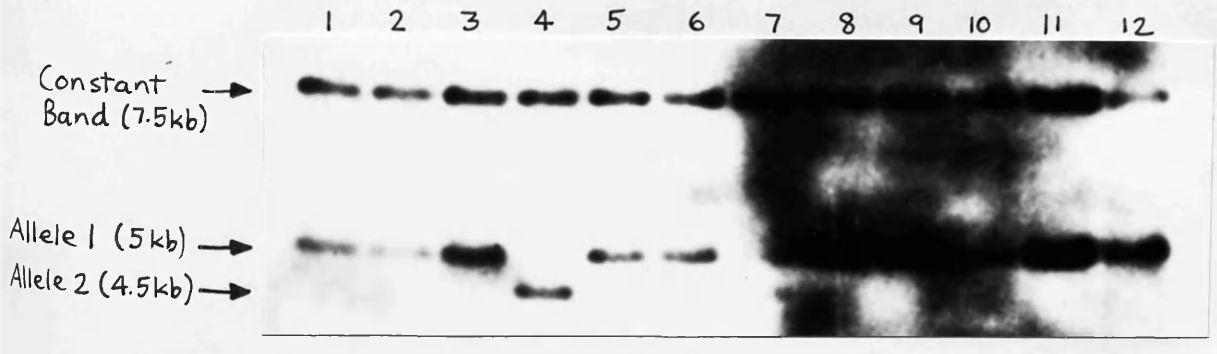
The restriction enzyme Eco RV was introduced into the RFLP screening panel at this stage, and its inclusion allowed the detection of another RFLP with probe JG81, as shown in Figure 18c. There is a constant band at 7.5kb, and polymorphic alleles at 5.0 and 4.5kb, respectively.



(a)



(b)

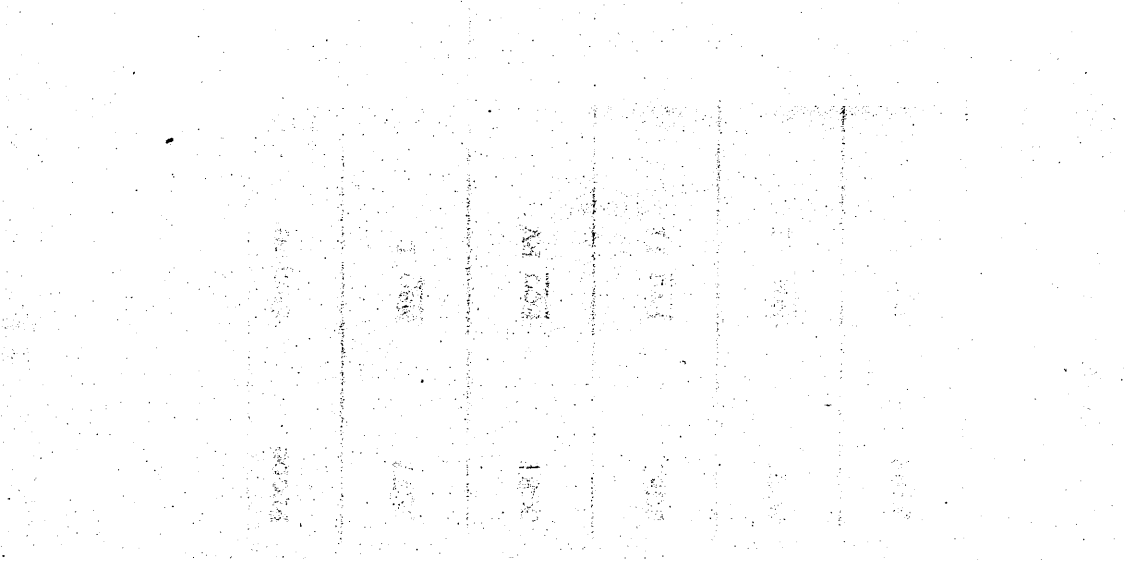


(c)

Figure 18 : Autoradiographs demonstrating the JG81/Pvu II polymorphism on (a) 10cm and (b) 20cm long gels; (c) demonstrates the JG81/Eco RV polymorphism on long (20cm) gel.

3.3.4 Summary of RFLP Screening

Table 4 gives statistical data on the 5 RFLPs detected during this project. The JG77/*Msp* I polymorphism was used to analyse Down's syndrome families to determine the origin of the extra chromosome, as described in section 3.4. This explains the greater number of individuals tested with this RFLP. Also shown in Table 4 is the polymorphism information content (PIC) for each probe/enzyme combination. This is a measure of the "usefulness" of a particular polymorphic DNA locus, and in the present context estimates the likelihood of it being informative in the analysis of Down's syndrome families. (The formula for derivation of the PIC is presented in Skolnick & White, 1982).



Probe	Enzyme	Constant Band (kb)	Allele	Length (kb)	Frequency	Individuals Tested	PIC
JG77	Msp I	5.0	1 2	6.0 3.0	0.67 0.33	62	0.34
JG81	Eco RV	7.5	1 2	5.0 4.5	0.88 0.12	19	0.20
JG81	Pvu II	3.5	1 2	8.5 8.0	0.83 0.17	12	0.24
JG90	Nde II	1.2	1 2	2.2 1.8	0.70 0.30	20	0.33
JG99	Pst I	-	1 2	7.0 6.6	0.16 0.84	16	0.23

Table 4 : Details of RFLPs detected during this study

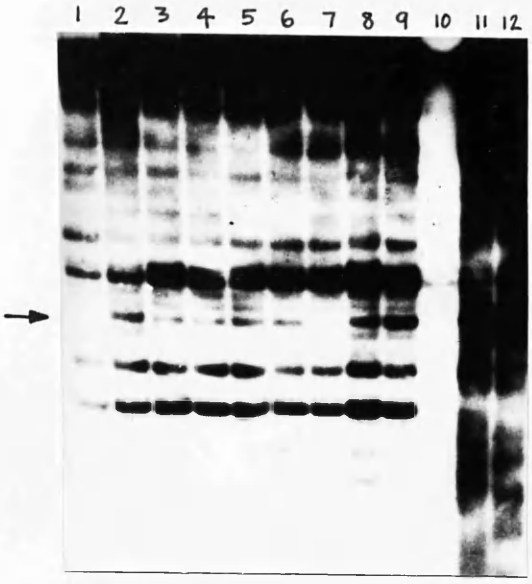
(PIC : Polymorphism Information Content - see results section 3.3.4)

Following Page 96

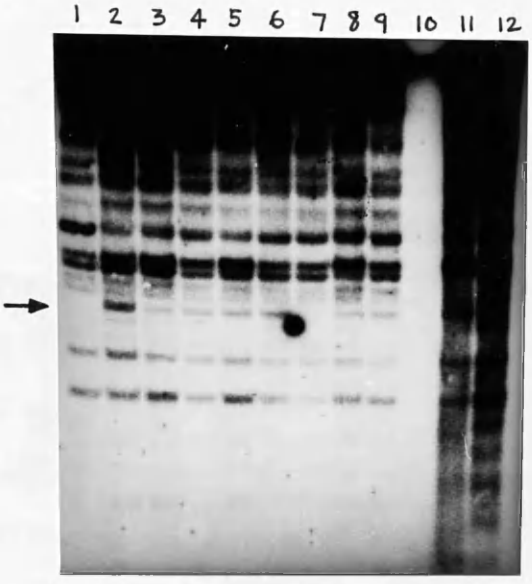
3.3.5 Hybridisation of Mild Repeat Probes to the Long RFLP Screening Panel

There were two reasons for hybridising probes JG51 and JG73 to this panel: (i) to screen for RFLPs in the same manner as for the single-copy probes, and (ii) since it appears that these probes display very similar patterns of hybridisation to human genomic and to somatic cell hybrid DNAs digested with the enzyme Hind III (Figure R14), this was also an attempt to determine whether other restriction enzymes showed up any differences in the chromosomal organisation of these probes.

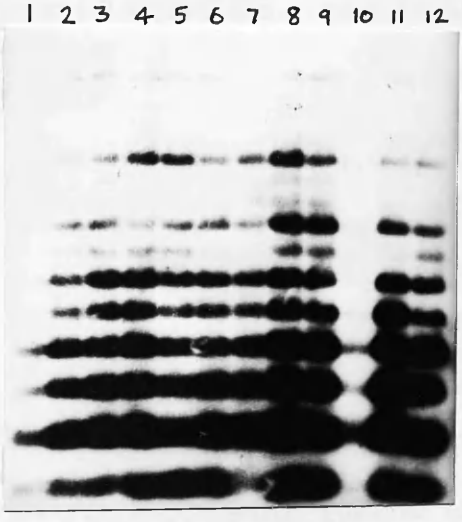
This latter question is easily answered - for all 20 enzymes tested, both probes gave identical band patterns. Examples are shown in Figure 19 for the enzymes Pvu II and Hae III. This suggests that these two probes share extensive sequence homology.



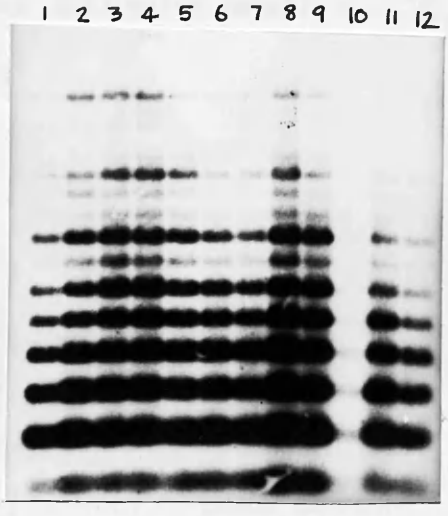
JG51/Pvu II



JG73/Pvu II



JG51/Hae III



JG73/Hae III

Figure 19 : Hybridisation patterns of probes JG51 and JG73 to human genomic DNA samples digested with restriction enzymes Pvu II and Hae III.

(Arrows adjacent to Pvu II photographs indicate possible RFLP - see section 3.3.5)

The question of whether or not these two probes detect RFLPs is more difficult. It is certainly the case that some restriction enzymes produce variations in band pattern between different individuals. For example, in Figure 19(a), which shows hybridisation to DNA samples digested with the enzyme Pvu II, lanes 1, 7 and 12 are lacking a band at the point indicated on the film. If this is an RFLP, then it is impossible to determine the position of the other allele(s) on the filter. Another possibility perhaps is that the individuals represented in these three tracks are deleted for the particular region of DNA represented by this band.

Hybridisation to DNA samples digested with the enzyme Xmn I (Figure 20a - results shown for JG73 only) reveals a very interesting band pattern. At approximately 8/9kb, there appears to be a simple, two-allele RFLP, with some individuals homozygous for the upper band (lanes 6,7,8,9 and 12), some heterozygous (lanes 1,3,4,5 and 11) and one person homozygous for the lower band (lane 2). However, if one considers that lanes 1 to 5 and lane 11 contain DNA from normal males, while the other lanes contain female DNA, another explanation for the observed pattern of bands is possible. It might be the case that the lower of the two bands is male-specific, representing hybridisation to the Y chromosome, while the upper band is merely deleted in the DNA of the individual in lane 2, in a similar way as described above for the enzyme Pvu II.

Support for this is provided by examination of the band patterns obtained with these probes and the enzymes Hind III, Eco RI and Eco RV (Also shown in Figure 20). In each case a strong signal indicating hybridisation to a fragment of about 8kb is visible in the lanes of the filter containing DNA from males; this band does not appear in DNA samples from the females used here. It is interesting to note that for all four probe/enzyme combinations shown in Figure 20, the putative Y-band is the same size. Further experiments are necessary to clarify this situation, and there was unfortunately insufficient time for these to be carried out.

In conclusion, it is unlikely that any variations in DNA sequence between individuals detected using these mild repeat probes could be used to follow the inheritance of chromosome 21 within families until the particular fragments involved were assigned exclusively to that chromosome.

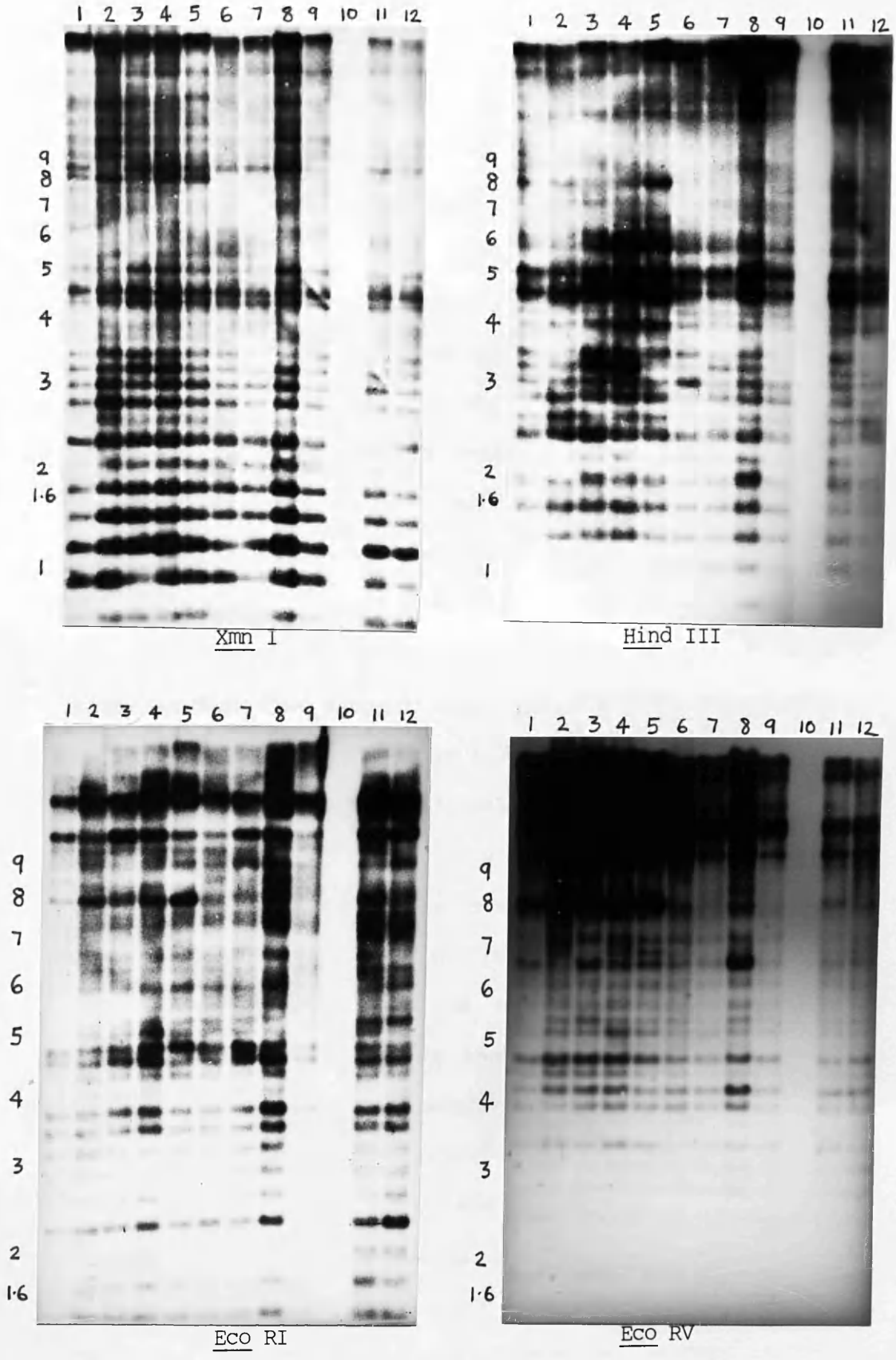


Figure 20 : Hybridisation patterns of probe JG73 on genomic DNA samples digested with restriction enzymes Xmn I, Hind III, Eco RI and Eco RV. Fragment sizes (in kb) are indicated to the left of the photographs.

3.4 Analysis of Nondisjunction in Down's Syndrome Families using RFLP Markers and Cytogenetic Heteromorphisms

As mentioned previously, chromosome 21-specific DNA probes which detect RFLPs are potentially of great value in determination of the parental origin of nondisjunction in Down's syndrome families. If one considers a Down's syndrome nuclear family consisting of father (F), child (C) and mother (M) (these abbreviations are used in Figures 21 to 30, inclusive), and the analysis of such a family with a simple two-allele polymorphic probe, then there are essentially only eight possible band patterns which will reveal the origin of the extra chromosome. These are illustrated in Figure 21.

In the first type of informative pattern (Figure 21a to d), both parents are homozygous for different alleles. The child has two copies of one allele, and one copy of the other. For example, if the band pattern was found to be that shown in Figure 21b, then it is obvious that because the child has two copies of the lower allele, which the father does not have, the extra chromosome must have come from the mother. Given only this information it is impossible to tell at which meiotic division nondisjunction occurred.

In the second type of band pattern (Figure 21e to h), one parent is heterozygous and the other is homozygous. The child has two copies of the allele not present in the homozygous parent, and one copy of the other allele. Considering the pattern shown in Figure 21e, and following the same reasoning as used above for Figure 21b it is

possible to say that the extra chromosome is derived from the mother. In this example it is also possible to tell that since the mother has two different chromosome 21s in relation to this marker, the only way in which she could pass on two identical copies of the chromosome would be if nondisjunction took place at the second meiotic division. Nondisjunction in this case can be localised to the second maternal meiotic division. It should be noted, however, that recombination between the centromere and DNA markers on the long arm of the chromosome can lead to misinterpretation of RFLP results in which nondisjunction is localised to a specific meiotic division. An example of this is presented in section 3.4.5 for family 25-26-27 and a consideration of this problem is presented in section 4.4.6 of the discussion.

A total of 33 Down's syndrome nuclear families consisting of father, mother and affected child (or fetus in some cases) were tested using five probe/enzyme systems to determine the origin of nondisjunction. To ensure confidentiality, each family was allocated a number. In addition to the JG77/Msp I polymorphism, four polymorphic probes supplied by other workers were used. (See Table 5, which also shows the number of families tested and the number informative for each marker. Five of the Down's syndrome families were also analysed using Q-banding heteromorphisms* and in three cases the origin of nondisjunction was determined. Details are given below, along with the RFLP results for each of the informative families.

(* - Cytogenetic studies performed by Dr. Elizabeth Boyd)

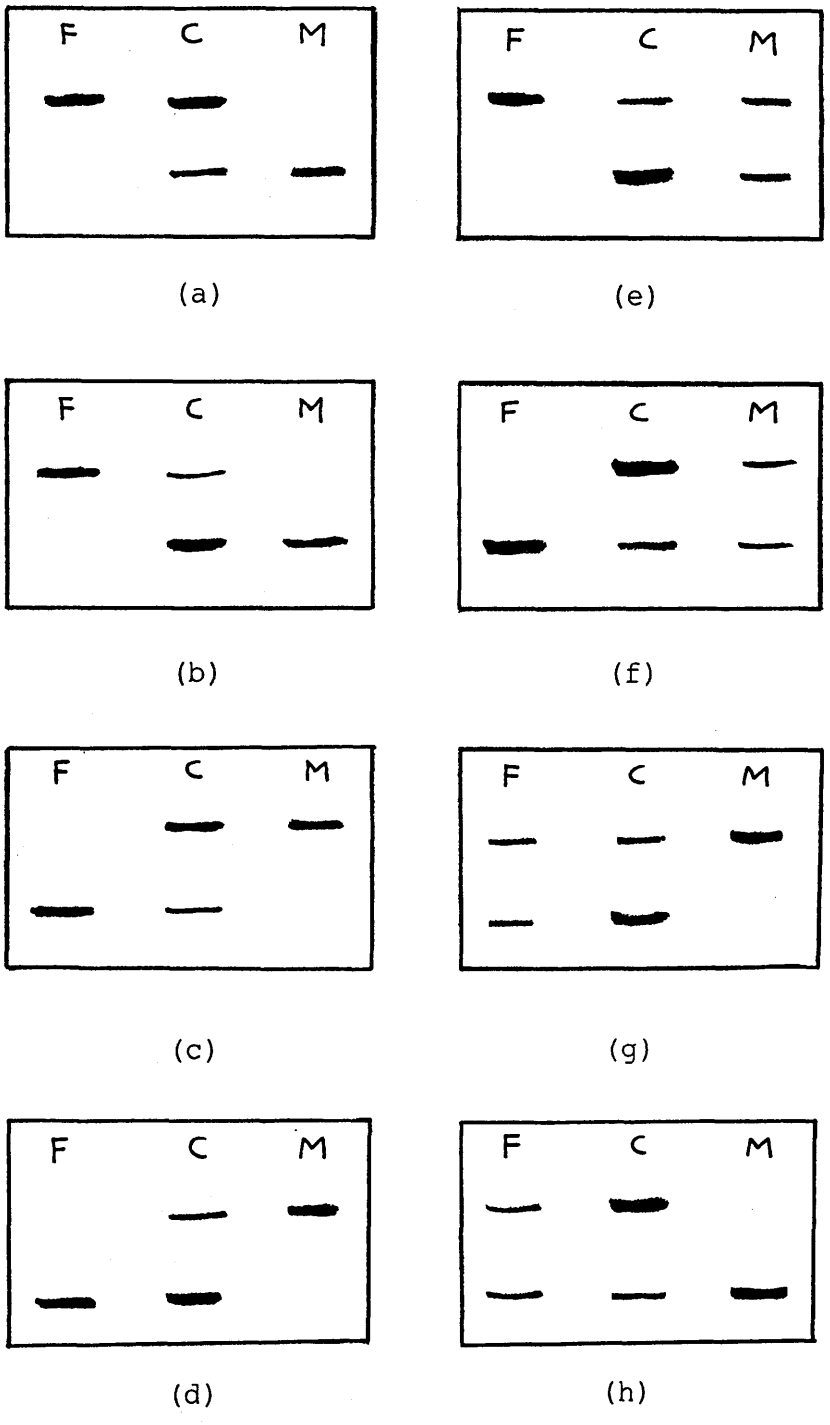


Figure 21 : Diagram showing possible informative band patterns for a two-allele RFLP in Down's syndrome nuclear families.

(F - Father, C - Child, M - Mother)

Probe Name	HGM Designation	Enzyme	Constant Band(s) (kb)	Allele Sizes (kb)	Frequency	PIC	Number of Families Tested	Number of Informative Families (%)
E8	D21S15	Msp I	3.0, 1.5	4.1 3.4, 0.7	0.54 0.46	0.37	19	3 (16%)
H8	D21S17	Bgl II	-	18.5 12.3	0.57 0.43	0.37	27	3 (11%)
D21K9	D21S13	Taq I	2.9, 2.0 1.6, 0.4	7.0 6.0	0.71 0.29	0.33	14	2 (14%)
10.2	D21S25	Hind III	-	9.5 8.5	0.40 0.60	0.36	26	7 (27%)
JG77	D21S93	Msp I	5.0	6.0 3.0	0.67 0.33	0.34	14	2 (14%)

Table 5 : Details of RFLP analysis of Down's syndrome families

Following Page 101

3.4.1 Family 1-2-3 (Figure 22)

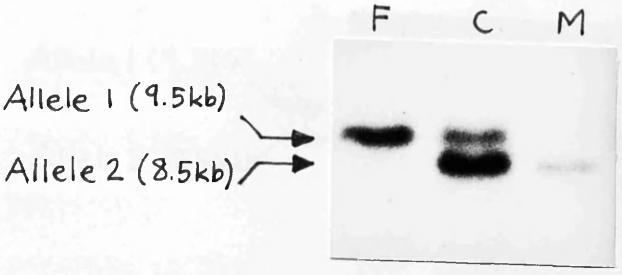
Examination of the band pattern with the 10.2/Hind III RFLP shows that the father and mother are homozygous for the 9.5 and 8.5kb alleles, respectively. The child has two copies of the 8.5kb allele and one copy of the 9.5kb allele. Since neither of the father's chromosome 21s has the 8.5kb allele, the extra chromosome in this case must therefore be derived from the mother. The band pattern with the D21K9/Taq I marker localises the nondisjunctional event to the second maternal meiotic division, since this is apparently the only‡ way in which the mother can donate two identical chromosome 21s to her offspring. (‡ - Recombination, though unlikely, may still have occurred).

3.4.2 Family 4-5-6 (Figure 23)

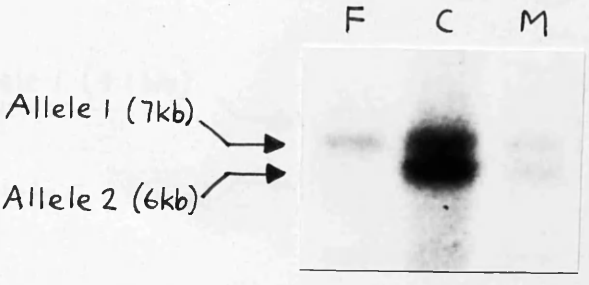
The patterns for the 10.2/Hind III and E8/Msp I RFLPs* shown here are essentially identical to those shown in Figure 22 for probes 10.2 and D21K9 with family 1-2-3. The interpretation of the results is therefore the same, and demonstrate maternal nondisjunction at the second maternal meiotic division.

(* - Results in which the meiotic stage of nondisjunction is defined by probes E8, H8, JG77 and 10.2 are subject to the effects of recombination and cannot be considered definite at this stage).

Following
Page 102



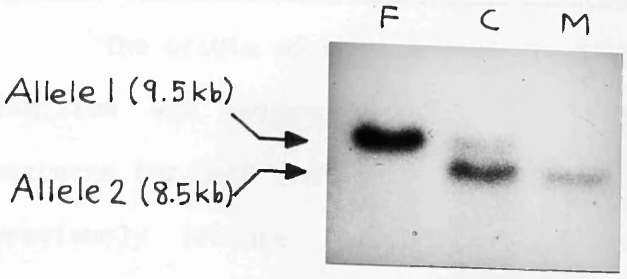
10.2/Hind III



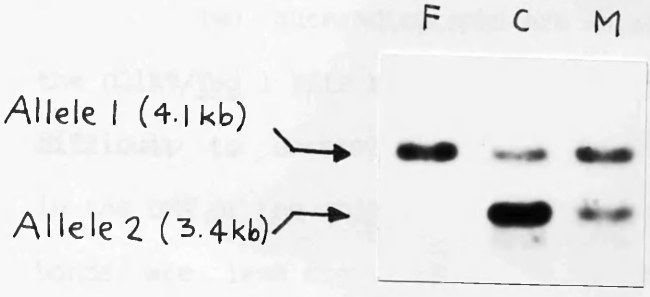
D21K9/Taq I

Figure 22 : RFLP results for family 1-2-3

Note : In this and subsequent figures, only the polymorphic bands for the D21K9/Taq I RFLP are shown. The sizes of the constant bands are given in Table 5.



10.2/Hind III



E8/Msp I

Figure 23 : RFLP results for family 4-5-6

Note : In this and subsequent figures, the 0.7kb band for the E8/Msp I RFLP is not shown, as its presence or absence from the filter can be inferred indirectly from the 3.4kb band.

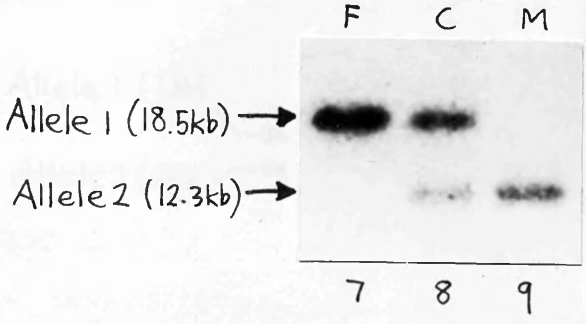
3.4.3 Families 7-8-9 and 13-14-15 (Figure 24)

The origin of the extra chromosome in both of these families was determined using the H8/Bgl II RFLP. The band patterns for both families are similiar to those discussed previously (Figure 21a and c, respectively), and it is possible to determine that the extra chromosome 21 was of paternal origin in family 7-8-9 and of maternal origin in family 13-14-15. These band patterns do not provide any information about the meiotic division at which nondisjunction occurred.

3.4.4 Family 16-17-18 (Figure 25)

Two autoradiographs are shown for this family with the D21K9/Taq I RFLP because in one of these (a) it is very difficult to determine which allele is present in duplicate in the DNA of the child. In the other film (b), although the bands are less distinct, it is clear that the child has two copies of the 7.0kb band, which shows that the additional chromosome was derived from the mother. This was confirmed by Q-banding studies, which also localised the nondisjunctional event to the first maternal meiotic division.

Following
Page 103



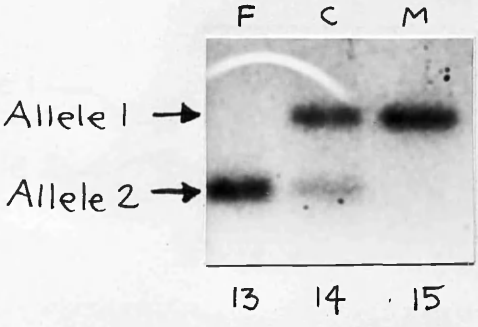
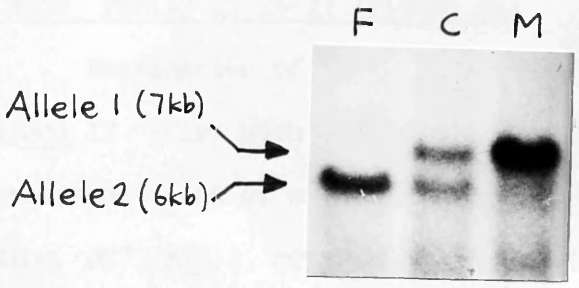


Figure 24 : RFLP results for families 7-8-9 and 13-14-15 with the H8/Bgl II RFLP.

Following
Page 103



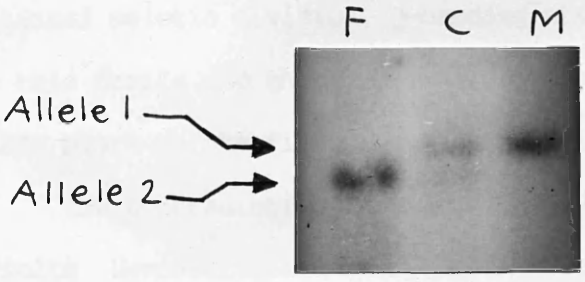


Figure 25 : RFLP results for family 16-17-18 with the D21K9/Taq I RFLP.

3.4.5 Family 25-26-27 (Figure 26)

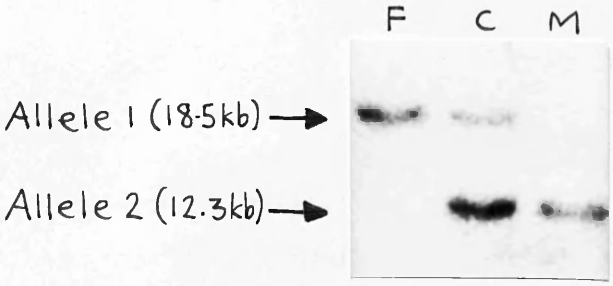
Examination of the band pattern obtained using the H8/Bgl II RFLP with this family shows that the additional chromosome 21 is of maternal origin. The results obtained using JG77/Msp I confirm maternal origin of nondisjunction and show this to have occurred at the second meiotic division. The hybridisation pattern obtained with the D21K9/Taq I marker provides no information regarding the parental origin of the extra chromosome; given the prior knowledge that maternal nondisjunction has occurred in this family, however, the band pattern with this RFLP marker demonstrates that nondisjunction took place at the first maternal meiotic division. Q-banding studies were informative in this family and these also showed nondisjunction to have taken place at the first maternal meiotic division.

The contradiction between the JG77 and D21K9/Q-banding results demonstrates that recombination has occurred between these two markers prior to nondisjunction at the first maternal meiotic division.

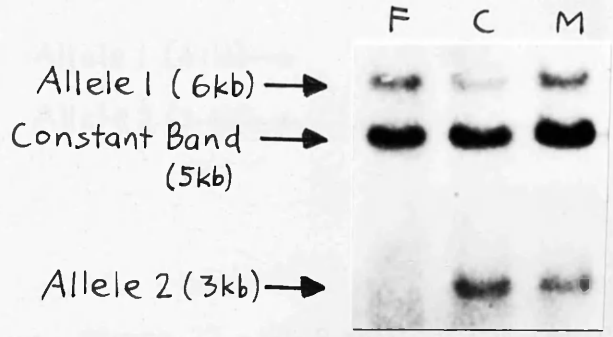
3.4.6 Family 28-29-30 (Figure 27)

The origin of the additional chromosome 21 in this family was found to be maternal by examination of the results with E8/Msp I. Studies of the cytogenetic heteromorphisms confirmed this and showed that the nondisjunctional event occurred during the first meiotic division in the mother.

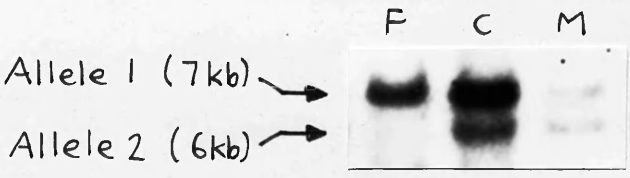
Following
Page 104



H8/Bgl II



JG77/Msp I



D21K9/Taq I

Figure 26 : RFLP results for family 25-26-27

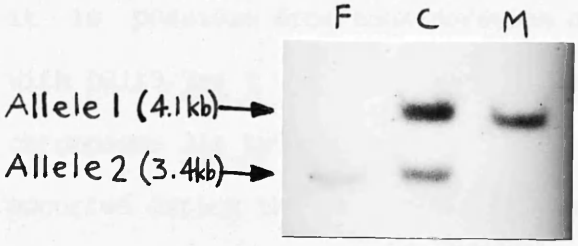


Figure 27 : RFLP results for family 28-29-30 with the E8/Msp I polymorphism

3.4.7 Family 31-32-33 (Figure 28)

Results for this family are presented for JG77/Msp I, 10.2/Hind III and D21K9/Taq I. The polymorphic alleles for the first two are identical, and show that the extra chromosome is of paternal origin. Taking this into account, it is possible from consideration of the band pattern found with D21K9/Taq I to state that since none of the child's chromosome 21s have the 6.0kb allele nondisjunction must have occurred during the second paternal meiotic division.

3.4.8. Family 37-38-39 (Figure 29)

Results obtained in this family with JG77/Msp I, 10.2/Hind III and H8/Bgl II are shown. Interpretation of the pattern with the first probe/enzyme system is straightforward and shows that the extra chromosome was derived from the mother. The band pattern obtained with probe 10.2/Hind III in this family is very interesting. It appears that one of the chromosomes in the father has the 9.5kb allele, but his other chromosome shows a band which lies somewhere between 9.5 and 8.5kb. This is probably the result of a DNA rearrangement in one of the father's chromosome 21s, leading to a larger or smaller fragment of DNA becoming homologous to the probe used. This does not affect the interpretation of the band pattern with respect to determination of the origin of nondisjunction, however, since it is evident that the father

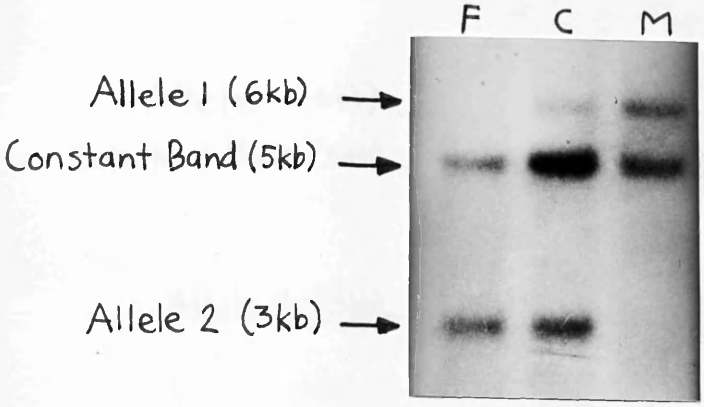
has not passed on the chromosome carrying the unusual fragment. The father has transmitted his "normal" 9.5kb allele to the child, while the mother has obviously donated two copies of the 8.5kb allele. This confirms the maternal origin of the extra chromosome defined by JG77/Msp I above.

Given the knowledge that the extra chromosome was derived from the mother, the band pattern obtained with H8/Bgl II shows that the nondisjunctional error occurred at the first maternal meiotic division.

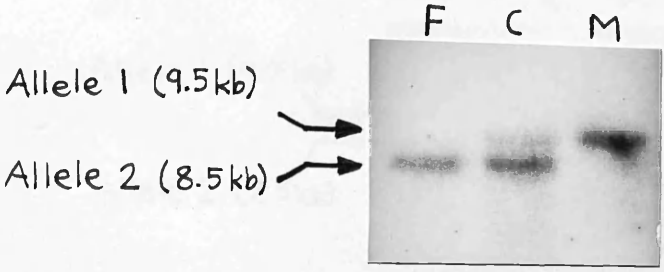
3.4.9 Families 90-92, 135-137 and 153-155 (Figure 30)

Each of these families proved to be informative for the 10.2/Hind III polymorphism. The results are explained as follows : In the first family (90-91-92) the child has two copies of the upper (9.5kb) allele and one copy of the lower (8.5kb) allele. The 9.5kb allele is not present on either of the mother's chromosome 21s, and so the extra copy of chromosome 21 must be of paternal origin. These results also indicate that nondisjunction occurred during the second meiotic division, since this seems to be the only way in which the child could receive two copies of the 9.5kb allele from the father. Following similar reasoning, the band pattern in family 135-136-137 demonstrates that nondisjunction must have occurred during the second maternal meiotic division. The results for the third family (153-154-155) are identical to others presented above and show only that the extra chromosome was maternal in origin.

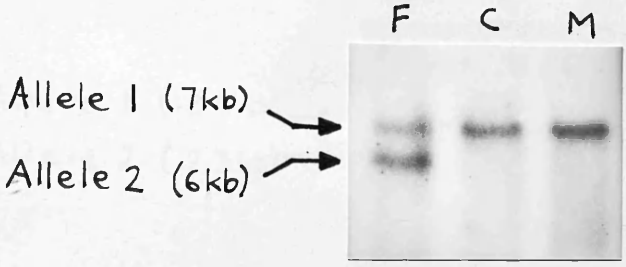
Following Page 106



JG77/Msp I

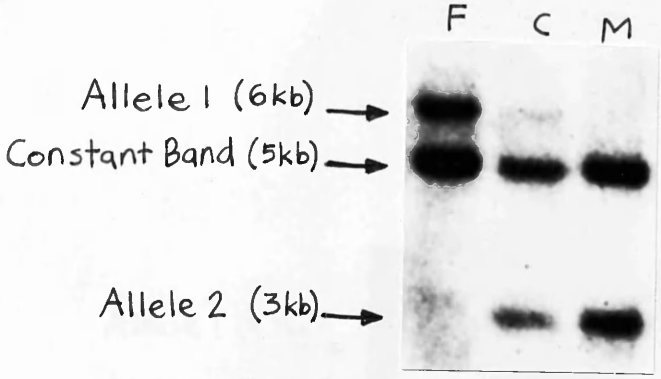


10.2/Hind III

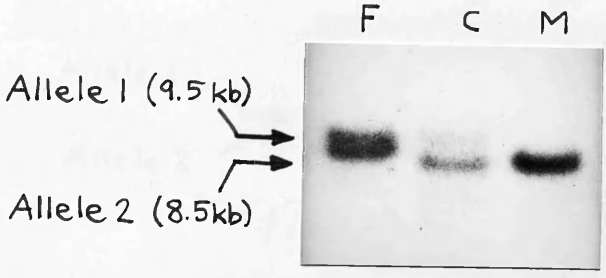


D21K9/Taq I

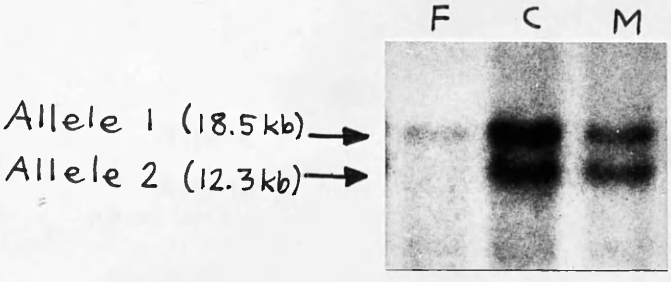
Figure 28 : RFLP results for family 31-32-33



JG77/Msp I

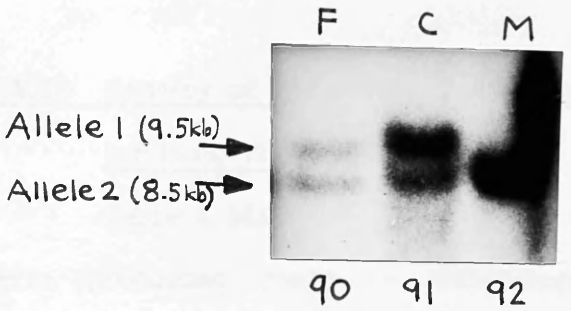


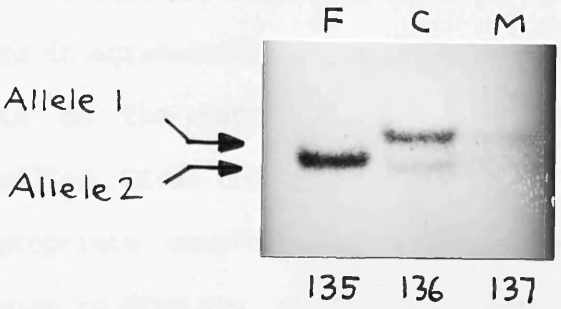
10.2/Hind III



H8/Bgl II

Figure 29 : RFLP results for family 37-38-39





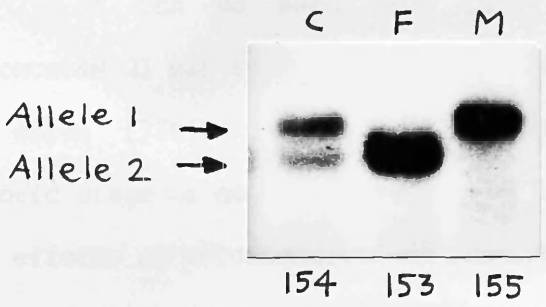


Figure 30 : RFLP results for families 90-91-92, 135-136-137 and 153-154-155 with the 10.2/Hind III RFLP.

3.4.10 Summary of Nondisjunction Analysis of Down's Syndrome Families

Table 6 lists the families in which the origin of the extra chromosome could be determined and summarises the cytogenetic and RFLP results for these families. In the three cases where Q-banding results allowed the parental origin of the additional chromosome 21 to be determined, these results were in agreement with the RFLP analyses. Table 7 presents data on the parental ages in each of the informative families. Given the small sample size, and the lack of an appropriate control population, however, it was considered unwise to draw any conclusions regarding the relationship between parental age and nondisjunction based on these results.

It can be seen from Table 6 that the additional chromosome 21 was shown to be of maternal origin in 9 of the 12 cases (75%), and of paternal origin in 3 (25%). The meiotic stage of nondisjunction is also shown in Table 6, but the effects of recombination on the definition of the meiotic stage of nondisjunction by polymorphic DNA markers should be noted at this point. For example, it may be the case that in those families apparently showing nondisjunction at the second meiotic division, the event might have actually taken place at the first meiotic division, following recombination between the centromere and the polymorphic marker in question, as described for family 25-26-27.

In five families, however, the meiotic stage of nondisjunction is defined either by cytogenetic analysis of Q-banding heteromorphisms, or by the pericentromeric RFLP marker D21S13/Taq I, or both, and can therefore be localised with confidence to a particular meiotic division. Of these, three families (16-17-18, 25-26-27 and 28-29-30) show nondisjunction at the first maternal meiotic division, while the other two show nondisjunction at the second maternal (Family 1-2-3) and second paternal (Family 31-32-33) meiotic divisions, respectively. The ratio of nondisjunction occurring at each meiotic division in these families is as follows :

Maternal I	Maternal II	Paternal I	Paternal II
0.6	0.2	0	0.2

All other results presented in Table 6 concerning localisation of nondisjunction to a specific meiotic division must be considered as provisional until they can be confirmed or corrected using pericentromeric markers.

Following Page 108

Family	Cytogenetic Analysis	Locus: Probe: Enzyme: Location:	D21S13 D21K9 Taq I q11.1	D21S93 JG77 Msp I (q22.1 -	D21S25 10.2 Hind III q22.2)	D21S17 H8 Bgl II q22.3	D21S15 E8 Msp I q22.3	Meiotic division at which nondisjunction occurred
F 1	-		11	11	11	12	-	
C 2	-		122*	112	122*	111	-	Maternal II
M 3	-		12	12	22	11	-	
F 4	ab		12	12	11	11	11	
C 5	aaa		112	122	122*	111	122*	Maternal II
M 6	aa		12	12	22	11	12	
F 7	-		22	12	22	11	12	
C 8	-		222	122	222	122*	122	Paternal
M 9	-		12	12	22	22	12	
F 13	ab		-	11	22	22	12	
C 14	aab		-	112	222	112*	112	Maternal
M 15	ac		-	12	22	11	11	
F 16	ab		22	11	-	12	-	
C 17	bcd*		112*	111	-	112	-	Maternal I
M 18	cd		11	11	-	12	-	
F 25	ab		11	11	-	11	-	
C 26	acd*		112§	122*	-	122*	-	Maternal I
M 27	cd		12	12	-	22	-	
F 28	ab		12	12	-	12	22	
C 29	bcd*		112	112	-	122	112*	Maternal I
M 30	cd		11	11	-	12	11	
F 31	-		12	22	22	12	-	
C 32	-		111§	122*	122*	122	-	Paternal II
M 33	-		11	11	11	12	-	
F 37	-		-	11	13	11	-	
C 38	-		-	122*	122*	112§	-	Maternal I
M 39	-		-	22	22	12	-	
F 90	-		-	-	12	-	-	
C 91	-		-	-	112*	-	-	Paternal II
M 92	-		-	-	22	-	-	
F 135	-		-	-	22	-	-	
C 136	-		-	-	112*	-	-	Maternal II
M 137	-		-	-	12	-	-	
F 153	-		-	-	22	-	-	
C 154	-		-	-	112*	-	-	Maternal
M 155	-		-	-	11	-	-	

Table 6 : Summary of Cytogenetic and RFLP Analysis of Down's syndrome Families

Notes:
1. F - Father, C - Child, M - Mother
2. Cytogenetic markers are arbitrarily assigned as a, b, c or d
3. Different alleles for RFLP markers are denoted by 1 and 2, respectively, except in the case of Family 37-38-39 with the 10.2/Hind III marker, where the father's rare allele is labelled "3"
4. The map position 21q22.1 - q22.2 is given for both JG77 and 10.2; the relative order of these probes on the chromosome is not known
5. * indicates results in which parental origin can be determined
6. § indicates results in which meiotic stage of nondisjunction can be inferred

Meiotic Stage of Nondisjunction	Number of Cases	Mean Maternal Age	Mean Paternal Age
Maternal I	4	32.5	34.5
Maternal II	3	39	40
Maternal I or II	2	35.5	37.5
All Maternal Nondisjunctions	9	35	37
Paternal I	0	-	-
Paternal II	2	35.5	34
Paternal I or II	1	27	29
All Paternal Nondisjunctions	3	32	33

Table 7 (b) - Parental age data on Down's syndrome families

Following Page 108

Family	Father's Age	Mother's Age	Origin of Nondisjunction
1-2-3	41	42	Maternal II
4-5-6	42	37	Maternal II
7-8-9	29	27	Paternal
13-14-15	32	30	Maternal
16-17-18	43	33	Maternal I
25-26-27	31	34	Maternal I
28-29-30	34	30	Maternal I
31-32-33	27	26	Paternal II
37-38-39	30	33	Maternal I
90-91-92	44	42	Paternal II
135-136-137	37	37	Maternal II
153-154-155	43	41	Maternal
Mean :	34	36	

Table 7a: Parental age data on Down's syndrome families

(Ages given are at birth of affected child or at termination of fetus diagnosed as having Down's syndrome)

3.5 Testing of Chromosome 21 DNA Probes for Expression using Northern Blots

RNA samples were prepared from age- and sex-matched normal and Down's syndrome fetal brain tissues and used to prepare Northern blot filters. These were hybridised with probes JG72, JG77, JG90, JG99 and JG108.

The actin gene probe pAM91 (Minty et al. 1982) was used as a control probe for these filters to ensure that the same amount of RNA was added to each lane, but pAM91 in fact showed a 3:2 dosage ratio of hybridisation signal between Down's syndrome and normal RNA (results not shown). This probe was subsequently hybridised to chromosome 21 mapping panel 6, and a signal was evident in the lane containing DNA from the 21-only hybrid, THYB133R (results not shown), indicating the presence of sequences homologous to this probe on chromosome 21 and explaining the observed increase in dosage of hybridisation.

Regarding the testing of the chromosome 21-specific sequences : probe JG99 did not hybridise to either RNA sample, suggesting that this clone is not part of an expressed sequence. Figure 33 shows the hybridisation patterns of the other four probes on the Northern blot filters. Probes JG72 and JG108 seem to be expressed at a significant level only in Down's syndrome brain. JG77 and JG90 are expressed in both normal and trisomy 21 brain tissue, and at a much higher level in the latter. Densitometry analysis (results not shown) of the

autoradiographs for these two probes showed a five-fold increase in signal intensity between normal and Down's syndrome brain samples.

Figure 31 : Autoradiographs showing Northern blot analysis of probes JG72, JG77, JG90 and JG108

(N - normal, D - Down's syndrome RNA samples)

Following Page 110

CHAPTER FOUR

DISCUSSION

The discussion is divided into 5 main sections :

Section 4.1 presents an evaluation of the different recombinant libraries and screening methods used in the current project.

Section 4.2 discusses the regional mapping of recombinants isolated during the present study and considers the usefulness of somatic cell hybrids for this purpose.

Section 4.3 is concerned with the screening of recombinant probes for the ablity to detect restriction fragment length polymorphisms (RFLPs), and evaluates the effectiveness of different RFLP screening strategies.

Section 4.4 discusses the results obtained using chromosome 21-linked RFLP and cytogenetic markers to study nondisjunction in Down's syndrome families.

Section 4.5 presents a brief summary of recent developments in mapping of genes to chromosome 21, in particular those involved in Alzheimer's disease, and considers the possible relevance of DNA sequences isolated during the present study to the pathogenesis of Down's syndrome and Alzheimer's disease.

4.1 Isolation of Recombinant Clones from Sorted Chromosome Libraries - Evaluation of the Different Screening Methods Used

4.1.1 Preassociation/cDNA screening method

The first method, used with the sorted chromosome 21 library of Young et al. (1983), involved the prehybridisation of plate filters with non-radioactive human genomic DNA, followed by hybridisation with labelled cDNA prepared from Down's syndrome fetal liver mRNA. It was hoped that this technique would select for single- or low-copy number, expressed DNA sequences, as these are more likely to represent parts of genes. All six of the clones isolated (J21.1, J21.2, J21.3, J21.4, J21.5, J21.6) contained highly repeated DNA, however, demonstrating the failure of this method to pick out low copy number sequences.

It was considered likely that due to the large insert sizes found in these six clones (which range from 9.5kb to 13.5kb), they might contain interspersed single-copy and repetitive human DNA sequences. This possibility was tested using the pre-reassociation technique (Sealy et al. 1985) and two of the clones, J21.1 and J21.2, were shown to hybridise with non-repetitive DNA sequences. Regarding clone J21.1, an attempt was made to separate this part of the cloned DNA from the repetitive DNA also present, but this proved impossible.

The single-copy human DNA sequence in clone J21.2 was found to hybridise with sequences on other human chromosomes,

but not to chromosome 21. This is not surprising, given that in the library used only 60% of the human DNA inserts are derived from chromosome 21, while the remaining 40% map to chromosome 22 (B.Young, personal communication).

Due to the lack of success with clones J21.1 and J21.2, the four remaining clones were not tested for the presence of single-copy human DNA sequences and because of the repetitive nature of all six inserts, none were tested for expression. As such it was not possible to evaluate the degree of success of this method in isolating expressed sequences.

The cDNA screening technique described in this project was used successfully with a similar recombinant library and allowed the isolation of five single-copy DNA sequences from the human Y chromosome (Florentin 1987). For all five of these probes, however, this involved subcloning of the single-copy sequence from a larger original clone which also contained repetitive DNA sequences, as attempted here for clone J21.1. This is however a difficult and time-consuming process.

4.1.2 Selection of plaques which failed to hybridise with labelled human genomic DNA

A chromosome 21-specific flow-sorted recombinant DNA library supplied by the National Gene Library Project (Lawrence Livermore laboratory) was also screened during the current project. This library was prepared by digestion of flow-sorted chromosome 21 DNA with the restriction enzyme

Hind III and subsequent cloning into the Hind III site of the bacteriophage vector Charon 21A.

This library has two main advantages over that described above (Young et al. 1983) : firstly, it is estimated to contain over 95% chromosome 21 inserts (Information supplied with library), increasing the likelihood of isolating DNA sequences from chromosome 21. Secondly, the vector used will only accept for cloning fragments of less than 9.1kb (Fuscoe et al. 1987) this relative reduction in the size of human DNA inserts increases the probability of isolating clones containing only single-copy DNA sequences.

The method used for the selection of recombinants from this library involved the isolation of plaques failing to show hybridisation with radioactively labelled human genomic DNA, in the hope that these would contain human DNA inserts of low copy-number. This method had been used previously with the sorted chromosome 21 library mentioned above (Young et al. 1983) to isolate five chromosome 21-specific DNA probes (Stewart 1984, Stewart et al. 1985, Davies et al. 1984). Three of these five recombinants have subsequently been used to isolate homologous clones from a cDNA recombinant library (Neve et al. 1986), which shows that they represent expressed sequences. This demonstrates that it is possible to pick out single-copy, expressed sequences from this library and suggests a possible flaw in the cDNA method described for the selection of such recombinants.

An obvious problem with the selection of plaques failing to hybridise with human genomic DNA is that a significant proportion of plaques picked will not contain any human DNA. This was found to be the case here, with 80% of the plaques examined falling into this category. Of the 24 clones with human DNA inserts isolated, however, two-thirds contained single or low copy-number human DNA sequences, demonstrating the usefulness of this technique.

The main advantage of this method over the previous one is that it allows rapid isolation and characterisation of recombinant clones : following the initial selection of non-hybridising plaques, it is a relatively simple matter to purify the bacteriophage DNA and digest with a restriction enzyme to test for the presence of human insert DNA. The current practice of radioactively labelling DNA samples excised from LMP agarose directly allows inserts to be tested immediately to establish whether they contain single-copy sequences. As described in the text, these can then be used in Southern blot experiments, allowing them to be regionally mapped on the chromosome and tested for the presence of RFLPs, and on Northern blots to determine whether they represent expressed sequences.

Although it is not possible to compare the two screening strategies directly, as they were not used to screen the same recombinant library, the main objectives of isolating (i) single-copy and (ii) expressed sequences were more easily accomplished using the second technique described

(i.e. selection of plaques failing to hybridise with human genomic DNA), and as this method was the more practical and successful of the two it is recommended for this type of work.

Another method potentially of great value in the screening of such recombinant libraries is the use of an oligonucleotide probe complementary to the insertion site of the vector, as descibed by Fuscoe et al. (1987). This probe hybridises strongly to non-recombinants, but less stably when a cloned insert is present, and could be used in the selection of recombinant clones.

4.2 Regional Mapping of DNA Sequences on Chromosome 21

4.2.1 Consideration of the Usefulness of Somatic Cell Hybrids

Advances in the development of methods for the construction and characterisation of somatic cell hybrids have been one of the major contributing factors in the recent rapid evolution of the molecular genetic map of chromosome 21. Use of hybrids containing translocation derivatives of chromosome 21 in mapping experiments, as performed in the current study, has led to the accurate regional assignment of numerous DNA sequences (Van Keuren et al. 1986a, Korenberg 1987).

The utility of such chromosome 21 hybrid panels is limited, however, by the different translocation chromosomes available for study. This dependance on naturally occurring rearrangements limits the variety of hybrids that will be found and excludes many potentially informative chromosomal rearrangements. In addition, the presence of non-chromosome 21 human material in many of these hybrids confounds certain types of analyses, especially those involving repetitive sequences.

These problems have been overcome by some workers who have constructed somatic cell hybrids containing artificially fragmented pieces of chromosome 21 (Graw et al., 1985, Graw et al., 1988). The method used involves X-ray irradiation of whole cells to fragment the genome.

The segment of interest is then rescued by fusion of the irradiated cells to rodent cells and application of selective pressure. The authors have used this technique with some success in constructing a panel of Chinese hamster/human hybrids that contain segments of chromosome 21 centered about phosphoribosyl glycinamide synthetase (GARS), the selected marker.

Another possible problem associated with somatic cell hybrids is the occurrence of rearrangements between human and rodent chromosomes. These seem to occur frequently in interspecific somatic cell hybrids and the use of the differential G-11 stain to identify such recombinant chromosomes is advisable. Detailed molecular and cytogenetic characterisation of hybrids should also be carried out, due to the effects of deletion and other rearrangements. The hybrid cell lines used in the current project have been extensively characterised using both cytogenetic (including G-11 staining) and molecular methods (see Materials & Methods, Section 2.2). This allows the regional localisation of the probes presented here to be stated with a high degree of confidence.

4.2.2 Chromosomal Localisation of Probes Isolated During the Present Study

All 15 probes isolated during this study have been regionally mapped on chromosome 21 using a range of somatic cell hybrids containing different parts of the chromosome.

None of the sequences isolated from the recombinant library used here were derived from any other human chromosome. The contamination of such libraries with DNA sequences from chromosomes other than the desired one has been a problem in the past, but the development of bivariate flow-sorting of human chromosomes (Deaven et al. 1986) has almost eliminated this problem.

Five of the probes were mapped more accurately using additional hybrid cell lines which contained different parts of chromosome 21. Obviously, the greater number of hybrids containing different parts of chromosome 21 used, the more accurately probes can be mapped.

Another useful technique in the chromosomal assignment of human DNA sequences is that of in situ hybridisation, although regional localisation on a small chromosome like chromosome 21 is quite difficult. It was intended that this method should be used with the mild repeat probes JG51 and JG73, since it is likely that this would demonstrate clearly whether or not these probes detected homology with sequences on human chromosomes other than chromosome 21. Unfortunately time did not allow these experiments to be carried out.

As shown in Figure 15, all of the probes isolated here map on the long arm of chromosome 21, half above and half below the q2.1/q22.1 junction defined by hybrid ACEM2-90. None of the single-copy probes isolated here mapped to the short arm of chromosome 21. This is not surprising, because, to date, no single-copy or repeated probes specific to the

short arm of chromosome 21, or any other acrocentric chromosome, have been isolated (HGM 9, 1988). It is likely that the difficulty in isolating such sequences arises from the occurrence of somatic recombination between rDNA sequences on non-homologous chromosomes (see, for example, Worton et al. 1987).

Probes JG51 and JG73 are of interest because of their multiple-band hybridisation patterns. It would appear that although originally isolated from a chromosome 21-specific recombinant library, these probes are closely related members of a mildy repetitive DNA sequence family, members of which are found on a number of human chromosomes including chromosome 21. The results obtained by hybridising these two probes to hybrid mapping panel 8 (Results Section 3.2.8, Figure 14) showed that a significant proportion of sequences homologous to these probes are present in the pericentromeric region of chromosome 21. This raises the possiblility that these sequences are members of the alphoid repeat family, members of which are present in the centromeric regions of all human chromosomes (Willard 1985, Willard et al. 1986). Definite proof of this would require the demonstration of cross-homology with known alphoid sequences, or detailed molecular analysis of these sequences, and there was unfortunately insufficient time for these experiments to be carried out.

A mildly repetitive sequence which shows a very similar hybridisation pattern to probes JG51 and JG73 was isolated

from a chromosome 21 recombinant library by virtue of its hybridisation to a repetitve sequence originally derived from chromosome 12 (Law & Van Keuren 1986). The authors demonstrate that such cloned mild repeat sequences can be used in the molecular characterisation of rearrangements of human chromosome 21, using a similar technique to that described for the characterisation of a range of somatic cell hybrids containing different parts of chromosome 11 (Gusella et al. 1982). Given the similarities in hybridisation pattern between probes JG51/JG73 and that described by Law & Van Keuren, it is possible that JG51/JG73 could also prove useful in such analyses.

4.3 Screening of Recombinants for Restriction Fragment Length Polymorphisms (RFLPs)

The usefulness of arbitrary DNA sequences in human genetic analysis relies mainly on their ability to detect restriction fragment length polymorphisms (RFLPs), as described previously (Introduction, Section 1.2.3). The probability that an RFLP will be found depends upon (i) the number of individuals tested, (ii) the number of restriction enzymes used, and (iii) whether the probe being tested is X-linked or autosomal (see Hofker et al. 1986).

Two different strategies are generally applied in screening DNA probes for RFLPs. The first uses a few DNA samples and a large number of restriction enzymes, with the intention of revealing polymorphisms with high rare allele frequencies. The second method uses numerous DNA samples, but only a few restriction enzymes, and is intended to find as many RFLPs as possible, regardless of their frequency.

4.3.1 RFLP Screening with Few DNAs, Numerous Enzymes

Obviously, the fewer individuals examined, the more frequent a polymorphism must be in order to be detected. RFLPs detected in this way are therfore more likely to be informative in the general population, since a greater number of individuals will be heterozygous. A study in which DNA probes from the X chromosome were screened in this way confirms the usefulness of this method (Aldridge et al. 1984).

In the present study, RFLP screening was initially carried out using DNA from 5 unrelated individuals digested with 15 different restriction enzymes. This revealed two RFLPs : JG77/Msp I and JG99/Pst I. Following further testing of these polymorphisms, the rare allele frequencies were found to be 0.33 and 0.16, respectively. It has been suggested that minor allele frequencies of less than 0.2 do not prove useful in the analysis of nondisjunction (Stewart et al., 1988). As such, only the JG77/Msp I polymorphism was used in the analysis of nondisjunction in Down's syndrome families. The origin of the additional chromosome was determined in 2 of the 14 families tested with this probe/enzyme combination, showing it to be of some value in this type of analysis.

4.3.2 RFLP Screening with Numerous DNAs, Few Enzymes

The second and most commonly used method to screen for RFLPs uses a large number of DNA samples digested with few restriction enzymes. This increases the probability of detecting RFLPs, but means that a significant proportion of those found will have low rare allele frequencies (Skolnick & White 1982). Since only two RFLPs were detected in the present study using 5 DNA samples, further testing was carried out using 7 different DNAs. This revealed the JG90/Nde II polymorphism. This RFLP has a minor allele frequency of 0.3, suggesting that it should prove useful in nondisjunction analysis. It should be noted that Nde II was

not used in the original, five-member screening panel, and it is likely that the relatively high frequency of this RFLP would have allowed its detection using only five DNA samples. Unfortunately, time did not allow the use of this RFLP in nondisjunction analysis.

4.3.3 RFLP Screening using Longer Gels

Another possible factor in RFLP screening, apparently neglected by many workers in the field, is that certain RFLPs may avoid detection because the difference in size between the polymorphic alleles is insufficient to allow their resolution on the agarose gels normally used in Southern blot experiments, which typically range from about 7cm to 12cm in length. This was confirmed in the current project, where a slight variation in the band pattern found with probe JG81/*Pvu* II on normal (10cm long) gels was shown, using longer (20cm) gels, to be an RFLP in which the polymorphic alleles were 8.5 and 8.0kb, respectively. The resolution of these fragment sizes on a normal gel is insufficient to allow accurate interpretation of the allele(s) present in any given individual, and the use of long gels for the analysis of this RFLP is necessary. The other polymorphism revealed during this project was for probe JG81 with the restriction enzyme *Eco* RV. The allele sizes in this case were 4.5 and 4.0kb, respectively, suggesting that although only tested here on long gels, results with this RFLP would be easily interpreted using normal gels.

As mentioned above, RFLPs detected using a large number of DNA samples have relatively low rare allele frequencies. This was confirmed here, as the JG81/Pvu II and JG81/Eco RV RFLPs have frequencies of 0.17 and 0.12, respectively. Given these values, it is unlikely that these RFLPs would be of great value in nondisjunction analysis. It could be argued that frequencies may vary in populations of different ethnic origin, but the 12 individuals used in the long RFLP screening panel came from widely varying geographic locations, suggesting that this possibility does not apply to these polymorphisms.

4.3.4 Summary of RFLP Screening

In the present study, five RFLPs were discovered with four of the recombinant probes tested. Only two of these have rare allele frequencies of greater than 0.2. The usefulness of one of these polymorphisms in nondisjunction analysis has been demonstrated.

Some of the chromosome 21 probes isolated in this project have not yet been screened for RFLPs, while others have been tested with only a limited number of restriction enzymes. The possibility therefore exists that additional polymorphisms may be detected using these chromosome 21-specific probes.

4.4 Determination of Parental Origin of Nondisjunction in Down's Syndrome Families

As mentioned in the introduction (Section 1.2.2), almost all of the information to date on the parental origin of nondisjunction in Down's syndrome has come from studies of chromosome heteromorphisms. Some of the problems inherent in such analysis can be overcome using RFLPs, and one of the aims of the current project was to use chromosome 21-linked RFLPs to determine the parental origin of the additional chromosome 21 in Down's syndrome families.

4.4.1 Determination of Parental Origin Using Polymorphic DNA Markers

During the course of the current project a total of 33 Down's syndrome families were examined using 5 chromosome 21-specific RFLP markers, and although it was not possible to test each family with all 5 probes, the parental origin of the additional chromosome was determined in 12 families (36%). The majority of cases showed maternal nondisjunction (9 families - 75%) as opposed to paternal nondisjunction (3 families - 25%).

Four other groups have published data on the analysis of nondisjunction in trisomy 21 using RFLP markers. The results obtained in each of these studies are summarised in Table 8, which also provides for comparison results obtained using cytogenetic methods. It is obvious from this table that the limited number of results available from RFLP studies of

nondisjunction in trisomy 21 confirm the findings of the numerous cytogenetic studies to date, which show that 80% of nondisjunction errors occur in the mother, while the remaining 20% take place during paternal meioses.

4.4.2 Studies of Nondisjunction Using a Combination of Cytogenetic and RFLP Techniques

Analysis of Q-banding heteromorphisms was also carried out during this study. Due to the constraints of time and resources, however, only five families were tested in this way. Of these, three proved to be informative and showed nondisjunction at the first maternal meiotic division. In each case the cytogenetic results were in agreement with those obtained using RFLPs, and in two families they provided information on the meiotic division responsible for the nondisjunctional error which was not provided using RFLP markers alone.

Results of a similar study of trisomy 13 were recently published by Hassold et al. (1987), who used a combination of Q-banding and RFLP analysis, and in all seven cases examined in this way, the parental origin of the additional chromosome was determined (5 maternal, 2 paternal).

Cytogenetic and molecular methods have also been combined successfully by Stewart et al. (1988) in a pilot study of five Down's syndrome families. They used probes of 16 polymorphic DNA loci from chromosome 21 in conjunction with Q- and NOR-banding techniques. This allowed the

determination of the parental origin of the extra chromosome in all five cases (4 maternal, 1 paternal). In both of these studies, neither the cytogenetic nor the molecular methods alone revealed the origin of the additional chromosome in every case, and a combination of both was required to achieve this. It is evident from all three studies that the efficiency with which the origin of nondisjunction can be determined has been greatly increased by the use of RFLP analysis. The study of Q-banding heteromorphisms allows the origin of the extra chromosome 21 to be determined in 50% of cases; when combined with the examination of the NOR heteromorphisms revealed by silver staining, this figure rises to over 80% (Mikkelsen et al. 1980).

It is obvious that if a sufficient number of RFLP markers were used to examine nondisjunction in Down's syndrome families, the origin of the error should be found in every case. (A statistical consideration of this topic is given in Stewart et al. 1988). The studies mentioned above by Hassold et al. (1987) and Stewart et al. (1988) suggest that a different strategy would be just as productive, since they have used only a fraction of the available polymorphic chromosome markers in conjunction with Q- and NOR-banding to achieve this 100% success rate.

Following Page 128

	Reference		Parental Origin of Additional Chromosome 21	
			Maternal	Paternal
Studies using cytogenetic markers	Mikkelsen et al.	(1980)	61	12
	Magenis & Chamberlin	(1981)	151	46
	Jongbloet et al.	(1981)	51	13
	Hatcher et al.	(1982)	24	3
	Ayme et al.	(1986)	416	98
	Total		703	172
	Percentage		80%	20%
Studies using RFLP markers	Davies et al.	(1984)	1	0
	Antonarakis et al.	(1986)	22	3
	Stewart et al.	(1988)	4*	1
	Rudd et al.	(1988)	7	2
	Present study	(1988)	9	3
	Total		43	9
	Percentage		83%	17%

Table 8 : Comparison of cytogenetic and RFLP studies of nondisjunction in Down's syndrome families

(* - In one of the cases described by Stewart et al. (1988), RFLP analysis failed to reveal the parental origin of nondisjunction, and cytogenetic techniques provided this information)

4.4.3 Localisation of Nondisjunction to a Specific Meiotic Division

Although any chromosome 21 marker can provide information on the parental origin of nondisjunction in Down's syndrome, markers for the pericentromeric region are essential to determine at which meiotic stage the error occurred. This is because recombination can occur between the centromere and polymorphic markers, which may lead to anomalous interpretation of results.

Use of markers closely linked to the centromere (preferably on both sides) would minimise the effects of such recombination. There are two types of useful pericentromeric marker available : (i) the cytogenetic heteromorphisms of Q- and NOR-banding on the short arm of chromosome 21, and (ii) polymorphic DNA markers which map in the proximal long arm (band 21q11). Although crossing-over could theoretically occur between the centromere and these pericentromeric markers, the chromosomal region involved is very small and the probability of recombination taking place is low. As the use of cytogenetic heteromorphisms in the analysis of nondisjunction are discussed extensively elsewhere (e.g. Magenis & Chamberlin 1981), no further mention of these markers is made here.

4.4.4 Polymorphic DNA Markers Close to the Centromere

The D21S13/Taq I marker used in this project (detected by probe D21K9; Davies et al. 1984) is currently the most proximal marker on the linkage map of chromosome 21 (R.Tanzi, personal communication), and as such is the most useful marker available for the determination of the meiotic stage of nondisjunction in trisomy 21.

Another pericentromeric marker, D21S110/Msp I (detected by probe p21-4U; D.Kurnit, personal communication) is tightly linked to D21S13, as shown by the fact that there were no recombination events between these two markers in 29 informative meioses (R.Tanzi, personal communication). The combined use of these two RFLP markers allows chromosome 21 homologues to be distinguished in a significant number of subjects and these markers represent a highly informative pericentromeric haplotype (Stewart et al. 1988).

In the study by Antonarakis et al. (1986; results also presented in Warren et al. 1987), the authors constructed a linkage map of chromosome 21 in which they favoured the relative order cen - D21S111 - D21S13 - (D21S1/D21S11) over cen - D21S13 - D21S111 - (D21S1/D21S11), and gave the distance between D21S111 and D21S13 as 14cM. On the basis of this data they used the D21S111/Sst I polymorphism (identified by probe CW21pc) as a pericentromeric marker to determine the meiotic division at which nondisjunction occurred (details in section 4.4.5). The number of informative meioses used in the derivation of this linkage

order was small (Warren et al. 1987), however, and a more extensive study (R.Tanzi, personal communication) showed D21S111 to be at least 15cM distal to D21S13. The order of markers on 21q is therefore : cen - (D21S13/D21S110) - 15cM - D21S111, placing D21S111 at a substantial distance from the centromere. Results obtained using this RFLP marker to determine the meiotic stage of nondisjunction are therefore open to some dispute, since there is a distinct possibility that recombination might take place between the centromere and this marker.

Studies of chromosome-specific alphoid repeat sequences (see for example Willard et al. 1986) may provide useful polymorphic markers for the centromere of chromosome 21, but as yet no such probe has been isolated.

4.4.5 Evaluation of Results obtained using RFLP Markers to Determine the Meiotic Stage of Nondisjunction

In the present study, the meiotic stage of nondisjunction was defined in four families using probes which map distal to the q21/q22.1 junction (see Table 6 for details). As such, there is a significant probability that recombination might have occurred between the centromere and these RFLP markers. These results on the meiotic stage of nondisjunction must therefore be regarded as "provisional", until they can be confirmed or corrected using appropriate pericentromeric markers.

In the other five cases in which the meiotic stage of nondisjunction was determined, this information was provided by the pericentromeric RFLP marker D21S13/Taq I and/or by analysis of cytogenetic heteromorphisms on 21p. These results can be considered reliable, and showed that nondisjunction took place at maternal meiosis I in 3 cases and at maternal meiosis II and paternal meiosis II, respectively, in the other two cases. Identical results were obtained by Stewart et al. (1988) in their pilot study of five families.

In the recent paper by Rudd et al. (1988), the authors used chromosome 21-linked RFLP markers to study the parental origin of nondisjunction in 20 Down's syndrome families. They did not attempt to determine the meiotic stage of origin, however, and in fact selected parental RFLP patterns in which it would be impossible to do so. Also, the problems caused by the use of D21S111 as a pericentromeric marker effectively nullify the results presented by Antonarakis et al. (1986) regarding the localisation of nondisjunction to a particular meiotic division. It is interesting to note that these workers also used the D21S13/Taq I RFLP in their analysis of Down's syndrome families, but did not think it closely linked to the centromere, and did not use the results obtained with this probe to localise nondisjunction to a specific meiotic division. As such, the results presented in this study (for families 1-2-3, 16-17-18, 25-26-27, 28-29-30 and 31-32-33) and in that of Stewart et al. (1988) are the only reliable results available in which RFLP and cytogenetic

markers have been combined successfully to localise nondisjunction in Down's syndrome families to a specific meiotic division. The ratio of nondisjunction occurring at each meiotic division in these 10 families can therefore be summarised as follows :

Maternal I	Maternal II	Paternal I	Paternal II
0.6	0.2	0	0.2

These values comply almost exactly ($X^2_3 = 0.13$, $p=0.95$) with the maximum likelihood estimates for nondisjunction at each meiotic division calculated by Hassold et al. (1984), based on the results obtained in cytogenetic analyses :

Maternal I	Maternal II	Paternal I	Paternal II
0.67	0.13	0.07	0.13

This demonstrates the reliability of using pericentromeric RFLP and cytogenetic markers in the localisation of nondisjunction to specific meiotic division in the parents of Down's syndrome families.

4.4.6 Effects of Recombination on RFLP Analysis of Nondisjunction

Crossing-over between two nondisjoined chromosomes can only be detected when the parent of origin is heterozygous for two or more markers. An example of this type of pattern was described previously for family 25-26-27 (Results Section 3.4.5.). It can be stated that in this family a crossover event must have occurred between the markers D21S13/Taq I and JG77/Msp I, followed by nondisjunction at maternal meiosis I.

Stewart et al. (1988) describe similar crossover events in two of the five families in their pilot study. In one example cytogenetic markers and a polymorphic DNA marker on the proximal short arm of chromosome 21 are consistent with a maternal meiosis I error, while two more distal RFLP markers indicate a maternal meiosis II error. Examination of the band patterns for these probe/enzyme combinations demonstrates that nondisjunction took place at maternal meiosis I, and must have been preceded by a crossover between two of the polymorphic markers. In the other example, the converse is true, with a recombination event occurring prior to nondisjunction at maternal meiosis II. Recombination was also demonstrated in four of the seven cases of trisomy 13 described by Hassold et al. (1987). Crossing over occurred prior to nondisjunction at maternal meiosis I in two cases, and at maternal meiosis II and paternal meiosis I in the other two cases. These results demonstrate that recombination between the centromere and RFLP markers on the long arm of

chromosome 21 can complicate the analysis of nondisjunction using polymorphic DNA probes. The combination of pericentromeric cytogenetic and RFLP markers with polymorphic markers on the long arm of the chromosome should eliminate the confounding effects of such recombination.

4.4.7 Factors Affecting Nondisjunction - Recent Work

1. Possible Involvement of Risosomal RNA Genes in Nondisjunction

The ribosomal RNA gene cluster on chromosome 21 (RNR4) is located in the secondary constriction, or "stalk" region, in band 21p12. These secondary constrictions associated with cell nucleoli are called nucleolar organising regions (NORs). A recent study of the inheritance of NOR variants (visible after silver staining) in trisomy 21 families suggested that a particular type of heteromorphism, called a double NOR (dNOR) may play a role in meiotic nondisjunction (Jackson-Cook et al. 1985). To date, however, no other group (e.g. Spinner et al. 1986, Hassold et al. 1988) has been able to confirm a high frequency of dNOR variants in Down's syndrome subjects and families.

Also, the tendency of ribosomal genes to undergo non-homologous recombination has been proposed as a possible mechanism, via the formation of dicentric chromosomes, for promoting meiotic nondisjunction (Schmickel et al. 1985).

2. Possible Effects of Aberrant Recombination Levels on Nondisjunction

As mentioned in the Introduction (Section 1.2.5), aberrant levels of recombination have been proposed as a possible factor influencing the normal segregation of chromosomes at meiosis. Use of the centromere mapping technique (Ott et al 1976) allows the possible effects of aberrant levels of recombination on nondisjunction in Down's syndrome to be evaluated by estimating the levels of recombination on chromosomes 21 involved in normal meioses with those undergoing nondisjunction.

This approach was used recently by Antonarakis et al. (1986), in a study of 34 Down's syndrome families, their parents and normal sibs. They reported statistically significant reductions in the map distances associated with trisomy 21 and stated that recombination involving the two nondisjoined chromosomes was rarely detected. From these results it was suggested that recombination is reduced on chromosome 21s that undergo nondisjunction and that defective pairing may consequently be responsible for a large proportion of cases of trisomy 21.

Apart from small sample size, there are some technical limitations to this study, however. Firstly, they used markers that did not span the terminal third of chromosome 21. Since the linkage map of chromosome 21 (R.Tanzi, personal communication) shows a high frequency of terminal chiasmata on 21q, markers from this part of the chromosome should also

be included in any such study. Secondly, they used D21S111 as a pericentromeric marker and, as described previously (Section 4.4.4) this locus is at a substantial distance from the centromere, and as such is not the ideal choice for determination of the meiotic stage of nondisjunction.

The results presented in the present study, and by other workers (Hassold et al. 1987, Stewart et al. 1988), demonstrating recombination between nondisjoined chromosomes in a significant proportion (44%*) of cases suggest that pairing failure is not the only cause of autosomal trisomy. In order to fully investigate the possible relevance of aberrant recombination levels on nondisjunction, a large sample size should be studied using both pericentromeric cytogenetic and RFLP markers in conjunction with RFLP markers spanning the whole length of the long arm of chromosome 21.

(* - As mentioned above (Section 4.4.6), crossing-over between two nondisjoined chromosomes can only be detected when the parent of origin is heterozygous for two or more markers. Only cases in which these conditions are satisfied are included in the calculation of this figure. Details as follows : Present Study - 1 out of 5 cases; Stewart et al. (1988) - 2 out of 5 cases; Hassold et al. (1987) - 4 out of 6 cases)

4.5 Genes Mapped to Chromosome 21 - Recent Work

As with other human chromosomes, recent advances in molecular genetic techniques, particularly in the field of somatic cell genetics, have led to the assignment of additional genes to chromosome 21 and to the more accurate localisation of genes previously mapped to this chromosome (for details see HGM 9, 1988). The number of anonymous DNA sequences localised to chromosome 21 has also increased enormously : from 9 at the beginning of this project to the present total of 99 (see HGM9). In fact, this number does not include the 13 single-copy chromosome 21-specific DNA sequences isolated during the present study. These therefore represent almost 12% of the arbitrary DNA sequences so far mapped to this chromosome, and as such are a major contribution to the molecular map of chromosome 21.

The regional localisation of polymorphic DNA probes on chromosome 21 and their incorporation into genetic linkage maps of the chromosome has shown that the physical and genetic maps of chromosome 21 are in close agreement (see, for example, Watkins et al. 1987).

Some of the most exciting recent developments in the molecular analysis of chromosome 21 concerned the localisation to this chromosome of genes involved in Alzheimer's disease; these are summarised in section 4.5.1. The possible relevance of the DNA probes isolated in the present study to Down's syndrome and Alzheimer's disease is considered in section 4.5.2.

4.5.1 Recent Developments in the Study of the Molecular Biology of Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by widespread functional disturbance of the human brain. The fact that almost all Down's syndrome patients over the age of 40 display this characteristic neuropathology (Wisniewski et al 1985) suggested a possible link between the two disorders. This theory is supported by recent developments in the study of AD at the molecular level, as follows.

1. Localisation of the Amyloid Plaque Protein (A4) to Chromosome 21

A major structural component of the neuritic plaques found in the brain of AD patients is a substance called amyloid. The major subunit of this is an insoluble highly aggregating small polypeptide called amyloid beta protein A4. Three different groups of researchers used synthetic oligonucleotides deduced from the sequence of the A4 polypeptide to isolate cDNA clones encoding the complete A4 subunit protein from foetal brain cDNA libraries (Goldgaber et al. 1987, Kang et al. 1987, Tanzi et al. 1987a). All three groups mapped the amyloid beta peptide (AP) gene to chromosome 21 using somatic cell hybrids, while Tanzi et al. (1987a) regionally localised the gene to 21q11.2 - 21q21.

2. Localisation of the Gene for Familial Alzheimer's Disease (FAD) to Chromosome 21

Several large families have been reported in which autosomal dominant transmission of AD is apparent (Nee et al. 1983, Goudsmit et al. 1981). Given the suggested link between AD and Down's syndrome and the availability of numerous polymorphic DNA markers on chromosome 21, one group of researchers decided to test for co-segregation of the gene for FAD and several anonymous chromosome 21 DNA markers (St George-Hyslop et al. 1987a). Positive LOD scores were obtained for linkage of FAD with the DNA markers D21S16 ($z = 2.32$ at $\theta = 0.00$) and D21S1/D21S11 ($z = 2.37$ at $\theta = 0.08$), which map in the region 21q11.2 - 21q21. The LOD scores for neither probe were greater than three, however, and could not by themselves be considered as proof of linkage. Conclusive evidence of the localisation of the FAD gene to chromosome 21 was provided by three point analysis of FAD, D21S16 and D21S1/D21S11, which provided a peak LOD score of 4.25. Testing of additional DNA markers from this region on these and other FAD pedigrees should allow a more accurate location for the FAD gene to be defined.

---

3. Does Duplication of Chromosome 21 Genes Cause Alzheimer's Disease?

One group of workers claimed to have shown that the AP gene is duplicated in patients with Alzheimer's disease (Delabar et al. 1987) and suggested that the neuro-

pathological changes observed in aged Down's syndrome individuals might be caused by over-expression of the amyloid beta peptide (AP) gene due to the presence of the extra copy of chromosome 21 in these patients. Recent reports have shown no evidence of duplication of chromosome 21 genes in individuals with Alzheimer's disease (St George-Hyslop et al. 1987b, Tanzi et al. 1987c, Podlisny et al. 1987) and suggest that the results obtained by Delabar et al. (1987) are an artifact of the experimental methods used. It would appear, therefore, that duplications in the AP gene, the FAD gene or the Down's syndrome critical region (all of which were tested by St George-Hyslop et al. 1987b) are not common events in either sporadic or familial Alzheimer's disease.

4. Is the Amyloid Beta Protein Gene the Source of the Genetic Defect in Familial Alzheimer's Disease?

Mapping of the genes for AP and FAD into the q11.2 - q21 region of chromosome 21 raised the possibility that the AP gene might represent the primary genetic defect in Alzheimer's disease. However, two independant groups of researchers, using frequent RFLPs of the A4-amyloid gene to study large FAD pedigrees, found recombination between the Alzheimer's disease locus and the AP gene on several occasions (Tanzi et al. 1987b, Van Broeckhoven et al. 1987). The former group excluded the FAD gene from a region of approximately 8×10^6bp of DNA on either side of the AP locus. This demonstration of non-linkage between FAD and the

AP gene suggests that alterations in the AP gene are not the primary defect causing Alzheimer's disease.

4.5.2 Relevance of Probes Isolated During the Current Study to Down's Syndrome and Alzheimer's disease

1. Down's syndrome

As mentioned previously (Introduction, Section 1.1.3), there is a lack of agreement over the definition of the critical region of chromosome 21 involved in Down's syndrome. Most workers agree that bands 21q22.1 and 21q22.2 are necessary to develop the Down's syndrome phenotype, but other groups suggest that band 21q22.3 should also be included in the pathogenic segment. It can be seen from Table 3 and Figure 15 that six of the single-copy probes isolated in the current project map into the 21q22.1 - 21qter region (JG12, JG22, JG24, JG63, JG81 and JG108), and one (probe JG77) to the region 21q22.1 -21q22.2. These chromosome 21-specific DNA sequences may therefore be involved in the pathogenesis of Down's syndrome. Probe JG77 is of further interest because it was shown to be expressed in Northern blot experiments (Results, Section 3.5). This raises the possibility that this sequence might represent part of a gene involved in the pathogenesis of Down's syndrome.

2. Alzheimer's disease

Localisation of the genes for familial Alzheimer's disease (FAD) and amyloid beta protein (AP) to the 21q11.2 - 21q21 region confirmed what scientists had suspected for some time - that a connection existed between Down's syndrome and Alzheimer's disease. This finding is of particular importance to the present study, as any DNA markers assigned to this chromosomal region might be linked to, or might even represent part of either of these genes. It should be noted, however, that this chromosomal region is large enough to contain around 500 genes (Barnes 1987), and presumably a large number of DNA markers localised to this region of chromosome 21 will not show linkage to either gene.

A further cause for optimism, however, is provided by the relatively new technologies of "walking" (Steinmetz et al. 1984) and "jumping" (Collins & Weissman 1984, Poustka et al. 1987) the chromosome. As mentioned in the Introduction (Section 1.4), these techniques allow the use of DNA sequences not closely linked to an original clone for the isolation of genes and/or sequences important in the pathogenesis of inherited disorders. Application of these methods to the study of Alzheimer's disease could therefore allow the primary genetic defect involved to be identified using DNA probes mapped to the 21q11.2 - 21q21 region.

Five of the single-copy probes isolated during the current study map into this chromosomal region (JG21, JG62, JG72, JG90 and JG99), and it is possible that these sequences

might either be directly involved in the pathogenesis of Alzheimer's disease, or may lead to the isolation of sequences important in the disorder. In fact, the JG90/Nde II marker is currently being tested for possible linkage with either the AP gene or the gene for familial Alzheimer's disease (A.Vandenberghe, personal communication).

4.6 Conclusions

Although the chromosomal abnormality for Down's syndrome has been known for over 25 years the biochemical and molecular basis for the expression of the phenotype is still poorly understood. It has been shown that the q22 region of chromosome 21 is responsible for the Down's syndrome phenotype. The isolation of DNA probes from this part of the chromosome, as described in the current project, provides a possible means of investigating the molecular pathogenesis of Down's syndrome by defining the number of genes involved, their function and temporal expression during development.

The most important applications of arbitrary DNA sequences relate to their ability to detect restriction fragment length polymorphisms (RFLPs). Polymorphic DNA probes from chromosome 21 have three important uses : (i) to follow the inheritance of chromosome 21 in Down's syndrome families, and in this way permit the parental origin, and in some cases the meiotic stage, of nondisjunction to be defined; (ii) to show linkage with genetic disorders of unknown chromosomal origin, as recently demonstrated in the localisation of familial Alzheimer's disease to chromosome 21; (iii) in the construction of a genetic linkage map of chromosome 21. This last objective, in conjunction with the construction of similar linkage maps of all other human chromosomes, has the ultimate aim of allowing any genetic disease loci to be mapped to a particular chromosome by virtue of linkage with arbitrary polymorphic DNA markers.

The combination of regional mapping of DNA sequences using somatic cell hybrids with the recently developed technique of pulse field electrophoresis allows the construction of a detailed physical map of huamn chromosomes. Several laboratories have in fact already begun such research programs, and it has been predicted that future research may eventually result in the sequencing of the entire human genome, which should provide the answers to numerous currently intractable questions in the field of human genetics.

In the absence of an understanding of the molecular pathogenesis of Down's syndrome, no rational therapy is available, and current emphasis must therefore lie with prevention of the disorder. Lack of knowledge about the mechanisms involved in nondisjunction means that secondary prevention by detection and selective termination of pregnancy is currently the main focus of effort. To facilitate this the identification of risk factors for Down's syndrome such as increased maternal age and reduced levels of maternal serum alpha-fetoprotein is clearly important.

Polymorphic DNA probes as described in this study might also be of use in the definition of risk factors in families showing recurrent trisomy 21 - by comparing pericentromeric RFLP haplotypes in affected family members it could be determined whether or not all centromeres are equally predisposed to nondisjunction. Furthermore, DNA probes may also be useful for diagnosis of Down's syndrome either by

in situ hybridisation to interphase nuclei or by dosage studies in fetal tissues, which might be obtained either by conventional prenatal diagnosis techniques or by isolation of fetal cells from maternal blood.

To summarise, the results obtained in this project represent a valuable contribution to the molecular analysis of chromosome 21 and Down's syndrome, and should prove useful in future research into many different aspects of the molecular biology of chromosome 21-linked disorders.

BIBLIOGRAPHY

ANTONARAKIS, S.E., CHAKRAVATI, A., WARREN, A.C. et al. (1986) Reduced recombination rate on chromosomes 21 that have undergone nondisjunction. Cold Spring Harbor Symposium Quant Biol 51:158-190

ARNHEIM, N., KRYSTAL, M., SCHMICKEL,R., WILSON, G., RYDER, O., ZIMMER, E. (1980) Molecular evidence for exchanges among ribosomal genes on nonhomologous chromosomes in man and apes. Proc Natl Acad Sci U.S.A. 77:7323-7327

AULA, P., LEISTI, J., von KOSKULL, H. (1973) Partial trisomy 21. Clin Genet 4:241

AYME, S. and LIPPMAN-HAND, A (1982) Maternal age effect in aneuploidy : does altered embryonic selection play a role ? Am J Hum Genet 34:558-565

BAIKIE, A.G. (1971) Banding patterns of metaphase chromosomes in Down's syndrome. Lancet 2:494

BARNES, D.M. (1987) Defect in Alzheimer's is on chromosome 21. Science 235:846-847

BENTON, W.D. and DAVIS, R.W. (1977) Screening lambda-gt recombinant clones by hybridisation to single plaques in situ. Science 196:180-182

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

BOUCHE, J.P. (1981) The effect of spermidine on endonuclease inhibition by agarose contaminants. Analyt Biochem 115:42-45

BOUE, J.G. & BOUE, A. (1973) Increased frequency of chromosomal anomalies after induced ovulation. Lancet 1:679-680

CAMERON, J.R., PHILIPPSON, P., DAVIS, R.W. (1977) Analysis of chromosomal integration and deletions in yeast plasmids. Nucl Acids Res 4:1429-1448

CAROTHERS, A.D. (1983) Evidence that altered embryonic selection contributes to maternal age effect in aneuploidy : a spurious conclusion attributable to pooling of heterogeneous data. Am J Hum Genet 35:1057-1059

CASPERSSON, T., ZECH, L., JOHANSSON, C., MODEST, E.J. (1970) Identification of human chromosomes by DNA-binding fluorescent agents. Chromosoma 30:215-227

CERVENKA, J., GORLIN, R.J., DJAVADI, G.R. (1977) Down syndrome due to partial trisomy 21q. Clin Genet 11:119-121

CLARKE, C.M., EDWARDS, J.H., SMALLPIECE, V. (1961) 21 Trisomy/normal mosaicism in an intelligent child with mongoloid characters. Lancet 1:1028

COLLINS, F.S. & WEISSMAN, S.M. (1984) Directional cloning of DNA fragments at a large distance from an initial probe : a circularization method.
Proc Natl Acad Sci U.S.A. 81:6812-6816

CREMER, T., LANDEGENT, J., BRUCKNER, A. et al. (1986) Detection of chromosome aberrations in the human interphase nucleus by visualisation of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques : diagnosis of trisomy 18 with probe L1.84.
Hum Genet 74:346-352

CUCKLE, H.S., WALD, N.J., LINDENBAULM, R.H. (1984) Maternal serum AFP measurement - a screening test for Down's syndrome.
Lancet (Apr 28) : 926-929

DANIEL, A., STEWART, L., SAVILLE, T. et al. (1982) Prenatal diagnosis in 3000 women for chromosome, X-linked, and metabolic disorders. Am J Med Genet 11:61-75

DAVIES, K.E., HARPER, K., BONTHRON, D. et al. (1984) Use of a chromosome 21 cloned DNA probe for the analysis of non-disjunction in Down's syndrome. Hum Genet 66:54-56

de GROUCHY, J. & TURLEAU, C. (1977) Clinical Atlas of Human chromosomes. John Wiley & Sons, New York.

DELABAR, J-M., GOLDGABER, D., LAMOUR, Y. et al. (1987) Beta amyloid gene duplication in Alzheimer's disease and karyotypically normal Down's syndrome. Science 235:1390-1392

DRABKIN, H.A., DIAZ, M., BRADLEY, C.M., LE BEAU, M.M., ROWLEY, J.D., PATTERSON, D. (1985) Isolation and analysis of the $21q^{+}$ chromosome in the acute myelogenous leukemia 8;21 translocation : evidence that c-mos is not translocated. Proc Natl Acad Sci U.S.A. 82:464-468

EPSTEIN, C.J., COX, D.R., EPSTEIN, L.B. (1985) Mouse Trisomy 16 : An animal model of human trisomy 21 (Down syndrome) in "Molecular structure of the number 21 chromosome and Down syndrome" (G.F.Smith, Ed.) Ann N Y Acad Sci 450:157-168

ERICKSON, J.D. (1978) Down syndrome, paternal age, maternal age and birth order. Ann Hum Genet 41:289-298

ERICKSON, J.D. (1981) Paternal age and Down syndrome. Am J Hum Genet 31:489-497

EUNPU, D.L., McDONALD, D.M., ZACKAI, E.H. (1986) Trisomy 21 : rate in second-degree relatives. Am J Med Genet 25:361-363

FEINBERG, A.P. & VOGELSTEIN, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analyt Biochem 132:6-13

FERGUSON-SMITH, M.A. and YATES, J.R.W. (1984) Maternal age specific rates for chromosome aberrations and factors influencing them : report of a collaberative European study on 52,965 amniocenteses. Prenatal Diagnosis 4:5-44

FIALKOW, P.J., BLUMBERG, S.B., LOUNDON, W.T., SUTNIK, A.I., THULINE, H.C. (1971) Thyroid antibodies and Australia antigen in Down's syndrome. J Ment Defic Res 15:177-180

FLORENTIN, L. (1987) Isolation and characterisation of DNA sequences from the human Y chromosome.
Glasgow University PhD Thesis (No.7679)

FORD, C.E. & HAMERTON, J.L. (1956) The chromosomes of man. Nature 168:1020

FRANCHI, L., MANDL, A.M., ZUCKERMAN, S. (1962) The Ovary, Academic Press, London

FUSCOE, J.C., CLARK, L.M., VAN DILLA, M.A. (1986) Construction of fifteen human chromosome-specific DNA libraries from flow-purified chromosomes. Cytogenet Cell Genet 43:79-86

FUSCOE, J.C. (1987) Human chromosome-specific DNA libraries : use of an oligodeoxynecleotide probe to detect non-recombinants. Gene 52:291-296

GIBSON, D. & POZSONYI, J. (1965) Morphological and behavioural consequences of chromosome subtype in mongolism. Amer J Ment Defic 69:801

GOODFELLOW, P., BANTING, G., LEVY, R., POVEY, S. McMICHAEL, P. (1980) A human X-linked antigen linked by a monoclonal antibody. Somat Cell Genet 6:777-787

GOODFELLOW, P., DAVIES, P.K., ROPERS, H.H. (1985) Report of the committee on the genetic constitution of the X and Y chromosomes. Cytogenet Cell Genet 40 (HGM 8):296

GOLDGABER, D., LERMAN, M.J., McBRIDE, O.W., SAFFIOTTI, U., GAJDUSEK, D.C. (1987) Characterisation and chromosomal localisation of a cDNA encoding brain amyloid of Alzheimer's disease. Science 235:877-880

GOUDSMIT, J., WHITE, B.J., WEITKAMP, L.R. et al. (1981) Familial Alzheimer's disease in two kindreds of the same geographic and ethnic origin. J Neurol Sci 49:79-89

GRAW, S.L., PATTERSON, D., DAVIDSON, J.N., GUSELLA, J., WATKINS, P. (1985) Construction and characterisation of hamster/human hybrids containing fragments of human chromosome 21. Am J Hum Genet 37:231A

GRAW, S.L., DAVIDSON, J.N., GUSELLA, J. et al. Irradiation reduced human chromosome 21 hybrids (Somatic Cell Molec Genet 1988, in press)

GUSELLA, J.F., JONES, C., KAO, F.T., HOUSMAN, D., PUCK, T.T. (1982) Genetic fine structure mapping in human chromosome 11 by use of repetitive DNA sequences.
Proc Natl Acad Sci U.S.A. 79:7804-7808

GUSELLA, J.F., WEXLER, N.S., CONNEALLY, P.M. et al. (1983) A polymorphic marker genetically linked to Huntingdon's disease. Nature 306:234-238

GUSELLA, J.F., TANZI, R.E., ANDERSON, M.A. et al. (1984) DNA markers for nervous system disorders. Science 225:1320-1326

GUSTAVSEN, K., -H. (1964) Down's syndrome - A clinical and cytological investigation. Almgvists & Wiksells, Uppsala, Sweden

HADEBANK, M. & RODEWALD, A. (1982) Moderate Down's syndrome in three siblings having 21q22.3-qter and therefore no SOD-1 excess. Hum Genet 60:74-77

HAGEMEIJER, A. and SMITT, E.M.E. (1977) Partial trisomy 21 - further evidence that trisomy of band 21q22 is essential for Down's phenotype. Hum Genet 38:15-23

HALBERTSMA, T. (1923) Mongolism in one of twins and the etiology of mongolism. Am J Dis Child 25:350

HARLAP, S., SHIONO, P.H., PELLEGRIN, F. et al. (1979) Chromosome abnormalities in oral contraceptive breakthrough pregnancies. Lancet 1:1342-1343

HASSOLD, T.J. and JACOBS, P.A. (1984) Trisomy in man. Ann Rev Genet 18:69-97

HATCHER, N.H., HEALY, N.P., HOOK, E.B., WILLEY, A.M. (1982) Unexpected high proportion of first meiotic nondisjunction in trisomy 21. Am J Hum Genet 34:127A

HASSOLD, T.J., JACOBS, P.A., LEPPERT, M., SHELDON, M. (1987) Cytogenetic and molecular studies of trisomy 13. J Med Genet 24:725-732

HASSOLD, T.J., JACOBS, P.A., PETTAY, D. (1988) Analysis of nucleolar organizing regions in patients of trisomic spontaneous abortions. (Hum Genet, in press)

HENDERSON, S.A. & EDWARDS, R.G. (1968) Chiasma frequency and maternal age in mammals. Nature 218:22-28

HERZENBERG, L.A., BIANCHI, D.W., SCRODER, J., CANN, H.M., IVERSON, G.M. (1979) Fetal cells in the blood of pregnant women : detection and enrichment by fluorescence activated cell sorting. Proc Natl Acad Sci U.S.A. 76:1453-1455

HESTON, L.L. (1984) Down's syndrome and Alzheimer's dementia : defining an association. Psychiatr Dev 4:287-294

HEYMAN, A., WILKINSON, W.E., HURWITZ, B.H. et al. (1983) Alzheimer's disease: genetic aspects and associated clinical disorders. Ann Neurol 14:507-515

HGM 7 (7th International Human Gene Mapping Conference), Los Angeles 1984. Cytogenet Cell Genet, Vol 37

HGM 8 (8th International Human Gene Mapping Conference), Helsinki, 1985. Cytogenet Cell Genet, Vol 40

HGM 9 (9th International Human Gene Mapping Conference), Paris, 1987. (Cytogenet Cell Genet, in press)

HOFFMAN, E.P., BROWN, R.H. (Jnr), KUNKEL, L.M. (1987) Dystrophin - The protein product of the Duchenne Muscular Dystrophy locus. Cell 51:919-928

HOFKER, M.H., SKRAASTAD, M.I., BERGEN A.A.B. et al. (1986) The X chromosome shows less genetic variation at restriction sites than the autosomes. Am J Hum Genet 39:438-451

HOLMES, B.L. (1978) Genetic counselling for the older pregnant women : new data and questions.
N Engl J Med 298:1419-1421

HOOK, E.B., CROSS, P.K., LAMSON, S.H., REGAL, R.R., BAIRD, P.A., UH, S.H. (1981) Paternal age and Down syndrome in British Columbia. Am J Hum Genet 33:123-128

HYMAN, R.L. & STALLINGS, V. (1974) Complementation patterns of Thy-1 variants and evidence that antigen loss variants "pre-exist" in the parental population.
J Natl Cancer Inst 52:429-436

JACOBS, P.A. & MAYER, M. (1981) The origin of human trisomy : A study of heteromorphisms and satellite associations.
Ann Hum Genet 45:49-57

JACKSON-COOK, C.K., FLANNERY, D.B., COREY, L.A., NANCE, W.E., BROWN, J.A. (1985) Nucleolar organising region variants as a risk factor for Down's syndrome. Am J Hum Genet 37:1049-1061

JONGBLOET, P.H., FRANTS, R.R., HAMERS, A.J. (1981) Parental alpha-1-antitrypsin (PI) types and meiotic nondisjunction in the etiology of Down's syndrome. Clin Genet 20:304-309

JUBERG, R.C. & MOWREY, P.N. (1983) Origin of non-disjunction in trisomy 21 syndrome : all studies compiled, parental age analysis, and international comparisons.
Am J Med Genet 16:111-116

KANG, J., LEMAIRE, H-G., UNTERBECK, A. et al. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell surface receptor. Nature 325:733-736

KAUFMAN, M. (1983) Ethanol-induced chromosomal abnormalities at conception. Nature 302:258-260

KLINE, J., LEVIN, B., SHROUT, P., STEIN, Z., SUSSER, M., WARBURTON, D. (1983) Maternal smoking and trisomy among spontaneously aborted conceptions. Am J Hum Genet 35:421-431

KNOWLTON, R.G., COHEN-HAGUENAUER, O., VAN CONG, N. et al. (1985) A polymorphic marker linked to cystic fibrosis is located on chromosome 7 Nature 318:380

KOENIG, M., HOFFMAN, E.P., BERTELSON, C.J., MONACO, A.P., FEENER, C., KUNKEL, L.M. (1987) Complete cloning of the Duchenne Muscular Dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell 50:509-517

KORENBERG, J.R., CROYLE, M.L., COX, D.R. (1987) Isolation and regional mapping of DNA sequences unique to human chromosome 21. Am J Hum Genet 41:963-978

KRYSTAL, M., D'EUSTACHIO, P., RUDDLE, F.H., ARNHEIM, N. (1981) Human nucleolus organizers on nonhomologous chromosomes can share the same ribosomal gene variants. Proc Nat Acad Sci 78:5744-5748

KUNKEL, L.M., SMITH, K.D., BOYER, S.H. et al. (1977) Analysis of human Y-chromosome specific reiterated DNA in chromosome variants. Proc Natl Acad Sci U.S.A. 74:1249-1254

LAW, M.L. & VAN KEUREN, M.L. (1986) Application of molecular and somatic cell genetics to the study of chromosome 21. Ann N Y Acad Sci 477:151-158

LEJEUNE, J., GAUTIER, M., TURPIN, R. (1959a) Etudes des chromosomes somatiques de neuf enfants mongoliens. C R Acad Sci 248:1721

LEJEUNE, J., GAUTIER, M., TURPIN, R. (1959b) Les chromosomes humaines en culture de tissus. C R Acad Sci 248:602

LEVAN, A. and HSU, T.C. (1960) The chromosomes of a mongoloid female, mother of a normal boy. Hereditas (Lund) 46:770

LEVANON, D., LIEMAN-HURWITZ, J., DAFNI, N. et al. (1985) Architecture and anatomy of the chromosomal locus in human chromosome 21 encoding the Cu/Zn superoxide dismutase. Embo J 4(1):77-84

LIEMAN-HURWITZ, J., DAFNI, N., LAVIE, V., GRONER, Y. (1982) Human cytoplasmic SOD cDNA clone : a probe for studying the molecular biology of Down's syndrome. Proc Nat Acad Sci 79:2808-2811

MAGENIS, R.E. & CHAMBERLIN, J. (1981) Parental origin of nondisjunction in trisomy 21. In "Trisomy 21 (Down syndrome) : Research prespectives. (de la Cruz, F.E. & Gerald, P.S., eds.) University Park Press, Baltimore. pp77-93

MANIATIS, T., FRITSCH, E.F., SAMBROOK, J. (1982) Molecular Cloning : A Laboratory Manual. Cold Spring Harbor

MATTEI, J.F., MATTEI, M.J., BAETEMAN, M.A., GIRAUD, F. (1981) Trisomy 21 for the region 21q22.3 : identification by high resolution R-banding patterns. Hum Genet 56:409-411

MERKATZ, I.R., NITOWSKY, H.M., MACRI, J.N., JOHNSON, W.E. (1984) An association between low maternal serum AFP and foetal chromosomal abnormalities.
Am J Obstet Gynacol 148(7):886-893

MIKKELSEN, M., HANNE, P., JORGEN, G., AKSEL, L. (1980) Nondisjunction in trisomy 21 : Study of chromosomal heteromorphisms in 110 families. Ann Hum Genet 44:17-28

MINTY, A.J., ALONSO, S., CARAVATTI, M., BUCKINGHAM, M.E. (1982) A fetal skeletal muscle actin mRNA and its identity with cardiac actin mRNA. Cell 30:185-192

MONACO, A.P., NEVE, R.L., COLLETTI-FEENER, C.A. et.al. (1986) Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. Nature 323:646-650

MOTTIRONI, V.D., HOOK, E.B., WILEY, A.M., PORTER, I.H., SWIFT, R.V., HATCHER, N.H. (1983) Decreased HLA heterogeneity in parents of children with Down syndrome.
Am J Hum Genet 35:1289-1296

NEE, L.E., POLINSKY, R.J., ELDRIDGE, R., WEINGARTNER, H., SMALLBERG, S., EBERT, M. (1983) A family with histologically confirmed Alzheimer's disease. Arch Neurol 40:203-208

NEVE, R.L., STEWART, G.D., NEWCOMB, P. et al. (1986) Human chromosome 21-encoded cDNA clones. Gene 49:361-369

NIEBUHR, E. (1974) Down's syndrome - the possibility of a pathogenic segment on chromosome no. 21. Humangenetik 21:99-10

OTT, J., LINDER, D., McCAW, B.K., LOVRIEN, E., HECHT, F. (1976) Estimating distances from the centromere by means of benign ovarian teratomas in man. Ann Hum Genet 40:191-196

PAGE, D.C., MOSHER, R., SIMPSON, E.M. et al. (1987) The sex-determining region of the human Y-chromosome encodes a finger protein. Cell 51:1091-1104

PATTERSON, D., JONES, C., SCOGGIN, C., MILLER, Y.E., GRAW, S. (1982) Somatic cell genetic approaches to Down's syndrome. Annals N Y Acad Sci 396:69-81

PATTERSON, D., VAN KEUREN, M.L., DRABKIN, H., WATKINS, P.C., GUSELLA, J.F., SCOGGIN, C.(1985) Molecular analysis of chromosome 21 using somatic cell hybrids. Ann N Y Acad Sci 450:109-120

PENROSE, L.S. (1933) The relative effects of paternal and maternal age in mongolism. J Genet 27:219

PENROSE, L.S., ELLIS, J.R., DELHARTY, J.D.A. (1960) Chromosomal translocations in mongolism and in normal relatives. Lancet 2:409

PODLISNY, M.B., LEE, G., SELKOE, D.J. (1987) Gene dosage of the amyloid beta precursor protein in Alzheimer's disease. Science 238:669-671

POISSONIER, M., SAINT-PAUL, B., DUTRILLAUX, X., CHAISSAIGNE, P., GRUYER, P., BLIGNIERIES-STROUCK, G. (1976) Trisomie 21 Partielle 21q21(21q22.2) Ann Genet (Paris) 79:69-73

POLANI, P.E., BRIGGS, J.H., FORD, C.E., CLARKE, C.M. BERG, J.M. (1960) A mongol girl with 46 chromosomes. Lancet 1:721

POUSTKA, A., POHL, T.M., BARLOW, D.P., FRISCHAUF, A-M., LEHRACH, H. (1987) Construction and use of human chromosome jumping libraries from Not I-digested DNA. Nature 325:353-355

REEDERS, S.T., BREUNING, M.H., DAVIES, K.E. et al. (1985) A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. Nature 317:542

REUBEN, M.S. & KLEIN, S. (1926) Mongolian idiocy in both twins. Arch Pediatr 43:552

RIDLER, M.A.C. (1971) Banding patterns of metaphase chromosomes in Down's syndrome. Lancet 2:603

RIGBY et al. (1977) - see Page 174

ROBAKIS, N.K., WISNIEWSKI, H.M., JENKINS, E.C. et al. (1987) Chromosome 21q21 sublocalisation of a gene encoding B-amyloid peptide in cerebral vessels and neuritic (senile) plaques of people with Alzheimer disease and Down's syndrome. Lancet 1:384-385

ROBERTSON, W.R.S. (1916) Chromosome studies I. J Morphol 27:178

RUDD, N.L., DIMNIK, L.S., GREENTREE, C., MENDES-CRABB, K., HOAR, D.I. (1988) The use of DNA probes to establish parental origin in Down syndrome. Hum Genet 78:175-178

SANDLER, L. (1981) The meiotic nondisjunction of homologous chromosomes in Drosophila. In "Trisomy 21 (Down Syndrome) - Research Perspectives" (de la Cruz, F. & Gerald, P., eds.) University Park Press, Baltimore

SCHMICKEL, R.D., GONZALEZ, I.L., ERICKSON, J.M. (1985) Nucleolus organising genes on chromosome 21 : recombination and nondisjunction Ann N Y Acad Sci 450:121-131

SEALEY, P.G., WHITTAKER, P.A., SOUTHERN, E.M. (1985) Removal of repeated sequences from hybridisation probes. Nucleic Acids Research 13(6):1905-1922

SINET, P.M. (1982) Metabolism of oxygen derivatives in Down's syndrome. Annals N Y Acad Sci 396:83-94

SINET, P.M., COUTURIER, J., DUTRILLAUX, B., et al. (1976) Trisomie 21 et superoxide dismutase-1 (IPO-A). Exp Cell Res 97:47-55

SKOLNICK, M.H., WHITE, R. (1982) Strategies for detecting and characterising restriction fragment length polymorphisms (RFLPs). Cytogenet Cell Genet 32:58-67

SMITH, G.F. & BERG, J.M. (1976) Down's Anomaly, 2nd Edition, Churchill Ltd., London

SMITH, G.F. & WARREN, S.T. (1985) The biology of Down syndrome. In "Molecular structure of the number 21 chromosome and Down syndrome" (G.F.Smith, Ed.) Ann N Y Acad Sci 450:1-10

SOUTHERN, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517

SPINNER, N.B., EUNPE, D.L., SCHMICKEL, R.D., ZACKAI, E., BUNIN, G., EMANUEL, B.S. (1986) The role of cytologic and molecular NOR variants in trisomy 21. Am J Hum Genet 39(Suppl):A133

STEIN, Z, STEIN, W., SUSSER, M. (1986) Attrition of trisomies as a meternal screening device. Lancet 1:944-946

STEINMETZ, M., MINARD, K., HORVATH, S. (1982) A molecular map of the immune response region from the major histocompatibility complex of the mouse. Nature 300:35-42

STENE, J. & STENE, E. (1977) Statistical methods for detecting a moderate paternal age effect on incidence of a disorder when a maternal one is present. Ann Hum Genet 40:343-353

STENE, J. & STENE, E. (1978) On data and investigations on parental age effects. Ann Hum Genet 41:465-468

STENE, J., FISCHER, G., STENE, E., MIKKELSEN, M., PETERSEN, E. (1977) Paternal age effect in Down's syndrome. Ann Hum Genet 40:299-306

STENE, J., STENE, E., STENGEL-RUTKOWSKI, S., MURKEN, J.-D. (1981) Paternal age and Down's syndrome - data from prenatal diagnoses (DFG). Hum Genet 59:119-124

STEWART, G.D. (1984) The isolation and characterisation of cloned DNA from a chromosome 21 enriched DNA library. Glasgow University PhD Thesis (No. 6925)

STEWART, G.D., HARRIS, P., GALT, J., FERGUSON-SMITH, M.A. (1985) Cloned DNA probes regionally mapped to human chromosome 21 and their use in determining th origin of non-disjunction. Nucleic Acids Research 13(11):4125-4132

STEWART, G.D., HASSOLD, T.J., BERG, A., WATKINS, P.C., TANZI, R., KURNIT, D.M. (1988) Trisomy 21 (Down syndrome): Studying nondisjunction and meiotic recombination by using cytogenetic and molecular polymorphisms that span chromosome 21. Am J Hum Genet 42:344-346

STEWART, G.D., HASSOLD, T.J., KURNIT, D.M. (1988) Trisomy 21 : Molecular and cytogenetic studies of nondisjunction. (Review paper, Adv Hum Genet, in press)

ST.GEORGE-HYSLOP, P.H., TANZI, R.E., POLINSKY, R.J. et al. (1987a) The genetic defect causing familial Alzheimer's disease maps on chromosome 21. Science 235:885-890

ST.GEORGE-HYSLOP, P.H., TANZI, R.E., NEVE, R.L. et al. (1987b) Absence of duplication of chromosome 21 genes in familial and sporadic Alzheimer's disease. Science 238:664-666

SUMMITT, R.L. (1981) Chromosome 21 - specific segments that cause the phenotype of Down syndrome. In Trisomy 21 (Down Syndrome) Research Perspectives. de la Cruz, F.F. & Gerald, P.S., Eds. 225-235 University Park Press, Baltimore

SVED, J.A. & SANDLER, L. (1981) Relation of maternal age effect in Down syndrome to nondisjunction. In Trisomy 21 (Down Syndrome) Research Perspectives. de la Cruz, F.F. & Gerald, P.S., Eds. 95-98 University Park Press, Baltimore

TAMAREN, J, SPUHLER, K., SUJANSKY, E. (1983): Risk of trisomy 21 among second- and third-degree relatives of a proband with trisomy 21. Am J Med Genet 15:393-403

TAN, Y.H., TISCHFIELD, I., RUDDLE, F.H. (1973) The linkage of genes for the human interferon-induced anti-viral protein and indophenol oxidase-B traits to chromosome G-2.
J Exp Med 137:317-330

TAN, Y.H., SCHNEIDER, E.L., TISCHFIELD, I., RUDDLE, F.H. (1974) Human chromosome 21 dosage : Effect on the expression of the interferon-induced antiviral state. Science 185:61-63

TANZI, R.E., GUSELLA, J.F., WATKINS, P.C. et al. (1987a) Amyloid beta protein gene : cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science 235:880-884

TANZI, R.E., ST.GEORGE-HYSLOP, P.H., HAINES, J.L. et al. (1987b) The genetic defect in familial Alzheimer's disease is not tightly linked to the amyloid B-protein gene. Nature 329:156-157

TANZI, R.E., BIRD, E.D., LATT, S.A., NEVE, R.L. (1987c) The amyloid beta protein gene is not duplicated in brains from patients with Alzheimer's disease. Science 238:666-669

TJIO, J.H. and LEVAN, A. (1956) The chromosome number of Man. Hereditas 42:1

TURPIN, R. & LEJEUNE, J. (1961) Chromosome translocation in man. Lancet I:616-617

UCHIDA, I. (1979) Radiation-induced nondisjunction. Envir Health Perspec 31:13-18

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

VAN KEUREN, M.L., DRABKIN, H.A., HART, I., HARKER, D., PATTERSON, D., VORA, S. (1986b) Regional assignment of human liver-type 6-phosphofructokinase to chromosome 21q22.3 by using somatic cell hybrids and a monoclonal anti-L antibody. Hum Genet 74:34-40

VAN BROECKHOVEN, C., GENTHE, A.M., VANDENBERGHE, A. et al. (1987) Failure of familial Alzheimer's disease to segregate with the A4-amyloid gene in several European families. Nature 329:153-155

VIERA, J. & MESSING, J. (1982) The pUC plasmids, an M13mp7-derived system fo insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268

VOGEL, F. & MOTULSKY, A.G. (1986) Human Genetics : Problems and Approaches Springer-Verlag, Berlin

WAARDENBURG, P.J. (1932) Das menschliche Augen und seine Erbanlagen. Haag:Martinus Nijhoff

WAHL, G.M., STERN, M., STARK, G.R. (1979) Efficient transfer of large DNA fragments from agarose gels to diazobenzylooxymethyl paper and rapid hybridisation using dextran sulphate. Proc Natl Acad Sci U.S.A. 76:3683-3687

WAINWRIGHT, B.J., SCAMBLER, P.J., SCHMIDTKE, J. et al. (1985) Localization of cystic fibrosis locus to human chromosome 7cen-q22 Nature 318:384

WARBURTON, D., STEIN, Z., KLINE, J. (1983) In utero selection against fetuses with trisomy. Am J Hum Genet 35:1059-1064

WARREN, A.C., CHAKRAVATI, A., WONG, C. et al. (1987) Evidence for reduced recombination on the nondisjoined chromosomes 21 in Down syndrome. Science 237:652-654

WATKINS, P.C., TANZI, R.E., GIBBONS, K.T. et al. (1985) Isolation of polymorphic DNA segments from human chromosome 21. Nucleic Acid Res 13 (17):6075-6088

WATKINS, P.C., TANZI, R.E., CHENG, S.V., GUSELLA, J.F. (1987) Molecular genetics of human chromosome 21.
J Med Genet 24:257-270

WATKINS, P.C., TANZI, R.E., STUART, N., STANISLOVITIS, P., GUSELLA, J.F. (1987) A cosmid clone genetic linkage map of chromosome 21 and localisation of the breast cancer estrogen-inducible (BCEI) gene. HGM9, Paris 1987, Abstract No. 214 (Cytogenet Cell Genet, in press)

WERNER, W., HERRMANN, F.H., JOHN, B. (1982) Cytogenetic studies of a family with trisomy 21 mosaicism in two successive generations a the cause of Down's syndrome. Hum Genet 60:202-204

WHITE, M.J.D. (1954) Animal cytology and evolution, 1st Edition. London:Cambridge University Press

WHITE, M.J.D. (1973) Animal cytology and evolution, 3rd Edition. London:Cambridge University Press

WHITE, R. (1985) DNA sequence polymorphisms revitalise linkage approaches in human genetics. Trends in Genetics (June):177-181

WILLARD, H.F. (1985) Chromosome-specific organisation of human alpha satellite DNA. Am J Hum Genet 37:524-532

WILLARD, H.F., WAYE, J.S., SKOLNICK, M.H., SCHWARTZ, C.E., POWERS, V.E., ENGLAND, S.B. (1986) Detection of restriction fragment length polymorphisms at the centromeres of human chromosomes using chromosome-specific alpha satellite DNA : implications for the development of centromere-based genetic linkage maps. Proc Natl Acad Sci U.S.A. 83:5611-5615

WILLIAMS, J.D., SUMMITT, R.L., MARTENS, P.R., KIMBRELL, R.A. (1975) Familial Down syndrome due to t(10;21) translocation : Evidence that the Down phenotype is related to trisomy of a specific segment of chromosome 21. Am J Hum Genet 27:478-485

WISNIEWSKI, K.E., DALTON, A.J., CRAPPER McCLACHLAN, D.R., WEN, G.Y., WISNIEWSKI, H.M. (1985) Alzheimer's disease in Down's syndrome : Clinicopathological studies. Neurology 35:957-961

WORTON, R.G., SUTHERLAND, J., BODRUG, S. et al. (1987) Orientation of ribosomal RNA genes and isolation of acrocentric specific probe ACR-1 mapping distal to the ribosomal RNA genes. HGM 9, Paris 1987, Abstract No. 652 (Cytogenet Cell Genet, in press)

YOUNG, B.D., JEANPIERRE, M., GOYNS, M.H., STEWART, G.D., ELLIOT, T., KRUMLAUF, R. (1983) Construction and characterisation of chromosomal DNA libraries. Haematology and Blood Transfusion 28:301-309

RIGBY, P.W.J., DIECKMANN, M., RHODES, C., BERG, P. (1977) Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113:237-251

Publications and Meeting presentations :

1. Stewart, G.D., Harris, P., Galt, J., Ferguson-Smith, M.A. (1985) : "Cloned DNA probes regionally mapped to human chromosome 21 and their use in determining the origin of nondisjunction" Nucleic Acids Research 13:4125-4132

2. Galt, J. - "Use of chromosome 21 DNA probes in the analysis of nondisjunction in Down's syndrome families" Presented at the annual conference of the Association of Clinical Cytogeneticists, Newcastle, July 1985

3. Galt, J., Morrison, N. and Ferguson-Smith, M.A. - "Parental origin of nondisjunction in Down's syndrome using DNA probes" 7th International Congress of Human Genetics, Abstracts, Part I:CI.35

4. Manuscript in preparation : Stefani, L., Galt, J., Palmer, A., Ferguson-Smith, M.A. and Nevin, N.C. "Expression of chromosome 21 specific sequences in normal and Down's syndrome tissues" (These results were presented at HGM 9 (Paris), 1987)