THE MAPPING OF HUMAN CHROMOSOMES BY FLUORESCENCE IN SITU HYBRIDIZATION

BY

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'nihil est in intellectu quod non antea fuerit in sensu'

'there is nothing in the intellect which was not previously in the senses'

John Locke

TABLE OF CONTENTS

THESIS SUMMARY 8

DECLARATION 9

ACKNOWLEDGEMENTS 10

LIST OF FIGURES 12

CHAPTER 1

HISTORICAL BACKGROUND AND LITERATURE REVIEW

1.1 HUMAN CYTOGENETICS 15

1.2 CHROMOSOME BANDING 15

1.3 ISOTOPIC IN SITU HYBRIDIZATION 16

1.4 FLUORESCENCE IN SITU HYBRIDIZATION 18

1.5 APPLICATIONS OF FISH 18

1.5.1 Gene Mapping and Ordering 18

1.5.2 Clinical Applications of Interphase FISH 21

1.5.3 Clinical Applications of Metaphase FISH 26

1.6 EXTENDED CHROMATIN TECHNIQUES 27

1.7 A PUTATIVE BREAST CANCER GENE ON HUMAN CHROMOSOME 16q24 30

1.8 FRAGILE SITES ON HUMAN CHROMOSOMES 31

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS 36

2.1.1 Chemicals and Reagents 36

2.1.2 Enzymes 36
2.1.3 Buffers 36
2.1.4 DNA Probes 37
2.1.5 Human Experimental Material 37
2.1.6 Kits 37
2.1.7 Photographic Materials 37
2.1.8 Media and Solutions 37
2.1.8.1 Cell Culture 37
2.1.8.2 In Situ Hybridization 37
2.2 METHODS 38
2.2.1 Lymphocyte Culture 38
2.2.2 Cell Synchronization ('TdC' method) 38
2.2.3 Fragile Site Induction 38
2.2.4 Chromosome Harvest 38
2.2.5 Fibroblast Culture 38
2.2.6 Trypsin Treatment of Fibroblast Culture 38
2.2.7 Fibroblast Harvest 38
2.2.8 Preparation of Total Human DNA 38
2.2.9 Preparation of Sonicated Salmon Sperm DNA 40
2.2.10 Nick Translation 40
2.2.11 Random Primer Labeling 40
2.2.12 Slide Preparation - Metaphases and Nuclei 40
2.2.13 Slide Preparation - Extended Chromatin Fibres (ECFs) 40
2.2.14.1 Protocol 1 (for live cells) 41
2.2.14.2 Protocol 2 (for fixed cell suspensions) 41
2.2.15 Fluorescence in situ Hybridization (FISH) 41
2.2.15.1 General Conditions 41
2.2.15.2 Probe Detection 42

CHAPTER 3

LOCALISATION OF SIX SINGLE COPY GENES ON HUMAN CHROMOSOMES

3.1 INTRODUCTION 45
3.2 MATERIALS 47
3.2.1 Probes 47
3.2.2 Cell Lines 47
3.3 METHODS 48
3.3.1 Probes 48
3.3.2 Slide Preparation 48
3.3.3 Probe Mix 48
3.3.4 Hybridization 48
3.3.5 Detection and Amplification 48
3.3.6 Scoring of slides 49
3.3.7 Photography 49
3.4 RESULTS AND COMMENTS 49
3.4.1 Sulphamidase 50
3.4.2 Grb 14 52
3.4.3 NPY 54
3.4.4 Brn-2 56
3.4.5 NKB1 59
3.4.6 Humly 9 61
3.5 DISCUSSION 63

CHAPTER 4

DEVELOPMENT OF A METHOD FOR THE DIAGNOSIS OF CHARCOT MARIE TOOTH SYNDROME TYPE 1A (CMT1A) AND HEREDITARY NEUROPATHY WITH LIABILITY TO PRESSURE PALSIES (HNPP)

4.1 INTRODUCTION 65
4.2 MATERIALS 67
4.2.1 Probes 67
4.2.2 Photography 68
4.3 METHODS 68
4.3.1 Probe Labeling 68

4.3.2 Cell Culture 68
4.3.3 Testing the Parameters of Method B 68
4.3.4 FISH procedure: pVAW409R1 69
4.3.5 FISH procedure: c132G8, c103B11 69
4.4 RESULTS AND COMMENTS 70
4.4.1 Parameters of Cell Culture Method B 70
4.4.2 Results of Optimization of FISH Procedure: pVAW409R1 72
4.4.3 Results of Optimization of FISH Procedure: c132G8, c103B11 74
4.5 DISCUSSION 83

CHAPTER 5
METAPHASE AND INTERPHASE ORDERING OF COSMID PROBES WITHIN CHROMOSOME INTERVAL 16q24.3

5.1 INTRODUCTION 86
5.2 METHODS 86
5.2.1 Slide Preparation 86
5.2.2 Probe Description 88
5.2.3 Probe Labeling 88
5.2.4 Hybridization of Single Cosmid Probes to Metaphases 88
5.2.5 Dual Hybridization of Differentially-Labeled Cosmid Probes to Metaphases 88
5.2.6 Hybridization of Biotin-Labeled and (separately) DIG-Labeled Cosmid Pairs to Interphase Nuclei 88
5.2.7 Triple Hybridization of Differentially-Labeled Cosmid Probes to Interphase Nuclei 90
5.2.8 Statistical analysis 90
5.2.9 Photography 90
5.3 RESULTS 90
5.3.1 Hybridization of Single Cosmid Probes to Metaphases 90
5.3.2 Dual Hybridization of Differentially-Labeled Cosmid Probes to Metaphases 91

5.3.3 Hybridization of Biotin-Labeled and (separately) DIG-Labeled Cosmid Pairs to Nuclei 94
5.3.4 Triple Hybridization of Differentially-Labeled Cosmid Probes to Interphase Nuclei 95
5.4 DISCUSSION 97

CHAPTER 6
DEVELOPMENT : PROBE HYBRIDIZATION TO EXTENDED CHROMATIN FIBRES

6.1 INTRODUCTION 100
6.2 METHODS 100
6.2.1 Slide Preparation 1 / Slide Preparation 2 100
6.2.2 Probe Description 100
6.2.3 Probe Labeling 100
6.2.4 Single Hybridization of Satellite DNA Probes 101
6.2.5 FISH Conditions - Single Hybridization of Satellite DNA Probes 101
6.2.6 Differential Hybridization of Satellite DNA Probes 101
6.2.7 FISH Conditions - Differential Hybridization of Two Satellite DNA Probes 101
6.2.8 Hybridization of P1 Genomic Probe 101
6.2.9 Differential Hybridization of Two Cosmid Probes 101
6.2.10 Photography 102
6.3 RESULTS AND COMMENTS 102
6.3.1 Slide Preparation 102
6.3.2 Probe Concentration and Detection 103
6.3.3 Appearance of Signal 103
6.3.4 Results of differential Hybridization of Satellite DNA Probes 105
6.3.5 Results of Hybridization of P1 Genomic Probe 105
6.3.6 Results of Differential Hybridization of Two Cosmid Probes 105
6.4 DISCUSSION 108

CHAPTER 7

ESTIMATION OF FRAGILE SITE EXPANSION BY HYBRIDIZATION OF LAMBDA PROBES TO EXTENDED CHROMATIN FIBRES (ECFs) FROM FRA16B NUCLEI

7.1 INTRODUCTION 110

7.2 METHODS 111

7.2.1 Cell Culture & Slide Preparation 111

7.2.2 Probe Description 111

7.2.3 Probe Labeling 111

7.2.4 FISH Conditions 111

7.2.5 Photography 113

7.2.6 Gap Measurements 113

7.3 RESULTS 113

7.4 DISCUSSION 118

SUMMARY 121

BIBLIOGRAPHY 122

APPENDIX A Papers published and accepted for publication during the course of this thesis 139

MINOR AMMENDMENTS

pp15, 30 : The correct localization of the BR1 gene is 13q14.3 not 13q13.

p95 : Table 5.3, experiment 3, c177D112 should read c177D12.

INTRODUCTION (Chapter 1) : For all references to Ph^1 read Ph.

THESIS SUMMARY

The aim of this study was to develop and utilize fluorescence in situ hybridization (FISH) for various gene mapping applications. Techniques were developed which enabled the hybridization of a wide range of probes to interphase nuclei, to chromosomes, and to extended chromatin fibres. Single and dual colour systems were utilized to detect this hybridization.

A comprehensive human gene map has the potential to solve many diagnostic problems in medical genetics. In this study, original localizations were made for six unique sequences. The gene for Sanfilippo A syndrome (sulphamidase) was localized to chromosome 17q25.3. *GRB14*, the gene for the SH2 domain-containing protein Grb 14, was assigned to chromosome 2q23. The neuropeptide Y gene, *NPY*, was localized to chromosome 7p15.1. The *brn-2* gene was localized to chromosome 6q16 and its behaviour in melanoma cell lines was observed. A natural killer cell receptor for HLA-B allotypes, *NKB1*, was assigned to chromosome 19q13.4, and the membrane antigen *Humly 9* was localized to chromosome 1q22.

To increase the reliability of diagnosis in the autosomal dominant conditions Charcot Marie Tooth syndrome Type 1A (CMT1A) and Hereditary Neuropathy with Liability to Pressure Palsies (HNPP), a FISH test for these syndromes was developed. The test is rapid, reliable and fits with little disruption into the normal procedures of the clinical cytogenetic laboratory.

Data was generated for a research project which aims to clone and identify a putative breast cancer gene. Twenty-one cosmids within a region of chromosome band 16q24.3 which shows loss of heterozygosity (LOH) in some breast cancers, were tested for single copy status by FISH. The contigs and singletons represented by these cosmids were ordered by two-colour hybridization with two differentially labeled probes on metaphase chromosomes, or with three differentially labeled probes on interphase nuclei.

Fragile sites have been shown to occur on chromosomes when a naturally-occurring repeated sequence undergoes a significant expansion in copy number. Lambda clones located each side of the rare distamycin A - inducible fragile site FRA16B were hybridized to extended chromatin fibres from FRA16B nuclei in an attempt to assess the size of the repeat expansion in these cells.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

All of the work presented in this thesis was carried out by me or under my direct supervision, except where otherwise acknowledged.

I consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Elizabeth Baker

8th November, 1996

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LIST OF FIGURES

figure **page**

CHAPTER 3

3.1 Metaphase showing FISH with the 3.6kb sulphamidase probe 51

3.2a Signal distribution following localization of *GRB14* to metaphases from the first normal male 53

3.2b Signal distribution following localization of *GRB14* to metaphases from the second normal male 53

3.3 Metaphases showing FISH with the *NPY* genomic probe 55

3.4 Metaphase showing FISH with the *brn-2* genomic probe 57

3.5 Hybridization of the *brn-2* genomic probe to metaphases from melanoma cell lines 58

3.6 Metaphase showing FISH with the 1.8kb *NKB1* cDNA probe 60

3.7 Metaphase showing FISH with the 1.9kb *humly9* cDNA probe 62

CHAPTER 4

4.1a Mechanism of the CMT1A duplication/HNPP deletion within human chromosome band 17p11.2 66

4.1b Genomic organization within the CMT1A duplication/HNPP deletion critical region within chromosome band 17p11.2 66

4.2 Nuclei present following overnight culture of peripheral blood leukocytes 73

4.3 Metaphase showing FISH with the pVAW409R1 probe 75

4.4 Nuclei showing FISH with the pVAW409R1 probe 75

4.5 Metaphase showing hybridization with cosmids c103B11 and c132G8 77

4.6 Nuclei from an overnight culture showing FISH with cosmids c103B11 and c132G8 77

4.7 Nuclei from a CMT1A patient showing FISH with the cosmid probes 103B11 and 132G8 80

4.8 Example of 'doubling' aretfact following FISH with the cosmid probes 80

4.9 Nuclei from cultured CVS showing FISH with cosmid probes 103B11 and 132G8 82

figure **page**

CHAPTER 5

5.1 Physical map of the region of LOH within chromosome band 16q24.3 87

5.2 Metaphase showing FISH with cosmid 425E4 92

5.3 Metaphase showing FISH with cosmid 323B6 92

5.4 Metaphase showing FISH with differentially labeled cosmid probes (1) 93

5.5 Metaphase showing FISH with differentially labeled cosmid probes (2) 93

5.6 FISH with three differentially labeled cosmid probes in interphase nuclei 96

CHAPTER 6

6.1 Examples of extended chromatin fibres 104

6.2 Differential hybridization of two satellite DNA probes to ECFs 106

6.3 FISH with P1 genomic probe to ECFs 107

6.4 FISH with two differentially-labeled cosmid probes to ECFs 107

CHAPTER 7

7.1 Cytogenetic appearance of the rare distamycin A - inducible fragile site, FRA16B 112

7.2 Structure of the lambda contig spanning the FRA16B fragile site on chromosome 16 112

7.3 Hybridization of lambda clones from either side of FRA16B to extended chromatin fibres 114

7.4 Gap lengths from LCL(1) - normal individual 115

7.5 Gap lengths from LCL(2) - individual heterozygous for FRA16B 116

7.6 Gap lengths from LCL(3) - individual homozygous for FRA16B 117

CHAPTER 1

HISTORICAL BACKGROUND AND LITERATURE REVIEW

This chapter describes the history and methods of in situ hybridization to the beginning of the project. It also provides the background of the research projects to which the methodology was applied.

1.1 HUMAN CYTOGENETICS

The science of human cytogenetics began in the early 1950s with the development of hypotonic treatment techniques for the preparation of human chromosomes (Hsu, 1952) and the establishment of the correct diploid number of 46 chromosomes for humans (Tjio and Levan, 1956). The subsequent correlation of trisomy 21 with Down syndrome (Lejeune et al, 1959) trisomy 18 with Edwards syndrome (Edwards et al, 1960) and trisomy 13 with Patau syndrome (Patau et al, 1960) stimulated the progress of medical cytogenetics, and cytogenetic techniques rapidly became routine tools for diagnosis and counseling in clinical practice.

1.2 CHROMOSOME BANDING

The advent of chromosome banding in the late 1960s was a landmark development in cytogenetics (Hsu,1979). It subdivided each chromosome into visually recognizable zones or bands. This allowed recognition of individual human chromosomes and consequently the detection and identification of smaller chromosome aberrations (Caspersson et al, 1970). With the subsequent introduction of cell synchronization procedures and prometaphase banding by Yunis in 1976, the resolution level of cytogenetic analysis achieved marked improvement. Prometaphase banding enabled recognition of micro-deletions causing specific clinical syndromes such as del(11p13) for aniridia/Wilms tumour, del(13q13) for retinoblastoma, del(15q13) for Prader-Willi and Angelman syndromes, and del(17p15) for Miller-Dieker syndrome (Gardner and Sutherland, 1996). However, a significant technical limitation of conventional cytogenetic techniques at this time was their inability to accurately characterize all cytologically recognizable chromosomal rearrangements. This is because many chromosome bands are indistinguishable from each other, thus their origin in a structurally abnormal chromosome can not be assigned with certainty.

The next and most dramatic development in cytogenetic detection methodology had its inception in 1969-1970. The technique was called **in situ hybridization** and it allowed the unequivocal visual identification of specific chromosomes and chromosomal segments through light microscopy (Pardue and Gall, 1970; Jones,

1970). In situ hybridization combined the methods of cytogenetics and molecular genetics into a new field - molecular cytogenetics.

1.3 ISOTOPIC IN SITU HYBRIDIZATION

The process of isotopic in situ hybridization (IISH) required a segment of probe DNA specific for a desired target chromosome to be chemically modified (labeled) with radioactive molecules, such as 3H, 14C or 35S (Malcolm et al, 1986). The target DNA was usually fixed to a microscope slide in a cytologically recognizable form ie the hybridization occurred *in situ*. Both the target and the probe DNA were denatured causing dissociation of the two strands of the DNA molecule. The target and probe DNA were mixed and the strands were allowed to recombine. Under appropriate conditions the probe annealed by hydrogen bonding to the target at sites where the base pairs were complementary, forming a double stranded 'hybrid' molecule. Excess DNA was then removed from the slide and visualization was by autoradiography.

The first three groups of researchers to perform isotopic in situ hybridization (Gall and Pardue, 1969; John et al, 1969; Buongiorno-Nardelli and Amaldi, 1970) detected highly repeated sequences such as satellite DNA and ribosomal RNA, in cytologic material. Molecular cloning was not yet possible and in situ hybridization was therefore restricted to those sequences that could be purified and isolated by conventional biochemical methods. The first groups used tritium detected by autoradiography using silver halide emulsion. After development, precipitated silver grains formed along the track of the beta particles emitted at the time of radioactive decay. With tritium, these grains were concentrated within several microns of the target sequence. Visible microscopically, this silver 'signal' had to be differentiated statistically from a considerable level of 'background' noise, much of which was caused by hybrid formation at sites of similar but not exact sequence homology. The low level of specific activity obtained for radio-labeled DNA probes compared to RNA probes resulted in less efficient detection (Hsu, 1979), however both were used successfully in early in situ hybridization experiments (Jones, 1970). The low sensitivity of the technique was compensated for by choosing target sequences that contained many hundreds

of copies, and many repeated DNA sequences were successfully localized on human chromosomes in this way (Jones et al, 1973; Gosden et al, 1975).

The advent of DNA cloning in the mid-1970s, a consequence of the discovery of restriction enzyme specificity, dramatically improved the potential of the methodology (Cohen et al, 1973). A clone is a fragment of DNA which has been inserted into a vector then used to transfect bacteria. The DNA of interest is amplified many times during bacterial growth and later extracted for use as a probe. Labeled, cloned DNA probes provide sequences free from other contaminating sequences. Since clones were relatively easy to prepare (Malcolm et al, 1977), high probe concentrations could be used: this offset such problems as the partial reannealing of complementary DNA in probe solutions following denaturation.

By the early 1980s the advent of cloned probes plus modifications to the isotopic in situ hybridization techniques were permitting rapid, regional mapping of single-copy genes. Using a method which became a standard in situ hybridization technique, Harper and Saunders (1981) utilized dextran sulphate in the hybridization solution which they used to successfully localize a 14.9kb single copy cloned probe. Dextran sulphate is an anionic polymer, and its effect in hybridization solution is the result of the exclusion of DNA from the volume occupied by the polymer, ie there is effectively an increase of DNA concentration at the surface of the hybridizing material (Nakamura, 1990). This property had previously been utilized in molecular genetics, where the hybridization of DNA probes to DNA on filters (Southern blots) was found to be greatly enhanced by its use (Southern, 1975). An added advantage that the polymer provided for in situ hybridization was that it promoted the formation of probe/vector networks, so that efficiency was increased without a concomittant increase in background (Harper, 1983). Harper and Saunders (1981) also provided a method for identifying the location of signal with great specificity by using high resolution chromosomes banded following the hybridization procedure. Similar techniques soon followed (eg Zabel et al, 1983).

A number of researchers saw the potential advantages of using non-radioactive labels including hazard reduction and a decrease in the lengthy exposure time required for autoradiography. Non-isotopic hybridization also had the potential to give a greater precision of signal localization. When detecting a radioactive isotope the resolution is limited by the scattering distance in the emulsion (1-2 microns for tritium). However, light emitted subsequent to an excitation process such as fluorescence arises from molecules directly attached, or close to, the probe (less than a few hundred Angstroms in indirect labeling methods) (Coppey-Moisan, 1994).

1.4 FLUORESCENCE IN SITU HYBRIDIZATION

When fluorescence is used in signal detection the process is called fluorescence in situ hybridization (FISH). The method of detecting labeled sites is dependant upon the nature of the original label attached to the probe. A molecule with fluorescent properties may be bound directly to the probe DNA (**direct labeling**) and the resultant hybrid molecule can be visualized microscopically immediately after hybridization. The probe may be modified with a hapten which is recognized after hybridization by a fluorescently-tagged binding protein (**direct detection**), or modified with a hapten which requires a series of binding proteins, the last of which is fluorescently tagged (**indirect detection**).

The first use of FISH was published in 1977 when Rudkin and Stollar used fluorescent antibodies to DNA-RNA hybrids to detect the RNA in cells. With this approach the probe, unlabeled, was detected after hybridization with highly specific affinity reagents that produced a fluorescent precipitate on the specimen. In 1980 Bauman et al detected target DNA sequences in situ by using direct fluorochrome labeled probes. Although indirect procedures yield a higher spatial resolution and sensitivity than direct, since significantly smaller probes are needed, applications within research and clinical cytogenetics make both procedures extremely useful.

1.5 APPLICATIONS OF FISH

1.5.1 Gene Mapping and Ordering

The production of accurate maps of the genome is critically dependant on the ability to finely map probes either along a chromosome arm, ie metaphase mapping, or within non-dividing cells, ie interphase mapping. Metaphase and interphase mapping have several clear advantages over other forms of mapping. Genetic mapping relies upon the availability of polymorphic markers and large pedigrees; physical mapping by pulsed-field gel electrophoresis (PFGE) of DNA cut with rare-cutting restriction enzymes depends upon the presence or methylation status of rare restriction sites; hybrid mapping with interspecific somatic cell hybrids containing various subsets or segments of human chromosomes (Callen et al, 1992b) relies heavily on the sometimes crude identification of breakpoints in chromosomes of poor morphology or banding patterns. Metaphase and interphase mapping are not hampered by these features.

Metaphase Mapping

Probes have been ordered by fractional length measurements of their signals on unbanded chromosomes (Lichter et al, 1990), by their locations on banded chromosomes (Lawrence et al, 1990) or by differential labeling and detection (Dauwerse et al, 1992). Fluorescent light exhibits spectral properties of both excitation and emission. Thus **differential labeling** refers to the direct labeling of probes with molecules which can be distinguished because they fluoresce in different colours, or to the indirect labeling of probes with haptens detected by antibody-conjugated fluorophores of various colours. The range of genomic distances that can be studied by FISH in this way is dictated by several factors. The first is the minimum distance at which two fluorescent dots can be completely resolved with a high quality fluorescent microscope. **Resolution** is expressed as the ability to separate the images of two neighbouring object points. Because of the undulatory nature of light, the spatial resolution is limited by diffraction, about 0.2 microns for blue light (wavelength ~510nm) with a 1.3 numerical aperture objective lens (Trask et al, 1989; Coppey-Moisan, 1994). This limitation is somewhat reduced by differential labeling, since fluorescent dots of different colours can be more easily distinguished (even when slightly overlapping) than dots of the same colour. The second factor is the need to deposit a sufficiently large cluster of fluorescent molecules at the site of hybridization for visualization: this requires a large single copy probe or a

multiple copy target. The third factor is the degree of compaction of the chromatin fibre. FISH to high resolution metaphase preparations provides at best a physical mapping technology with a resolution of approximately one megabase, if differentially labeled probes are used (Trask et al, 1991). If the distance between two probes is less than 1Mb there is insufficient separation of the hybridization signals and only one is detected.

Interphase Mapping

Lawrence et al (1988) used fluorescence detection of biotinylated probes hybridized to interphase nuclei of the Namalwa cell line in order to investigate the integration of the Epstein-Barr virus (EBV). The sensitivity and low background of the method allowed them to successively detect down to 5kb of the EBV genome in a single interphase nucleus. They concluded from their measurements that the condensation of DNA was at least 10-fold less in chromatin than in chromosomes. Combined with the greater resolution of fluorescent signal, FISH to nuclei provided a 200-fold greater resolution than autoradiographic techniques on chromosomes. Sequences separated by as little as 50kb were shown to be resolvable in interphase nuclei using FISH (Lawrence et al, 1988; Trask et al, 1989) and the relative distance between DNA sequence probes in interphase was shown to be related in a simple way to the distance between the sequences on the linear DNA molecule (Trask et al, 1989).

Van den Engh expanded this work in 1992, showing that the sites of DNA sequences separated by 100 to 2000kb followed a random walk model. This model provides simple, statistical rules which relate the separation of DNA sequences along the linear DNA strand to the observed separation of DNA sequences in interphase chromatin. Van den Engh used 13 cosmids from a 4Mb region of human chromosome 4p16.3. Because of their close proximity, these cosmids could not be resolved on metaphase chromosomes. They were hybridized in differentially labeled pairs to interphase nuclei and 100-200 measurements were made between each set. The measurements yielded both probe order and estimates of the genomic distance between the probes. Although the error of **individual** pairwise measurements was still relatively large, the confidence of distance estimates was substantially increased by measuring the

average distance between any given probe and several other probes, ie building a consensus map based on **numerous** pairwise combinations.

Not all combinations of probes allowed an order to be derived in this way. To deduce order from interphase measurements, the distances between pairs of probes needed to be similar. It was difficult, for example, to orient two closely spaced markers relative to a third over a megabase away (Shipley, 1993). Also, accuracy of linear relationship was shown to decrease significantly between 1 and 2Mb, with the linear correlation essentially lost above this. This arises from several factors. (1)Nuclei which *in vivo* are arranged in three dimensions are now swollen, fixed, squashed and viewed in two dimensions. (2)DNA denaturation, incubation at high temperatures and with formamide, are likely to randomize some levels of chromatin structure. (3)Evidence exists for the presence in interphase of specific tertiary structure, in the form of chromosome folding, condensation and nuclear matrix association (Den Dunnen et al, 1992).

The ability to provide relative orders for DNA sequences in chromosome regions of known gene density is important in the positioning of loci which then become candidates for diseases assigned to that region. Trask (1991) used FISH to interphase chromatin to derive the order of DNA sequences in a 2-3Mb region of human chromosome band Xq28. Trask used two methods to obtain this order; the relative distance measured between pairs of probes, and the order of colours in nuclei hybridized with three or more differentially-labeled probes. These methods have since been utilized to ascertain probe order in many regions of interest, eg the region surrounding the Ewing sarcoma breakpoint on chromosome 22 (Shipley et al, 1993), the human major histocompatability complex (*MHC*) class 11 region within chromosome band 6p21 (Senger et al, 1993), and the breast cancer (*BRCA1*) region on chromosome 17q12-21 (Flejter et al,1993).

1.5.2 Clinical Applications of Interphase FISH

Cytogenetic banding techniques have facilitated the location of the gene defects in several clinical syndromes, eg the deletion of chromosome 15 causing Prader-Willi syndrome (Gregory et al, 1990) and the identification of cytogenetic

deletions or rearrangements in regions of the X chromosome causing Duchenne muscular dystrophy (Monaco et al, 1986), choroideraemia (Cremers et al,1990) and Kallmann syndrome (Legouis et al, 1991). At the same time, molecular techniques have highlighted the existence of cytogenetically undetectable rearrangements in a number of single gene disorders, eg the duplication in chromosome 17p11.2 causing Charcot Marie Tooth disease Type 1A (Lupski et al, 1991) and the small deletions in chromosome 16p13 causing thalassemia-associated mental retardation (Wilkie et al, 1990). FISH is ideally placed to provide the rapid and reliable detection of many such anomalies for the clinical cytogenetic laboratory.

The research work in interphase mapping rapidly led to clinical application of the methodology. There were several perceived advantages in interphase cytogenetics, compared to standard metaphase analysis. The technique was rapid, because it did not require dividing cells: routine cytogenetic analysis required 48-72 hours of tissue culture for blood chromosome analysis and 7-21 days for pre-natal studies. The interphase FISH method also seemed to lend itself to automated analysis, offering the possibility that it might be cheaper to perform. Trisomy of chromosomes 13, 18, 21, and aneuploidy of the X or Y chromosomes, together account for >97% of chromosome abnormalities identified during pre-natal karyotyping (Ledbetter et al, 1992). With the continued development of probe labeling and detection systems the possibility existed that these chromosomes could be visualised simultaneously following in situ hybridization, and in fact by 1992 Ried et al had reported simultaneous multicolor fluorescence detection of probe sets for those five chromosomes in uncultured amniotic fluid cells. The technique could also be used with specimens which were traditionally difficult to analyze cytogenetically because of poor morphology and low to non-existent mitotic indices. These were most often from leukaemic specimens, from solid tumors (where the cytogenetically abnormal cell line was often only a proportion of the cell population), and from patients on chemotherapy whose cells do not readily undergo mitosis (Smit et al, 1991; Tiainen et al, 1992).

There were three types of probes utilized for interphase cytogenetics: whole chromosome probes, chromosome specific repeats, and probes to specific loci. While each of these probe types theoretically could be used to identify the presence of a particular chromosome, different parameters affected the ability of each to detect more subtle chromosomal abnormalities (Klinger et al, 1992).

Whole chromosome probes label ('paint') the entire chromosome (or large chromosomal segments) and are useful in detecting structural rearrangements. The probes are generally composed of DNA extracted from flow-sorted chromosome-specific libraries (Voojis et al,1993), and the addition of unlabeled human genomic or cot-1 DNA can be used to block repetitive sequence elements from binding to non-target chromosomes (Sealy et al, 1985). With each chromosome occupying a distinct focal domain in the interphase nucleus, a discrete hybridization signal should theoretically be obtained in most nuclei for each specific chromosome present. Lichter et al (1988b) reported rapid detection of numerical and structural aberrations in interphase cells using a chromosome 21 paint. Pinkel et al (1988) detected translocations and aneuploidy involving both chromosome 4 and chromosome 21 using paints for these chromosomes. However, disadvantages soon became apparent. The large and complex probes generated a very large diffuse signal, making the edges of the signal very difficult to delineate, and overlap of the hybridization domains was common. This overlap was exacerbated by the positioning of the nucleolar organizing regions (NORs) of the short arms of the acrocentric chromosomes, which frequently results in the close proximity of homologs within this group. In practice this commonly resulted in frequencies as low as 10-50% for trisomic amniocyte nuclei showing three hybridization domains (Yu et al, 1990; Kuo et al, 1991).

Chromosome-specific repeat probes target the repetitive alpha-satellite sequences found in the peri-centromeric regions of human chromosomes, and the satellite 11 and satellite 111 sequences found in the heterochromatic regions of chromosomes 1,9,16 and the Y chromosome. These probes produce very strong and relatively punctate signals in interphase nuclei. They are the probes of choice for the rapid diagnosis of aneuploidy, for the detection of cytogenetically distinct

cell populations (eg in solid tumours) and for the detection of rare abnormal cells indicating residual disease. However, difficulties also existed in the clinical utilization of these probes. The chromosomal specificity of the repetitive probes was very sensitive to hybridization conditions (Klinger et al, 1992) and where sequence homology was very high - eg between chromosomes 13 and 21, or 14 and 22 - differentiation was generally impossible. This resulted in both pairs of chromosomes showing signal in an interphase nucleus. Further, the centromeric position of the probes limited identification of Robertsonian translocations (where acrocentric chromosomes are joined at their centromeres), affecting the detection of Down syndrome. Similar to the problems of chromosome painting, the clustering of acrocentric chromosomes around NORs could obscure resolution of the centromeric probes.

Complicating the analysis further, signal size is related to length polymorphisms of the pericentromeric alphoid repeats. Hence a normal individual may have a relatively small array of alpha satellite DNA on a chromosome which consequently remains undetected by interphase FISH. Failure to detect one of the chromosome 21 pair following interphase FISH of a chorionic villus sample was reported by Mizunoe and Young (1992). When FISH was performed on chromosomes from the parents of the pregnancy as well as several close relatives, this discrepancy was explained by striking heteromorphism at the chromosome 21 centromeres. Only one chromosome 21 centromere fluoresced brightly. The other showed a very small fluorescent signal easily overlooked in the interphase cell nucleus. A similar undetected repeat region was the possible cause of the pre-natal diagnosis error of Benn et al (1992), who wrongly interpreted their inability to detect a Y chromosome (part of a 13, 18, 21, X and Y probe set) as indicative of a 45,X (Turner) genotype. Verlinsky et al (1995) studied 516 amniotic fluid samples using alphoid repeat probes. There were four cases of discrepancy between FISH results and follow-up cytogenetic analysis. In three cases the results were false negative, ie the 13/21 alpha satellite probe failed to detect the presence of trisomy 21 (Down syndrome). The fourth case was a false positive diagnosis of Down syndrome, made when the same probe cross-hybridized to chromosome 22 alpha satellite DNA.

The main problem in producing **locus specific probes** for clinical FISH is that each must contain enough genomic sequence from a particular chromosome region so that hybridization is consistent and reliable: most commonly several tens to hundreds of kb. Such probes avoid many of the problems of paint or repeat probes in that they are less subject to population polymorphisms and the signals are very punctate. Ried et al (1992b) reported a method for building cosmid contigs of ~100kb for any chromosome region, allowing a precise selection of probes from the critical region of a disorder. A **cosmid contig** is a series of cosmids each one of which partially overlaps at least one other; a contig can therefore span a long continuous stretch of genomic DNA. Contigs of euchromatin have been used for the interphase diagnosis of Down syndrome (Klinger et al, 1992; Zheng et al,1992; Davies et al, 1994), but this approach to diagnosis requires stringent methodology. Klinger et al (1992) constructed DNA probe sets based on cosmid contigs that were specific for chromosomes 21, 18, 13, X and Y. In a blind interphase FISH analysis of 117 uncultured amniotic fluid samples they correctly identified 21 of 21 abnormal samples, but noted that hybridization efficiency was, on average, lower in trisomic samples, necessitating stringent cutoff levels in the performance criteria used to distinguish abnormal (trisomic) cases from normal (disomic) cases. A discussion of this approach is included in Chapter 4 of this thesis which describes the use of a small cosmid contig in the development of a method for the diagnosis of Charcot Marie Tooth syndrome Type 1A.

The problems posed by these three types of probes (ie whole chromosome paints, chromosome specific repeat probes, and locus specific probes) were seen as less important by cancer cytogeneticists who were delighted to have a methodology that could be applied to cells that produced infrequent metaphases of poor quality, and in which many chromosome abnormalities would have remained undetected but for interphase FISH. With chromosome paints, complex translocations were revealed (Speleman et al, 1992). With repeat probes, abnormal copy numbers of targeted chromosomes were detected in interphase nuclei in bone marrow cells, blood cells, fine-needle aspirations and tissue sections. Locus specific probes were used for detection of specific tumor suppressor genes, oncogenes and amplified genes. Two-colour FISH was used

with cosmid probes to portions of the *BCR* and *ABL* genes (35kb and 200kb probes respectively) to detect the *BCR/ABL* fusion in nuclei from patients with chronic myeloid leukemia (CML) (Tkachuk et al, 1990). In leukemic cells from CML patients there is typically a rearrangement of chromosomes 9 and 22 which produces a small fusion chromosome called the Philadelphia (or Ph') chromosome. This fusion event was detected in all samples analyzed by FISH, including a small proportion which were cytogenetically Ph' negative. An advantage of the interphase method was that genotype analysis could be associated with cell phenotype (judged by morphology and other markers), permitting study of the lineage specificity, as well as the frequency, of cells carrying the CML genotype.

1.5.3 Clinical Applications of Metaphase FISH

Although FISH did not replace chromosome banding, it greatly enhanced the capacity for accurate cytogenetic interpretation of metaphase chromosomes. One example is the identification of the origin of small, extra, 'marker' chromosomes. The first tools of identification were the chromosome specific repeat probes (Callen et al, 1991, 1992a). Later studies have used microdissection and PCR amplification of the marker DNA, followed by 'reverse painting' of the resultant DNA preparation onto normal human chromosomes: the sites of hybridization indicate the origin of the marker (Fang et al, 1995).

Whole chromosome paints were used in specific translocation detection, especially where the banding pattern in the regions involved was ambiguous (Brandt et al, 1994). Paints also facilitated the detection of cryptic translocations (Kohler et al, 1994) and complex translocations(Chu et al, 1993). Locus specific probes were developed and made commercially available for the detection of the partial deletions of chromosome 5p in Cri du Chat syndrome, of 4p in Wolf-Hirshhorn syndrome, 7q in Williams syndrome, 17p in Miller-Dieker syndrome and 22q in the CATCH 22 group (ONCOR, 1996).

While clinical cytogeneticists enthusiastically applied FISH technology, those involved in the construction of physical maps of the human genome began an adaption of the method which they hoped would facilitate the ordering of

sequences (1)less than 50kb apart and (2)between 1 and 3 Mb apart. Resolution demands of greater than 3Mb were being met by FISH to prometaphase chromosomes, while resolutions between 50kb and 1Mb could be achieved on G0 interphase fibroblast nuclei.

1.6 EXTENDED CHROMATIN TECHNIQUES

The methods that were developed disrupted higher-order chromatin structure so that the simple linear relationship between interphase distance and genomic separation would hold over a much greater distance range. The type of denaturant and denaturing procedure varied, but resulted in decondensed chromatin fibres present on a slide as either DNA free of its higher-order structure but retained within a nuclear envelope; as DNA extending from a remnant nuclear structure; or as DNA completely free of nuclear structure.

Brandriff et al (1991) performed FISH on the highly decondensed chromatin of sperm pronuclei, following fusion of human sperm and hamster oocytes. Hamster egg cytoplasm processed the tightly packaged sperm DNA into large diffuse networks of chromatin fibre bundles, providing hybridization targets more extended than those available in somatic interphase cell nuclei. They used known cosmids and cosmid pools from the factor V111/colour vision pigment gene region of human chromosome band Xq28. Cosmid pairs appeared as two resolved fluorescent dots whose distance apart increased with increasing distance between the probes on the linear DNA molecule. Tracks of signals produced by pools of cosmids formed either a straight line of fluorescent dots or more complicated patterns. The mean pronuclear distances between hybridization sites were about three times greater than those measured in interphase cells for equivalent genomic distances and although the method had the disadvantage of being very laborious, requiring growing cells and a very specific expertise, they were able to achieve a resolution of 20-800kb. Florijn et al (1995) achieved a resolution of 1Mb using nuclear halos to map cosmid contigs, with an average fibre condensation of 0.33um/kb.

Wiegant et al (1992) hybridized alphoid and cosmid DNAs to fibroblasts grown on microscope slides. They utilized detergent and high salt extraction followed

by intercalating dye/UV light treatment. The procedure generated many DNA loops surrounding the remaining nuclear matrix in a halo-like fashion. The hybridization signals appeared as strings of fluorescent dots. The number of fluorescent signals on the strings varied from cell to cell and ranged from 10-20 for all the cosmids tested. No consistent pattern for the signal distribution along the string was visually or statistically apparent for any of the cosmids tested. The length of the string of signals was typically 10 microns for a 35-40kb cosmid. The signal from the hybridization of alphoid DNA was also visible as beads-on-a-string. If all DNA is available for hybridization then a continuous length of alphoid DNA should produce a continuous fluorescent line. Since this was not the case, and since the pattern of dots was not identical from one hybridization site to another, factors such as DNA loss or random association of hybridizing probe fragments were assumed to play an important role in the signal pattern. The resolution range of the technique was 10-200kb, and required growing cells for its utilization.

Heng et al (1992) employed the topoisomerase inhibitor *N*-[4-(9-acridinylamino)-3-methoxyphenyl]methane-sulfonamide (*m*-AMSA), which is believed to be necessary for chromosome condensation, although the actual mechanism is not fully understood. They achieved consistent results with the growing cells of lymphocyte cultures and using a simple alkaline lysis procedure produced homogenous spreads of free chromatin. Hybridization of a 40kb cosmid onto the fibres produced a fluorescent dot rather than the expected 'string'. This may have been due to the signal detection being amplified through a secondary antibody, or the concentration of signal being highest in the centre of the hybridization region, causing the signal to become rounded in appearance. The best resolution achieved with this technique was 10kb.

Parra and Windle (1993) released DNA from cells at harvest using detergent lysis. The stretching of the duplex DNA strands was accomplished by the movement of the aqueous, viscous, lysed drop of cells across the surface of a glass slide, causing threads of the DNA to be pulled out in a long stream. The predicted span per basepair for relaxed duplex DNA is 0.34nm. With this technique the stretched DNA occasionally extended up to twice the expected

length. The highest mapping resolution was in fact obtained when the DNA was stretched from 100-200% of the expected length. Naming the method 'DIRVISH' DNA (DIRect VISual Hybridization), they achieved a remarkable resolution of <5-700kb.

Fidlerova et al (1994) used buffered formamide, and alkaline treatment with sodium hydroxide (in two separate methods), to release chromatin from routinely harvested and fixed cells from lymphocyte cultures. These were the first methods available to be used with stored, unique specimens. Contact of cells with sodium hydroxide produced immediate disruption of nuclei spread on a slide. The resultant free DNA fibres stretched down the slide during a rinse with methanol. Formamide-treated nuclei were not disrupted until they came subsequently into contact with the methanol: the released chromatin was simultaneously fixed onto the slide. With formamide treatment the borders of most of the disrupted nuclei could still be defined, allowing hybridization signals from the one nucleus to be identified. Fidlerova et al (1994) performed FISH with various combinations of differentially labeled cosmid probes from the HLA class 11 region of human chromosome band 6p21.31. Regions of overlap of the red- and green-labeled probes were seen as a change of colour to yellow. The lengths of the strings of signal from any one probe varied considerably but were consistently twice as extended (0.38um/kb) on the alkaline lysis slides as on the formamide treated slides (0.2um/kb) (Senger et al, 1994). A resolution of several kb was achieved with this method.

Haaf and Ward (1994a,b) extended chromatin mechanically by cyto-centrifugation of unfixed hypotonically-treated metaphase chromosomes. Stretching was caused by shear forces generated during cytocentrifugation, with chromosomes being extended from 5 to 20 times their normal length. The chromosomes became highly deformed and metaphase groups were broken up, so that localization of new sequences on specific chromosomes was not possible. However, the procedure permitted mapping in the 1-3Mb resolution range, plus ordering of clones separated by as little as 170kb. Since the telomeres of the chromosomes were generally identifiable, the orientation of probes along a chromatid was also possible.

Table 1 lists the genomic distances over which various FISH mapping techniques are informative.

Table 1 : RESOLUTION OF FISH MAPPING TECHNIQUES

Technique	*Resolution Range (kb)*	*Cell population*
chromosomes	>1000	mitotic (fixed)
stretched chromosomes	>170	mitotic (unfixed)
interphase nuclei	50-1000	G0/G1 (fixed)
interphase pronuclei	20-800	sperm pronuclei (unfixed)
halo DNA	10-200	any (unfixed)
DIRVISH DNA	<5-700	any (unfixed)
m-AMSA free chromatin	10-350	any (unfixed)
NaOH or FA free chromatin	3-500	any (fixed)

The described procedures for the production of extended chromatin fibres considerably enlarged the strategies available for mapping and ordering by FISH. They form part of the method development in this thesis (Chapter 6).

1.7 PUTATIVE BREAST CANCER GENE ON HUMAN CHROMOSOME 16

A region of the long arm of chromosome 16 has been identified as containing a tumour suppressor gene likely to be involved in the latter stages of the pathway leading to invasive breast cancer. One approach to the identification of genes involved in the neoplastic pathway is by loss-of-heterozygosity (LOH) studies. With this approach, the genome is screened and regions are identified where a genetic marker which is heterozygous in normal tissue is homozygous in tumour tissue, ie it has lost its heterozygosity.

Tumour suppressor genes have been shown to be inactivated by two mutational 'hits', for example the retinoblastoma gene *RB1* located on chromosome 13q13. The initial gene mutation may be constitutional or, more usually, somatic. The

second hit is very often a chromosomal mechanism, always occurring somatically, the effect of which is that the cell becomes homozygous for the initial (first hit) mutation in the tumour suppressor gene. The second hit may include whole chromosome loss through mitotic non-disjunction (possibly followed by reduplication of the remaining homolog), or partial chromosome loss through intra-chromosomal deletion or mitotic recombination. All these mechanisms lead to LOH at or around the tumour suppressor gene.

Non-random LOH is widely assumed to imply the presence of a tumour suppressor gene. A number of studies have confirmed the frequent (40-60%) LOH of the long arm of chromosome 16 in primary breast cancer (Larsson et al, 1990; Sato et al, 1990; Harada et al, 1994). Tsuda et al (1994) reported LOH of the 16q24-qter region in 52% of the 234 tumours in their study. In a study of LOH in tumours from patients with familial breast cancer, Lindblom et al (1993) found that the LOH on 16q in patients at time of operation was significantly correlated with the occurrence of subsequent distant metastases 1 to 13 years later, suggesting that LOH on 16q may be involved relatively late in the oncogenic pathway.

The region of 16q24.3 to which LOH in tumours has been localized is estimated to be 2-3Mb in length. Chapter 5 of this thesis describes the use of FISH methods to provide data on the fidelity and orientation of cosmid clones assigned to this region, thus assisting in the early steps toward the cloning and identification of the putative breast cancer gene.

1.8 FRAGILE SITES ON HUMAN CHROMOSOMES

A concise summary of the nature, methods of induction and clinical significance of fragile sites can be found in Sutherland et al (1996 - APPENDIX A). A description of those areas relevant to this thesis follows.

Fragile sites are specific points on chromosomes that show a nonrandom tendency to reveal a gap or a break when the cells from which the chromosomes were prepared are exposed to a specific chemical agent or condition of tissue

culture. Hence a fragile site is an area of chromatin that is not compacted when viewed during mitosis (Sutherland et al, 1996 - APPENDIX A). Fragile sites can be divided into two main categories, rare or common, according to their frequency within the population; they can be further sub-classified according to the conditions of tissue culture under which they are expressed. Rare fragile sites are seen at a specific location on 1 in 40 chromosomes at most. Common fragile sites are probably present on all chromosomes and as such are normal components of chromosome structure.

Each fragile site has a gene symbol *'FRA'*, followed by the number of the chromosome on which the site is observed, then an assigned letter of the alphabet. Thus the rare folate-sensitive fragile site on chromosome 12 at band q13.1 is written *FRA12A*.

The first description of a rare fragile site on a human chromosome (possibly *FRA9A* on band q32 of chromosome 9) was by Dekaban (1965), while Lejeune et al (1968) were the first to demonstrate their heritable nature. Sutherland (1979) identified the relationship between a large group of rare fragile sites and conditions of folic acid and thymidine deprivation in tissue culture.

The Rare Folate-Sensitive Fragile Sites

Only fragile sites from this group have so far been characterized at the molecular level. These are *FRAXA, FRAXE, FRAXF, FRA11B* and *FRA16A*. Their common feature is a dynamic mutation of a CCG trinucleotide repeat that occurs naturally at the sites. The CCG repeat is polymorphic in copy number in the normal population. A small increase above the normal range of copy numbers is termed a premutation. Individuals with the premutation do not express a fragile site but may have children in whom the repeat copy number has increased even further. At a certain level of increased copy number, the DNA in the area becomes subjected to CpG methylation. Once this has happened, the fragile site can be induced to appear in the chromosome and any gene in which the fragile site is located could be expected to be inactivated.

Since Dekaban's 1965 publication, 21 other rare folate-sensitive fragile sites have been described. Three of these - *FRAXA*, *FRAXE* and *FRA11B* - have been associated with human disease; *FRAXA* with fragile X syndrome, the most common familial form of intellectual handicap (Kremer et al, 1991); *FRAXE* with a mild form of mental retardation (Chakrabarti et al, 1996; Gecz et al, 1996); and *FRA11B* with a mental retardation/malformation condition known as Jacobsen syndrome (Jones et al, 1995 - APPENDIX A).

The Rare Distamycin A - Inducible Fragile Sites

The fragile sites in this category are induced by distamycin A and by a number of related compounds that bind externally and without intercalation to the minor groove of the double stranded DNA molecule in AT-rich regions. Daunomycin and quinacrine mustard, both AT-specific compounds, interact with DNA by intercalation (Waring, 1970), but neither agent induces the expression of *FRA16B*, one of the sites in this category (Schmid et al, 1986).

There are two subgroups in the category. Subgroup A, which contains only *FRA16B* and *FRA17A*, can be distinguished from subgroup B because the former can also be induced by the addition of BrdU to the culture medium. The fragile sites in subgroup B have been described only in the Japanese population (Takahashi et al, 1988).

FRA16B at chromosome band 16q22.1 and *FRA17A* at chromosome band 17p12 are often expressed spontaneously, although they require induction for maximum expression. Both have been observed in homozygous expression without apparent phenotypic effect. *FRA16B* is the most frequent of the rare fragile sites, being carried by $\sim$1 in 20 individuals of the German population (Schmid et al, 1986). The difference in folate-sensitivity and induction requirements makes it highly unlikely that the distamycin A-inducible fragile sites will have the same DNA structure as the folate sensitive fragile sites.

The Rare Bromodeoxyuridine-Requiring Fragile Sites

The two fragile sites in this group - *FRA10B* and *FRA12C* - are induced only by BrdU and the related compound BrdC. *FRA10B*, at chromosome band 10q25.2,

is the second most frequent of the rare fragile sites being present in 1 in 40 individuals of the Australian population. *FRA10B* has also been seen in homozygous expression without clinical consequence (Sutherland, 1981).

The Common Fragile Sites

Common fragile sites are a constant feature of the genome, being present on all chromosomes as part of normal chromosome structure. Their role, if any, in genome organization is presently unknown although their ubiquitous nature suggests a conservation of function, if a function can be shown (Wilke et al, 1996). The ~90 common fragile sites form three groups on the basis of their induction by either aphidicolin, 5-azacytidine or bromodeoxyuridine. It is not known what relationship exists between the chemistry of this induction and DNA composition at the sites, although the inducers are all agents that perturb DNA replication.

The aphidicolin-inducible fragile sites form the major block of common fragile sites. They are seen at low levels of expression under the same conditions that induce the rare folate-sensitive fragile sites but can be specifically induced by aphidicolin, a compound that inhibits DNA polymerase α (Glover et al, 1984). Ethanol has a synergistic effect upon fragile site induction by aphidicolin (Kuwano and Kajii, 1987).

The Significance of Fragile Sites

Fragile sites are of interest for several reasons. There is association of three rare folate-sensitive fragile sites with human disease. Although this is the only link so far observed between fragile sites and clinical phenotype, the role of fragile sites in oncogenesis is an ongoing debate of much controversy.

Gene mappers have used the rare fragile sites as genetic markers for linkage studies (Sutherland et al, 1982), and both the rare and common fragile sites have been used to improve the resolution of chromosomal in situ hybridization (Baker et al, 1992; Lapsys et al, 1992). Chapter 7 of this thesis describes the use of FISH to investigate the expansion of sequences around the rare distamycin A-inducible fragile site *FRA16B*.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Chemicals and Reagents

acetic acid glacial (UNIVAR, Ajax chemicals)

anti-digoxigenin-rhodamine (Boehringer Mannheim)

anti-rabbit IgG-rhodamine (Boehringer Mannheim)

avidin-FITC (Vector)

berenil (diminazine aceturate) (Calbiochem)

biotinylated goat anti-avidin (Vector)

bromodeoxyuridine (BrdU) (Sigma)

BSA (bovine serum albumin) (Sigma)

colchicine (Sigma)

deoxycytidine (Sigma)

DABCO (diazabicyclooctane) (Sigma)

DAPI (diamidino phenylindole dihydrochloride) (Sigma)

dextran sulfate (Pharmacia)

EARTH rubber cement (Malvern Star)

ethanol (Ace)

formamide (Fluka) :deionized with mixed bed resin before use

methanol (UNIVAR, Ajax Chemicals)

mixed bed resin AG 501-X8(D) (Bio-rad)

potassium chloride (UNIVAR, Ajax Chemicals)

propidium iodide

rabbit anti-sheep IgG (Vector)

SDS (Sigma)

thymidine (Sigma)

2.1.2 Enzymes

RNaseA, from bovine pancreas (Boehringer Mannheim) :heat denatured at 100°C for 10min to inactivate any contaminating DNases.

2.1.3 Buffers

SSC :prepared as 20X, diluted and adjusted to pH7.0 for use.

(1XSSC = 150mM NaCl, 15mM sodium citrate)

TE :10mM Tris-HCl pH8.0, 1mM EDTA

PBS: (Cytosystems)

2.1.4 DNA Probes

C1, C2 and RR6 : a gift from Helen Eyre; P1.1 : a gift from Erica Woollatt; from the Dept of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, Adelaide.

D1, D4 (ONCOR)

2.1.5 Human Experimental Material

Human fibroblast and LCL cultures were a gift from Rosalie Smith; human lymphocyte cultures used as normal control material were a gift from Trudy Hocking; both from the Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, Adelaide.

2.1.6 Kits

DIG DNA labeling kit (Boehringer Mannheim)

BIO-Nick nick translation system (Gibco BRL)

2.1.7 Photographic Materials

KODAK Ektachrome 160 T colour slide film (KODAK)

KODAK Royal Gold 1000 colour print film (KODAK)

2.1.8 Media and Solutions

2.1.8.1 Cell Culture

All media were sterile filtered after preparation.

MEM-FA (CSL) (Sutherland et al, 1979)

RPMI 1640 powdered medium(GIBCO)

fetal calf serum (FCS) (CSL)

phytohaemagglutinin (PHA), M form (GIBCO)

trypsin-EDTA (Trace)

2.1.8.2 In situ Hybridization

antifade mounting medium - 90% glycerol, 10% 200uM TRIS pH7.5, 2%(w/v)DABCO

blocking solution - 4XSSC (pH7.0), 1%BSA

DNA (sodium salt) from human placenta (total human DNA) (Sigma)

hybridization mixture - 2XSSC, 10%(w/v)dextran sulfate, 50% deionized formamide, 1%(v/v)Tween 20

salmon sperm DNA (Calbiochem)

2.2 METHODS

2.2.1 Lymphocye Culture

Up to 8 drops of heparinized venous blood were grown in 5ml culture medium MEM-FA or RPMI 1640 containing 5%FCS, 0.5 units/ml heparin and 2%(v/v)PHA, for 3 days. (Adapted from Moorhead et al, 1960)

2.2.2 Cell Synchronization ('TdC' method)

Lymphocyte cultures were prepared as in 2.2.1 and grown for 3-4 days. Twenty-two hours before harvest, cells were blocked in the S phase by the addition of 0.3mg/ml thymidine. Five and three quarter hours before harvest the block was released by replacement with fresh medium and the addition of 30ug/ml deoxycytidine and 10ug/ml BrdU (Adapted from Wheater and Roberts, 1987).

2.2.3 Fragile Site Induction

The distamycin A-inducible rare fragile site FRA16B was induced in lymphocytes by the addition of 150ug/ml Berenil 24hrs before harvest (Sutherland, 1984).

2.2.4 Chromosome Harvest

Colchicine, 55ul of 0.1mg/ml, was added to 5ml unsynchronized lymphocyte cultures 1hr before harvest, and to synchronized lymphocyte cultures 20min before harvest.

Cells were spun (all centrifugation was at 1000rpm for 5min), the supernatant was removed, and the pellet was resuspended in 0.075M KCl to 9ml.

After 25min the suspensions were mixed with 1ml fixative (3:1 methanol:acetic acid), centrifuged, the supernatant removed and the pellet resuspended in 10ml fresh fixative. The centrifugation and resuspension were repeated a further two times.

Harvested cells were stored at an appropriate concentration in fixative at -20°C until spread. (Adapted from Moorhead et al, 1960)

2.2.5 Fibroblast Culture

Cells were grown in 5ml RPMI 1640 supplemented with 5% FCS in T25 tissue culture flasks at 37°C. The medium was changed twice weekly and cultures were gassed with 5% carbon dioxide in medical air prior to incubation. If confluent nuclei at G0/G1 phase were required, cells were cultured until they completely covered the base of the flask then were grown for a further 3-4 days. Cells were grown to confluence because the pattern of signal given by hybridization to G0/G1 cells is less complex than that given by cells in late S or G2. The former give rise to a single signal whereas the latter can give doublet signals (one for each chromatid). (Adapted from Rooney and Czepulkowski, 1986)

2.2.6 Trypsin Treatment of Fibroblast Cultures

All medium was poured off the fibroblast cultures and retained in labeled centrifuge tubes. Cells were rinsed in 2ml Ca++ and Mg++ free PBS, and 1.5ml trypsin-EDTA was added. Cultures were incubated at 37°C until the cells began to detach from the base of the flask. The trypsin/cell suspension was then transferred to the matching tube of medium: this halted the proteolytic action of the trypsin. If trypsinized cells are to be used for FISH to extended chromatin fibres then prolonged trypsinization should be avoided, as trypsin weakens the nuclear envelope. This will affect later spreading of the fibres. (Adapted from Rooney and Czepulkowski, 1986)

2.2.7 Fibroblast Harvest

Trypsin-treated fibroblast cultures were harvested as for 2.2.4, with 3hrs colchicine treatment prior to harvest (55ul 0.1mg/ml colchicine per 5ml culture) if metaphases were required, or with omission of the colchicine treatment if G0/G1 nuclei were being collected. (Adapted from Rooney and Czepulkowski, 1986)

2.2.8 Preparation of Total Human DNA

To prepare competitor DNA, placental DNA was dissolved to 10mg/ml in TE overnight, microwaved 3min and heated to 100°C for 8hrs. The resulting DNA fragments were up to 700bp long with a majority at 300bp. (Sealey et al, 1985)

2.2. 9 Preparation of Salmon Sperm DNA

Desiccated salmon sperm DNA was dissolved to 25mg/ml in double distilled water, sonicated for 4min at the highest limit and diluted to 10mg/ml. The resultant DNA fragments were ~50-400bp long. (Maniatis et al, 1982)

2.2.10 Nick Translation

Biotin-14-dATP was incorporated into double stranded DNA by nick translation using the BRL BIO-nick labeling system. The protocol was as recommended by the supplier except that salmon sperm DNA (250X) was added as carrier DNA during ethanol precipitation of the labeled DNA.

2.2.11 Random Primer Labeling

Double stranded DNA was random primer labeled with DIG-11-dUTP according to the protocol of the supplier (Boehringer Mannheim). The amount of DNA in each labeling reaction was ~500ng, incubation was overnight at 37°C, and salmon sperm DNA (250X) was added as carrier during the ethanol precipitation of the labeled DNA. The ratio of DIG-labeled uridine to dTTP in the kit produces DNA with a DIG-labeled nucleotide incorporated every 20^{th} to 25^{th} nucleotide. This labeling density will permit optimal steric interaction between the hapten and the fluorophore-conjugated anti-DIG antibodies, for the conjugates are large enough to cover about 20 nucleotides.

2.2.12 Slide Preparation - Metaphases and Nuclei

Fixed cell suspensions were brought from -20°C storage and allowed to come to room temperature for 1hr. Single drops at appropriate concentrations (determined visually under an inverted microscope) were dropped onto dry, ethanol-cleaned slides in a controlled atmosphere of 46-48% humidity at 24°C. Spread slides were stored for 1-4 days in a box with desiccant prior to FISH.

2.2.13 Slide Preparation - Extended Chromatin Fibres

Several ECF procedures reported in the literature (particularly Parra and Windle, 1993, and Fidlerova et al, 1994) were modified to form **Protocol 1** which utilizes live cells and **Protocol 2** which utilizes fixed cell suspensions. The appearance and concentration of fibres on the first slide prepared by either method were checked under an inverted

microscope, or by mounting the slide in DAPI-containing antifade, before the remainder of the slides for the experiment were made.

2.2.14.1 Protocol 1 (for live cells)

A Ficoll-Paque lymphocyte suspension (optimum concentration 1000-2000cells/ul) or cells collected by centrifugation of cell cultures may be used.

Prepare lysing solution : 0.5%SDS, 50mM EDTA, 200mM Tris (pH7.4)

Use dry, ethanol-cleaned glass slides. Pipette 1.5ul suspended cells in a short straight line at one end of a slide laying flat. Air dry. Immediately distribute 10ul lysate over the cells and leave undisturbed for 5min. Tilt the slide with a steady hand so that the DNA strands run in vertical streams down the length of the slide. Air dry for 3min (excess drying can cause the DNA to become inert). Fix strands to the slide by immersing in a coplin jar of 3:1 methanol:acetic acid for 5min (excess fixation can cross-link DNA so that it becomes difficult to denature, or becomes totally inert). Air dry. (Adapted from Parra and Windle, 1993)

2.2.14.2 Protocol 2 (for fixed cell suspensions)

Prepare lysing solution : 5:2 5M NaOH:absolute ethanol (mix just before use).

Bring stored, fixed cell suspensions to room temperature.

Use dry, ethanol-cleaned glass slides. With a transfer pipette, drop one drop of cell suspension onto one end of a slide laying flat. As evaporation becomes visible ($\sim$10secs) place slide into coplin jar of 1XPBS to rehydrate. After 30secs drain slide briefly and lay flat on paper towelling. Immediately pipette 50ul lysate to the short side of the cells, take up a large glass coverslip and very gently drag the lysate with a steady hand across the cells and along the length of the slide. Drop 2 drops methanol onto the short side of the cells - the methanol will be seen to travel along the slide. Continue adding single drops until the slide is flooded. Leave slide horizontal for a further 1min then transfer to a coplin jar of methanol for 5min fixation. Air dry. (Adapted from Fidlerova et al, 1994)

2.2.15 Fluorescence in situ Hybridization (FISH)

2.2.15.1 General conditions:

Biotin- and DIG- labeled probes were hybridized in situ to metaphase, nuclei and ECF preparations. The method is summarized in Callen et al, 1990, although several further modifications have been made. These are covered in the following method description.

Pretreatment: Nuclei and ECF slides were pretreated by baking at 60° C for 5min.

RNaseA treatment: Slides were incubated in DNase-free RNase A (100ug/ml) in 2XSSC, pH7.0 at 37° C for 60min then passed through a dehydrating alcohol series before being airdried in a vertical position.

Denaturation: Chromosomal DNA was denatured by incubating the slides in a solution of 70% formamide/2XSSC, pH7.0 for 3min at 70°C followed by immediate application of the denatured probe mixture.

Probe mix: Probe mix concentrations are given in the text.

For simultaneous hybridization of biotin- and DIG-labeled probes, DNAs were either (1) combined before probe desiccation or (2) desiccated separately, resuspended in hybridization mixture and pre-reassociated separately, then combined just before application to a slide.

Probe Mix Preparation: Labeled probe DNA was combined with the total human DNA and desiccated to zero volume. The DNA was resuspended in 10ul hybridization mixture, denatured at 70°C for 5min, and if appropriate allowed to pre-reassociate (for the removal of repetitive sequences) for 50min at 37°C. Following pre-reassociation the probes were applied to briefly-drained, denatured slides, covered with a coverslip and sealed at the corners with rubber cement. Incubation was overnight at 37° C in a humid chamber.

2.2.15.2 Probe detection:

The biotin- and DIG- detection systems detailed below were determined in part by the particular filter sets available at the commencement of the project and in part by the availability of relevant antibody conjugates.

Post Hyb Washes: Coverslips were shaken off and slides were put through 2 changes of 50%formamide/2XSSC, pH7.0 at 43°C for 10min each, 2 changes of 2XSSC, pH7.0 at RT for 5min each, and one wash of 1XSSC at RT for 10min.

[All subsequent incubations were under parafilm in a moist chamber at RT, and all washes after antibody incubations were in 4XSSC/0.05%Tween 20, twice for 5min each.]

Following the post-hybridization washes, the slides were equilibrated for 3min in 4XSSC/0.05%Tween 20, then incubated for 10min in a blocking solution of 4XSSC/1%BSA. (Adapted from Kievits and Wiegant (1988), personal communication).

Detection & Amplification:

For biotin detection, slides were incubated in avidin-conjugated FITC diluted to 5ug/ml with blocking solution for 20min. The slides were washed. An amplification step of

biotinylated anti-avidin diluted to 1ug/ml with blocking solution was applied to the slides for 20min followed by a further layer of avidin-FITC. Slides were washed, given final rinses in 2XSSC and 1XPBS, and mounted in an antifade solution containing propidium iodide (as counterstain) and DAPI (for chromosome identification) - see concentrations below.

For DIG detection, slides were incubated for 20min in rhodamine-conjugated sheep anti-DIG diluted to 0.7ug/ml with blocking solution. Slides were washed. Amplification was by a 20min incubation in rabbit anti-sheep IgG diluted to 15ug/ml with blocking solution, followed by washes and a 20min incubation in rhodamine conjugated anti-rabbit IgG diluted to 0.7ug/ml in blocking solution. Slides were washed, rinsed in 2XSSC and 1XPBS, and mounted in antifade solution containing propidium iodide and DAPI - see concentrations below.

For simultaneous biotin and DIG detection, slides were exposed to one biotin detection procedure followed by one DIG detection procedure. This was found to be more successful than simultaneous application of the two antibody systems. Slides were mounted as for DIG detection.

Antifade solutions:

FITC detection (chromosome preparations): 0.08ug/ml PI , 0.6ug/ml DAPI

Simultaneous FITC & TRITC detection (chromosomes & nuclei): 0.001ug/ml PI , 1ug/ml DAPI

FITC detection (nuclei): 0.03ug/ml PI , 0.4 ug/ml DAPI

Simultaneous FITC & TRITC detection (ECF preparations): no PI, 1ug/ml DAPI.

NB: Slides of fibre preparations were mounted in antifade solution containing DAPI only, as even low concentrations of propidium iodide tended to partially obscure rhodamine signal on the fibres.

Microscopy: Slides were viewed under an Olypus BX40 microscope fitted with UV, FITC and TRITC single pass filters and an FITC/TRITC dual pass filter (Chroma).

CHAPTER 3

LOCALIZATION OF SIX SINGLE COPY GENES TO HUMAN CHROMOSOMES

The gene localizations described in this Chapter have all been published during the course of the project and are appended (Appendix A).

The purpose of mapping a gene by FISH is to identify the precise physical position the gene occupies on a chromosome. In clinical cytogenetics there is the potential to relate that information to the existence of cytogenetic aberrations in patients with specific clinical disorders. For example, a disease may be co-segregating with a small chromosome deletion indicating that the disease gene is located in the missing part of that chromosome. Or the gene may be located in the breakpoint of a chromosome translocation, with the break inactivating gene function. Observations such as these have been used to identify about half of the 40 genes for known diseases so far cloned, eg neurofibromatosis and Duchenne muscular dystrophy (Francek et al, 1985; Cawthon et al, 1990).

Gene mapping information is also vital in providing candidate genes for diseases assigned by linkage analysis to a particular chromosomal interval. Known as positional cloning, this strategy requires statistically significant linkage to be established between a disease occurrence and DNA markers located in a particular chromosomal interval. The interval is refined by analysis of recombinant families, with markers that are not included in the crossing over being placed closer to the mutation. A search can then be made amongst genes localized to that reduced interval for any whose function might be relevant to the disease phenotype, with the possibility that sequencing such a gene may reveal mutations in affected family members.

The isolation and mapping of human genes has provided a wealth of ready-made material for those who work with other species. Comparative mapping has revealed distinct regions of conservation as well as evolutionary rearrangements between humans and other species (Eppig and Nadeau, 1995; McConkey et al, 1996 - Appendix A). Well developed physical maps of human chromosomes are a useful tool for linking cloned genes to phenotypes in many species, and particularly in elucidating mouse models of human diseases.

One example is the mapping of the *Pax3* gene to the 'Splotch' locus in mice. When Waardenburg Syndrome Type 1 (WS1) in humans was mapped to the homologous region of the human genome (chromosome 2q37), *Pax3* became a potential candidate for WS1.

A subsequent molecular study showed mutations in the gene both in 'Splotch' mice and WS1 individuals (Eppig and Nadeau, 1995). Similarly the *trembler* (*Tr*) mouse serves as a model for human Charcot Marie Tooth disease type 1A (CMT1A) based on its neurological phenotype. The mouse *Pmp-22* gene was mapped to the genetically defined *Tr* locus on mouse chromosome 11, and point mutations found in two domains. Human *PMP22* clones were isolated and the gene found to map to human chromosome 17p11.2 in a region of conserved synteny with mouse chromosome 11. The *PMP22* gene has since been shown to be duplicated, but not disrupted, in CMT1A patients (see Chapter 4 of this thesis).

In order to extend the human gene map, six genes were hybridized by FISH to human chromosomes. The six genes encoded the following proteins:

Sulphamidase: Sulphamidase is the enzyme deficient in the autosomal recessive disorder Sanfilippo A syndrome. Sanfilippo A syndrome is one of four recognized Sanfilippo sub-types (A,B,C and D) that result from deficiencies of different enzymes involved in the lysosomal degradation of heparan sulphate. The Sanfilippo sub-types are also known as mucopolysaccharidosis (MPS) type 111 (ie MPS-111A,B,C and D) and are part of the large group of lysosomal storage disorders (LSD) (Scott et al, 1995 - Appendix A).

Grb 14: Grb 14 is a novel SH2 domain-containing adapter. The SH2 domain-containing proteins consist largely of non-catalytic protein modules and function downstream of tyrosine kinases, linking receptors or other tyrosine phosphorylated proteins to separate effectors (Baker et al, 1996 - Appendix A). Grb 14 is related in molecular architecture and sequence homology to Grb 7 and Grb 10, the genes for which are situated close to the genes which encode members of the erbB family of receptor tyrosine kinases.

NPY: Neuropeptide Y, NPY, is one of the most highly conserved peptides known. It acts both centrally and peripherally to regulate the cardiovascular system, and moderates a wide range of other important physiological activities (Baker et al, 1995 - Appendix A).

Brn-2: Brn-2 protein is expressed in the adult brain in a subset of neurons, the hippocampus and the hypothalamus. cDNA molecules encoding the Brn-2 protein have been associated with N-Oct-3 and N-Oct-5 activities in the human brain. The *brn-2* gene

encodes the binding activities of the N-Oct-3 and N-Oct-5 octamer transcription factors, and may play a role in the specification of melanocytic cell fate, differentiation and in the oncogenic potential of melanoma (Angus et al, 1995 - Appendix A). Therefore, in addition to localization of the *brn-2* gene, its presence in four melanoma cell lines was investigated by FISH.

NKB 1: The surface antigen, NKB 1, is expressed on T and natural killer (NK) cell subsets. It is a putative inhibitory receptor for HLA-B class 1 molecules possessing the Bw4 serological epitope (Litwin et al, 1995; Baker et al, 1995 - Appendix A).

Humly 9: Humly 9, the human homolog of mouse Ly 9, is a cell membrane antigen restricted in expression to leucocytes. It belongs to an immunoglobulin-superfamily subgroup which includes CD48, CD2 and LFA-3 (Sandrin et al, 1995 - Appendix A).

3.2 MATERIALS

3.2.1 Probes

Sulphamidase: 3.6kb clone, insert only, containing the coding sequences plus some intronic sequences of the human sulphamidase gene.

Grb 14: 1.7kb cDNA clone in plasmid vector, containing the entire Grb 14 transcript.

NPY: 15kb genomic fragment in lambda vector, containing the entire human NPY transcript.

Brn-2: 15kb genomic fragment in lambda vector, containing the entire human Brn-2 transcript.

NKB1: 1.8kb cDNA clone in plasmid vector, containing the entire human NKB1 transcript.

Humly 9: 1.9kb cDNA clone, insert only, containing all but the 5' sequences encoding the first domain of the human Humly 9 transcript.

3.2.2 Cell Lines

Venous blood cultures from normal males were used for all single copy gene localizations.

The human melanoma cell lines used for study with *Brn-2* have been described by Sturm et al, 1991. They were MM418, derived from a primary human melanoma, and MM96L, A2058 and MM138, established from secondary melanomas.

3.3 METHODS

3.3.1 **Probes**

All probes were biotin labeled by nick-translation with biotin-14-dATP (2.2.11).

3.3.2 **Slide Preparation**

Lymphocytes: Metaphase preparations were obtained from peripheral blood lymphocyte cultures from normal human males by the 'TdC' method (2.2.2) and were spread for FISH as described in 2.2.12.

Fibroblasts: Metaphase preparations were obtained as described in 2.2.5, 2.2.6 and 2.2.7 (Fibroblast Culture, Trypsin Treatment, and Harvest)

3.3.3 **Probe Mix**

A set of four probe mixes was prepared from each probe: these consisted of 50ng, 100ng, 150ng, and 200ng of labeled DNA per 10ul hybridization mixture plus 150-200x total human DNA as competitor. Where the probe was described as cDNA the 50ng mix did not have total human DNA added. Probe mixes (1) and (3) were applied to metaphases from one normal male. Probe mixes (2) and (4) were applied to metaphases from a second normal male.

For *brn-2* analysis of melanoma cell lines, a second probe was added to the Probe Mix to unequivocally identify the normal or structurally altered chromosomes 6 in the analyzed metaphases. P308 - a probe which hybridizes to the pericentromeric alphoid repeat sequences of chromosome 6 - was biotinylated, denatured separately and added to the Brn-2 probe mix just before hybridization. The final concentration of p308 on the slide was 0.004ng/ul. The very low concentration was necessary because the Brn-2 cDNA probe with which it was coupled was undergoing two rounds of amplification.

3.3.4 **Hybridization**

Hybridization conditions are described in 2.2.15.1.

3.3.5 **Detection and Amplification**

Detection and amplification were as described in 2.2.15.2 except that the first post-hybridization wash was at 45°C and two rounds of amplification followed the first avidin-FITC detection step.

3.3.6 **Scoring of slides**

Slides were scored by plotting all signal which touched the chromosomes within a metaphase onto an ideogram of the human karyotype. A minimum of 20 metaphases was scored in this way for the slide with the highest signal and lowest background; a minimum of 15 metaphases was scored on a second slide from a different individual to check the first result.

3.3.7 **Photography**

The representative metaphases shown in this study were photographed using KODAK Ektachrome 160T colour slide film. Colour or black and white prints were prepared from the colour slides, and high resolution photocopies of these used for the thesis, without signal enhancement or background reduction.

3.4 RESULTS AND COMMENTS

The results of the six localizations are presented in Table 2.

Table 2 : Localization conditions for six single copy probes

gene	probe description	optimal probe conc on slide	ratio of total human DNA : probe DNA	localization
sulphamidase	3.6kb genomic	5ng/ul	200:1	**17q25.3**
GRB 14	1.7kb cDNA	15ng/ul	150:1	**2q23**
NPY	15kb genomic	5ng/ul	200:1	**7p15.1**
Brn-2	15kb genomic	5ng/ul	200:1	**6q16**
NKB1	1.8kb cDNA	5ng/ul	none	**19q13.4**
Humly 9	1.9kb cDNA	5ng/ul	none	**1q22**

3.4.1 **Sulphamidase**

Results: Analysis of 20 metaphases from the first normal male showed signal at 17q25 in all cells: 61% of this signal was at 17q25.3. Only one non-specific background dot was seen in these 20 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from the second normal male. A representative metaphase is shown in figure 3.1.

The optimum concentration of probe DNA amongst those tested was 5ng/ul, with 200x total human DNA. Signal was observed on four chromatids (ie both arms of both homologs) in 2 out of 20 metaphases; on 3 chromatids in 6 metaphases, on 2 chromatids in 8 metaphases and on one chromatid in 4 metaphases. The non-specific background level rose to unacceptable levels as the probe concentration increased.

Discussion: The localization of sulphamidase to 17q25.3 was interesting in that attempts to obtain a 'whole chromosome' localization by PCR analysis of somatic cell hybrids using the NIGMS Human/Rodent somatic cell hybrid mapping panel #2 were unsuccessful, despite good control reactions (Scott et al, 1995 - Appendix A). The implication is that the chromosome 17 in that panel may be missing part of band 17q25.

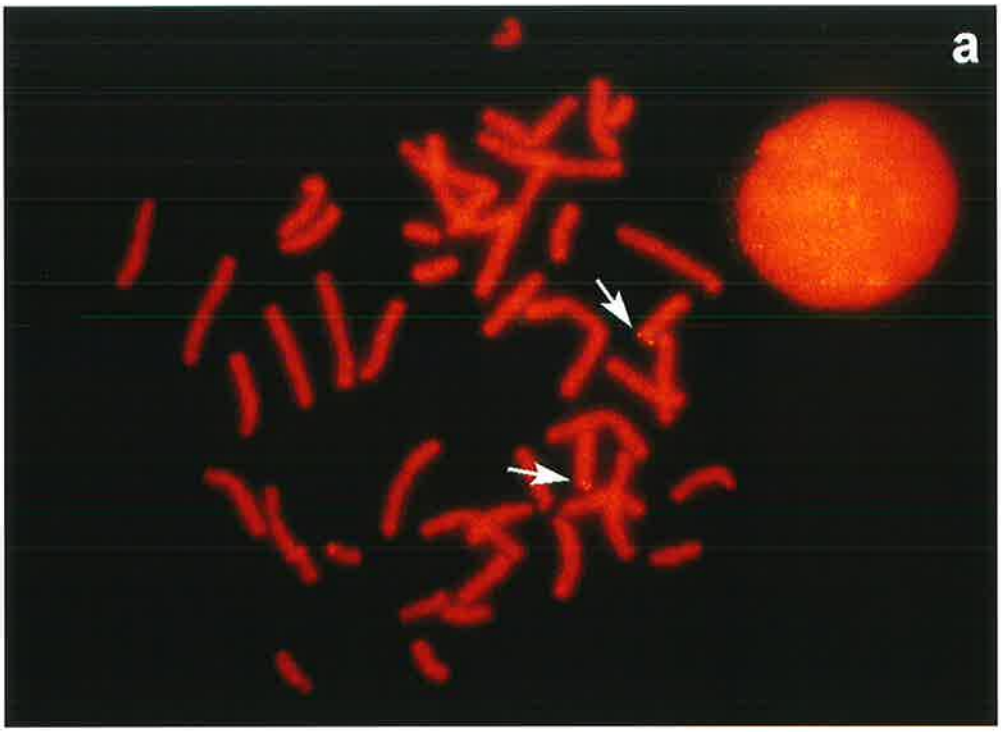
The only other disease phenotype presently mapped to 17q25 is Russell-Silver syndrome (MIM#180860), a form of dwarfism with an unknown pattern of inheritance. Due to differences in clinical phenotype, and the well known pathophysiology of LSD and function of sulphamidase, Russell-Silver syndrome and Sanfilippo A syndrome are unlikely to be allelic. The gene coding for α-N-acetylglucosaminidase, which is deficient in MPS-111B has been mapped to 17q11-21, a considerable genomic distance from 17q25.

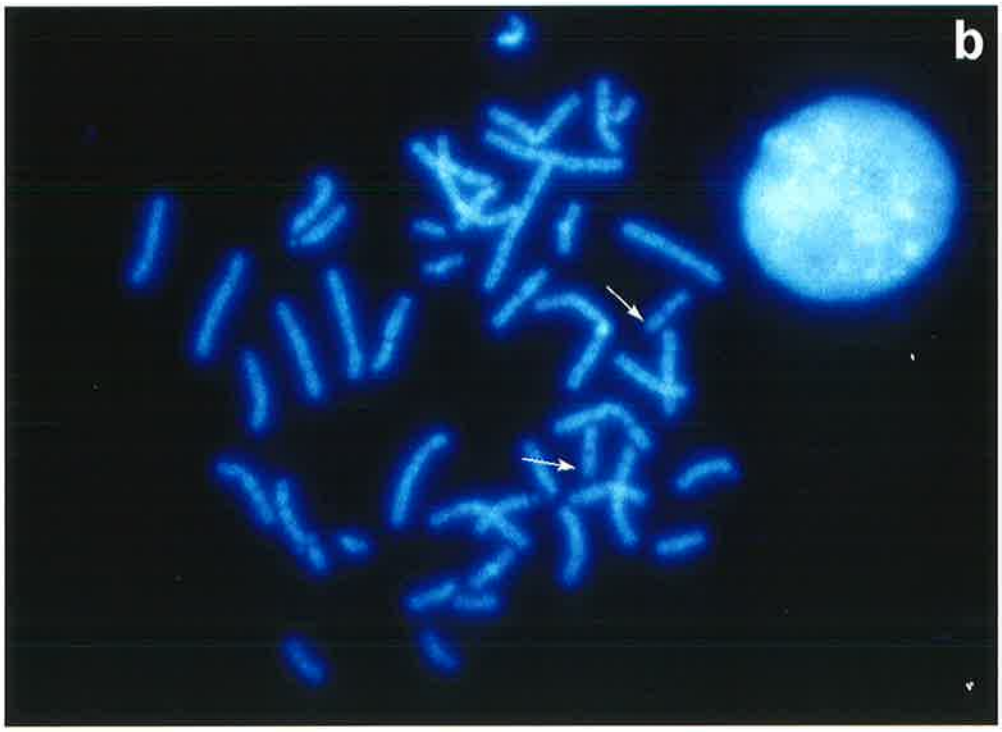
Figure 3.1 : METAPHASE SHOWING FISH WITH THE 3.6kb SULPHAMIDASE PROBE

(a) Normal male chromosomes stained orange with propidium iodide. Yellow fluorescent signal from hybridization of the probe to chromosome band 17q25.3 is indicated by arrows.

(b) The same metaphase as (a) stained blue with DAPI for chromosome identification. Each chromosome 17 is indicated by an arrow.

NB The photocopies of these two images were of poor quality, therefore the colour photographic slides were scanned as a computer file and printed on a high quality (dye sublimation) printer. There was no signal enhancement or background reduction.


a


b

3.4.2 **Grb 14**

Results: Analysis of 30 metaphases from the first normal male showed signal at 2q22-q24 in 18 of the 30 cells: 82% of this signal was at 2q23. Five of the metaphases also showed signal at 12q13 while five showed signal at 6q27. In addition, 31 non-specific background dots were observed in these 30 metaphases, none of which occurred at the same site more than once. The ideogram of this localization is shown in figure 3.2a.

Because of the appearance of minor peaks in this localisation, 56 metaphases were scored for the second normal male. Twenty-eight of the 56 cells showed signal at 2q23-q24: 79% of the signal was at 2q23. Seven of the 56 metaphases also showed signal at 12q13-q24.1 while 6 showed signal at 6q26-27. There were 52 non-specific background dots seen in these 56 metaphases, none of which occurred at the same site more than twice. The ideogram of the second localization is shown in figure 3.2b.

The optimum concentration of probe DNA amongst those tested was 15ng/ul with 150x total human DNA. Signal was seen on 4 chromatids of chromosome 2 in 1 of the 30 metaphases scored for the first normal male; on 3 chromatids in 4 metaphases; on 2 chromatids in 8 metaphases and on one chromatid in 7 metaphases. Concentrations below 15ng/ul gave poorer signal, while 20ng/ul showed increased background.

Discussion: This study localizes the *GRB14* gene to chromosome 2q23. The *GRB7* and *GRB10* genes are situated close to genes which encode members of the erbB family of receptor tyrosine kinases. The closest known *ERBB* family gene to 2q23 is that encoding erbB4 at chromosome 2q33.3-q34. Consequently the *GRB14* and *ERBB4* genes appear to have co-segregated during evolution, in a similar manner to *GRB7* and *ERBB2* on chromosome 17.

The *ERBB3* gene has been localized to 12q13 (Kraus et al, 1989), so it is of interest that one of the minor peaks of hybridization for *GRB14* was at the same locus. Grb 14 bears ~50% amino acid identity with Grb 7 and Grb 10 over the central region of these proteins and ~70% amino acid identity in the C-terminal SH2 domain. The evidence of minor localization peaks at 12q13 and 6q27 is therefore highly suggestive of the existence of further *GRB7*-related genes at these loci.

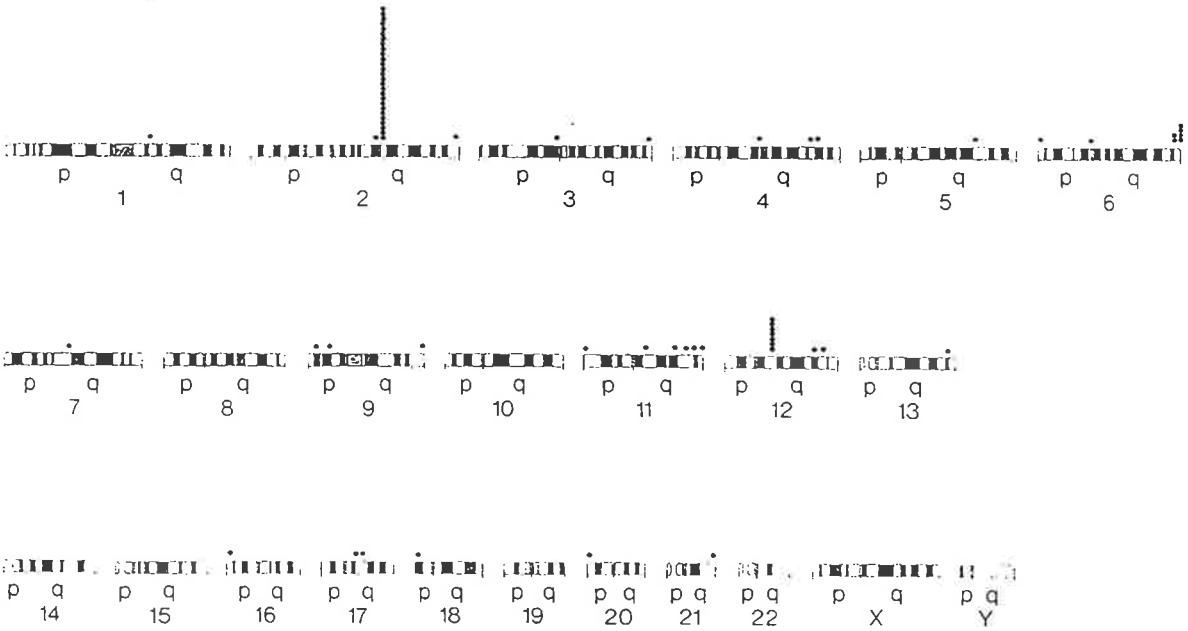
Figure 3.2a : SIGNAL DISTRIBUTION FOLLOWING LOCALIZATION OF *GRB14* TO METAPHASES FROM THE FIRST NORMAL MALE.

All fluorescent dots on, or touching, the chromosomes from 30 metaphases were plotted on an ideogram of the human karyotype at the 550-band level of resolution. A major peak of signal is evident at chromosome band 2q23. Minor peaks are present at 6q27 and 12q13.

Figure 3.2b : SIGNAL DISTRIBUTION FOLLOWING LOCALIZATION OF *GRB14* TO METAPHASES FROM THE SECOND NORMAL MALE.

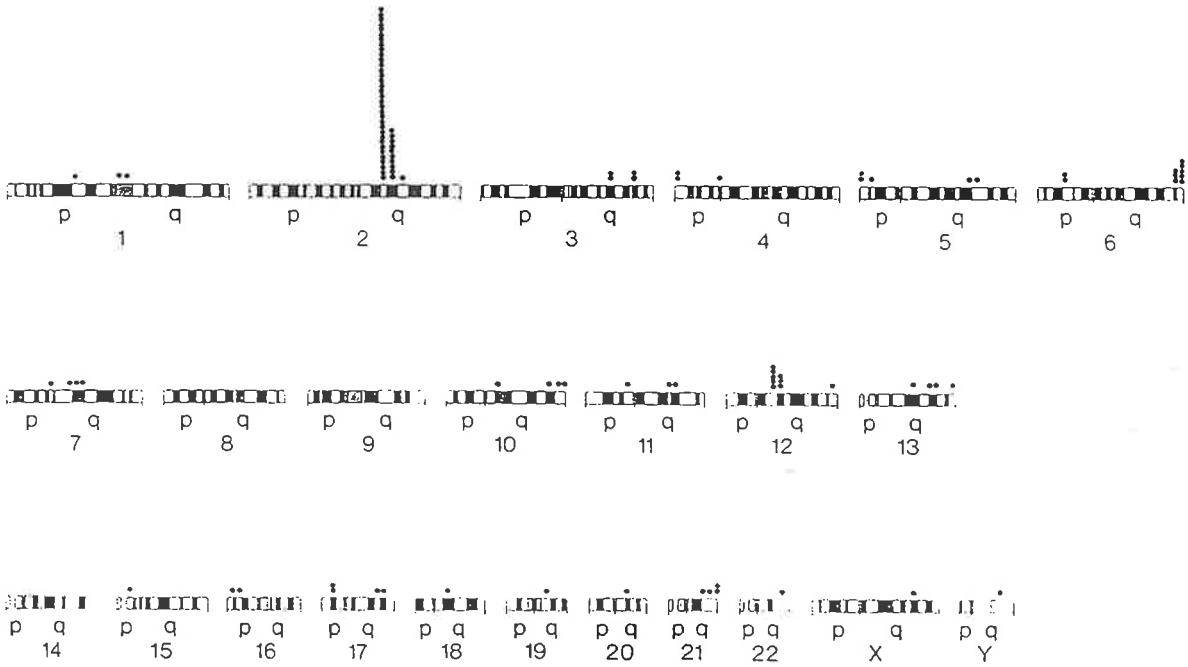
Scoring of fluorescent signal from 56 metaphases was as described in Fig. 3.2a. A major peak of signal is present at chromosome band 2q23. Minor peaks are again present at 6q27 and 12q13.

FIGURE 3.2a



Chromosomes

FIGURE 3.2b



Chromosomes

3.4.3 **NPY**

Results: Analysis of 20 metaphases from the first normal male showed signal at 7p14-p15.3 in all cells: 60% of this signal was at 7p15.1. There were 5 non-specific background dots seen in these 20 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from the second normal male. A representative metaphase is shown in figure 3.3.

The optimum concentration of probe DNA amongst those tested was 5ng/ul with 200x total human DNA. Signal was observed on 4 chromatids of chromosome 7 in 5 of the 20 metaphases; on 3 chromatids in 7 metaphases; and on 2 chromatids in 8 metaphases.

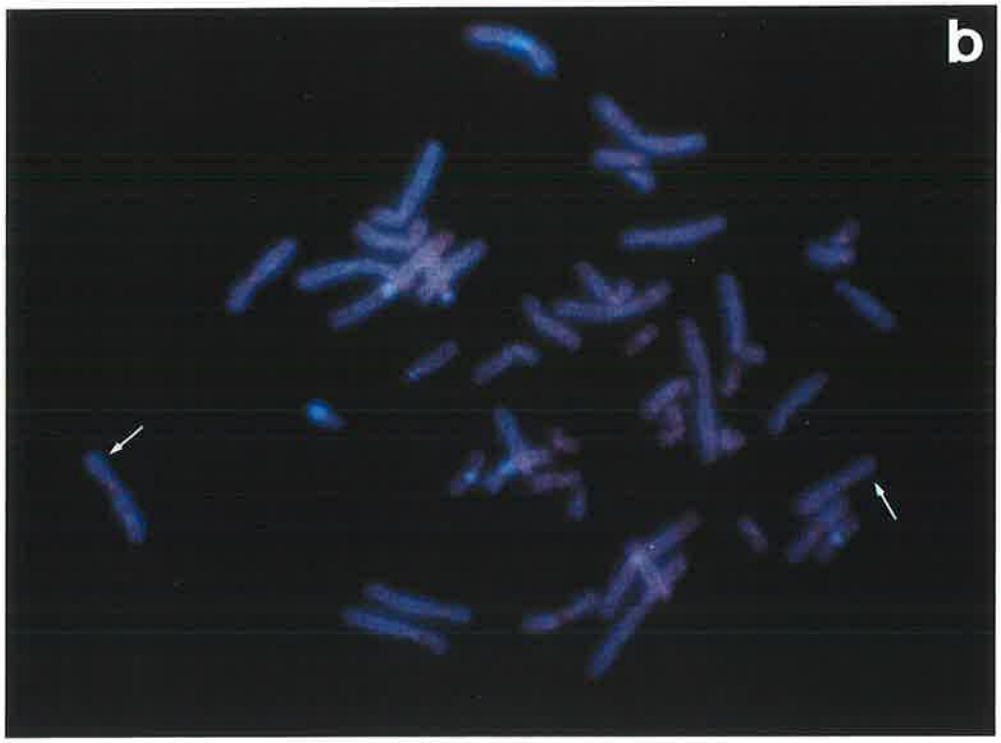
Discussion: NPY is a member of a gene family that also includes peptide YY (PYY) and pancreatic polypeptide (PPY). The PPY and NPY genes were previously assigned to chromosomes 17 and 7 by Takenchi et al (1986). Analysis of their structure and localization suggested that these genes arose from an initial gene duplication event that generated the NPY and PYY genes, followed by a duplication of the PYY gene to create the PPY gene (Hort et al, 1995 - Appendix A). The human PYY and PPY genes are only 10kb apart on chromosome 17q21.1. It is of interest that some members of other gene families (eg the homeobox gene HOXA) segregate with chromosome 7 and others (eg HOXB) with chromosome 17 in a manner similar to NPY and PYY. The implication is that these gene families arose from the duplication of an ancient linkage group, followed by chromosomal translocation.

Figure 3.3 : METAPHASE SHOWING FISH WITH THE *NPY* GENOMIC PROBE.

(a) Normal male chromosomes stained with propidium iodide. Fluorescent signal from hybridization of the probe to chromosome band 7p15.1 is indicated by arrows.

(b) The same metaphase as in (a) stained with DAPI for chromosome identification. Each chromosome 7 is indicated by an arrow.


a


b

3.4.4 **Brn-2**

Results (Localization): Analysis of 20 metaphases from the first normal male showed signal at 6q15-q21 in all cells: 93% of this signal was at 6q16. There was a total of 19 non-specific background dots seen in these 20 metaphases. A similar result was obtained from hybridization of the probe to 20 metaphases from the second normal male. A representative metaphase is shown in figure 3.4.

The optimum concentration of probe DNA was 5ng/ul with 200x total human DNA. Bright signal was seen on 4 chromatids in 7 cells; on 3 chromatids in 4 cells; on 2 chromatids in 4 cells and on one chromatid in 5 cells.

Discussion (Localization): Localization of the human *brn-2* gene to chromosome band 6q16 corresponds with the location of mouse *brn-2* on mouse chromosome 4, 3.6cM proximal to the reference locus Thyrotropin α, the human homolog of which lies at human 6q12-21 (Avraham et al, 1993).

The localization of *brn-2* to 6q16 placed it within a region (6q16-q21) commonly deleted or rearranged in melanomas. For this reason the *brn-2* gene was hybridized to four melanoma cell lines.

Results (Melanoma Cell Lines): FISH analysis revealed that the MM96L cell line had 2 copies of chromosome 6 but only one copy of *brn-2*. Deletions and rearrangements of chromosome 6 were observed in some MM96L cells (12/22 examined) but in every cell at least one copy of *brn-2* on a chromosome 6 was observed (fig. 3.5a).

Cell line MM138 had 6 copies of chromosome 6, all showing *brn-2* signal, in 23/33 cells examined (fig. 3.5b). In the remaining 10/33 cells an apparently structurally normal chromosome 6 was present which had lost *brn-2*. At least three other copies of chromosome 6 retained *brn-2* within these 10 cells.

Cell lines MM418 and A2058 had the same number of chromosomes 6 to copies of *brn-2*, ie 3 copies and 2 copies respectively.

Discussion (Melanoma Cell Lines): The results suggest that although the *brn-2* gene lies within, or adjacent to, the region of chromosomal deletion in melanoma, it is not itself totally deleted from, or rearranged, in melanoma cells. It has been shown that loss of *brn-2* leads to the complete loss of the tumour-forming ability of the Brn-2 negative cells in immunocompromised mice (Trent et al, 1990). This suggests that *brn-2* plays an essential, positive role in melanoma oncogenesis.

Figure 3.4 : METAPHASE SHOWING FISH WITH THE *brn-2* GENOMIC PROBE.

(a) Normal male chromosomes stained with propidium iodide. Fluorescent signal from hybridization of the probe to chromosome band 6q16 is indicated by arrows.

(b) The same metaphase as (a) stained with DAPI for chromosome identification. Each chromosome 6 is indicated by an arrow.


a

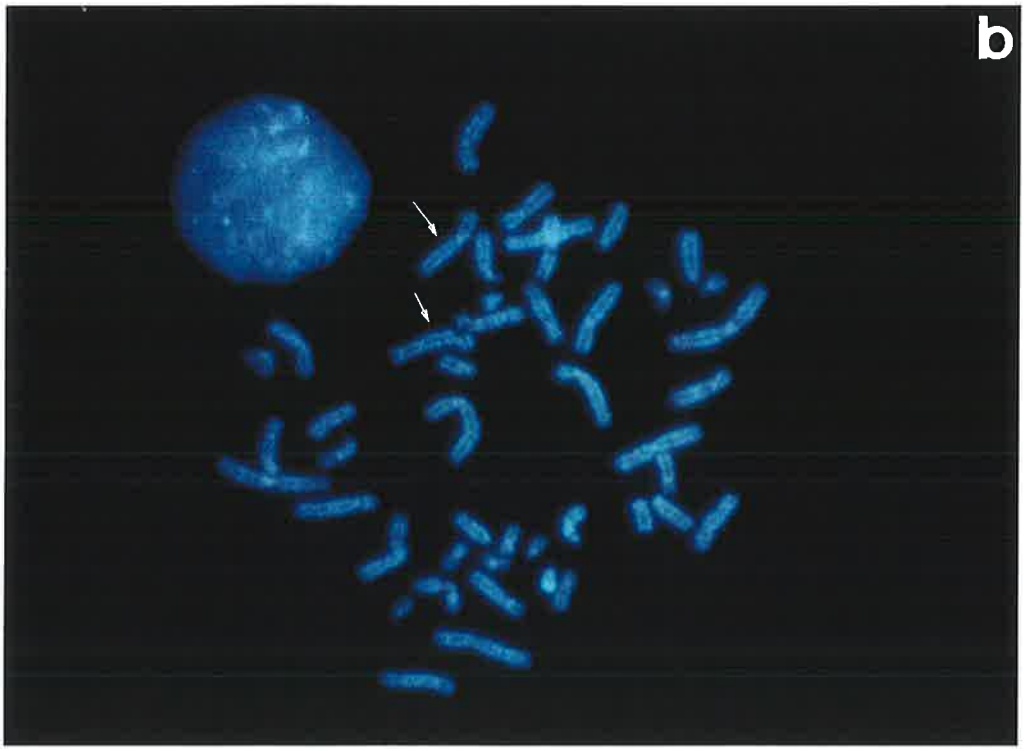
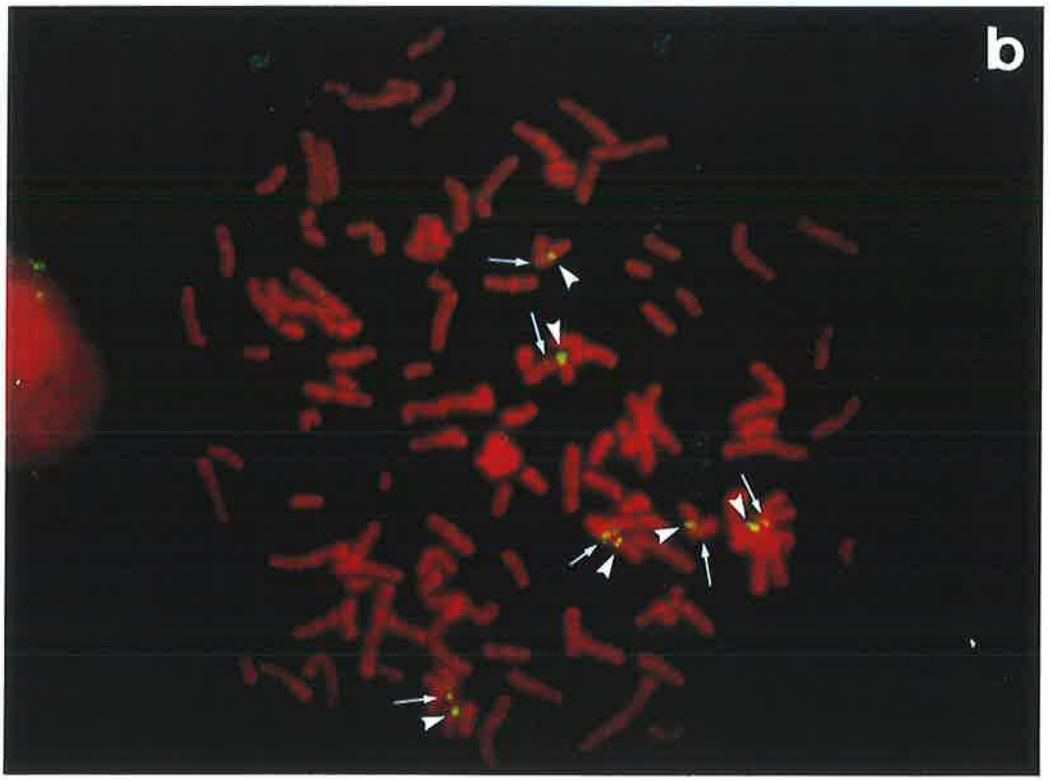

b

Figure 3.5 : HYBRIDIZATION OF THE *brn-2* GENOMIC PROBE TO METAPHASES FROM MELANOMA CELL LINES.

(a) Metaphase from the secondary melanoma MM96L, stained with propidium iodide. Fluorescent signal from the chromosome 6 -specific centromere probe p308 is present at the centromere of chromosome 6 and indicated by an arrowhead. Fluorescent signal from hybridization of the *brn-2* probe to chromosome band 6q16 is indicated by an arrow. There is only one copy of chromosome 6 in this metaphase: this chromosome retains a copy of *brn-2*.

(b) Metaphase from the secondary melanoma MM138, stained with propidium iodide. Chromosome 6 centromere-specific fluorescent signal from probe p308 is indicated by arrowheads. Fluorescent signal identifying hybridization of the *brn-2* probe is indicated by arrows. There are six copies of chromosome 6 in this metaphase, each of which contains a copy of *brn-2*.


a


b

3.4.5 **NKB1**

Results: Analysis of 20 metaphases from the first normal male showed signal at 19q13.4 in all cells. Two non-specific background dots were seen in these 20 metaphases. A similar result was obtained from hybridization of the probe to 20 metaphases from the second normal male. A representative metaphase is shown in figure 3.6.

The optimum probe DNA concentration for this localization was 5ng/ul with no prereassociation needed. Although the probe was only a 1.8kb cDNA, good signal was seen on 4 chromatids in 9 metaphases; on 3 chromatids in 5 metaphases; and on 2 chromatids in 6 metaphases.

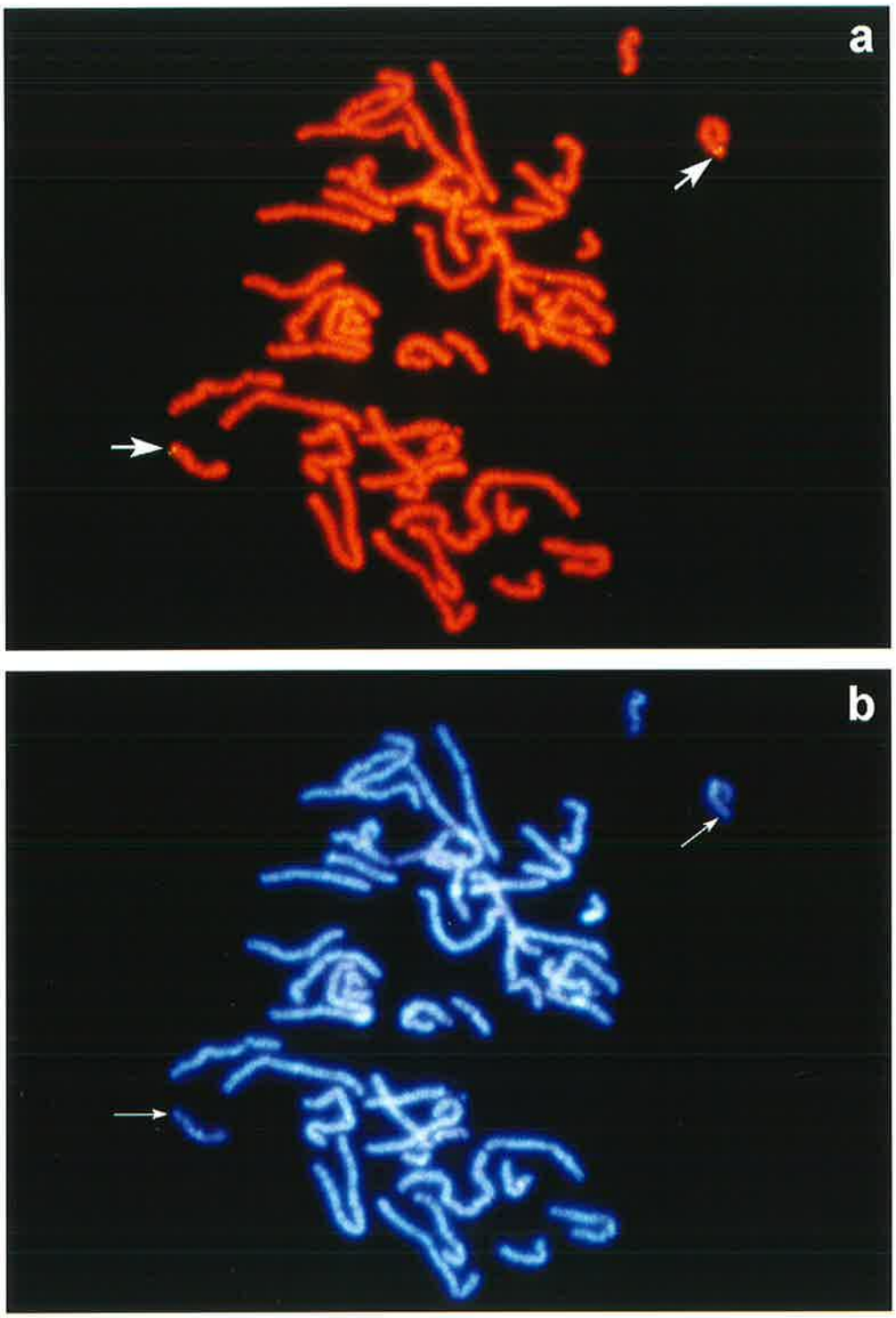
Discussion: The localization of the NKB1 cDNA sequence to 19q13.4 was consistent with results previously obtained from PCR analysis of a panel of human/hamster somatic hybrid cell lines using primers specific for the first Ig domain in the NKB1 cDNA sequence (Litwin et al, 1995).

Figure 3.6 : METAPHASE SHOWING FISH WITH THE 1.8kb NKB1 cDNA PROBE.

(a) Normal male chromosomes stained with propidium iodide. Fluorescent signal from hybridization of the probe to chromosome band 19q13.4 is indicated by arrows.

(b) The same metaphase as (a) stained with DAPI for chromosome identification. Each chromosome 19 is indicated by an arrow.

NB The photocopies of these two images were of poor quality, therefore the colour photographic slides were scanned as a computer file and printed on a high quality (dye sublimation) printer. There was no signal enhancement or background reduction.


a
b

3.4.6 **Humly 9**

Results: Analysis of 25 metaphases from the first normal male showed signal at 1q21-1q24 in all cells: 86% of this signal was at 1q22. There was a total of four non-specific background dots seen in these 25 metaphases. A similar result was obtained from hybridization of the probe to 20 metaphases from the second normal male. A representative metaphase is shown in figure 3.7.

The optimum probe DNA concentration among those tested was 5ng/ul, with no pre-reassociation needed. Signal was seen on 4 chromatids in 2 of the 25 cells; on 3 chromatids in 2 cells; on 2 chromatids in 16 cells and on one chromatid in 5 cells.

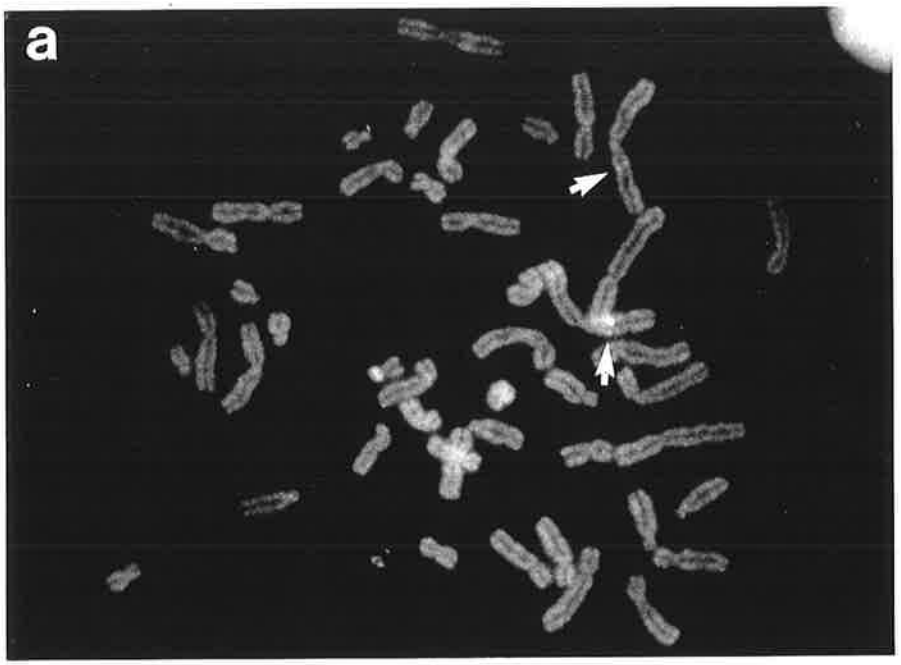
Discussion: Localization of the Humly 9 cDNA probe to human chromosome band 1q22 was supported by Southern blot analysis of a panel of human/hamster hybrid cells using the same probe. (Sandrin et al, 1995 - Appendix A).

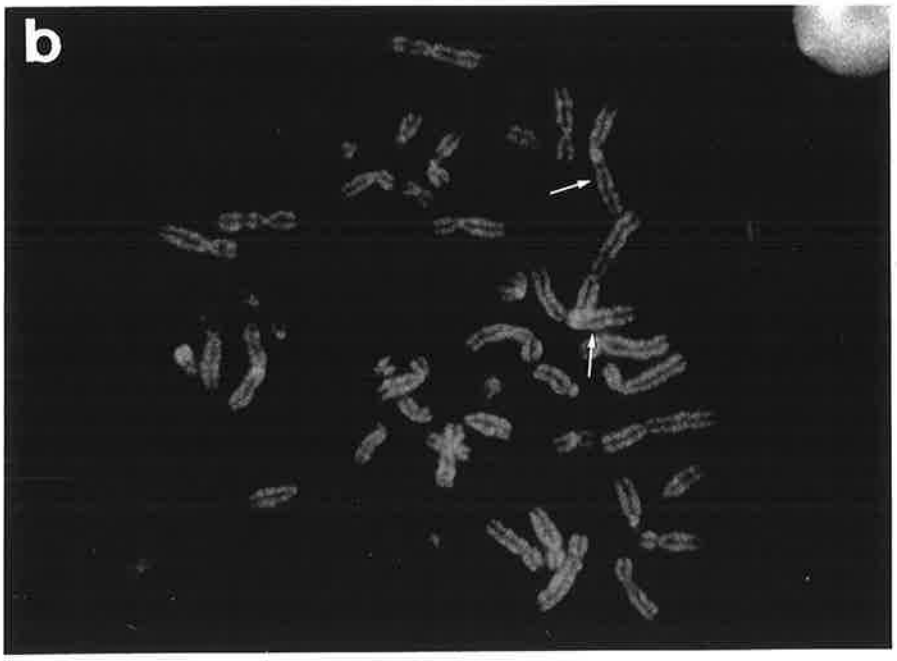
Sandrin et al (1992) suggested that a common ancestor duplicated and diverged to give rise to the precursors of mouse Ly 9 and the homolog of human CD48 in the mouse - BCM1. This was followed by a second duplication event which produced the 4 domain structure of Ly 9. Given the similarities of the amino acid sequences of Ly 9 and Humly 9, and the mapping of the *humly9* gene to the same chromosomal location as the *CD48* gene (ie 1q22), it can be postulated that the duplication event which gave rise to the four domain structure must have occurred before mouse/human divergence.

Figure 3.7 : METAPHASE SHOWING FISH WITH THE 1.9kb *humly9* cDNA PROBE.

(a) Normal male chromosomes stained with propidium iodide. Fluorescent signal from hybridization of the probe to chromosome band 1q22 is indicated by arrows.

(b) The same metaphase as in (a) stained with DAPI for chromosome identification. Each chromosome 1 is indicated by an arrow.


a


b

The method described for the localization of single copy genes has sufficient adaptability to cope with a range of probe sizes, with cDNA and genomic DNA, with probes still in their vectors and excised inserts. The low level of the secondary antibody (biotinylated goat anti-avidin), when combined with the high stringency washes, permits a detection step plus two rounds of amplification without a prohibitive level of fluorescent background developing on the slide.

An important factor in the scoring of small (800bp - 2000bp) single copy probes is the necessity to score **all** fluorescent dots on, or touching, the chromosomes. This is because such small probes do not always, or even often, give doublet signals on both homologs. The plotting of signal from successive metaphases onto an ideogram of the human karyotype will readily eliminate non-specific background, and is an ideal way of displaying secondary peaks of localization. These may usefully indicate the existence and location of pseudogenes, or gene family members.

CHAPTER 4

DEVELOPMENT OF A METHOD FOR THE DIAGNOSIS OF CHARCOT MARIE TOOTH SYNDROME TYPE 1A (CMT1A) AND HEREDITARY NEUROPATHY WITH LIABILITY TO PRESSURE PALSIES (HNPP).

4.1 INTRODUCTION

Charcot Marie Tooth disease (CMT) has a prevalence rate of 1/2500 and is the most common inherited peripheral neuropathy in humans involving both motor and sensory nerves (Patel et al, 1992). The most common subtype Charcot Marie Tooth disease Type 1A (CMT1A; hereditary motor and sensory neuropathy 1) is an autosomal dominant demyelinating peripheral neuropathy characterized by chronic, progressive distal muscle atrophy, sensory impairment and symmetrically decreased peripheral nerve conduction velocities (Kiyosawa et al, 1995). Hereditary neuropathy with liability to pressure palsies (HNPP; also called tomaculous neuropathy) is an autosomal dominant disorder characterized by diverse sensory or motor nerve palsies often precipitated by minor trauma and displaying characteristic focal sausage-shaped thickenings of the myelin sheath (tomacula) in numerous internodes in peripheral nerve biopsy (Windebank, 1993).

CMT1A is associated with a submicroscopic tandem duplication involving 1.5Mb of sequence (a genetic distance of ~6cM) on the short arm of chromosome 17, while HNPP is associated with a 1.5Mb deletion at the same locus (Chance and Fischbeck, 1994). These mutational events, frequently *de novo*, are responsible for the overwhelming majority of patients with the respective phenotypes. The peripheral myelin protein 22 gene (*PMP22*) which is located within the region duplicated in CMT1A patients and deleted in HNPP patients is proposed to play a crucial gene dosage role in the aetiology of both disorders via trisomic overexpression in CMT1A and hemizygous underexpression in HNPP (Valentijn et al, 1992). Only a few CMT1A and HNPP cases have been shown to be due to other mechanisms, eg point mutations in *PMP22* (Chance and Fischbeck, 1994).

The 1.5Mb CMT1A chromosomal segment duplicated in CMT1A and deleted in HNPP is flanked by a ~30kb complex low-copy repeat (the CMT1A-REPs - figs 4.1a,1b). Three copies of the CMT1A-REP repeat are present on the stably inherited CMT1A duplication chromosome (Pentao et al, 1992) while only one copy is present on the deleted HNPP chromosome (Chance et al, 1994), supporting the model that the duplicated chromosome in CMT1A and the deleted chromosome in HNPP are the reciprocal products of unequal crossover occurring through misalignment of the CMT1A-REP homologs (Chance et al,

Figure 4.1a : MECHANISM OF THE CMT1A DUPLICATION / HNPP DELETION WITHIN HUMAN CHROMOSOME BAND 17p11.2

The 1.5Mb critical region which is duplicated in CMT1A and deleted in HNPP is flanked by the proximal and distal CMT1A-REP repeat units. Correct alignment of the repeat units, followed by crossing over, results in two normal derivatives. Misalignment of the repeat units, followed by crossing-over, results in one derivative which is duplicated for the 1.5Mb critical region, and one derivative which is deleted for the 1.5Mb critical region.

Figure 4.1b : GENOMIC ORGANIZATION WITHIN THE CMT1A DUPLICATION / HNPP DELETION CRITICAL REGION WITHIN CHROMOSOME BAND 17p11.2

The 1.5Mb critical region duplicated in CMT1A and deleted in HNPP contains the peripheral myelin protein (*PMP22*) gene whose triple (CMT1A) or single (HNPP) dosage is believed to be pathogenic. Probe VAW409 is located approximately 50kb 5' of *PMP22*. Genomic clones c132G8 and c103B11 encompass the *PMP22* gene.

Figure 4.1a : MECHANISM OF THE CMT1A DUPLICATION / HNPP DELETION

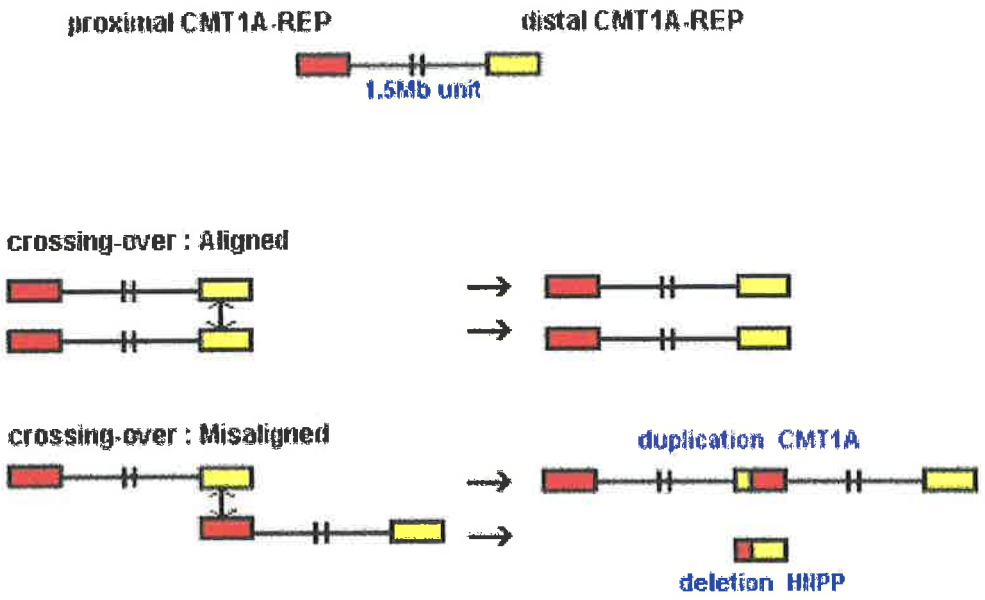
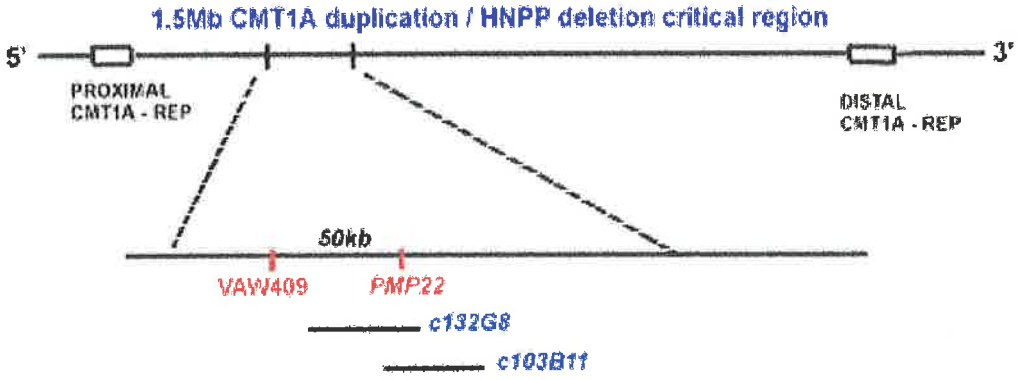


Figure 4.1b : GENOMIC ORGANIZATION OF THE CMT1A/HNPP REGION



1994) (fig 4.1a). This misalignment appears to be predominantly male specific; in a study of the parental origin of the duplication in nine genetically sporadic CMT1A patients, Palau et al (1993) demonstrated that in all cases the mutation was the product of an unequal exchange that had occurred during spermatogenesis.

Diagnosis of CMT1A has until recently been limited to dosage studies on Southern blots following DNA extraction of patients' cells. Given the high incidence of the disorder, the need to differentiate Type 1A from other CMTs, and the unavailability of a commercial FISH probe for the disease, it was decided that an attempt should be made to develop a rapid FISH method which would aid in the diagnosis of these two disorders.

Since HNPP is most often due to a deletion of chromosome 17p, it is suitable for assessment by FISH to metaphase chromosmes. However, the duplication within 17p in CMT1A is tandem, and does not permit resolution of two signals (one for each duplicated segment) on the chromosome arm: the signal appears as a single bright dot or group of dots whether there is one copy of the monomer present or two. The method developed for HNPP FISH was therefore standard metaphase FISH, but the method for CMT1A had the following intentions: (1) to provide a reliable, rapid test (3-6 days total time) (2) to use FISH to interphase cells in G0/G1 (3) to fit the method with as little adaption as possible into existing cytogenetic technology.

4.2 MATERIALS

4.2.1 Probes

VAW409R1: 10kb plasmid, specific for chromosome 17p11.2, located approximately 50kb 5' of *PMP22* within the CMT1A duplication/ HNPP deletion critical region (Patel et al, 1992)(fig 4.1b).

132G8: ~35kb cosmid, located within the duplication/deletion critical region and containing part of the 5' region of the *PMP22* gene (Murakami and Lupski, 1996; Patel et al, 1992) (fig 4.1b).

103B11: ~25kb cosmid, located within the duplication/deletion critical region and containing the third exon of the *PMP22* gene (Patel et al, 1992) (fig 4.1b).

NB: Cosmid 132G8 overlaps cosmid 103B11 by ~11kb (Patel et al, 1992), therefore total genomic coverage of these two cosmids is ~50kb.

4.2.2 Photography

The images shown in this study are high resolution photocopies of colour prints prepared from colour slides. There is no image enhancement.

4.3 METHODS

4.3.1 Probe Labeling

All probes were separately labeled by nick translation with biotin-14-dATP

4.3.2 Cell Culture

Blood cultures were prepared by two separate methods.

Method A (for HNPP): as detailed in 2.2.1 and 2.2.4. In brief, PHA-stimulated lymphocytes were grown for 72hrs in tissue culture medium and treated with colchicine prior to harvest, to arrest cells in metaphase. Harvest included 25min hypotonic treatment with 0.075M KCl. The fixed cell suspensions were either spread immediately or stored at -20°C in excess fixative until required for FISH. Slides intended for FISH were spread as outlined in 2.2.12.

Method B (for CMT1A): The purpose of Method B was to provide nuclei in G0/G1 rather than S or G2. This is because hybridization to G0/G1 cells usually gives a single signal per homolog whereas hybridization to cells in late S or G2 can give a complex pattern, as the replicated chromatids frequently give double signals.

Method B was as for Method A except that cells were cultivated for only 11-24hrs, and were harvested without colchicine treatment.

Cell suspensions were either spread immediately or were stored at -20°C in excess fixative until required for FISH.

4.3.3 Testing the parameters of Method B

The following variables were assessed:

(1)Volume of blood added to culture:

5, 8 or 10 drops of blood from a pasteur pipette were added to the culture medium

(2) With or without PHA:

Blood was cultured in medium with and without PHA.

(3) With or without heparin:

Blood was cultured in medium with and without heparin.

(4) Duration of culture:

Cells were cultured for 11hrs, 18hrs, 20hrs or 24hrs.

(5) Storage of blood culture before incubation:

Two blood samples were added to culture medium containing PHA, refrigerated for 3 nights at 4°C, and cultured for 20hrs at 37°C before harvest. This procedure was designed to mimic a long-weekend delay in culture.

(6) Storage of blood samples before their addition to culture medium:

Blood was stored at 4°C for 1 day, 2 days or 9 days before initiation of culture.

(7) Density of cells on slide:

Slides for FISH were spread at different densities (from very thin to quite thick as assessed under an inverted microscope).

4.3.4 FISH Procedure: pVAW409R1

Probe pVAW409R1 was hybridized to metaphases and nuclei collected by Method A. Hybridization and detection are detailed in 2.2.15.1 and 2.2.15.2 with the following variations attempted in the technique:

(1) Separate slides were incubated (just prior to the RNAase step) for 0min, 5min, 15min and 20min respectively in a 60°C oven.

(2) Final probe concentrations of 10ng/ul and 20ng/ul with 200x total human DNA were applied to separate slides; these slides received one detection (avidin-FITC) step plus one amplification. Final probe concentrations of 30ng/ul and 40ng/ul with 200x total human DNA were applied to separate slides; these slides received one detection step only.

(3) Post hybridization washes of 42°C in 50%FA/2xSSC (2 x 5min) or washes of 70°C in 0.1xSSC (2 x 5min) were compared with the standard 44°C in 50%FA/2xSSC (2 x 10min).

(4) A series of five cases, two of which were known CMT1A, were scored blind to assess the adequacy of the fluorescent signal on metaphases and nuclei from 3 day cultures.

4.3.5 FISH procedure: c132G8, c103B11

(1) The cosmids were hybridized both separately and in combination to metaphases as described in 2.2.15.1, to check their specificity and sensitivity.

(2) The cosmids were hybridized as a contig to nuclei as described in 2.2.15.1. Final probe concentration was 20ng/ul for each probe with 200x total human DNA. Pre-reassociation was for 20min, 50min and 80min for separate slides.

(3) Post hybridization washes of 42°C in 50%FA/2xSSC (2 x 5min) were used.

(4) A range of propidium iodide concentrations in the antifade mounting medium was assessed.

(5) A series of four cases (diagnosis unknown to the investigator) was scored to assess the adequacy of signal on nuclei from one-day cultures and to devise a suitable scoring method.

(6) A series of three cases (diagnosis again unknown) was scored by the investigator and, separately, slides were prepared and scored by two other people to assess the reproducibility of the method and to finalize the most effective scoring method.

(7) A further seven cases were scored independently by the investigator and other people using the amended scoring method.

(8) Two suspensions obtained from harvest of chorionic villus (CVS) cells were spread and FISHed with the cosmid contig.

4.4 RESULTS AND COMMENTS

4.4.1 Parameters of cell culture Method B

(1) Volume of blood:

The addition of 8 drops of blood to 5ml of culture medium gave the optimum suspension for spreading slides.

COMMENT: There was occasional clumping of cells at the first-fix stage of harvest when a 10-drop culture was being harvested. There was little difference in the appearance of nuclei on slides spread from either 5- or 8-drop cultures, therefore the latter was chosen as the standard as it gave more working suspension at the end.

(2) With and without PHA:

There was little difference in the appearance of nuclei collected from cultures which had PHA in the medium and those which did not. There was no difference noted in the appearance of fluorescent signal after FISH.

COMMENT: This is not surprising as the first 24hrs of a cell culture is a lag period and in general the first round of replication is not until 48hrs after culture initiation. Since one of the design aims of the test was to have it fit in with standard cytogenetic procedures, it was decided that medium **with** PHA would be used as the standard: the same flask of medium could then be used to set up a CMT1A culture as other blood cultures.

(3) With and without heparin:

There was a noticeable difference in the appearance of cultures prior to harvest when the culture medium did not contain anticoagulant.

COMMENT: Although all blood samples were collected in heparinized tubes there was still evident clumping of cells both in the culture tube and on the slides spread after harvest when the culture medium did not have anticoagulant added. This led to areas of very high background fluorescence and many overlapping (therefore unscoreable) nuclei following FISH. Since the standard medium for setting up blood cultures for cytogenetic analysis contains 0.5 units/ml heparin (2.2.1) it was also chosen as the best medium for standard CMT1A analysis.

(4) Duration of culture:

There was no difference in the appearance or hybridization efficiency of cells which had been cultivated for 11hrs, 18hrs, 20hrs or 24hrs.

COMMENT: This was a useful finding as it meant that a culture for CMT1A investigation could be set up at any time during the day before a standard cytogenetic harvest. It probably also means that culture medium innoculated with blood in the morning would provide nuclei suitable for FISH analysis if harvested late the same afternoon. However, this procedure would be **less** convenient in terms of fitting in with routine harvesting arrangements, so was not attempted.

(5) & (6) Storage of cultures before incubation/ Storage of bloods before culture:

The two blood cultures placed in refrigeration for 3 nights followed by 20hrs incubation at 37°C gave an interesting result. A small number of metaphases were observed on one of the slides, indicating that even at 4°C some cells had proceeded through the cell cycle. The blood samples kept for 1 and 2 days before culture initiation produced nuclei of good morphology and fluorescent signal after FISH. The blood sample kept at 4°C for 9 days before culture initiation gave an extremely poor result. Necrotic nuclei covered most of

the slide after FISH with only an occasional nucleus able to be scored (although the signal present in the scoreable nuclei was good).

COMMENT: It was decided that blood samples which arrived on a day which did not preceed a standard harvest day could safely be stored at 4°C for 1-2 days before being set up as cultures, but that this storage time should be kept to a minimum.

(7) Thin, average and thickly spread cell suspensions all hybridized well after FISH with little difference in non-specific background fluorescence.

COMMENT: An average spread of nuclei on a slide is that concentration which would normally be considered optimal for metaphase FISH (assessed under an inverted microscope). It was anticipated that a thick suspension, ie one with twice as many cells as usual, would give a higher background fluorescence than a thin spread but this was not the case. In fact the thick suspensions were vastly easier (because faster) to score, and any areas where cells overlapped were easily disregarded during scoring. Figure 4.2 shows a good density spread.

It was noted that two slides which had been spread to optimum density by the dropping of one thin drop over another, gave a poor result after FISH. These slides showed a marked increase in non-specific background fluorescence, presumably due to the overlay of cellular material on the slide, ie the increase in cellular proteins and matrix. It is obviously preferable, if a suspension is too dilute, to respin the tube and correct the density by removing some of the supernatant fixative.

4.4.2 Results of optimization of FISH procedure: pVAW409R1

(1) The fluorescent signal seen on nuclei from 3-day cultures after hybridization with pVAW409R1 was more punctate (less diffuse) if slides had received at least 5min of pretreatment at 60°C before hybridization. Baking for 20min diminished the signal.

(2) & (3) The optimum concentration for pVAW409R1 hybridization to both metaphases and nuclei was 10ng/ul with 200x total human DNA. Combined with a post-hybridization wash of 42°C in 50%FA/2xSSC (2 x 5min) it gave the strongest signal (100% efficiency) with the lowest background.

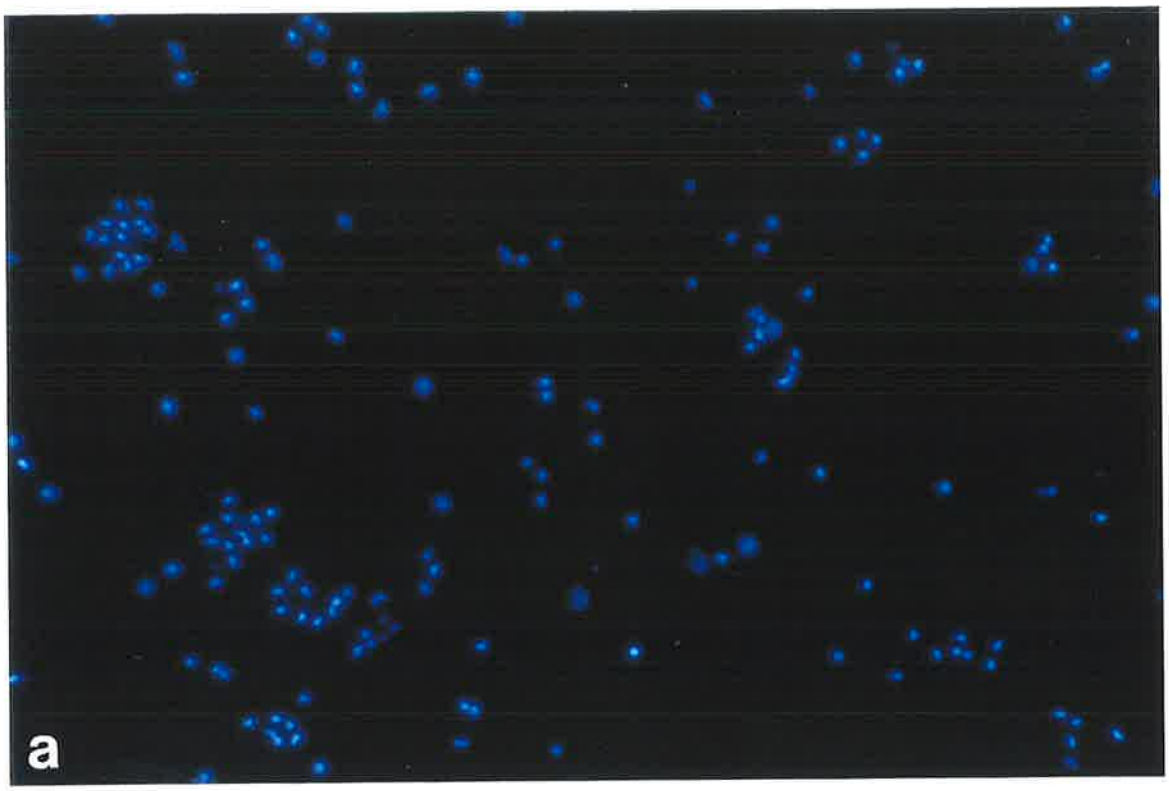
(4) The results of scoring blind a series of 6 cases, 2 of which were diagnosed CMT1A by DNA analysis, are shown in Table 4.1.

Figure 4.2 : NUCLEI PRESENT FOLLOWING

OVERNIGHT CULTURE OF PERIPHERAL BLOOD LEUKOCYTES.

(a) The figure shows nuclei spread at a good density for scoring. Objective magnification is x10.

(b) As above, with an objective magnification of x25. A range of leukocytes is present. T-lymphocytes are indicated by a short arrow. Multilobed neutrophils are indicated by an arrowhead.


a

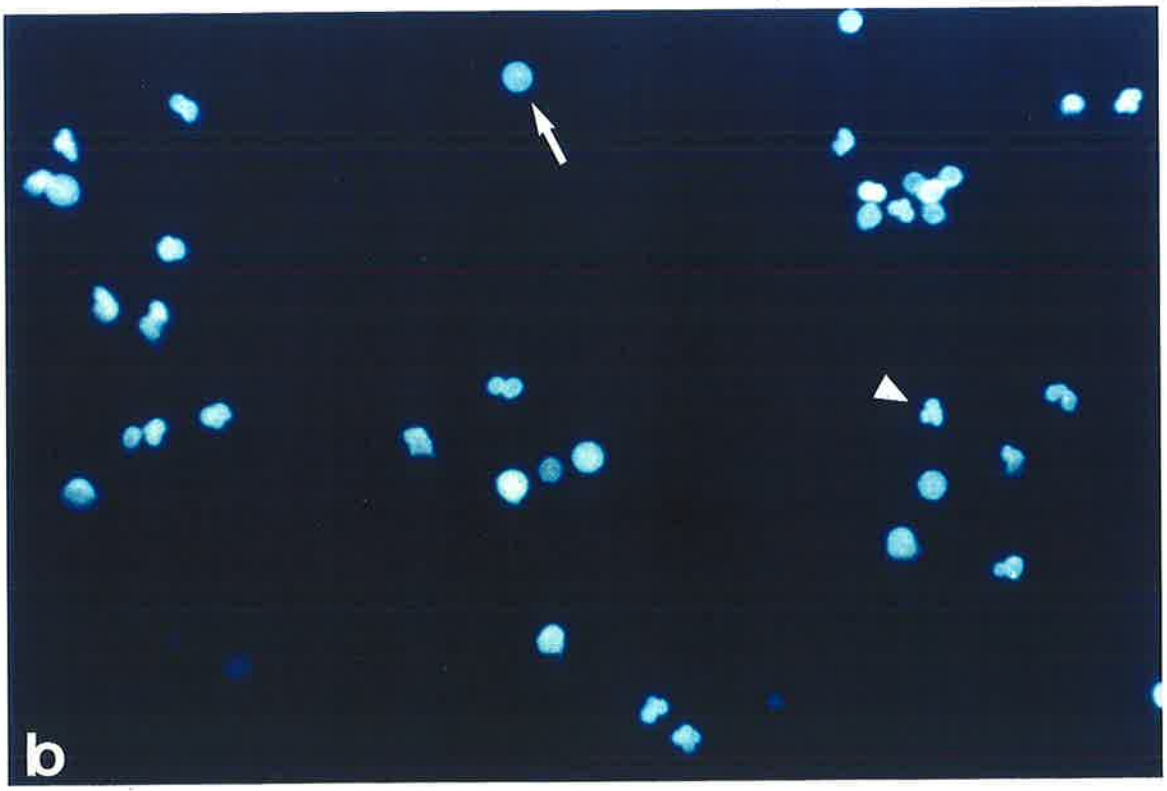

b

Table 4.1 : Signal distribution in 100 nuclei from each of six cases using pVAW409R1

CASE (known N or CMT1A)	*specific signals per nucleus*			*not evaluable*
	1	*2*	*3*	
1 (N)	25	36	11	28
2 (N)	12	27	1	60
3 (CMT1A)	13	14	12	61
4 (CMT1A)	11	13	11	65
5 (N)	22	34	3	41
6 (N)	35	27	1	35

COMMENT: An example of the signal observed on metaphases using the optimum conditions for probe pVAW409R1 is shown in figure 4.3. The probe was considered very suitable for scoring cases diagnosed as HNPP. However, the probe was not considered suitable for scoring nuclei for CMT1A. This is because of a slight but constant level of background dots in the nuclei (fig 4.4) which meant that a very high proportion of nuclei was rejected in each case as unscoreable (up to 65% - refer Table 4.1).

There were two possible ways to resolve this problem. The first was to add further pretreatment steps to the FISH procedure, eg treatment with acetic anhydride or Proteinase K. The second was to try a larger probe. Since the first option required steps that were not normally part of the diagnostic FISH procedure, the second option was preferred. A further influencing factor was the report of three rare CMT1A patients in whom there appeared to be a smaller duplication, still including the PMP22 gene (Ionasescu et al, 1993; Palau et al, 1993; Valentijn et al, 1993). The cosmid contig, containing *PMP22* cDNA, was considered preferable to pVAW409 (which was located ~50kb 5' of the gene). Hybridization conditions for the two cosmids 103B11 and 132G8 were therefore optimized.

4.4.3 **Results of optimization of FISH procedure: c132G8, c103B11**

(1) Hybridization of cosmids 132G8 and 103B11 to normal male metaphases at 20ng/ul (each probe) with 200x total human DNA showed the following:

c132G8: signal on both #17s in 14/20 metaphases, and on one #17 in 6/20 metaphases

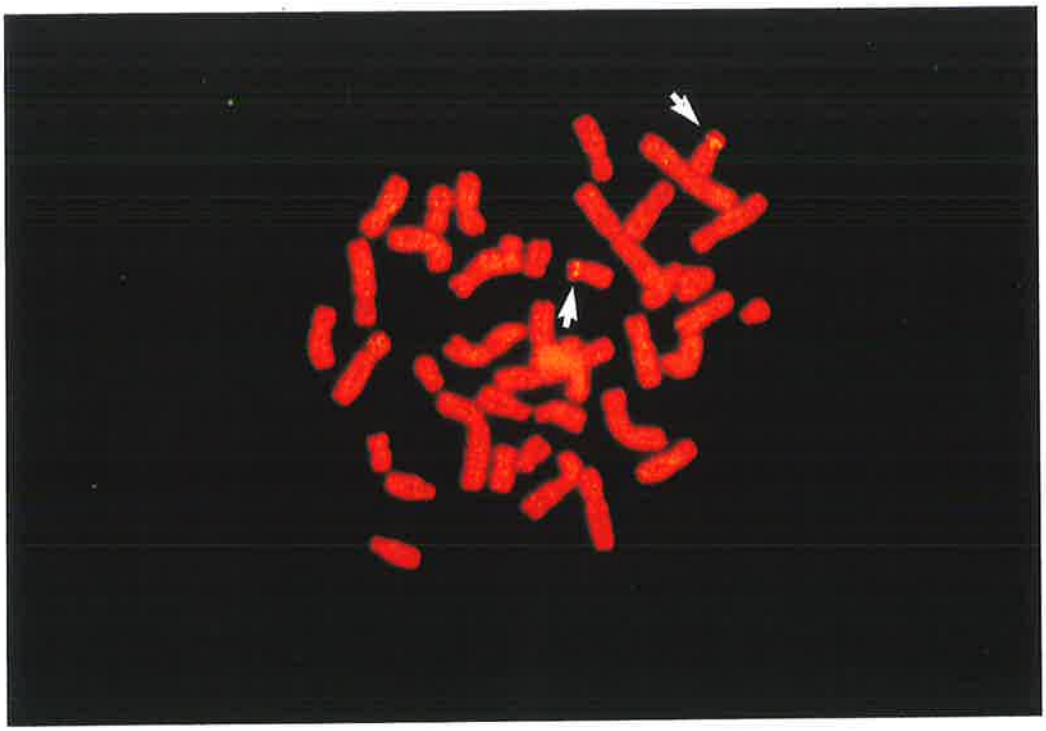
c103B11: signal on both #17s in 18/20 metaphases, and on one #17 in 2/20 metaphases

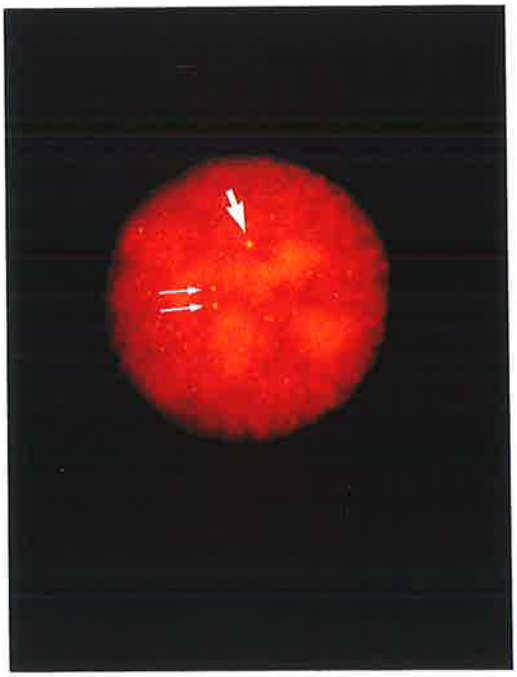
Figure 4.3 : METAPHASE SHOWING FISH WITH THE pVAW409R1 PROBE.

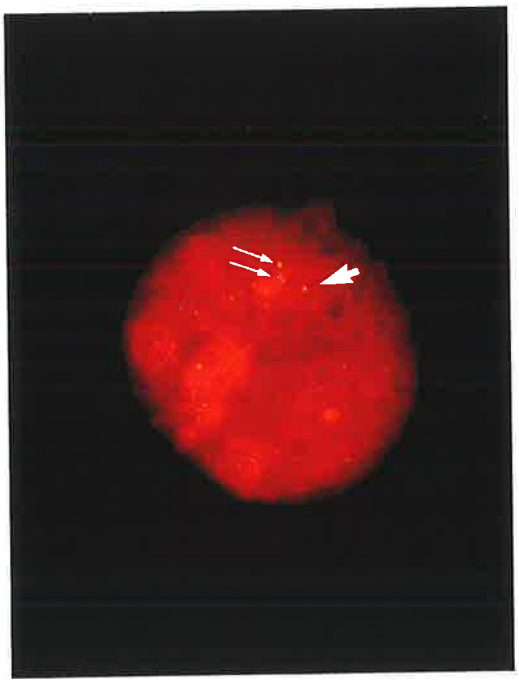
Normal human chromosomes hybridized with the pVAW409R1 probe. Hybridization sites on chromosome band 17p11.2 are indicated by arrows. There is signal on both chromatids of both chromosome 17 homologs, confirming that the deletion associated with HNPP is not present.

Figure 4.4 : NUCLEI SHOWING FISH WITH THE pVAW409R1 PROBE

Nuclei from a confirmed case of CMT1A hybridized with the probe pVAW409R1. Although the duplication associated with CMT1A can be seen, there is enough non-specific fluorescent background present to make confident scoring of the nuclei very difficult. The single arrow indicates the normal homolog; small double arrows indicate the homolog with the duplicated segment.







c132G8 & c103B11 (combined): signal on both #17s in 20/20 metaphases (fig 4.5)
COMMENT: There was excellent signal with little to no background in metaphases hybridized with the combined cosmid probes. Specificity and sensitivity of the combined cosmids probe was therefore very suitable for analysis of HNPP on metaphase chromosomes. A recent report (Nelis et al, 1996) describes a smaller deletion than the usual 1.5Mb in a rare HNPP case, with the *PMP22* gene still included in the area of deletion. The cosmids are possibly preferable to pVAW409R1 for this reason, since they directly encompass areas of the crucial *PMP22* gene.

(2) & (3) Pre-reassociation of the cosmids with total human DNA for 80min, combined with a post-hybridization wash of 42°C in 50%FA/2xSSC (2 x 5min) gave the cleanest signal in nuclei from the overnight cultures (fig 4.6).

(4) A very low (0.001 ug/ml) concentration of propidium iodide in the antifade mounting medium enabled a very bright signal to be seen in nuclei from the overnight cultures hybridized with the cosmid probes . However, it often allowed a little background fluorescence to become visible, therefore a concentration of 0.05ug/ml was chosen as the standard. Figures 4.9a and 4.9b show the effect of lowering then adjusting upward the concentration of propidium iodide used to stain nuclei from a CVS preparation. The final concentration used for nuclei is lower than the concentration (0.08ug/ml) of propidium iodide used for metaphase preparations (fig 4.3).

(5) The four cases scored blind to assess the adequacy of the technique are summarized in Table 4.2. The percentage of 'non-evaluable' nuclei is not included in this table as it did not exceed 10% in any one case.

Figure 4.5 : METAPHASE SHOWING HYBRIDIZATION WITH COSMIDS c103B11 AND c132G8

Normal human chromosomes hybridized with the combined cosmid probes 103B11 and 132G8. Hybridization sites on chromosome 17 are indicated by arrows. The propidium iodide concentration used to stain this metaphase was very low (0.001ug/ml), therefore the chromosomes are pale.

Figure 4.6 : NUCLEI FROM AN OVERNIGHT CULTURE SHOWING FISH WITH COSMIDS c103B11 AND c132G8.

Nuclei from a normal male blood sample cultured overnight and hybridized by FISH with c103B11 and c132G8. The two signals in each nucleus represent the two normal chromosome 17 homologs. The duplication associated with CMT1A is not present.

It can be seen that all forms of leukocytes hybridize equally well.

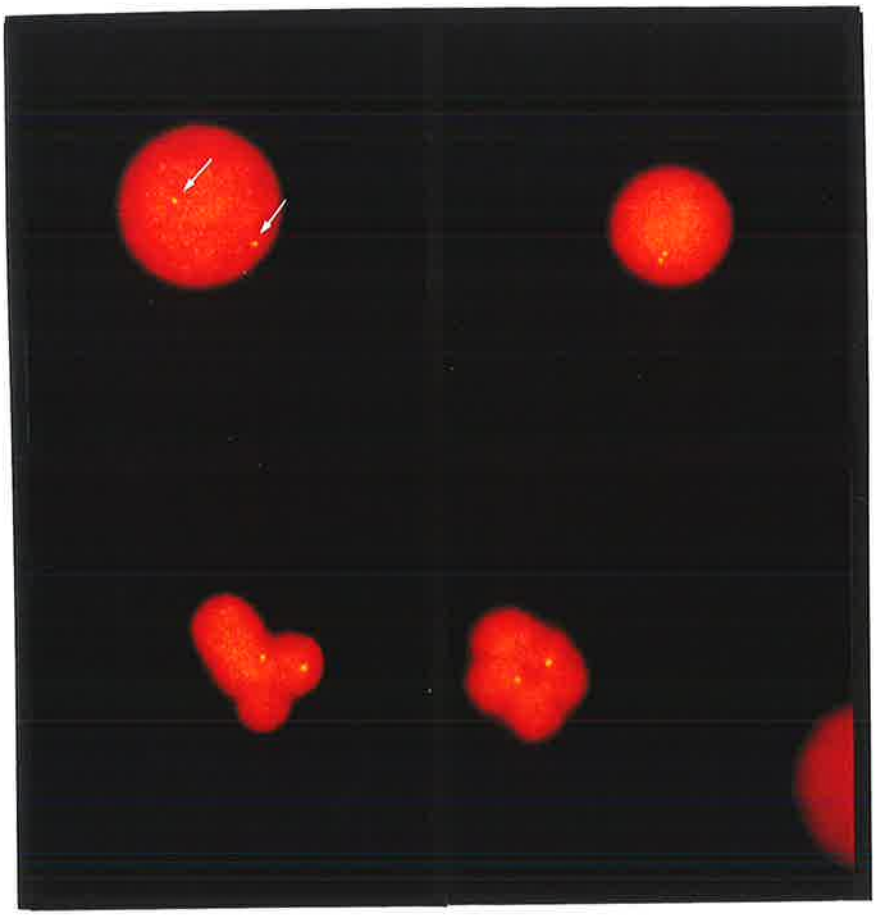


Table 4.2 : Signal distribution in 100 nuclei from each of four cases using combined cosmids 103B11/132G8

CASE	CLINICAL DIAGNOSIS	*specific signals per nucleus*			FISH DIAGNOSIS
		1	*2*	*3*	
1	N	15	80	5	N
2	N	30	68	2	N
3*	CMT ?type	5	21	74	CMT1A
4* **	CMT ?type	6	25	69	CMT1A

* Case 3 & Case 4 were later confirmed as CMT1A by DNA analysis **Case 4 is the father of Case 3

COMMENT: At this stage the scoring procedure was as follows:

- signal present as one domain (ie only one #17 detected)
- signal present as two domains (ie normal)
- signal present as three domains (one domain plus two closely spaced domains) (ie CMT1A). Figure 4.7 provides examples of CMT1A signal appearance using the cosmid probes.
- Only intact and non-overlapping nuclei were scored.
- Nuclei in which the domain definition of the signal was difficult (diffuse, very pale, very small) were rejected.

It was of interest that the overnight blood cultures provided a range of leucocytes available for scoring, eg T-lymphocytes and neutrophils of various configurations (see figs 4.2, 4.7).

(6) The three cases scored blind by the investigator and two other people to assess the reproducibility of results led to an amendment of the scoring procedure.

COMMENT: It was noted that the fluorescent signal was occasionally doubled in appearance (and very occasionally tripled). That is, one or more of the signals in a nucleus would appear as two small fluorescent dots lying very closely together (<1 domain diameter apart). Figure 4.8a provides an example of this doubling effect. If this was scored as **two** domains, then a result suggesting CMT1A was sometimes obtained (see Table 4.3, Case 1). Table 4.3 shows the signal distribution in three cases scored independently by three individuals before the problem caused by the doubling effect was resolved.

Table 4.3 : Comparison of signal distribution in 100 nuclei from each of three cases scored independently by three people using combined cosmids 103B11/132G8.

CASE	CLINICAL DIAGNOSIS	SCORER	*specific signals per nucleus* 1	2	3	FISH DIAGNOSIS
1	N	A	15	49	37	CMT?
		B	18	47	34	CMT?
		C	28	52	20	CMT?
2	CMT1A	A	12	27	61	CMT
		B	5	47	48	CMT
		C	12	50	36	CMT
3	N	A	26	66	13	Not CMT1A
		B	27	56	17	Not CMT1A
		C	47	46	6	Not CMT1A

There is variation evident from one scorer to another. Part of this variation was inevitable, due to slide variation (variation in spreading and hybridization efficiency), but a part would also have been caused by differing frequencies and interpretation of the 'doubling' artefact. The doubling of the dot should not have been due to chromatid replication as the nuclei had not been in culture for a long enough period. It was therefore assumed to be an amplification artefact. The problem was addressed by dividing the scoring of such doubled dots into those of <1 domain diameter and those of ≥ 1 domain diameter (fig 4.8b). This division solved the problem by placing artefacts into the first category and genuine duplications into the second. There was inevitably some overlap, but Table 4.4 shows the satisfactory result of re-scoring cases in this way.

Table 4.4 : Comparison of original (O) and amended (A) scoring procedures.

CASE	CLINICAL DIAGNOSIS	SCORING PROCEDURE	*specific signals per nucleus* 1	2	3	FISH DIAGNOSIS
1	N	O	15	49	37	CMT?
1(rescored)	N	A	22	73*	5**	Not CMT1A

* includes nuclei with doubling artefact of <1 domain diameter ** includes nuclei with doubled dot ≥1 domain diameter

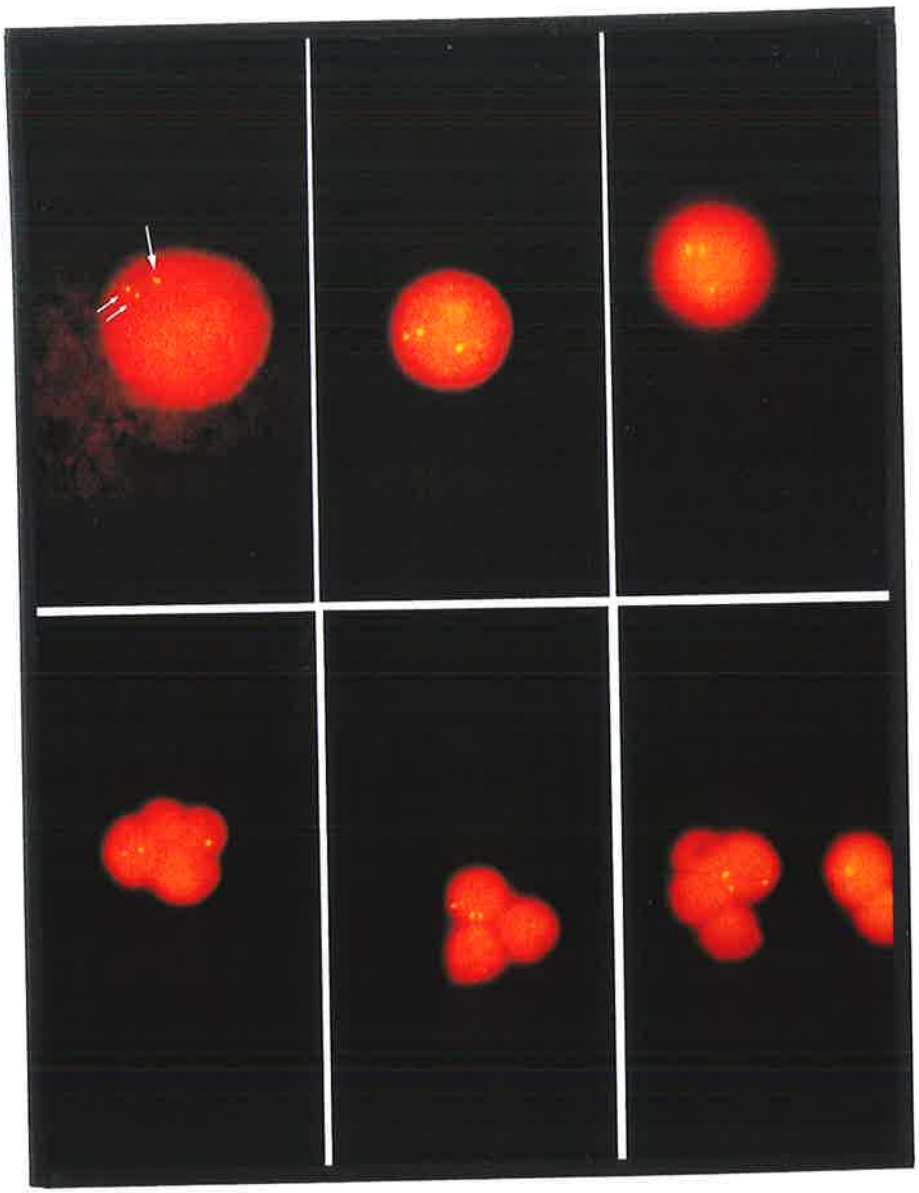
Figure 4.7 : NUCLEI FROM A CMT1A PATIENT SHOWING FISH WITH THE COSMID PROBES 103B11 AND 132G8.

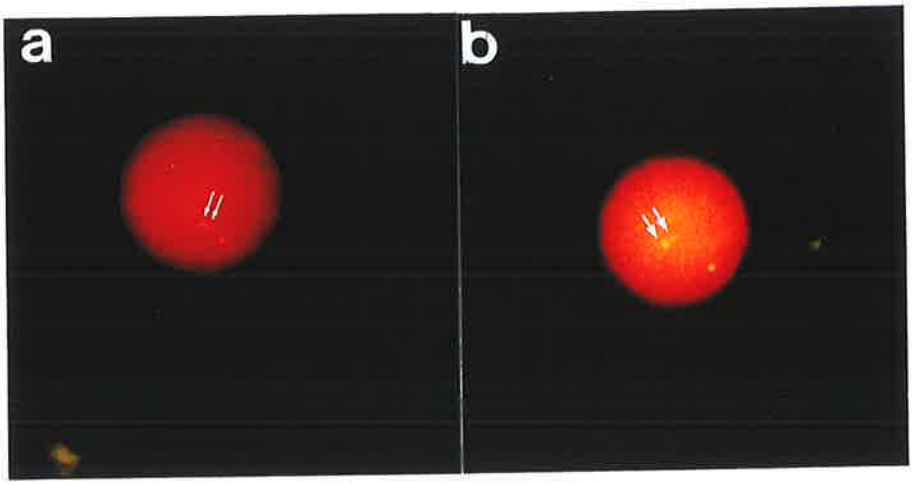
Nuclei from an overnight culture of peripheral blood leukocytes from a confirmed CMT1A patient. In the first nucleus, fluorescent signal from hybridization of the probe to the normal chromosome 17 homolog is indicated by a long arrow. Signal from the homolog carrying the duplication is indicated by two shorter arrows. There is little discernible non-specific fluorescent background in these nuclei; they are consequently very easy to score.

Figure 4.8 : EXAMPLE OF 'DOUBLING' ARTEFACT FOLLOWING FISH WITH THE COSMID PROBES.

(a) Nucleus from an overnight culture of peripheral blood leukocytes from a normal male control showing a closely-spaced double dot at one site of hybridization (2 small arrows). The dots are spaced less than one domain diameter apart; therefore in the final standardized method the nucleus would have been scored as having a total of **two** sites of hybridization only.

(b) Nucleus from the overnight culture of peripheral blood leukocytes from a confirmed CMT1A patient showing closely spaced dots at one site of hybridization (2 arrows). However, the dots are spaced greater than one domain diameter apart; therefore in the final standardized method the nucleus would have been scored as having a total of **three** sites of hybridization.




a
b

Once the amended scoring procedure became the standard, the results obtained by independent scorers of a further seven cases were more similar (Table 4.5).

Table 4.5 : Results obtained by independent people scoring 100 nuclei from each of five cases using combined cosmids 103B11/132G8 and amended scoring procedure.

CASE	CLINICAL DIAGNOSIS	SCORER	*specific signals per nucleus* 1	2	3	FISH DIAGNOSIS
1	N	A	15	78	7	Not CMT1A
		B	15	82	3	Not CMT1A
2	CMT ?type	A	2	24	74	CMT1A
		B	4	20	76	CMT1A
3	CMT ?type	A	6	92	2	Not CMT1A
		B	13	82	5	Not CMT1A
4	CMT ?type	A	13	85	2	Not CMT1A
		B	14	84	2	Not CMT1A
5	CMT ?type	A	20	80	0	Not CMT1A
		B	13	85	2	Not CMT1A

(7) The hybridization of the cosmid probes to CVS cells gave an excellent signal with little to no background, using the same hybridization and detection procedure as applied to overnight blood cultures (fig 4.9). Table 4.6 shows the signal distribution in two cases.

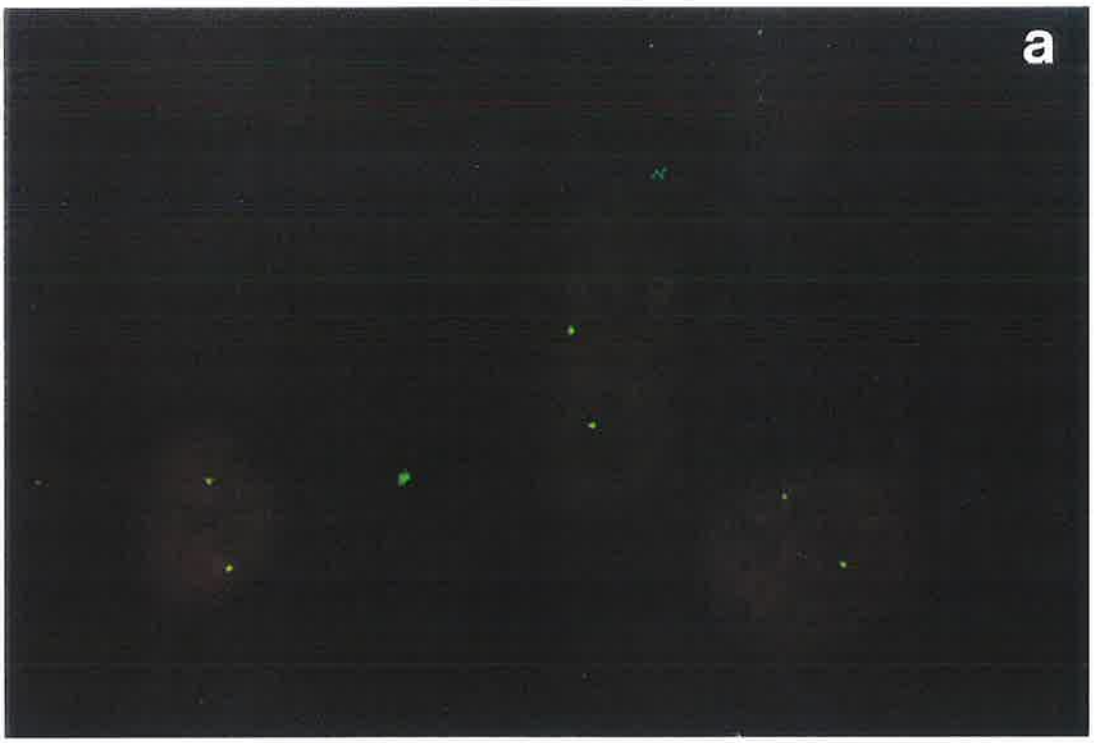
Table 4.6 : Signal distribution in 100 nuclei from two CVS samples using combined cosmids 103B11/132G8 and amended scoring procedure.

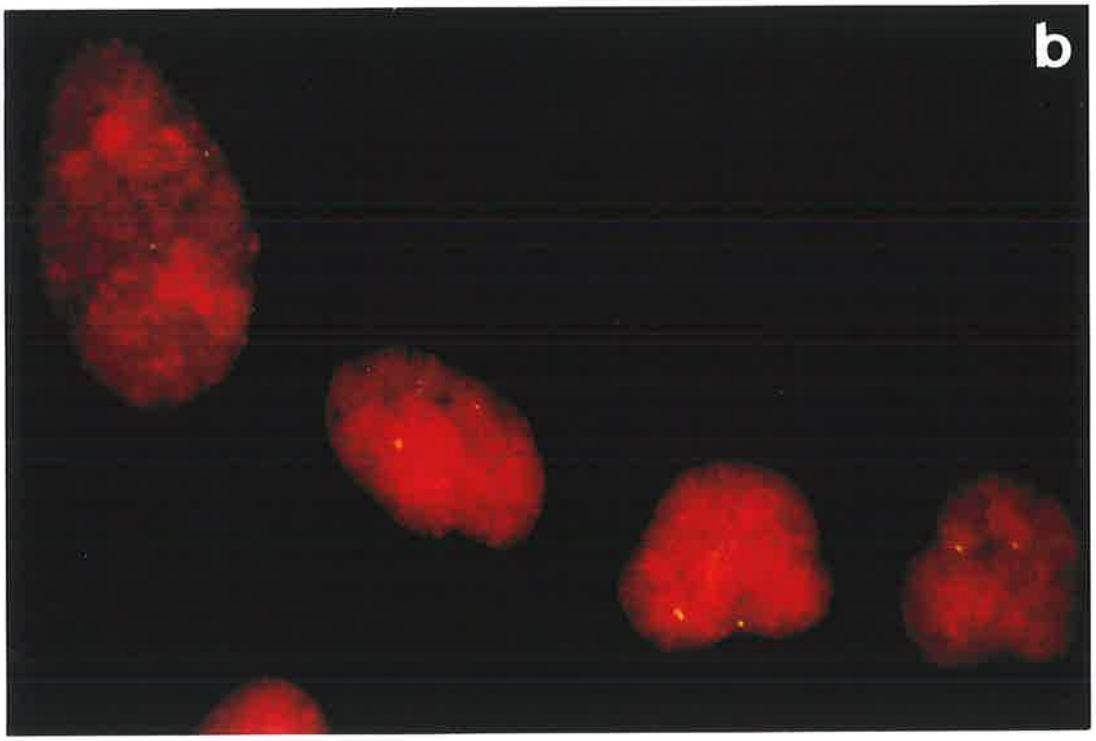
CASE	CLINICAL DIAGNOSIS	*specific signals / nucleus* 1	2	3	FISH DIAGNOSIS
1	N	5	90	5	Not CMT1A
2	N	19	78	3	Not CMT1A

Figure 4.9 : NUCLEI FROM CULTURED CHORIONIC VILLUS CELLS SHOWING FISH WITH THE COSMID PROBES.

(a) Nuclei from a CVS culture from a normal control hybidized with cosmids 103B11 and 132G8. Two sites of hybridization are evident in each nucleus. These nuclei have been stained with a very low concentration of propidium iodide (0.001ug/ml). Although the nuclei in the figure are free of non-specific fluorescent background, other nuclei within the same preparation were unscorable because the low level of propidium iodide was insufficient to block out the low levels of background signal which were occasionally present within nuclei. For this reason a higher concentration of propidium iodide (0.05ug/ml) was chosen as the standard, shown in figure 4.9b.

(b) Nuclei showing optimum concentration of propidium iodide for the visualization of cosmid probes 103B11 and 132G8 without unwanted fluorescent background.


a


b

COMMENT: It is useful to know that a method for prenatal diagnosis of CMT1A is available if required. The cultured villus cells hybridized well with a bright clean signal. In Table 4.2, case 4 is the father of case 3 and both were diagnosed as CMT1A; similarly, Navon et al (1995) reported a prenatal diagnosis requested because the father and two children in the family were affected with CMT1A.

4.5 DISCUSSION

The methods described in this chapter and summarized in Appendix B are fast and efficient in the detection of the duplication of chromosome 17p11.2 associated with Charcot Marie Tooth syndrome Type 1A. The blood culture requirements are undemanding in that they fit easily into the existing routine of a clinical cytogenetic laboratory with adjustments only to culture time and colchicine treatment.

The plasmid probe pVAW409R1 proved unsuitable for detection of CMT1A with nuclei as it gave a low level of non-specific fluorescent background despite attempts to increase the stringency of the procedure. The cosmid probes c103B11 and c132G8, when combined in a small contig, provided an excellent signal on interphase nuclei with negligible interference from non-specific bckground. The FISH procedure with these cosmids requires only a close attention to the details of slide spreading, genomic probe pre-reassociation with competitor DNA, relatively low stringency washes and counterstain concentrations. The experience with the cosmid probes vs the plasmid probe indicates the need for high quality optimized probes in clinical diagnostic work. Probes must be composed of a reasonable-sized subregion of the target chromosome area so that unambiguous discrimination can be made between normal and duplicated samples.

The method described for the detection of the deletion of chromosome 17p11.2 associated with Hereditary Neuropathy with liability to Pressure Palsies requires standard three-day blood cultures and normal cytogenetic harvesting methods. FISH requirements vary little from standard procedures with plasmid and cosmid probes. Both pVAW409R1 and the c103B11/c132G8 contig are suitable for detecting the HNPP deletion by FISH; the only

assessment to be made is whether the presence of rare cases of HNPP caused by a smaller deletion than the common 1.5Mb indicates that the cosmid probes (which encompass the *PMP22* gene) are preferable.

Both the CMT1A and the HNPP methods work well with nuclei from cultured chorionic villus samples and are therefore available for early pre-natal diagnosis of the disorders.

CHAPTER 5

METAPHASE AND INTERPHASE ORDERING OF COSMID PROBES WITHIN CHROMOSOME INTERVAL 16q24.3

5.1 INTRODUCTION

A region of the long arm of chromosome 16 (band 16q24.3) has been identified by LOH screening as containing a tumour suppressor gene. The gene is likely to be involved in the transition from in situ to metastatic breast cancer. In order to clone and identify this gene it is necessary to construct a cosmid-based physical map of the region. Once this has been done the region of LOH can be refined, and candidate genes can be isolated and tumour material screened for mutations.

A mouse/human somatic cell hybrid panel has been generated from chromosome 16. The average distance between the breakpoints on this panel is ~1Mb (Callen et al, 1992b). Two of the hybrids - A and B in figure 5.1 - contain only the most distal tip of the long arm of chromosome 16. They form the basis for the physical mapping of 16q24.3, for the LOH studies have identified the region between the polymorphic probes APRT (in interval A) and D16S303 (in interval B) as the current region of smallest LOH overlap (fig 5.1) (Cleton-Jansen et al, 1994).

A chromosome 16 cosmid library is available for this region, and many of the cosmids have been linked into contigs (Doggett et al, 1995). Using FISH to high resolution chromosomes and to interphase nuclei, an attempt was made to provide useful data regarding the fidelity, the ordering and the orientation of cosmids within intervals A and B.

5.2 METHODS

5.2.1 Slide Preparation

Fixed cell suspensions of metaphases and interphase nuclei from normal individuals were brought to room temperature and spread as outlined in 2.2.12.

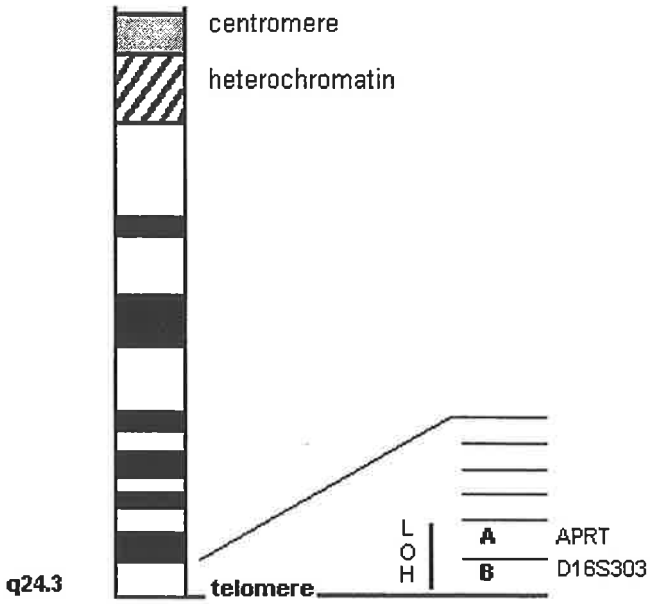
5.2.2 Probe description

Table 5.1 (p.89) shows the cosmids used in this study, their status as singletons or as part of a contig, and the intervals to which the (unordered) contigs and singletons were assigned at the commencement of this study.

Figure 5.1 : PHYSICAL MAP OF THE REGION OF LOH WITHIN CHROMOSOME BAND 16q24.3

Ideogram of the long arm of chromosome 16 showing the position of hybrid intervals within chromosome band 16q24.3, and the region of LOH in breast cancer. The two most distal long-arm hybrids of the chromosome 16 mouse / human somatic cell hybrid panel are shown as A and B. The polymorphic probes APRT in interval A and D16S303 in interval B defined the smallest region of LOH overlap at the commencement of this project.

Figure 5.1


centromere
heterochromatin
L
O
H
A
B
APRT
D16S303
q24.3
telomere

5.2.3 Probe Labeling

All cosmids were nick translated with biotin-14-dATP and, separately, random primer labeled with DIG-11-dUTP, as outlined in 2.2.10 and 2.2.11.

5.2.4 Hybridization of Single Cosmid Probes to Metaphases

Twenty-one biotin-labeled and 18 DIG-labeled cosmid probes from intervals A and B of chromosome band 16q24.3 were hybridized separately to 39 metaphase preparations using FISH method 2.2.15 to ascertain their suitability for use, ie their single copy status and the level of signal and non-specific background each type of label produced for each cosmid.

5.2.5 Dual Hybridization of Differentially-Labeled Cosmid Probes to Metaphase Preparations

To determine probe order in metaphase preparations it is necessary only to visualize one biotin-labeled plus one DIG-labeled probe together on a chromosome. Probe order can then be determined by the alignment of colours with respect to the telomere or centromere of the chromosome.

Nine biotin- or DIG-labeled probes representing six contigs and two singletons from intervals A and B were hybridized in differential pairwise combinations to metaphase preparations according to FISH method 2.2.15. To determine the order of singleton cosmids and cosmid contigs, FITC and rhodamine signals were simultaneously detected through a dual band-pass filter.

5.2.6 Hybridization of Biotin-Labeled and (separately) DIG-Labeled Cosmid Pairs to Interphase Nuclei

Eight biotin-labeled probes were hybridized in pairs to interphase nuclei to check the level of fluorescent signal and interference from background fluorescence that was present in nuclei after dual hybridizations, using FISH method 2.2.15. Eight DIG-labeled probes were similarly treated.

Table 5.1 : RESULTS OF FISH TO METAPHASE CHROMOSOMES USING 16q24.3 COSMID PROBES FROM 10 CONTIGS AND 4 SINGLETONS

Probe	singleton or contig name	interval	biotin signal at 16q24.3	DIG signal at 16q24.3	comment
309A3	S	A	+++*	+*	+*= ave. signal +++*= excellent signal
303B4	S	A	+	++	high BGD - DIG
313G3	S	A	+	+	not used further - signal also at 16p11
32D9	S	A	-	-	not used further - signal at 16q21 not 16q24
340E8	cAPRT	A	+	+	high BGD - DIG
310D1	c10.2	A	+	not done	
365A7	c113	A	++	not done	
434D8	c113	A	++	not done	
301F4	c639	A	+	+	high BGD - biotin and DIG
310H9	c76.1	A	+	++	
317E5	c317E5	B	++	+	
408G10	c317E5	B	+++	++	
383H6	c317E5	B	+	+++	high BGD - biotin
425E4	cAc6.26	B	+++	+	not used further - contains subtelomeric repeat sequences
435H5	cRDP	B	++	+	
336A8	c177D12	B	++	++	
398E11	c177D12	B	+	+	
323B6	c177D12	B	+++	+	
377F1	c177D12	B	+	++	high BGD - biotin
361G2	cLL64	B	++	+	
352A12	cLL64	B	+++	1+	

5.2.7 Triple Hybridization of Differentially-Labeled Cosmid Probes to Interphase Nuclei

To establish probe order in interphase nuclei it is necessary that one of the triplet of cosmids is already established as lying completely proximal or distal to the other two. Ten cosmid probes were hybridized 3 at a time in 6 differentially labeled combinations to preparations of interphase nuclei using FISH method 2.2.15. In one hybridization, two small overlapping cosmids from one contig (365A7 and 434D8 from contig 113), were combined to make a single probe for that contig.

To determine the order of the 3 probes, FITC and rhodamine signals were simultaneously detected through a dual band-pass filter.

5.2.8 Statistical Analysis

The statistical significance of interphase and metaphase results was determined by calculating the cumulative binomial distribution (displayed as *P* value in Tables 5.2, 5.3).

5.2.9 Photography

The images shown in this study are high resolution photocopies of colour prints prepared from colour slides. There is no image enhancement.

5.3 RESULTS

5.3.1 Hybridization of Single Cosmid Probes to Metaphases

Since the cosmids were anonymous DNA segments with high variability in the percentage of length that contained repetitive elements, all probes were labeled separately with both biotin and DIG in the expectation that one form of labeling might give a better signal-to-noise ratio than the other for individual probes. The results of the hybridizations of 21 cosmids labeled with biotin, and 18 of the same 21 labeled with DIG, are shown in Table 5.1.

In summary, of the 21 cosmids tested:

- five cosmids (303B4, 340E8, 301F4, 383H6, and 377F1) had a level of background fluorescence with either or both forms of labeling that was unacceptable for nuclei experimentation, where there is no possibility of distinguishing a bright background dot

from a target signal. These 5 cosmids were, however, retained for possible use in metaphase hybridizations where a low level of background on other chromosomes does not interfere with the interpretation of signal on the target chromosome.

- cosmid 32D9 was discarded as it gave signal at 16q21.1 not 16q24.
- cosmid 313G3 gave excellent signal at 16q24.3 but was discarded as it also hybridized to sequences at 16p11.
- cosmid 425E4 gave excellent signal at 16q24.3 but also detected subtelomeric sequences at 16pter, 3qter, 4qter, 7pter, 9qter and 18pter (fig 5.2). This was a useful hybridization, however, as the possession of subtelomeric sequences indicates that the contig to which this cosmid belongs (cAc6.26) is likely to be the most telomeric of the contigs in interval B.

As a result there were 17 cosmids which could be chosen on their signal brightness and contig position for use in metaphase ordering, and 13 cosmids which could be used for ordering in nuclei (fig 5.3).

5.3.2 Dual Hybridization of Differentially-Labeled Cosmid Probes to Metaphases

The results of 9 pairwise hybridizations are shown in Table 5.2.

Chromosome 16s exhibiting two different coloured signals from a differentially-labeled pair of cosmids were examined. The order of signal colours relative to the telomere of the chromosome was scored. Figures 5.4 and 5.5 give representative examples of these.

From 20 to 62 chromosomes were recorded for each pair, and the order was expressed as three numbers, ie FOR a particular order : AGAINST a particular order : or NO PARTICULAR ORDER (where the two coloured signals were on top of, or side by side, each other).

The statistical significance of the metaphase results was determined by calculating the exact cumulative binomial distribution. To determine whether, in a sample of n trials of which there were k 'againsts' and $(n\text{-}k)$ 'fors', the result is significantly different from random ($p = q = 0.5$) the exact cumulative binomial probability can be calculated and compared with the significance level α. If $P\ (x \leq k \,/\, p = 0.5,\ n) < \alpha$ then the hypothesis $P\ (\text{'fors'}) = P(\text{'againsts'}) = 0.05$ is assumed false. On this basis, the results for experiment 9 failed to be significant and have thus not been included in the conclusions.

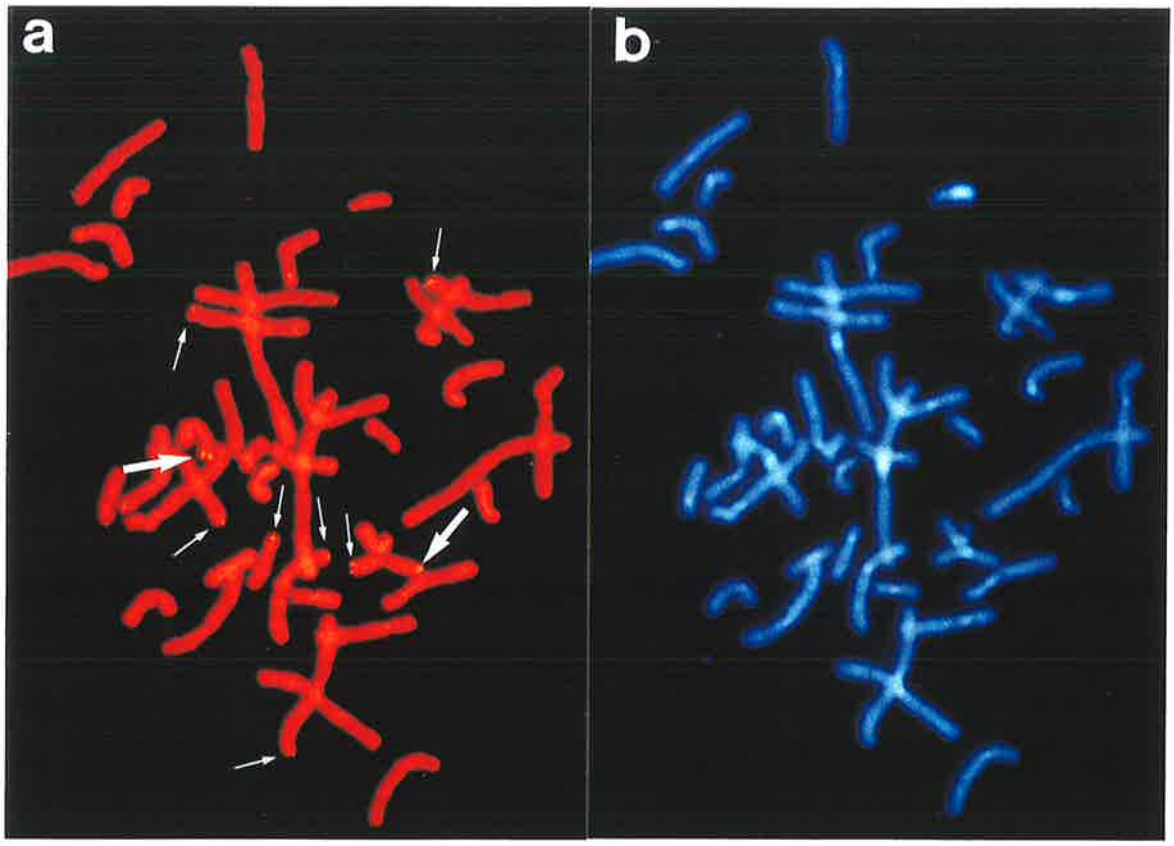
Figure 5.2 : METAPHASE SHOWING FISH WITH COSMID 425E4

(a) Normal male chromosomes stained with propidium iodide. Fluorescent signal from hybridization of the cosmid probe 425E4 is evident on chromosome 16q24.3 (large arrow), but is also present within the subtelomeric regions of chromosomes 16p, 3q, 4q, 7p, 9q and 18p (small arrows).

(b) The same metaphase as (a) stained with DAPI for chromosome identification.

Figure 5.3 : METAPHASE SHOWING FISH WITH COSMID 323B6

Representative metaphase showing FISH with a cosmid which can be used as a probe for either chromosomes or nuclei. Fluorescent signal on chromosome 16q24.3 from hybridization of cosmid probe 323B6 is indicated by arrows. There is no non-specific fluorescent background present.


a
b

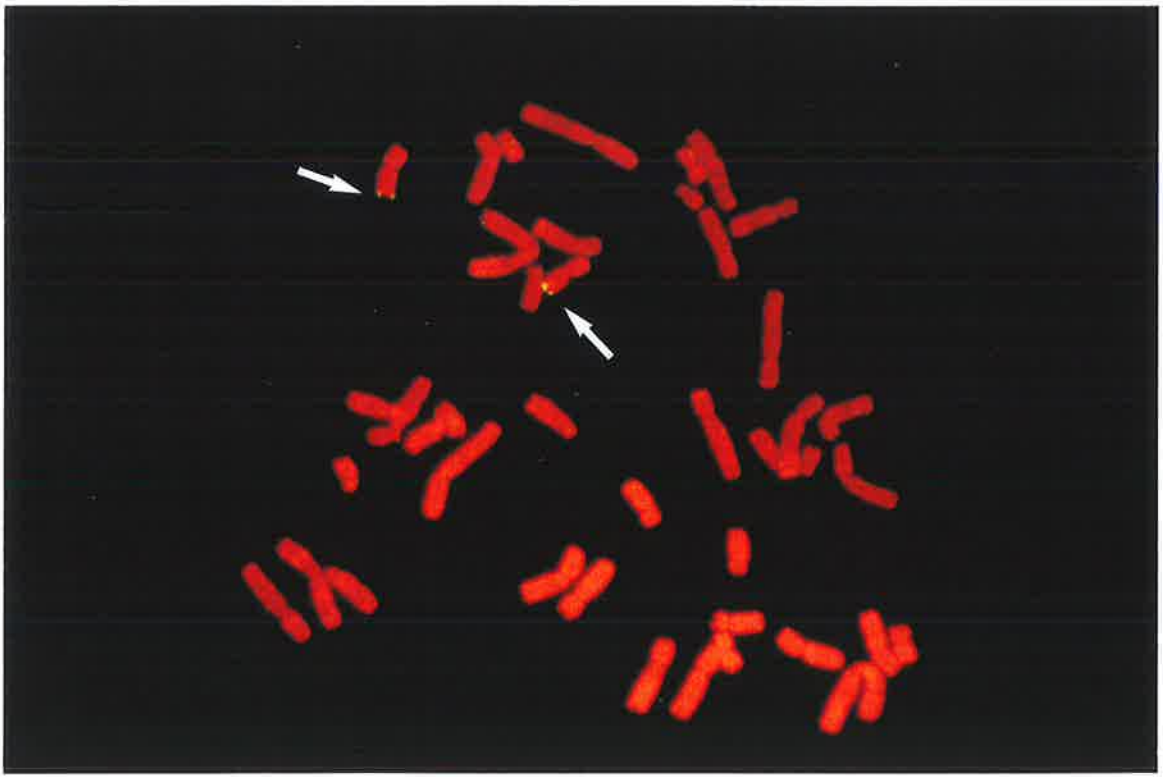


Figure 5.4 : METAPHASE SHOWING FISH WITH DIFFERENTIALLY LABELED COSMID PROBES (1)

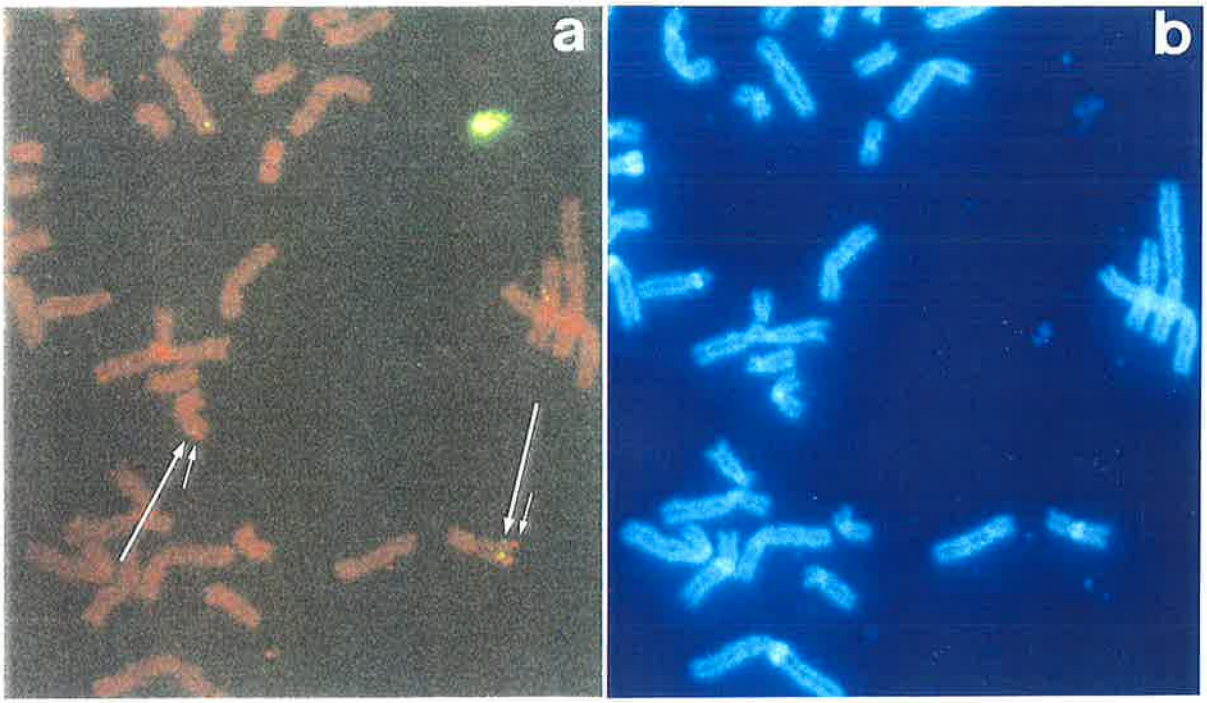
(a) Normal male chromosomes counterstained with propidium iodide. Fluorescent signals from cosmid 310D1 (labeled with biotin and detected with FITC) and cosmid 383H6 (labeled with DIG and detected with TRITC) are evident at chromosome band 16q24.3. The signals are present in the order : yellow (large arrow) - red (small arrow) - telomere.

(b) The same metaphase as (a) stained with DAPI for identification of chromosome 16.

Figure 5.5 : METAPHASE SHOWING FISH WITH DIFFERENTIALLY LABELED COSMID PROBES (2)

(a) Normal male chromosomes stained with propidium iodide. Fluorescent signal from cosmid 301F4 (labeled with biotin and detected with FITC) and cosmid 340E8 (labeled with DIG and detected with TRITC) are evident in chromosome band 16q24.3. The signals are present in the order : yellow (large arrow) - red (small arrow) - telomere.

(b) The same metaphase as (a) stained with DAPI for identification of chromosome 16.


a
b

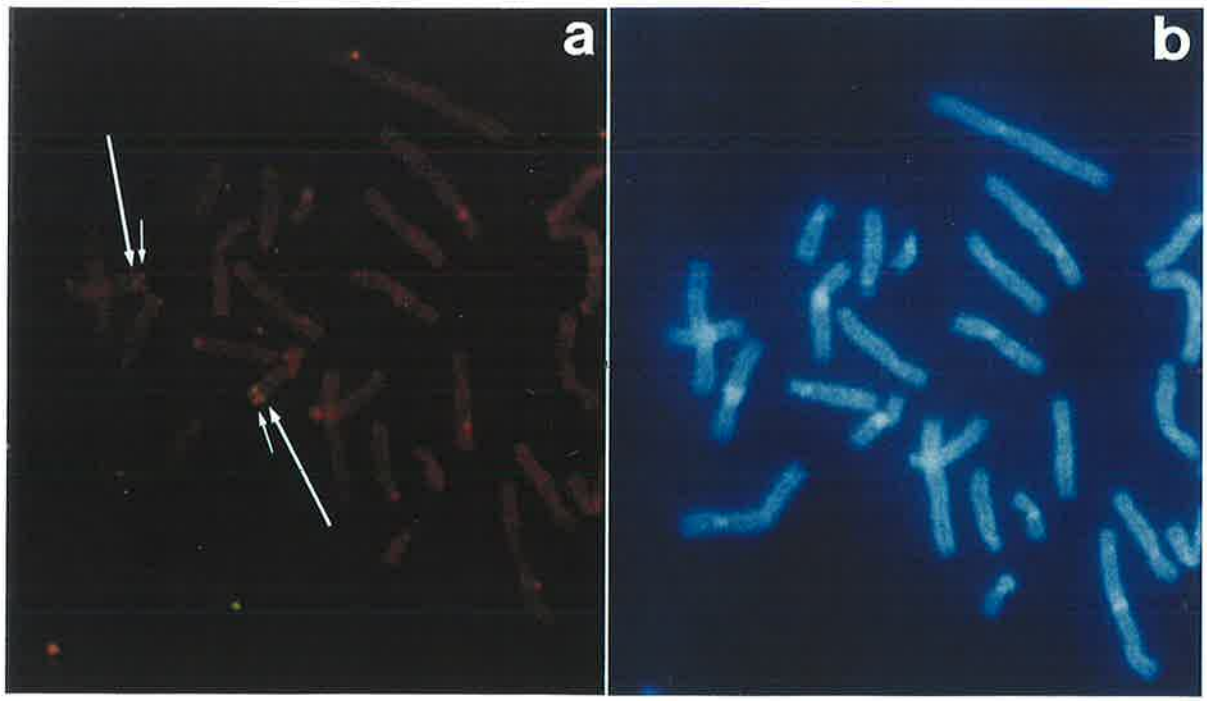

a
b

Table 5.2 : RESULTS OF FISH WITH DIFFERENTIALLY LABELED COSMID PAIRS TO METAPHASE CHROMOSOMES

experi-ment	colour order	scoring for:against:no order	interpretation of probe (ie contig) order	'P' value
1	Y - R - tel	20 : 0 : 0	cen - 310D1 (c10.2) - 383H6 (c317E5) - tel	9.5367×10^{-7}
2	Y - R - tel	17 : 0 : 9	cen - 310D1 (c10.2) - 340E8 (cAPRT) - tel	7.6294×10^{-6}
3	Y - R - tel	24 : 7 : 31	cen - 301F4 (c639) - 340E8 (cAPRT) - tel	1.6634×10^{-3}
4	Y - R - tel	10 : 2 : 8	cen - 310D1 (c10.2) - 301F4 (c639) - tel	1.9287×10^{-2}
5	Y/R - tel	signal overlap	cen - 383H6 (c317E5)/377F1 (c177D12) - tel	
6	Y/R - tel	signal overlap	cen - 317E5 (c317E5)/377F1 (c177D12) - tel	
7	Y - R - tel	12 : 1 : 18	cen - 309A3 (singleton) - 340E8 (cAPRT) - tel	1.7089×10^{-3}
8	Y - R - tel	21 : 7 : 28	cen - 303B4 (singleton) - 340E8 (cAPRT) - tel	6.2705×10^{-3}
9	Y - R - tel	7 : 4 : 13*	*cen - 310H9 (c76.1) - 340E8 (cAPRT) - tel	*0.27441

*results not statistically significant

Cosmids 383H6 and 317E5 from either end of the c317E5 contig were both tested against 377F1 from the c177D12 contig, for the orientation of the two contigs was unknown. Given the length of the c317E5 contig (9 overlapping cosmids covering ~60kb), it was possible that the distance provided by one of these tested pairs may have been sufficient to give discrete signals with FISH. However, in experiments 5,6 and 9 the order of pairs (383H6 and 377F1), (317E5 and 377F1) and (310H9 and 340E8) could not be determined and the two probes within each set were therefore assumed to be <1Mb apart from each other.

The order of contigs determined in this way was :

cen - c10.2 - c639 - cAPRT (interval A) ‖ (interval B) c 317E5/c177D12 - tel

The order of singletons determined in this way (relative to the contigs) was :

centromere - 309A3/303B4 - cAPRT - telomere

5.3.3 Hybridization of Biotin-Labeled and (separately) DIG-Labeled Cosmid Pairs to Nuclei

Eight biotin-labeled cosmids which gave a good signal when hybridized singly to chromosomes, gave a slightly lower signal but little to no background when hybridized in

pairs to nuclei. Eight DIG-labeled cosmids hybridized in pairs to nuclei gave a slightly lower signal and a higher background level than desirable when hybridized in pairs to nuclei. Because of this it was decided that triple hybridizations of differentially-labeled probes should if possible contain one DIG- and two biotin-labeled probes.

5.3.4 Triple Hybridization of Differentially-Labeled Cosmid Probes to Interphase Nuclei

Eight separate triple hybridizations were made. Nuclei exhibiting three coloured signals from a triplet of cosmids were examined and the order of colours noted. Between 15 and 47 sets of signals were recorded for each hybridization.

In two of the eight hybridizations the probes gave signal too sporadic to be scoreable. The DIG-labeled probes were in general less consistent than the biotin-labeled probes.
Two hybridizations were performed before it was known that the contigs RDP and 317E5 were overlapping. The overlap explains why three distinct signals were never observed for these two experiments.
The results of the remaining four separate triple hybridizations are shown in Table 5.3, and representative nuclei are shown in figure 5.6. The statistical significance of the interphase results was determined by calculating the exact cumulative binomial probability, expressed as *P* in Table 5.3.

Table 5.3 : RESULTS OF FISH WITH DIFFERENTIALLY LABELED COSMID TRIPLETS TO INTERPHASE NUCLEI

exper-iment	colour order	scoring for:against: no order	interpretation of probe (ie contig or singleton) order	'P' value
1	Y - Y - R	10 : 1 : 4	cen - 309A3 (singleton) - 361G2 (cLL64) - 336A8 (c177D12) - tel	5.8×10^{-3}
2	Y - R - Y	27 : 6 : 14	cen - 309A3 (singleton) - 352A12(cLL64) - 323B6 (c177D12) - tel	1.62×10^{-4}
3	R - R - Y	20 : 4 : 9*	352A12 (cLL64)/398E11 (c177D112) - 435H5 (cRDP) *	7.72×10^{-4}
4	Y - Y - R	20 : 4 : 1	cen - 365A7+434D8 (c113) - 408G10 (c317E5) - 352 A12 (cLL64) - tel	7.72×10^{-4}

*orientation in regard to telomere not established

Figure 5.6 : FISH WITH THREE DIFFERENTIALLY LABELLED COSMID PROBES TO INTERPHASE NUCLEI

(a) - (e) : Nuclei from several hybridizations showing signal from three differentially labeled cosmids within chromosome region 16q24.3. Signal is present in the following order

(a) Yellow (large arrow) - Red (small arrow) - Yellow(large arrow)

(b) Y - Y - R

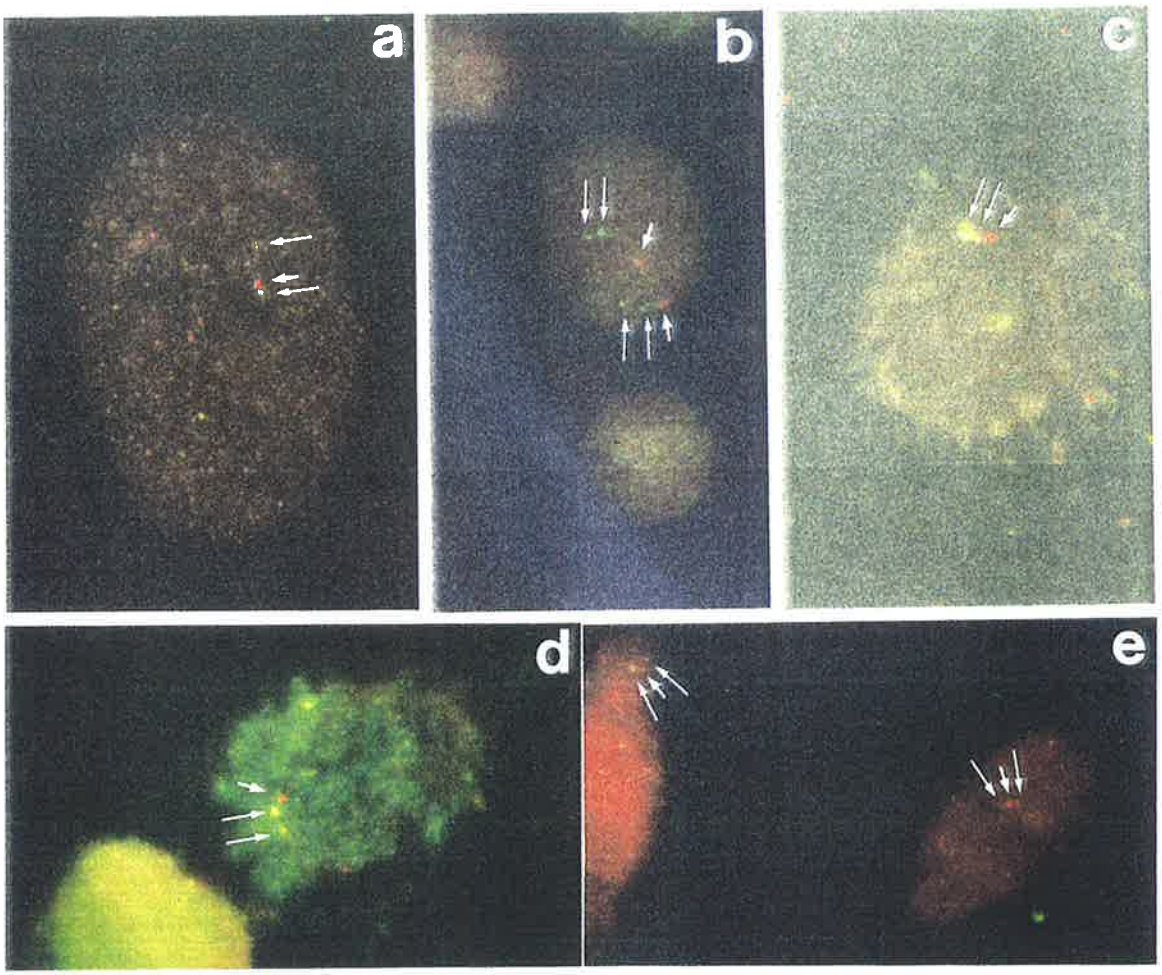
(c) Y - Y - R

(d) Y - Y - R

(e) Y - R - Y

(f) Nucleus showing two different probe orders within the one cell,

ie Y - R - Y and Y - Y - R


a
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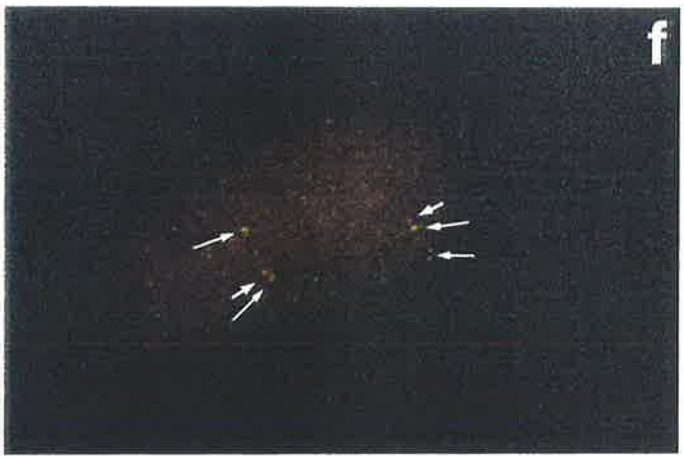

f

In experiment three the two DIG-labeled probes both lay to one side of 435H5 and were therefore unable to be ordered with respect to each other. Also, since all three probes were from Interval B, the orientation of the group could not be established by this experiment. However, other data available at this time (Whitmore, personal communication) had established an overlap between contigs cRDP and c317E5. Therefore, since the FISH data placed c317E5 proximal to cLL64 within interval B (experiment 4), cRDP could also be placed proximal to cLL64.

The order of singletons and contigs determined in this way was:
cen - 309A3 - cLL64 - c177D12 - tel
and cen - c113 - c317E5 - cLL64 - tel

When the data from Tables 5.2 and 5.3 are combined, the final order of contigs is :

	INTERVAL A	INTERVAL B	
cen -	**c10.2 - c639 - cAPRT -**	**c317E5/cRDP - cLL64 - c177D12 -**	**tel**

5.4 DISCUSSION

Metaphase and interphase mapping are relatively rapid and straightforward techniques for the ordering of DNA sequences. Probe order is derived from the order of red and yellow fluorescent signals after two-colour hybridization with two probes on metaphase chromosomes, or with three probes on interphase nuclei.

The sensitivity and specificity of the probes must be tested on metaphase chromosomes prior to the mapping. This is especially important in interphase mapping where there is no inbuilt control - such as chromosome morphology - for the identification of the site of hybridization. Nuclei arrested in G0/G1 are the preferred target for interphase mapping, where a doubling of signals caused by DNA replication at the target site of a given cosmid can be confused with signals from two probes labeled with the same

fluorochrome, especially if one of those probes is hybridizing with low efficiency. Double dots may also be an artefact of the nature of avidin accumulation at the hybridization site.

In this study there was no attempt to assess map **distances**. Probes hybridized to some regions of chromosomes -including the centromere and telomeres - often show a higher variability in map position than probes to other chromosomal locations, indicating that local chromatin structure plays a significant role in determining signal position. For example, the T2AG3 oligomer specific for human telomeres often appears proximal to the visible end of metaphase chromosomes after FISH. Chromatin folding near the telomere may also account for some of the high 'no particular order' scores obtained with several of the cosmid combinations in this study. The final order of cosmid probes obtained for the telomeric chromosome band 16q24.3 was therefore considered to be a useful indication of probable contig order rather than an irrefutable order.

The information obtained by the metaphase and interphase ordering and orientation of cosmid probes, has furthered the construction of the physical map of chromosome 16 in the region of LOH within chromosome band 16q24.3.

CHAPTER 6

DEVELOPMENT : PROBE HYBRIDIZATION TO EXTENDED CHROMATIN FIBRES

6.1 INTRODUCTION

Before attempting to localize lambda probes to extended chromatin fibres it was necessary to establish optimum spreading and detection conditions. This was done using the more easily hybridized satellite DNA probes, followed by hybridization with P1 then cosmid probes.

6.2 METHODS

6.2.1

Slide Preparation 1

Two ml of well growing LCL cells were centrifuged and resuspended to 1500cells/ul in medium. Extended chromatin fibre preparations were made using ECF Protocol 1 (for live cells) (2.2.14.1).

Slide Preparation 2

Cells from a 3-day lymphocyte culture which had been harvested, fixed and stored at -20°C for 3 weeks were brought to room temperature. Extended chromatin fibre preparations were made using ECF Protocol 2 (for fixed cells) (2.2.14.2).

6.2.2 Probe Description

All probes used in this development chapter were first checked for specificity and sensitivity on metaphase chromosomes.

D1 and D4 are alpha satellite DNA probes specific for the pericentromeric regions of chromosomes 1 and 4 respectively.

RR6 is a satellite DNA probe specific for human 1q heterochromatin.

P1.1 is an ~80kb single copy P1 clone.

C1 and C2 are two 35-40kb cosmid clones which hybridize to chromosome band 4q31.1.

6.2.3 Probe Labeling

RR6 and C2 were random primer labeled with DIG-11-dUTP.

D1, D4, P1.1 and C1 were nick translated with biotin-14-dATP.

6.2.4 Single Hybridization of Satellite DNA Probes

Probes D1 and D4 (biotin-labeled) and RR6 (DIG-labeled) were individually hybridized to ECF preparations according to the FISH method described in 2.2.15, except as detailed below in 6.2.5.

6.2.5 FISH Conditions - Single Hybridization of Satellite DNA Probes

A range of concentrations (from 0.01 - 0.5ng/ul) was used for each probe.

Slides were not baked prior to hybridization.

Detection of biotin-labeled probes was attempted with one detection step and, separately, with one detection plus one amplification.

Detection of the DIG-labeled probe was with one detection step.

Slides were mounted in antifade solution which contained a low concentration of propidium iodide as well as DAPI.

6.2.6 Differential Hybridization of Satellite DNA Probes

Probes D1 (biotin-labeled) and RR6 (DIG-labeled) were hybridized simultaneously to ECF preparations according to the FISH method described in 2.2.15, except as detailed below in 6.2.7.

6.2.7 FISH Conditions - Differential Hybridization of Two Satellite DNA Probes

Slides were not baked prior to hybridization.

The biotin and DIG detection systems were performed simultaneously, or with biotin detection first followed by DIG detection.

Slides were mounted in antifade solution which contained propidium iodide as well as DAPI.

6.2.8 Trial Hybridization of P1 Genomic Probe

A biotin-labeled P1 clone was hybridized at 10ng/ul (with 500:1 total human DNA as competitor) to ECFs prepared according to Protocol 2, and detected with one detection step plus one amplification.

6.2.9 Differential Hybridization of Two Cosmid Probes

Cosmid probes C1 (biotin-labeled) and C2 (DIG-labeled) were simultaneously hybridized at 15ng/ul each (with 500:1 total human DNA as competitor) to ECFs prepared by

Protocol 2. In one hybridization the two cosmids were pre-reassociated at the same time with the total human DNA; in a separate hybridization the cosmids were pre-reassociated separately with total human DNA then combined just before hybridization. Detection consisted of one detection step plus one amplification for each hapten.

6.2.10 Photography

The images shown in this study are high resolution photocopies of colour prints prepared from colour slides. There is no image enhancement.

6.3 RESULTS AND COMMENTS

6.3.1 Slide Preparation

When prepared from a cell suspension at too high a density, free chromatin fibres tend to aggregate in thick ropes of strands across the slide (ECF Protocol 1) or in spider-web-like networks (ECF Protocol 2). A network or thick rope of fibres makes interpretation of probe signal difficult and potentially misleading.

Protocol 1:

The duration of the alkaline treatment is important. Under-treatment produces nuclei but few free fibres on the slide; over-treatment produces areas of aggregated fibres or can remove DNA from the slide completely.

Protocol 2:

The original Protocol (Fidlerova et al, 1994) required that the lysing solution be composed of 5:2 **0.07M** NaOH:absolute ethanol. The substitution of a range of NaOH molarities for the 0.07M showed that the use of **5M** NaOH in the same proportions left more fibres on the slide and gave more controllable stretching. As 5M was high compared with that recommended, the result was checked by repeating the procedure twice.

The timing ratio of PBS : NaOH : Methanol is critical. The nuclei are disrupted by the alkali treatment and stretched by the wash of methanol down the slide, but the length of the original PBS exposure determines the sensitivity of the nuclei to the NaOH. The original protocol used a 60sec PBS exposure. A 30sec exposure was found to be more manageable.

An optimal slide is one which still contains a number of intact nuclei, has long straight fibres extending from the remnants of nuclei, and has areas of very fine stranding (not networks) when observed under high power microscopy. The intact nuclei are useful for checking the integrity and the background level of fluorescent signal produced during hybridization. The fibres extending from nuclear remnants are useful for checking the single cell origin of the observed signal. The areas of very fine free chromatin provide the highest resolution on the slide. Figure 6.1 provides examples of ECFs spread by Protocol 2. Protocol 2 provided the desired variations more often than Protocol 1, and conditions of spreading were more easily controlled with Protocol 2. For these reasons, plus the advantage of being able to use stored, fixed cell suspensions, Protocol 2 became the method of choice.

6.3.2 Probe Concentration and Detection

Optimum probe concentrations for the single hybridizations of satellite DNA probes were:
D1 : 0.04ng/ul with one detection step.
D4 : 0.01ng/ul with one detection step. Even at this low concentration (the lowest attempted) other centromeric regions partially homologous to chromosome 4 were beginning to be detected. This proved the value of having some intact nuclei on the slide for these indicated not only bright signal from the chromosome 4 centromeres but also less bright signal from the centromeres of other chromosomes. Thus the many strings of signal present on the free fibres could be interpreted as being derived from more than the desired target sequence on chromosome 4.
RR6 : 0.4ng/ul with one detection step.

6.3.3 Appearance of Signal

The appearance of individual probe signals as a single dot, a short string of dots, or extended strings of dots, generally varied according to the condensation of the nuclear structure to which the probe had hybridized. However, the **intensity** of the signal thus produced did not diminish greatly, ie the string of signal from a highly extended fibre was occasionally as bright as the single dot of signal within a highly condensed nucleus. It is possible that this unexpected phenomenon is due to the fact that the binding of avidin or of intermediate and fluor-conjugated antibodies is less encumbered by steric hindrance when hybridizing to ECFs than it is to the relatively inaccessible target of intact nuclear DNA, which is contracted and surrounded by extensively cross-linked proteins.

Figure 6.1 : EXAMPLES OF EXTENDED CHROMATIN FIBRES

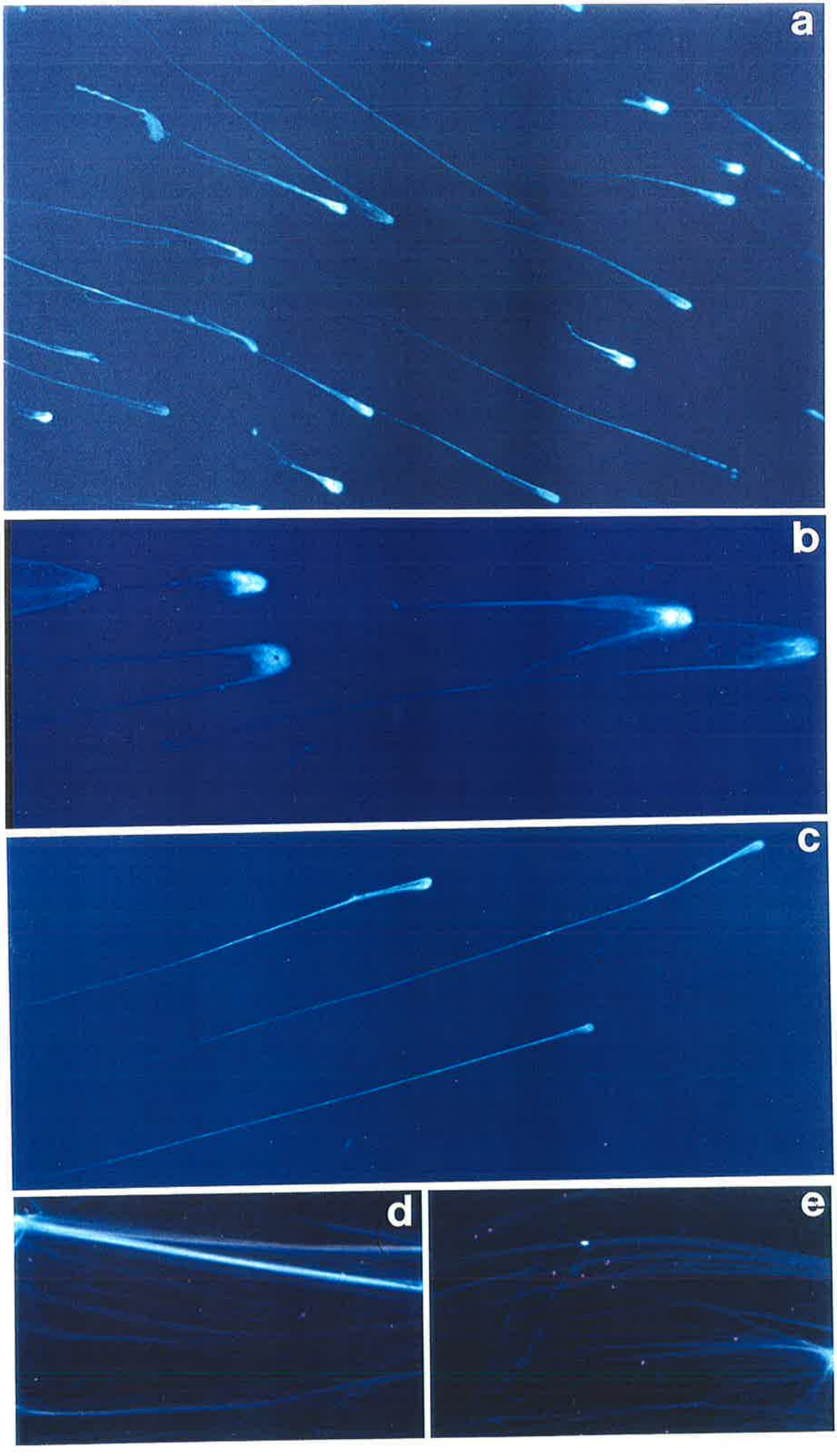
DAPI - stained extended chromatin fibres prepared by ECF Protocol 2.

(a) Nuclei showing a range of fibre extensions (magnification = x32)

(b) Nuclei with a short fibre extension (magnification = x80)

(c) Nuclei with a long fibre extension (magnification = x32)

(d) (e) Nuclei with a long fibre extension showing very fine fibres (magnification = x80)


a
b
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d
e

The strings of signal seen following differential hybridization were typically thicker for DIG-labeled probes than for biotin-labeled probes. This variation is possibly related to the different proteins used for detecting each hapten.

The presence of propidium iodide as counterstain in the antifade solution was discontinued after these trial ECF experiments, as even a low concentration tended to partially obscure the red rhodamine signal. Fibre morphology could be monitored by changing to the DAPI wavelength to check signal and fibre integrity as necessary.

6.3.4 Results of Differential Hybridization of Satellite DNA Probes

An example of the simultaneous hybridization of bio-D1 and DIG-RR6 to ECFs is shown in figure 6.2. Detection of the biotin-labeled probe first, followed by detection of the DIG-labeled probe, gave a cleaner in situ result ie less non-specific background and a brighter signal, than simultaneous detection. The two-step procedure was therefore adopted as the standard.

6.3.5 Results of Hybridization of P1 Genomic Probe

Examples of the hybridization of the 80kb genomic P1 probe is shown in figure 6.3. The high concentration of competitor DNA efficiently blocked the binding of repetitive DNA sequences.

A photographic slide of a stage micrometer was compared with photographic slides of P1 probe signals on ECFs. The lengths of the signals were measured in microns. The theoretical length of one basepair of DNA is 3.4Å. The maximum signal length from an 80kb probe should therefore be ~27.2um. Measurements of 10 signals from P1.1 varied in length from 19.5 - 33um, with a mean of 26.25um. The average extension of fibres on that slide was therefore ~3kb/um.

6.3.6 Results of Differential Hybridization of Two Cosmid Probes

Examples of the hybridization of the probes C1 and C2 are shown in figure 6.4. The two probes could not be visualized separately on metaphase chromosomes. Hybridization to ECFs shows that the cosmids are overlapping, with signal from the DIG-labeled cosmid C2 extending slightly beyond the biotinylated C1.

A cleaner FISH result (lower non-specific background) was achieved when the two probes were pre-reassociated with total human DNA separately before hybridization.

Figure 6.2 : DIFFERENTIAL HYBRIDIZATION OF TWO SATELLITE DNA PROBES TO ECFs

(a) ECFs stained with DAPI to show strand morphology.
(magnification = x320, single band pass UV filter)

(b) The same fibres as (a) showing hybridization with the biotinylated satellite DNA probe D1.
(single band pass FITC filter)

(c) The same fibres as (a) showing hybridization with the DIG-labeled satellite DNA probe RR6.
(single band pass TRITC filter)

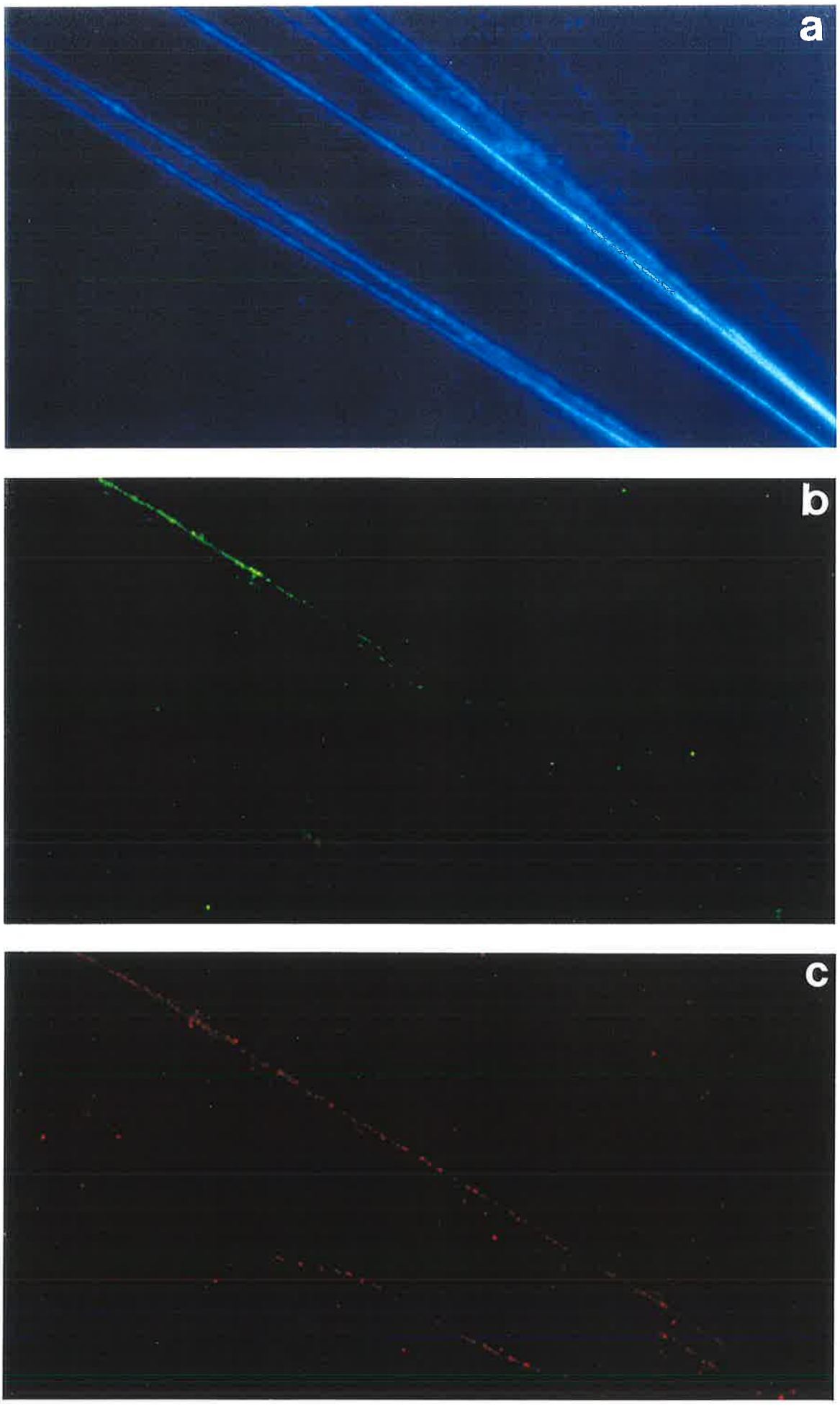
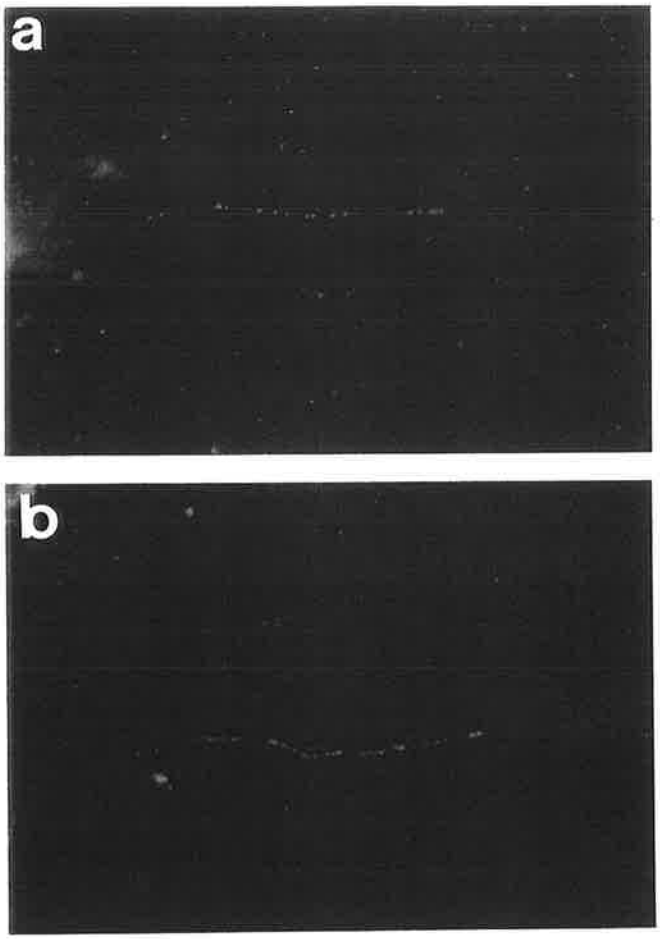

a
b
c

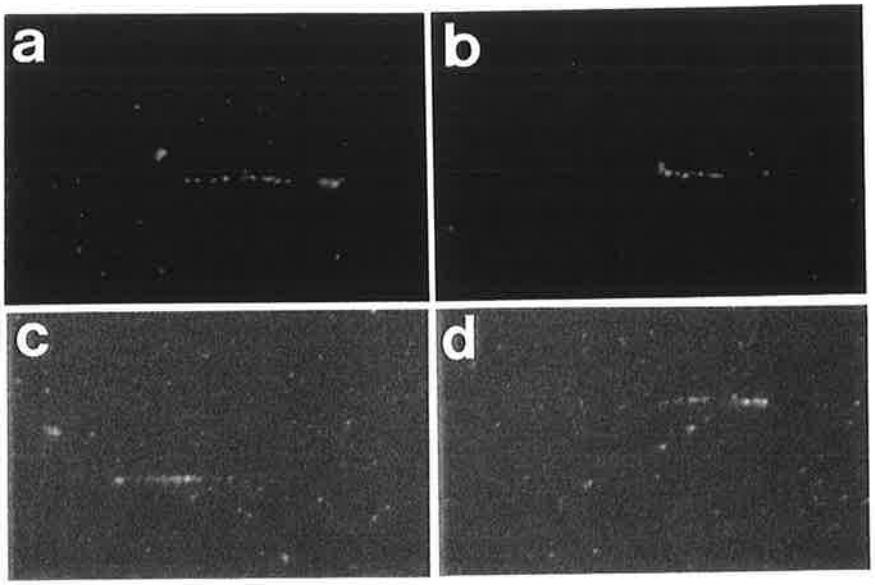
Figure 6.3 : FISH WITH P1 GENOMIC PROBE TO ECFs

(a) (b) Hybridization signal from biotinylated P1 clone (P1.1) on extended chromatin fibres. (magnification = x320)

Figure 6.4 : FISH WITH TWO DIFFERENTIALLY-LABELED COSMID PROBES TO ECFs

(a) - (d) Simultaneous hybridization of biotinylated cosmid C1 and DIG-labeled cosmid C2 to extended chromatin fibres. Several different fibre extensions are apparent but the pattern of hybridization remains the same, ie the red signal from the DIG-labeled C2 overlaps and extends slightly beyond the signal from C1. (magnification = x320)


a
b


a
b
c
d

It is not difficult to determine whether successful hybridization has been achieved with extended chromatin fibres. If fibres have been spread at an optimum concentration on the slide, so that they can be determined as coming from one nucleus as opposed to another, then signal patterns of similar length can be seen within groups of fibres as the slide is scanned. Non-specific fluorescent background tends to give much brighter dots than true signal, and can occasionally be visualized on more than one wavelength.

As a result of the method development it was decided that :

(1) ECF spreading Protocol 2 would be used as the standard method.

(2) Differentially-labeled probes would be pre-reassociated separately before hybridization.

(3) Detection and amplification of differential signal would be sequential rather than simultaneous.

CHAPTER 7

ESTIMATION OF FRAGILE SITE EXPANSION BY HYBRIDIZATION OF LAMBDA PROBES TO EXTENDED CHROMATIN FIBRES (ECFs) FROM FRA16B NUCLEI

7.1 INTRODUCTION

Fragile sites are specific gaps and breaks that are visible in metaphase chromosomes under light microscopy. The rare distamycin A - inducible fragile site FRA16B is located at the interface of chromosome bands 16q21-q22 (fig 7.1). It is often expressed at low levels spontaneously in lymphocyte culture but can be induced to extremely high levels of expression (~95%) with AT - sequence DNA binding agents (eg distamycin A, berenil, Hoechst) (Sutherland et al, 1996 - APPENDIX A).

All the fragile sites characterized thus far at the molecular level have been rare folate-sensitive fragile sites, a different class from the one containing FRA16B. The cause of fragility in the rare folate-sensitive fragile sites is the dynamic mutation of a naturally-occurring CCG trinucleotide repeat (Kremer et al, 1991). Perfect repeat copies predispose to expansion of the sequence. Once the repeat has undergone a sufficient expansion in copy number, it is possible to induce the fragile site in chromosomes.

A chromosome 16 lambda contig which extends several hundred kilobases across the FRA16B region is now available. The fragile site itself has been shown to be associated with the expansion of a naturally-occurring, AT-rich, 33bp minisatellite repeat, present in low copy number in normal chromosomes (Yu et al, 1996 - APPENDIX A). The appearance of the repeat sequence after pulsed-field gel electrophoresis (PFGE) suggests that the size of the repeat expansion in some FRA16B individuals may be up to 70kb. However, it is not known whether the structure of the repeat has any effect on the rate at which it migrates in a gel, a factor which would affect the estimation of repeat expansion size.

Therefore, using FISH with lambda clones that map to each side of FRA16B, an attempt was made to ascertain the size of the expansion occurring at this fragile site by hybridization to extended chromatin fibres prepared from LCL nuclei with (1) two normal chromosome 16s, (2) one normal chromosome 16 and one FRA16B chromosome, and (3) two FRA16B chromosomes. The size of the repeat expansion in the latter two individuals had already been assessed by PFGE.

7.2.1 Cell Culture & Slide Preparation

LCL cultures from three individuals were harvested:

LCL(1) contains two normal chromosome 16s.

LCL(2) contains one normal chromosome 16 and one FRA16B chromosome with a repeat expansion estimated at 70kb by PFGE.

LCL(3) contains two FRA16B chromosomes, both with a repeat expansion estimated at 30kb by PFGE.

10ml aliquots of these LCLs were harvested without colchicine treatment as described in 2.2.4.

ECF slides were prepared from fixed cell suspensions of nuclei as optimized in Chapter 6 and according to 2.2.14.2 (ECF Protocol 2).

7.2.2 Probe Description

All probes were checked for specificity, sensitivity and optimum hybridization concentrations on metaphase chromosomes before being used with ECF preparations.

The probes used in this study were lambda clones λ688, λ33, λ582 and λ322.

λ688 and λ33 overlap, and together cover a genomic distance of 26kb, beginning ~14kb distal of FRA16B (fig 7.2).

λ582 and λ322 overlap, and together cover a genomic distance of 15.4kb, ending ~36kb proximal of FRA16B (fig 7.2).

7.2.3 Probe Labeling

The four lambda clones were labeled by nick translation with biotin-14-dATP.

7.2.4 FISH Conditions

λ33, λ688 and λ582 were hybridized at a final concentration of 20ng/ul for each probe. λ322 was hybridized at 10ng/ul because the higher concentration produced a low level of non-specific background on metaphase chromosomes. Pre-reassociation with total human DNA was kept at 200:1 as the total concentration of DNA would otherwise have become too high to dissolve in the 10ul of hybridization mix. Signal was detected with one detection step plus one amplification.

Figure 7.1 : CYTOGENETIC APPEARANCE OF THE RARE DISTAMYCIN A - INDUCIBLE FRAGILE SITE, FRA16B

(a) Leishman - stained partial metaphase showing a chromosome 16 with FRA16B (arrowed).

(b) Leishman - stained partial metaphase showing a FRA16B chromosome (arrowed) in a tiradial configuration (ie duplication if the fragment distal to the fragile site).

(c),(d) G-banded pairs of chromosome 16s from two metaphases showing the breakpoint of FRA16B at 16q22.1 (arrowed).

Figure 7.2 : STRUCTURE OF THE LAMBDA CONTIG SPANNING THE FRA16B FRAGILE SITE ON CHROMOSOME 16

The overlapping lambda clone pairs 322 + 582, and 33 + 688, are separated by ~50kb genomic DNA across the FRA16B region.

P = proximal to FRA16B

D = distal to FRA16B

Fig 7.1

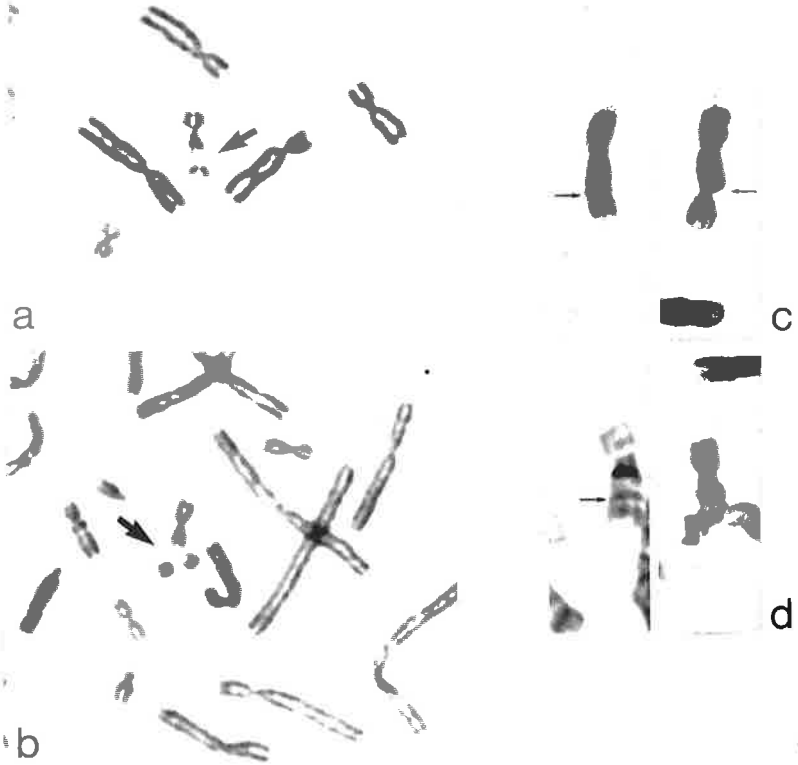
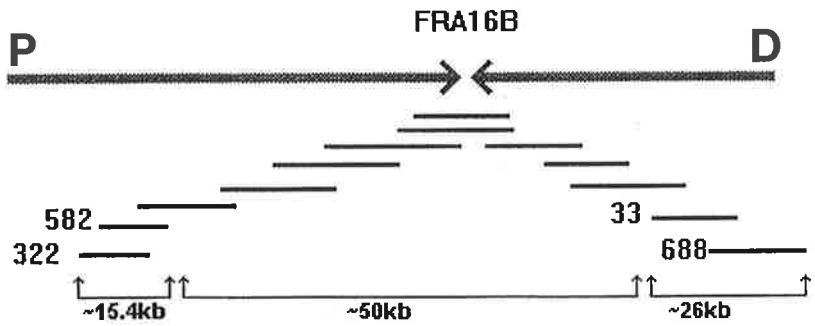


Fig 7.2



7.2.5 Photography

The images shown in this study are high resolution photocopies of colour prints prepared from colour slides. There is no image enhancement.

7.2.6 Gap measurements

Photographic slides of the hybridizations were projected onto a wall. Since the degree of fibre extension varied across each slide, the larger (26kb) of the lambda pairs was normalized by moving the projector closer to or further from the wall. The gap between the lambda pairs was then measured and the known 26kb length was used as an internal standard for calibrating measurements to kilobases.

RESULTS

Representative hybridizations following FISH with the two pairs of lambda clones to ECFs from the three LCLs are shown in figure 7.3. The accumulated gap measurements are shown in figures 7.4, 7.5 and 7.6.

LCL(1) : two normal chromosome 16s

The gap measurements from the hybridization of the lambda clone pairs to ECFs prepared from LCL(1) are shown in figure 7.4. The gap between the two lambda pairs is shown in the figure as 'length in kb'. Fourteen hybridizations are represented, with gap measurement lengths ranging from 40.4 - 65kb. The mean gap length was 53.1kb (Table 7.1).

LCL(2) : one normal chromosome 16 plus one FRA16B

The gap measurements from the hybridization of the lambda clone pairs to ECFs from LCL(2) are shown in figure 7.5. Fifty-four hybridizations are represented. The cut-off point for the normal allele was assessed subjectively at 69.3kb, the first major break in the series of gap measurements. The remaining measurements were assumed to comprise the range for the FRA16B allele, except for the two highest outliers which were not included because the lengths were so great the signals may have been derived from different fibres. Although the cut-off point was determined subjectively, this decision was supported by considering the 95% confidence limits for each group of data (Table 7.1)

Figure 7.3 : HYBRIDIZATION OF LAMBDA CLONES FROM BOTH SIDES OF FRA16B TO EXTENDED CHROMATIN FIBRES

Examples of hybridization of two pairs of overlapping lambda clones to extended chromatin fibres from individuals who are normal, heterozygous or homozygous for FRA16B. Clones were labeled with biotin and detected with FITC.
(magnification = x320)

One pair of overlapping lambda clones extends 26kb, and its hybridization is indicated by large arrows.
The other pair extends 15.4kb and its hybridization is indicated by small arrows.
The gap between the two pairs was measured and converted to kilobases by comparison with the known 26kb length.

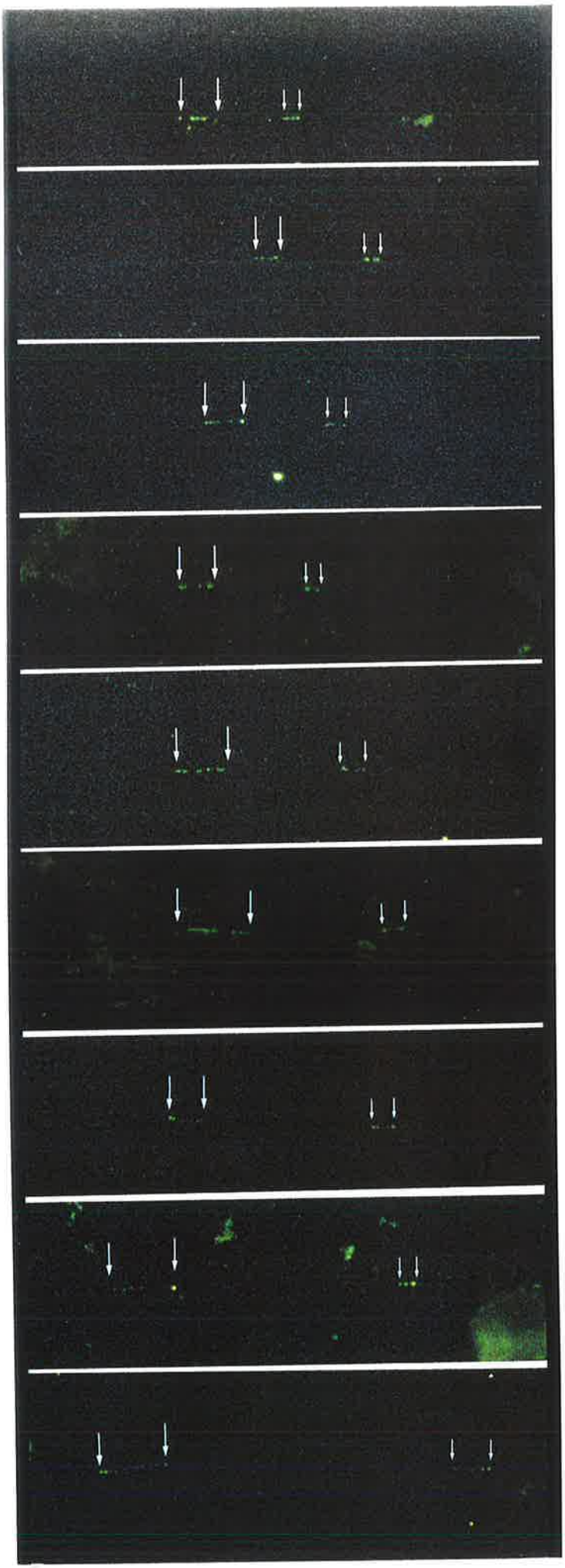


Figure 7.4 : GAP MEASUREMENTS FROM LCL(1)

The bars in the figure represent individual gap lengths measured between the hybridization sites of lambda pairs (688 + 33) and (582 + 322) located on either side of FRA16B. The biotin-labeled probes were hybridized to ECFs from an individual with 2 normal chromosome 16s, and detected with FITC. Gap measurements were converted to kilobases using the 26kb probe pair as an internal standard.

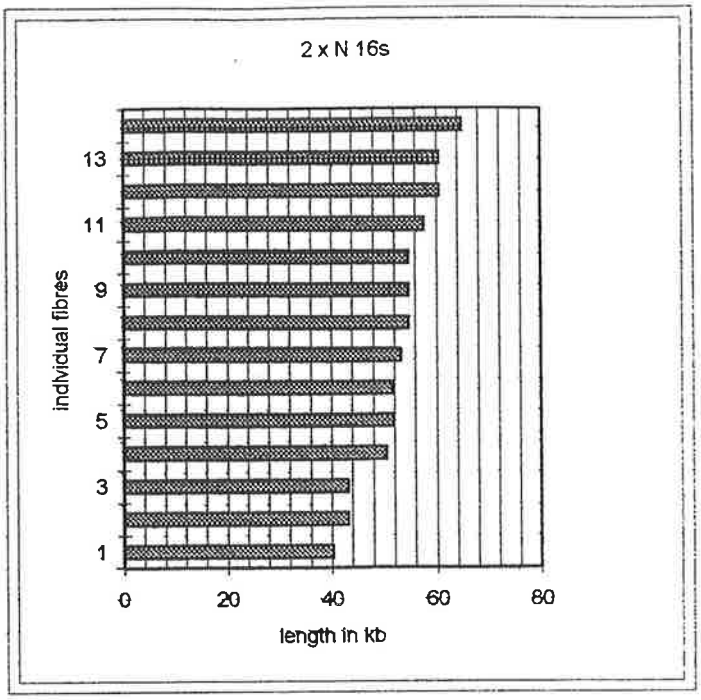

2 x N 16s
13
11
9
7
5
3
1
individual fibres
0
20
40
60
80
length in kb

Figure 7.5 : GAP MEASUREMENTS FROM LCL(2)

The bars in the figure represent individual gap lengths measured between the hybridization sites of lambda pairs (688 + 33) and (582 + 322) located on either side of FRA16B. The biotin-labeled probes were hybridized to ECFs prepared from an individual heterozygous for the fragile site, ie with one normal chromosome 16 and one FRA16B. The hybridization was detected with FITC. Gap measurements were converted to kilobases using the 26kb probe pair as an internal standard.

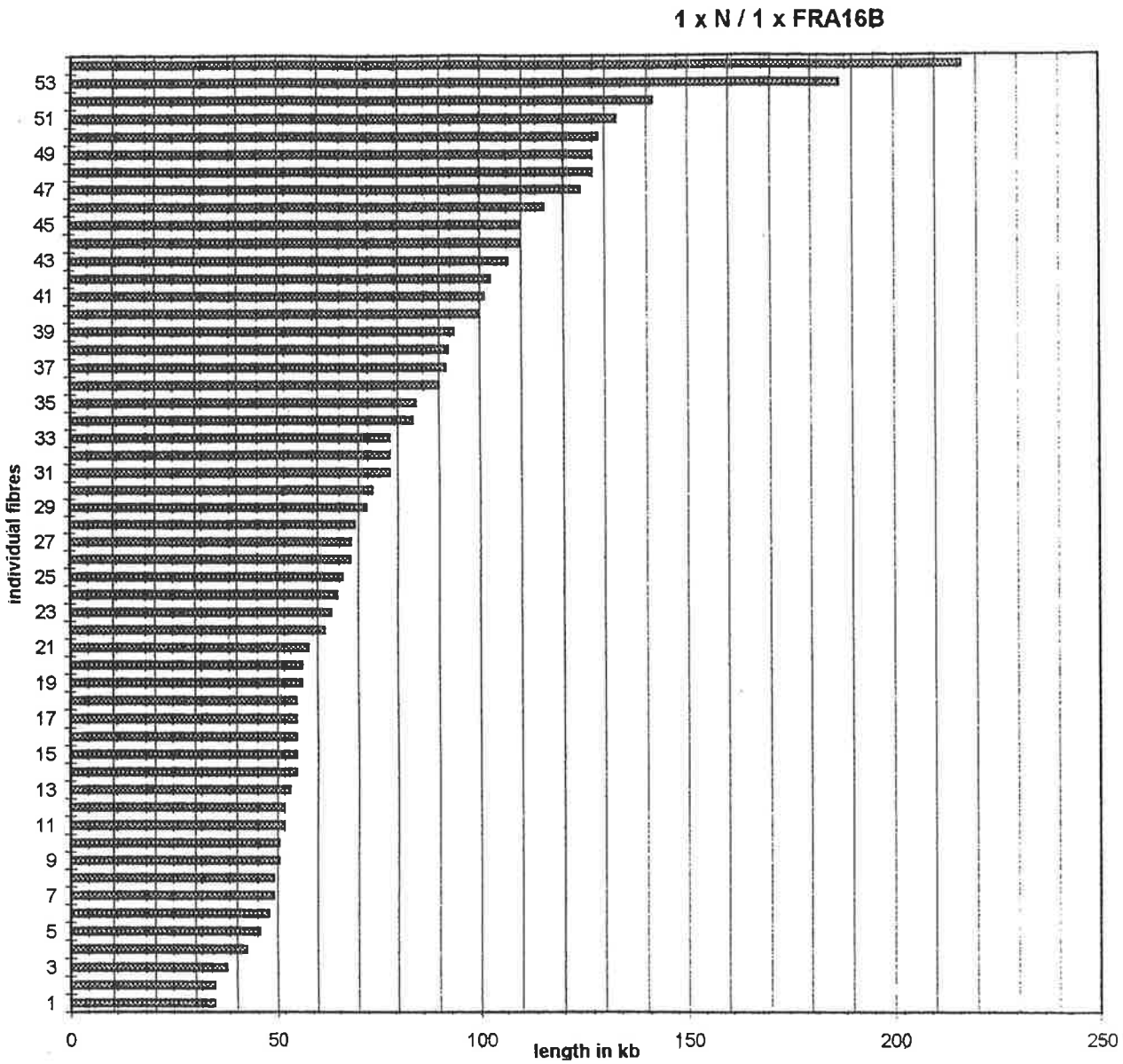
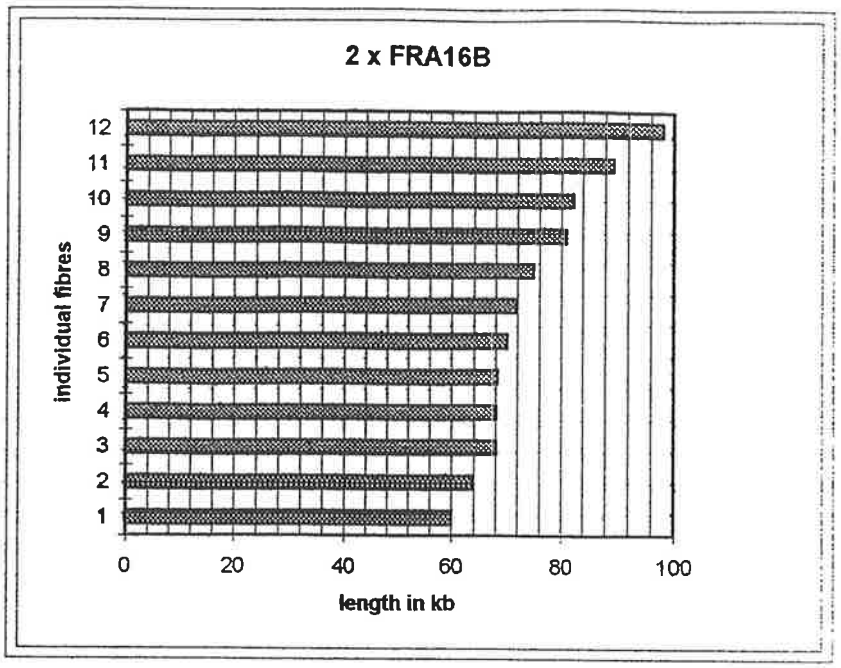

1 x N / 1 x FRA16B
individual fibres
53
51
49
47
45
43
41
39
37
35
33
31
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
0
50
100
150
200
250
length in kb

Figure 7.6 : GAP MEASUREMENTS FROM LCL(3)

The bars in the figure represent individual gap lengths measured between the hybridization sites on lambda pairs (688 + 33) and (582 + 322) located on either side of FRA16B. The biotin-labeled probes were hybridized to ECFs prepared from an individual homozygous for the fragile site (ie with two FRA16Bs), and the hybridization was detected with FITC. Gap measurements were converted to kilobases using the 26kb probe pair as an internal standard.


2 x FRA16B
individual fibres
12
11
10
9
8
7
6
5
4
3
2
1
0
20
40
60
80
100
length in kb

There were therefore 28 hybridizations representing the normal allele, with gap measurement lengths ranging from 34.7 - 69.3kb, and a mean gap length of 53.9kb (Table 7.1). There were 24 hybridizations representing the FRA16B allele, with gap measurement lengths ranging from 72.2 - 141.6kb, and a mean gap length of 101.8kb (Table 7.1).

LCL(3) : two FRA16B chromosomes

The gap measurements following hybridization of the lambda pairs to ECFs prepared from LCL(3) are shown in figure 7.6. Twelve hybridizations are represented. The gap measurements range from 60.1 - 98.2kb, with a mean gap length of 74.8kb (Table 7.1).

Table 7.1 : Gap length measurements

LCL	Range of gap lengths (kb)	Mean length (kb)	S.D.
(1) 2 x N16	40.4 - 65	53.1	7.1 (n = 14)
(2) 1 x N16	34.7 - 69.3	53.9	9.5 (n = 28)
1 x FRA16B	72.2 - 141.6	101.8	20.6 (n = 24)
(3) 2 x FRA16B	60.1 - 98.2	74.8	11.1 (n = 12)

DISCUSSION

The restriction map of the lambda subclones spanning the FRA16B site showed a physical distance of 50kb between the two lambda pairs in the normal allele (fig 7.2). This is very comparable to the 53.1kb distance shown by ECF measurements from the normal alleles of LCL(1). However, there are two factors which should be considered:

(1) All signals achieved by ECF FISH are not entirely contiguous, ie they contain gaps of various sizes (see fig 7.3). It is possible that hybridization to the ends of the lambda pairs in this study was occasionally incomplete, leading to a lack of fluorescent signal and thus a larger gap estimate once the (assumed) 26kb probe length was normalized.

(2) The unstable region associated with the fragile site is composed of several types of repeated sequence including minisatellites with 32, 33 and 37 base pair repeat motifs (Yu et al, 1996). The fragile site itself has been shown to be composed of an amplification of the 33bp repeat sequence. Although the 32bp and 37bp repeats have been eliminated as

candidates for the FRA16B expansion, they may contribute to smaller length variations between individuals within this region.
Assuming that the hybridization of the lambda clones was generally consistent however, a mean gap length of 53.1kb was chosen as the normal base line from which to judge any increases in gap distances in the FRA16B alleles. This also compares well with the mean gap length seen in the normal allele of the heterozygote LCL(2), ie 53.9kb.

The size of the repeat expansion of the FRA16B allele in LCL(2) was estimated by PFGE to be ~70kb. The expansion size by ECF measurement was the mean total gap length (101.8kb) less the normal allele size (53.1kb), ie 48.7kb. This is approximately a 21kb difference in length estimate between techniques. The size of the repeat expansion of the FRA16B alleles in the FRA16B homozygote LCL(3) was estimated by PFGE to be ~30kb. The expansion size estimated by ECF measurement was the mean total gap length (74.8kb) less the normal allele size (53.1kb), ie a 21kb expansion. This is a 9kb difference in estimate between the two techniques.
It is possible that the DNA at the unstable AT-rich region (within which FRA16B is located) runs atypically in a gel, producing a lagging band which has been interpreted as being of larger size than it actually is. Heiskanen (1995) found that the observed variation in measured distances within the subcentromeric region of chromosome 4q was twice as high as in other regions (although the mean was not affected), indicating that chromatin structure may have an impact on DNA stretching. It is therefore also possible, but perhaps less likely, that the differences in PFGE and ECF measurements are due to highly repetitive DNA at the FRA16B site stretching differently from normal DNA during preparation of ECFs.

The relative changes between the techniques are identical, ie 70kb (PFGE)→ 48.7 (ECF) is proportionate to 30kb (PFGE) → 21kb (ECF), indicating that whatever factors are causing the differences in size estimation, they are consistent.

A variation in measured gap distances across a number of ECFs has been reported for all regions examined by ECF FISH. Senger et al (1994) determined sizes of gaps between probes in the HLA Class 11 region on chromosome 6p. The standard error in their calculations ranged from 3.3 to 8.2kb for gaps of 14 to 76kb. They stated that probe pairs with greater gaps showed a wider **range** of gap distances than probe pairs with smaller

gaps. An increase in standard error also occurred in the present study as the gap distances increased, possibly complicated by the chromatin structure of the DNA in the region. Other contributing factors would be incomplete hybridization as mentioned previously, variable decondensation along the relevant stretch of chromatin, and inappropriate selection of signals which, although in close association, were not in fact from the same fibre.

Heiskanen et al (1996) recommended that 10-15 separate fibre strands should be analysed to obtain an accurate estimate of mean signal length or gap size. With very large gap sizes such as those encountered at the LCL(2) fragile site, it is probably advisable to increase the sample of hybridizations in order to increase confidence in estimation of the gap sizes involved.

Under optimal circumstances, ECF FISH permits the accurate assessment of gap sizes between two probes in 3 days. ECF FISH is suited to the high resolution mapping of genomic areas in the resolution range of several kb to 500kb, and to specialized applications such as that described in this chapter.

SUMMARY

This thesis describes the modification, extension and development of a number of fluorescence in situ hybridization techniques for research projects requiring the mapping of genes and cloned DNA segments to human chromosomes:

- Six new single copy gene localizations were achieved with probes ranging in size from 1.7kb - 15kb. The adjustment of stringency conditions and antibody concentrations permitted a standard protocol to be developed.

- A method was developed for the diagnosis by FISH of the genetically complementary syndromes CMT1A and HNPP which involve respectively a 1.5Mb duplication or deletion of chromosome 17p. The test for CMT1A was facilitated by the use of a small cosmid contig containing the critical *PMP22* gene. The probe had good sensitivity without background interference in interphase nuclei. The nuclei were collected in G0/G1 phase by culturing for 12-24hrs in tissue culture medium without colchicine treatment. The test for HNPP was detected as a deletion on metaphase chromosome preparations by standard FISH methods, using either the same cosmid probe as in CMT1A, or probe pVAW409R1 located 50kb 5' of the *PMP22* gene.

- Cosmid probes were ordered and oriented within chromosome interval 16q24.3 as part of a research project attempting to isolate a tumor suppressor gene within this region. Physical mapping by FISH in this study included the ordering of cosmid contigs and singleton clones by dual colour FISH to metaphase chromosomes and interphase nuclei.

- The size of the amplification of repetitive sequences at the rare distamycin-A inducible fragile site FRA16B was determined by the modification of a method which produces extended chromatin fibres. Comparative measurements were made of gap distances between lambda contigs which hybridized to each side of the fragile site. The results showed that the measurement of repeat expansion by pulsed-field gel electrophoresis results in an increased estimate of the length of repetitive DNA present at the site compared to the FISH approach.

BIBLIOGRAPHY

Avraham K B, Cho B C, Gilbert D, Fujii H, Okamoto K, et al. (1993) Murine chromosomal location of four class 111 POU transcription factors. Genomics **18**:131-133.

Baker E, Sutherland G R, Jiang W-M, Yuan Q, et al. (1992) Mapping of the human integrin ß7 gene (*ITGB7*) to 12q13.13 by non-isotopic in situ hybridization. Mammalian Genome **2**:272-273.

Barker D, Wright E, Nguyen K, Cannon L, Fain P et al. (1978) Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. Science **236**:1100-1102

Bauman G J, Wiegant J, Borst P, van Duijn P. (1980) A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochrome-labelled RNA. Exp Cell Res **128**:485-490.

Benn P, Ciarleglio L, Lettieri L, Rodis J, Greenstein R. (1992) A rapid (but wrong) prenatal diagnosis. New Engl J Med **326**:1638-1639.

Brandriff B, Gordon L, Trask B. (1991) A new system for high resolution DNA sequence mapping in interphase pronuclei. Genomics **10**:75-82.

Brandt C A, Lyngbye T, Pedersen S, Bolund L, Friedrich U. (1994) Value of chromosome painting in determining the chromosomal outcome in offspring of a 12;16 translocation carrier. J Med Genet **31**:234-237

Buongiorno-Nardelli N, Amaldi F. (1970) Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. Nature **225**:946-948.

Callen D F, Baker E, Eyre H J, Chernos J E, Bell J A, Sutherland G R. (1990) Reassessment of two apparent deletions of chromosome 16p to an ins(11;16) and a t(1;16) by chromosome painting. Ann Genet **33**:219-221.

Callen D F, Eyre H J, Ringenbergs M L, Freemantle C J, Woodroffe P, Haan E A. (1991) Chromosomal origin of small ring marker chromosomes in man: characterization by molecular genetics. Am J Hum Genet **48**:769-782.

Callen D F, Eyre H, Yip M-Y, Freemantle J, Haan E A. (1992a) Molecular cytogenetic and clinical studies of 42 patients with marker chromosomes. Am J Med Genet **43**:709-715.

Callen D F, Doggett N A, Stallings R L, Chen L Z, Whitmore S A, et al. (1992b) High-resolution cytogenetic-based physical map of human chromosome 16. Genomics **13**:1178-1185.

Caspersson T, Zech L, Johanson C. (1970) Differential banding of alkylating fluorochromes in human chromosomes. Exp Cell Res **60**:315-319.

Cawthon R M, Weiss R, Ganfeng X, Viskochil D, Culver M, et al. (1990) A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure and point mutations. Cell **62**:193-201.

Chakrabarti L, Knight S J L, Flannery A V, Davies K E. (1996) A candidate gene for mild mental handicap at the FRAXE fragile site. Hum Mol Genet **5**:275-282.

Chance P F, Abbas N, Lensch N W, Pentao L, Roa B B, et al. (1994) Two autosomal dominant neuropathies result from reciprocal DNA duplication/deletion of a region on chromosome 17. Hum Mol Genet **3**:223-228.

Chance P and Fischbeck K H. (1994) Molecular genetics of Charcot-Marie-Tooth disease and related neuropathies. Hum Mol Genet **3**:1503-1507.

Chu T W, Teebi A S, Gibson L, Breg W R, Yang-Feng T L. (1993) C phenotype with a complex chromosome rearrangement resulting in partial trisomy 13 and partial tetrasomy 13 delineated by FISH analysis. Am J Hum Genet **53** (suppl):534.

Cleton-Jansen A-M, Moerland E, Kulpers-Dijkshoorn N, Callen D F, et al. (1994) At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. Genes Chrom Cancer **9**:101-107.

Cohen S N, Chang A C Y, Boyer H W, Helling R B. (1973) Construction of biologically functional bacterial plasmids *in vitro*. Proc Natl Acad Sci **70**:3240.

Coppey-Moisan M, Delic J, Magdelenat H, Coppey J. (1994) Principle of digital imaging microscopy. From: Methods in Molecular Biology, vol 33: In situ Hybridization Protocols, K H A Choo (ed), Humana Press Inc, Totowa, NJ.

Cremer T, Lichter P, Borden J, Ward D C, Manuelidis L. (1988) Detection of chromosome aberrations in metaphase and interphase tumor cells by in situ hybridization using chromosome-specific library probes. Hum Genet **80**:235-246.

Cremers F P, van de Pol D J, van Kerkhoff L P, Wieringa B, Ropers H H. (1990) Cloning of a gene that is rearranged in patients with choroideraemia. Nature **347**:674-677.

Dauwerse J G, Wiegant J, Raap A K, Breuning M H, Van Ommen G J B. (1992) Multiple colors by fluorescence in situ hybridization using ratio-labelled DNA probes create a molecular karyotype. Hum Mol Genet **1**:593-598.

Davies A F, Barber L, Murer-Orlando M, Bobrow M, Adinolfi M. (1994) FISH detection of trisomy 21 in interphase by the simultaneous use of two differentially labelled cosmid contigs. J Med Genet **31**:679-685.

Dekaban A. (1965) Persisting clone of cells with an abnormal chromosome in a woman previously irradiated. J Nucl Med **6**:740-746.

Den Dunnen J T, Grootscholten P M, Dauwerse J G, Walker A P, Monaco A P, et al. (1992) Reconstruction of the 2.4Mb human DMD-gene by homologous YAC recombination. Hum Mol Genet **1**:19-28.

Doggett N A, Goodwin L A, Tesmer J G, Meincke L J, Altherr M R, et al. (1995) An integrated map of human chromosome 16. Nature **377** (supp):375

Edwards J H, Harnden D G, Cameron A H, Crosse V M, Wolff O H. (1960) A new trisomic syndrome. Lancet **1**:787-790.

Fang Y-Y, Eyre H J, Bohlander S K, Estop A, McPherson E, et al. (1995) Mechanisms of small ring formation suggested by the molecular characterization of two small accessory ring chromosomes derived from chromosome 4. Am J Hum Genet **57**:1137-1142.

Eppig J T and Nadeau J H. (1995) Comparative maps: the mammalian jigsaw puzzle. Current Opinion in Genet and Devnt **5**:709-716.

Fidlerova H, Senger G, Kost M, Sanseau P, Sheer D. (1994) Two simple procedures for releasing chromatin from routinely fixed cells for fluorescence in situ hybridization. Cytogenet Cell Genet **65**:203-205.

Flejter W L, Barcroft C L, Guo S-W, Lynch E D, Boehnke M, et al. (1993) Multicolor FISH mapping with *Alu*-PCR-amplified YAC clone DNA determines the order of markers in the *BRCA1* region on chromosome 17q12-q21. Genomics **17**:624-631.

Florijn R J, Bonden L A J, Vrolijk H, Wiegant J, Vaandrager J W, et al. (1995) High-resolution DNA fiber-FISH for genomic DNA mapping and colour bar-coding of large genes. Hum Mol Genet **4**:831-836.

Francek U, Ochs H D, de Martinville B, Giacalone J, Lindgren V, et al. (1985) Minor Xp21 chromosome deletion in a male associated with expression of Duchenne muscular dystrophy, chronic granulomatous disease, tetinits pigmentosa, and McLeod syndrome. Am J Hum Genet **37**:250-267.

Gall J G, Pardue M L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Nat Acad Sci USA **63**:378-383.

Gardner R J M, Sutherland G R. (1996) Chromosome Abnormalities and Genetic Counseling II. Oxford Uni Press, New York.

Gecz J, Gedeon A K, Sutherland G R, Mulley J C. (1996) Identification of the gene *FMR2*, associated with FRAXE mental retardation. Nature Genet **13**:105-108.

Glover T W, Berger C, Coyle J, Echo B. (1984) DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. Hum Genet **67**:136-142.

Gosden J R, Mitchell A R, Buckland R A, Clayton R P, Evans H J. (1975) The location of four human satellite DNAs on human chromosomes. Exp Cell Res **92**:148-158.

Gregory C A, Kirkilionis A J, Greenberg C R, Chudley A E, Hamerton J L. (1990) Detection of molecular rearrangements in Prader-Willi syndrome using genomic probes recognizing four loci within the PWCR. Am J Med Genet **35**:536-545.

Haaf T, Ward D C. (1994a) High resolution ordering of YAC contigs using extended chromatin and chromosomes. Hum Mol Genet **3**:629-633.

Haaf T, Ward D C. (1994b) Structural analysis of α-satellite DNA and centromere proteins using extended chromatin and chromosomes. Hum Mol Genet **3**:697-709.

Harada Y, Katagiri T, Ito I, Akiyama F, Sakamoto G, et al. (1994) Genetic studies of 457 breast cancers. Cancer **74**:2281-2286.

Harper M E, Saunders G F. (1981) Localization of single copy DNA sequences on G-banded human chromosomes by in situ hybridization. Chromosoma **83**:431-439.

Harper M E. (1983) Chromosome mapping of single-copy sequences by in situ hybridization. In: Banbury Report **14** : Recombinant DNA Applications to Human Disease. Caskey C T and White R L (eds). Cold Spring Harbour Laboratory.

Heiskanen M, Kallioniemi O, Palotie A. (1996) Fiber-FISH: experiences and a refined protocol. Genet Analysis: Biomol Engineering **12**:179-184.

Heiskanen M. (1995) Fiber-FISH: new strategy for positioning and ordering of clones. Academic dissertation, Dept Clin Chem, Univ of Helsinki, Finland.

Heng H H Q, Squire J, Tsui L-C. (1992) High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. Proc Natl Acad Sci USA **89**:9509-9513.

Hsu T C. (1952) Mammalian chromosomes in vitro. I. The karyotype of man. J Hered **43**:167-172.

Hsu T C. (1979) Human and mammalian cytogenetics. An historical perspective. Springer-Verlag, New York.

Ionasescu V V, Ionasescu R, Searby C, Barker D F. (1993) Charcot-Marie-Tooth neuropathy type 1A with both duplication and non-duplication. Hum Mol Genet **2**:405-410.

John H A, Birnstiel M L, Jones K W. (1969) RNA-DNA hybrids at the cytological level. Nature **223**:582-587.

Jones K W. (1970) Chromosomal and nuclear location of mouse satellite DNA in individual cells. Nature **225**:912-915.

Jones K W, Prosser J, Corneo G, Ginelli E. (1973) The chromosomal location of human satellite DNA 111. Chromosoma **42**:445-451.

Kievits T, Wiegant J. (1988) Method for in situ hybridization with cosmids. Personal Communication.

Kiyosawa H, Lensch M W, Chance P. (1995) Analysis of the CMT1A-REP repeat: mapping crossover breakpoints in CMT1A and HNPP. Hum Mol Genet **4**: 2327-2334.

Klinger K, Landes G, Shook D, Harvey R, Lopez L, et al. (1992) Rapid detection of chromosome aneuploidies in uncultured amniocytes by using fluorescence in situ hybridization (FISH). Am J Hum Genet **51**:55-65.

Kohler A, Hain J, Muller U. (1994) Familial half cryptic translocation t(9;17). J Med Genet **31**:712-714.

Kraus M H, Issing W, Miki T, Popescu N C, Aaronson S A. (1989) Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci USA **86**:9193-9197.

Kremer E J, Pritchard M, Lynch M, Yu S, Holman K, et al. (1991) Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n. Science **252**:1711-1714.

Kuo W-L, Tenjin H, Segraves R, Pinkel D, Golbus M S, Gray J. (1991) Detection of aneuploidy involving chromosomes 13, 18, or 21, by fluorescence in situ hybridization (FISH) to interphase and metaphase amniocytes. Am J Hum Genet **49**:112-119.

Kuwano A, Kajii T. (1987) Synergistic effect of aphidicolin and ethanol on the induction of common fragile sites. Hum Genet **75**:75-78.

Lapsys N M, Layfield R, Baker E, Callen D F, et al. (1992) Chromosomal location of the human transketolase gene. Cytogenet Cell Genet **61**:274-275.

Larsson C, Brystrom C, Skoog L, Rotstein S, Nordenskjold M. (1990) Genomic alterations in human breast carcinomas. Genes Chrom Cancer **2**:191-197.

Lawrence J B, Villnave C A, Singer R H. (1988) Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell **52**:51-61.

Lawrence J B, Singer R H, McNeil J A. (1990) Interphase and metaphase resolution of different distances within the human dystrophin gene. Science **249**:928-932.

Ledbetter D H, Zachary J M, Simpson J L, Golbus M S, Pergament E, et al. (1992) Cytogenetic results from the U.S. collaborative study on CVS. Prenatal Diagnosis **12**:317-345.

Legouis R, Hardelin J P, Levilliers J, Claverie J M, Compain S, et al. (1991) The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. Cell **67**:423-435.

Lejeune J, Gautier M, Turpin R. (1959) Etude des chromosomes somatiques de neuf enfants mongoliens. Compt Rend **248**:1721-1722.

Lejeune J, Dutrillaux B, Lafourcade J, Berger R, Abonyi D, et al. (1968) Endoréduplication sélective du bras long du chromosome 2 chez une femme et sa fille. C R Acad Sc Paris **266**:24-26.

Lichter P, Cremer T, Borden J, Manuelidis L, Ward D C. (1988a) Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. Hum Genet **80**:224-234.

Lichter P, Cremer T, Tang C-J C, Watkins P C, Manuelidis L, et al. (1988b) Rapid detection of human chromosome 21 aberrations by in situ hybridization. Proc Natl Acad Sci USA **85**:9664-9668.

Lichter P, Tang C-J C, Call K, Hermanson G, Evans G A, et al. (1990) High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science **247**:64-69.

Lindblom A, Rotstein S, Skoog L, Nordenskjold M, Larsson C. (1993) Deletions on chromosome 16 in primary familial breast carcinomas are associated with development of distant metastases. Cancer Res **53**:3701-3711.

Litwin V, Gumperz J, Parham P, Phillips J H, Lanier L L. (1994) NKB1: A natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. J Exp Med **180**:537-543.

Lupski J R, Montes de Oca-Luna R, Slangenhaupt S, Tentao L, Guzetta V, et al. (1991) DNA duplication associated with Charcot-Marie-Tooth disease Type 1A. Cell **66**:219-232.

Malcolm S, Williamson R, Boyd E, Ferguson-Smith M A. (1977) A comparison of in situ hybridization techniques for gene localization. Cytogenet Cell Genet **19**:256-261.

Malcolm S, Cowell J K, Young B D. (1986) Specialist techniques in research and diagnostic clinical cytogenetics, in Human Cytogenetics, a practical approach, Rooney D E and Czepulkowski B H (eds), IRL Press Ltd, Oxford.

Maniatis T, Fritsch E F, Sambrook J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory.

Mizunoe T, Young S R. (1992) Low fluorescence alpha satellite region yields negative result. Prenatal diagnosis **12**:549-550.

Monaco A P, Neve R L, Colletti-Feener C, Bertelson C J, Kurnit D M, et al. (1986) Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. Nature **323**:646-650.

Moorhead P S, Newell P C, Mellman W J, Battips D M, Hungerford D A. (1960) Chromosome preparations of leukocytes cultured from human peripheral blood. Exptl Cell Res **20**:613-616.

Murakami T, Lupski J R. (1996) A 1.5Mb cosmid contig of the CMT1A duplication/HNPP deletion critical region in 17p11.2-p12. Genomics **34**:128-133.

Nakamura R M. (1990) Overview and Principles of In-Situ Hybridization. Clin Biochem **23**:255-259.

Navon R, Timmerman V, Lofgren A, Liang P, Nelis E, et al (1995) Prenatal diagnosis of Charcot-Marie-Tooth disease type 1A (CMT1A) using molecular genetic techniques. Prenatal Diagnosis **15**:633-640.

Nelis E, Van Broeckhoven C, et al. (1996) Estimation of the mutation frequencies in Charcot-Marie-Tooth disease type 1 (CMT1) and hereditary neuropathy with liability to pressure palsies (HNPP): A European collaborative study. Eur J Hum Genet **4**:25-33.

ONCOR, Inc. 1996 Product Profile. Gaithersburg, MD 20877, USA.

Palau F, Lofgren A, De Jonghe P, Bort S, et al. (1993) Origin of the *de novo* duplication in Charcot-Marie-Tooth disease type 1A: Unequal nonsister chromatid exchange during spermatogenesis. Hum Mol Genet **2**:2031-2035.

Pardue M L, Gall J G. (1975) Nucleic acid hybridization to the DNA of cytological preparations. Methods Cell Biol **10**:1-16.

Parra I, Windle B. (1993) High resolution visual mapping of stretched DNA by fluorescent hybridization. Nature Genet **5**:17-21.

Patau K A, Smith D W, Therman E M, Inhorn S L, Wagner H P. (1960) Multiple congenital anomoly caused by an extra autosome. Lancet **1**:790-793.

Patel P I, Roa B B, Welcher A A, Schoener-Scott R, Trask B J, et al. (1992) The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A. Nature Genet **1**:159-165.

Pentao L, Wise C A, Chinault A C, Patel P I, Lupski J R. (1992) Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5Mb monomer unit. Nature Genet **2**:292-300.

Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, et al. (1988) Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. Proc Natl Acad Sci USA **85**:9138-9142.

Ried T, Landes G, Dackowski W, Klinger K, Ward D C. (1992a) Multicolor fluorescence in situ hybridization for the simultaneous detection of probe sets for chromosomes 13,18,21,X and Y in uncultured amniotic fluid cells. Hum Mol Genet **1**:307-313.

Ried T, Baldini A, Rand T C, Ward D C. (1992b) Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital imaging microscopy. Proc Natl Acad Sci USA **89**:1388-1392.

Rooney D E, Czepulkowski B H. (eds) (1986) Human Cytogenetics: a practical approach. IRL Press Ltd, Oxford.

Rudkin G T, Stollar B D. (1977) High resolution detection of DNA-RNA hybrids in situ by indirect immunofluoresc-ence. Nature **265**:472-473.

Ruttledge M H, Xie Y-G, Han F-Y, Giovannini M, Janson M, et al. (1994) Physical mapping of the NF2/meningioma region on human chromosome 22q12. Genomics **19**:52-59.

Sandrin M S, Gumley T P, Henning M M, Vaughan H A, Gonez L J, et al. (1992) Isolation and characterization of cDNA clones for mouse Ly 9. J Immunol **149**:1636-1641.

Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F. (1990) Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res **50**:7184-7189.

Schmid M, Feichtinger A, Jessberger J, Kohler J, Lange R. (1986) The fragile site (16)(q22). 1. Induction by AT-specific DNA-ligands and population frequency. Hum Genet **74**:67-73.

Sealey P G, Whittaker P A, Southern E M. (1985) Removal of repeated sequences from hybridisation probes. Nucleic Acids Res **13**:1905-1922.

Senger G, Ragoussis J, Sheer D. (1993) Fine mapping of the MHC class 11 region within 6p21 and evaluation of probe ordering using interphase fluorescence in situ hybridization. Cytogenet Cell Genet **64**:49-53.

Senger G, Jones T A, Fidlerova H, Sanseau P, Trowsdale J, et al. (1994) Released chromatin: linearized DNA for high resolution fluorescence in situ hybridization. Hum Mol Genet **3**:1275-1280.

Shipley J M, Jones T A, Patel K, Kiely F M, De Stavola B L, et al. (1993) Ordering of probes surrounding the Ewing's sarcoma breakpoint on chromosome 22 using fluorescence in situ hybridization to interphase nuclei. Cytogenet Cell Genet **64**:233-239.

Smit V T H B M, Wessels J W, Mollevanger P, Dauwerse J G, van Vliet M, et al. (1991) Improved interpretation of complex chromosomal rearrangements by combined GTG banding and in situ suppression hybridization using chromosome-specific libraries and cosmid probes. Genes, Chromosomes and Cancer **3**:239-248.

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

Speleman F, Van Roy N, Wiegant J, Dierick A M, Uyttendaele D, et al. (1992) Molecular cytogenetic analysis of a complex t(10;22;11) translocation in Ewing's sarcoma. Genes, Chromosomes and Cancer **4**:188-191.

Sturm R A, Bisshop F, Takahashi H, Parson P G. (1991) A melanoma octamer binding protein is responsive to differentiating agents. Cell Growth and Differ **2**:519 - 524.

Sutherland G R. (1979) Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am J Hum Genet **31**:125-135.

Sutherland G R. (1981) Heritable fragile sites on human chromosomes. VII. Children homozygous for the BrdU-requiring fra(10)(q25) are phenotypically normal. Am J Hum Genet **33**:946-949.

Sutherland G R, Baker E, Mulley J C. (1982) Genetic length of a human chromosomal segment measured by recombination between two fragile sites. Science **212**:373-374.

Sutherland G R, Jacky P B, Baker E. (1984) Heritable fragile sites on human chromosomes. XI. Factors affecting expression of fragile sites at 10q25, 16q22 and 17p12. Am J Hum Genet **36**:110-122.

Takahashi E, Hori T, Murata M. (1988) Population cytogenetics of rare fragile sites in Japan. Hum Genet **78**:121-126.

Takenchi T, Gumucio D L, Yamada T, Meister M H, Minth C D, et al. (1986) Genes encoding pancreatic polypeptide and neuropeptide Y are on chromosome 17 and 7. J Clin Invest **77**:1038-1041

Tiainen M, Popp S, Parlier V, Emmerich P, Bellomo M J, et al. (1992) Chromosomal in situ suppression hybridization of immunologically classified mitotic cells in hematologic malignancies. Genes, chromosomes and Cancer **4**:135-140.

Tkachuk D C, Westbrook C A, Andreeff M, Donlon T A, Cleary M L, et al (1990) Detection of *bcr-abl* fusion in chronic myelogeneous leukemia by in situ hybridization. Science **250**:559-562.

Tjio J H, Levan A. (1956) The chromosome number of man. Hereditas **42**:1-6.

Trask B, Pinkel D, ven den Eng G. (1989) The proximity of DNA sequences in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs. Genomics **5**:710-717.

Trask B J, Massa H, Kenwrick S, Gitschier J. (1991) Mapping of human chromosome Xq28 by two-color fluorescence in situ hybridization of DNA sequences to interphase cell nuclei. Am J Hum Genet **48**:1-15.

Trent J M, Stanbridge E J, Heyoung L M, Meese E U, Casey G, et al.(1990) Tumorigenicity in human mekanoma cell lines controlled by introduction of human chromosome 6. Science **247**:568-571.

Tsuda H, Callen D F, Fukutomi T, Nakamura Y, Hirohashi S. (1994) Allele loss on chromosome 16q24-qter occurs frequently in breast cancers irrespectively of differences in the phenotype and extent of spread. Cancer Res **54**:513-517.

Valentijn L J, Bolhuis P A, Zorn I, Hoogendijk J E, van den Bosch N, et al. (1992) The peripheral myelin gene, *PMP22/GAS-3* is duplicated in Charcot-Marie-Tooth disease type 1A. Nature Genet **1**:166-170.

Valentjin L J, Baas F, Zorn I, Hensels G W, et al. (1993) Alternatively sized duplication in Charcot-Marie-Tooth disease type 1A. Hum Mol Genet **2**:2143-2146.

van den Engh G, Sachs R, Trask B J. (1992) Estimating genomic distance from DNA sequence location in cell nuclei by a random walk model. Science **257**:1410-1412.

Verlinsky Y, Ginsberg N, Chmura M, Freidine M, White M, et al. (1995) Cross-hybridization of the chromosome 13/21 alpha satellite DNA probe to chromosome 22 in the prenatal screening of common chromosomal aneuploidies by FISH. Prenat Diag **15**:831-834.

Vogelstein B, Pardoll D M, Coffey D S. (1980) Supercoiled loops and eucaryotic DNA replication. Cell **22**:79-85.

Vooijs M, Yu L C, Tkachuk D, Pinkel D, Johnson D, et al. (1993) Libraries for each human chromosome, constructed from soter-enriched chromosomes by using linker-adaptor PCR. Am J Hum Genet **52**:586-597.

Waring M.(1970) Drugs and DNA : uncoiling of the DNA double helix as evidence of intercalation. Hum Genet **9**: 234-236.

Wheater R F, Roberts S H. (1987) An improved lymphocyte culture technique: deoxycytidine release of a thymidine block and use of a constant humidity chamber for slide making. J Med Genet 24: 113-115.

Wiegant J, Kalle W, Mullenders L, Brookes S, Hoovers J M N, Dauwerse J G, van Ommen G J B, Raap A K. (1992) High-resolution in situ hybridization using DNA halo preparations. Hum Mol Genet **1**:587-591.

Wilke C M, Hall B K, Hoge A, Paradee W, Smith D I, et al. (1996) FRA3B extends over a broad region and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. Hum Mol Genet **5**:187-195.

Wilkie A O M, Buckle V L, Harris P C, Lamb J, Barton N J, et al. (1990) Clinical features and molecular analysis of the α-thalassemia/ mental retardation syndromes. 1. Cases due to deletions involving chromosome band 16p13.3. Am J Hum Genet **46**:1112-1126.

Windebank A. (1993) Inherited recurrent focal neuropathies. In: Dyck P, Thomas P, Griffin J, Low P, Poduslo J (eds) Peripheral neuropathy, 3rd ed. W B Saunders, Philadelphia, pp1137-1148.

Yunis J. (1976) High resolution of human chromosomes. Science **191**:1268-1270.

Yu L-C, Bryndorf T, Christensen B, Segraves R, Kuo W-L, et al. (1990) Enumeration of specific chromosomes in uncultured fetal cells using fluorescence in situ hybridization (FISH). Am J Hum Genet **47** [Suppl]: A287.

Zabel B U, Naylor S L, Sakaguchi A Y, Bell G E, Shows T B. (1983) High-resolution chromosomal localization of human genes for amylase, proopiomelanocortin, somatostatin, and a DNA fragment (D3S1) by in situ hybridization. Proc Natl Acad Sci USA **80**:6932-6936.

Zheng Y L, Ferguson-Smith M A, Warner J P, Ferguson-Smith M E, Sargent C A, et al. (1992) Analysis of chromosome 21 copy number in uncultured amniocytes by fluorescence in situ hybridization using a cosmid contig. Prenat Diagn **12**:931-943.

APPENDIX A

PAPERS PUBLISHED AND ACCEPTED FOR PUBLICATION DURING THE COURSE OF THIS THESIS

The human FISH component of these papers was done by me. I wrote the relevant sections of the methods and results, and in some instances the discussion.

Angus J, Thompson F, Murphy K, **Baker E**, Sutherland G R, Parsons P G, Sturm R A. (1995) The *brn-2* gene regulates the melanocytic phenotype and tumorigenic potential of human melanoma cells. Oncogene **11**:691-700.

Baker E, Hort Y J, Ball H, Sutherland G R, Shine J, Herzog H. (1995) Assignment of the human neuropeptide Y gene to chromosome 7p15.1 by non isotopic *in situ* hybridization. Genomics **26**:163-164.

Baker E, D'Andrea A, Phillips J H, Sutherland G R, Lanier L L. (1995) Natural killer cell receptor for HLA-B allotypes, NKB1. Map position 19q13.4. Chromosome Res **3**:511.

Baker E, Sutherland G R, Sutherland R L, Daly R J. (1996) Assignment of the human *GRB14* gene to chromosome 2q22-q24 by fluorescence *in situ* hybridisation. Genomics **36**:218-220.

Gedeon A K, Keinänen M, Adès L C, Kääriänen H, Gész J, **Baker E**, Sutherland G R, Mulley J C. (1995) Overlapping submicroscopic deletions in Xq28 in two unrelated boys with developmental disorders: Identification of a gene near FRAXE. Am J Hum Genet **56**:907-914.

Gibson L, Holmgreen S P, Huang D C S, Bernard O, Copeland N G, Jenkins N A, Sutherland G R, **Baker E**, Adams J M, Cory S. (1996) *bcl-w*, a novel member of the *bcl-2* family, promotes cell survival. Oncogene **13**:665-675.

Gibson M A, Hatzinikolas G, Davis E C, **Baker E**, Sutherland G R, Mecha M. (1995) Bovine latent TGF-beta binding protein-2: molecular cloning, identification of tissue isoforms and immunolocalisation to the elastin-associated microfibrils. Molec Cell Biol **15**:6932-6952.

Handford C A, Lynch J W, **Baker E**, Webb G C, Ford J H, Sutherland G R, Schofield P R. (1996) The human glycine receptor ß subunit: Primary structure, functional characterisation and chromosomal localisation of the human and murine genes. Molec Brain Res **35**:211-219.

Hort Y, **Baker E**, Sutherland G R, Shine J, Herzog H. (1995) Gene duplication of the human peptide YY gene (*PYY*) generated the pancreatic polypeptide gene (*PPY*) on chromosome 17q21.1. Genomics **26**:77-83.

Jones C, Penny L, Mattina T, Yu S, **Baker E**, Voullaire L, Langdon W Y, Sutherland G R, Richards R I, Tunnacliffe A. (1995) Association of a chromosome deletion syndrome with a fragile site within the proto-oncogene *CBL2*. Nature **376:**145-149.

Krozowski Z, **Baker E**, Obeyesekere V, Callen D F. (1995) Localization of the gene for human 11ß hydroxysteroid dehydrogenase type 2 enzyme to chromosome 16q22. Cytogenet Cell Genet **71**:124-125.

Lei F, Townsend-Nicholson A, **Baker E**, Sutherland G R, Schofield P R. (1996) Characterization and chromosomal localization of the human A2a adenosine receptor subtype gene: *ADORA2A*. Biochem and Biophys Research Communications **223**:461-467.

McConkey E H, Menon R, Williams G, **Baker E**, Sutherland G R. (1996) Assignment of the gene for beta-casein to 4q13-21 in humans and 3p12-13 in chimpanzees. Cytogenet Cell Genet **72**:60-62.

Mulley J C, Yu S, Loesch D Z, Hay D A, Donnelly A, Gedeon A K, Carbonell P, Lopez I, Glover G, Gabarron J, Yu P W L, **Baker E**, Haan E A, Hockey A, Knight S J L, Davies K E, Richards R I, Sutherland G R. (1995) FRAXE and mental retardation. J Med Genet **32**:162-169.

Sandrin M S, Henning M M, Lo M F, **Baker E**, Sutherland G R, Mc Kenzie I F C. (1995) Isolation and characterisation of cDNA clones for Humly-9: the human homolog of murine Ly-9. Immunogenet **43**:13-19.

Scott H S, Blanch L, Guo X-H, Freeman C, Orsborn A, **Baker E**, Sutherland G R, Morris C P, Hopwood J J. (1995) Cloning of the sulphamidase gene and

identification of mutations in Sanfilippo A syndrome. Nature Genet **11**:465-467.

Sturm R A, **Baker E**, Eyre H J, Sutherland G R. (1995) The human OFT1 locus which overlaps the CD3Z gene is located at 1q22-q23. Cytogenet Cell Genet **68**:231-232.

Sutherland G R, **Baker E**, Richards R I. (1996) Fragile Sites. In, Encyclopedia of Molecular Biology and Molecular Medicine, Meyer R A (ed), VCH, New York, pp313-318.

Tavaria M, Gabriele T, Anderson R L, Mirault M-E, **Baker E**, Sutherland G R, Kola I. (1995) Localization of the gene encoding the human heat shock cognate protein, HSP73, to chromosome 11. Genomics **29**:266-268.

Townsend-Nicholson A, **Baker E**, Schofield P R, Sutherland G R. (1995) Localisation of the adenosine A1 receptor subtype gene (*ADORA1*) to chromosome 1q32.1. Genomics **26**:423-425.

Townsend-Nicholson A, **Baker E**, Sutherland G R, Schofield P R. (1995) Localization of the adenosine A2b receptor subtype gene (*ADORA2B*) to chromosome 17p11.2-p12 by FISH and PCR screening of somatic cell hybrids. Genomics **25**:605-607.

Walker M E, **Baker E**, Wallace J C, Sutherland G R. (1995) Assignment of the human pyruvate carboxylase gene (*PC*) to 11q13.4 by fluorescence in situ hybridization. Cytogenet Cell Genet **69**:187-189.

Yu S, Manglesdorf M, Hewett D, Hobson L, **Baker E**, Eyre H J, Lapsys N, Lane S, Le Paslier D, Doggett N A, Sutherland G R, Richards R I. (1996) Human chromosomal fragile site *FRA16B* is an amplified AT-rich minisatellite repeat. Cell (submitted).

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