FLUORESCENT MOLECULAR CYTOGENETICS:

PREIMPLANTATION DIAGNOSIS, COLORECTAL CANCER AND MAPPING CHROMOSOME 9q

by

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ABSTRACT

The technique of fluorescent *in-situ* hybridisation (FISH) on to metaphase and interphase nuclei has many applications. In this thesis, FISH has been used in three areas of research:- Preimplantation diagnosis, genetics of colorectal cancer and early stages in mapping the terminal region of chromosome 9q to search for the tuberous sclerosis locus (TSC1).

Linkage data has suggested possible tuberous sclerosis loci on chromosomes 9, 11, and/or 12. In this study a hybrid was screened by FISH and found to contain only chromosome 9q. Fragment hybrids made from this were also screened. Phage clones isolated from a hybrid containing three small human pieces and clones linked to TSC1 were mapped to chromosome 9q. Cosmid clones isolated from the original hybrid were also mapped.

Colorectal cancer progression is a multi-stage process and genes on 17p (P53), 18q (DCC), 12p (*K-ras*) and 5q (MCC and APC - the familial polyposis gene) have been isolated and specifically implicated. In this study two colon cancer cell lines were examined. The first "LIM1215" was confirmed to contain only extra material derived from chromosome 1q32-qter. The second "AA/C1/SB10" (experimentally derived from an adenoma line) was found to have three copies of a segment of terminal 1q, two chromosome 1 centromeres, four chromosome 18 centromeres but no intact chromosome 18. A patient with a deleted segment around the APC region was examined by FISH using cosmid clones linked to APC. Progress towards a molecular map of the region and a precise cytogenetic location for the APC gene using FISH has hence been made. Clones expressed in normal colonic mucosa and mapping to chromosome 5 were also mapped.

Preimplantation diagnosis is carried out on the embryo prior to implantation into the mother's uterus. In this study FISH approaches for the sexing of human preimplantation embryos have been developed. FISH has been put into clinical practice for the sexing of preimplantation embryos in families at risk of transmiting X-linked diseases to their male offspring. Initial research into the possible screening of chromosomal translocations in preimplantation embryos has been investigated.

ACKNOWLEDGEMENTS

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I would like to express my gratitude to all the other people I have collaborated with in the course of this study namely Dr Richard Penketh, Dr Jonathan Wolfe, Jude Fitzgibbon, Nick Hornigold, Dr Patrick Klein, Dr Kiran Gulati, Simon Gayther, Dr Begona Cachon, Sarah Leigh, Karen Woodward, the team of Dr Mary Lucas and to all the people (mentioned in the appendix) who provided materials for this study (hope I didn't miss anyone). Especial thanks to Miss Jane Catherall for her professional drawings included in this thesis.

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This thesis is dedicated to all the women under the care of the Hammersmith Hospital Obstetrics and Gynaecology unit.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

TABLES:

INTRODUCTION

EMBRYOGENESIS AND CYTOGENETICS

HISTORICAL ASPECTS

"From the conjugation of blood and semen, the embryo comes into existence. During the period favourable for conception after sexual intercourse, it becomes a 'Kalada' (one day old embryo)..... During the seventh month it becomes endowed with life."

circa 1416 B.C. (ancient Hindu Sanskrit).

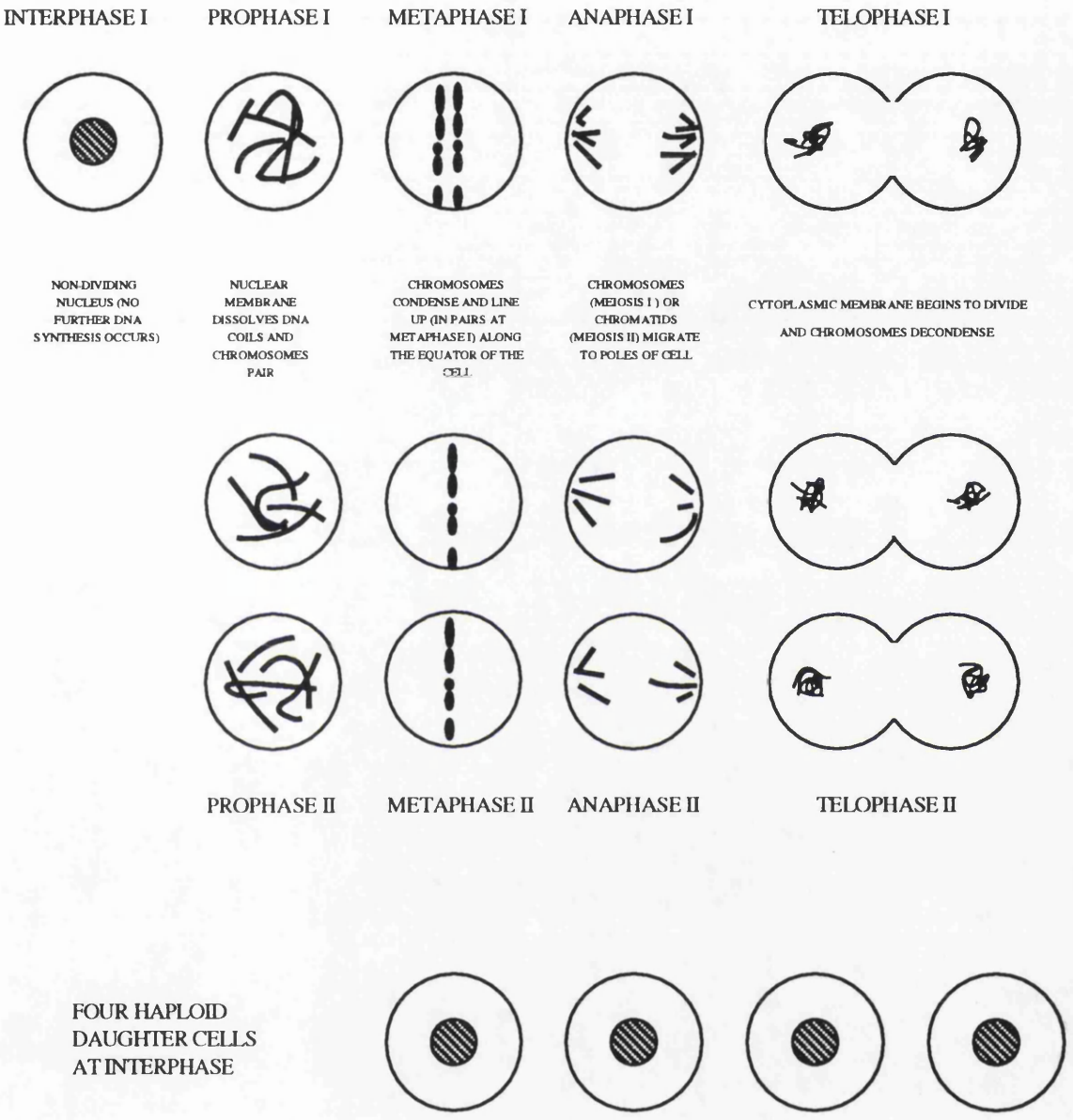
Acquiring knowledge concerning the study of embryology has been the pursuit of scientists and philosophers for centuries. The books of Hippocrates (fifth century B.C.) effectively began descriptive embryology yet it is Aristotle (fourth century B.C.) who first produced a complete treatise describing the development of chick and other embryos. He suggested the same erroneous idea of embryogenesis via the union of semen and menstrual blood proposed by the ancient Hindus centuries earlier. Despite this, he is widely credited with being the "founder of embryology." A major breakthrough in the advancement of embryonic research was the invention of the microscope (first applied to embryology by Harvey in 1651). Discovery of the sperm soon followed (Hamm and Leeuwenhoek., 1677) however it was not until 1827 that von Baer discovered the oocyte. He also described dividing zygotes and provided much knowledge regarding organ and tissue origin. Because of his far reaching contributions, he was later dubbed "the father of *modern* embryology." (All the above reviewed in Moore., 1988).

ORIGIN OF GERM CELLS

Meiosis is the process whereby cells halve their chromosomal complement and recombine their genetic material. Meiosis consists of two divisions, from one cell, four daughter cells emerge and the process can be subdivided into stages according to the state of the chromosomes. These are i) interphase where DNA replication, transcription and other normal functions of the cell not associated with division take place. DNA replication is complete by pre-meiotic interphase; ii) prophase where the DNA begins to condense to form chromosomes; iii) metaphase where the chromosomes line up along the equator of the cell; iv) anaphase where the chromosomes migrate to the poles; and v) telophase where the cytoplasmic membrane divides. Chromosomes exist in alike pairs or "homologues." Meiosis has two stages namely meiosis I and meiosis II. At prophase I homologues "pair up" and exchange genetic material:- so called genetic recombination or crossing over. At metaphase I chromosomes remain paired, pairing continues and chromosomes condense and line up along the equator of the cell as bivalents. At anaphase I homologous chromosomes separate and migrate to opposite poles of the cells and hence, at this stage, and telophase I (where the cell membrane divides) the genetic complement of the cell is halved. Meiosis II is similar to normal cell division (mitosis). At metaphase II chromosomes line up (not in pairs) along the equator of the cell and at anaphase II sister chromatids migrate to the poles. Thus, at meiosis II, the number of chromosomes is not halved. Four haploid daughter cells hence emerge.

Fig 1.1:

MEIOSIS



Meiosis is symmetrical in males (spermatogenesis) but asymmetrical in females (oogenesis). This is illustrated in the following diagram (taken from Moore., 1988).

Fig 1.2:

A: Spermatogenesis

In spermatogenesis (taking place in the testis) a spermatogonium forms a primary spermatocyte at prophase I, meiosis I continues to form two secondary spermatocytes. Meiosis II gives rise to spermatids which then (via spermiogenesis) form small mature sperm.

B: Oogenesis

Oogenesis takes place in the ovary and oviduct. A primary oocyte is surrounded by follicular cells. These follicular cells divide and form a cavity known as the antrum. A protective layer: the "zona pellucida" forms. Meiosis I occurs and is asymmetrical forming the large secondary oocyte and the "1st polar body." The secondary oocyte is then released from the follicular cells and ovary into the oviduct surrounded by a layer of cells the "corona radiata." It is then approached by the sperm (if present). When a sperm has entered, the secondary oocyte (fertilisation), meiosis II occurs, thus forming a mature oocyte and a small 2nd polar body.

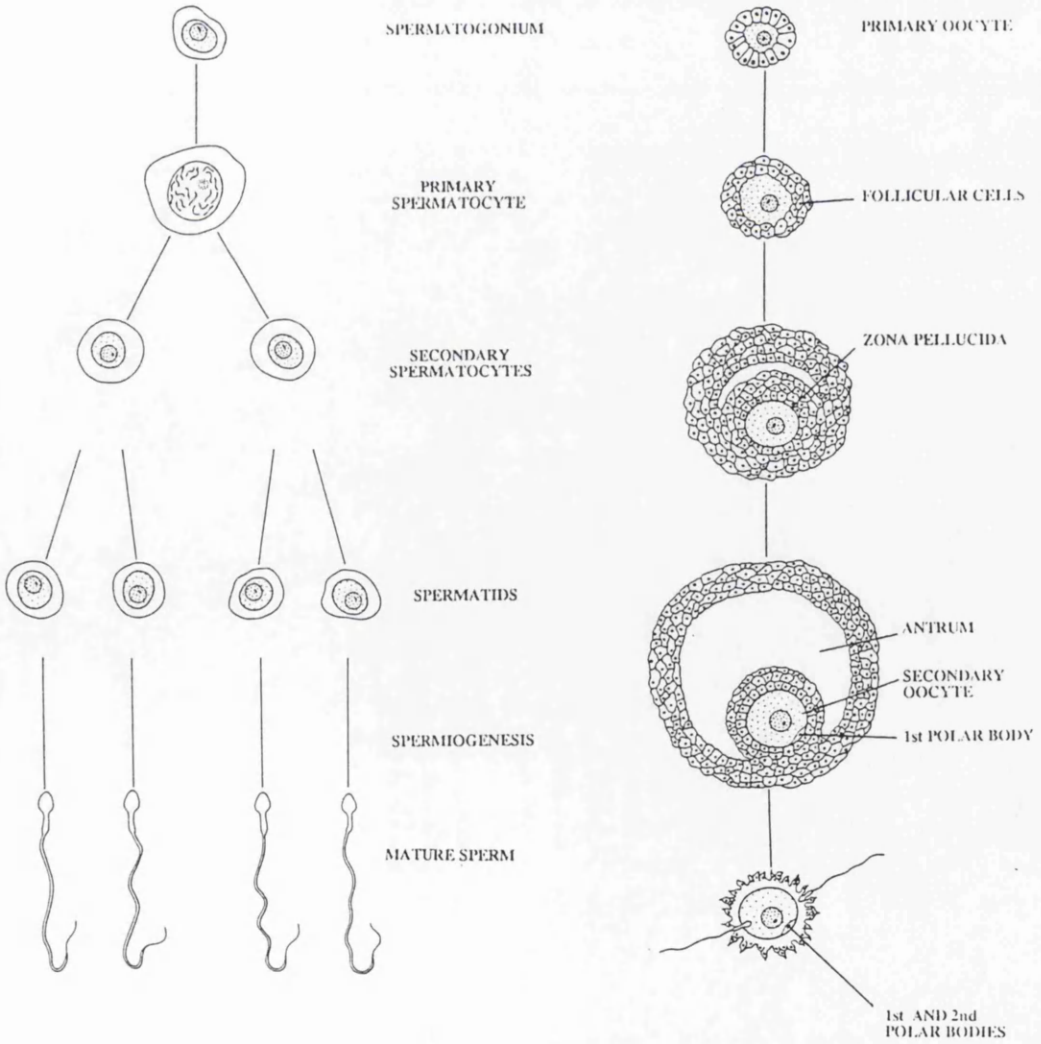
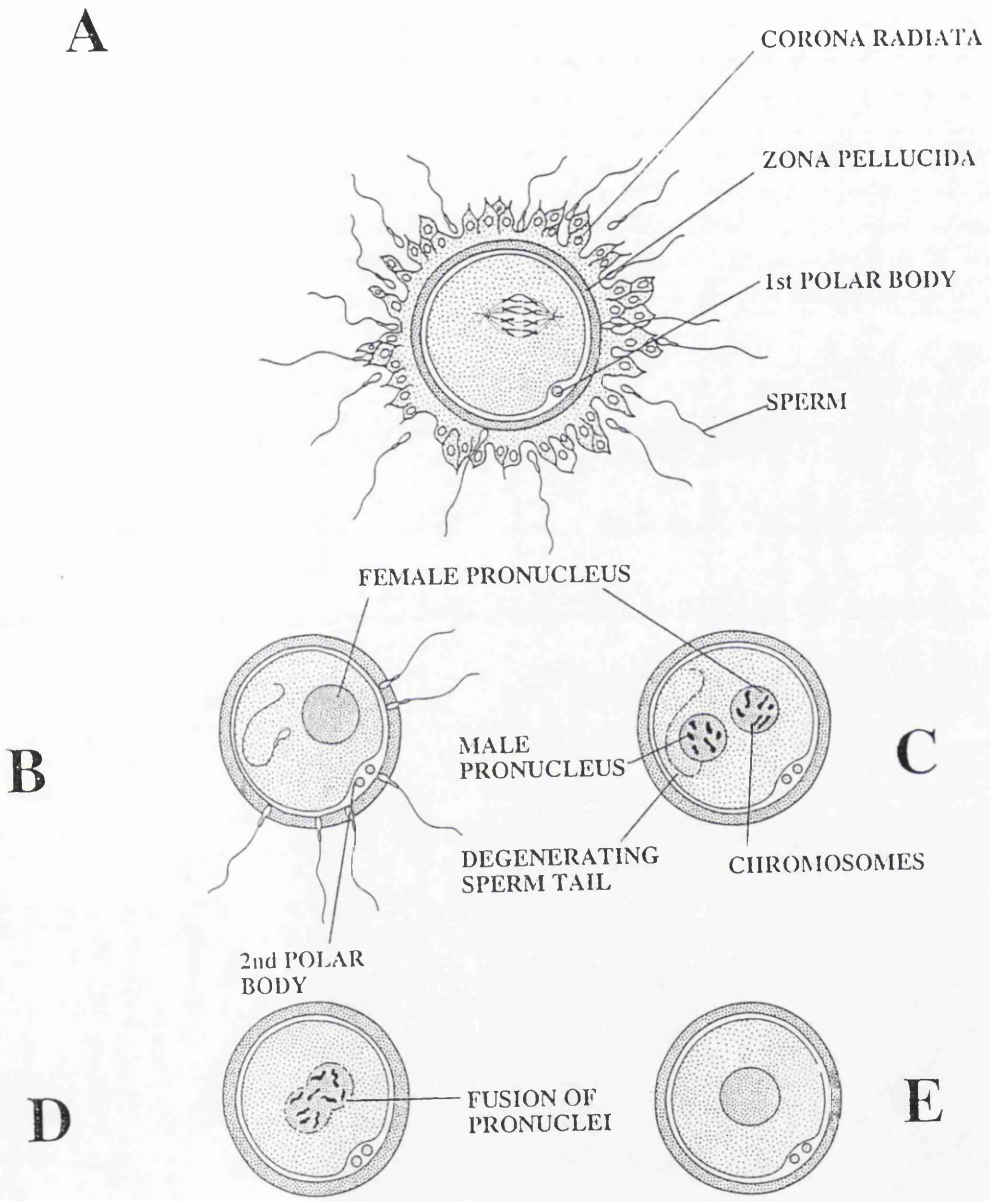


Fig 1.3:- **Fertilisation** occurs when the sperm and oocyte fuse thus (Moore., 1988):



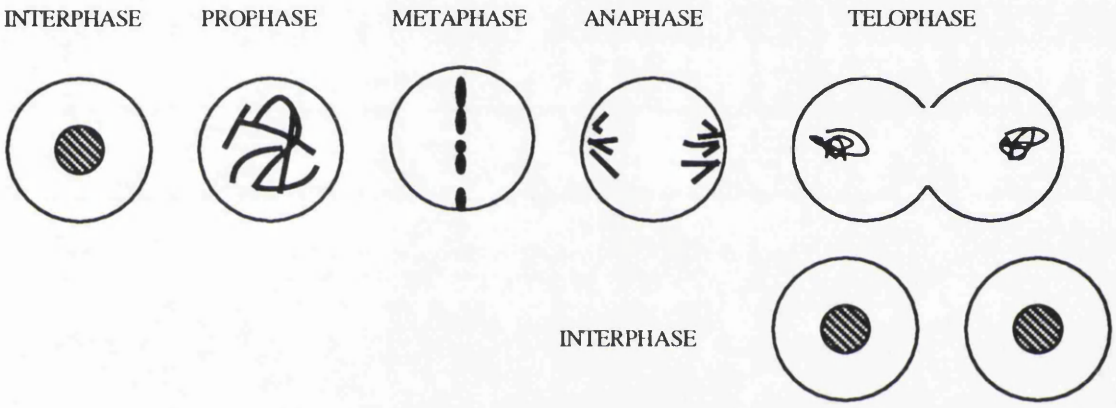
A. Sperm approach the corona radiata. 1st polar body is visible. Oocyte is in metaphase II.
B. A single sperm enters the oocyte, corona radiata dissolves. Meiosis II continues in the oocyte and the 2nd polar body is visible.
C. Sperm tail degenerates, sperm head enlarges and both pronuclei enter prophase.
D. Chromosomes mix (and line up in homologous pairs).
E. One-cell embryo (day-1) with two polar bodies visible.

Hence the new individual with a unique genetic complement is formed.

MITOSIS

The new cell undergoes ordinary cell division or "mitosis". Mitosis is a simpler process than meiosis but is subdivided in a similar way. Chromosomes condense at prophase, line up along the equator at metaphase, sister chromatids separate and migrate to the poles at anaphase and the cytoplasmic membrane divides at telophase.

Fig 1.4:
MITOSIS



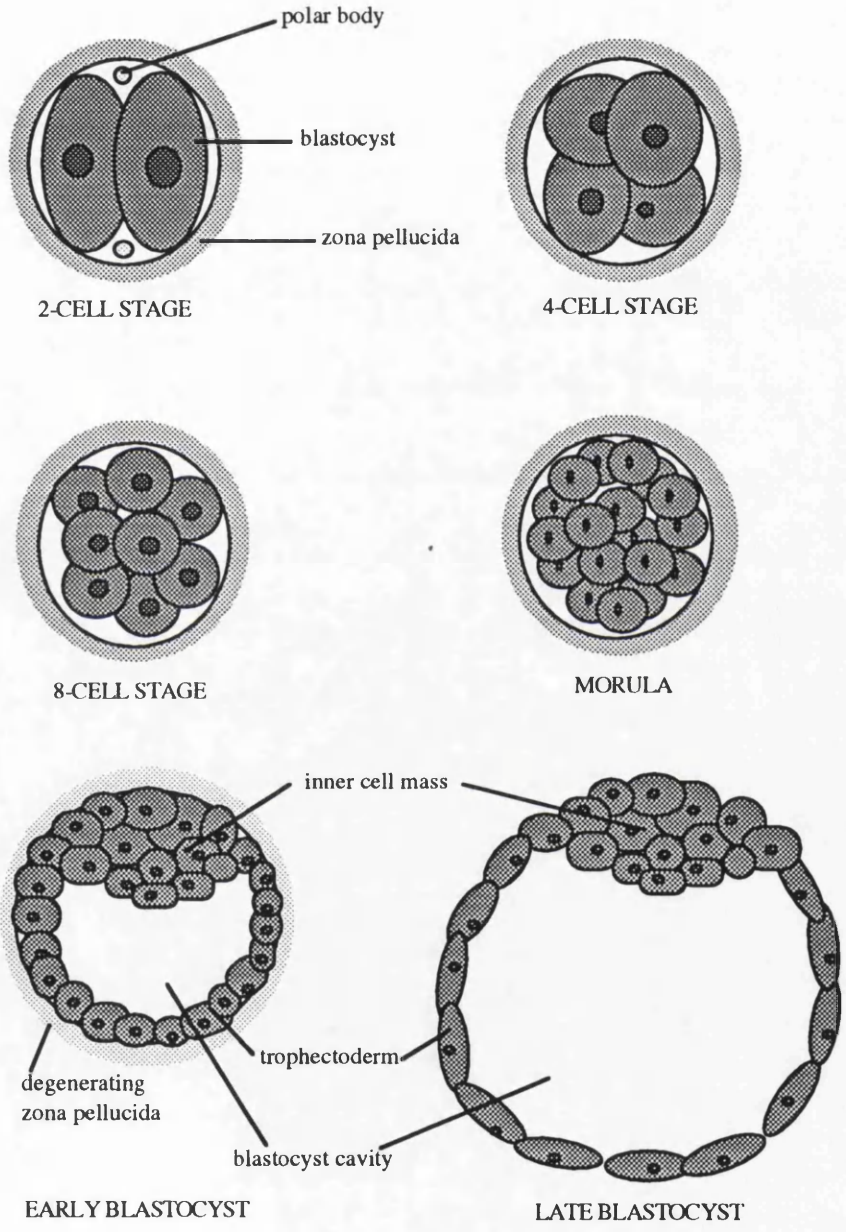
In this case, only two daughter cells result.

At prophase the chromosomes begin to appear, the number and nature of them is consistent from cell to cell and can be analysed at metaphase by karyotyping. Chromosomes occur in homologous pairs, one donated from each parent. The only unalike pair of chromosomes are the X and Y in males. These are referred to as the sex chromosomes or gonosomes and hence the embryo's sex is determined by whether it inherits the paternal X or Y chromosome. Thus an embryo is male if it results from the fusion of oocyte and a Y chromosome carrying sperm and female if it results from fusion with an X chromosome carrying sperm.

EARLY EMBRYOLOGY

Mitotic division in the early embryo or "cleavage" is illustrated in the following diagram (Moore., 1988):

Fig 1.5: - CLEAVAGE DIVISIONS

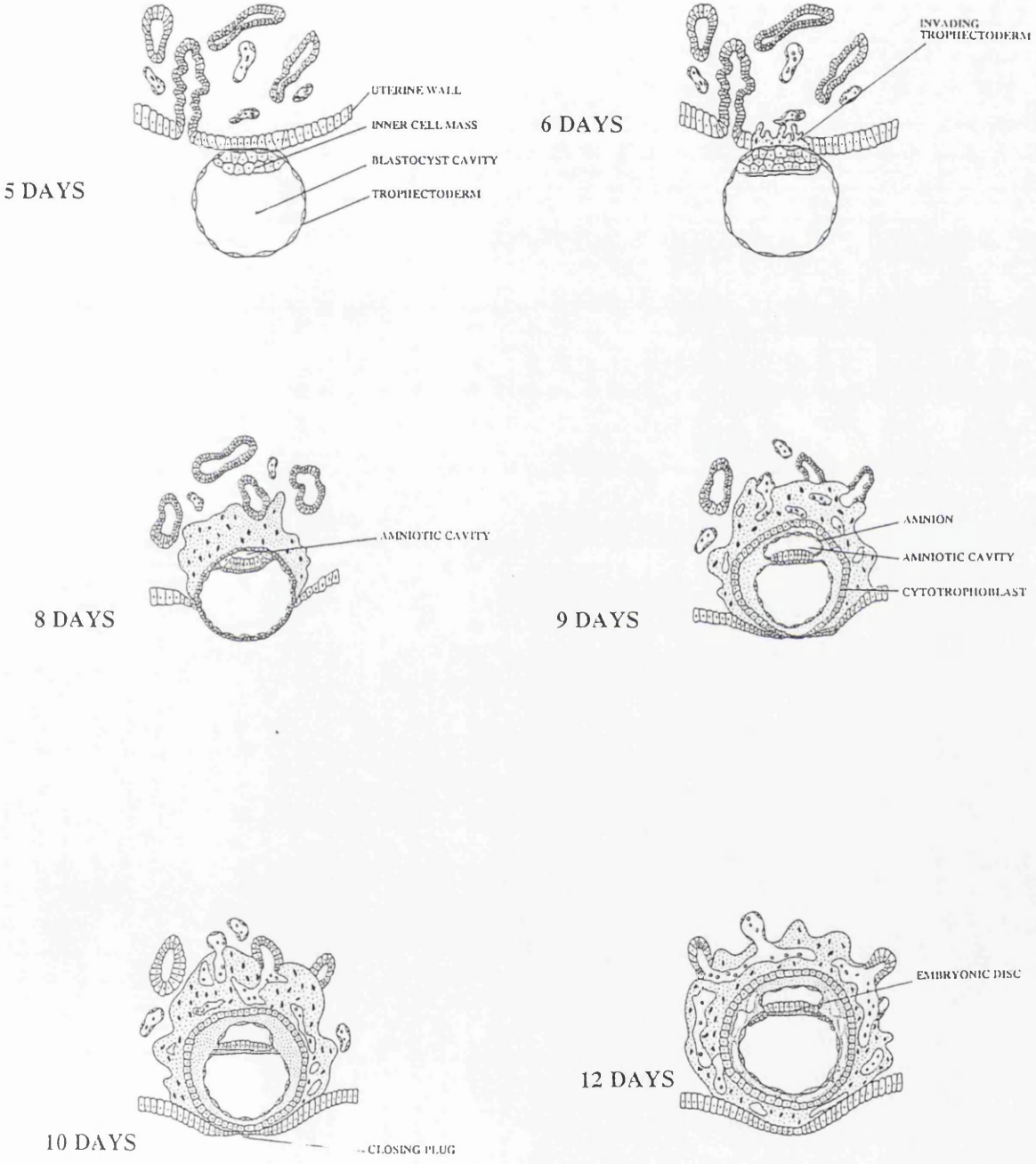


Although, after each cleavage the number of cells (or "blastomeres") increases, the size of the embryo remains constant until the zona pellucida degenerates. In early embryonic stages therefore after each division, blastomeres get smaller. Once the zona

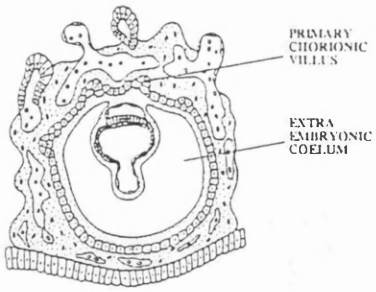
has disappeared, the blastocyst increases rapidly in size. It is the inner cell mass (ICM) that will give rise to the fetus and the trophectoderm (TE) that will give rise to the surrounding tissues.

On the fifth day post-fertilisation, the embryo approaches the uterus wall, on the sixth day post- fertilisation it undergoes implantation into it. As implantation progresses, a small space between the inner cell mass and invading trophectoderm develops namely the amniotic cavity. This becomes surrounded by specialised cells to form the amnion (around day-9). Also on day-9 a layer known as the cytotrophoblast is visible. On day-10 the uterus wall closes around the embryo and the embryonic disc (which will give rise to the fetus) is clearly visible. The end of the embryo's 2nd week is characterised by the first appearance of the "chorionic villi" and the "primitive streak." The primitive streak is a slit like feature appearing at the caudal end of the embryonic disc. Its appearance is significant in a number of ways: Firstly it marks the beginning of the third week of gestation, secondly it coincides with the first missed menstrual period and lastly the number of primitive streaks that form determine the number of fetuses that will develop (i.e. if two primitive streaks form, twinning occurs), thirdly the primitive streak is the first evidence of the development of a nervous system and thus before this stage the embryo is unlikely to experience any form of sensation. It is because of this that experimentation on human embryos is allowed, by law, up until primitive streak formation i.e 14 days post-fertilisation (as recommended by the Warnock committee and debated in parliament., April 1990). At 16 days the secondary chorionic villi are visible as are the tertiary villi at 21 days. The following diagrams show embryogenesis from 5 days to 21 days post-fertilisation (Moore., 1988).

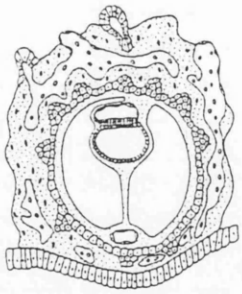
Fig 1.6: - EARLY EMBRYOLOGY

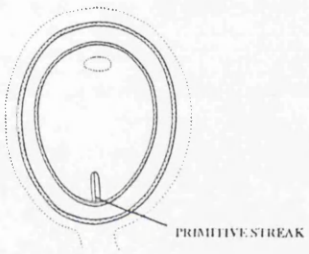


13 DAYS



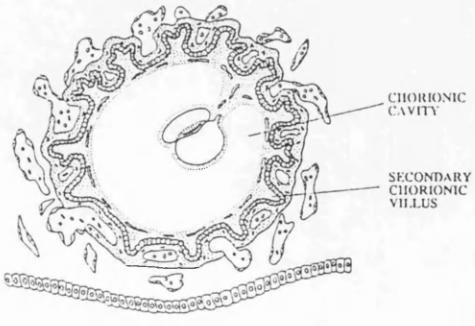
14 DAYS



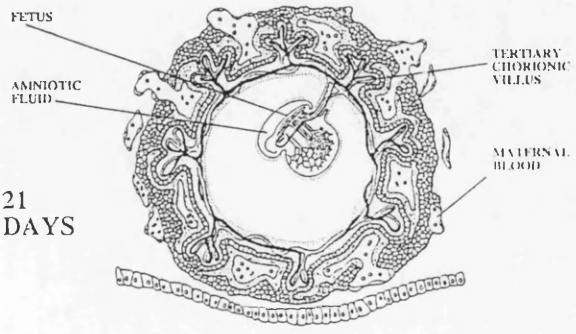


EMBRYONIC DISC AT 14 DAYS

16
DAYS



21
DAYS



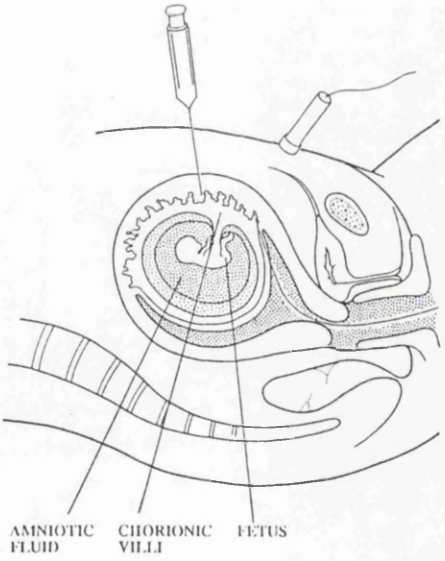
PRENATAL DIAGNOSIS

SAMPLING APPROACHES

At eight and a half weeks, the tissues of the embryo have formed and the developing fetus is clearly visible. At 15-16 weeks the amniotic cavity is very large. Prenatal sampling (in both cases) involves the insertion of a needle or catheter (shown in the diagrams below). At eight and a half weeks samples can be taken from the chorionic villi. At 15-16 weeks samples of the amniotic fluid surrounding the fetus can be taken. Once the sample has been taken, as the tissues are fetally derived, the chromosomal complement of these tissues and hence of the fetus can be determined.

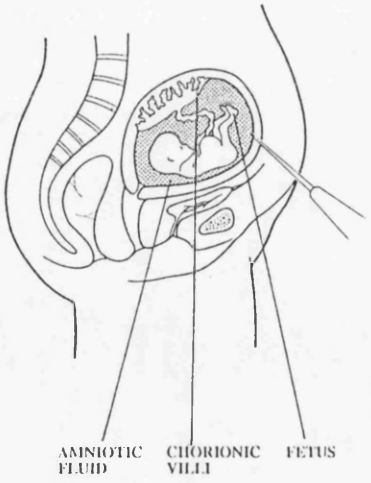
Fig 1.7:

CHORIONIC VILLUS SAMPLING



8-9 WEEKS

AMNIOCENTESIS



15-16 WEEKS

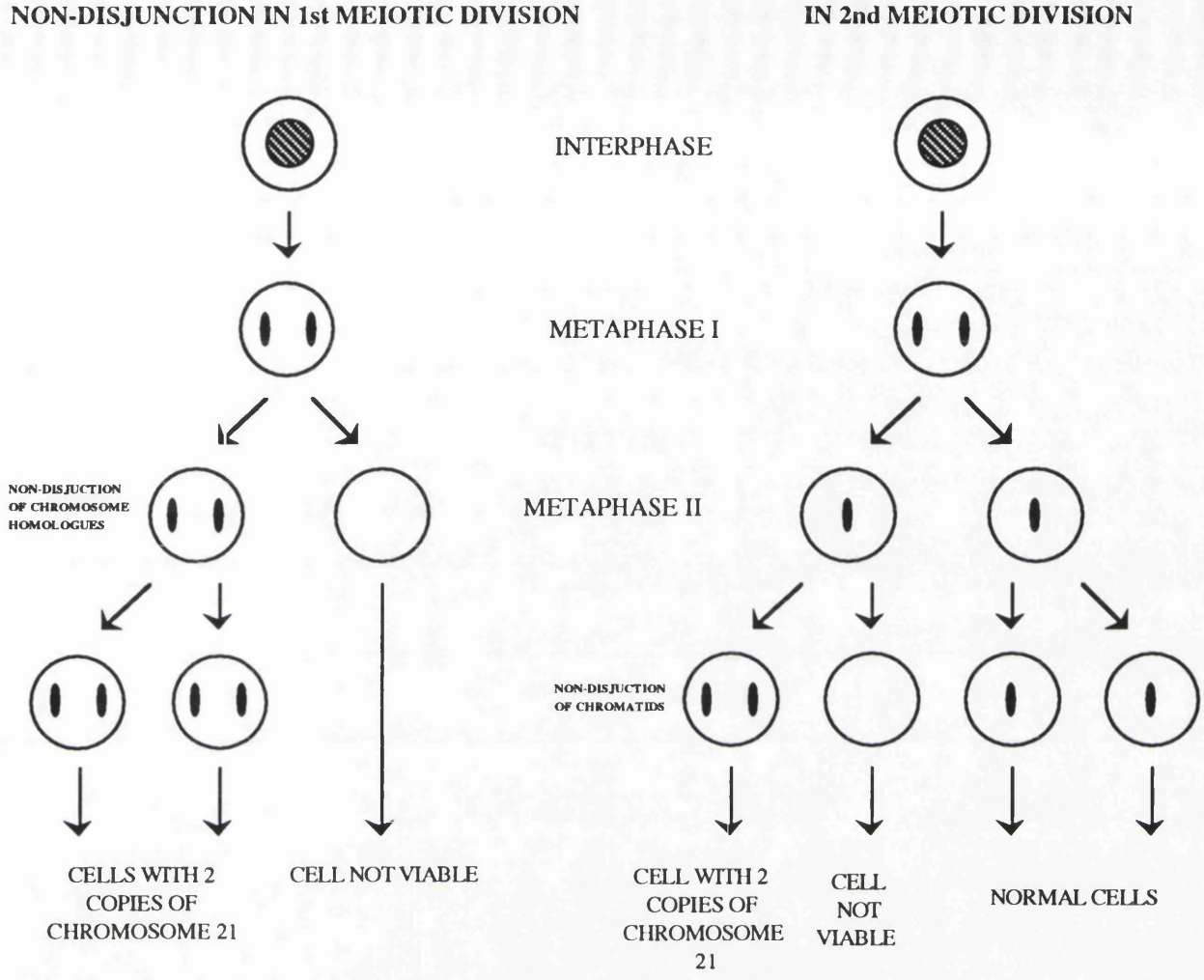
CHROMOSOMAL PREPARATION

In order to prepare chromosomes, cells from the amniotic fluid need to be cultured over a period of two weeks. CVSs can also be cultured, however an advantage of CVS is that chromosomes can be obtained directly without the need for culturing and hence a diagnosis can be achieved with speed. Direct CVS chromosome preparations are derived from the outer epithelial layer of the villus and, in general, give metaphases. Chromosome spreads can be difficult to analyse completely however and therefore are, in most diagnostic labs, merely homogeneously stained and analysed for sex and aneuploidy alone. Cultured CVS preparations are derived from the inner mesenchymal core of the villus and chromosome spreads prepared from these are of much better quality. It is because of this that it is highly recommended for these laboratories to use both methods so that a confident diagnosis can be made for each patient (M. Lucas., personal communication). The aim of prenatal chromosomal diagnosis is to ascertain the chromosomal complement of the embryo and offer selective termination of abnormal fetuses.

CHROMOSOME ABNORMALITIES

Chromosomal abnormalities can be numerical or structural. Numerical abnormalities involve a whole extra (or one less) chromosome in the complement and arise in the fetus by a process known as "meiotic non-disjunction." This is illustrated in the following diagram and more commonly occurs at metaphase I where homologues do not separate and both enter one daughter cell. It can also occur at metaphase II however when sister chromatids do not separate and both enter one daughter cell. Each scenario is illustrated below for non-disjunction of chromosome 21.

Fig 1.8:

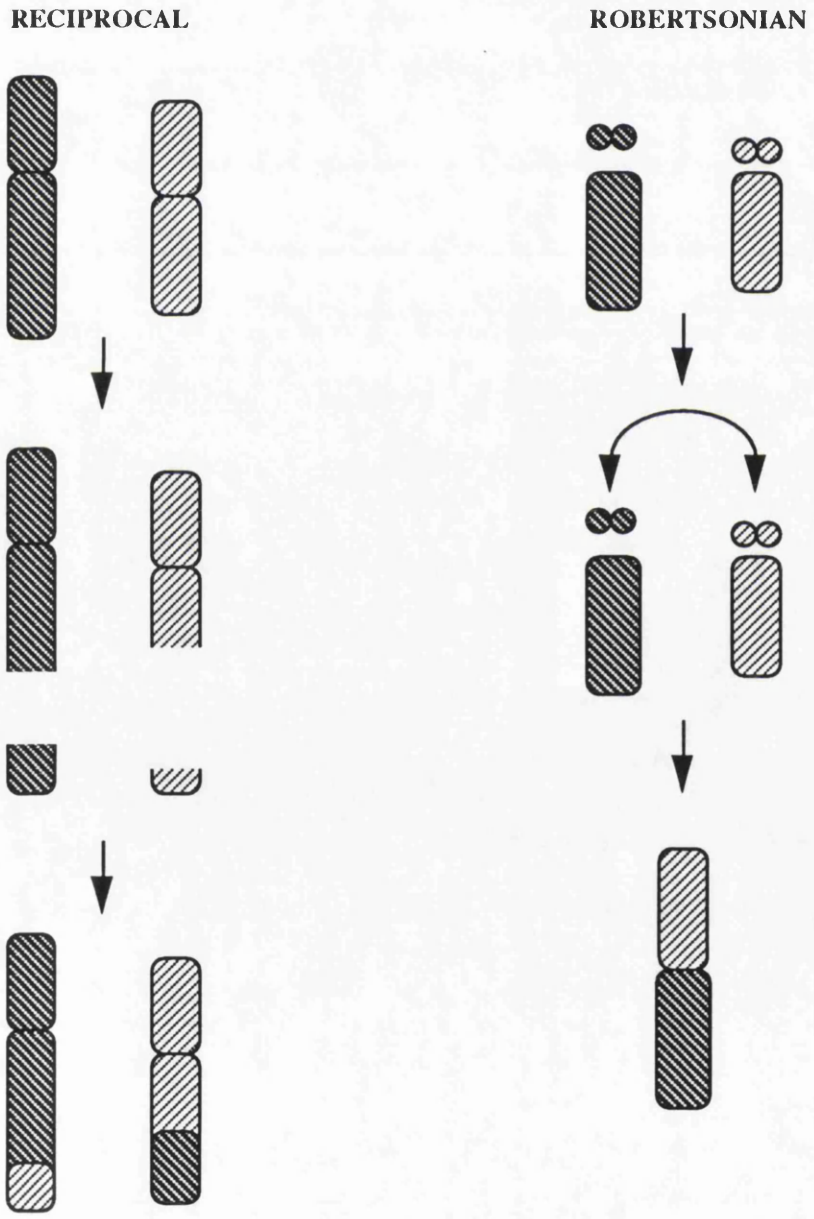


Resulting fetuses then have three copies (trisomy) of, in this example, chromosome 21. Trisomy 21 or "Down's syndrome" is the most common of all human autosomal trisomies at birth however live births with trisomy 18 and 13 also occur. Non-disjunction can also occur during mitosis. This could then lead to chromosomal "mosaicism" (i.e. some embryonic tissues with one chromosomal complement and some with another). Aneuploidy of the sex chromosomes is also common. Females with five, four, three or one (Turner's syndrome) have been reported whereas in males XXY, XYY, XXYY, XXXY, XXXYY and XXXXY individuals exist.

Structural abnormalities can be divided into seven groups:

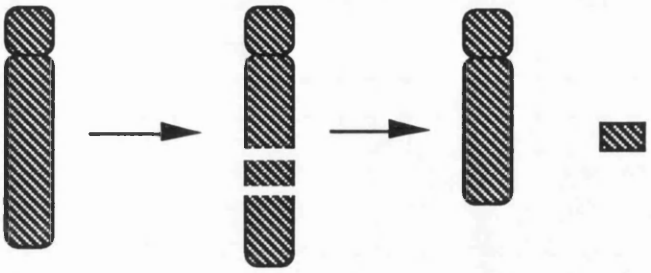
i) Translocation - the transfer of a piece of chromatin on to another chromosome. Balanced carriers of the rearrangement can be normal as long as no material is lost or duplicated. Their offspring however run a high risk (25-33%) of being abnormal. Translocations can be reciprocal (where two chromosomes have an interstitial break and exchange pieces) or Robertsonian (where chromosomes, usually acrocentrics, fuse after breaks form in the region of the centromere).

Fig 1.9a:



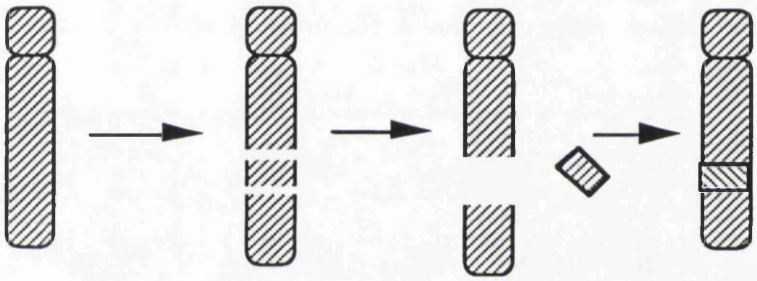
ii) Deletion - two interstitial breaks in a chromosome leading to loss of a portion of the chromosome. If constitutional it results in a severely affected individual.

Fig 1.9b:



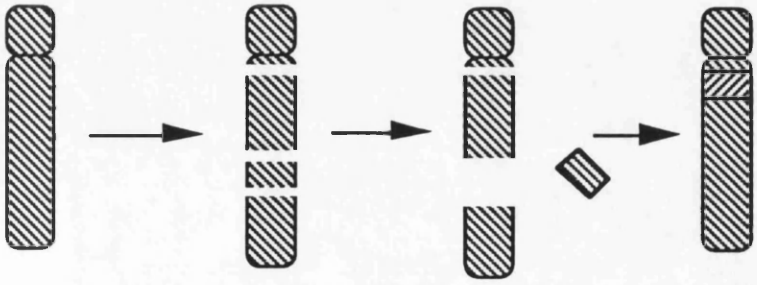
iii) Inversion - where a chromosome has two interstitial breaks, the fragment is inverted and re-attaches itself in the reverse orientation. As with translocation, carriers need not be affected but offspring can be at risk.

Fig 1.9c:



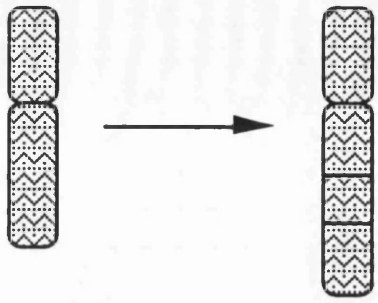
iv) Insertion - where three interstitial breaks are involved either all on one chromosome or two on one and one on another. The two breaks result in a fragment which detaches and then inserts in the space left by the third break either in the same or in the reverse orientation. Again carriers may not be affected but offspring are usually at risk.

Fig 1.9d:



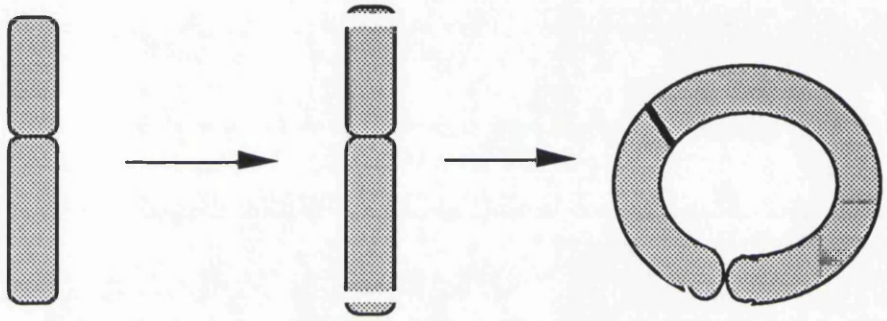
v) Duplication - Extra material in the karyotype, either within the same chromosome, attached to another chromosome or appearing as a separate fragment. If constitutional, the individual is usually affected but generally less seriously than with a deletion as no material is lost.

Fig 1.9e:



vi) Ring chromosome - where terminal p and q arm fuse to form a ring. Carriers are usually severely affected as the process involves loss of the termini of both chromosome arms.

Fig 1.9f:



vii) Isochromosome - where the chromosome appears as two joined copies of one arm of the chromosome due to it dividing transversely instead of longitudinally at meiosis II or mitosis. The other arm is lost.

MOLECULAR ANALYSES

Many single gene disorders e.g. muscular dystrophy, haemophilia, cystic fibrosis, beta thallasemia and alpha 1 antitrypsin deficiency can be diagnosed prenatally by extraction of the DNA from the fetal (CVS) sample followed by analysis using techniques of molecular biology such as Southern blotting and polymerase chain reaction (PCR).

RELATIVE MERITS OF CVS AND AMNIOCENTESIS

CVS is performed usually at 8-9 weeks gestation whereas amniocentesis is performed at 15-16 weeks. Brambati and Lucia (1990) have reported CVS taken as early as the 6th week of gestation and Smith *et al* (1990a) report that amniocentesis can be performed at 11 weeks (bringing it into the first trimester). Rapid results mean that therapeutic abortion can be offered quickly which ultimately leads to less trauma for families having to make the difficult decision of whether to proceed with a termination. Hence CVS gained rapid popularity initially following it's development.

Recent studies have however, implicated amniocentesis as the preferable approach for chromosomal diagnosis. Firstly 91% of women allocated to amniocentesis gave birth to a live baby compared with 86% for CVS (MRC working party., 1991) hence suggesting that CVS is riskier for the fetus. Secondly many reports have appeared of limb and facial abnormalities associated with CVS. Thirdly the great advantage of an earlier result associated with CVS is balanced by the fact that CVS reveals mosaic results more often than amniocentesis since amniocentesis analyses cells directly shed from the fetus. Lastly because amniocyte chromosomes are more easily analysable than those from direct CVS, then the former are more likely to reveal more subtle chromosomal abnormalities (Lilford., 1991).

PROSPECTS FOR PREIMPLANTATION DIAGNOSIS (PID)

Preimplantation diagnosis (PID) is carried out on an embryo prior to implantation into the mother's uterus. Although considerable advances have been made in prenatal diagnosis, selective termination of affected offspring is unacceptable in some cases. The need (at least initially) to develop PID hence, is not as a working alternative or replacement for prenatal diagnosis but to alleviate the suffering of many special case families.

Penketh and McLaren (1987) report one thallasemia family who had one unaffected child after seven pregnancies four of which were terminated. Another woman whose brother died of Duchenne Muscular Dystrophy (DMD) has two teenage nephews with the condition and is a carrier herself. She had two terminations from her first marriage which broke down as a result. In her second marriage she, following second trimester prenatal diagnosis, has had three terminations of male fetuses and consequently has been advised to refrain from further conception until research in DMD has reached a more advanced stage.

In some families, selective abortion is morally or religiously unacceptable. For instance Catholic, Muslim and orthodox Askenazi Jew (Penketh and McLaren., 1988; Winston., 1987) faiths all prohibit terminations of pregnancy. Clearly families such as these would benefit from PID. It should be noted however that the Catholic faith has not embraced even this approach.

STRATEGIES FOR PID

Prospectively, PID can be approached in one of four ways. Each can involve either *in-vitro* fertilised (IVF) embryos (Steptoe and Edwards., 1978) or embryos fertilised normally and flushed from the mother's uterus (uterine lavage) (Buster *et al.*, 1985): These are a) nutrient measurement of intact embryos, b) removal and diagnosis of a polar body, c) removal and diagnosis of trophectoderm material, d) removal and diagnosis of single blastomeres at early cleavage stages. It is the latter that has aroused the most interest in this field. (Reviewed in Adinolfi and Polani., 1989).

NUTRIENT MEASUREMENT OF WHOLE EMBRYOS

This is a non-invasive strategy. When an embryo grows, it takes up nutrients. Gardner and Leech (1986) reported measurement of pyruvate and glucose uptake in

mouse preimplantation embryos. Hardy *et al* (1989) made a similar study in human oocytes and preimplantation embryos. Accurate measurement of uptake of pyruvate, glucose and perhaps other nutrients could help select healthy embryos (i.e. those taking up nutrients normally and hence not displaying potential metabolic defects) for the purposes of selective transfer.

POLAR BODY BIOPSY

This involves physical removal of a polar body (usually the second polar body appearing post-fertilisation) from an IVF embryo at the two-cell stage. This could be applicable for both DNA and chromosomal analyses. It, of course, only assesses the contribution of the maternal genome but in the case of recessive disorders only the contribution of one of the parents need be controlled. Genetic analysis results are further clouded by the process of meiotic "crossing over" where homologous chromosomes exchange genetic material at metaphase I. In the case of chromosome analysis, assessing the contribution of only one parent is obviously restrictive despite the fact that 80% of all Down's syndrome cases arise from an extra chromosome 21 donated by the mother.

TROPHECTODERM BIOPSY

The third approach involves biopsy of material from the trophectoderm (TE) when the embryo is at the blastocyst stage and subsequent chromosomal or DNA analysis. The first report of preimplantation diagnosis (diagnosing sex in rabbits) was using this approach (Gardner and Edwards., 1968). Micromanipulation techniques were used to remove the outer layer of TE. Sexing was achieved by detecting Barr Bodies (inactive X chromosomes visible only in female interphases). Embryos were transferred into the mother's uterus and sex confirmed later in gestation or at birth. TE biopsy at the blastocyst stage has some advantages: a) The maximum number of cells are available for diagnosis making any test more reliable than if fewer cells were available. b) TE cells are extraembryonic and contribute only to the tissues surrounding the fetus. c)

By the blastocyst stage, embryonic gene expression is likely to be well established especially in the TE making sensitive biochemical assays possible (Handyside and Delhanty., 1992). The major problem with applying this strategy clinically (at least using IVF) is the unexpectedly low pregnancy rate following IVF and transfer at this stage (Dawson *et al.*, 1988, Bolton *et al.*, 1991).

CLEAVAGE STAGE BIOPSY

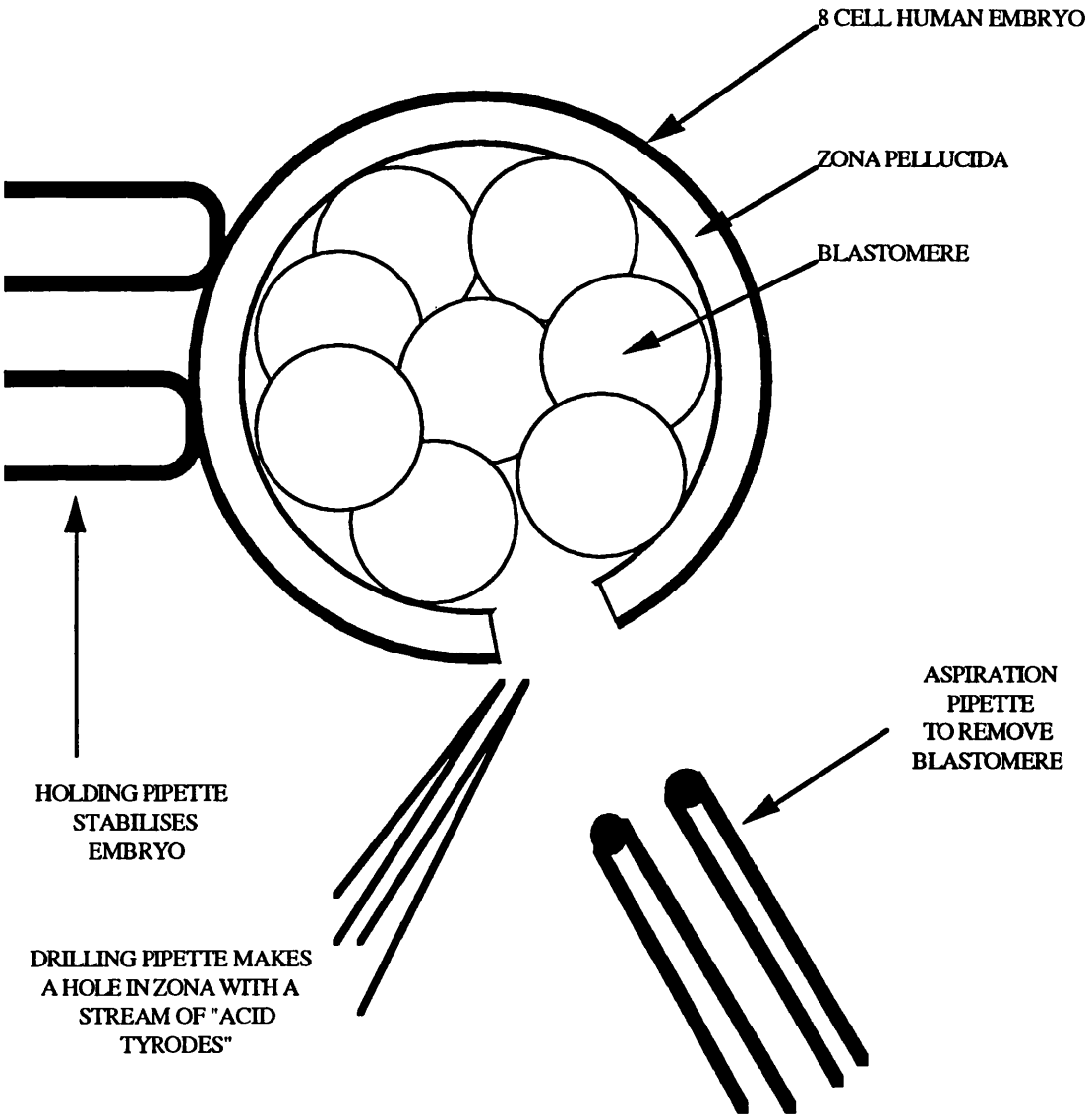
This involves removal of one or more blastomeres at the 4-16 cell stage embryo. Diagnosis can be made on the biopsied cell(s) and the remainder implanted into the mother's uterus if necessary. At these early cleavage stages, mammalian blastomeres remain totipotent.

The biopsy technique (e.g. Handyside *et al.*, 1990) involves placing the embryo in a drop of medium under oil and placing under a dissecting microscope for micromanipulation. The embryo is immobilised by suction on a holding pipette, a small hole is drilled in the zona pellucida using a tiny pipette, a second larger pipette is then pushed into the hole to remove one or two cells. The remaining embryo is quickly returned to culture and the biopsied cell(s) prepared for analysis. This is illustrated in figure 1.10.

Fig 1.10:

BIOPSY OF 8 CELL HUMAN EMBRYO PRIOR TO PID

HANDYSIDE *et al.*, 1990,1991.



Preimplantation development is not adversely affected by biopsy at the 8 cell stage. This was confirmed by Hardy *et al* (1990) who took 1 and 2 cell biopsies and found that glucose and pyruvate uptake only decreased in proportion to reduction in cellular mass and furthermore over half hatched out of the zona pellucida *in-vitro*.

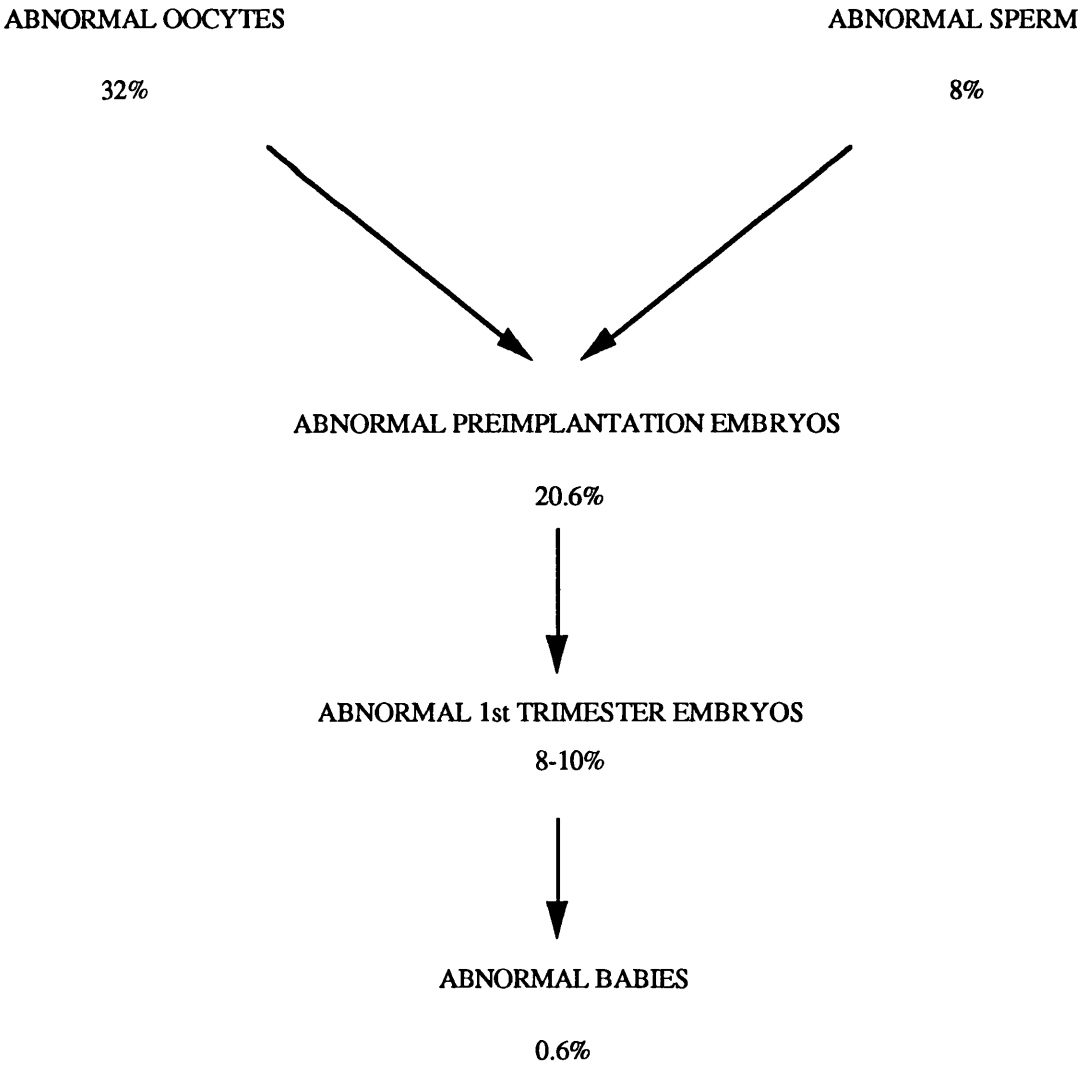
Monk *et al* (1987) successfully measured production of the enzyme HPRT in one biopsied blastomere of 8-cell mouse preimplantation embryos recovered by uterine lavage. Since the parents were of a strain of HPRT deficient mice, it followed that about a quarter should not produce HPRT even at this early developmental stage. This was the case. Then the remaining seven cells were transferred into the mother's uterus and all those not producing HPRT developed into HPRT deficient individuals. Similarly, those that produced HPRT developed into normal individuals. HPRT deficiency in man is a sex-linked condition known as Lesch-Nyhan syndrome hence a working model for human preimplantation diagnosis had been demonstrated.

A cytogenetically based strategy in mouse was produced by Bacchus and Buselmaier (1988): They employed a uterine lavage approach to recover four-cell embryos. One blastomere was removed from each by dissagregation. The removed cell was induced into metaphase and limited karyotypic analysis performed on the basis of an easily identifiable chromosome translocation present in the male parent. The remaining three cells from the embryo were transferred and some developed. The studies of Wilton and colleagues (1989-1990) describe a complete mouse model of cytogenetic PID employing embryo biopsy, diagnosis of a single blastomere, transfer and normal fetal development *in-utero* : Wilton and Trounson (1989) described the biopsy technique i.e. removal of one blastomere from a four cell embryo (recovered by uterine lavage) utilising micromanipulation technology. It was found that this procedure had little or no effect on *in-vitro* development of the resulting embryo. Furthermore following embryo transfer into a pseudopregnant female the fetal development rate was not significantly altered however implantation rate was reduced in comparison with controls. Wilton *et al* (1989) adapted the above method of biopsy and discovered that this adaptation allowed biopsy and transfer without significant reduction in the implantation rate. They further went on to demonstrate successful transfer and subsequent pregnancy using embryos which had been biopsied and cryopreserved. Kola and Wilton (1990) described an efficient approach of embryo biopsy, arrest of a

single blastomere in metaphase, cytogenetic preparation, analysis, transfer and subsequent *in-utero* development. In all cases PID was fully predictive of subsequent fetal karyotype. In this case a Robertsonian translocation containing chromosome 16 (the mouse equivalent of chromosome 21) was detected.

Human chromosomal preimplantation studies have been performed on IVF embryos. Plachot *et al* (1987) successfully obtained analysable metaphase spreads from human embryos. They report that 21-23% of these were abnormal and present the following model (also based on oocyte studies) for the natural selection against chromosome abnormalities in human development.

Fig 1.11: NATURAL SELECTION AGAINST CHROMOSOME ABNORMALITIES



Although around 90% of human cleavage stage cells can be arrested in metaphase following overnight treatment with colchicine,analysable chromosome spreads are however universally very difficult to obtain as chromosomes are often short and clumped together (L.J. Wilton., personal communication). Since karyotype analysis of CVS involves looking at 20-30 metaphases selected for optimal spreading (M.Lucas., personal communication) it is unlikely that PID via karyotyping alone is, at present, a feasible strategy.

Genetic analysis of tiny amounts of DNA is possible by gene amplification via the polymerase chain reaction (PCR) (Saiki *et al.*, 1985). This approach was first achieved in human blastomeres by Handyside *et al* (1989) reporting the successful amplification of Y chromosome specific sequences and hence providing a quick, efficient method of sexing human preimplantation embryos. Amplification could be consistently achieved on 2pg of male DNA (the equivalent of a diploid cell) and on single cells also. Coutelle *et al* (1989) have amplified sequences from both cystic fibrosis and muscular dystrophy genes in human oocytes thereby demonstrating the possibility for diagnosis in single preimplantation cells and an animal model was provided by Holding and Monk (1990) amplifying the beta-thallasemia gene in single mouse blastomeres.

PID of sex in humans was first reported by Handyside *et al* (1990). Eight couples at risk of transmitting various X-linked diseases (including X-linked mental retardation, Lesch-Nyhan syndrome, adrenoleukodystrophy, retinitis pigmentosa, hereditary motor neurone disease type II and Duchenne muscular dystrophy) were counselled as to the possibility of PID of sex following IVF and selective transfer of female embryos (Handyside *et al.*, 1990, 1991). After routine assessment of each couple for IVF, women were induced to superovulate hormonally, oocytes were recovered and fertilised *in-vitro* by standard methods employed for infertile couples. Embryos were biopsied early on day-3 (6-10 cell stage), this is the latest day on which embryos have

been routinely transferred without affecting pregnancy rates (Handyside and Delhanty., 1992) thus allowing a maximum of 12 hours to biopsy and analyse the embryo before transfer. Biopsy was performed as already described. Since between 5% of cells from good quality embryos and 15% from poor quality ones have no nucleus (Hardy *et al.*, 1991), each cell was checked for the presence of a nucleus before proceeding with DNA analysis. On average, about five embryos were biopsied so that it was likely that at least two would be diagnosed as female. In each case, two embryos were transferred as pregnancy rates are significantly higher than after transfer of one. Transfer of three is considered to be too many because of the maternal health risks associated with multiple pregnancies and as the necessary confirmation of results by CVS is feasible with twins but not triplets Handyside *et al* (1990, 1991).

DNA amplification by PCR was performed in the earlier patients on a Y-specific sequence repeated 800-5000 times on the long arm of the Y chromosome (Cooke *et al.*, 1982) but later using a repeat sequence for the centromere of the chromosome (Witt and Erickson., 1989). Diagnosis was made on the basis of presence or absence of an amplified fragment as visualised on a polyacrylamide gel.

Five out of the eight women became pregnant after a total of 13 treatment cycles. The first two were both twin pregnancies and the other three singletons. The sex of each of the seven fetuses was checked by CVS and karyotyping at 10-11 weeks. All proved to be female except for one singleton pregnancy which was male and subsequently terminated. The remaining pregnancies went to term and all were apparently healthy girls with one exception (the second child of the second set of twins) which was stillborn (Handyside and Delhanty., 1992).

Misidentification of sex in the one case illustrates the problem of relying on the presence or absence of an amplified fragment and hence a negative result for diagnosis, simple failure of amplification could, and did, lead quite easily to misdiagnosis. The incidence of a stillbirth was, after detailed post-mortem examination, thought to be an

obstetric complication "intrapartum anoxia" prior to Caesarian delivery (Handyside and Delhanty., 1992).

A number of studies involving preimplantation embryos and the technique known as *in-situ* hybridisation (ISH) have been reported. This is covered in a subsequent section.

ASPECTS OF COLORECTAL CANCER

GENERAL FEATURES

"An optimistic outlook is that the colorectal model will prove relevant to other common neoplasms in which tumour development is less well defined and tumours of varying stage are difficult to study. According to this optimistic outlook, the pathogenesis of human neoplasia is a puzzle that might prove solvable in the coming decades."

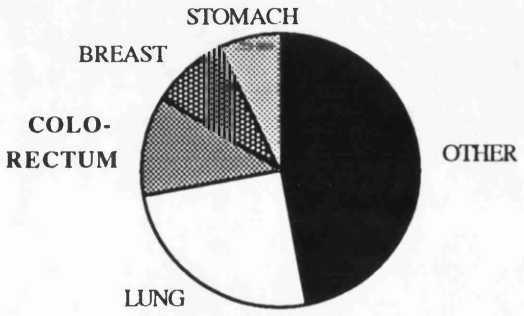
Fearon and Voglestein (1990).

When the exquisite controls regulating cell division break down, a cell can begin to ignore its usual boundaries. When daughter cells of such a cell inherit the propensity to ignore cellular boundaries also, they can divide profusely and form a clone of considerable size ultimately leading to a mass or tumour.

It is estimated that one in three of the country's population develop some form of cancer and one in five die of it. Cancer of the colon and rectum represents the second most common cause of death from cancer in the U.K. (Cancer Research Campaign fact sheets 1990).

Fig 1.12:

CANCER MORTALITY U.K. 1988



Study of colorectal cancer provides an excellent model system in which to to examine human neoplasia in general for a number of reasons: Firstly histopathological and clinical evidence indicates that the vast majority (if not, the entirety) of malignant colorectal tumours arise from pre-existing benign polyps (adenomas)(Sugarbaker., 1985). Secondly, all forms of polyps and tumours can be readily obtained by surgery - this is not always the case with other tumour types. Finally, inherited and somatic genetic alterations can be studied as both hereditary and environmental factors contribute to development of the cancer.

Colorectal polyps (adenomas) are lesions which project from the mucosa of the intestine into the lumen. The number of polyps in the colorectum is described as either a) a solitary polyp, b) multiple polyps (usually 2-20 but can approach 100) or c) polyposis (100+ polyps, usually many hundreds). Their size is highly variable ranging from barely visible polyps several millimetres in diameter to a diameter of several centimetres. Their shape also varies and can be described as pedunculated, semi-pedunculated, sessile, smooth or papilliary.

Early clinical diagnosis of colorectal cancer is difficult as symptoms are usually confined to bleeding and alterations in bowel habits. Anemia, weight loss, cramps, narrowing of the stool and burning pains are all indicative that the disease has reached its later stages.

Hereditary predisposition of colon cancer is divided into two types i.e with or without preceding polyposis. It is on the polyposis type that this thesis will dwell. Familial Polyposis Coli (FPC) was first described by Bussey (1975). Characteristics of this condition are hyperproliferation of mesenchymal and epithelial tissue leading to the appearance of hundreds of adenomatous colorectal polyps between the ages of 10 and 20. Unless surgery is performed progression to malignancy inevitably ensues (Muto *et al.*, 1977). This condition affects 1 in 10,000 individuals (Reed and Neel., 1955) and is of great clinical importance as studies of families with an inevitable

predisposition to colorectal cancer provide an excellent model for studying that particular cancer and carcinogenesis as a whole.

CYTOGENETIC ASPECTS

Regarding human solid tumours, colorectal adenocarcinomas are one of the most widely studied cytogenetically. This fact can be attributed largely to the relative ease in which analysable metaphases can be obtained. On the basis of direct karyotype studies, tumours can be divided into four categories (Muleris *et al.*, 1990a,b) namely: 1) Monosomic-type near-diploid tumours (MD), 2) Monosomic-type polyploid tumours (MP) which have a pattern of chromosome imbalance similar to the MD type and are derived from these by endoreduplication, 3) Trisomic type tumours (TT), 4) Tumours with normal karyotypes (NT).

MD tumours vary in chromosome number between 41 and 49 with a mode of 44. They can be characterised further by virtue of the fact that they simultaneously lose a whole chromosome 18 and the short arm of chromosome 17. Other cytogenetic abnormalities also exist with varying frequencies as shown in table 1.1. MP tumours are more heterogeneous than MD tumours and this is reflected in their greater variation in chromosome number (58 to 129). Again, loss of 18 and 17p is found exclusively. The pattern of losses and gains is indeed very similar to that of MD tumours and a correlated molecular and cytogenetic study indicates that MP tumours arise from MD tumours and undergo endoreduplication to achieve MP status (Reichmann., 1982a,b). TT tumours range in chromosome number from 47 to 58 with a mode of 50 and, in contrast to MD and MP types, do not exclusively lose both 17p and 18. The most consistent abnormalities are gains of whole chromosomes, most frequently 7, 13, 12, 20 and early replicating X chromosome (Xe). Polyploid sidelines have never been seen in these tumours indicating that they do not undergo endoreduplication. Finding colorectal tumours with normal karyotypes is not unknown (NT tumours): Reichmann

et al (1982) report 3 out of 31. Muleris *et al* (1990b) report 7 out of 100. These cells, in both cases, were confirmed as cancerous and not stromal or inflammatory cells by flow cytometry. Occasionally atypical cells, i.e. ones which do not fit easily into any of these categories, appear.

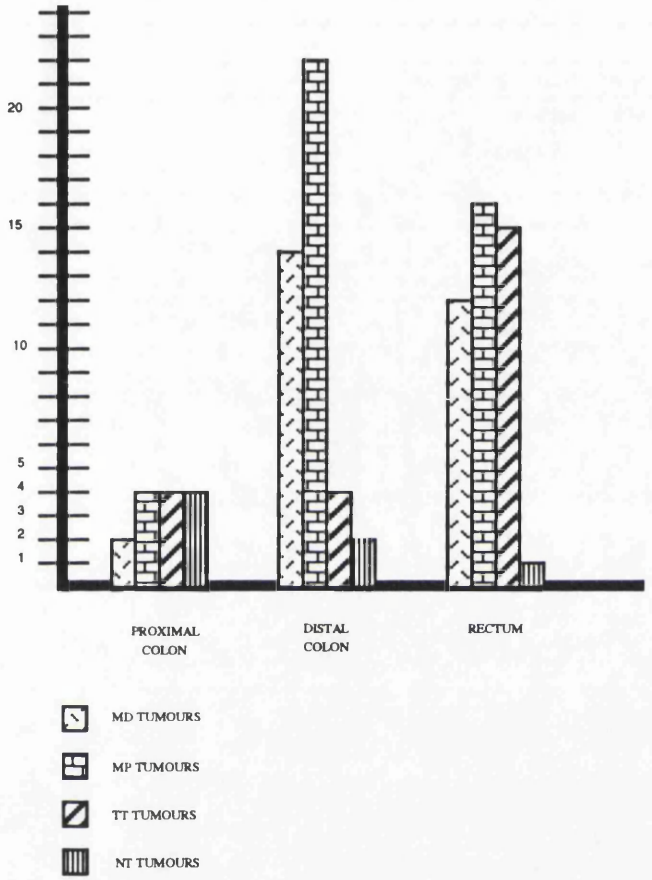
Table 1.1:- FREQUENCY OF THE MOST RECURRENT CHROMOSOME ABNORMALITIES OBSERVED IN A SERIES OF 100 COLORECTAL TUMOURS. (Muleris *et al.*, 1990).

	% OF TUMOURS WITH THE ABNORMALITY				
CHROMOSOME IMBALANCES	MD	MP	MEAN MD &MP	TT	GENERAL MEAN
del(1)(p3)	61	74	69	18	53
-4q	43	74	61	4	44
del(5)(q2)	32	78	60	18	47
-6q	18	52	38	0	27
+7	21	17	19	86	33
-8p	61	70	66	18	51
+8q	50	30	38	54	40
-9q	7	70	45	9	33
del(10)(q2)	32	87	65	14	49
-11	18	52	38	4	28
+12	4	4	4	64	17
+13	46	48	47	77	51
-14	39	83	65	0	47
-15	36	74	59	0	42
-17p	100	100	100	27	76
-18	100	100	100	32	78
+20	46	35	39	68	43
-21	14	91	60	9	44
-22	29	61	48	4	35
+Xe, F	42	56	50	62	43
+X, M	60	85	74	62	59
-Xl, F	17	87	57	25	41
-Y	40	85	65	37	49

Quantitative analyses reveal that chromosomal mutagenesis is two to three fold higher in MD and MP tumours than in TT tumours.

The distribution of these tumours throughout the colorectum is not uniform and this is illustrated by the following graph.

Fig 1.13:- DISTRIBUTION OF TUMOURS THROUGHOUT THE COLORECTUM (Muleris *et al.*, 1990).



One exceptional difference between the studies of Muleris *et al* and Reichmann and co-workers (Reichmann *et al.*, 1980, 1981, 1982a,b; Reichmann and Levin., 1982; Levin and Reichmann., 1986) involves the appearance of "double minutes". Muleris *et al* do not mention their existence however in Reichmann and co-workers' studies they figure significantly. Double minutes (DM's) are small paired chromatin bodies whose number varies from cell to cell. Their presence (and that of homogeneously staining regions (HSR's)) suggests the existence of oncogene amplification (Fearon and Voglestein., 1990). Levin and Reichmann (1986) among others report HSR's and Reichmann and Levin (1982) describe premature chromosome condensation in some cells. A possible explanation for the discrepancy between the two groups' results is

that Muleris *et al* use R-banding when analysing their results whereas other groups (e.g. Reichmann *et al*) use G-banding. DMs stain positive with G-banding and negative with R-banding.

CHROMOSOMAL STUDIES OF FPC PATIENTS

Several works have implicated chromosome instability in FPC patients. Delhanty *et al* (1983) studied lymphocytes, skin fibroblasts, colon fibroblasts and colon epithelial-like cells in 17 patients. It was found that, in all cases, lymphocytes, early-passage fibroblasts and colon fibroblasts showed an increase in all classes of chromosome aberrations when compared controls. On the other hand epithelial-like colon cells were initially remarkably stable. Brondom-Nielsen *et al* (1985) reported no increase of structural aberrations, and no heritable chromosomal fragile sites in five FPC patients studied. In this paper however structural analysis was limited to study merely of chromosome breaks.

MOLECULAR ASPECTS

Colorectal tumorigenesis is a multistage process involving a series of genetic changes to which both hereditary and environmental factors contribute. Tumours arise as a result of the mutational activation of oncogenes and the mutational inactivation of tumour suppressor genes. Mutation of at least four or five genes is required for a malignant tumour (less for a benign one) and, although these changes do occur in some semblance of order, it is the accumulation of mutations and not necessarily their sequence that leads to tumorigenesis (Fearon and Voglestein., 1990).

Mutational activation of the *ras* oncogene (on chromosome 12p) is one identified in colorectal tumours. Around 50% of carcinomas and also adenomas greater than 1cm

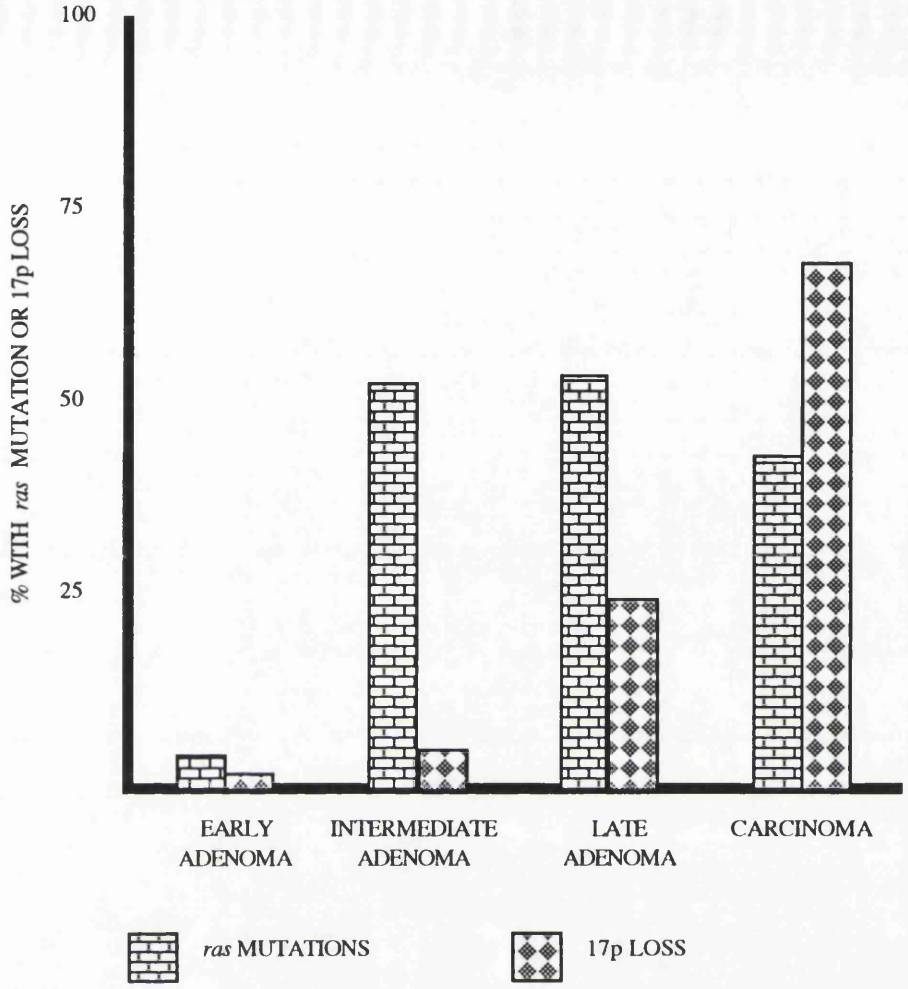
in size have been shown to have *ras* mutations. In contrast less than 10% of adenomas less than 1cm in size have such mutations (reviewed in Fearon and Voglestein., 1990).

Loss of alleles occurs frequently in colorectal neoplasms. Generally, the losses involve only one of the two chromosomes and thus render the cell hemizygous. These allele losses have been interpreted as evidence that the region in question contains a tumour suppressor gene (or genes). It is the products of tumour suppressor genes that normally regulate differentiation and growth and thus suppress tumour development. Restriction fragment length polymorphism studies have been used to determine whether one or both of the parental alleles is lost in the tumour DNA.

Losses on chromosome 5q have been observed in 20-50% of carcinomas and 30% of adenomas in patients with non-polyposis colorectal cancer (Voglestein *et al.*, 1988; Sasaki *et al.*, 1989). Losses in patients with FPC is covered in a subsequent section.

The loss of a portion of chromosome 17p is reported in over three quarters of colorectal carcinomas (Voglestein *et al.*, 1988; Delattre *et al.*, 1989). The common region of loss on 17p is known to be at the site of the p53 gene (Baker *et al.*, 1989). Where the p53 allele is lost, amino acid substitutions have been reported in the remaining allele in many colorectal cancers. Furthermore *in-vitro* evidence suggests that p53 can function as a tumour suppressor gene (Finlay *et al.*, 1989). This hence supports the hypothesis that p53 normally inhibits colorectal tumour growth and hence a mutant p53 can lead to tumour formation (Fearon and Voglestein., 1990).

Fig 1.14:- THE FREQUENCY OF *ras* MUTATIONS AND 17p LOSSES IN COLORECTAL TUMOURS (Fearon and Voglestein., 1990)



Early adenomas were described as less than 1cm in size, intermediate adenomas were greater than 1cm and did not contain foci of carcinoma, late adenomas are also greater than 1cm but did contain foci of carcinoma (in the latter case the foci of carcinoma were removed before study). A carcinoma was described as such because infiltration into the submucosa had occured.

The second most common region of loss is on chromosome 18q. This region shows loss in 70% of carcinomas and nearly 50% of late adenomas (Voglestein *et al.*, 1988; Delattre *et al.*, 1989). Within this region, a contiguous 370kb region containing coding sequences has been cloned (Fearon *et al.*, 1990). This potential tumour supressor gene has been termed "DCC" (Deleted in Colon Cancer) and contains at least eight exons. DCC is expressed in normal colonic mucosa, however its expression is

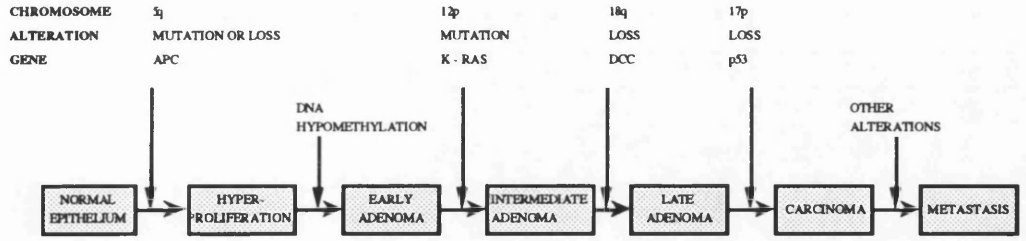
absent or greatly reduced in most colorectal carcinomas. Mutation within DCC in colorectal cancers included a homozygous deletion at the 5' end, a point mutation in one of the introns and ten DNA insertions within a 0.17kb fragment. Predicted amino acid sequences suggest an homology with neural cell adhesion molecules and other related cell surface glycoproteins, hence it is possible that DCC could have a role in pathenogenesis of colorectal neoplasia perhaps via alteration in cell/cell and/or cell/extracellular matrix interactions.

In addition to 5q, 17p and 18q deletions, other allelic losses have been reported in colon cancer. Regions from 1q, 4p, 6p, 6q, 8p, 9q and 22q were lost in 25-50% of cases and with the exception of the acrocentric arms, losses on each of the other chromosome arms have been identified. This could be explained in one of two ways: a) These other losses may exert no phenotypic effect but may arise coincidentally with the other genetic alterations perhaps in a complex mitotic event which aberrantly segregated numerous chromosomes. b) There may be many tumour suppressor genes in the genome and each chromosome region lost (e.g. on 1q, 4p, 6p, etc) could contain such a gene. This could explain the heterogeneity in the biological properties of tumours from different patients.

Further somatic mutations in colorectal tumours are reported e.g. significant loss of methyl groups even in very small adenomas. This may lead to inhibition of chromosome condensation, mitotic non-disjunction and ultimate genome instability. Expression or activity of genes or gene products have been noted. These include high expression of *c-myc*, elevated tyrosine kinase activities and increased expression of glycoconjugates (reviewed in Fearon and Voglestein., 1990).

Fearon and Voglestein have proposed a model for the development of colorectal tumours and this is illustrated in the figure below:

Fig 1.15:- COLORECTAL TUMOUR PROGRESSION



The above figure depicts the relative timing of genetic changes in colorectal tumorigenesis and although these alterations tend to occur in characteristic phases of tumour progression, no given alteration is restricted to one particular phase. Furthermore, it has been possible to study different stages of neoplasia in the same tumour specimen by isolating DNA from both adenomatous and carcinomatous regions of the same tumour (Voglestein *et al.*, 1988). Hence, it seems clear that rather than the sequence of events, it is their accumulation that is important. Indeed, in certain cases, it has been demonstrated that the genetic changes in tumour has occured in a completely different order than the one shown (Fearon and Voglestein., 1990).

MAPPING THE APC GENE REGION

The locus responsible for the FPC condition is referred to as **APC** (adenomatous polyposis coli). The condition known as Gardner's syndrome (GS) is one clinically similar to FPC but patients however typically display extra-colonic manifestations. Both clinical and genetic evidence suggests that the two may not be easily distinguishable and are probably allelic (Nakamura *et al.*, 1988). Hence, when referring to FPC and GS collectively, the abbreviation **FAP** is sometimes used.

CYTOGENETIC AND ALLELE LOSS STUDIES

Herrera *et al* (1986) described a patient with mental retardation and GS. The patient had a deletion on chromosome 5q namely 5q13-15 or 5q15-22 (the two bands being indistinguishable cytogenetically). Hockey *et al* (1989) reported a deletion of 5q15-22 in two patients with FPC thus further implicating chromosome 5 in FAP.

According to Knudson's hypothesis (Knudson., 1971) inheritance of one mutant allele should be followed by loss or inactivation of the second in order to initiate tumour formation. This was exemplified in the example of hereditary retinonblastoma (RB) (Cavenee *et al.*, 1983). Here, the loss of both alleles at the RB locus (on chromosome 13q14) the first in the germ line, the second a somatic mutation in the retinal cell leads to tumour formation. RB is said to be a paradigm to which all heritable and sporadic forms of cancer including colon cancer are analogous (Reviewed in Marshall., 1991). It hence follows that adenomas from patients with FPC should show allelic losses on chromosome 5q. Solomon *et al* (1987), Voglestein *et al* (1988) and Sasaki *et al* (1989) initially reported that 5q adenomatous losses were not seen in FPC however Rees *et al* (1989) described 5q losses in three out of 35 informative FPC adenomas using a probe near the q-terminus of the chromosome. Since then Okamoto *et al* (1990) have also reported 5q losses in patients with FPC and GS. They went on to demonstrate further that allele loss was greater in FAP patients when clones that showed linkage to APC were used. Thus Knudson's hypothesis was supported for APC as it was for the paradigm of retinoblastoma.

LINKAGE STUDIES

Bodmer *et al* (1987) and Leppert *et al* (1987) were the first to localise the APC gene. Bodmer *et al* demonstrated linkage between APC and a marker C11P11. C11P11 was mapped by *in-situ* hybridisation to chromosome 5q21-22. Leppert *et al* also showed linkage to C11P11 analysing DNA from five kindreds. Aldred *et al* (1988) reported a

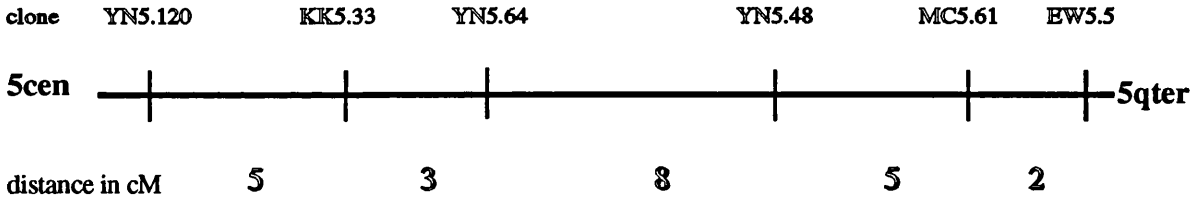
recombinant between C11P11 and APC hence suggesting that it was not quite as close to the gene as was originally hoped. Meera Khan *et al* (1989) reported linkage of a probe Pi227 which was more informative than C11P11 as it recognised 4 restriction fragment length polymorphism sites exhibiting a total of 9 alleles. This is in comparison to C11P11 which has a two allelic system and low heterozygosity.

Fig 1.16:- PROBE ORDERS RELATIVE TO THE APC LOCUS:

Dunlop *et al* (1989) performed linkage studies on the two probes in relation to APC in six Scottish families and proposed a gene order:

cen - Pi227 - C11P11 - APC - tel

Nakamura *et al* (1988) isolated six polymorphic DNA markers around the APC region. Linkage analysis was performed yielding the following map:



APC was said to be most closely linked to clone YN5.48 in the interval YN5.64-YN5.48.

Dunlop *et al* (1990) performed more linkage analysis with existing clones and three further ones namely ECB27 (Murday *et al*,. 1989), L5.62 and EF5.44 (no reference given). Analysis was performed on nine Scottish families and the following map suggested:

cen - Pi227 - C11P11 - ECB27 - L5.62 - APC - EF5.44 - YN5.48 - tel

No recombination was found between APC and EF5.44 or YN5.48.

Friedl *et al* (1991) proposed the map:

cen - Pi227 - C11P11 - ECB27 - KK5.33 - APC - tel

Finally, Cachon-Gonzalez *et al* (1991) proposed the following map based on 20 U.K. families:

cen - Pi227 - C11P11 - ECB27 - APC - YN5.48 - tel

ISOLATION OF THE APC GENE

The first candidate gene for APC was cloned by Kinzler *et al* (1991a). Using DNA probes known to be around the APC gene, a panel of sporadic colorectal carcinomas was studied. One probe which was named cosmid 5.71 detected a rearranged restriction fragment in the DNA of one tumour. Further studies indicated that the sequences within 5.71 were highly conserved in rodent DNA. A gene "MCC" (Mutated in Colorectal Cancer) was identified from these sequences and it was found that it encoded a 829 amino acid protein with a region of similarity to a G-protein. It was established that the rearrangement found in the tumour disrupted the gene's coding region and furthermore two other tumours displayed point mutations in MCC. At this time, MCC had not been tested in the germline of FAP patients so it was not certain whether MCC was indeed APC. Bourne (1991) suggested two hypotheses for the action of the MCC protein and how it might lead to colon cancer. The first was that it binds to and inhibits G-proteins through the calcium/phosphonositide pathway, which is mitogenic in many cells. The second, and preferred, hypothesis was that of the coiled coil. Here, the protein would form a structure where long regions of alpha helix wrap around each other (hence-a coiled coil). Coiled coils, in general, tend to serve as spacers between other proteins which bind to the globular domains at either end. Cell proliferation is frequently regulated by cell shape thus one can imagine a filamentous cellular structure disrupted by a coiled coil may somehow derepress a mitogenic signal.

Three further candidate genes around the APC region were isolated by Joslyn *et al* (1991) using nested deletions. The first had a sequence identical to "SRP19" a gene coding for part of a ribosomal signal recognition particle. The second was termed DP1 (deleted in polyposis 1), this was transcribed in the same orientation as MCC. Two other stretches of DNA were found and termed DP2 and DP3, however these were found to overlap and hence be a single gene DP2.5 transcribed in the same orientation as SRP19. The small nested deletions (100-260kb) in two patients with FPC encompassed these 3 genes and it was established that MCC was outside this deleted region. All three genes were sequenced.

Discovery that MCC was outside the deleted region shed doubt on its role in FPC despite the fact that its 3' end had not been isolated. It was postulated that the deletion mutations could alter or remove the regulatory elements affecting MCC expression. Alternatively, MCC alterations could be a second step in tumorigenesis. It is, of course, possible that these alterations play no part in tumour formations and are just coincidental. In an accompanying paper, Groden *et al* (1991) identified that it was the DP2.5 that was indeed the APC gene. Single strand conformation polymorphism (SSCP) studies identified four alterations of DP2.5 exons unique to FPC patients. Two of these mutations were base substitutions leading to a change from amino acid to a stop codon, the other two were small deletions leading to frameshifts.

In tandem with the above two papers Kinzler *et al* (1991b) and Nishisho *et al* (1991) (accompanying papers) also identified the APC locus. A portion of the region closely linked to APC (between the markers YN5.48 and YN5.64) was saturated with yeast artificial chromosome (YAC) cloned contiguous stretches of DNA (contigs). Six contigs were isolated in total spanning approximately 5.5Mb of DNA. Three of these encompassing ~4Mb were contained within the central portion of this region. In the first, the gene "FER" was found and in the second, the gene "TB1" (identified by cross hybridisation between human and rodent DNA's). The third contig was the one

of most interest, it was initiated from the MCC gene and hence contained it. It contained nine overlapping YACs (also one cosmid centromeric of the MCC gene called "L5.79") and within this region, four genes were identified, the first was MCC. The second "TB2" was identified, it showed no similarity to any existing sequence and there was no evidence linking it to colorectal tumorigenesis in any way. The third "SRP19" was an already cloned and sequenced gene (see above). The final gene was found to be mutated in the germ line of FAP patients and in sporadic colorectal cancers and was hence identified as the APC gene.

DNA from colorectal tumours and normal colonic mucosa was examined with clones from the genes FER, TB1, TB2, SRP19, MCC and APC (Nishisho *et al.*, 1991). Only MCC and APC seemed to be implicated in colorectal neoplasia. Then the germ line DNA of FPC and GS patients was examined for subtle alterations in MCC and APC. Unique point mutations of the APC gene only were found in five out of 103 FAP patients-four of which were a change from an amino acid to a stop codon, the fifth an Arg-Cys change. APC and MCC are very close (~150kb apart). This, and the fact that both are implicated in colorectal tumorigenesis seems to suggest an interaction between the two. Furthermore the protein encoded by the APC gene (consisting of 2,843 amino acids), like that of the the MCC gene (829 amino acids), is predicted to have coiled coil regions (Kinzler *et al.*, 1991b).

TUBEROUS SCLEROSIS AND GENE MAPPING BY IRRADIATION-FUSION HYBRIDS

CLINICAL FEATURES OF TUBEROUS SCLEROSIS

"It is likely that we will not pass into the third stage of the disease, one of absolute confidence, until the gene locus has been characterised and patients with these manifestations can be tested to to whether they do indeed have TS. In the meantime we have to rely on clinical judgement based on careful observation."

Osborne and Fryer 1990.

Tuberous sclerosis (TS) was first described by Bourneville (1880) recognising white tumours or "tubers" and ares of sclerosis in patients with epilepsy and mental retardation.

The clinical features associated with TS are many and no one symptom is present in all patients. Skin signs commonly presented in TS patients are as follows: "Angiofibromatosis" a facial skin rash is found in 85% of cases (though is rarely obvious before the age of 2) and, from a distance gives an appearance similar to acne. "Hypomelanic patches" are depigmented areas of skin present in 80% of cases. They are unclear in normal light but show up brightly under U.V. They vary in size from one to several centimetres, are commonly oval in shape and occasionally affect the hair, eyebrows, eyelashes and iris. The "forehead fibrous plaque" is a smooth, raised, red, waxy lesion and can be present early in life occasionally beneath the hair. "Shagreen patches" - large areas of thick, discoloured skin commonly over the lower back (40% of TS patients present these). "Fibromas" brown lesions growing from toe and fingernails occur 50% of the time and are sometimes the only diagnostic feature of TS. Also reported are "skin tags" (30% of cases), "pitted teeth" and "gum fibromas."

Phakomas of the retina (50% of TS cases) are initially transparent and difficult to see but frequently calcify making visualisation easier. Neurological features of TS include "siezures in" 50% of cases. Scans of the brain reveal the classical "subepenchymal glial nodules" (80% of cases) and benign "giant cell astrocytomas" (the tubers described by Bourneville in 1880). Physical handicap (usually secondary to mental retardation) is found 20% of the time. TS also affects the organs of the body. The heart is commonly affected with "rhabdomyomas." The kidneys are affected in 60% of cases - "Angiomyolipomas" are the most common sometimes causing haemorrhage, pain or renal enlargement. Bony changes include cysts, periosteal new bone and areas of sclerosis. Cystic disease of the lung is uncommon but reported (more so in females). Finally colorectal polyps are common but theses are non-adenomatous and asymptomatic.

Table 1.2:- DIAGNOSTIC FEATURES OF TS

PRIMARY FEATURE (A definite diagnosis can usually be made if only one of these is present):

Classical shagreen patches, Fibroma of the toe and fingernails, Retinal phakomas, Facial angiofibromas, Subepedymal glial nodules, Renal angiomyolipomata.

SECONDARY FEATURE (Two or more features are required to make a diagnosis)

Hypomalanic macules, Polycystic kidneys, Cardiac rhabdomyoma, Cortical tubers, Cystic lungs, Infantile spasms, Siezures, Forehead fibrous plaque, Giant cell astrocytoma, Atypical shagreen patch, Isolated renal angiomyolipoma, First degree relative with TS.

GENETICS OF TS

The clinical heterogeneity of TS is matched only by its genetic heterogeneity. The hereditary nature of TS was first reported in 1913 however it was Bundey and Evans (1969) who first recognised that this disease was, in fact, autosomal dominant. It was not until 1987 however, that evidence first came to light that the gene resided on chromosome 9.

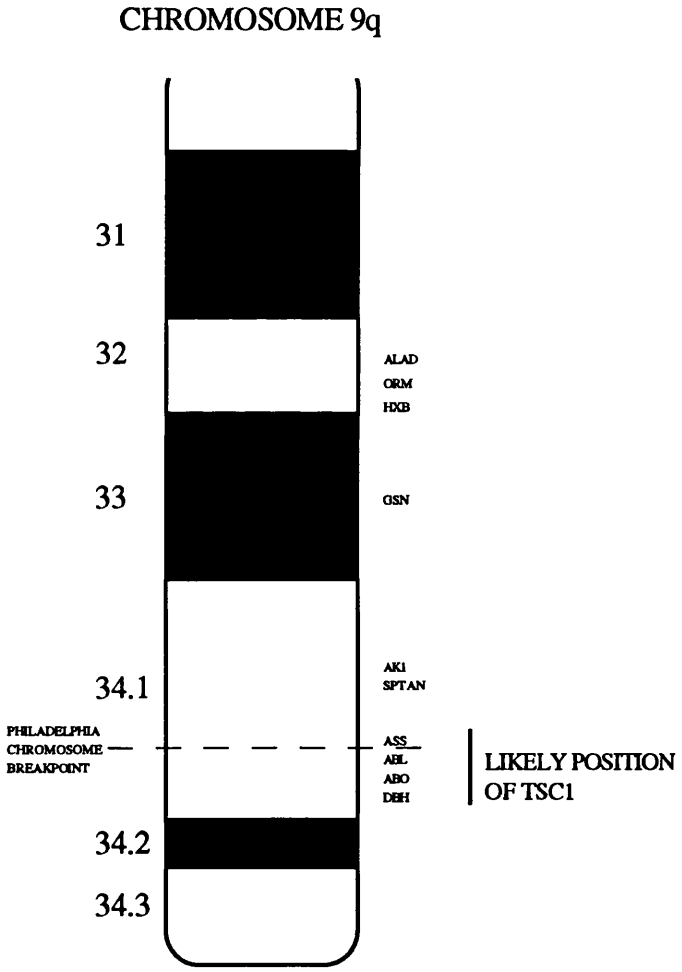
Fryer *et al* (1987) and Connor *et al* (1987) found encouraging lod scores and hence linkage between TS and the blood group locus ABO and also between TS and the red cell enzyme polymorphism adenylate kinase (AK1) and an RFLP at the Ableson oncogene locus (ABL). Since then, other groups have also reported linkage between the TS locus (TSC) and ABO: (Northrup *et al* ., 1987; Renuich., 1987; Kandt *et al* ., 1988; Smith *et al* ., 1987b) and TSC and ABL (Povey *et al.*, 1988). All the markers ABO, AK1 and ABL have been mapped to the chromosome band 9q34 - the q-terminal band of chromosome 9.

When an infant with trisomy 11q23-qter was reported with TS and mental retardation, the authors suggested the possibility of a second TS locus on chromosome 11 (Clark *et al.*, 1988). Furthermore Smith *et al* (1990) established linkage (in 15 American TS families) to probes MCT128.1 and the tyrosonase probe "TYR" which map in the region 11q14-11q23.

Very recent studies (Fashold *et al.*, 1991) on a patient with a chromosome translocation t(3:12)(p26.3:q23.3) and TS prompted search for a TSC locus on chromosome 12. Linkage analysis in the appropriate region of chromosome 12 (12q23) indicate that a third locus may be present on this chromosome.

The locus on 9q34 has been given the name TSC1; it is on this locus that this thesis will dwell. There have, of course, been many DNA markers or "clones" assigned to chromosome 9q and current research (summarised from the first international workshop on chromosome 9, 1992) has suggested the following positions of selected markers. The markers ABO, ASS and ABL are included.

Fig 1.17:- SELECTION OF MARKERS ON CHROMOSOME 9q



ALAD - aminolevulinate, delta, dehydratase; ORM - orosmomucoid; HXB - hexabracion; GSN - gelsolin; SPTAN - spectrin, alpha, non-erythrocytic 1; DBH - dopamine beta-hydroxylase; (taken from HGM11).

In addition to TSC1, there are also two uncloned genes present around this general area. These are the gene NSP1 (nail-patella syndrome) which is closely linked to AK1 (Schleurterman., 1969), and DYT1 (torsion dystonia) which is closely linked to ASS (Kramer *et al.*, 1990).

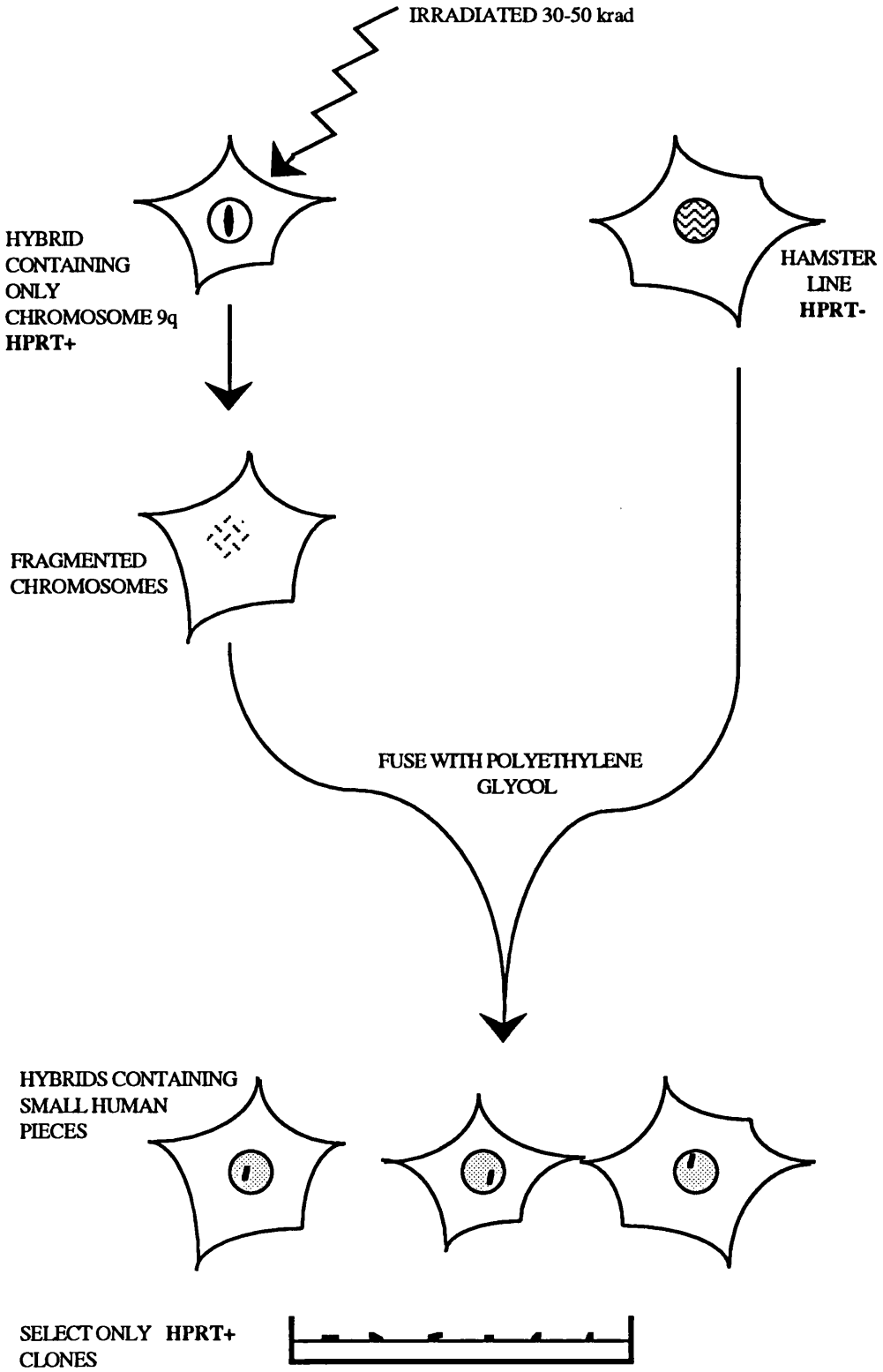
IRRADIATION HYBRID GENE MAPPING STRATEGY

Generating irradiation-fusion hybrids involves the fragmentation of human DNA from a somatic cell hybrid into smaller segments more amenable to analysis and the rescue of these by fusion to a viable rodent cell (Goss and Harris., 1975).

Hybrids used in this study were generated using a selection system based around the enzyme hypoxanthine phosphoribosyl transferase (HPRT) (Benham *et al.*, 1989 and Goodfellow *et al.*, 1990). In the presence of methotrexate, normal DNA synthesis is blocked, however cells synthesizing HPRT and thymidine kinase (TK) can by-pass the normal pathway by making use of a "salvage pathway" which involves converting hypoxanthine in the medium to inositol monophosphate (HPRT) and thymidine to thymine (TK). Thus in the presence of hypoxanthine, methotrexate and thymine (HMT), cells which synthesize HPRT and TK (i.e. those which are HPRT+ and TK+) will survive and those which do not produce either or both of these enzymes (i.e. are HPRT- and/or TK-) will not.

Irradiation-fusion hybrids were prepared in the following way: An HPRT+ hybrid (the *parent*) containing a single human piece was irradiated with 30-50 krad which has the effect of fragmenting the chromosomes and is lethal to the cells. This was fused with a HPRT- hamster cell line and then grown in HMT. Thus the HPRT+ parent hybrid does not survive because of the irradiation dose, the HPRT- hamster cell line does not survive as it is in HMT and only fused cells with DNA fragments containing a functional HPRT gene from the parent hybrid (on the hamster X chromosome) survive (Fig 1.18). Other fragments from the parent hybrid are also retained, some of which will be human. Large doses of irradiation such as 30-50 krad produce hybrids with small fragments from the parent whereas smaller doses produce larger ones. It is with the screening of such irradiation-fusion hybrids containing human elements that this study is concerned.

Fig 1.18:
IRRADIATION - FUSION STRATEGY



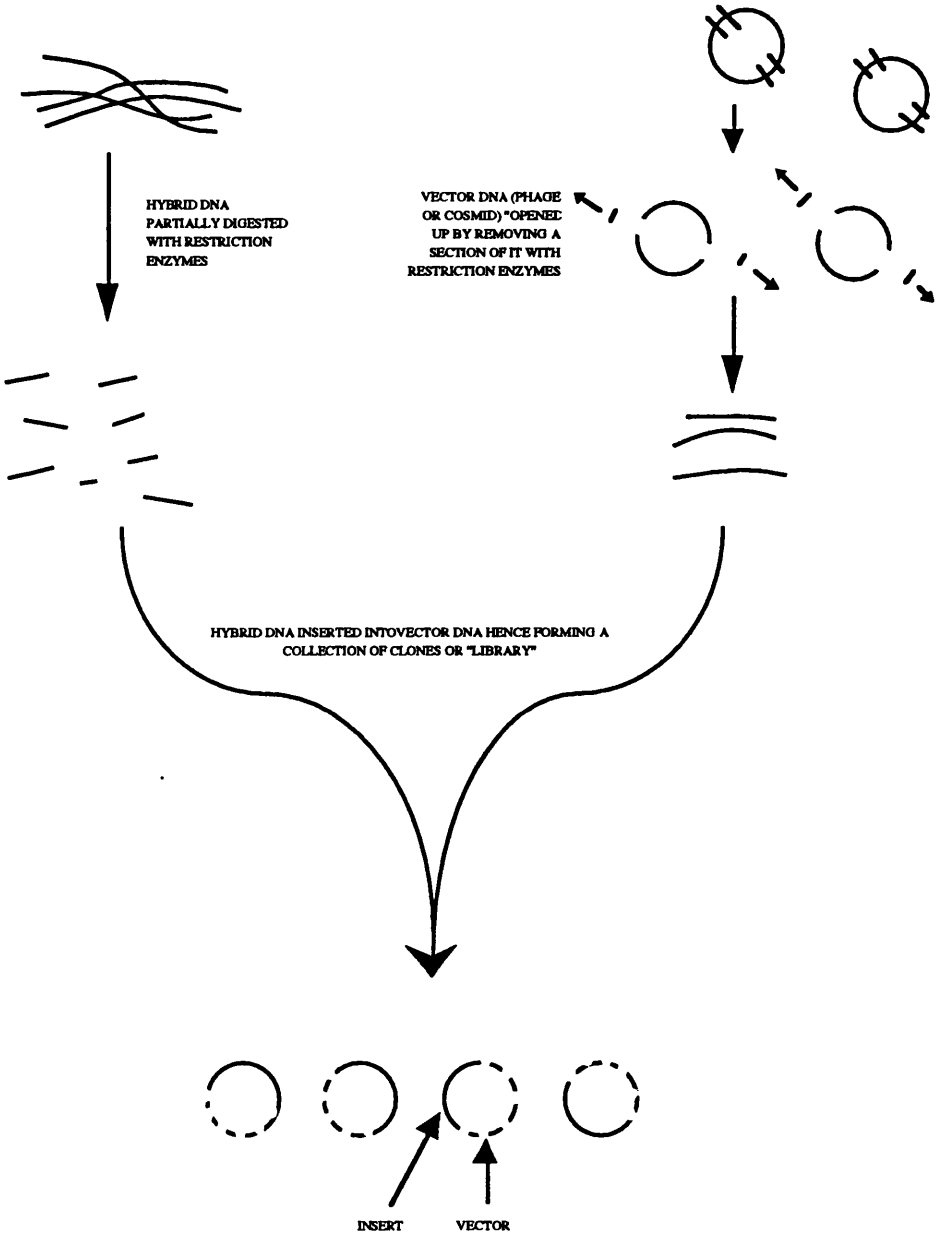
Once hybrids are made, information about the human DNA contained within them needs to be ascertained. Two pieces of information are essential; a) does the hybrid contain the region of interest in the genome? (i.e. are the closely linked markers present in the hybrid?), and b) how many human pieces are contained within the hybrid? The first can be determined by molecular techniques such as PCR and Southern Blotting. It is with the second that this study is concerned.

SUBSEQUENT STEPS IN THE MAPPING PROCEDURE

Once suitable hybrids are selected, they can either be subcloned to produce hybrids with fewer or smaller fragments or used directly to make a "library" i.e. a collection of clones specifically from the hybrid either in "phage" or "cosmid" vectors. The basic strategy for this is illustrated in figure 1.19. Firstly DNA from the hybrid of interest is partially digested with restriction enzymes making it of suitable size to be inserted into a vector. Vector DNA (phage vectors contain only phage DNA, cosmids contain bacterial and phage DNA) is also treated with a restriction enzyme in order to remove small pieces and make the vector able to receive the digested hybrid DNA or "insert." The two are then exposed to each other and the insert DNA thus incorporates itself into the the vector. Once inserted into a vector, the DNA is said to be "cloned." Cloned DNA can be manipulated and amplified with relative ease.

Fig 1.19:

MAKING A LIBRARY OF CLONES FROM A FRAGMENT HYBRID



When a library is made, single clones can be selected from it and tested for the presence of human material. Phage clones are technically easier to make using this procedure. Cosmids, on the other hand, are technically easier to manipulate once made and have the added advantage that they hold more insert. Using such a strategy, the

region of the genome encompassed by the hybrid and thus (hopefully) the region surrounding the disease locus can be saturated with cloned DNA (cf. the APC gene mapping strategy) which can then be tested for linkage to TSC1. Once the region in which the gene lies has been further refined, this area can then be saturated with contiguous clones and the search for the gene can begin in earnest.

FLUORESCENT *IN-SITU* HYBRIDISATION

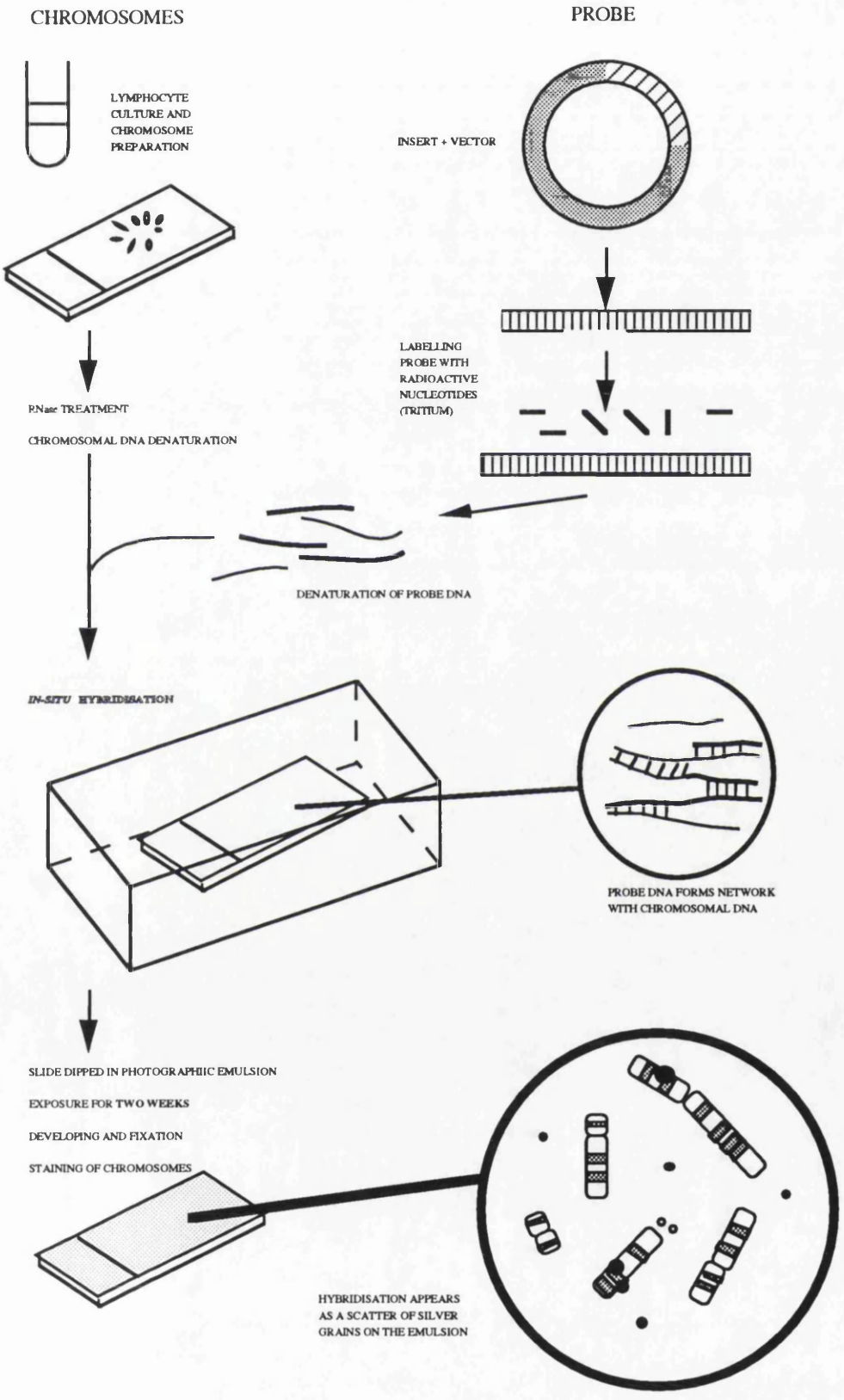
HISTORICAL ASPECTS

"With recent refinements in non-isotopic in-situ hybridisation methods a plethora of new applications are beginning to emerge."

Lichter and Ward (1990)

In-situ hybridisation (ISH) was first described by Gall and Pardue (1969) in a paper entitled "formation and detection of RNA-DNA hybrids in cytological preparations" detecting *Xenopus* rRNA genes. Techniques incorporating DNA-DNA hybrids and using chromosomal preparations soon followed. The original approach remains remarkably unchanged to the present day. The vast majority of cloned genes have been mapped directly to a particular chromosome band (reviewed in Malcolm *et al.*, 1986).

Fig 1.20:
RADIOACTIVE ISH (AFTER MALCOLM *et al* ., 1986)

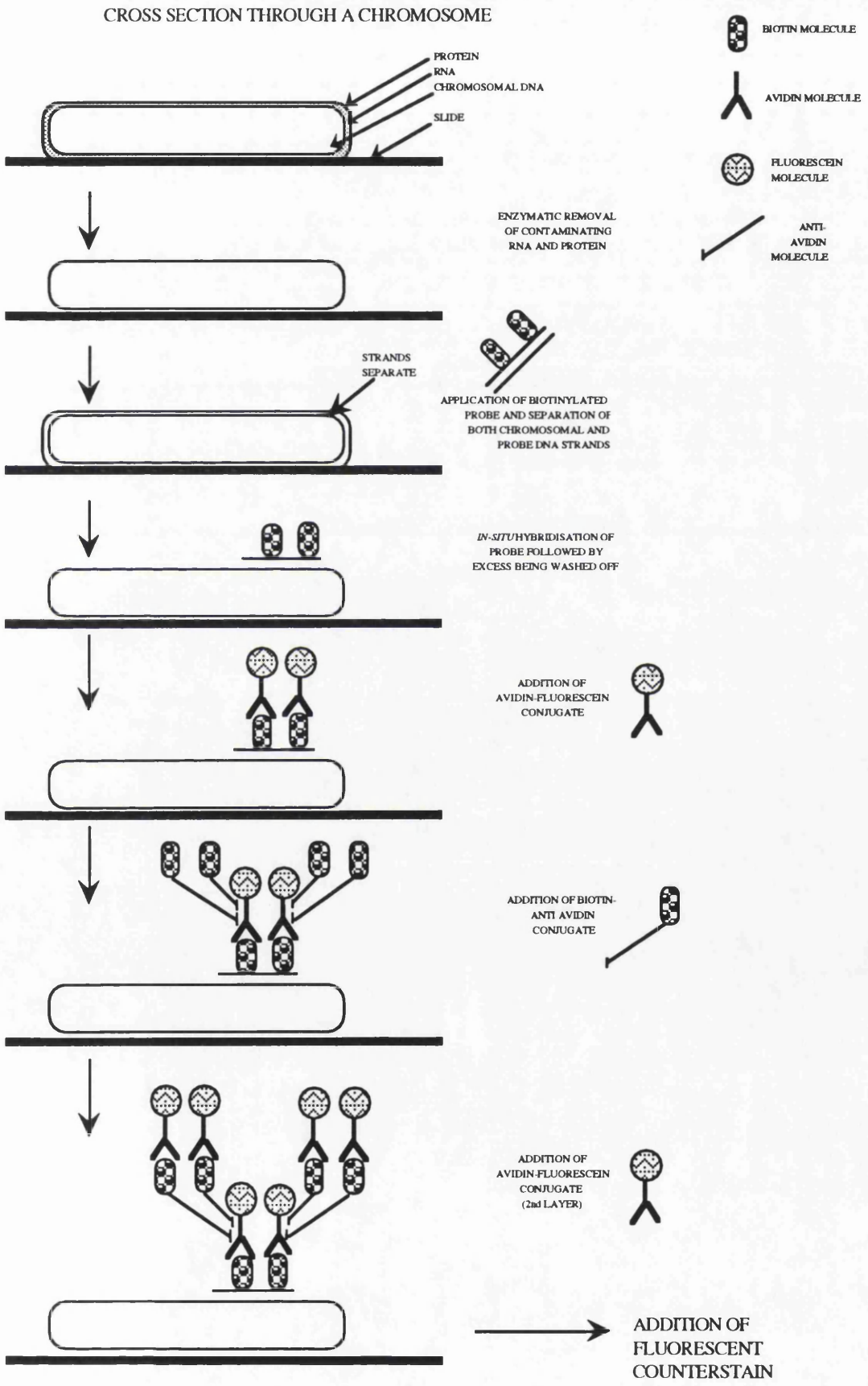


Isotopic ISH typically results in a scatter of silver grains over the chromosome spreads. These need to be analysed statistically by examining 50-100 metaphases producing a mode of hybridisation signals. Also, either pre- or post-hybridisation, chromosomes need to be banded in order to achieve accurate cytogenetic assignment. Furthermore, autoradiographic exposure takes around two weeks and the hazards associated with the handling of radioactive isotopes are well documented.

Fluorescent ISH (FISH) to chromosomes was first described by Rudkin and Stollar (1977) detecting rRNA genes in *Drosophila* using a fluorescent antibody to DNA-RNA hybrids as the detection system. Van Proojen-Knegt et al (1982) first visualised 18s and 28s rRNA genes on *human* metaphase spreads, again forming RNA-DNA hybrids and detecting via a fluorescent RNA-DNA antiserum. Detection using RNA-DNA hybrids proved to be insensitive for most applications in comparison to isotopic ISH and it became clear that a totally different approach would have to be developed.

Pinkel *et al* (1986) developed a system of FISH which involved directly labelling DNA probes with vitamin H (biotin) molecules. DNA-DNA hybrids are formed and biotin detection facilitated by means of fluorescently labelled "avidin" molecules which show an extremely high affinity for biotin. Furthermore, the fluorescent signal can be amplified by means of "biotinylated anti-avidin" followed by a second layer of fluorescent avidin. This method has since proved to be the most widely applicable of all FISH approaches and hence the vast majority of advances in this field have incorporated it. Briefly, cytogenetic preparations are made by routine protocols. Exogenous RNA and protein is enzymatically removed. Chromosomal and labelled probe DNA is denatured and allowed to hybridise *in-situ.* Fluorescent detection is achieved by means of a fluorescein/avidin conjugate. Sequential layers of biotin/anti-avidin conjugate followed by a second layer of avidin/fluorescein facilitate signal amplification. This amplification step is referred to as the *"Pinkel sandwich"* (Trask., 1991). Finally the chromosomes are counterstained with a fluorescent total DNA stain for relocation purposes. This is summarised in figure 1.21:

Fig 1.21:- **FISH** (after Pinkel *et al.*,1986)

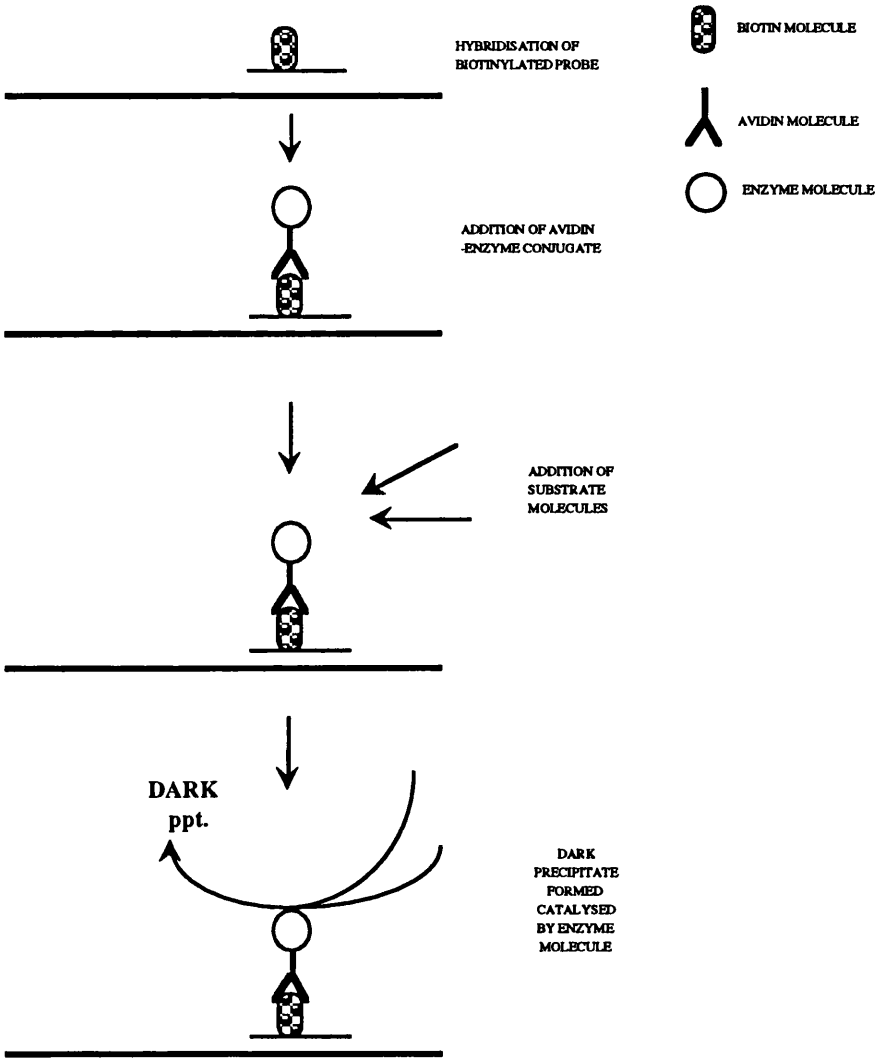


ENZYMATIC DETECTION OF BIOTINYLATED PROBES

Non-radioactive ISH (NISH) can also be achieved without the aid of fluorochromes and fluorescent microscopes. Enzymatic means of biotin detection have been described and involve use of an enzyme conjugated avidin molecule and substrate molecules which will form a dark precipitate when mixed and catalysed by that enzyme.

Fig 1.22:

BASIC ENZYMATIC NISH STRATEGY



Two different approaches have been described: Mannuelidis *et al* (1982) described the use of horseradish peroxidase as the enzyme and diaminibenzidine and hydrogen

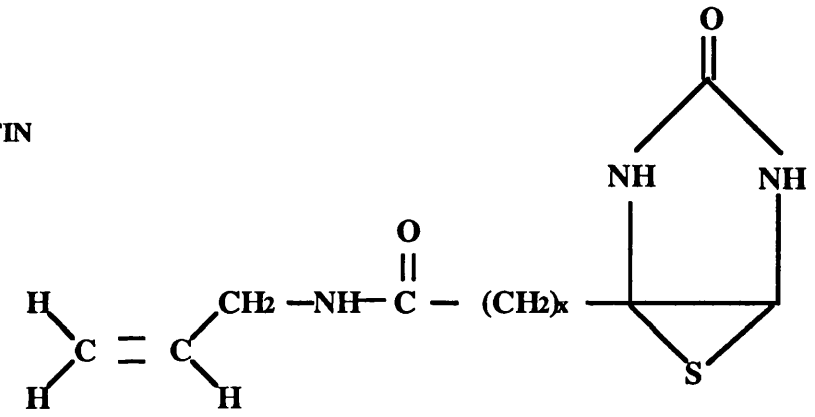
peroxide as the substrate molecules detecting satellite sequences in mouse. Garson *et al* (1987) described detection of nerve growth factor and n-*myc* oncogene sequences as small as one kilobase. The enzyme used was alkaline phosphatase and substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

STRUCTURAL AFFINITY OF BIOTIN AND AVIDIN

Biotin is an essential vitamin (vitamin H) and has a structure thus:

Fig 1.23:

BIOTIN

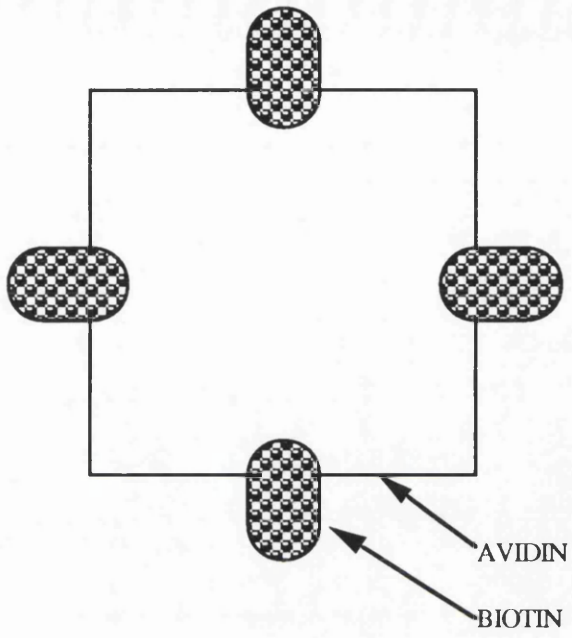


x = 4,7,11,16 or 21

Avidin is a tetrameric protein (M.W. 4x15,000) from avian egg white.

The two have a high affinity for each other (dissociation constant $Kd=10^{-15}M$). Four biotin molecules bind to each of the four residues of avidin and the bond is essentially irreversible.

Fig 1.24:- THE FOUR BIOTIN BINDING SITES OF AVIDIN



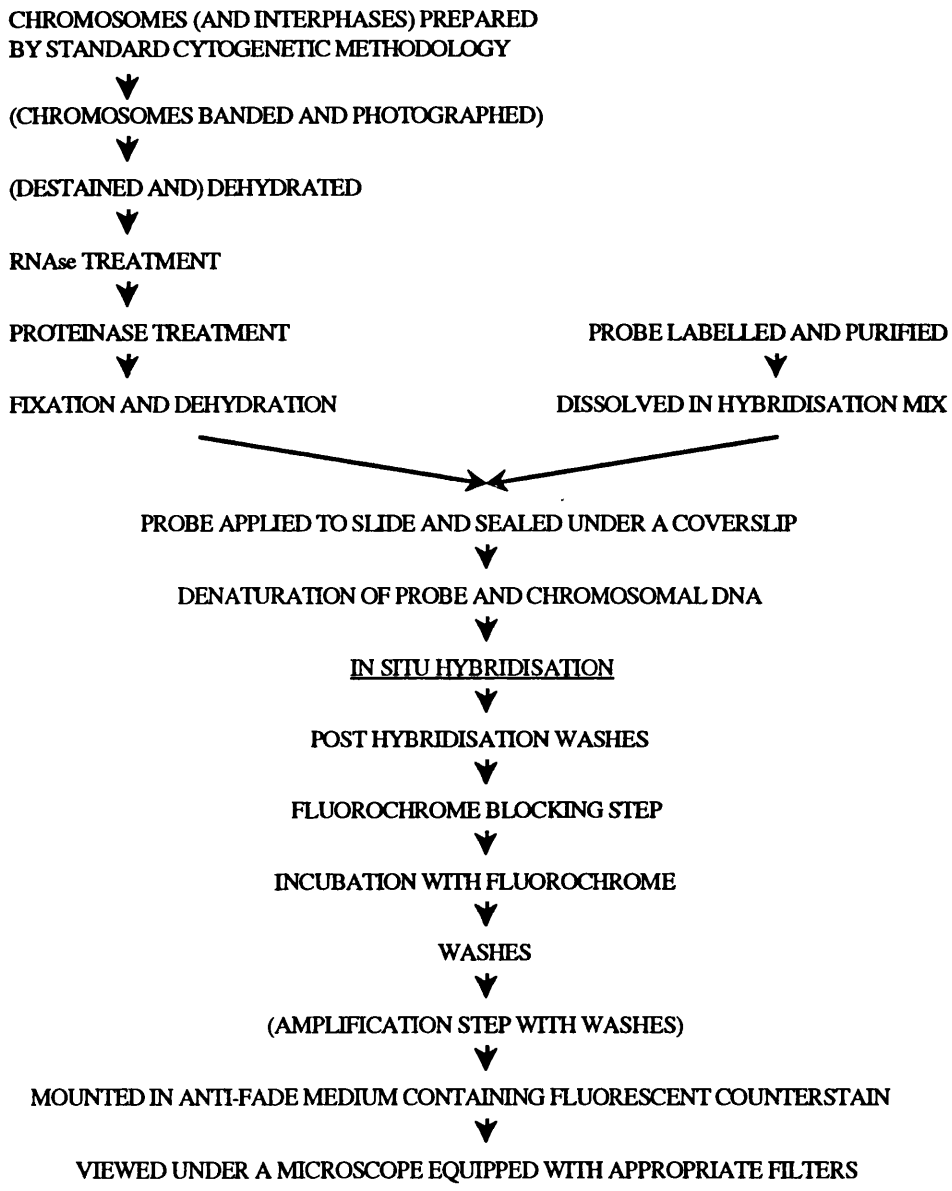
Binding is non-covalent but multiple hydrogen bonds and Van der Waal's forces, coupled with the fact that the biotin is physically buried in the protein interior ensure an attraction ~200 times stronger that any antibody-antigen relationship (Weber *et al.*, 1989).

TECHNICAL ASPECTS OF FISH

The following figure illustrates a technical breakdown of FISH based on Pinkel *et al* (1986) and on the University of Leiden practical course (Van der Ploeg *et al.*, 1990).

Fig 1.25:

THE FISH TECHNIQUE



Materials are prepared by standard cytogenetic methodology. Preparations are fixed in acid/alcohol and air dried on a glass slide. For optimal hybridisation, little or no cytoplasm must be surrounding the nuclei and chromosomes. Efficiency of hybridisation is also thought to decrease when slides are stored for a considerable length of time.

Chromosomal banding treatments prior to hybridisation (pre-banding) have been described in the literature. These include standard Giemsa-trypsin banding, lipsol banding, Wright's staining, Acridine orange R-banding and Quinacrine mustard Q-banding. Isotopic approaches frequently employed a pre-banding strategy i.e. chromosomes were banded using a method that would not cause them to be damaged beyond recognition when subsequent ISH was performed. Between 50 and 100 metaphases were then photographed, the ISH protocol followed then metaphases relocated and analysed. This approach was obviously tedious but necessary as post-banding techniques (i.e. banding the chromosomes after ISH) were often unreliable (Malcolm *et al.*, 1986).

There is still no universal, reliable post-FISH-banding approach (Trask., 1991), indeed Lichter *et al* (1990) did not attempt banding at all and assigned chromosomal location on the grounds of signal position relative to chromosome length. Other means include chromosomal identification by simultaneous hybridisation of probes known to hybridise to certain chromosomes such as the chromosome specific centromeric probes discussed in the next section (e.g. Kievits *et al.*, 1990b).

Several post-banding methods however have been described. These include propidium iodide R-banding (Cherif *et al.*, 1990), acridine orange R-banding (Malcolm *et al.*, 1986), replication Q-banding using actinomycin/DAPI staining (Tucker *et al.*, 1988), simultaneous hybridisation of Alu (R-banding) or LINE (G-banding) sequences, alkali or exonuclease treatment (R-banding) (Berube and Gagne., 1990), synchronising lymphocyte cultures with bromodeoxyuridine then using a combination of fluorescent counterstains (namely DAPI (giving a Q-band pattern) and PI (giving an R-band pattern by quenching fluorescence in G-band regions)) (Fan *et al.*, 1990), classical R-banding (Viegas-Peguinot *et al.*, 1989b) and trypsin-Giemsa (classical G-banding) (Smit *et al.*, 1990).

RNase treatment obviously removes exogenous RNA from the preparation and hence increases accessibility of DNA targets. Proteinase K treatment also increases target accessibility by digesting cytoplasmic and histone proteins.

Denaturation of probe and genomic DNA can be achieved separately or simultaneously. When the probe contains repetitive elements (e.g. "Alu" sequences) it must be denatured in the presence of excess unlabelled human DNA and preannealed (the nature of human repetitive DNA is covered in a later section). Chromosomal DNA can be separately denatured. When probe preannealing is unnecessary, both DNA's can be denatured simultaneously by heating.

Hybridisation cocktails for FISH generally contain formamide, monovalent cations (chiefly sodium), carrier DNA (usually salmon sperm), dextran sulphate and a buffer (usually phosphate). Formamide is an organic solvent which lowers the melting temperature of DNA. Lowering the DNA melting temperature is necessary as exposure of chromosomes to high temperatures for long periods of time may lead to significant deterioration in morphology. Monovalent cations interact electrostatically with the phosphate groups of nucleic acids. Consequently renaturation rates are partially dependant on sodium concentration. Carrier DNA (e.g. sonicated salmon sperm DNA) is thought to reduce signal to noise ratio by blocking aspecific hybridisation, yeast RNA is thought to have a similar effect. Dextran sulphate in the reaction mix has the effect of forming large networks of probe DNA at the site of hybridisation. The mix is buffered (usually with phosphate) as the pH can affect the rate of hybridisation.

Labelled probe invariably hybridises aspecifically to sequences to which they show some homology. Aspecific hybridisation can be dissociated by post-hybridisation washes. The stringency of these washes can be varied according to the nature of the probe. Increasing formamide concentration, decreasing salt concentration and increasing temperature all increase wash stringency.

For immunocytological detection, a blocking step prior to addition of fluorescent conjugate is used. Duhamel and Johnson (1985) report the use of non-fat dry milk as an excellent agent for blocking aspecific binding of avidin conjugates.

To prevent fading of flourescence, anti-fade agents are used. Two in common use are 1,4 diazabyclo-2,2,2-octane (DABCO) (Johnson *et al.*, 1982) and p-phenyl diamine dihydrochloride (pPD) (Johnson and Noguaria-Aruajo., 1981) and are carried in glycerol as a mounting medium. The mounting medium also contains a fluorescent DNA counterstain such as 4,6 diamidino-2-phenylindole (DAPI) which is a fluorescent blue colour and/or propidium iodide (PI) which emits red.

CHOICE OF HAPTENS

A hapten is the name given to the molecule which labels the DNA. Biotin is not the only hapten available but is the most common. Also described are digoxigenin, 2 acetlyaminofluorene (AAF), the sulphone radical and mercury.

Digoxigenin is a steroid derived from *Digitalis* plants and can be introduced into a probe in similar ways to biotin. It can be detected by fluorescent conjugated antibodies to it or to "digoxin" from which it is derived. Signal amplification can be achieved using secondary and tertiary fluorescent antibodies.

AAF can be used to label a probe by virtue of the fact that it (a carcinogen) binds covalently in the C8 (8th carbon atom) position of guanosine residues in DNA or RNA. Detection is via fluorescent antibodies. (Landegent *et al.*, 1984; Tchen *et al.*, 1984).

Mercury modification is one of the oldest of the non-radioactive approaches. Probe is chemically mercurated at the C5 (5th carbon atom) position of the pyrimidines. To facilitate detection, a mercury binding ligand is attached carrying a sulphydryl group on

one end and a hapten on the other. This "secondary hapten" can be trinitrophenol (TNP) or biotin both of which can be detected by fluorescent conjugates or a fluorescent material itself.(Hopman *et al.*, 1986; Raap *et al.*, 1990).

Labelling with sulphone radicals (sulphonation/transamination) relies on the fact that bisulphite reacts reversibly with the C5-C6 double bond of cytidine residues in DNA to give 5,6 dihydrocytidine-6-sulphonate. Detection can be achieved using fluorescent antibodies.

Of all these approaches, biotin and digoxigenin are thought to be the most sensitive (J. Wiegant., personal communication).

CHOICE OF FLUOROCHROMES AND FLUORESCENT MICROSCOPY

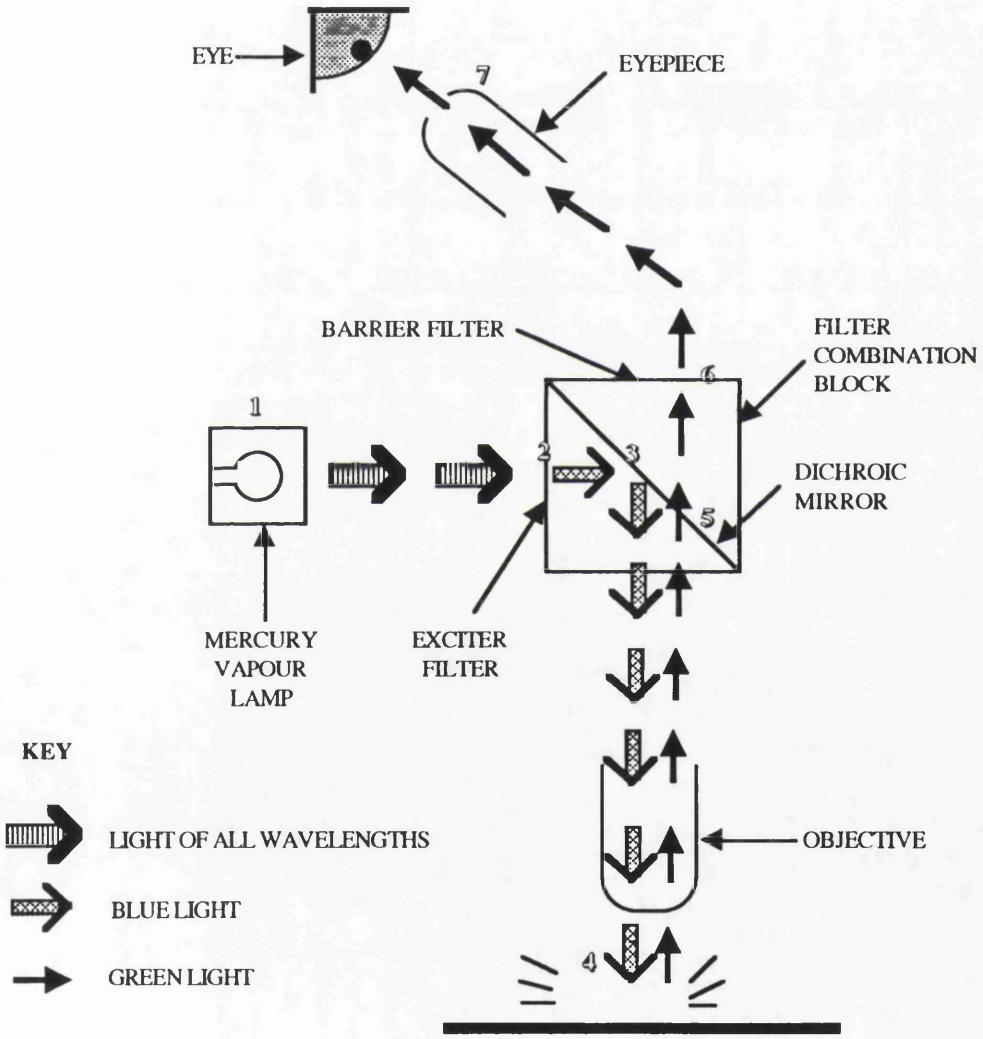
By definition, a fluorochrome is a material which absorbs light at one wavelength, becomes "excited" by this and then emits light at a higher wavelength. Fluorescent materials are therefore described as having absorption spectra (the range of wavelengths at which they excite) and emission spectra (the range of wavelengths at which they emit).

Fluorescein isothiocyanate (FITC) is the most commonly used. Also common is tetramethyl rhodamine isothiocyanate (TRITC). This excites in the green range and emits in the red. Texas red (or sulphorhodamine 101) excites in the green and gives a very deep red colour. A relatively new fluorochrome is "AMCA" (7-amino-4-methyl coumarin-3 acetic acid) (Khalfan *et al.*, 1986). It excites in the ultraviolet and emits in the blue range. Of the two fluorescent counterstains mentioned earlier, DAPI absorbs U.V. and emits blue, PI absorbs in violet-green wavelengths and emits red. A combination of both counterstains can be used with FITC preparations (hence red

chromosomes and yellow/green signal can be viewed simultaneously) but propidium iodide cannot be used with TRITC or Texas Red as it drowns out the signal.

Fluorescence microscopy is achieved using fluorescent filter blocks inserted into the microscope. A fluorescent filter block has three elements to it namely: 1) An exciter filter (EF), 2) A dichroic mirror and 3) A barrier filter. The microscope set up is as in the following diagram and demonstrates fluorescent viewing of an FITC signal:

Fig 1.26:
LIGHT PATH DOWN A FLUORESCENT MICROSCOPE



1) Mercury vapour lamp emits all wavelengths, 2) Exciter filter (EF) allows only blue light through (450-490nm), 3) Dichroic mirror (DM) reflects blue light (below

510nm), 4) Blue light is shone on specimen via microscope objective. FITC excites and emits green light (peak at 515nm). Green light passes back up objective, 5) Dichroic mirror (DM) allows green light (above 510nm) through, 6) Barrier filter (BF) filters out green light (above 515nm), 7) Fluorescent signals viewed.

The following table illustrates likely filter combinations for each fluorochrome. The figures given represent light wavelengths in nanometres allowed through by the filters and dichroic mirror.

Table 1.3:- FILTERS AND MIRROR USED FOR EACH FLUOROCHROME

	EF	DM	BF	
FITC (GREEN)[1]	450-490nm	>510nm	>515nm	as illustrated above
TRITC & TEXAS RED[1]	530-560nm	>580nm	>580nm	
AMCA (BLUE)[1,2]	340-380nm	>400nm	>420nm	

1. Will detect PI; 2. Will detect DAPI

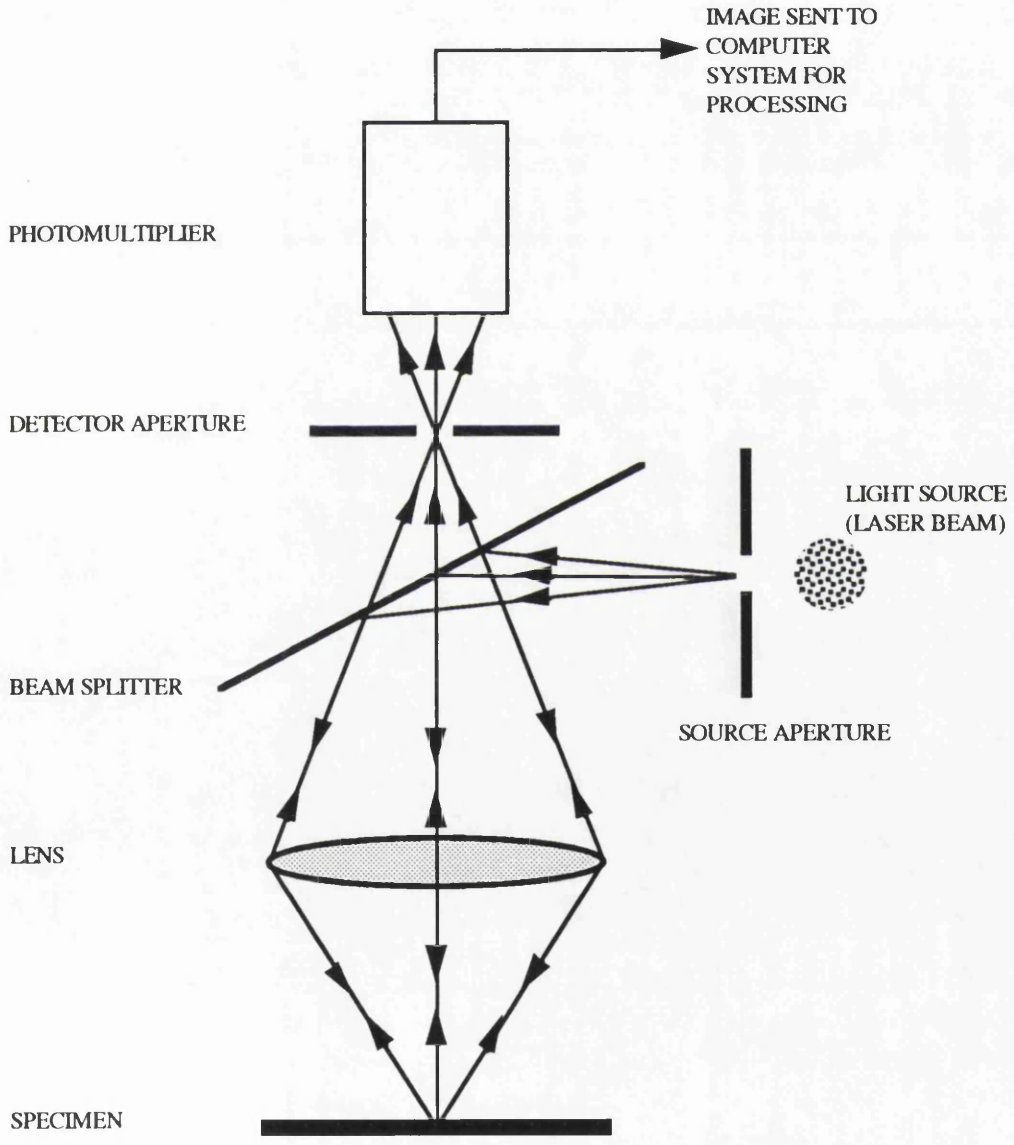
Recently, the possibility of labelling probes directly with the fluorochrome has become apparent. Labelling is achieved by similar means as biotin and digoxigenin, signal amplification can be achieved using anti-fluorochrome antibodies. Fluorecein-12-dUTP is marketed by Boehringer-Mannheim and its applicability has been assessed by Wiegant *et al* (1991). They found the sensitivity to be 50-100kb when the signal is unamplified and as low as 1-5kb when amplified. Using this approach also has the advantage that a low background yield is obtained.

Given that a number of probe labels and fluorochromes exist, the possibility of multiple labelling (i.e. simultaneous hybridisation and detection of two or more probes on the same cytological preparation) arises. Applications of this are discussed in subsequent sections.

CONFOCAL MICROSCOPY

The principle feature of confocal microscopy is that detection of the fluorescent image is limited strictly to what is in focus. Whereas in conventional fluorescent microscopy, out of focus image appears as fluorescent "glare", in confocal microscopy all out of focus image appears black.

Fig 1.27:
LIGHT PATH OF A CONFOCAL MICROSCOPE



A confocal imaging system does not obtain a complete optical image of the specimen. It is therefore necessary for the light source (i.e. laser) to scan the specimen and build

up an image which is processed by the computer. Fluorescence is detected by inserting a filter in the system similar to one employed in conventional fluorescent microscopy.

The chief application of a confocal microscope therefore is to collect optical sections of specimens while keeping them intact and hence building up three dimensional images of them. Although the majority of ISH specimens are essentially two dimensional, a confocal microscope can be an invaluable tool in this field because of its image analysis capabilities. Albertson *et al* (1991) compared conventional and confocal microscopy in the context of FISH and found that although the sensitivity did not vary greatly between the two, the greater resolution provided by the confocal microscope enabled acquisition of sharper images especially when analysing chromosome bands. The confocal microscope also has added advantages in that the computer software can manipulate, enhance and measure images as required by the operator.

NATURE OF PROBES USED IN FISH

Probe DNA in molecular biology is packaged into unrelated "vectors". Such vectors include plasmids, phages, cosmids and yeast artificial chromosomes (YACs). Therefore the whole probe is said to have two elements namely the vector and the "insert" (i.e. the complementary human DNA sequence). Unlike in classical molecular biology techniques, the vector is not cut from the insert before labelling as residual attached labelled vector DNA is thought to increase the signal size when present (Joop Wiegant., personal communication). Plasmid vector DNA is, on average, around 2kb in size and the insert it carries can vary from around 500 base pairs to around 5kb. Phage clone probes have an insert size of 3-20kb and a vector size of around 40kb; cosmids: insert ~ 40kb, vector ~5-6kb; YACs: insert 500+ kb, vector ~ 7-8 kb.

HUMAN REPETITIVE DNA

It is estimated that around 40% of the human genome is made up of sequences that are present in more than one copy i.e repetitive sequences (Britten and Davidson., 1971). These sequences are classified according to their organisation (either tandem i.e. in long head to tail arrays or interspersed within unique sequences), copy number (which can be between 10 and 10,000 copies) and length of the repeating unit (between 2 base pairs and 5kb). At present, there is no clear agreement as to why these sequences persist in eukaryotic genomes.

Interspersed sequences are divided into two families namely short interspersed repeated elements (SINEs) or long interspersed repeated elements (LINEs) (Singer., 1982). the most highly repeated SINE is the "Alu" repetitive DNA family (Schmidt and Jelinek., 1982). These repeating units are ~ 300bp long and are repeated throughout the genome. They are found between genes and within introns but never within exons (Moyzis *et al.*, 1989). LINE repeats are around 5kb or longer. The most common of these are the L1 elements present in approximately 50,000 copies per haploid genome and are actively transcribed. Predicted protein products resemble retroviral proteins (Fanning and Singer., 1987).

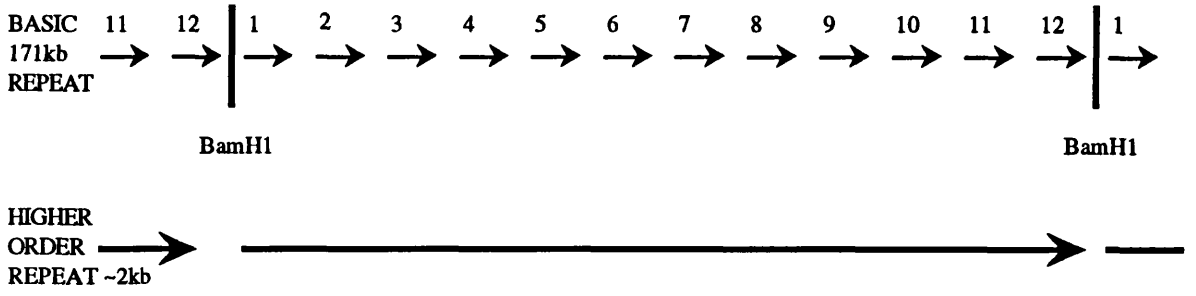
Tandemly repeated DNA is found in several different organisations:

1) Multigene families consist of virtually identical gene copies arranged head to tail,for instance the clusters of ribosomal RNA detected in early ISH studies. 2) The repeating unit may consist only of $(CA)_n$. So called CA repeats (or GTn polymorphisms) are known to be highly polymorphic in the population and are used in the study of family lineages. 3) Short stretches of tandem repeats 15-70kb long repeating 10-200 times and varying from individual to individual (Jeffreys *et al.*, 1985). These are termed mini-satellite regions. Such "hypervariable" sequences are the ones exploited in genetic fingerprinting experiments. Isotopic ISH studies indicate that these repeats

tend to cluster around the pro-terminal regions of certain autosomes (Royle *et al.*, 1988). 4) Classical human satellite DNA is found within specific constitutive heterochromatic regions of eukaryotic chromosomes. Four classes of satellite DNA are identified in man namely satellites I, II, III, IV and these regions constitute 6% of the human genome. The long arm of the Y chromosome and the chromosome 9 C-band are the major sites of localisation for classical satellite DNA. Eleven chromosomes (including the X) however, do not seem to contain appreciable amounts (Gosden *et al.*, 1975). 5) Alpha satellite or "alphoid" DNA consists of tandem repeats but is not regarded as classical satellite DNA as it can be isolated as a distinct buoyant density fraction in isopycnic centrifugations. Alphoid repeats constitute a group of related, highly divergent sequences each approximately 171kb in length. These sequences show 20-40% divergence from one another. Arrays of alphoid repeats are found exclusively around the centromeric regions of all the chromosomes. Tandem arrays of these monomer units show chromosome specific higher order repeat units and are revealed by restriction endonucleases. The following diagram shows the hierarchical order of repeat units for chromosomes X, 7 and 10.

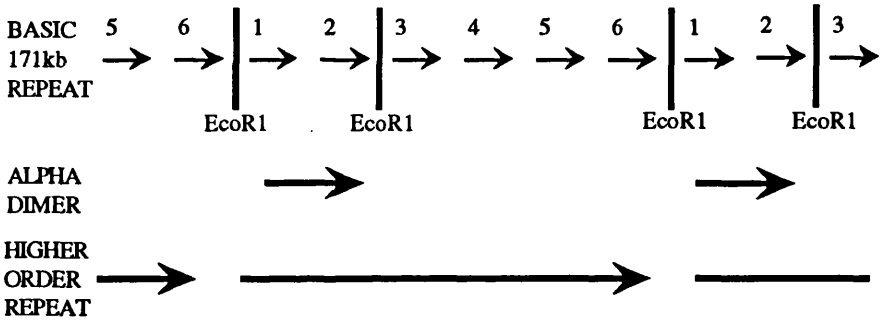
Fig 1.28:- HIGHER ORDER REPEATS IN THREE CHROMOSOMES

X CHROMOSOME



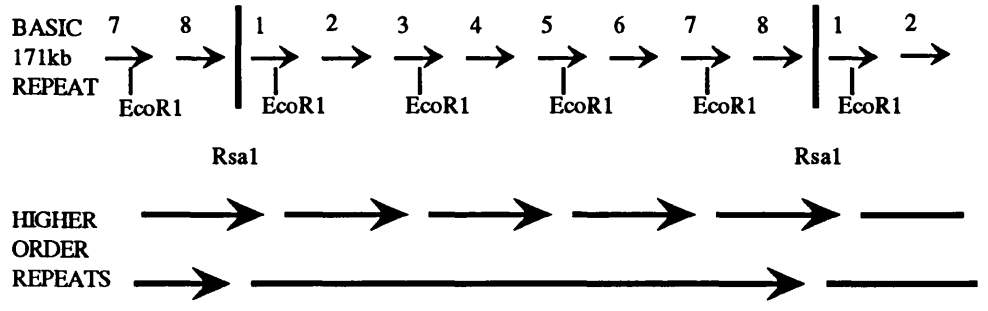
(Willard & Waye.,1987)

CHROMOSOME 7



(Willard & Waye.,1987)

CHROMOSOME 10



(Devilee *et al.*,1988)

As mentioned, those higher order repeats are largely chromosome specific and the exploitation of them for "interphase cytogenetics" is discussed in a later section.

CISS

Interspersed throughout the whole genome are highly repeated sequences of which the "Alu" sequences are the most common (see previous section). Probe inserts (invariably YACs and cosmids, frequently phages and occasionally plasmids) contain these and other repeats. Were these probes to be applied directly on to chromosomes via FISH, the whole chromosome complement would light up due to these repeats finding complementary sequences all over the genome. As mentioned in the section "TECHNICAL ASPECTS" in order to inactivate this and allow the unique portion of the probe to find its complementary sequence, the probe must be preannealed with total unlabelled DNA in excess. Such an approach was first described for FISH by Lichter *et al* (1988b) and Cremer *et al* (1988a) (accompanying paper) and is known as competitive *in-situ* suppression or **CISS.** Chromosomal DNA is denatured separately then preannealed probe is applied leaving unique sequences free to hybridise.

FISH - APPLICATIONS AND PREVIOUS WORK

DETECTION OF SINGLE COPY PLASMID CLONED PROBES

In general, the mapping of single copy plasmid clones has not been as popular using FISH as has the mapping of larger cloned sequences. Notable exceptions to this are Viegas-Peguinot *et al* (1989b) successfully mapping the human desmin gene (from which they had cloned a 3kb fragment) to band 2q35. Later they mapped 6kb and 2.3kb clones to chromosomes 18 and 22 respectively and furthermore described a method producing an R-banding pattern post hybridisation. Cherif *et al* (1989) reported mapping 1.3kb and 1.8kb clones derived from the proto-oncogene c-ets1 to

chromosome 21 while Fan *et al* (1990) successfully mapped clones as small as one kilobase to chromosome 11 on banded chromosome spreads.

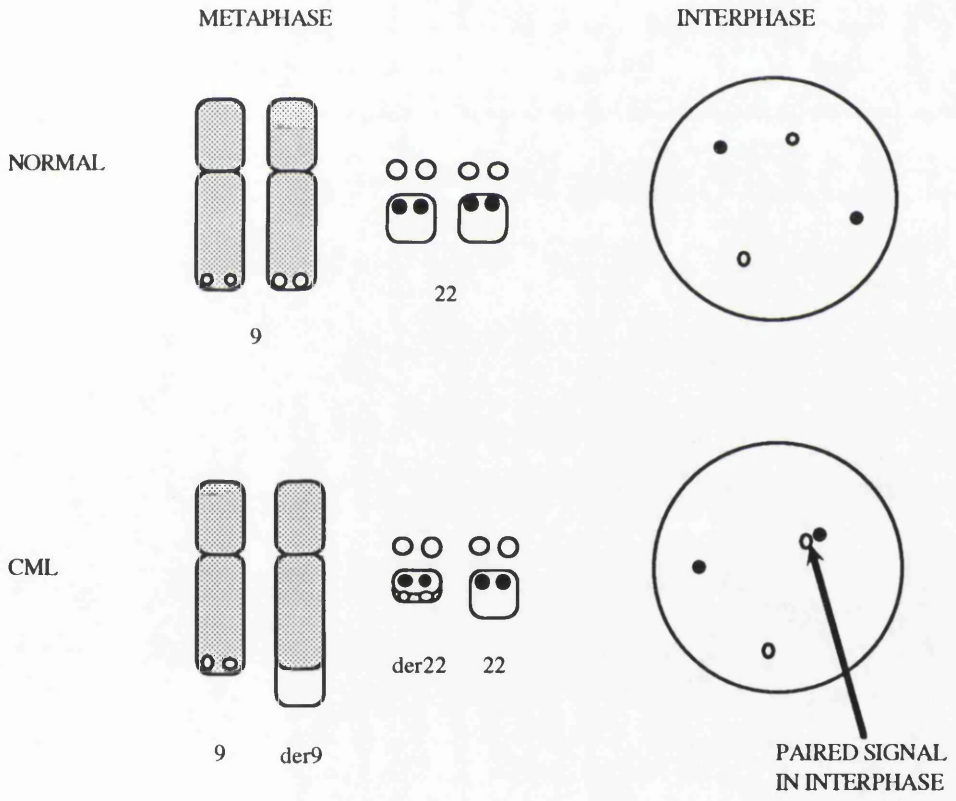
MAPPING OF LARGER CLONED SEQUENCES

In the field of gene mapping, it is in the direct visualisation of larger cloned sequences on chromosomes namely phages, YACs and more commonly cosmids that most progress has been made. Mapping of longer sequences by FISH invariably requires the employment of the CISS approach.

Lichter *et al* (1990) were the first to describe the mapping by FISH of cosmids on chromosome 11. They used confocal microscopy for single and double labelling (biotin and digoxigenin labels were employed). Since the realisation that cosmids could be mapped using FISH, the majority of interest in this field has been in that direction. Signals are typically visible on both chromatids of both homologues. When conditions are optimal, a cosmid signal can be visualised in the interphase nucleus as well as on the metaphase chromosome. Using single colour FISH, signals between 25 and at least 250kb can be resolved in interphase nuclei. Furthermore, the distance between two clones can be ascertained solely by interphase mapping with an accuracy of within 40kb (Trask *et al.*, 1989; Bentley-Lawrence *et al.*, 1990). Using dual colour fluorescence, signals 50kb apart can be resolved at interphase and signals 3 megabases apart can be resolved at metaphase. Clones which are close together can furthermore be ordered with respect to one another by examining interphase nuclei. This is achieved by varying the hapten (usually biotin and digoxigenin are used) with which each of three or more probes are labelled (e.g. labelling two probes with biotin and the other with digoxigenin). The probes can be simultaneously hybridised on the same preparation then detected with red and green fluorochromes respectively. Simply scoring the order of red and green dots in a series of experiments where different combinations of haptens are used thus gives a physical order of clones. Such an approach is referred to as "interphase mapping" (Trask., 1991).

Chromosomal translocations can be mapped in interphase nuclei using dual colour interphase mapping technology. Both Arnoldus *et al* (1990) and Tkachuk *et al* (1990) have reported detection of the "Philadelphia chromosome" in the interphases of the bone marrow of patients with chronic myeloid leukemia (CML). The Philadelphia chromosome is the der(22) from a reciprocal translocation t(9;22)(q34:q11). Both groups using cosmid and phage clones detected the Philadelphia chromosome (diagnostic of CML) in interphase nuclei as well as metaphase chromosomes thus:

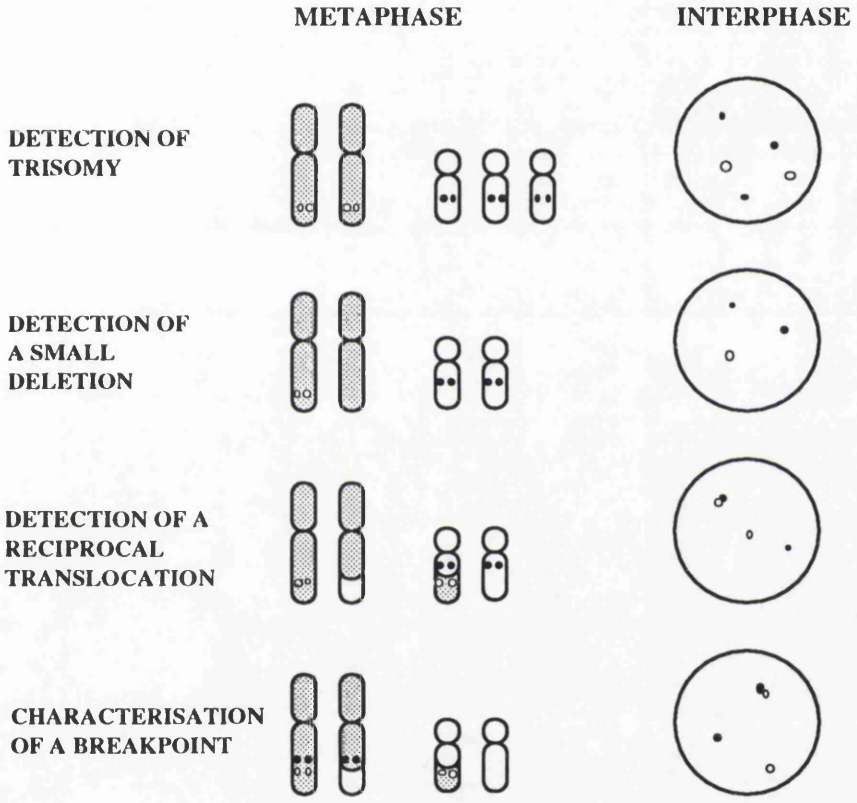
Fig 1.29:
DETECTION OF THE PHILADELPHIA CHROMOSOME IN INTERPHASE NUCLEI



Lichter and Ward (1990) present a model for the detection of chromosomal rearrangements and molecular characterisation of chromosomal breakpoints in interphase nuclei provided that suitable cosmids are available. This is illustrated in the following figure.

Fig 1.30:

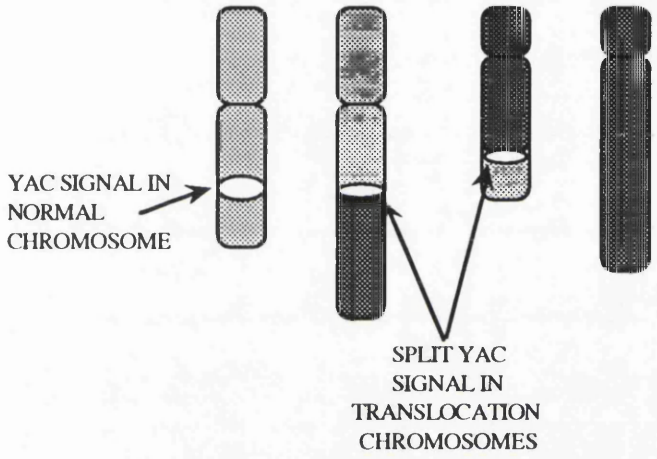
DETECTION OF CHROMOSOMAL ABERRATIONS AND BREAKPOINT CHARACTERISATION USING COSMIDS (Lichter and Ward., 1990)



Klinger *et al* (1991 (HGM11)) demonstrated for the first time simultaneous detection of probes for chromosomes 21, 18, 13, X and Y each in a different colour. The probes used were contig cosmid clones and five colour detection was facilitated by labelling each probe with either one or a different combination of two labels, detecting with appropriate fluorochromes, analysing each digitally for the presence of one or two colours then assigning each a pseudocolour using computer software.

The mapping of yeast artificial chromosomes (YACS) is also documented in the literature. Being of very large insert size YACs tend to give large signals however aspecific background signal on unrelated chromosomes is often a problem when hybridising (Riethman *et al.*, 1989; Wada *et al.*, 1990). YACs can be used to narrow down the molecular location of chromosomal breakpoints in translocations thus, this is illustrated below:

Fig 1.31:- DELINEATION OF A BREAKPOINT USING A SINGLE YAC



Visualising a split YAC signal hence indicates that the breakpoint must be encompassed within the few hundred kb to which that YAC hybridises (Rowley *et al.*, 1990).

EXPLOITING TANDEM REPEAT DNA

Classical satellite, mini-satellite and alpha-satellite sequences have been studied using ISH. Since these tandem repeat sequences are largely chromosome specific, CISS is not necessary prior to ISH however high stringency conditions are often needed to maintain chromosomal specificity.

It is in the exploitation of classical-satellite and alpha-satellite DNA that there has perhaps been the most interest in FISH. When conditions are ideal, these probes light up brightly specific regions (usually centromeres) of human chromosomes. Signals

are large enough to be seen and counted in interphase nuclei. Probes are now available for nearly all the human chromosomes including both sex chromosomes. Two notable exceptions are chromosomes 21 and 13. Probe L1.26 (Devilee *et al.*, 1986) recognises the centromeres of both chromosomes 13 and 21. These probes have a multitude of applications chiefly in fields where analysable metaphases can not always be obtained in cytogenetic preparations. Such fields include cancer cytogenetics, prenatal diagnosis and preimplantation diagnosis. Detection of chromosomes in interphase nuclei is referred to as ***"interphase cytogenetics."***

Many interphase cytogenetic studies of cancer preparations have appeared in the literature. Early breakthroughs in this field were Cremer *et al* (1988) studying aneuploidy in neuroectodermal tumour cells, Hopman *et al* (1988) studying bladder tumours, Viegas-Peguinot (1989c) detecting 1q polysomy in pseudodiploid breast and pseudotriploid colon tumours, Devilee *et al* (1988b) working on breast tumours, Nederlof *et al* (1989b) investigating colon tumour cell lines and patients with haematological disorders and Van Dekken *et al* (1990) who used 12 different chromosome specific probes in a variety of solid tumours.

In all the above studies both single and dual hybridisations and detections are used. Nederlof *et al* (1989a) were the first to achieve triple hybridisation and detection using centromere specific probes. Biotin, AAF and Mercury labelling was employed coupled with FITC, TRITC and AMCA fluorescent detection systems. Although a significant breakthrough, three colour flourescence has limited usefulness as the DNA usually needs to be counterstained fluorescently for location purposes hence using up the spectra of one fluorochrome.

These probes have also been used in prenatal diagnosis. Sexing of prenatal samples has been cited by a number of authors using ISH. Kozma and Adinolfi (1988) and Guyot *et al* (1988) both used FISH with probes specific for the long arm of the Y chromosome. West *et al* (1989) used tritium ISH with a probe pHY2.1 (Cooke *et al.*,

1982) recognising a sequence repeated on the long arm of the Y chromosome. This probe is still detectable using ISH technology in males who lack the Y chromosome fluorescent region (Gosden *et al.*, 1984). Human trisomies that result in live births are those of chromosomes 21, 18 and 13. Cremer *et al* (1986) described diagnosis of trisomy 18 in the interphase nuclei of prenatal samples using the probe L1.84 (specific for the centromere of chromosome 18) employing various ISH techniques. As mentioned however, there is no current chromosome satellite specific probe for chromosomes 21 or 13. The probes available detect both 21 and 13 together as the satellite DNA's are very similar (Willard., 1990).

These probes have been applied to the study of preimplantation embryos. Jones *et al* (1987) used both radioactive and non-radioactive approaches, the non-isotopic technique employed enzymatic (i.e. alkaline phosphatase) detection of a biotinylated probe. They reported good results using radioactivity though more ambiguous ones using biotin. West *et al* (1987, 1988) using radioactive means attempted sexing on 14 morphologically normal and 9 apparently abnormal embryos. All 14 apparently normal embryos were sexed with confidence however regarding the 9 abnormal ones, clear diagnosis of sex was not always possible. Angell (1987) report polyploidy in IVF embryos using an identical approach. Penketh *et al* (1989) used an alkaline phosphatase based detection system and biotinylated pHY2.1 and claimed a 66% success rate i.e. 2/3 of known male nuclei displayed a Y signal. Studies using FISH on this material are Grifo *et al* (1990) apparently detecting two Y chromosomes in a single human embryo and Pieters *et al* (1990) detecting chromosome 1 in a single human preimplantation embryo.

CHROMOSOME PAINTING

By using a collection of probes or "library" specific for a particular chromosome, it is possible to decorate or "paint" a whole chromosome. Since many of the clones in the library contain interspersed repeats CISS needs to be applied. Julien *et al* (1986) were

the first to report the painting of chromosome 21 creating chromosome 21 probes using a dual laser cell sorter and subsequently detecting trisomy 21 in prenatal samples. Cremer *et al* (1988) and Lichter *et al* (1988)(accompanying papers) describe the delineation of chromosome specific libraries, subsequent chromosome painting and detection of chromosome 1, 4, 7, 18 and 22 aberrations in tumour cells. Flow sorted human chromosome libraries were used here also. Pinkel *et al* (1989) detected trisomy 21 at interphase and chromosome 4 translocations using libraries obtained from the American Type Culture Collection which were cloned into "Bluescribe" plasmid vectors. An improvement in selecting library clones was demonstrated by Fuscoe *et al* (1989),they subcloned a chromosome 21 specific library into "Bluescribe" and selectively picked unique sequence inserts. The result was more intense signals when the library of clones was put through CISS and FISH. Very intense chromosome painting was also reported by Jauch *et al* (1990) who applied their studies to human sex chromosomes and amplified libraries in their original phage vectors. Currently, chromosome painting libraries are available for all human chromosomes.

Lichter *et al* (1990) have reviewed the feasibility of various chromosome 21 probes including 13/21 alphoid probes, chromosome painting libraries and cosmid clones and conclude that use of a single cosmid clone for the detection of chromosome 21 at interphase is the most applicable.

FISH FOR THE SCREENING OF SOMATIC CELL HYBRIDS

When somatic cell hybrids are made, it is essential to know two things about them namely a) how many human pieces are present on the rodent background? and b) what is the human chromosomal origin of these pieces?

Determining how many human pieces are present requires preparing chromosome spreads from the hybrid and a simple FISH experiment without CISS using labelled total human DNA as a probe (e.g. Pinkel *et al.*, 1986). The result is that human

elements from the spread are labelled and fluoresce whereas the rodent background does not.

In order to determine the chromosomal origin of human fragments "reverse chromosome painting" needs to be applied. Here, labelled total hybrid DNA is used as a probe on human chromosome spreads. CISS is necessary due to the human interspersed repetitive sequences present. This approach has been described by Kievits *et al* (1990). Because of the paucity of human in relation to rodent DNA and the need for CISS, this method of screening somatic cell hybrids is technically more difficult than the former however signal reliability can be improved by prior amplification of the human part by PCR of Alu sequences.

AIMS OF THIS STUDY

The purpose of this thesis is five-fold: Firstly FISH in relation to other forms of ISH is compared. Secondly, the application of chromosome specific repetitive probes is assessed using FISH. Thirdly, FISH is demonstrated as a powerful tool in a continuing study in the attempt to map the TSC1 locus on chromosome 9q. Fourthly, FISH is shown to be a useful vehicle to reconcile cytogenetic and molecular data in aspects of study of colorectal cancer. Lastly, FISH is applied in the field of preimplantation diagnosis.

1. ISH APPROACHES COMPARED.

The use of a satellite repetitive probe and a single copy plasmid cloned probe are compared using.tritium, biotin-alkaline phosphatase and biotin-fluorescein approaches. The relative merits of each method is discussed.

2. APPLICATION OF REPETITIVE PROBES

Both strategies for single and double labelling are compared probes using alphoid, classical satellite and VNTR probes. The applicability of certain probes in the field of prenatal diagnosis is assessed. Single and dual FISH on prenatal samples using X and Y specific probes is performed and assessed both as a method for screening prenatal samples and for ascertaining the relative XX/XY contents of a patient who is known to be XX/XY dispermic chimaera. The prospects for FISH in determining any chromosomal abnormality is discussed.

3. TS AND CHROMOSOME 9q

FISH is used in the initial stages of an irradiation-fusion gene mapping strategy in the search for the TSC1 locus. A parent hybrid and a panel of irradiation-fusion hybrids

isolated from it are screened. These results are compared with molecular data and hybrids are selected for the presence of markers known to be linked to TSC1 and for the presence of few (ideally 1) human piece(s). The sensitivity of this approach and its applicability in this and future gene mapping strategies is discussed in the light of the results obtained.

In a second set of experiments, a hybrid already considered to be of interest as it consists of small human fragments which contain markers linked to TSC1 is taken and screened. In a strategy to enrich the area with cloned DNA segments, phage clones isolated from the hybrid are taken and their position on a chromosome band noted by FISH. The efficacy of mapping phage clones by FISH is discussed. In addition a number of cosmids isolated from the parent hybrid CJ9q are mapped to chromosome 9q. Phage and cosmid mapping by FISH is compared. Current and future applications of FISH in the gene mapping strategy are discussed.

4. ASPECTS OF COLORECTAL CANCER

Chromosomes 1q, 5q and 18q have been all implicated in colorectal cancer and are concentrated upon in this study. Two cell lines are taken and analysed for the presence of regions of chromosomes 1 and 18. Analysis is firstly by classical G-banding and secondly by FISH using repetitive probes and cosmids. The role of chromosome 1 in cellular immortalisation is discussed as is the role of chromosome 18 and DCC in the adenoma-carcinoma sequence. A family with chromosome 5 rearrangements is examined using cosmids around the APC locus in an attempt to define in molecular terms the nature of those rearrangements using FISH. Clones on chromosome 5 expressed in normal colonic mucosa are mapped.

5. PREIMPLANTATION DIAGNOSIS

Methods of sexing preimplantation embryos are described using both single and dual FISH leading to clinical application for a number of patients. Further refinements to this approach and future applications are discussed.

The use of chromosome specific probes is described in patients carrying Robertsonian translocations with a view also to performing preimplantation diagnosis on them. The prospects for such an approach to screen out possible trisomies is assessed.

Finally the possible applicability of this approach in screening out any likely chromosome abnormality at the preimplantation stage is discussed.

MATERIALS AND METHODS

Accompanying this section are appendices 2 to 5. Appendix 2 describes the derivation of the cell lines used in this study. Appendix 3 lists the abbreviations and recipes of reagents mentioned in this section but not included here for the sake of continuity. Appendix 4 gives a list of probes used in this study along with references and sources for each probe. Appendix 5 gives the pedigree of the family investigated in this study with chromosome 5 rearrangements and details of the deletion of the proband studied.

MATERIALS

PROBES

Repetitive probes used in this study were supplied biotinylated by Oncor USA with the exception of the following gifts: pHY2.1 (Dr Howard Cooke), CY98 (Dr Jonathan Wolfe., UCL), pUC1.77 (Dr Joop Wiegant., Leiden University) αXT (Dr A.L. Bak Aarhus University, Denmark), λMS8 and λMS32 (Prof. Alec Jeffreys (Leicester University) and ICI diagnostics).

Single copy probe PGDH4 was isolated and supplied by Dr Patrick Klein (formerly of UCL). Single copy probes for the DCC gene (Josh4.4, Sam3.3 and P15-65) were supplied by Dr Bert Voglestein (Johns Hopkins Oncology Centre USA).

Chromosome 9 cosmids and phage clones were supplied by the team of Dr Jonathan Wolfe, namely Nick Hornigold, Jude Fitzgibbon, Fiorella Florian and Joseph Nahmias (UCL).

Cosmid ECB27 was isolated by Dr M. Begona Cachon-Gonzalez by screening a cosmid library with a single copy DNA fragment from ECB27. Cosmid L5-79 was

supplied by Mr Malcolm Dunlop (Edinburgh MRC human genetics unit). Probes λ5.3, cos 5.3 and cos 5.5 were isolated and supplied by Sarah Leigh, Simon Gayther and Kate Lawson (UCL).

A table of probes used in this study along with references and acknowledgemnents is given in appendix.4.

PRENATAL SAMPLES

All CVS and amniocyte materials were supplied by the clinical cytogenetic team of Dr Mary Lucas namely Dr Ros Hastings, Beverley Hamer and Tim Chamberlain (UCL).

CELL LINES

The hybrid cell line CJ9q was supplied by Dr Jonathan Wolfe (UCL). The details of its isolation are given in appendix 2.

All fragment hybrids were prepared and supplied by Jude Fitzgibbon, Nick Hornigold and Fiorella Florian (UCL). The details of fragment hybrid preparation are given in the introduction.

The cell line LIM1215 was supplied by Dr Robert Whitehead (Ludwig Institute, Melbourne). The details of its isolation are given in appendix 2.

The cell line AA/C1/SB10 was supplied by Dr Chris Paraskeva (Bristol University). The details of its isolation are given in appendix 2.

PATIENT MATERIALS

Blood and chromosome suspensions from the proband of the family with chromosome 5 rearrangments was supplied by Prof. John Burn, Dr Pam Chapman and Ian Cross (Newcastle University). Details of the pedigree of this family are given in appendix 5.

Blood from Roberstonian translocation patients was arranged by Prof. Robert Winston (Hammersmith Hospital) and Dr Joy Delhanty (UCL).

PREIMPLANTATION EMBRYOS

All preimplantation embryos were supplied by Dr Alan Handyside and Dr Richard Penketh (Hammersmith Hospital) by arrangement with Prof. Robert Winston. All embryos were surplus to requirements and were supplied with the patients' informed consent.

The author is grateful to all of the above.

All the embryo experimentation was approved by the research ethics committee of the Royal Post-graduate Medical School, Hammersmith Hospital (protocol number 89-3085) and by the Interim Licensing Authority for human *In-Vitro* Fertilisation and embryology.

PROBE LABELLING TECHNIQUES

A number of techniques were used in this study to incorporate labels into probes. The relative merits of each are compared in the discussion. Of the various nick translation protocols, in the early stages of this study, the Amersham kit was used for tritium and the BRL kit for biotin and digoxigenin as these were the ones recommended in the literature. Later, the literature and personal communications tended to favour the first principles approach. All these protocols are described.

NICK TRANSLATION

TRITIUM - AMERSHAM KIT:

In an Eppendorf tube, 100 picomoles of tritium labelled dCTP, 100 picomoles of tritium labelled dTTP were added and lyophilised for 1 hour. 3.3µl dATP and 3.3µl dGTP (from kit), 200ng of probe and sterile water to 40µl were then added. Incubation (15°C for 90 minutes) was followed by the tube being put on ice to stop the reaction. A column of Sephadex G50 fine (Sigma) in a 1ml syringe was made, placed in a centrifuge tube and spun 1500rpm for 6min. 50µl 5mM Tris pH 7.5 was put on this column which was spun 1500rpm, 3min. The syringe was transferred into a fresh centrifuge tube and 50µl of reaction mix + 50µl Tris pipetted on the column and this preceded spinning 1500rpm, 3 min. Incorporation of tritium was determined using a scintillation counter. The probe was then lyophilised and dissolved in an appropriate volume of TE buffer.

BIOTIN/DIGOXIGENIN - BRL KIT:

In an eppendorf tube the following were pipetted: 5µl solution A (A1 for dATPs, A4 for dUTPs), 1µg DNA, 2.5µl biotin/digoxigenin dNTP (BRL/Amersham), Water (solution E) to 45µl and 5µl solution C (DNAse/Polymerase enzyme mix). This was incubated at 15°C for 90 minutes after which 5µl solution D (stop buffer) was added. A column of fine Sephadex G50 fine (Sigma) was prepared in a glass pipette, filled up to the kink in the tube and flushed through with TE buffer. 55µl reaction mix was added to the top of the column followed by 545µl of TE buffer. A fresh eppendorf tube was put under the column, and 600µl TE added to elute the probe.

BIOTIN/DIGOXIGENIN - FROM FIRST PRINCIPLES:

In an eppendorf tube the following were added: 2µl 10 x salts solution, 2.5µl each 0.5mM dATP, dCTP, dGTP (Pharmacia), 2.5µl Biotin-16-dUTP or Digoxigenin-11-dUTP (Boehringer), 0.5-1.0µg DNA, 1µl DNAse I (Sigma) (1mg/ml diluted 1µl/500µl water). This was mixed and centrifuged to collect. 1µl DNA polymerase I (BRL) was added and mixed with a pipette. Incubation was 15°C-90 mins (preferable for biotin) or overnight 4°C (preferable for digoxigenin). 5µl stop buffer (300mM EDTA pH8), 1µl 5% SDS, 25µl TNE were then added. A "NICK" column (Pharmacia) was developed with TNE buffer, the probe mix added probe to column and eluted with TNE (probe came out in 2nd 400µl TNE).

OLIGOLABELLING

P^{32} OR BIOTIN:

6µl of probe (~5mg/ml) was boiled for 1 minute then incubated at 37°C for 10min. 11.5µl labelling solution (see appendix), 1µl 10mg/ml BSA, 3-6 µl P^{32} dUTP or Biotin dUTP and 0.5-1.0µl Klenow polymerase enzyme, were applied and the mix

incubated for 3-6 hours at room temperature. Purification was in a Sephadex column as for the nick translated probe.

PHOTOBIOTINYLATION

This technique was performed in the dark or in subdued lighting. In an eppendorf tube equal volumes of probe and photobiotin were mixed and placed on ice. Exposure to U.V. light (e.g. from the unscrewed objective of the fluorescent microscope) preceded incubation at room temperature for 15min (mixing every 5min). The volume was adjusted to 100µl with TE buffer and labelled DNA extracted with 2 x 100µl of butan-2-ol discarding upper layer each time.

PREPARATION OF LABELLED PROBE

WITHOUT "CISS":

To purified probe the following were added: 50 x salmon sperm DNA, 50 x yeast tRNA (Sigma), 0.1 x total volume 3M Ammonium acetate, 2.5 x volume ice-cold ethanol. Precipitation overnight at -20°C preceded centrifugation in a microcentrifuge on high speed for 30min at 4°C. The supernatant was drained and pellet lyophilised then resuspended in a hybridisation cocktail. Incubation was for 30min at 37°C mixing occasionally to ensure that the probe dissolved.

WITH "CISS":

To purified probe the following were added: 50 x salmon sperm DNA, 50 x yeast tRNA, 500 x sonicated human placental DNA **or** 50-100 x Cot1 DNA (BRL), 0.1 x volume 3M Ammonium acetate, 2.5 x volume ice-cold ethanol. Precipitation was 1 hour on ice (human placental DNA) or 2 hours at -70°C (Cot1 DNA). The pellet was

drained and lyophilised then resuspended in hybridisation cocktail by incubation for 30min at 37°C mixing occasionally to ensure that the probe dissolved.

ASSAYING FOR THE INCORPORATION OF HAPTENS

BIOTIN:

On nitrocellulose paper, 1µl of probe from the column was spotted followed by 3x10-fold serial dilutions (+ controls) and baked for 2 hours at 80°C. Using distilled not deionised water in the following: From kit, *(Vectastain ABC-Elite horseradish peroxidase kit (Vector)).* 1drop solution A and 1drop solution B in 5ml TNT were mixed well. Paper was washed 3 x 10 min in TNT (Tris\NaCl\Tween20 - see appendix), reagent made up 30 min earlier was added and incubated at room temp for 30 min. This had the effect of attaching strepavidin-peroxidase conjugate to the biotinylated probe. The paper was washed 3x 10 min TNT then transferred to a clean staining vessel. Colorimetric detection was achieved using a second kit *(Vector peroxidase substrate kit DAB (Vector)).* In a disposable Universal bottle: 5ml of water, and 2 drops of buffer stock solution were combined and mixed, 4 drops of DAB (diaminobenzidine) stock solution were added and mixed, 2 drops of Hydrogen Peroxide solution were added and mixed, 2 drops of Nickel solution were added and mixed. Incubation of the paper in staining solution continued until suitable staining developed. This preceded washing with 3 changes of distilled water and air drying.

DIGOXIGENIN:

Serial dilutions were spotted as for biotin but on nylon filter. This was baked at 80°C for 2 hours then washed briefly in TN. Pre-incubation in TNB for 30min, room temp preceded incubation with peroxidase conjugated anti-digoxigenin antibody (Boehringer) (1:5000 in TNB), 30min. Washes were 3x 10min TN. Colorimetric was continued as for biotin.using the Vector peroxidase substrate kit DAB (Vector).

CYTOGENETIC PREPARATION

BLOOD

CULTURE:

In a 25ml culture flask the following were measured: 1ml heparinised blood, 17ml Iscoves modified DMEM medium (Imperial) with 1% GPS (glutamine, penicillin, streptamycin), 2ml FCS (fetal calf serum) and 200µl PHA (phytohaemaglutinin). Incubation proceeded with the flask upright for 72 hours at 37°C. (See appendix for recipes).

HARVEST-BASIC METHOD:

100µl colchicine (Gibco)(1mg/ml) was added and incubation continued for 1 hour at 37°C. The flask was gently inverted to mix, then contents transferred to 2 pointed bottomed centrifuge tubes which were centrifuged 1000rpm for 5min and supernatant discarded. 0.075M KCl was added dropwise and the pellet vigorously resuspended. Incubation at room temperature (15min) was followed by centrifugation (1200rpm, 6 min) and supernatant discarded. The pellet was resuspended as before (vigorously and dropwise) in 3:1 methanol:glacial acetic acid fixative (made up freshly) and then centrifuged (1200rpm, 6 min) and supernatant discarded. This step was repeated until the pellet was white and supernatant was clear (usually 3 changes). Suspensions were stored at -20°C.

HARVEST-EXTENDED CHROMOSOME METHOD:

200µl of 30mg/ml thymidine (Sigma) was added to the culture and incubation continued for 18 hours at 37°C and the flask inverted to mix the contents. 200µl of 0.227mg/ml 2-deoxycytidine (Sigma) was then added and incubation continued for 3

hours 55 min at 37°C, the flask was then inverted, 200µl of 10µg/ml colcemid (Gibco) was added and incubated for 20min at 37°C and the flask was again inverted. The culture was transferred to 2 centrifuge tubes, spun 1000rpm for 5min and supernatant discarded. The pellet was resuspended dropwise, gently but thoroughly in 0.0375M KCl, incubated for 10min at room temperature, then centrifuged at 1200rpm for 6 min and supernatant discarded. The pellet was resuspended in the same manner as the KCl with 2:1 methanol:glacial acetic acid fixative (fresh) then centrifuged at 1200rpm for 6 min and supernatant discarded. This was repeated until pellet was white and supernatant was clear (usually 2 changes). Suspensions were stored at -20°C.

FIBROBLASTS

CULTURE:

From a skin biopsy 2-3mm across (full skin depth) pieces about 1mm x 1mm were cut in a petri-dish with some sterile medium (MEM). These were put into a Leighton tube under a long coverslip and a few drops of medium was added. This was changed weekly.

SUB-CULTURE:

Medium was removed and the culture washed in Hank's balanced salt solution. 8 drops of trypsin in versene were added and incubated at 37°C for 5-10 minutes until most of the cells had come off. These cells were transferred to a 25cm^2 flask containing 5ml medium.

HARVEST:

Sub-cultured cells were examined for divisions (rounded cells), 5µl of 10µg/ml colcemid was added and incubated for 1 hour at 37°C. The medium was removed, transferred to a sterile centrifuge tube, centrifuged 1200rpm for 6min and supernatant

discarded. Cells from the flask were washed with 5ml KHE (KCl/Hepes/EDTA) hypotonic solution and added to cells spun down. 5ml KHE hypotonic were put on the cells and both tube and flask incubated for up to 20min at 37°C. The suspension was removed from the flask and added to the tube which was centrifuged at 1200rpm for 6 min and the supernatant discarded. The pellet was resuspended in the liquid remaining (vigorously) and then vigorously resuspended in 3:1 methanol:glacial acetic acid fixative dropwise until the tube was full. Centrifugation proceeded at 1200rpm for 6 min. Suspensions were stored at -20°C.

HYBRIDS

CULTURE:

All hybrids in this study were prepared and cultured by Mr Jude Fitzgibbon and Mr Nick Hornigold to whom the author is very grateful.

HARVEST:

Preparations were harvested as for fibroblasts except that colcemid was not added and preparations were left in hypotonic solution for 10 min only.

PRENATAL SAMPLES

CULTURE:

All prenatal sample preparations (amniocytes and CVS) were prepared by the clinical cytogenetic team of Dr Mary Lucas to whom the author is very grateful.

HARVEST:

Samples were harvested as for fibroblasts except that colcemid was added for 2 hours (amnio) or 30 min (CVS).

SLIDE PREPARATION

Many slide making methods were performed in this study. After much experimentation, the following one was found to be the most effective prior to FISH:

Chromosome suspension was taken, supernatant discarded and the pellet resuspended in fresh fixative to achieve a milky suspension. Slides were soaked in methanol with a few drops of conc HCl. The slide was dried immediately prior to use with a lint-free cloth and breathed upon. 1 drop of chromosome suspension was placed on it and the slide shaken twice. 2 drops of fresh fixative (3:1) were put on the slide which was shaken to dry. At the point when the slide dried it was flooded with 70% glacial acetic acid, left for 10 seconds and dried thoroughly by shaking. The slide was examined under phase-contrast microscopy looking for a high mitotic index and preparations which had no visible cytoplasm around them. Only slides which fitted these criteria were used.

EMBRYO SPREADING

Although some embryo spreading was attempted in this study, none of the resulting preparations were processed any further. This author is eternally grateful to Dr Alan Handyside, Dr Richard Penketh and particularly to Dr Leeanda Wilton for making

cytogenetic preparations of human embryos. An account of the technique used by Dr Wilton is given in Griffin *et al* (1992).

CHROMOSOME BANDING

Several banding techniques were used in this study. G-banding was the preferred approach however Q-banding was often desirable because of the speed in which it could be performed. Lipsol banding was commonly used as a pre-ISH treatment as it was gentle on the chromosomes.

G-BANDING

If slides were freshly made they were incubated in distilled water at 60-65°C for 2 hours before beginning. Otherwise they were stored at room temperature for at least 2 weeks before beginning.

Incubation in 2xSSC at 60-65°C for 3 hours, was followed by washing briefly in 0.9% NaCl. Slides were incubated in Hank's balanced salt solution (see appendix)+ 0.006% trypsin (Koch-Light Ltd.) 10°C for 5 sec - 1 min then briefly washed in 0.9% NaCl. Staining was achieved using 10% Giemsa staining solution (BDH) in pH 6.8 buffer (filtered) for 10 min followed by a brief wash in pH6.8 buffer.

Slides were viewed under bright field microscopy (wet under a coverslip) using a green filter.

Q-BANDING

Slides were stained in (CMA)2S (dichloro methoxyacridine spermidine) solution for 5-10min then rinsed in Q buffer (see appendix). Slides were mounted wet under a coverslip, blotted and viewed under a fluorescent microscope using a blue or blue-violet filter.

LIPSOL BANDING

Slides were prepared the previous evening and stored at room temperature.

Treatment with 0.5% "Lipsol" (LIP Ltd) detergent in 0.9% NaCl 10-15 seconds was followed by 3 brief changes of 0.9% NaCl. Staining was achieved using 10% Giemsa staining solution (BDH) (in pH 6.8 buffer) for 10 min followed by a brief wash in pH6.8 buffer. Slides were mounted wet under a coverslip, blotted and viewed under bright field microscopy using a green filter.

NON-FLUORESCENT *IN-SITU* HYBRIDISATION

Two non-fluorescent approaches of ISH are described here. The results and discussion compare the relative merits of fluorescent and non-fluorescent methods.

TRITIUM ISH

Slides were destained through a 50-70-100% alcohol series and air dried. RNaseA (Sigma) (100µg/ml.2xSSC 200µl/slide - 1 hour, 37°C in a moist chamber) treatment

was followed by brief washes (4 changes of 2xSSC) and a second dehydration through a 50-70-100% alcohol series. Chromosomal DNA denaturation was achieved in 70% formamide (Fluka) 2xSSC 0.1mM EDTA pH7 at 65°C for 4 min then washing and dehydration proceeded as before. Probe DNA was denatured by boiling in hybridisation mix (see appendix) for 5 min followed by plunging on ice and brief centrifugation. 30µl of denatured probe was pipetted on to the prepared slide and sealed under a coverslip using rubber solution. Hybridisation proceeded overnight at 42°C in a moist chamber.

Coverslips were removed gently in 5xSSC. Post-hybridisation washes were 2xSSC (room temp, 4 hours), 2xSSC (60°C, 2x 30 min), 0.2xSSC (room temp, 30 min) and 0.1xSSC (room temp, 30 min). Dehydration through 50-70-100% alcohol series preceded air drying. Autoradiographic detection of tritiated probe was achieved (in the dark) by dipping the slides in 7ml water + 7ml K2 photographic emulsion (Ilford) 40°C. This was left to dry in a light-proof box for 1 hour then stored at 4°C (dessicated) in the dark for 2 weeks.

In the dark under a brown safelight slides were warmed to room temp for 1-2 hours then developing proceeded as follows: D19 developer (Ilford) 1:1 with distilled water (4 min), distilled water briefly, fixative (Hypam) 1:4 with distilled water + 2.5% rapid hardener (Ilford) (3 min), tap water 2-3 min. Slides were stained in 10% Giemsa for 30 min and destained in an alcohol series and restained if necessary. Slides were viewed using bright-field microscopy.

ALKALINE PHOSPHATASE BIOTIN ISH

The area on the slide to be hybridised (size of a large round coverslip) was located with a sufficient number of metaphases and marked on the underside of the slide with a glass cutter. Slides were dehydrated through a 50-70-100% alcohol series and air

dried. RNAseA treatment (100µg/ml, 2xSSC pH7, 200µl per slide, 37°C 1 hour, in a moist chamber) was followed by one wash in PBS. Dehydration in a 50-70-100% alcohol series preceded air drying. Biotinylated probe was dissolved in hybridisation mix (see appendix), put on the slide, covered with a large round coverslip and sealed with rubber solution. Probe and chromosomal DNA were denatured simultaneously by heating at 85°C for 10 min and hybridised overnight at 42°C in a moist chamber.

Rubber solution was removed and 2xSSC used to float off the coverslips. Post hybridisation washes were 30 min in 2xSSC at room temp, 30 min in 0.1xSSC at 42°C and 15 min in 2xSSC at room temp. Incubation in buffer 2 (see appendix) for 15 min was performed to block aspecific avidin binding. Slides were wiped dry of all but marked area and 100µl of 100ng/µl streptavidin conjugated alkaline phosphatase (in buffer 2) was pipetted on to the slide and incubated at room temperature for 20 min in a moist chamber. Slides were flushed with buffer 1, washed for 2 x 5 min in buffer 1 and washed for 10 min in buffer 3. Slides were wiped dry of all but marked area and colorimetric detection facilitated by pipetting on 100µl of substrate mix (see appendix). Incubation proceeded at room temp, in a moist chamber, in the dark for 2 hours. Slides were flushed with buffer 3, washed in buffer 4 for 5 min, mounted in "glycergel" mounting medium (DAKO), sealed with nail varnish and viewed under bright field and phase contrast microscopy.

FLUORESCENT *IN-SITU* HYBRIDISATION

PRE-PREPARATION OF SLIDES

To achieve some banding in the final analysis slides were made, stored for 2 weeks dessicated at 4°C then baked at 56°C for 2 hours.

Slides were often used fresh if visualisation of chromosome bands was not necessary but if slides were 1 day or more old, they were washed for 10 min in PBS at room temperature to remove any residual acetic acid.

Dehydration in a 70-90-100% alcohol series (5 min each change) preceded air drying. RNAseA (Sigma) treatment (100µg/ml, 100µl/slide, 37°C 1 hour in a moist chamber) was followed by washing twice in 2xSSC 5 min at room temp and a further wash in ProK buffer (see appendix) 5 min at 37°C. Exogenous protein was removed using an incubation of Proteinase K (Sigma) 10-1000 ng/ml, in ProteinaseK buffer, 37°C, 7 min. Washing briefly in PBS+1% $MgCl_2$ was followed by a fixation step in 1% acid free paraformaldehyde (Fluka) + 1% $MgCl_2$ for 10 min at room temp and another wash in PBS for 5 min, room temperature. Dehydration (70-90-100% alcohol series) was followed by air drying.

PROBE COCKTAILS

ALPHOID AND CLASSICAL-SATELLITE PROBES:

60-65% deionised formamide (BDH) 2xSSCP (2xSSC + phosphate - see appendix).

Labelled probe 2ng/µl (with 50x concentration salmon sperm DNA and yeast tRNA (Sigma)).

SINGLE COPY AND HYPERVARIABLE PROBES:

50% deionised formamide.2xSSCP, 10-20% dextran sulphate.

Labelled probe 2-10ng/µl (with salmon sperm DNA and yeast tRNA as above).

PROBES CONTAINING INTERSPERSED REPEATS (CHIEFLY COSMIDS AND PHAGES):

50% deionised formamide.2xSSCP, 10% dextran sulphate.

Labelled probe 10ng/µl (with salmon sperm DNA, yeast tRNA and **either** 500x concn. sonicated human placental DNA (Sigma) **or** 50-100x concn. Cot1 DNA (BRL).

DENATURATION OF DNAs AND HYBRIDISATION

IF PROBE DID NOT NEED TO BE PREANNEALED BY "CISS":

Probe was applied (20µl if mix contained dextran sulphate, 10µl if not) to slide under a large round coverslip sealed with rubber solution. Strands were separated by baking at 80°C for 3min and hybridisation continued at 37°C (1 hour - overnight for alphoid and classical satellite probes, overnight - 3 days otherwise).

IF PREANNEALING BY "CISS" WAS REQUIRED:

Strands were separated in the probe DNA (containing competitor) by heating for 5 min at 70°C then preannealling continued for at least 2 hours at 37°C.

Chromosomal DNA was denatured by applying 70% deionised formamide.2xSSCP (100µl) under a coverslip to the slides which were then baked at 80°C for 3 min. Following this the slides were plunged in ice cold 70% ethanol with coverslips on for 5 min then transferred to a separate vessel with ice cold 70% ethanol for 5 min; dehydration (90% ethanol for 5 min, 100% ethanol for 5 min) then preceded air drying.

20µl of preannealed probe was applied under a large round coverslip and sealed with rubber solution and hybridisation proceeded overnight (or longer) at 37°C.

POST HYBRIDISATION WASHES

FOR ALPHOID AND CLASSICAL-SATELLITE PROBES:

Rubber solution was removed from the slides and washes were: 1) 5 min in 60-65% formamide.2xSSC at 37°C ensuring that the coverslips floated off, 2) (in a fresh jar) 2 x 5 min 60-65% formamide.2xSSC at 37°C, 3) 2 x 5 min 2xSSC at room temp.

FOR SINGLE COPY AND HYPERVARIABLE PROBES:

Rubber solution was removed from the slides and washes were: 1) 5 min in 50% formamide.2xSSC at 42°C ensuring that the coverslips floated off, 2) (in a fresh jar) 2 x 5 min in 50% formamide.2xSSC at 42°C, 3) 5 x 2 min in 2xSSC at 42°C.

FOR COSMID AND PHAGE PROBES :

Rubber solution was removed from the slides and washes were: 1) 5 min in 50% formamide.2xSSC at 45°C ensuring that the coverslips floated off, 2) (in a fresh jar) 2 x 5 min in 50% formamide.2xSSC at 45°C, 3) 3 x 5min in 0.1xSSC at 60°C.

IMMUNOCYTOCHEMICAL DETECTION

BIOTIN:

Preliminary step: Washing for 5 min in SSCT (4xSSC + Tween20 - see appendix) at room temp was followed by incubation for 20 min in SSCM (4xSSC + milk - see appendix) at room temp to block aspecific avidin binding.

Step 1: Incubation for 20-30 min with fluorochrome conjugated avidin (Av) (Vector) 1:200 - in SSCM at room temp.

If signal amplification was not required slides were washed for 5 min in SSCT then 2 x 5 min in PBS.and steps 2 an 3 were not performed.

Otherwise washes were 3 x 5 min in SSCT.

Step 2: Incubation for 20-30 min with biotinylated anti avidin (BioαAv) (Vector) 1:100 - in SSCM at room temp. Washes were 3 x 5 min SSCT at room temp.

Step 3: Incubation for 20-30 min with fluorochrome conjugated avidin (Av) 1:200 as in step 1 then. Washes were 5 min in SSCT room temp then 2 x 5 min in PBS at room temp.

DIGOXIGENIN I (WITHOUT AMPLIFICATION):

Preliminary step: Washing for 5 min in TNT (Tris/NaCl/Tween20) followed by incubation for 20 min in TNB (Tris/NaCl/Blocking reagent) at 37°C (in a moist chamber) to block aspecific binding of the antibody.

Step 1: Incubation for 30min with fluorochrome conjugated sheep-anti-digoxigenin (ShαDig) (BCL), 1:1000 in TNB, 37°C (moist chamber). Washes were 5min TNT, room temp and 2 x 5 min in PBS at room temp.

DIGOXIGENIN II (WITH AMPLIFICATION):

Preliminary step: Washing for 5min in TNT at room temperature followed by incubation for 20min in TNB at 37°C (in a moist chamber) to block aspecific binding of the antibodies.

Step 1: Incubation for 30min in mouse anti-digoxin (MαDig) (Sigma) 1:1000 in TNB, 37°C (moist chamber) and washes were 3 x 5 min in TNT at room temp.

Step 2: Incubation for 30 min in fluorochrome conjugated rabbit anti-mouse antibody (RaαM) (Sigma), 1:1000 in TNB at 37°C (moist chamber) and washes were 3 x 5 min in TNT at room temp.

Step 3: Incubation for 30 min in fluorochrome conjugated goat anti-rabbit antibody (GαRa) (Sigma) 1:1000 in TNB at 37°C (moist chamber) and washes were 5 min in TNT at room temp and 2 x 5 min in PBS at room temp.

The non-amplification strategies were used for only a selection of alphoid or satellite repetitive probes.

STRATEGIES FOR DUAL LABELLING

With single labelling the green fluorochrome "FITC" (fluorescein isothiocyanate) was generally used, with double labelling FITC was used for one probe and a red fluorochrome (Texas red (Tr) or Tetramethyl rhodamine isothiocyanate (TRITC)) for the other probe. Two dual labelling/detection approaches were used chiefly in this study. The first amplifies both signals, this was used exclusively in the early stages of this study for all probes and for non-tandem repetitive probes later. The second does not include an amplification step for either probe and was used in the later stages of this study for simultaneously detecting two tandem-repetitive probes.

STRATEGY I: (both signals amplified)

Preliminary step: 5 min SSCT room temp, 20 min SSCM room temp.

Step 1: 20 min Tr-Av in SSCM room temp, 5 min SSCT room temp, 2 x 5 min TNT room temp.

Step 2: 30 min BioαAv + MαDig in TNB 37°C (moist chamber), 3 x 5 min TNT room temp.

Step 3: 30 min Tr-Av + FITC-RaαM in TNB 37°C (moist chamber), 3 x 5min TNT room temp.

Step 4: 30 min FITC-GαRa in TNB 37°C (moist chamber), 5 min TNT room temp, 2 x 5 min PBS room temp.

In certain cases FITC was used to detect biotin and TRITC used to detect Digoxigenin.

STRATEGY II: (neither step amplified)

Preliminary step: 5 min TNT room temp, 20 min TNB 37°C (moist chamber).

Step 1: 30 min ShαDig + FITC-Av in TNB 37°C, 5 min TNB room temp, 2 x 5 min PBS room temp.

COUNTERSTAINING AND ANTI-FADE MEDIA

In order to retard fluorescent fading, slides were mounted in anti-fade medium as follows: Slides were drained thoroughly but not dried. 50-100µl of anti-fade medium was put on a coverslip. The slide was pressed on and blotted (the edges could be sealed with nail varnish at this point). Anti-fade agents used in this study were 1,4 diazabicylo-2-2-2-octane (DABCO), and p-Phenyl diamine Dihydrochloride (pPD) dissolved in glycerol (for full recipes, see appendix). Fluorescent counterstains were carried in the anti-fade medium. For single labelling, DAPI or a combination of DAPI and PI was used, (a combination of DAPI and PI generally gave better banding than single counterstaining, DAPI gives replication Q-bands and PI gives replication R-bands. For double labelling, DAPI alone was used at all times as PI drowns the red signal.

MICROSCOPY AND PHOTOGRAPHY

FLUORESCENT MICROSCOPY

All direct fluorescence microscopy was performed on a Reichart Polyvar microscope equipped with fluorescence: The filter combinations shown in the following table were used:

Table 2.1: FILTER COMBINATIONS USED IN THIS STUDY

NAME	E. F.	D.M.	B. F.	DETECTS:
U1	330-380nm	420nm	>418nm	DAPI only
B1	450-495nm	510nm	>520nm	FITC and PI simultaneously (also Q-banding)
B2	450-495nm	510nm	520-560nm	FITC only (also Q-banding)
G2	520-560nm	580nm	>590nm	TRITC or Texas Red (also PI only)

(E.F. = exiter filter, D.M. = dichroic mirror, B.F. = barrier filter).

Figures given represent wavelength in nm of light allowed through by the filter or mirror. 330-380nm = U.V; 450-495nm = blue light; 520-560nm = green light; 590-610nm = red light.

PHOTOGRAPHY DOWN THE MICROSCOPE

FOR G-BANDING:

Film used was Kodak TP2415 monochrome film, and the camera set at: ASA 50, DIN 18 and automatic shutter speed. The light was turned up so that the exposure read 0.25-0.5 sec before pressing the shutter.

Developing of the film was using Ilford HC110 developer 1:32, 8min. Stop solution was dilute acetic acid, fixation was with Hypam fixer 1:4 for 2min and rinsing with tap water for 30 min.

Prints were developed using Ilford multigrade III paper, Ilford paper developer and Ilford paper fixer.

FOR FLUORESCENCE:

Films used were Scotch 640T transparency film or Kodachrome Ektachrome 1000 print film, ASA was set according to the rating of the film. Automatic shutter speed

was used except for red images when an exposure of 30 sec was used. Films were processed professionally (CPL, Warren street).

CONFOCAL MICROSCOPY

The following procedure was used to acquire images on the confocal microscope:

Channel 1(propidium iodide) image was collected first and stored, then channel 2 image (FITC) was collected,images were then merged and stored (mostly applicable for single labelling experiments). (The original stored channel 1 image was overwritten when the next picture was made). Neutral density filter was set to 1. The following steps were used. Words and letters in square brackets indicate keys on the computer, two keys separated by a stroke indicates that the keys should be pressed separately.

To collect image on channel 1:

Find and focus on image using conventional fluorescence microscopy,
"Gain" set around 5,"Black level" around 5, Aperture barely open,
[Alt]/[L] (to get into live menu),
[Enter] (to start laser),
[F4] (to switch to fast scan speed),
Image should appear on the screen, if not turn up gain and open aperture a little,
Centre specimen using vernier knobs on microscope and arrow keys on keyboard,
Use focus knob on microscope to get brightest possible image,
Adjust "Gain", "Black level" and aperture until image is suitable,
[F1] (to switch to slow scan speed),
[K] (to switch to "Kalman" filter),
Let scan 5-10 times then [Enter] (to stop laser),
[Alt]/[F] (to get into file menu),
[P] (to put this file in the memory) [A] (the name of the file)

--Channel 1 images were always called "A" and each time a new image was collected, the previous one was overwritten--.

To collect image on channel 2:

Do not touch focus knob, it should now be in the right place,

"Gain" ~9-10,"Black level" ~5, aperture half way open or more, low signal setting,

[Alt]/[L] (to get into live menu),

[Enter] to start laser,

[K] (to operate "Kalman" filter),

Let scan 10-20 times then [Enter] (to stop laser),

[Shift]/[>] (this enables the operator to issue a complex command),

[Shift]/[@] [n] (this brings the image to maximum brightness),

[Shift]/[>]

Then type:- "MERGE A" (this has the effect of merging FITC and PI image),

[Alt]/[C] (to get into contrast menu),

Adjust relative intensities of signals using arrow keys until a suitable picture is presented,

[Alt]/[F]

[P] (now name the file and press [Enter])

ARCHIVING CONFOCAL IMAGES

Images were stored with an IBM 3363 optical disk drive. Polaroid photographs were produced using a Mitsubishi colour video copy processor. Photographs directly from the screen were taken using a cowl around the video screen (Shackman), using a Kodak ASA 200 transparency film. Camera settings: aperture-5.6, shutter speed 0.5-1 sec.

FURTHER USE OF THE CONFOCAL MICROSCOPE ATTACHMENT

The image analysis and storage capabilities of the confocal microscope make it an invaluable tool when practising FISH. It should be noted however that it should only be used as an instrument to display what is already visible, though not necessarily photographable, down the microscope. It is possible to display many strange results if

these restrictions are not adhered to. It is even possible to selectively remove background hybridisation and to artificially draw hybridisation signals on chromosomes. All confocal images presented in this study represent true images as visualised down the microscope.

The confocal microscope could also be used for classical cytogenetics. It was particularly useful for Q-banding as fluorescent glare could be eliminated and difference in brightness between light and dark bands optimised. The software can be used for sorting chromosomes this eliminates time-consuming photography and subsequent cutting and pasting of prints in many cases.

PROBE PREPARATION

TRANSFORMATION (COSMIDS AND PLASMIDS)

PREPARATION OF COMPETENT CELLS:

The following was made: 10ml of L-broth in a 100ml conical flask, 200ml of L-broth (see appendix) in a 500ml conical flask and 600ml of 100mM $CaCl_2$, all were autoclaved. The 10ml culture was innoculated with "Sure" competent cells and incubated overnight at 37°C, shaking. The 10ml culture was innoculated into the 200ml L-broth and incubated for 90min at 37°C, shaking. The culture was put on ice and swirled to cool.then transferred to pre-chilled centrifuge bottles and centrifuged at 5000rpm for 10 min at 4°C. The supernatant was poured off and the pellet resuspended in 500ml of 100mM $CaCl_2$. This was incubated on ice for 30 min and centrifuged at 5000rpm for 10 min at 4°C and the supernatant discarded. To 40ml of 100mM $CaCl_2$, 6ml of glycerol was added and this was used to resuspend the cells.

1ml aliquots were kept on ice for 12-24 hours before use then dropped in liquid nitrogen to freeze and stored at -70°C. Cells remain competent for about a month.

TRANSFORMATION OF DNA INTO COMPETENT CELLS:

An aliquot of competent cells was thawed on ice. To 200µl of cells 100ng DNA was added, incubated on ice for 30 min, heat shocked at 43°C for 2 min and put back on ice. 0.8ml L-Broth was added and then incubated shaking at 37°C for 1 hour. Centrifugation on slow setting preceded removal of 900µl of supernatant then resuspension in the remainder. The suspension was plated out on agar plates (see appendix) with the appropriate antibiotic (usually ampicillin for plasmids and kanamycin for cosmids) and incubated overnight at 37°C.

GROWING CELLS AND PREPARATION OF GLYCEROL STOCKS:

A **single** colony was picked from the agar plate and innoculated into 10ml of sterile L-Broth. This was incubated for ~5 hours at 37°C.

Glycerol was added to fill the bottom sector of a 1ml sterile ampoules, this was topped up with cells from the culture and stored at -70°C. The rest of the culture was added to 400ml sterile L-Broth (for plasmids) or sterile "cosmic" broth (for cosmids) with appropriate antibiotic and incubated overnight before proceeding to extract DNA (maxi-prep).

MAXI-PREPS (COSMIDS AND PLASMIDS)

PREPARATION OF DNA:

The following was continued from the above section or, if starting from a glycerol stock, a few cells were scraped from the top, innoculated into a 10 ml culture for ~5 hours and that was used to seed a 400ml overnight culture.

The culture was centrifuged at 600rpm, 4°C for 10 min, the supernatant discarded and resuspended well in solution 1 (see appendix). 50mg lysosyme was added, mixed well and incubated at room temp for 5 min. Freshly made solution 2 was mixed gently with an innoculating loop and let stand on ice for 10 min. 15ml of **ice-cold** solution 3 was added and mixed with an innoculating loop. This was incubated on ice for 10-30 min then centrifuged for 9500rpm for 15 min at 4°C. The volume of supernatant was measured and transferred to a conical flask, 0.6 x volume of isopropanol was added, mixed then incubated at room temp for 10-20 min or 1 hour at 4°C. Centrifugation at 9500rpm at 20°C for 15 min was followed by the pellet being drained, 20ml of 70% ethanol was added and the suspension vortexed. Centrifugation (9500rpm, 20°C, 10 min) preceded draining of the pellet which was then dried under a vacuum for 10min and resuspended in 10xTE (11.5ml).

PROBE PURIFICATION

CAESIUM CHLORIDE GRADIENT:

To DNA in 11.5ml 10x TE: 0.46ml 0.2M K_2HPO_4 pH 7.5, 12g CsCl, 1.2ml 10mg/ml EtBr and 1ml paraffin oil were added. This was transferred to a "Sorvall" centrifuge tube which was plugged and crimped. Centrifugation was in a "Sorvall" centrifuge (45000rpm, 17 hours, 20°C). Under U.V. light, the tube was examined for bands of DNA. The lower band was drawn out using a wide bore needle. An equal volume CsCl saturated iso-amyl alcohol was added and shaken gently for 1min and centrifuged for 5min at 1000rpm and the top layer discarded. This was repeated three times and then DNA was dialysed against 2 changes of 2 litres distilled water for 4 hours. The volume of the solution was measured, half volume of 7.5M Ammonium acetate and twice total volume of absolute ethanol (ice cold) was added. Precipitation proceeded for at least 30 min at -20°C followed by centrifugation (10000rpm, 15 min,

4°C) discarding of the supernatant and lyophilising of the pellet which was then resuspended in TE buffer.

PHENOL-CHLOROFORM EXTRACTION:

To prepared probe DNA: An equal volume of phenol was added, shaken for 1 min gently and centrifuged 2 min on high speed. The lower layer was removed, phenol added, shaken, centrifuged and the lower layer removed as before. An equal volume of chloroform was added, shaken for 1 min gently, centrifuged for 2 min on high speed and the lower layer removed. 2 volumes of ice cold ethanol were added and precipitation continued at -20°C for 30 min. Centrifugation (10 min on high) preceded pouring off of the supernatant then 70% ethanol was added and the preparation centrifuged for 10 min on high speed. Finally, the supernatant was poured off, the pellet lyophilised and resuspended in TE buffer.

"GENE-CLEAN" PROCEDURE:

To ~5µg DNA: 3 volumes of NaI and 5µl of glass milk (from "gene-clean" kit (Bio 101 Inc.) was added and quenched on ice for 5 min. Centrifugation for 30 seconds on high was followed by the supernatant being pipetted off and discarded. 700µl of "New Wash" (Bio 101 Inc.) was added and the pellet resuspended thoroughly then centrifuged for 30 seconds on high. The supernatant was removed, resuspended in "New Wash", and centrifuged as before - **this step was repeated.** 20µl TE buffer was added, the pellet was resuspended thoroughly and incubated at ~50°C for 3 min. This was centrifuged for 30 seconds on high and the supernatant retained. The DNA was centrifuged before use.

MISCELLANEOUS TECHNIQUES

"Alu" PRIMED POLYMERASE CHAIN REACTION

In a small reaction tube the following was mixed: Primer - 1μM concentration, enzyme (Taq polymerase) (Cetus) - 0.6 units, reaction buffer 10x - 5μl, nucleotides 200μM each dATP, dCTP, dGTP, dTTP (Pharmacia), DNA template - 0.3-0.5μg, sterile water to 50μl. Three drops of paraffin oil was pipetted on top of the reaction mixture to prevent evaporation.

Table 2.2

PCR cycle (Hybaid thermal reactor):

1x	94°C	5min;					
10x	94°C	45sec;	56°C	1min;	72°C	3min;	
10x	94°C	45sec;	56°C	1min;	72°C	4min;	
15x	94°C	45sec;	56°C	1min;	72°C	5min;	
1x	72°C	10min;					

1 - 1.2% MINIGEL

In a 250ml conical flask the following were added: 50ml TE buffer and 0.5-0.6g agarose (Sigma) added. This was microwaved for 2 min on high to dissolve the agarose. 5μl 10mg/ml EtBr was added and the solution poured into a minigel tank with combs and left to set. When the gel was set, buffer was poured in (gel buffer - 50ml TE and 50μl Et Br) and combs removed. 10μl DNA was added to 1μl of 10% blue sucrose (Sigma) then loaded into wells and run at 25 - 50v.

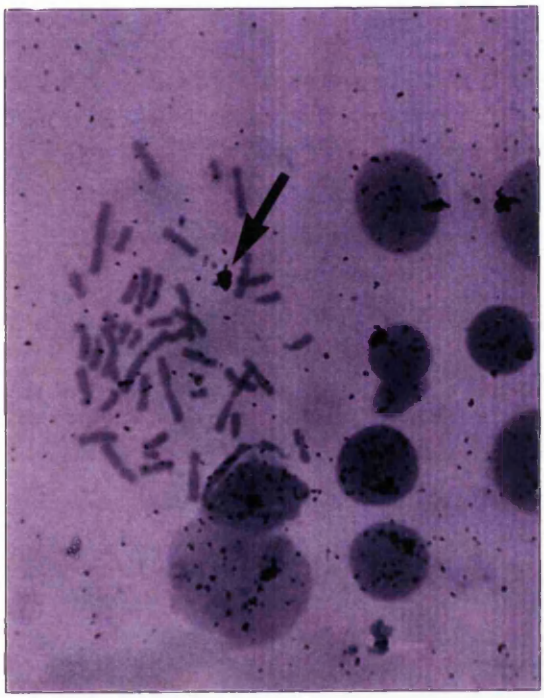
RESULTS

SECTION 1: APPROACHES COMPARED

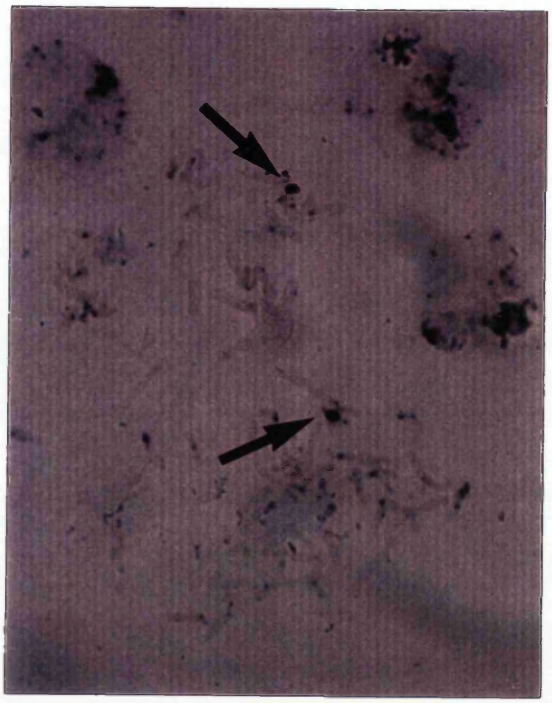
This section deals with the comparison of methods of ISH with a view to demonstrating the superiority of the FISH procedure. Two probes were compared using three different techniques. These were a) "pHY2.1" (Cooke *et al.*, 1982) a probe which recognises a 2.45kb repeated sequence of around 2000 copies on the long arm of the Y chromosome, and b) "pPGDH4" a single copy 1.7kb partial cDNA of the human 6-phosphogluconate dehydrogenase gene (Patrick Klein., personal communication). The probes were selected by virtue of the fact that they were working in the laboratory using the established radioactive ISH approach. The three methods compared were radioactive (tritium) ISH, biotin (alkaline phosphatase) ISH and biotin FISH. References for each of the two probes used are given in the appendix.

Fig. 3.1:- MALE HUMAN LYMPHOCYTE PREPARATIONS PROBED WITH pHY2.1.

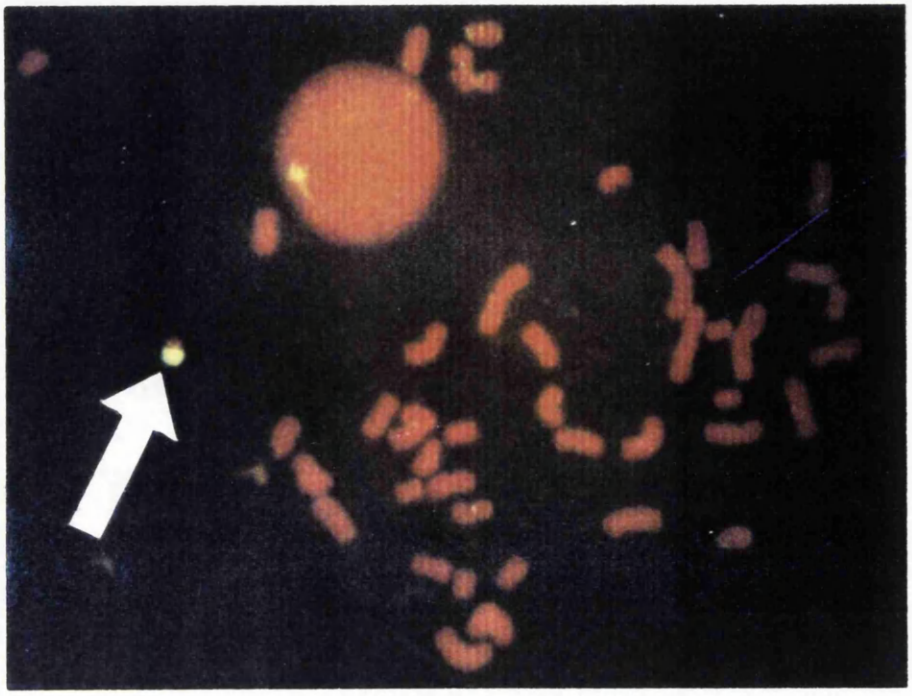
a) AUTORADOGRAPHIC DETECTION OF TRITIATED PROBE (METAPHASE Y CHROMOSOME ARROWED).



b) ALKALINE PHOSPHATASE DETECTON OF BIOTINYLATED PROBE (Y CHROMOSOMES ARROWED).



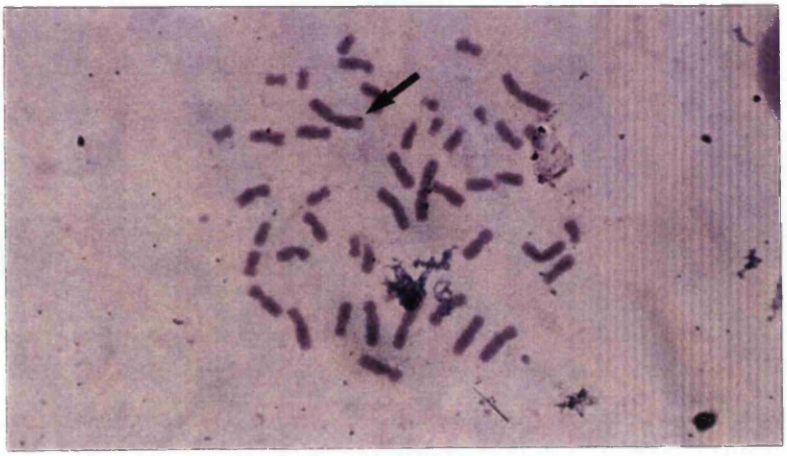
c) FLUORESCENT DETECTION OF BIOTINYLATED PROBE (Y CHROMOSOME ARROWED).



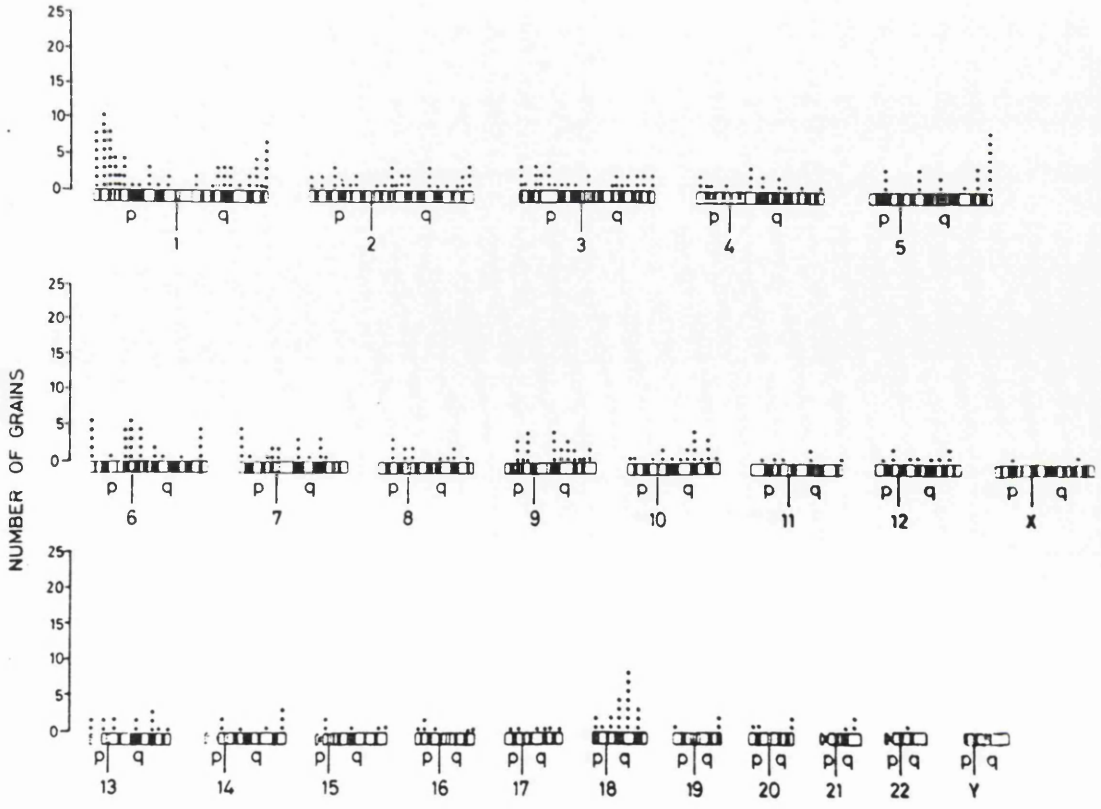
SINGLE COPY PROBE

Fig. 3.2:- MALE HUMAN LYMPHOCYTE PREPARATIONS PROBED WITH pPGDH4 AND IDEOGRAMS SHOWING PEAKS OF HYBRIDISATION (ACCURATE CYTOGENETIC ASSIGNMENT WAS ACHIEVED BY LIPSOL BANDING PRIOR TO ISH FOLLOWED BY PHOTOGRAPHY THEN RELOCATION OF METAPHASES POST-ISH AND COMPARISON OF PHOTOGRAPHS. HYBRIDISATION SIGNALS WERE THEN DOTTED ON TO A STANDARD IDEOGRAM). Performed by Kiran Gulati.

a) i) AUTORADIOGRAPHIC DETECTION OF TRITIATED PROBE (HYBRIDISATION SIGNAL ARROWED).

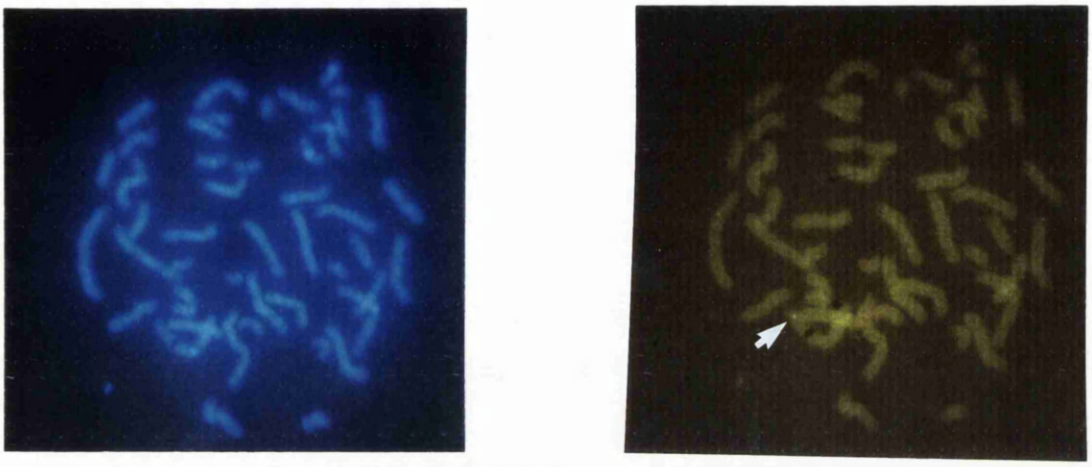


ii) IDEOGRAM SHOWING HYBRIDISATION PEAK (65 CELLS ANALYSED).

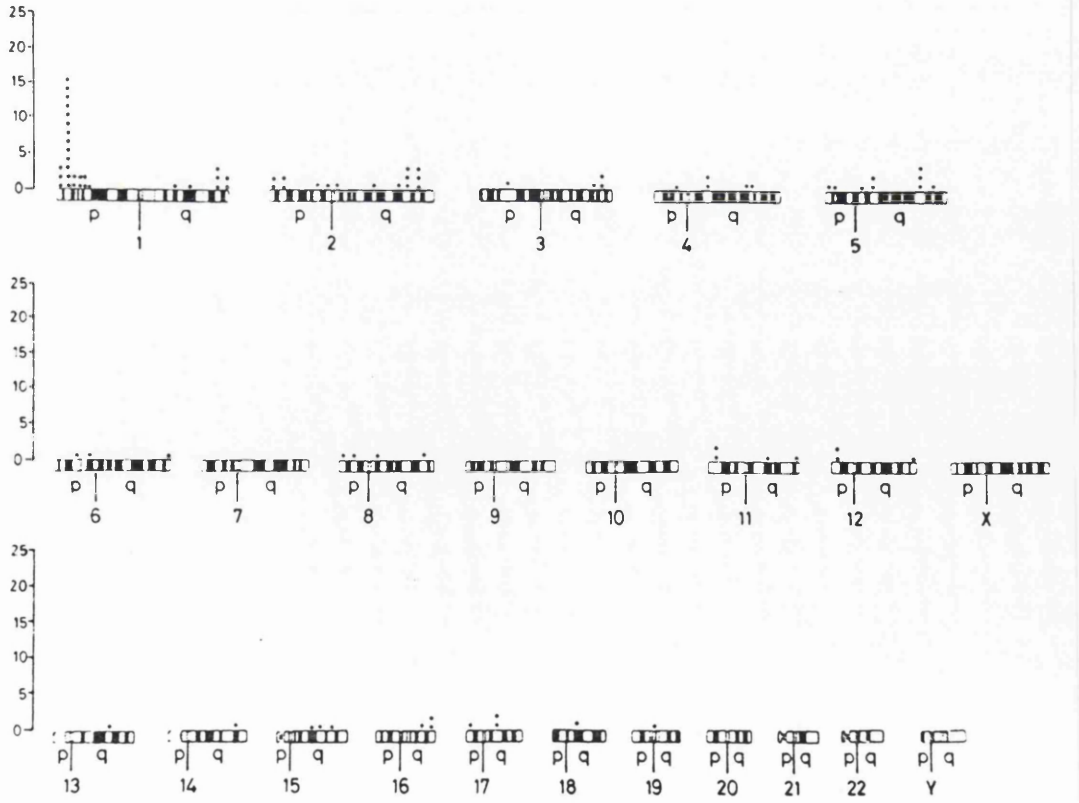


b) i) FLUORESCENT DETECTION OF BIOTINYLATED PROBE (HYBRIDISATION SIGNAL ARROWED).

In this case the probe was biotinylated using the BRL kit, hybridisation and washes were as described in the methods section for single copy probes. Detection was also as described for FITC detection of biotin using the "Pinkel Sandwich." Chromosomes were counterstained with DAPI only (left). Accurate band assignment was achieved as for the isotopic approach i.e. using pre-lipsol banding.



ii) IDEOGRAM SHOWING HYBRIDISATION PEAK (29 CELLS ANALYSED).



The alkaline phosphatase method did not work using probe PGDH4 despite a number of attempts.

In the case of the repetitive probe, there was less background hybridisation using the fluorescent technique as compared with the other two, this is illustrated in the photographs and was the case for both metaphase and interphase signals. Furthermore, scoring of interphase signals was more subjective using the tritium and alkaline phosphatase based techniques i.e. it was frequently difficult to ascertain what were true signals and what was background with alkaline phosphatase and tritium. This was not the case using FISH as signals were very specific. In the example of the single copy probe, metaphases needed to be analysed and a much greater proportion of the signals appeared on chromosome 1p36. The results in section 1 have hence demonstrated that, of the three approaches, FISH shows the greatest specificity and reliability. It was thus decided to use this approach in preference to the others for subsequent analyses in this study.

SECTION 2: SUCCESS OF REPETITIVE PROBES

This section deals with the general success of a selection of satellite repetitive probes on phytohaemaglutinin stimulated lymphocyte material. A photograph of each is presented as visualised on lymphocyte metaphases (a), followed by a statistical breakdown of its general success rate on interphase nuclei or, in the case of VNTR probes, an account of efficiency of labelling on metaphase chromosomes (b). Unless otherwise stated, the figures given represent biotin labelled probes (avidin-fluorescein detection system) not amplified via the "Pinkel sandwich approach." In some cases, the applicability of the probe in some form of research application (e.g. prenatal diagnosis) is dwelt upon. In the cases where the chromosomes appear red, they were stained with propidium iodide as well as DAPI. As mentioned in the introduction and methods, FITC and propidium iodide signals can be veiwed simultaneously. In the cases where the chromsomes appear as a faint green background, PI was not used.

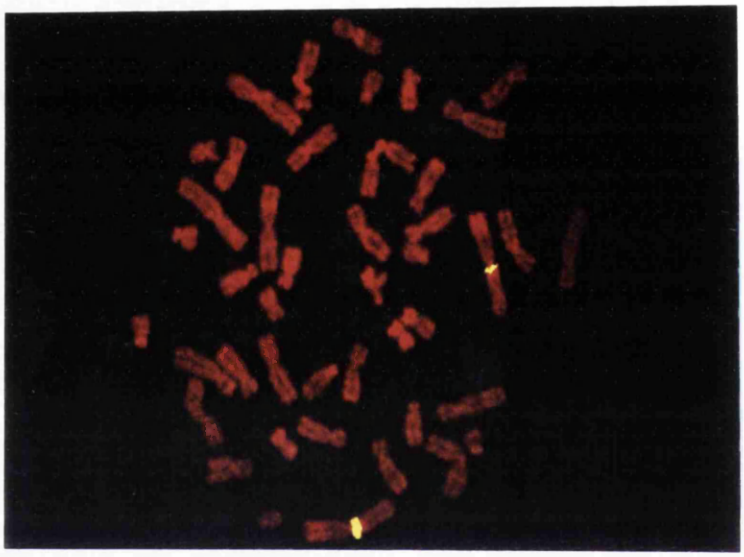
CRITERIA FOR SCORING INTERPHASE SIGNALS

A signal was only scored as such when it was appreciably brighter than any background fluorescence. Any dim "signal," i.e. one around 100-fold less bright than the true signal, was ignored. Often, when the nucleus had entered S-phase, two dots for each signal were seen. These two dots (or "split spots" as they were referred to) were always very close together and generally smaller than their "unsplit" counterparts. In order to resolve this problem, split spots were scored as one signal if they were less than one spot's diameter apart (Hopman *et al.*, 1988). If two large overlying foci could be clearly seen in two different planes of focus, then they were scored as two signals.

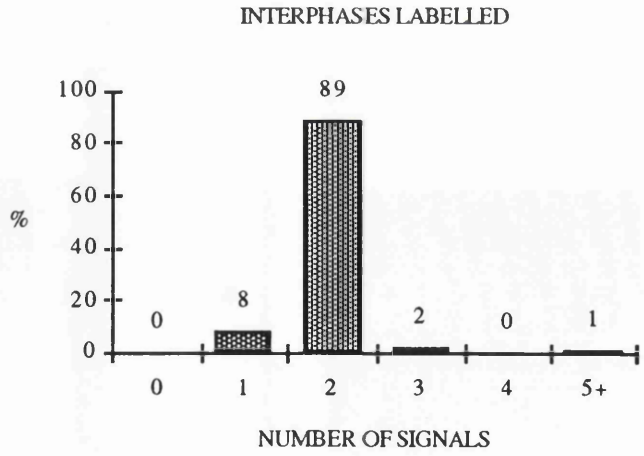
PUC1.77: Specific for the heterochromatic region of chromosome 1.

Fig. 3.3:

a



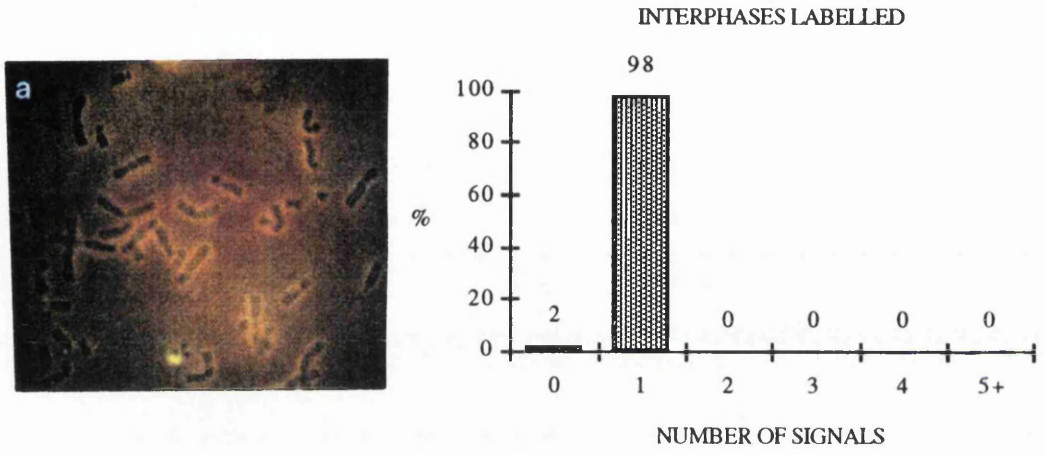
b



pHY2.1: Specific for the long arm of the Y Chromosome.

Fig. 3.4:

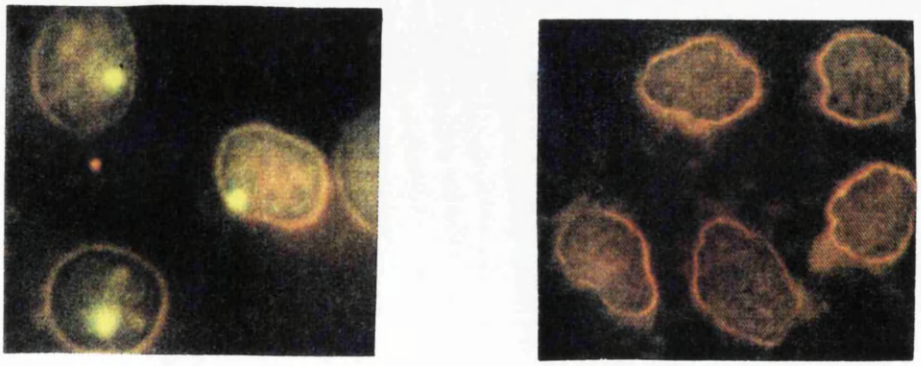
a b



pHY2.1 can be used to sex preparations of prenatal samples. The following photographs illustrate this on male CVS (left) and female CVS (right). In each case 100% of cells gave the expected number of signals and "Pinkel sandwich amplification" was used.

Fig. 3.5:

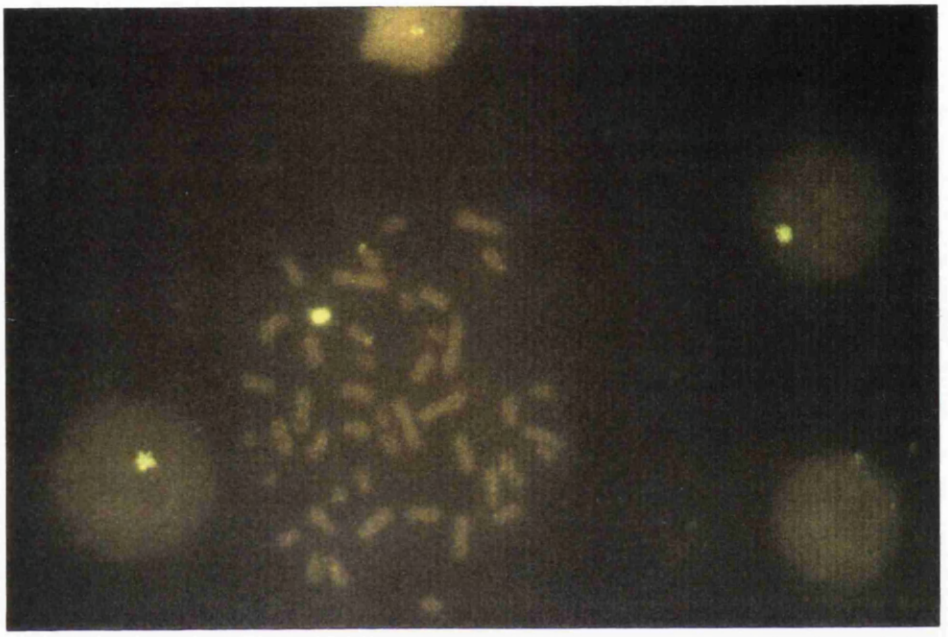
a) MALE CVS SEXED WITH pHY2.1 b) FEMALE CVS SEXED WITH pHY2.1



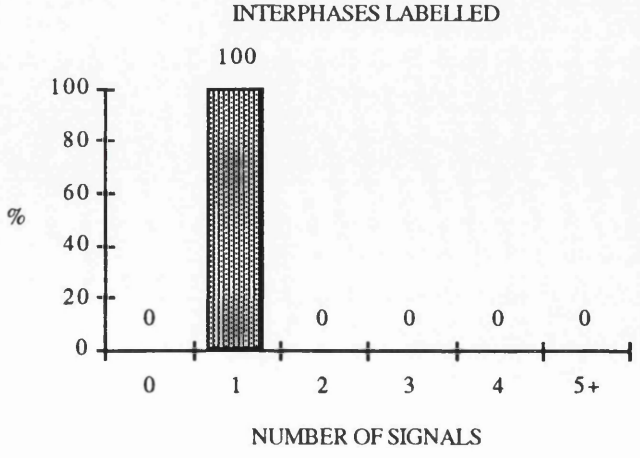
cY98: Specific for the long arm of the Y chromosome.

Fig. 3.6:

a



b



pBamX7: Specific for the centromere of the X chromosome.

Fig. 3.7:- On male cells.

a

b

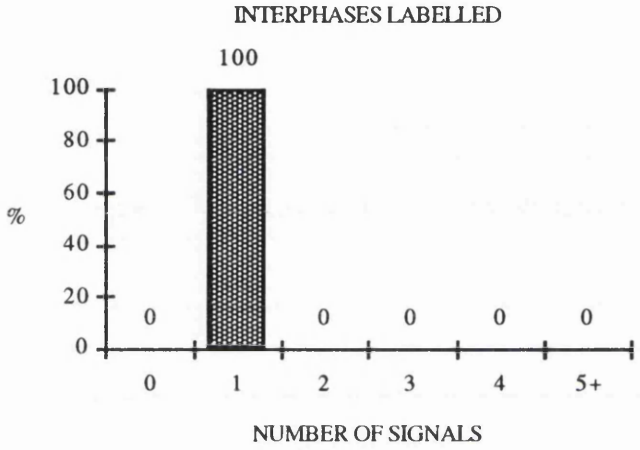
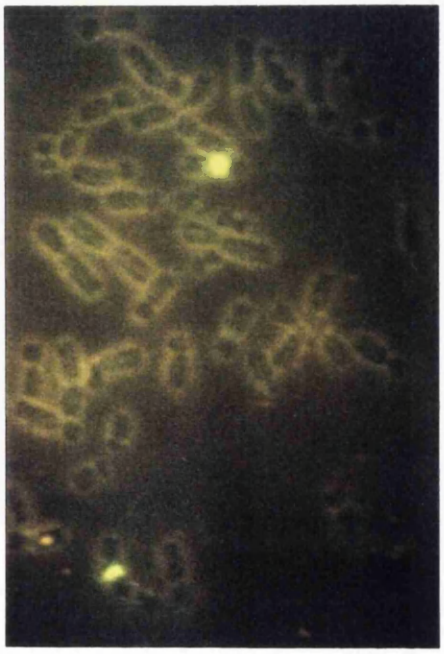
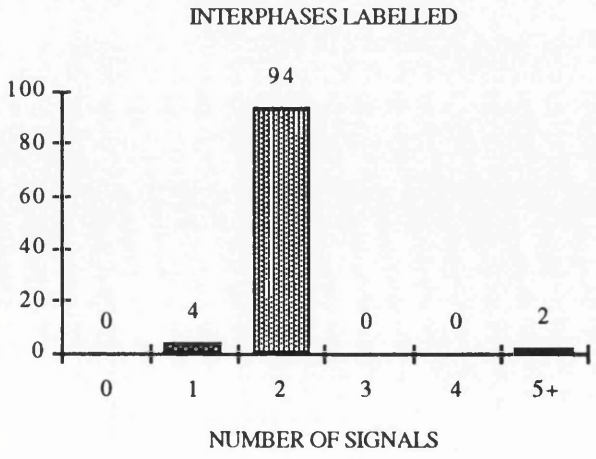


Fig. 3.8:- On female cells.

a



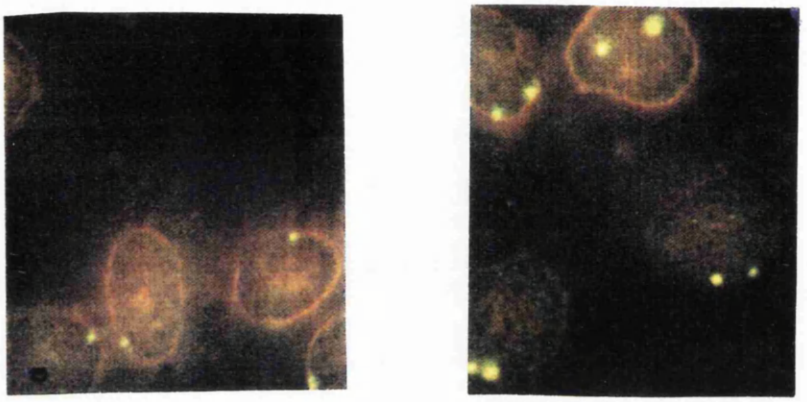
b



pBamX7 was used to sex prenatal samples; the following photographs illustrate this on male CVS (left) and female CVS (right).

Fig. 3.9:

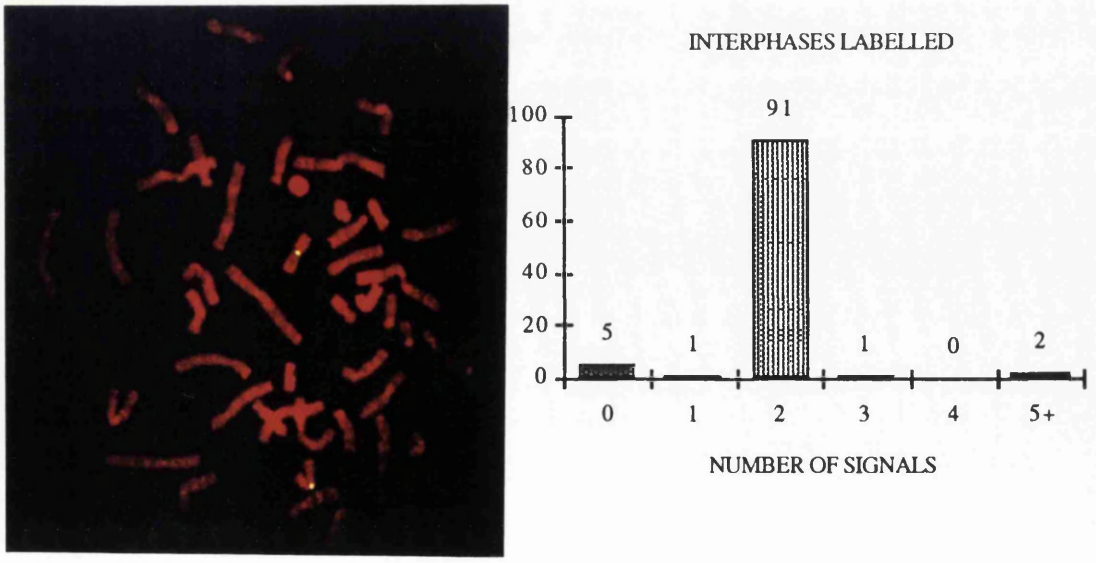
a) MALE CVS SEXED USING pBamX7 b) FEMALE CVS SEXED USING pBamX7



pSE16: Specific for the centromere of chromosome 16.

Fig. 3.10:

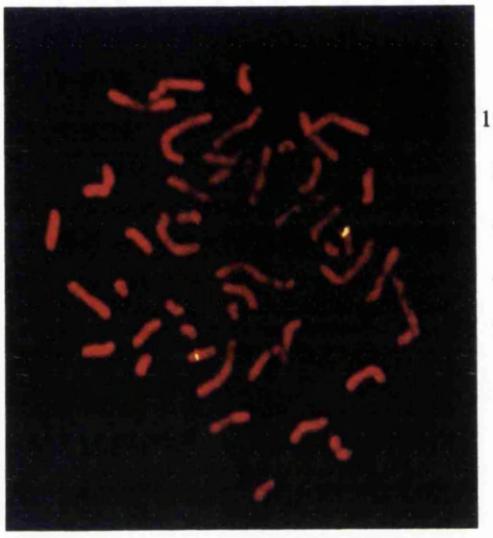
a b



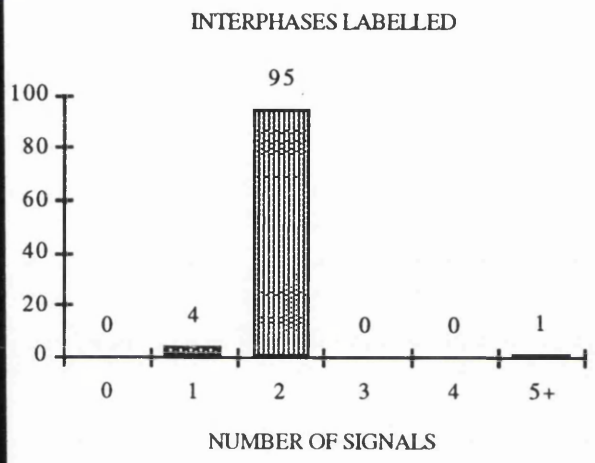
p17: Specific for the centromere of chromosome 17.

Fig. 3.11:

a



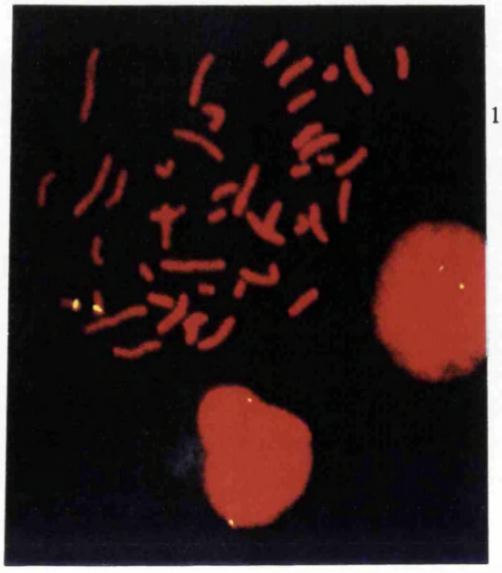
b



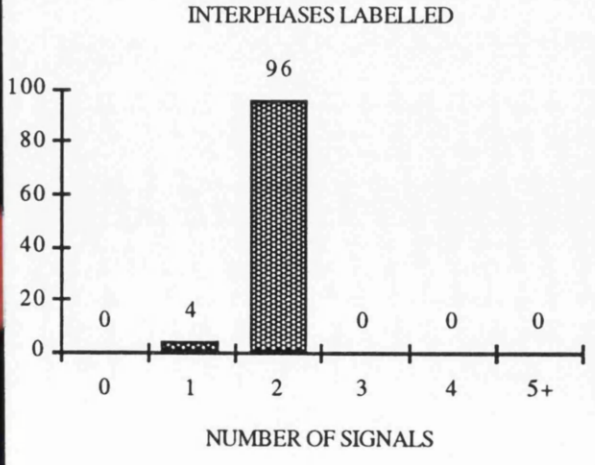
p18: Specific for the centromere of chromosome 18.

Fig. 3.12:

a



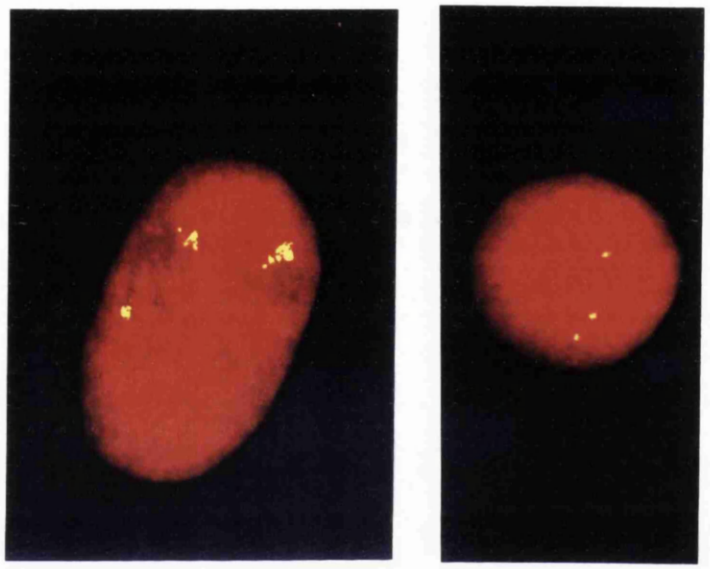
b



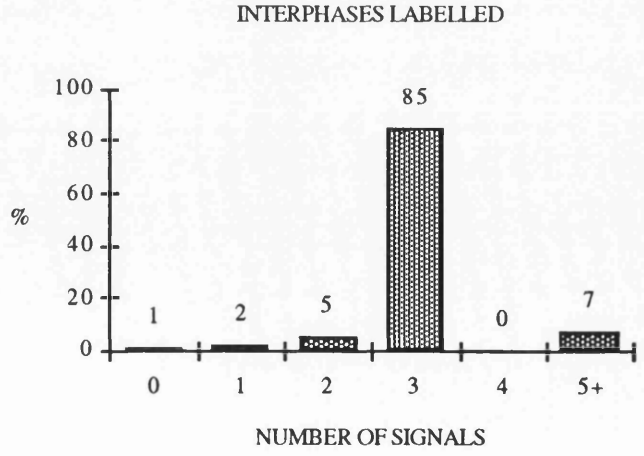
p18 was used to detect Edward's syndrome (trisomy 18) in prenatal samples. The following represents the success on this probe on an Edward's syndrome CVS. "Pinkel sandwich amplification" was used.

Fig. 3.13:

a



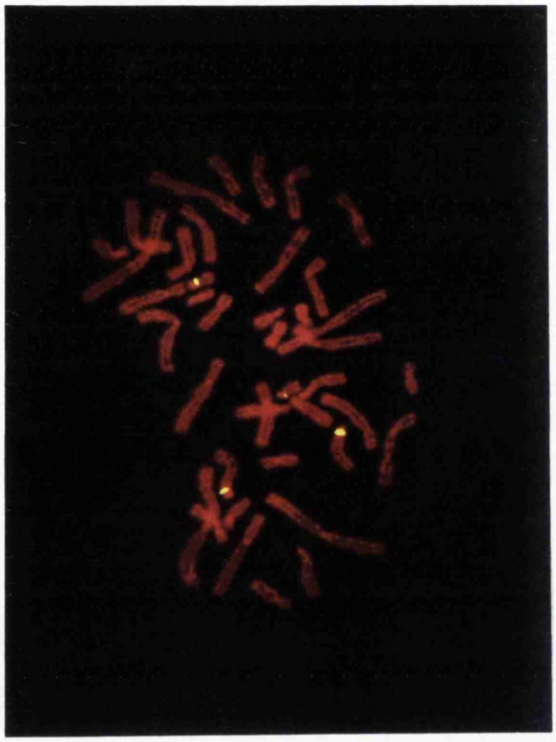
b



Certain probes showed limited specificity due to the fact that the alpha satellite DNA can be very similar on certain chromosomes, thus the probes used picked up two chromosomes simultaneously. Interphase counts were difficult to make as signal size and reliability was variable from individual to individual. Probes detecting chromosomes 13 and 21 (p31/21), 14 and 22 (αXT) are shown below:

Fig. 3.14:

a) p13/21



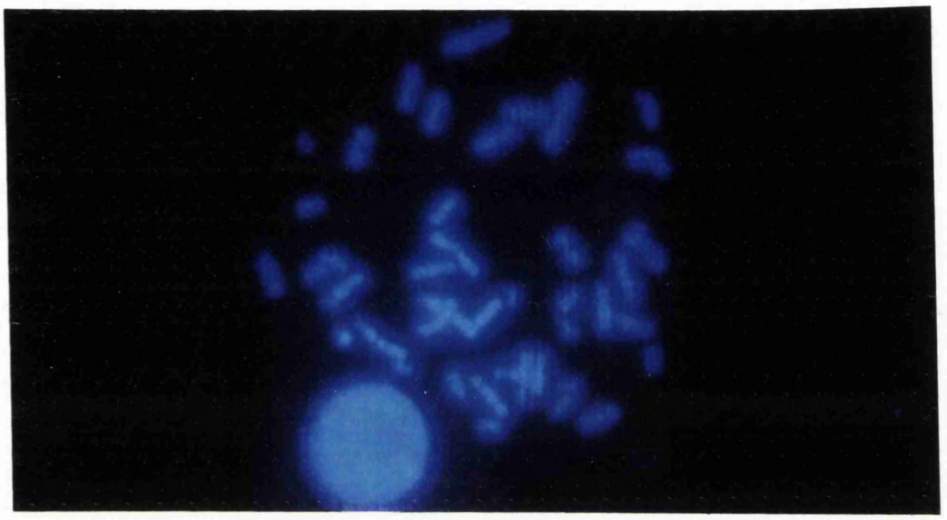
b) αXT

It is apparent therefore that alphoid and classical satellite DNA probes are useful tools in ascertaining chromosome number in the interphase nucleus. Hybridisation efficiencies are high (89-100%) and thus these probes have potentially a multitude of uses in various areas of human cytogenetics. Some of these uses are illustrated in the subsequent sections.

DOUBLE LABELLING WITH A COMBINATION OF pBamX7 (BIOTIN - FITC) AND pHY2.1 + CY98 (DIGOXIGENIN - TRITC)

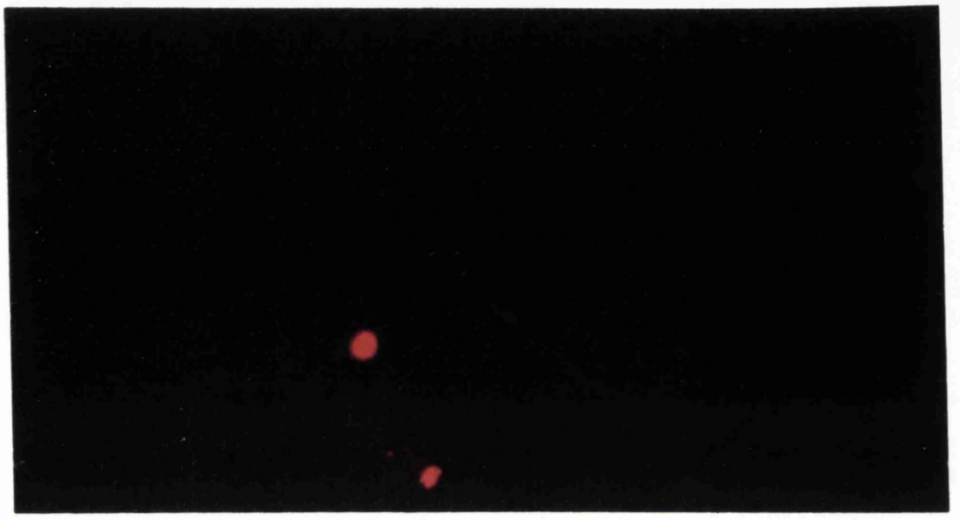
Fig. 3.15: - BOTH SIGNALS AMPLIFIED

a) DAPI COUNTERSTAIN



b) FITC (X CHROMOSOME) SIGNAL

c) TRITC (Y CHROMOSOME) SIGNAL



d) INTERPHASE COUNT

MALES (figures represent number of interphases)

NUMBER OF Y CHROMOSOME SIGNALS	NUMBER OF X CHROMOSOME SIGNALS 0	1	2	3	4	5+
0						
1		90			1	1
2						2
3						
4						1
5+		1				4

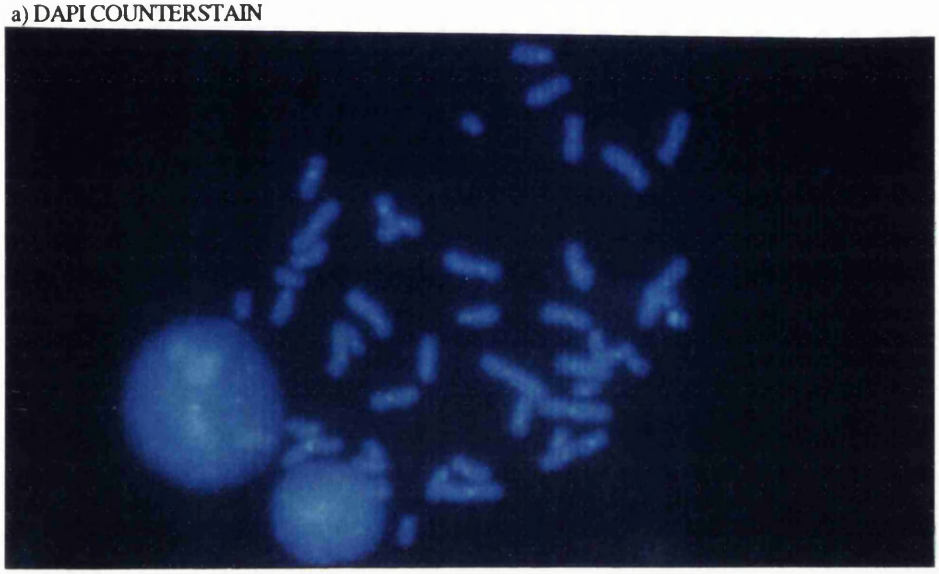
FEMALES (figures represent number of interphases)

NUMBER OF Y CHROMOSOME SIGNALS	NUMBER OF X CHROMOSOME SIGNALS 0	1	2	3	4	5+
0		7	83	1	1	6
1						
2						
3						
4						
5+			2			

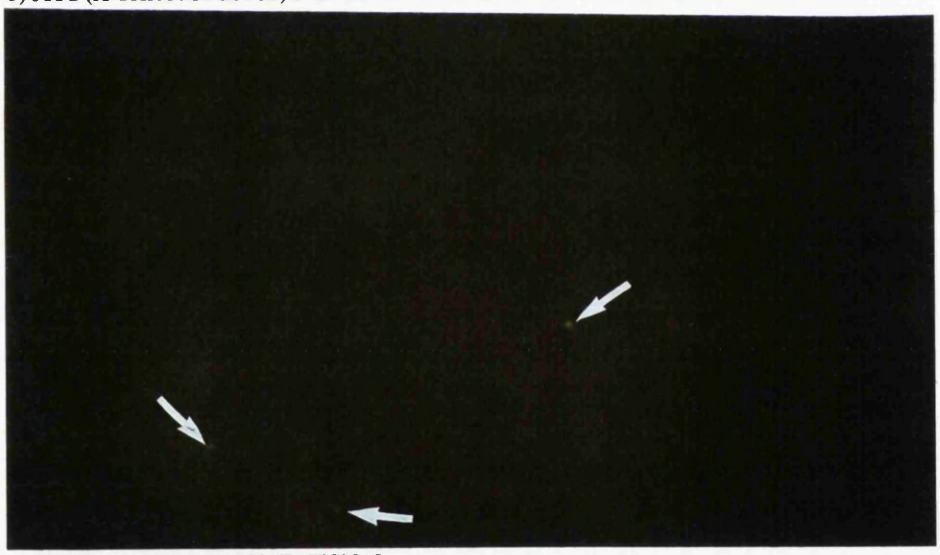
DOUBLE LABELLING WITH THE SAME PROBES AND FLUORCHROMES AS ABOVE BUT WITH NEITHER SIGNAL AMPLIFIED:-

Fig. 3.16:

a) DAPI COUNTERSTAIN



b) FITC (X CHROMOSOME) SIGNAL



c) TRITC (Y CHROMOSOME) SIGNAL

d) INTERPHASE COUNT

MALES (figures represent number of interphases)

NUMBER OF Y CHROMOSOME SIGNALS	NUMBER OF X CHROMOSOME SIGNALS 0	1	2	3	4	5+
0						
1		100				
2						
3						
4						
5+						

FEMALES (figures represent number of interphases)

NUMBER OF Y CHROMOSOME SIGNALS	NUMBER OF X CHROMOSOME SIGNALS 0	1	2	3	4	5+
0		4	94			2
1						
2						
3						
4						
5+						

This approach was used to ascertain the XX/XY cell ratio of a patient who was an XX/XY dispermic chimaera. (PHA stimulated lymphocytes).

Fig. 3.17:

a) ADJACENT CELLS WITH XX AND XY SIGNALS

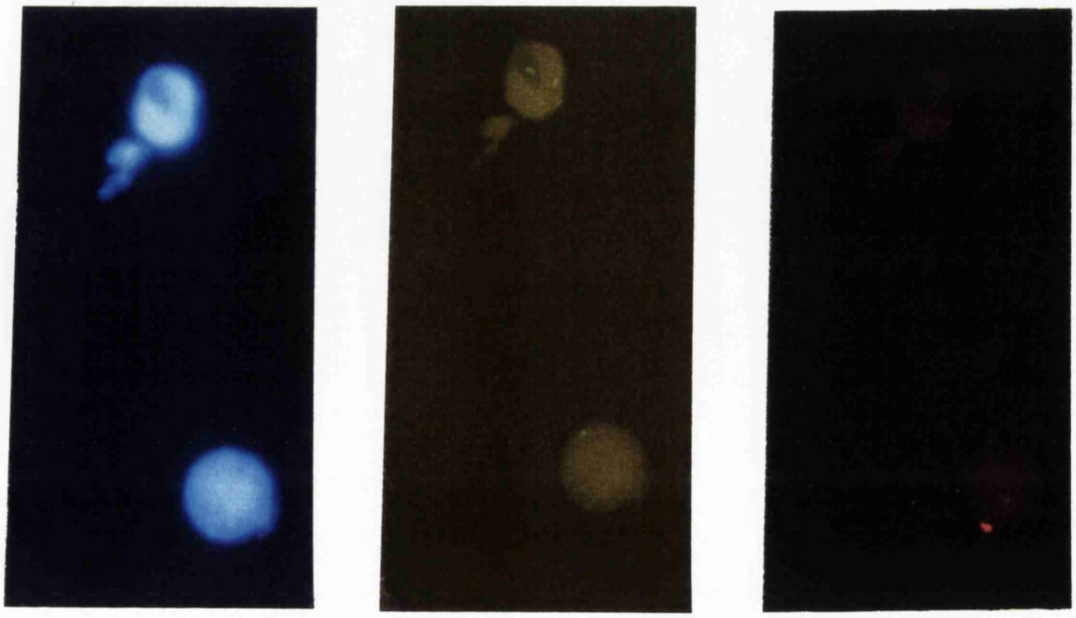
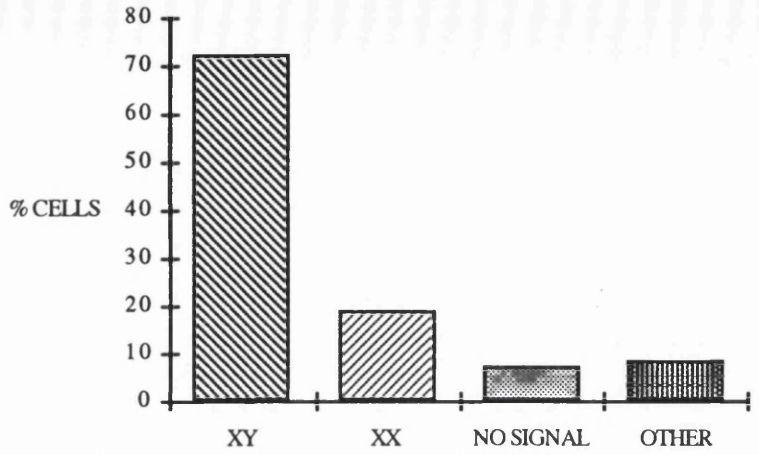


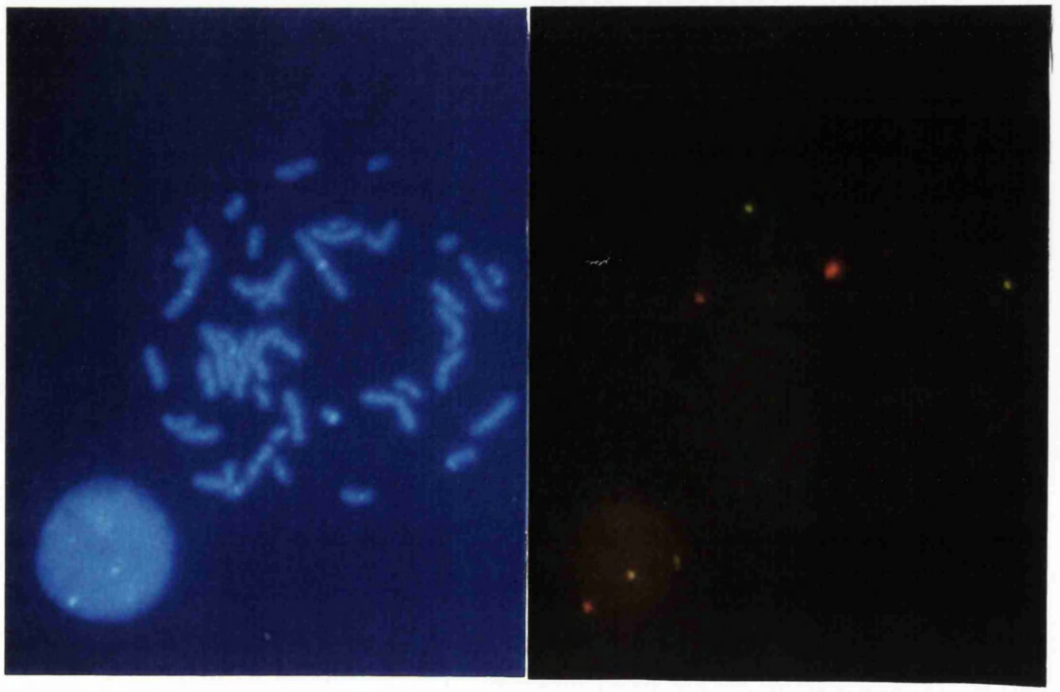
Fig 3.17 b):- INTERPHASE COUNT OF RELATIVE PROPORTION OF CELL TYPES IN DISPERMIC CHIMAERA



DOUBLE LABELLING WITH A COMBINATION OF p18 (BIOTIN - FITC) AND pUC1.77 (DIGOXIGENIN - TRITC).

NEITHER SIGNAL AMPLIFIED

Fig. 3.18:- NORMAL HUMAN LYMPHOCTYE MATERIAL.
a) INTERPHASES AND METAPHASE (DAPI COUNTERSTAIN)
b) RED SIGNAL (TRITC - CHROMOSOME 1) IS STRONG ENOUGH TO BE VISUALISED SIMULTANEOUSLY WITH GREEN SIGNAL (FITC - CHROMOSOME 18) ON B1 FILTER.



NUMBER OF CHROMOSOME 18 SIGNALS	NUMBER OF CHROMOSOME 1 SIGNALS					
	0	1	2	3	4	5+
0						
1		2	5			2
2		10	73			
3						
4						
5+			2			6

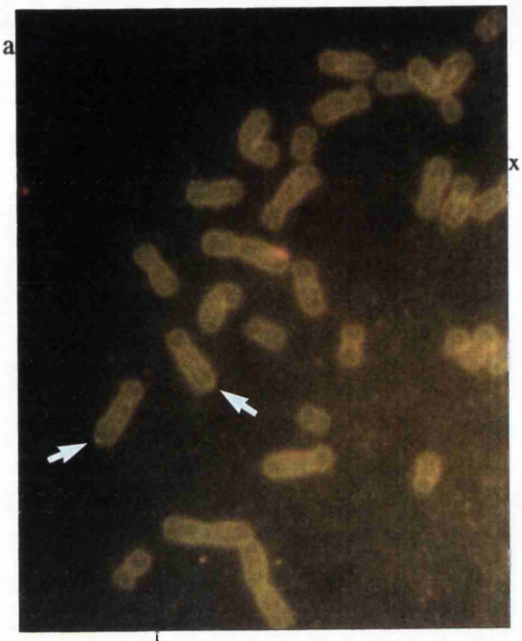
Double labelling strategies have the obvious advantage over single labelling in that two chromosomes can be simultaneously investigated on the same preparation. In the case of XY double labelling, the sex of a cell can be unequivocally determined. On any one cell, the hybridisation efficiency of one probe seemed to be independant of the other though in some cases (results not shown), when cytogenetic preparations were poor, both probes would show reduced efficiency.

MINI - SATELLITE "VNTR" PROBES

Two hypervariable probes were used in this study. For the first "λMS8" a photograph (fig 3.19a) and partial metaphase count (fig 3.19b) is given. For the second "λMS32" metaphases were pre-banded (using G-banding) and an ideogram peak is shown (fig 3.19c). Biotinylated probes were hybridised, washed and detected with FITC as for single copy probes (see methods); "Pinkel sandwich amplification" was used in both cases.

Fig. 3.19: NORMAL HUMAN LYMPHOCYE MATERIAL.

a

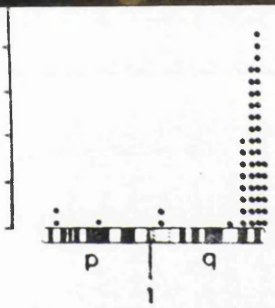


b

Metaphases with x number of signals on b group chromosome arms (50 counted)

x	0	1	2	3	4
	10	15	18	6	1

Fig. 3.19c:



The two probes λMS8 and λMS32 were supplied by Prof. Alec Jeffreys and ICI diagnostics to whom the author is very grateful.

The results in section 2 have demonstrated that alphoid and classical satellite probes give strong signals at both metaphase and interphase. Thus they are potentially useful in interphase cytogenetics whether using single labelling or double labelling strategies. It is apparent that non-amplification is generally preferable to amplification of signals because, although fluorescence was not as bright, a higher proportion of interphases with the correct number of signals were seen. Close observations of interphase nulcei in thse preparations revealed no signals sufficiently bright to count at interphase and this thus suggests (from the two probes tried) that VNTR probes are not useful for interphase cytogenetic applications.

SECTION 3: FISH APPROACHES FOR THE MAPPING OF CHROMOSOME 9q

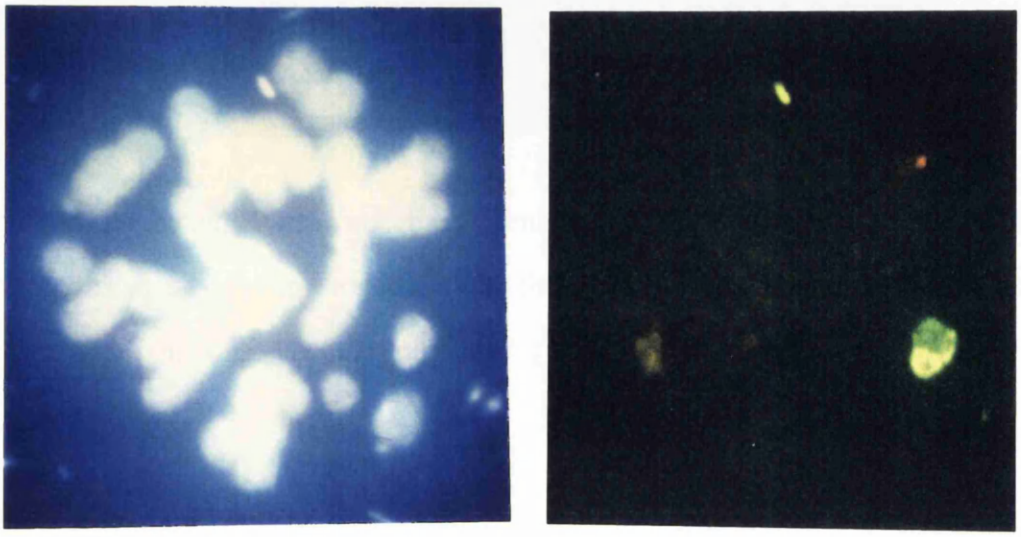
In this section FISH is shown to be a powerful tool for a gene mapping strategy in the search for the Tuberous Sclerosis locus "TSC1." Screening of somatic cell hybrids and the mapping of individual clones is demonstrated.

SCREENING THE PARENT HYBRID

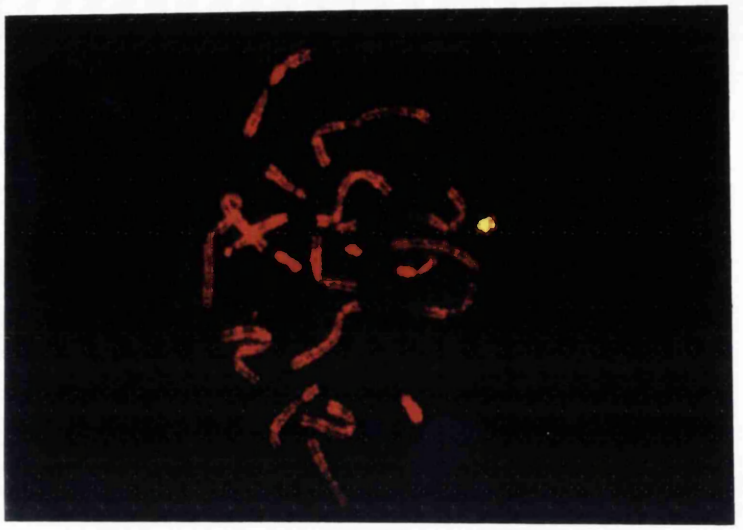
The parent hybrid "CJ9q" (see appendix) was screened by FISH a: to determine the number of human pieces it contained and b: to determine the human chromosomal origin of that (those) piece(s). In the first instance, chromosomal preparations of the hybrid were made and they were probed for human chromosomal content using sonicated, biotinylated human placental DNA as a probe (fig 3.20 a, b, c). In the second instance (fig. 3.21), human lymphocyte chromosome preparations were made and Alu PCR amplified, biotinylated CJ9q DNA was used as a probe incorporating CISS into the procedure (reverse chromosome painting).

Fig. 3.20:- Screening CJ9q for the presence of human material.

a) DAPI counterstain and b) fluorescein visualised signal (fluorescent microscopy).

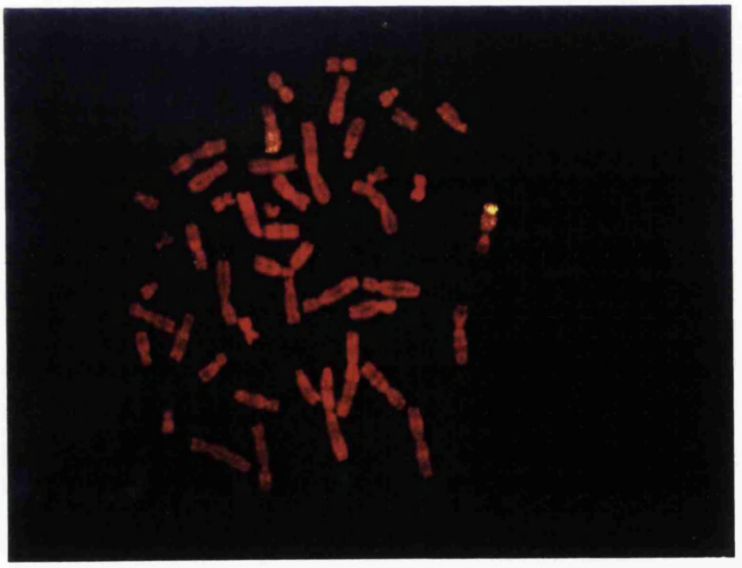


c) Simultaneous counterstain (red propidium iodide (PI)) and signal (yellow FITC)(confocal microscopy).



Note that CJ9q contains only one human piece and that the centromeric region fluoresces most brightly.

Fig. 3.21:- Screening CJ9q using reverse chromosome painting (FITC detection, PI counterstain)



Note that the only human material present is derived from chromosome 9q. Note also that the signal does not stretch as far as the centromere or heterochromatic region and that the G-light bands of chromosome 9q fluoresce more brightly than do the G-dark bands.

SCREENING OF FRAGMENT HYBRIDS

Irradiation-fusion fragment hybrids were made (by Mr Jude Fitzgibbon) from CJ9q using the approach summarised in the introduction.

Chromosome preparation from the hybrids were made as described and analysed by FISH using biotinylated human placental DNA as a probe.

In all, 22 hybrids were made and subsequently screened for the presence of human material. Human fragments were either integrated into a hamster chromosome or "solitary" (i.e. single chromosomes containing only human material and no visible hamster element). Results are as follows:

Table 3.1:

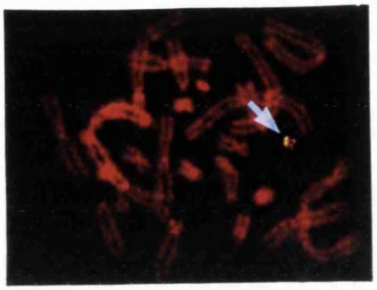
HYBRID NAME	No. OF HUMAN FRAGMENTS	TYPE OF FRAGMENT(S)	FREQUENCY IN METAPHASES 20 cells analysed	COMMENT
JJF1A	1	SMALL, SOLITARY	100%	? CENTROMERIC SEQUENCE IN FRAGMENT?
JJF2A	1	V. LARGE	80%	WHOLE CHROMOSOME PAINTED (REMAINING 20% OF METAPHASES - NO SIGNAL)
JJF2B	MULTIPLE			DIFFERENT CONTENT IN EACH CELL
JJF3A	5	2 X 2 SMALL AND CLOSE TOGETHER, 1 LARGE	80%	REMAINING 20% GAVE VARIABLE RESULTS
JJF3C	1	TINY AND INTEGRATED INTO A HAMSTER CHROMOSOME	60%	REMAINING 40% OF METAPHASES - NO SIGNAL
JJF6B	MULTIPLE			DIFFERENT IN EACH CELL
JJF6C	MULTIPLE			DIFFERENT IN EACH CELL
JJF7A	MULTIPLE			DIFFERENT IN EACH CELL
JJF7B	MULTIPLE			DIFFERENT IN EACH CELL
JJF8B	1	SMALL, SOLITARY	50%	? CENTROMERIC SEQUENCE IN FRAGMENT?

JJF9B	MULTIPLE			DIFFERENT IN EACH CELL
JJF12C	2	SMALL, VERY CLOSE TOGETHER, INTEGRATED INTO HAMSTER CHROMOSOME	100%	80% IN A METACENTRIC CHROMOSOME, 20% IN AN ACROCENTRIC CHROMOSOME
JJF13B	1	TINY, SOLITARY	30%	70% - NO SIGNAL
JJF14A	2	1 AVERAGE, INTEGRATED INTO TELOMERE OF AN ACROCENTRIC 1 TINY, INTEGRATED INTO TELOMERE OF AN ACROCENTRIC	90% 20%	ALL THOSE WITH TINY FRAGMENT HAD LARGER FRAGMENT ALSO
JJF15A	1	TINY, INTEGRATED INTO TELOMERE OF AN ACROCENTRIC	80%	20% - NO SIGNAL
JJF16C	0			PRESUMABLY NO HUMAN SEQUENCES PRESENT
JJF17A	1	SMALL, SOLITARY	80%	? CENTROMERIC SEQUENCE IN FRAGMENT? 20% - NO SIGNAL
JJF17B	1	TINY, INTEGRATED INTO SMALL CHROMOSOME	100%	
JJF18B	MULTIPLE			DIFFERENT IN EACH CELL
JJF19A	MULTIPLE			ONE FRAGMENT ONLY IN 40% OF CELLS,DIFFERENT IN ALL OTHER CASES
JJF20A	1	SMALL, SOLITARY	60%	? CENTROMERIC SEQUENCE IN FRAGMENT?
JJF21A	1	INTEGRATED INTO SMALL CHROMOSOME	25%	

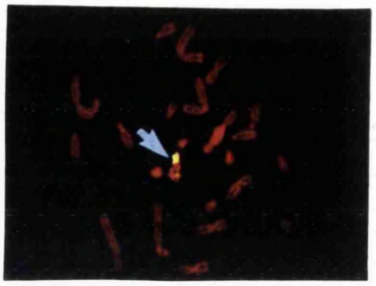
Of these hybrids the ones retaining 1 or 2 fragments were of most interest. Figs 3.22 and 3.23 show metaphases of each probed with total human DNA. Those in Fig 3.23 (namely 12C, 17A, 17B, 20A, and 21A) are particularly noteworthy as molecular (PCR and Southern blot) data has indicated that these contain clones which are linked to the TSC1 locus. This is illustrated in table 3.2 (page 153).

Fig. 3.22:- Photographs of FISH experiments of hybrids retaining 1 fragment.

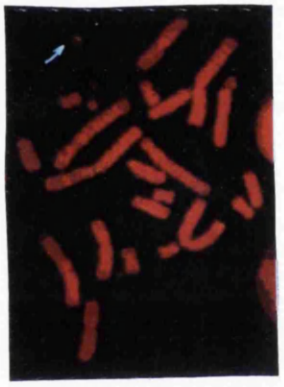
1A



2A

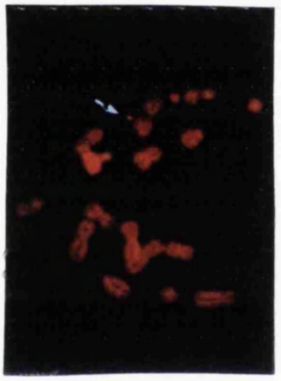


3C

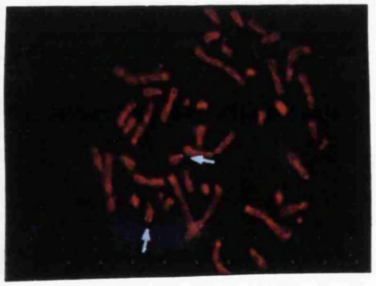


8B

13B



14A



15A

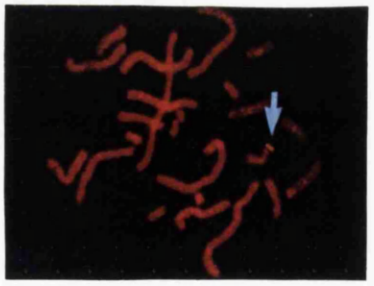
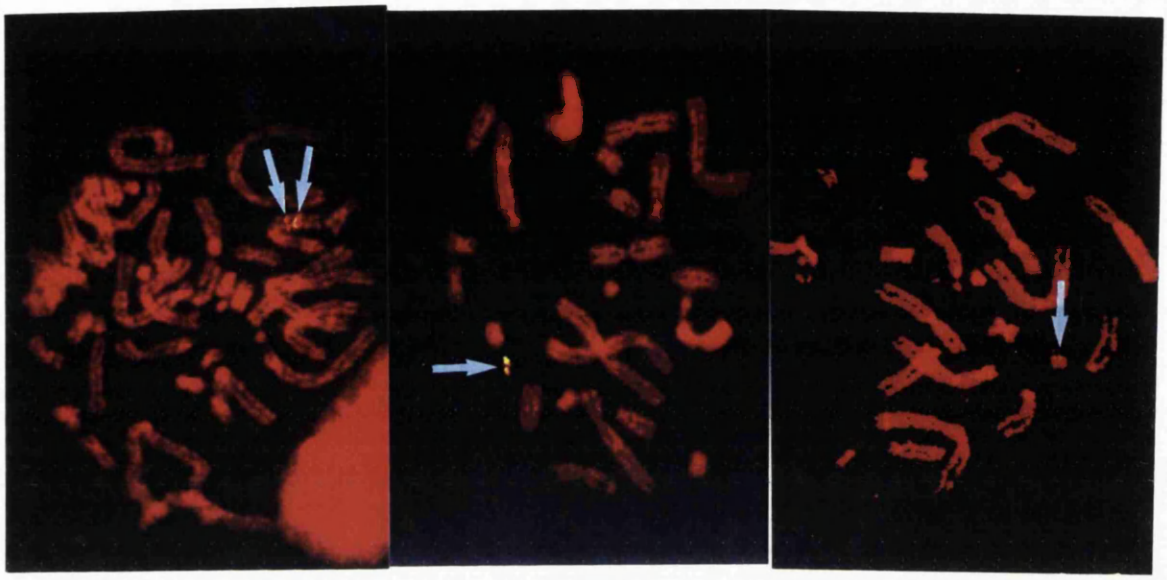


Fig 3.23:- Hybrids retaining one or two pieces and also containing clones linked to TSC1 (cf. Table 3.2).

12C 17A 17B



20A 21A

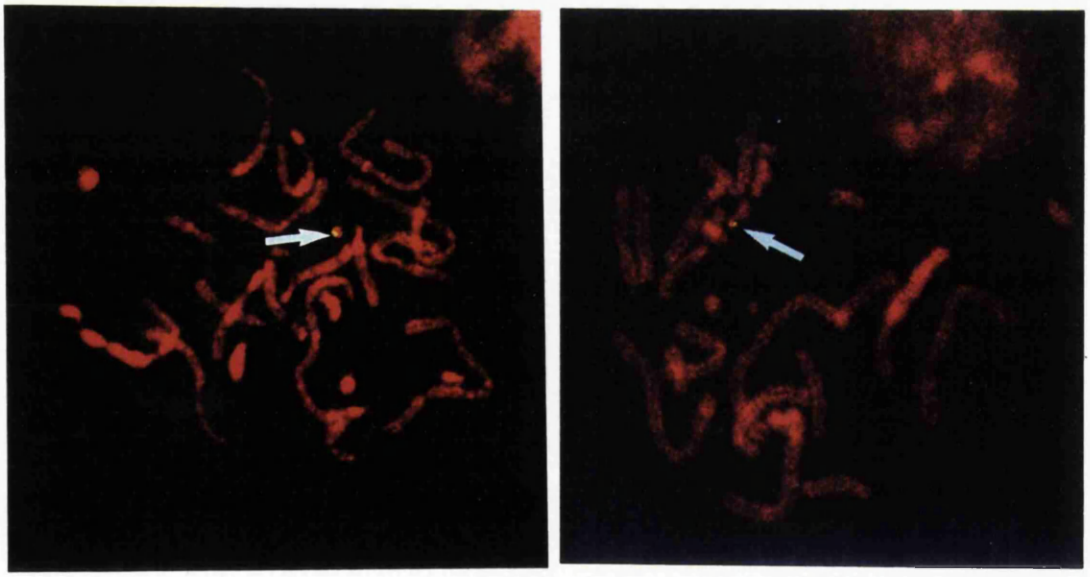


Table 3.2:- Presence or absence of chromosome 9q markers in hybrids.retaining 1 or 2 human fragments.

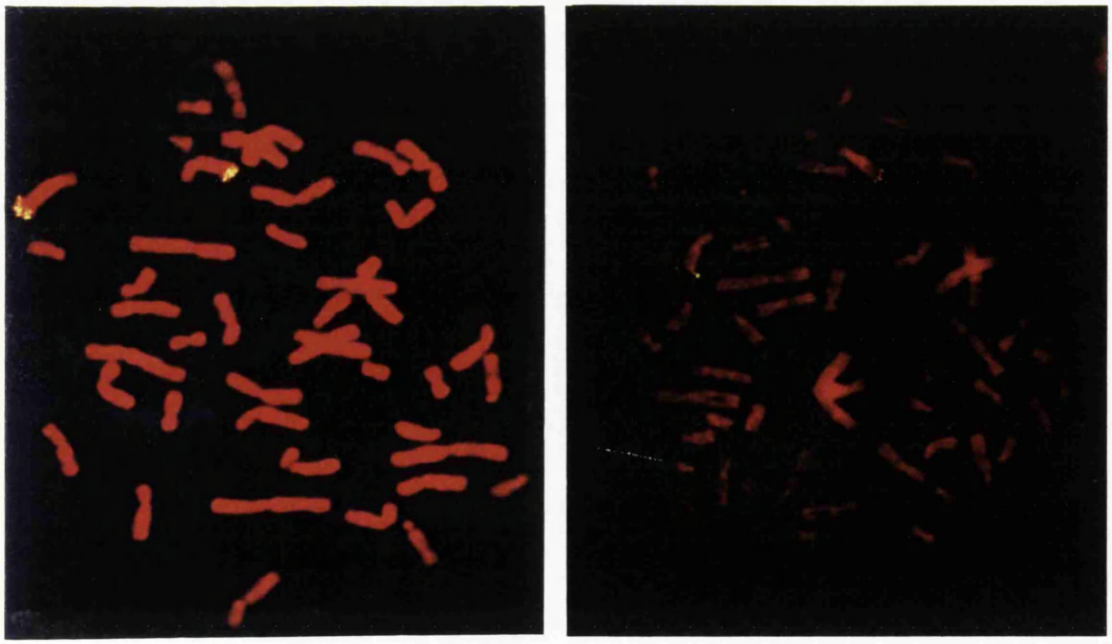
All markers were tested for all hybrids and the following order of probes is the one suggested at HGM11.

MARKER	HYBRID				
	12C	17A	17B	20A	21A
CEN					
D9S48		*			
GSN	*				
AK1	*				
ASS		*			
ABL	*	*			
ABO	*			*	
D9S10	*		*	*	
DBH	*		*	*	
D9S14			*		
D9S17	*			*	
D9S7	*				*
D9S11	*				
TEL					

TSC1 is thought to lie in the interval ASS-D9S14 (HGM11 collaborative effort).

All molecular analyses on these hybrids was performed by Mr Jude Fitzgibbon and Mr Josef Nahmias.

Fig 3.24:- Reverse chromsome painting of DNA from 12C and 17B onto normal human lymphocyte metaphases indicating both fragments map to 9q34.



MAPPING OF SHORT CLONES ISOLATED FROM AN IRRADIATION - FUSION HYBRID

In a separate set of experiments at a more advanced stage in the gene mapping strategy described in the introduction, phage clones were isolated from an existing hybrid "9a." This hybrid was of interest as it contained a marker linked to the TSC1 locus as revealed by molecular analysis.

Table 3.3:

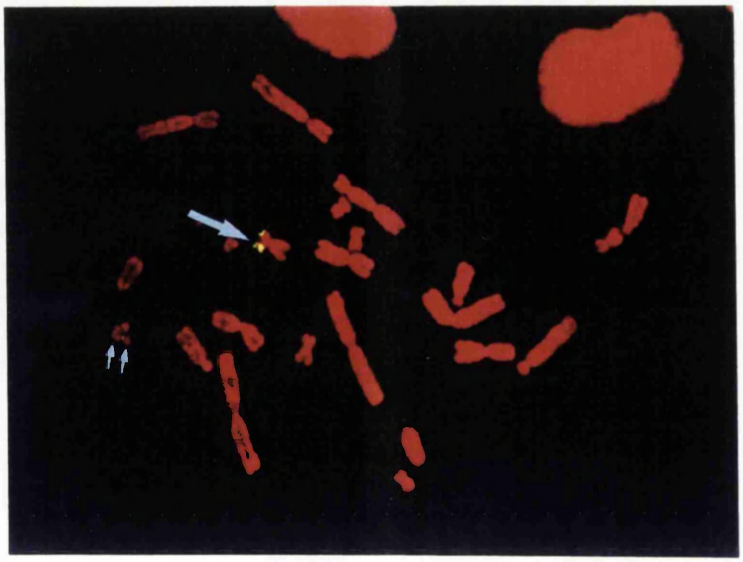
HYBRID	LOCUS								
	D9S48	SPTAN1	AK1	ABL	ASS	D9S10	DBH	D9S7	D9S11
9a	o	*	o	..	..	*	..	..	..

* = MARKER PRESENT .. = MARKER ABSENT o = MARKER NOT TESTED

Courtesy of Mr Nick Hornigold.

FISH analysis (human DNA as a probe on to hybrid metaphases) on this hybrid revealed one small and two tiny human fragments (Fig 3.25).

Fig 3.25:



This pattern was consistent in all metaphases analysed.

Five phage clones (insert size 15-20kb) were picked from this hybrid and mapping by FISH (using a biotinylated probe, CISS, FITC detection ("Pinkel sandwich") and PI counterstain; see methods section) was attempted for all of them (see table 3.4). Mr Nick Hornigold carried out the the clone isolation.

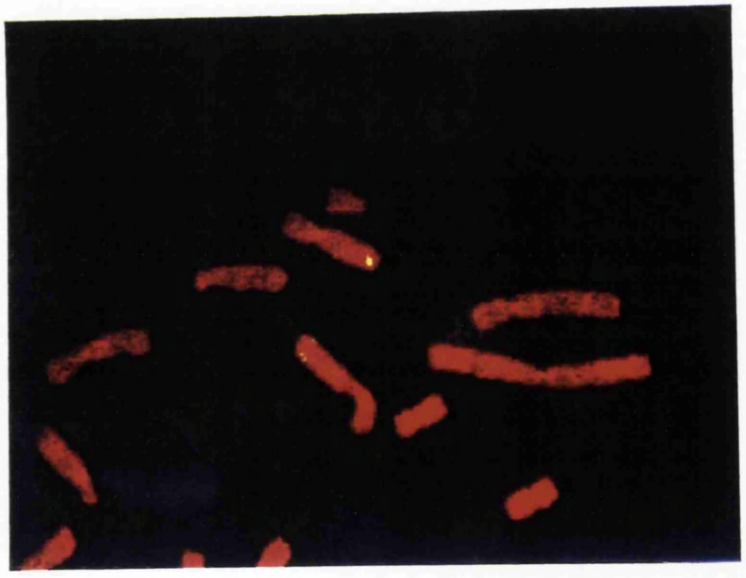
Table 3.4:- FISH mapping results of phage clones isolated from hybrid 9a.

Name	FISH RESULT	% METAPHASES WITH PAIRED SIGNAL: ON BOTH HOMOLOGUES	ON ONE HOMOLOGUE	ON NEITHER HOMOLOGUE	PAIRED SIGNALS ON OTHER CHROMOSOMES
9a1	Clone maps to 9q34.1	10	34	56	1(5q23),1(6p21), 1(9cen).
9a6	Clone maps to 9q31	2	29	69	
9a26	Clone maps to 9q34.1	6	39	55	
9a45	No clear signal				
9a50	No clear signal				

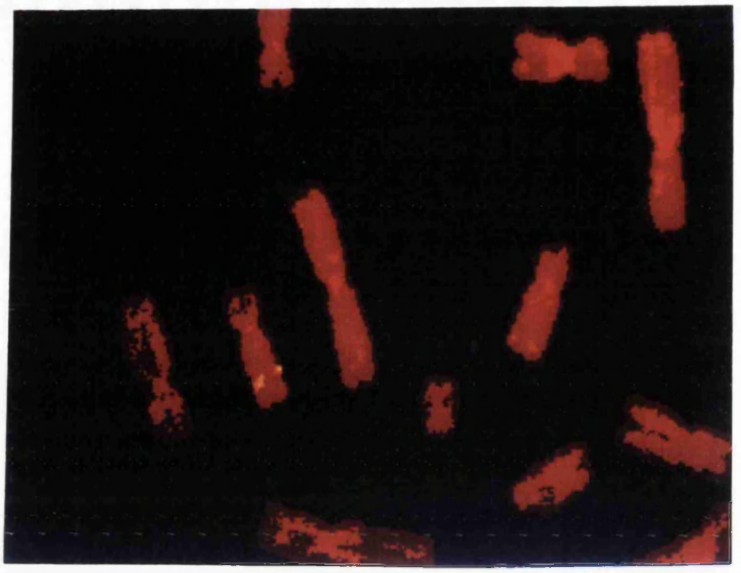
50 cells analysed in each case

Fig 3.26:- Photographs of representative metaphases showing FISH mapping of the above phage clones. (FITC signal, PI counterstain).

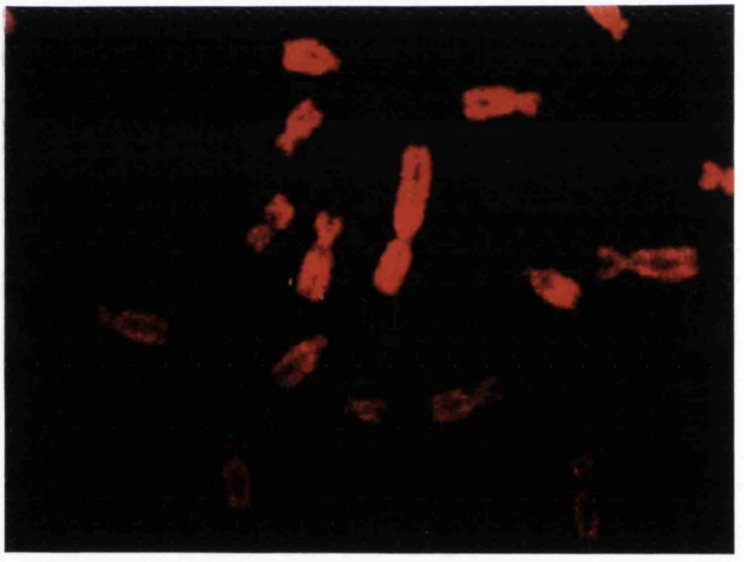
a) 9a1 mapping to 9q34.1.



b) 9a6 mapping to 9q31.

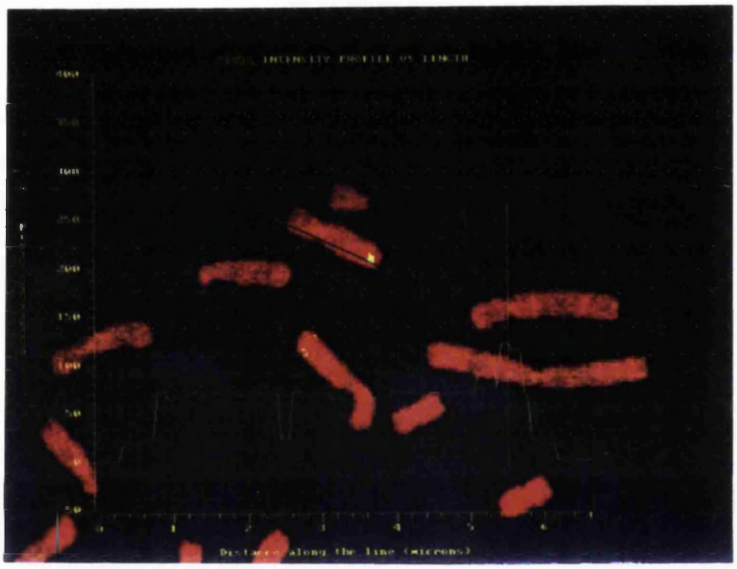


c) 9a26 mapping to 9q34.1.



Accurate band assignment was achieved in two ways: a) by comparison with DAPI bands (which give a pattern similar to Q-banding) and PI bands (which give a weak pattern similar to R-banding) both of which were visible in the preparation. b) By using the computer software to measure the distance of the signal from the p-terminus relative to the length of the whole chromosome thus:

Fig. 3.27:



In all cases both approaches were in complete concordance.

MAPPING COSMID CLONES ISOLATED FROM THE PARENT HYBRID

Five cosmid clones isolated from CJ9q by Jonathan Wolfe and Fiorella Florian were mapped in this study using biotinylated cosmid DNA as a probe on to normal human metaphases. FISH (inclucing CISS) was performed as described in the methods section and biotinylated probe detected using FITC and the "Pinkel sandwich." The results are summarised in the following table. Accurate chromosomal assignments were made as for the phage clones mapped in this study.

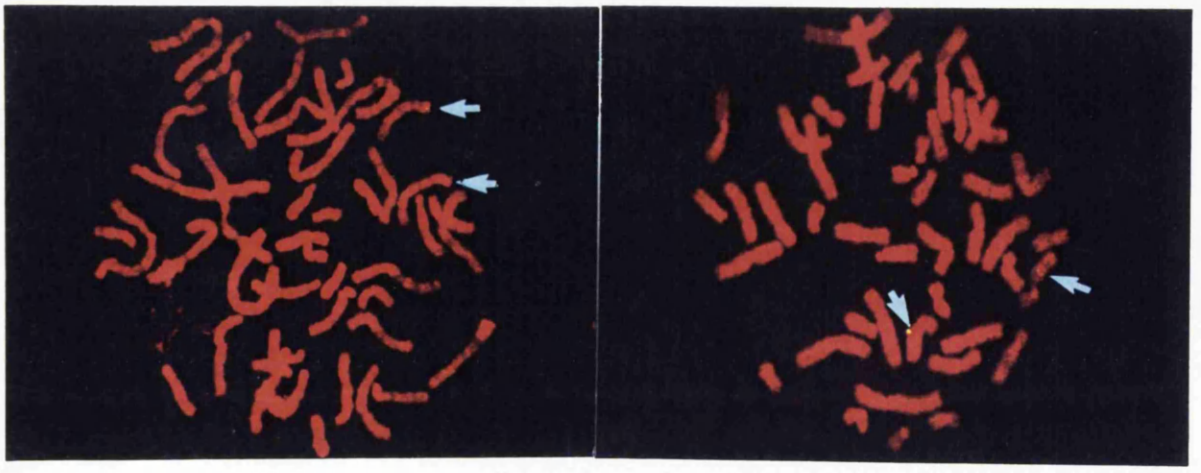
Table 3.5:-

COSMID NAME	MAP POSITION	EFFICIENCY OF HYBRIDISATION
cFF3	9q34	> 80% double signals on both homologues.
cFF40	9q34	> 80% double signals on both homologues.
cFF56	9q34	> 80% double signals on both homologues.
cFF59	9q13	> 80% double signals on both homologues.
cFF97	9q21	> 80% double signals on both homologues.

50 cells analysed in each case

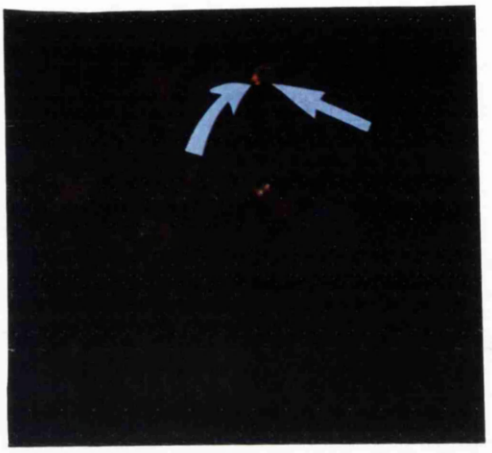
Fig. 3.28:-

a) cosmid cFF56 mapping to chromosome 9q34 b) cosmid cFF97 mapping to chromosome 9q31



c) dual labelling DBH (red - curved arrow) and cFF40 (green - straight arrow)) indicating DBH is more telomeric.

In this case DBH was labelled with digoxigenenin and CFF40 with biotin. Dual detection was achieved as described in the methods section (amplification of both probes was performed). Digoxigenin labelled probe was detected using TRITC and biotinylated probe using FITC. Chromosmes were not counterstained with PI as this would "drown" the TRITC signal. DAPI counterstained chromsomes of this spread are not shown as the confocal microscope cannot detect them.



The results in section 3 have demonstrated the application of FISH in a number of aspects of the gene mapping strategy including the screening of somatic cell hybrids and the mapping of individual clones. It is hence an invaluable approach in such a strategy.

SECTION 4: COLORECTAL CANCER STUDIES

This section deals with FISH studies on two colon cancer cell lines (LIM1215 and AA/C1/SB10 - see appendix 2 for details) and also an FPC patient carrying a deletion on one chromosome 5 (see appendix 5). Furthermore mapping of sequences known to be expressed in normal colonic mucosa to chromosome 5 is demonstrated.

LIM 1215

CYTOGENETIC STUDIES

Fig. 3.29 a,b shows two full karyotypes of LIM1215 and demonstrates the presence of the 13p+ marker. This marker was present in 100% of cells analysed and had been found to be the only karyotypic abnormality (Jenkyn *et al.*, 1987 - see appendix 2).

Fig. 3.29a:

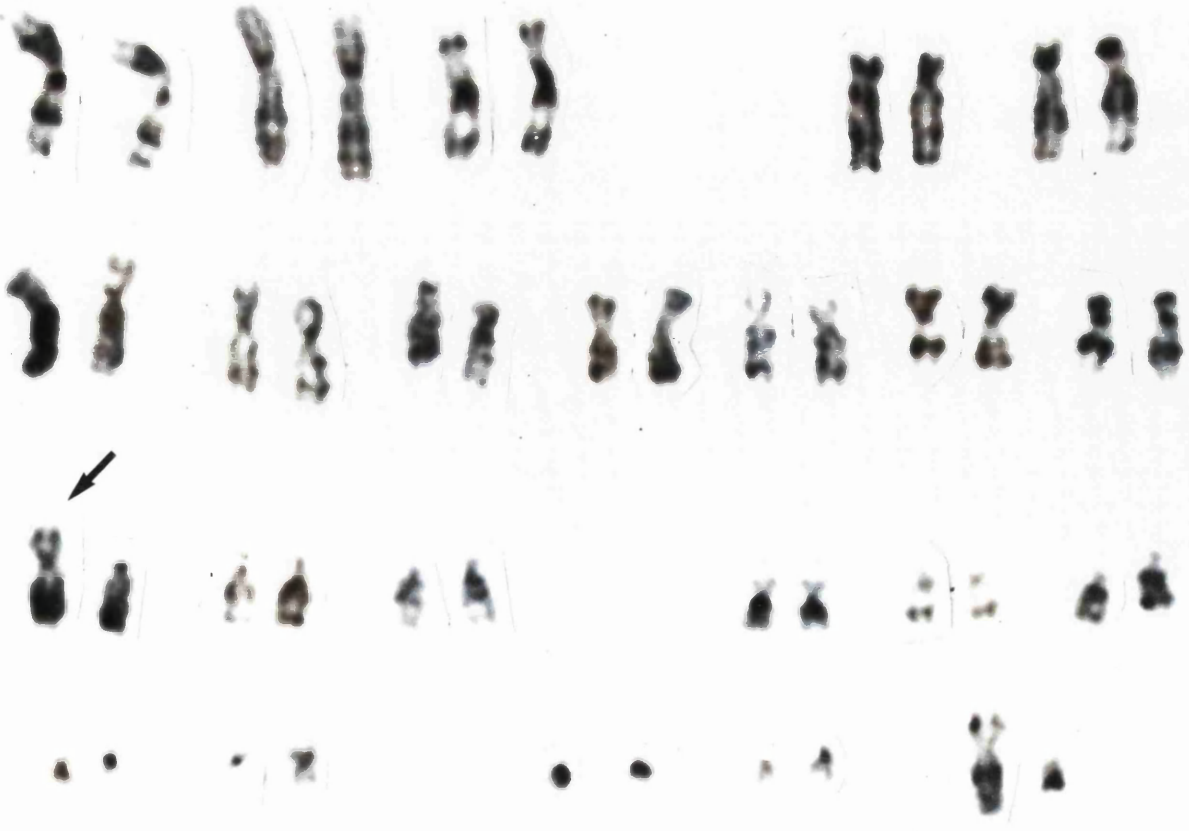


Fig. 3.29b:

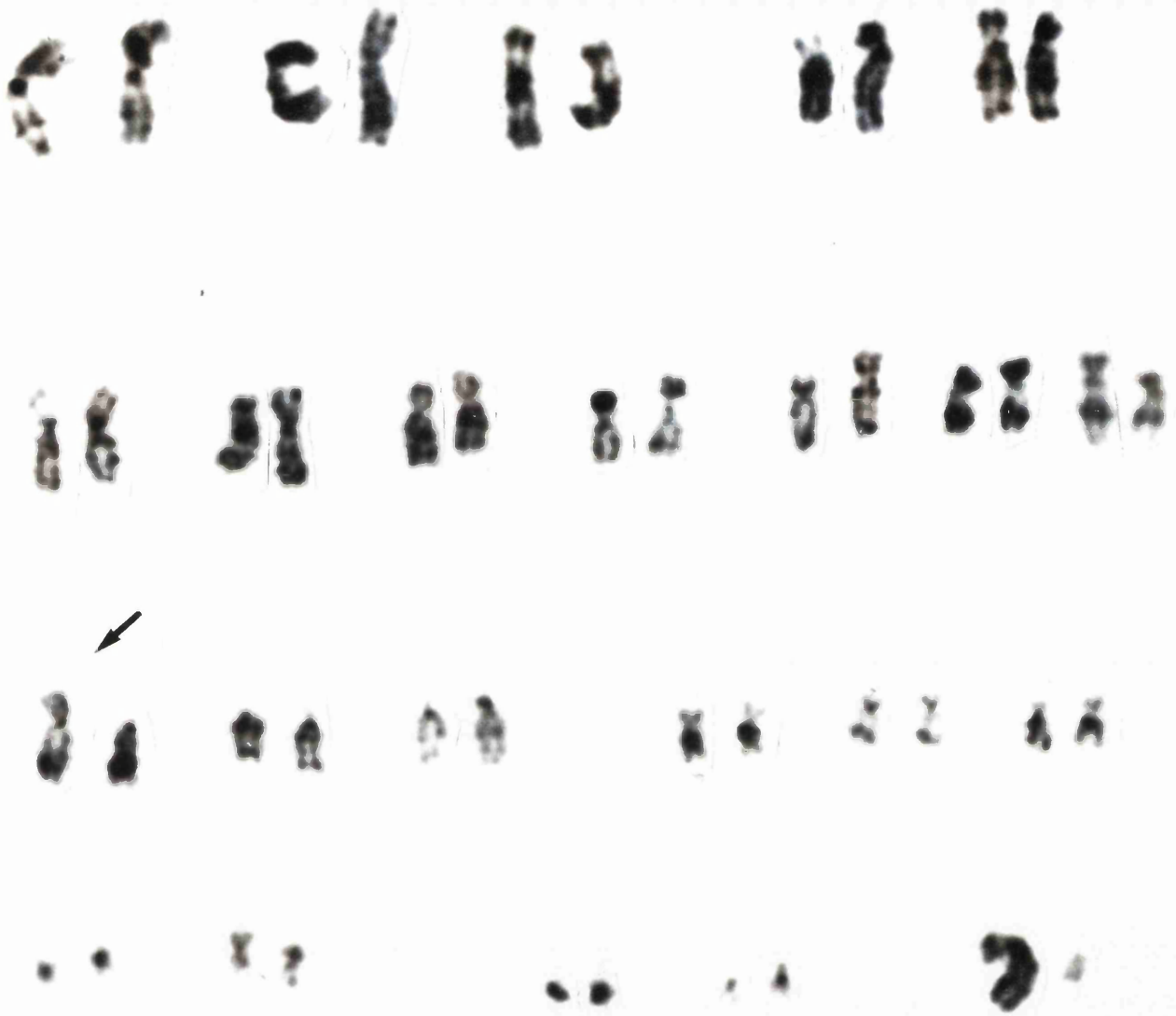


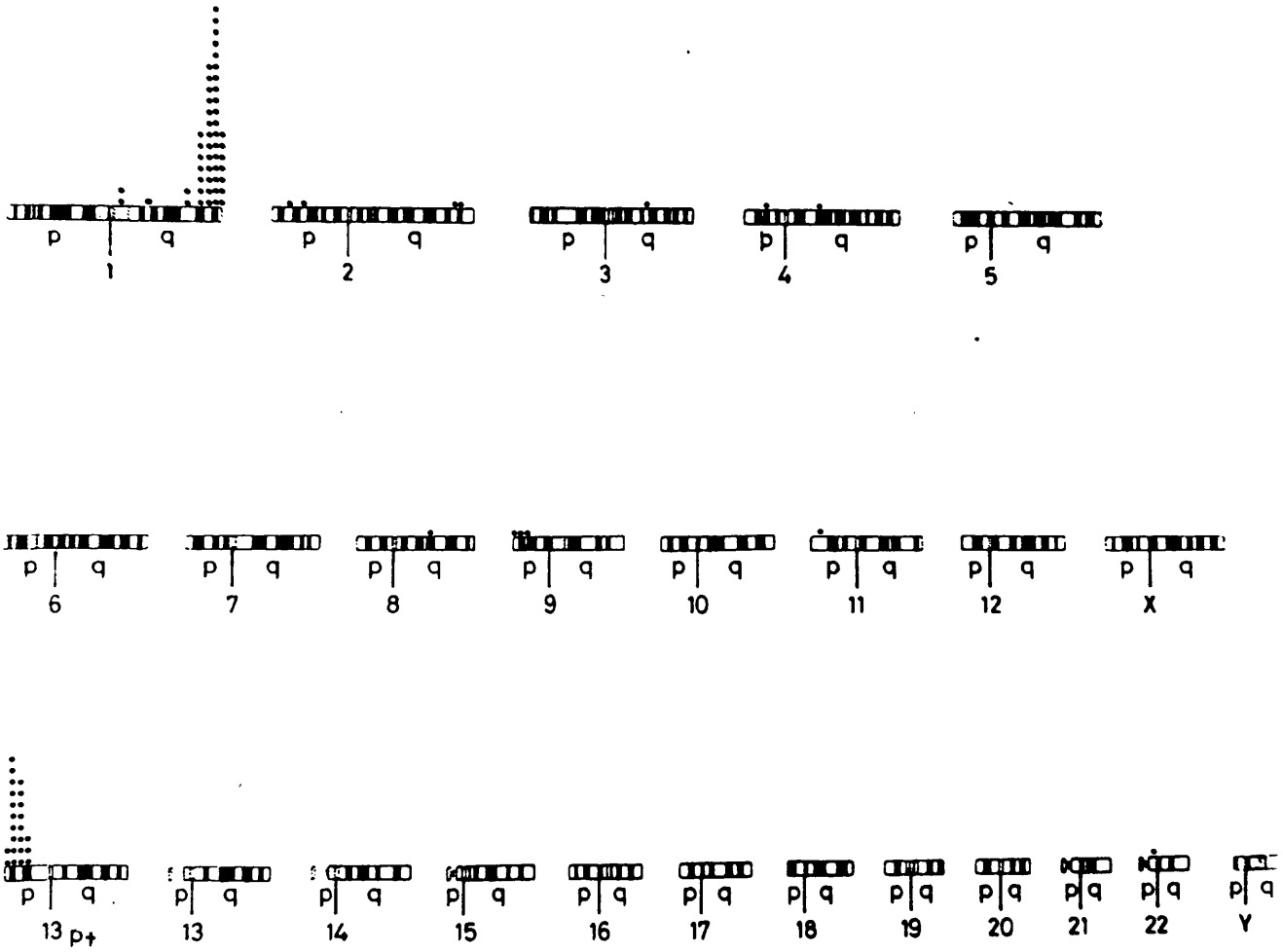
Fig 3.30 shows partial karyotypes in the light of results of Jenkyn *et al* (1987) comparing the short arm of 13p+ and 1q32-qter by G-banding homology. On the left is the normal chromosome 13, to the right of that is the 13p+ and to the right of that are the two chromosomes 1 (inverted). These results hence indicate the similarity of the bands on the p arm of 13p+ and of those on terminal 1q.

Fig. 3.30:

FISH STUDIES

Chromosome preparations were made from LIM1215 and banded using conventional trypsin-Giemsa banding (G-banding - see methods section). Analysable metaphases were photographed and their position noted. FISH was performed on this preparation using the VNTR probe λMS32 (biotinylated) which was detected using FITC and the "Pinkel sanwich" (see methods section for hybridisaton conditions for VNTR probes). Metaphases were relocated post-FISH and accurate cytogenetic assignment of visible signals achieved by comparison with photographs of G-banded metaphases. Hybridisation sites were noted by placing a dot on a standard ideogram leading to a peak of signals where hybridisation occured. The results show hybridisation peaks at 1q42-43 and also on the p-arm of 13p+ thus confirming trisomy of 1q32-qter in this line. 20 cells were analysed.

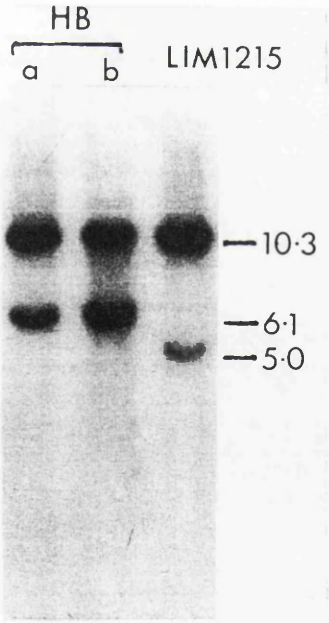
Fig 3.31:- Ideogram peak of λMS32 signals



MOLECULAR STUDIES

Southern blotting was also carried out (by Sarah Leigh) to confirm trisomy of the region of 1q by determining whether one allele as recognised by probe λMS32 was duplicated in the LIM1215 line. LIM1215 DNA was digested to completion with AluI and probed with P^{32} labelled λMS32. As the right-hand lane indicates, the 10.3kb allele is more intense than the 5.0kb allele. It is apparent therefore that it is this one which is duplicated. No normal material was available from this patient so a direct control could not be presented. For comparison however, samples from a patient "HB" are included. HBa shows a similar pattern to LIM1215 (i.e. one allele more intense than the other) because, as karyotypic data has shown, this material has an isochromsome 1q and hence trisomy for that region. HBb is from normal tissue and the bands are of equal intensity. The smaller bands are of different sizes in the two cases due to the highly polymorphic nature of the hypervariable probe.

Fig 3.32:



AA/C1/SB10

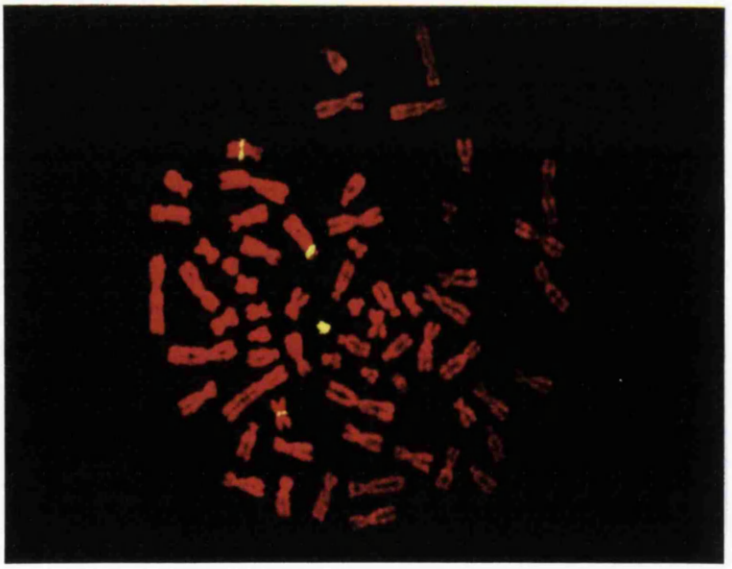
This cell line is an immortal tumorigenic cell line which was transformed *in-vitro* from an immortal non-tumorigenic cell line that was, in turn, derived from a premalignant adenoma of a patient with FPC. Further details are given in the appendix.

FISH, STUDIES

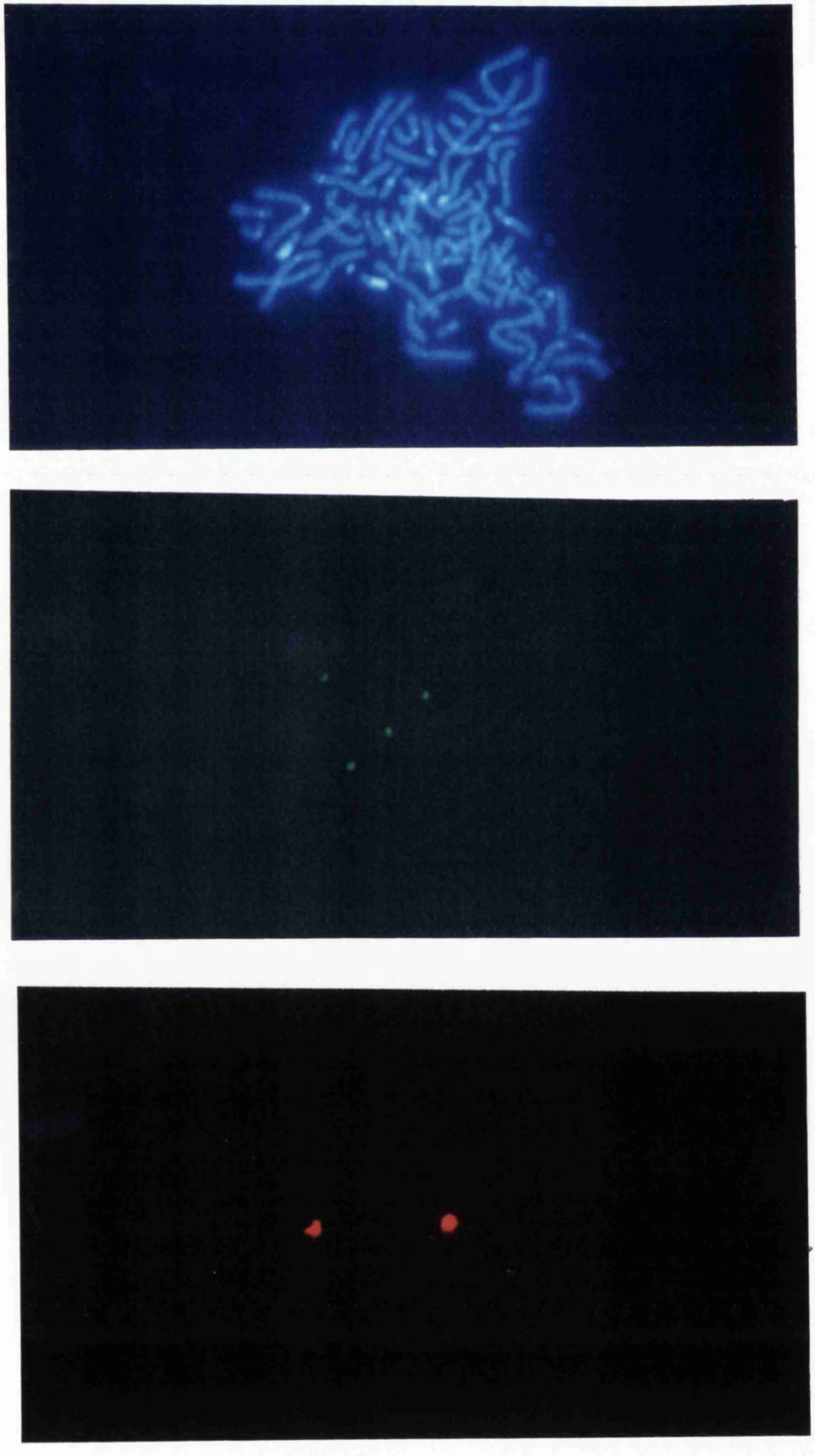
The repetitive probes p18 (specific for the centromere of chromosome 18) and pUC1.77 (specific for the heterochromatic region of chromosome 1) were used on this cell line to indicate the presence of these chromosomes. Single and dual labelling experiments have indicated the presence of four copies of the centromere of chromosome 18 and two of the centromere of chromosome 1 (Fig 3.33).

Fig. 3.33:-

a) Single FISH using biotinylated probe p18 (FITC detection, PI counterstain).

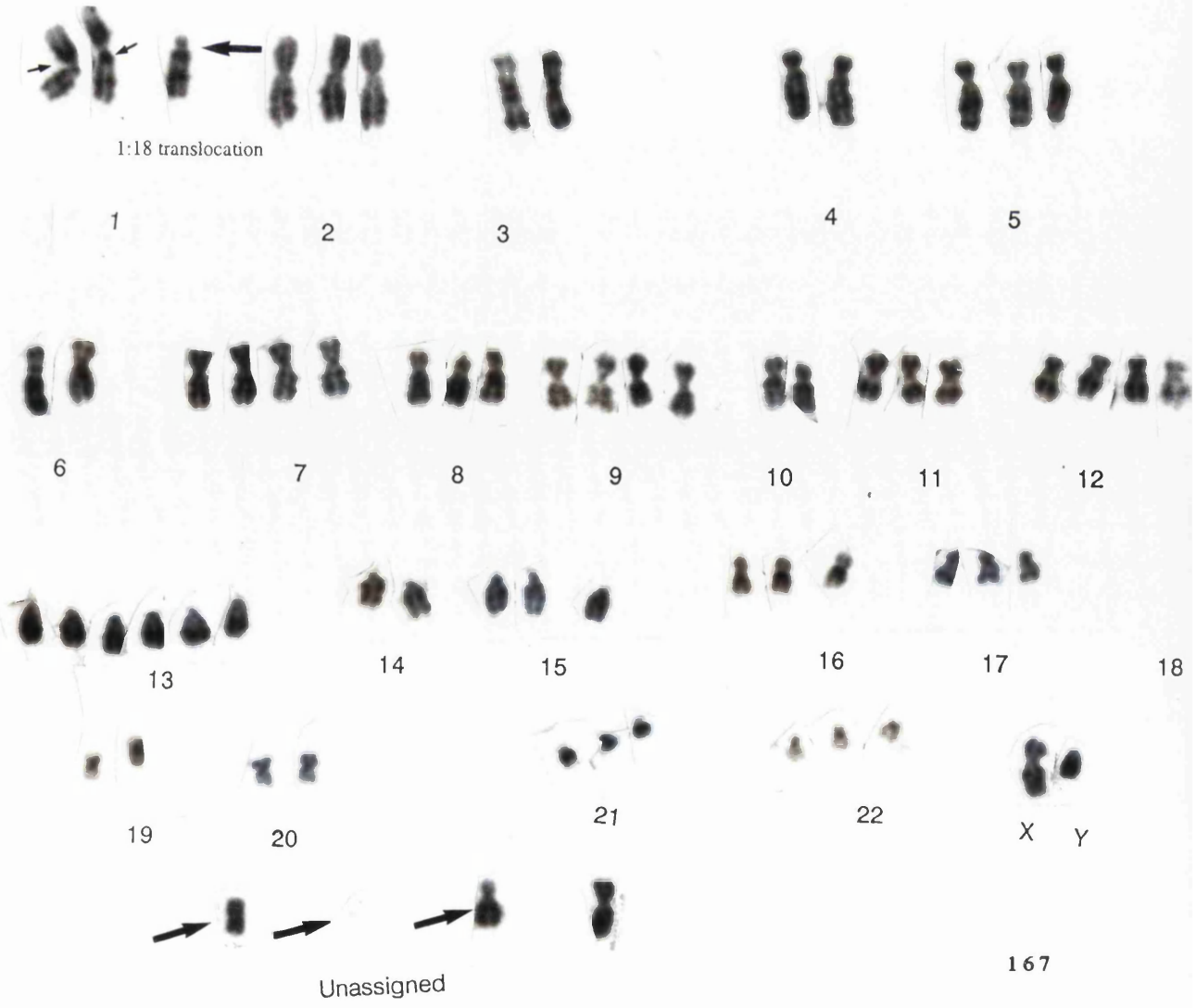


b) Dual FISH with probes p18 (biotin labelled and detected with FITC(green)) and pUC1.77 (digoxigenin labelled and detected with TRITC (red)). Neither signals were amplified.



Here, FISH data obtained in this study is compared with established G-banding data on the cell line. A recent G-banded karyotype of AA/C1/SB10 is shown (Fig 3.34). Three copies of chromosome 1q and no whole chromosome 18 are apparent. The larger arrows indicate the hybridisation sites of the chromosome 18 probe and the smaller arrows indicate the hybridisation sites of the chromosome 1 probe. This was worked out by examining the chromosomes with the fluorescent counterstains DAPI and PI in the above FISH experiments and equating them with the G-banded karyotype. Thus these experiments demonstrate the presence of four copies of the chromosome 18 centromere when G-banding alone suggested that their might be none. Also, an apparently deleted chromsome 1 with a pericentric inversion turned out to be a 1:18 translocation with the break in chromsome 18 in the region of the DCC gene.

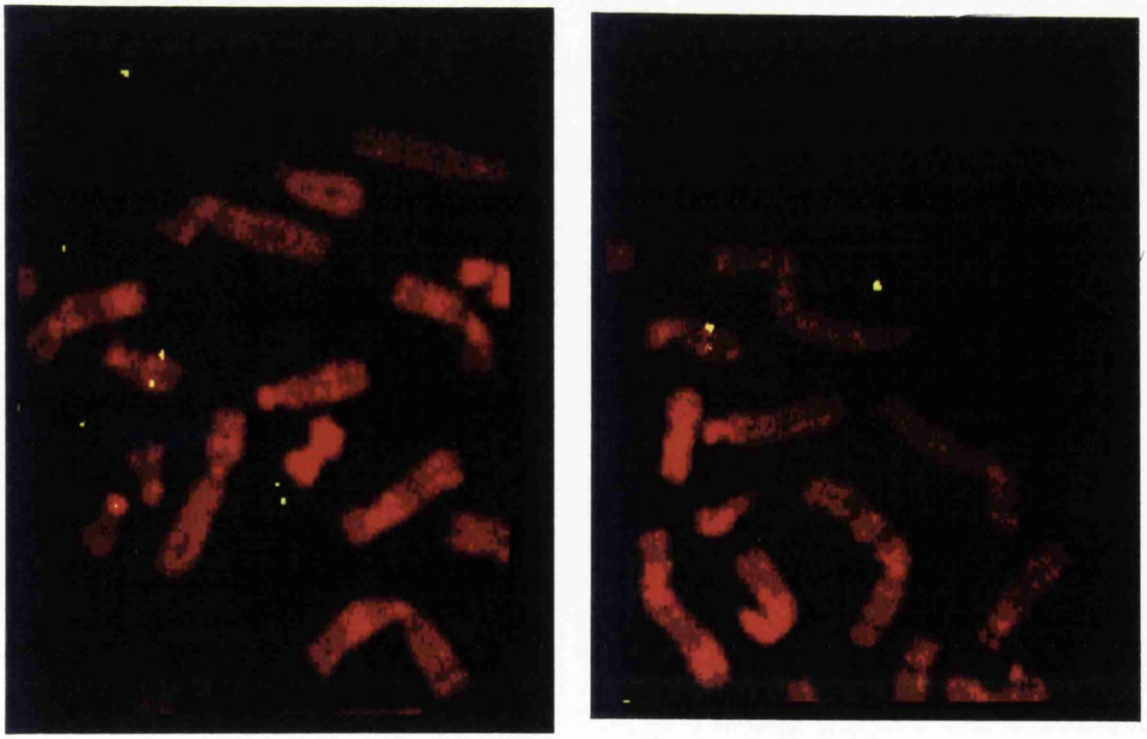
Fig 3.34:



Loss of chromosome 18 (or part of it) is thought to be an important event in the adenoma to carcinoma sequence of colorectal tumours (reviewed in Fearon and Voglestein., 1990). Thus it would have been reasonable to expect loss of chromosome 18 in this line. The common region of loss in colorectal cnacer on chromosome 18 is thought to be the DCC gene (Fearon *et al.*, 1990), thus attention in this study turned to this region of the genome.

Fig. 3.35 indicates the mapping of 3 cDNAs (p15-65, Josh 4.4 and Sam 1.1) from the DCC locus (total insert size around 5kb) on to normal human lymphocyte metaphases thus mapping the gene to chromosome region 18q21. Each probe was biotinylated (BRL kit) and the three were combined and used in a FISH experiment (the same approach as for cosmid mapping since there were some repetitive sequences in two of the probes). Biotinylated probe was detected using FITC ("Pinkel sandwich") and chromosomes were counterstained with PI.

Fig. 3.35:



The signals obtained in this experiment were very weak on control material and were not seen on both homologues in any metaphase examined. Hence mapping the cDNAs alone on the cell line AA/C1/SB10 was thought to be impracticable for investigating the presence of the DCC gene in this cell line. Thus attempts were made to screen a human genomic cosmid library with the probe p15-65 (this was the one which contained no repetitive elements). Resulting cosmids however did not produce signals on chromosome 18. Consequently research into the presence of the DCC gene in the cell line AA/C1/SB10 is ongoing.

EXAMINATION OF A CHROMOSOME 5 DELETION

In this study, one proband of a family with a rearranged chromosome 5 is examined. The pedigree of the family is shown in the appendix as are the proband's chromosomes 5 (he has a cytogenetically detectable deletion on one chromosme 5 (5q22-23.2., see appendix 5). The following figures demonstrate FISH cosmid experiments on the chromosomes 5 of that proband. Biotinylated cosmid DNA was detected with FITC and chromosomes counterstained with PI. Control experiments on normal lymphocyte metaphases indicated that both cosmids ECB27 and L5.79 mapped to 5q21-22 (> 80% of metaphases had double signals on both homologues).

Fig. 3.36a:- Cosmid probe ECB27 on lymphocyte metaphases of the proband with the deletion thus indicating that this clone is not within the deletion

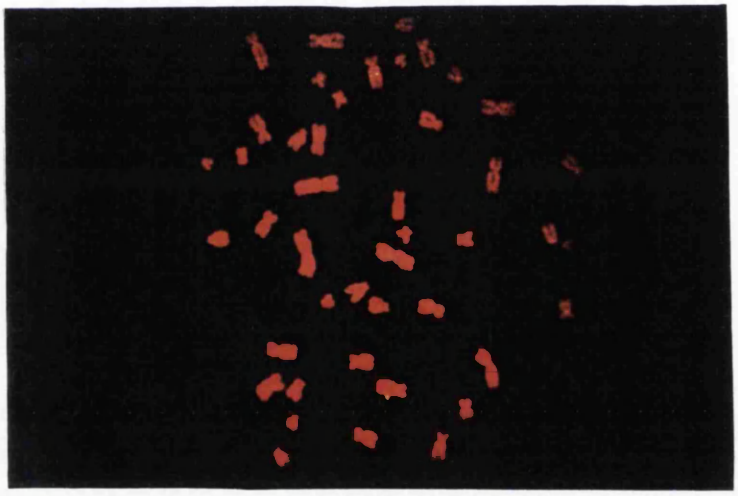
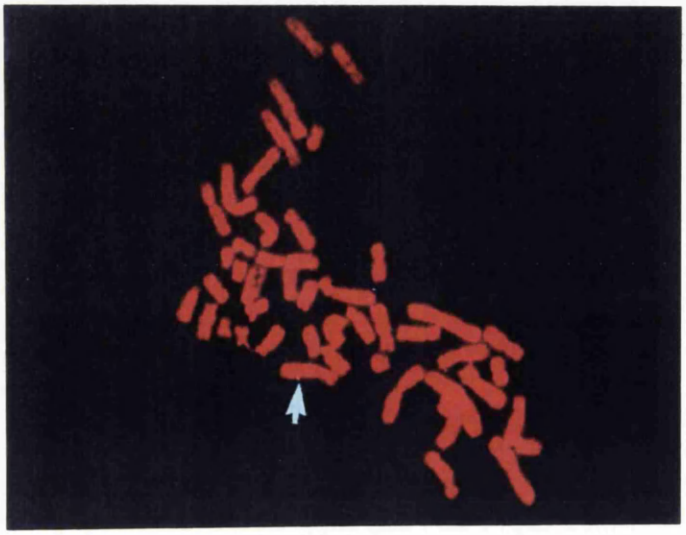


Fig. 3.36b:- Cosmid probe L5.79 on fibroblast metaphases of the proband indicating that this clone is not present on one of the chromosomes 5 and hence is contained within the deletion (20 cells examined).

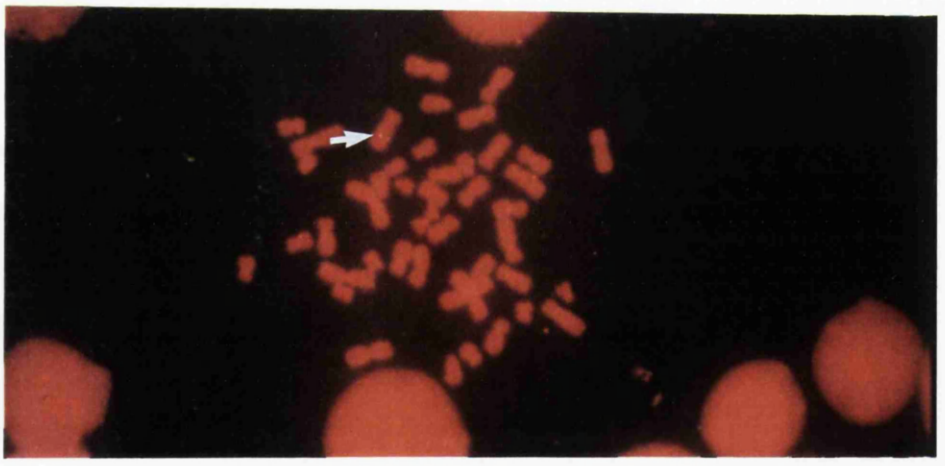


MAPPING OF SEQUENCES FOUND TO BE EXPRESSED ON CHROMOSOME 5

The following probes were isolated by Sarah Leigh, Simon Gayther and Kate Lawson. Clones expressed in normal colonic mucosa and mapping to chromosome 5 were isolated and cloned into λgt10 (phage vector). One of these (λ5.3 - insert size around 3kb) was successfully mapped by FISH to chromosome band 5q23-31.(Fig. 3.37a). A human genomic cosmid library was screened with this clone and another "λ5.5" (which was not successfully mapped by FISH) yielding clones "cos5.3iv" and "cos5.5iv" respectively. Both these were mapped to chromosome 5 by FISH (Fig. 3.37 b, c). In each case biotinylated probe was detected with FITC and chromosomes were counterstained with PI.

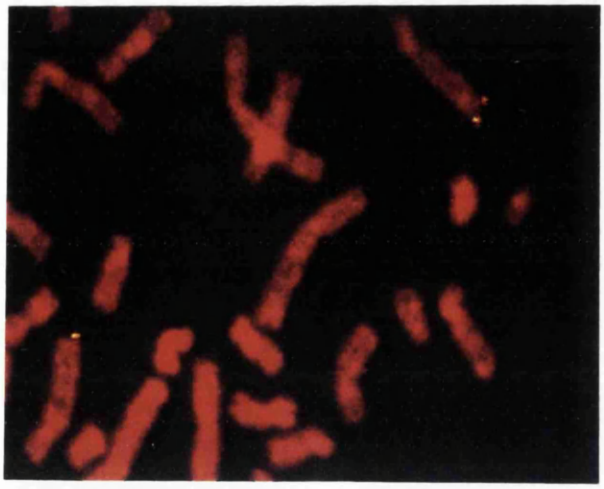
Fig 3.37:-

a) λ5.3 mapping to chromosome 5q23-31

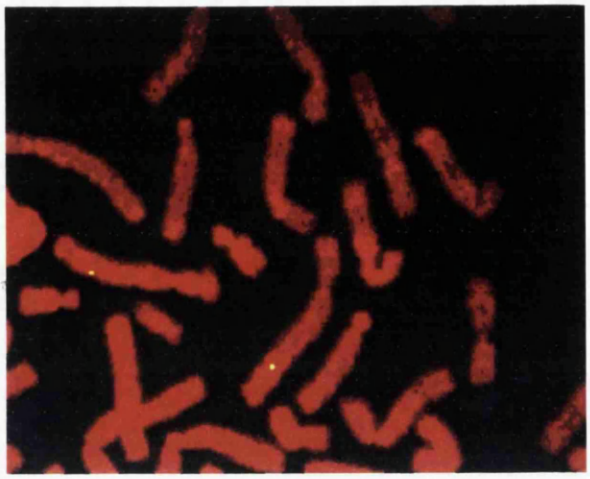


20 cells analysed

b) cos5.3iv mapping to chromosome 5q35



c) cos5.5iv mapping to chromosome 5q31



20 cells analysed

Cos 5.3 and λ5.3 were found to hybridise to each other (by Southern blotting) on two separate occasions. Hence it was certain that they contained the same sequence. Due to the discrepencies in the FISH results, they were both repeated (once for the phage, twice for the cosmid) to eliminate the possibility of sampling error. In each case they were found to give the same result as presented here.

The results in section 4 have hence illustrated how FISH can be used to add to and refine cytogenetic data by molecular means in the study of colorectal cancer. The study of cell lines and of patients is an approach which is applicable to all human neoplasms. Research in ongoing in the study of cell line AA/C1/SB10 and of individuals with reaaranged chromosomes 5.

SECTION 5: PREIMPLANTATION DIAGNOSIS BY FISH

SINGLE LABELLING FISH USING X AND Y CHROMOSOME SPECIFIC PROBES

This section deals with the prospects for preimplantation diagnosis of sex and chromosome abnormalities using FISH. In the case of sexing, research has reached the stage of a clinical trial. Embryonic nuclei were prepared for *single* labelling experiments by Dr Alan Handyside and Dr Richard Penketh. Y and X chromosome specific probes pHY2.1 and pBamX7 respectively were used separately on them employing FISH with a biotin-fluorescein detection system ("Pinkel sandwich" and DAPI counterstain). This is illustrated in the following figures.

Fig 3.38:- pHY2.1 sexing a male embryo (The "signal" with the small arrow in b) is an autofluorescent foreign body, see discussion)

a) b)

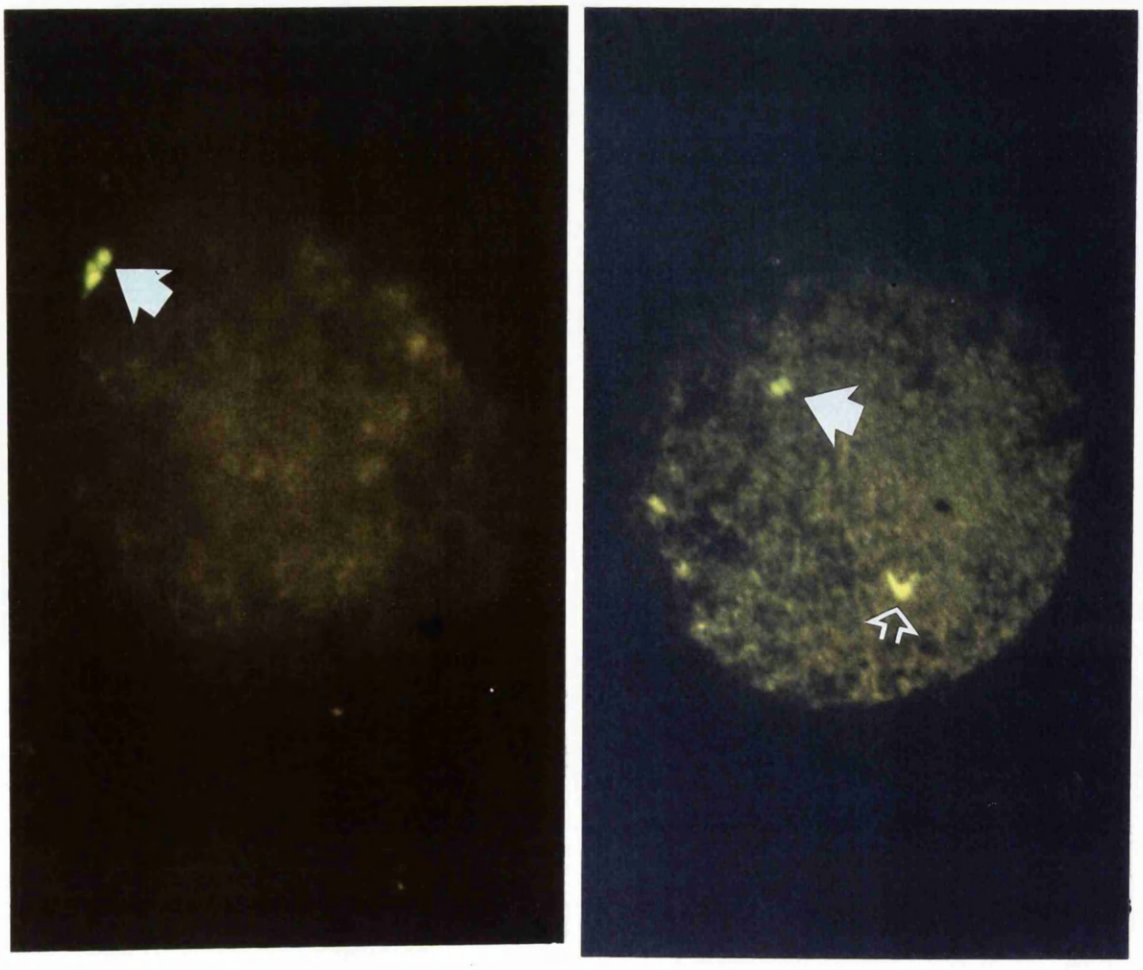


Fig 3.39:- pBamX7 sexing of male embryo cells

a) Two male cells (DAPI counterstain followed by amplified FITC signal)

b) Three male cells one of which is tetraploid (counterstain and signal as before).

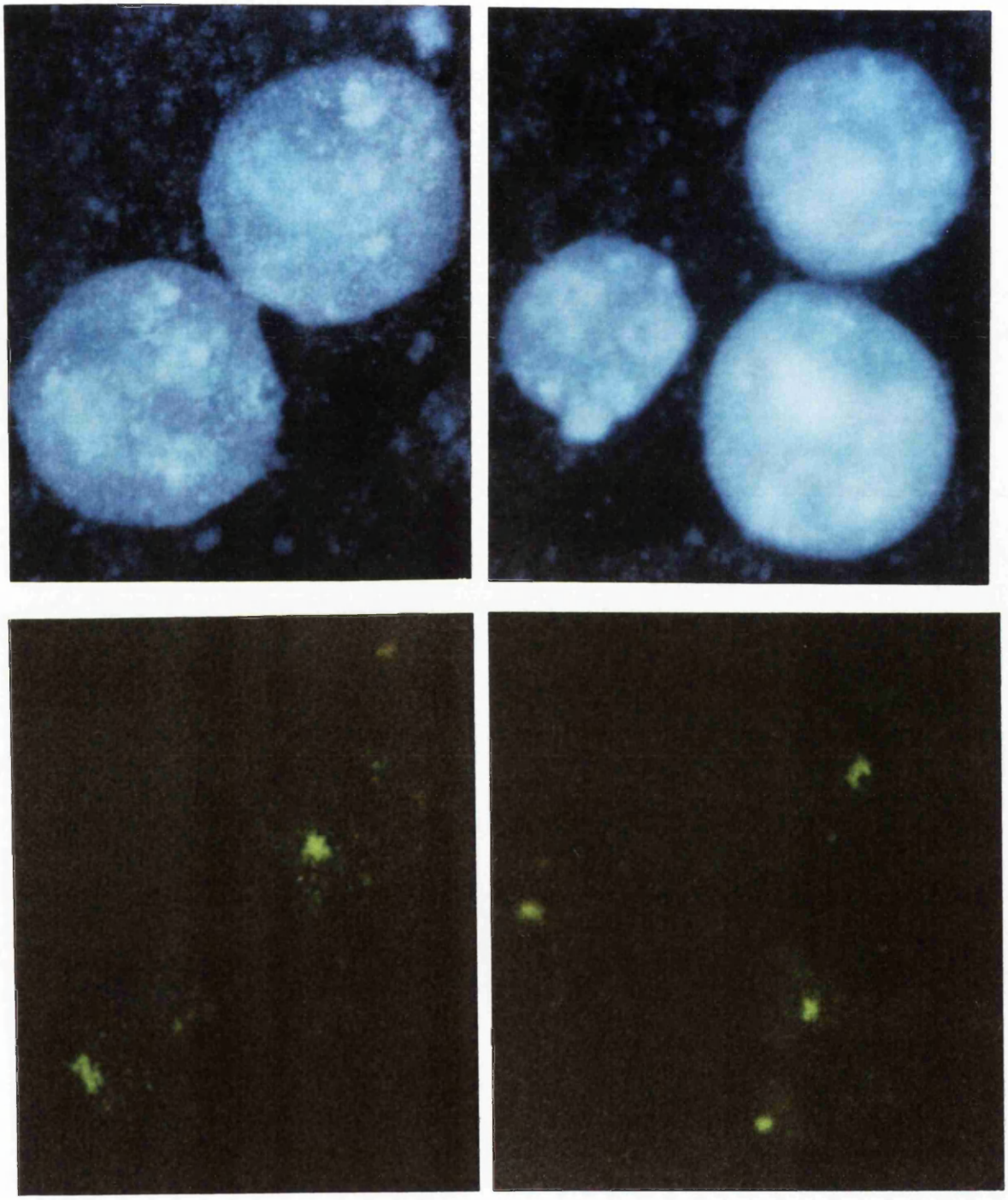


Table 3.6:- Efficiency of pHY2.1 probe on embryonic nuclei

Nuclei from a total of 11 embryos were examined using pHY2.1, three eight-cell embryos and groups of 1-4 cell from a series of clevage stage embryos. Using pBamX7, eight embryos at various stages between between days 3 and 5 with 6-19 nuclei per embryo. Classification of sex was made on the number of signals in the majority of nuclei.

		Number of nuclei		Total number of nuclei scored
		Number of hybridisation signals per nucleus		
		0	1	
Male embryos	a	1	7	8
	b	1	7	8
Male embryo cells	c	1	3	4
	d	1	1	2
	e	-	2	2
	f	-	1	1
Total		4	21	25
Female embryos	g	8	-	8
Female embryo cells	h	2	-	2
	i	2	-	2
	j	2	-	2
	k	1	-	1
Total		15	0	15

Table 3.7:- efficiency of pBamX7 probe on embryonic nuclei

		Number of nuclei						Total number of nuclei scored
		Number of hybridisation signals per nucleus						
		0	1	2	3	4	5+	
Male embryos	l	1	5	-	-	-	-	6
	m	2	4	-	-	-	-	6
	n	-	8*	2	-	-	-	10
	o	4	10*	3	-	-	2*	19
	p	2	12	2	-	1	-	17
	q	1	7	2	-	-	-	10
Total		10	46	9	0	1	2	68
Female embryos	r	4	-	5*	-	1	-	10
	s	1	1	5	-	-	-	7
Total		5	5	10	0	1	0	17

* includes at least one nucleus in metaphase

In both these cases, a proportion of males were incorrectly diagnosed as females. Thus, it was decided to proceed to the use of double labelling for both X and Y chromsomes to reduce the chance of misdiagnosis.

SIMULTANEOUS DETECTION OF X AND Y CHROMOSOME SPECIFIC PROBES USING DUAL FISH

All embryos were disaggregated on day 2 (~4-cell) and each cell spread separately by Dr Leeanda Wilton. Initially pHY2.1 (Y chromosome) and pBamX7 (X chromosome) were used as probes. Dual hybridisation was overnight in 62.5% formamide and no dextran suplphate, washes were in 62.5% formamide (37°C), both signals were amplified. pHY2.1 was labelled with digoxigenin and detected with FITC, pBamX7 was labelled with biotin and detected with Texas-red

Table 3.8:- Efficiency of pHY2.1 and pBamX7 dual labelling on embryonic interphase nuclei.

Embryo name	Number of cells tested	Signal			No result	Sex
		XX	XY	other	i.e. no signal	
A1	2	2	-	-	-	F
A2	2	-	-	-	2	?
A3	1	-	-	-	1	?
A4	1	-	1	-	-	M
A5	1	-	-	-	1	?
A6	2	-	-	-	2	?
A7	2	-	-	-	2	?
A8	1	-	1	-	-	M
Totals	12	2	2	0	8	

Then a third (digoxigenin labelled) probe was used namely cY98. Hybridisation, washing and detection conditions were as before.

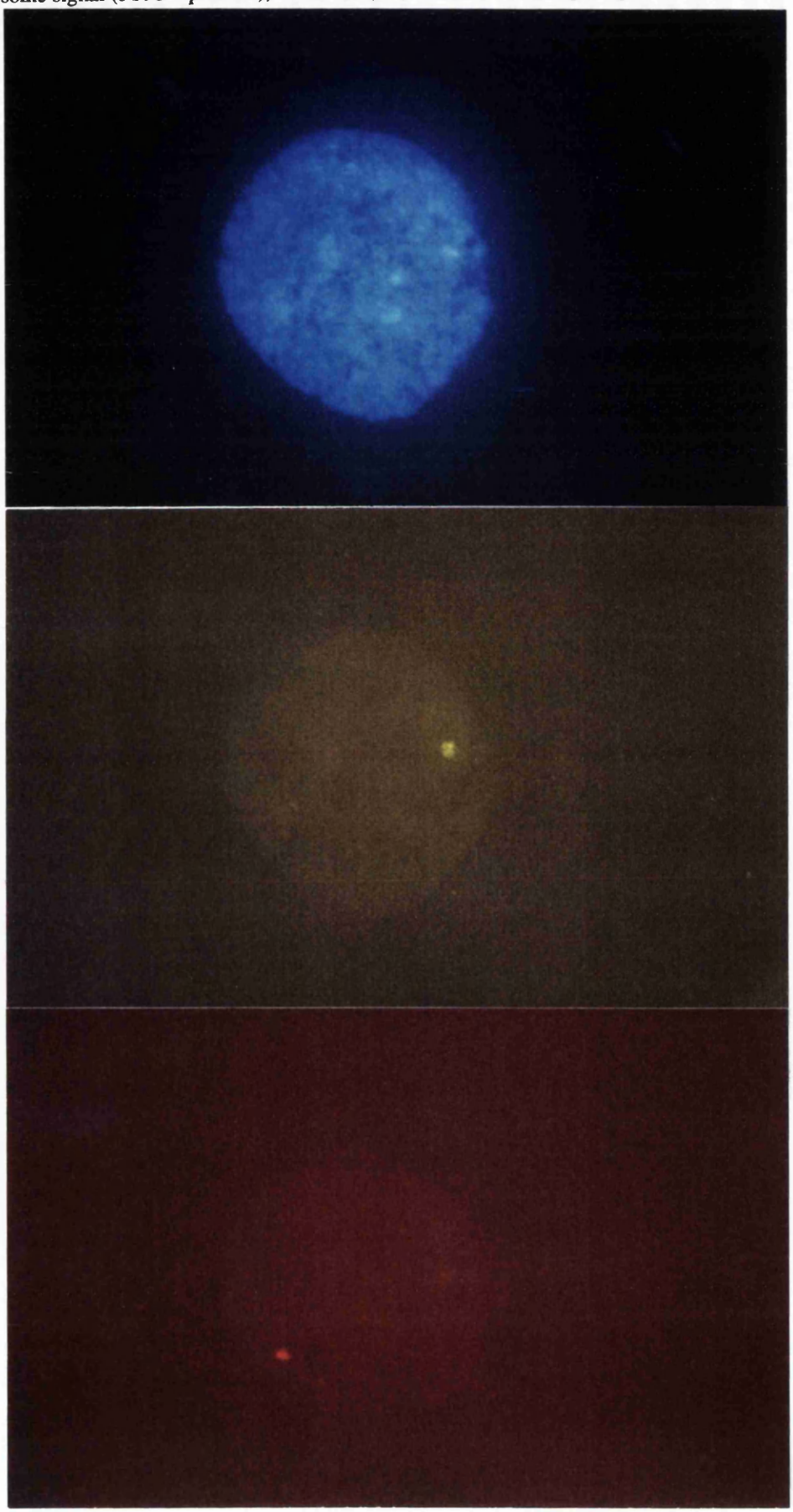
Table 3.9:- Efficiency of cY98, pHY2.1 and pBamX7 dual labelling on embryonic interphase nuclei.

Embryo name	Number of cells tested	Signal			No result	Sex
		XX	XY	other		
A1	1	-	-	-	1 c	?
A2	2	2	-	-	-	F
A4	1	-	1	-	-	M
A5	1	-	-	-	1 c	?
A6	2	-	2	-	-	M
A7	2	-	1	-	1 a	M
A8	2	-	2	-	-	M
B1	2	-	1	-	1 b	M
B2	1	-	-	1:YO	-	M
B3	2	1	1 d	-	-	F?
B4	2	-	2	-	-	M
Totals	18	3	10	1	4	

a. Cell lost in procedure, b. No hybridisation signal, c. Result obscured by autoflorescent debris, d. Misdiagnosis due to autofluorescent debris

These results show clearly the improvement in efficiency of labelling brought about by the introduction of probe cY98.

Fig 3.40:- Male interphase nucleus sexed by dual FISH: DAPI (blue) counterstain,FITC (green) Y chromosome signal (cY98 + pHY2.1), Texas red (red) X chromosome signal (pBamX7).



Identical experiments were performed on metaphase preparations of embryonic nuclei. These were also obtained from disaggregated 4-cell embryos and spread on to slides in the same way but, in this case, they were left in colchicine overnight in order to arrest the cells in metaphase. Nuclei were fixed on to glass by Dr Leeanda Wilton.

Table 3.10:- Efficiency of cY98, pHY2.1 and pBamX7 on embryonic metaphase nuclei.

Embryo name	Number of cells tested	Signal			No result	Sex
		XX	XY	other		
A1	1	1	-	-	-	F
C1	4	3	-	-	1 a	F
C2	3	-	3	-	-	M
C3	1	1	-	-	-	F
C4	3	-	3	-	-	M
C5	3	-	2	-	1 b	M
C6	4	-	3	1:XXY	-	M
D1	1	-	1	-	-	M
D2	2	2	-	-	-	F
D3	2	-	1	1:XO	-	M
D4	3	-	3	-	-	M
D5	1	1	-	-	-	F
Totals		28	8	16	2	2

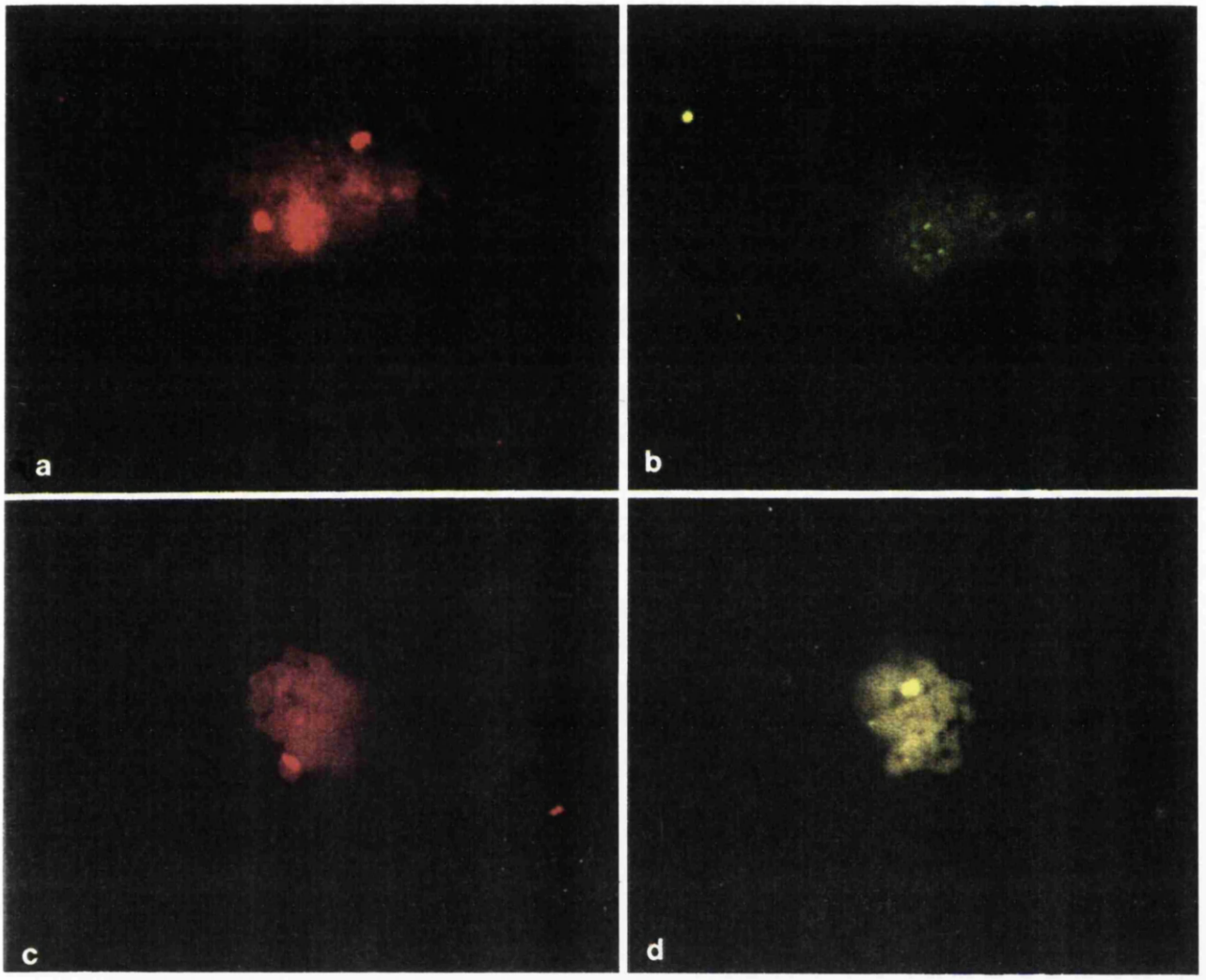
a. Cell lost in procedure, b. No hybridisation signal

The results indicate that use of pHY2.1 and pBamX7 alone on interphases leads to an efficiency of 33.3% (i.e only four out of 12 cells gave readable results). This is improved by introduction of probe cY98 to 77.8% and is further improved to 92.8% when metaphases are used.

Fig 3.41:- Embryonic metaphases sexed using dual labelling (probes were cY98 and pHY2.1 digoxigenin labelled and detected with FITC (green), pBamX7 biotin labelled and detected with Texas-red (red).

a), b) female: a) 2 X signals; b) No Y signal.

c), d) male: c)1 X signal ; d) 1 Y signal.



RAPID (6-7 HOUR) SEXING OF PREIMPLANTATION EMBRYONIC NUCLEI

In order for a preimplantation approach to be clinically applicable, biopsy and dignosis need to be performed within the same treatment cycle (see introduction). Thus 24 hours for FISH (as in the previous approach) is too long. A strategy taking 6-7 hours was hence developed.

ASSESESING THE FEASIBILITY OF THIS APPROACH FOR CLINICAL APPLICATION

Sexing was performed on interphase and metaphase preparation of embryonic nuclei in 6-7 hours using a 2 hour hybridisation period and detecting biotinylated X probe with FITC and digoxigenin labelled Y probes with TRITC.

Table 3.11:- Efficiency of rapid sexing on embryonic nuclei

a) Metaphases

Cell name	Comment prior to FISH	FISH result and comment	Sex of embryo
E1.1	Can't locate metaphase	Still can't find	?
E2.1	Can't locate metaphase	Still can't find	?
E2.2	Can't locate metaphase	Still can't find	
E3.1	Can't locate metaphase	Still can't find	?
E4.1	Clean metaphase	2 X signals	F

b) Interphases

E1.2	Clean interphase	2 clear X signals	
E1.3	Clean interphase	2 clear X signals	F
E1.4	Clean interphase	2 clear X signals	
E2.3	Clean interphase	2 clear X signals	
E2.4	Clean interphase	2 clear X signals	F
E2.5	Clean interphase	2 clear X signals	
E3.2	Clean interphase	2 clear X signals	
E3.3	Can't locate interphase	2 nuclei,both 2 X	F
E3.4	Clean interphase	2 clear X signals	
E3.5	Can't locate interphase	Still can't find	

PATIENT REPORTS

Since all visible interphase nuclei could be confidently sexed and as metaphases were not obtained when testing the feasibility of this approach, it was decided to proceed with a clinical trial using this approach on interphase nuclei.

In each case, patients were put through the IVF programme at the Hammersmith Hospital. Resulting embryos were numbered and suitable ones biopsied. Biopsy was on day 2. One cell was removed from the embryo (~4-cell) and this was allowed to divide overnight. On day 3 post-insemination the cells were spread on to glass slides (Griffin *et al.*, 1992) and the FISH sexing procedure performed. Embryos are named in this study firstly by the name of the patient (only two letters of the name are, in fact, used throughout) followed by the number of the embryo as assigned by the Hammersmith Hospital. Individual cells are named in this study firstly by the name of the embryo followed by a decimal point and a number. For instance "Wi.3.1" represents the first cell from the third embryo of Mrs Wi.

Mrs Wi. is a carrier of type II sensory motor neurone disease.

Five day-2 embryos were suitable for biopsy, namely numbers 3, 4, 6, 9, and 15.

Table 3.12a:- Mrs Wi embryo biopsy details.

Embryo name	No. of cells on day-2 and state of embryo	No. of cells biopsied	State of biopsied cells on day-3	Spreading comment
Wi.3	8 (good)	2	Still 2 cells	Each spread on separate slides
Wi.4	5 (uneven)	1	Cell divided into 2	Each spread on separate slides
Wi.6	4 (good)	1	Cell divided,1 binucleate	Spread separately but both lysed
Wi.9	4 (fragment)	1	Still 1 cell	Spread on 1 slide
Wi.15	8 (v. good)	2	Both divided,thus 4 cells	Two spread on separate slides Two spread on a single slide

With the exception of the cells that lysed, both biopsy and spreading procedures were not problematic.

Hence there were 9 slides.

Table 3.12b:- FISH sexing results on the embryos of Mrs Wi.

Cell name	Comment prior to FISH	FISH result and comment		Sex of embryo
Wi.3.1	Can't locate cell	Large nucleus,2 X signals	]_	F
Wi.3.2	Clean nucleus	2 X signals	]	
Wi.4.1	Can't locate cell	Still can't find	]_	M
Wi.4.2	Nucleus in metaphase	1X,1Y on metaphase	]	
Wi.6.1	Can't locate cell	Still can't find	-	?
Wi.9.1	Can't locate cell	Still can't find	-	?
Wi.15.1	Can't locate cell	Still can't find	]	
Wi.15.2	1 clean nucleus	1 X,1 Y signal	]--	M
Wi.15.3	2 nuclei with surrounding cytoplasm	a. No signal b. 1 X,1 Y signals	]	

The remaining cells from embryo 3 were transferred into the uterus of Mrs Wi.

Her hormone levels indicated that the embryo had implanted though the pregnancy did not continue further - a so-called "biochemical pregnancy."

Fig. 3.42:- Cells "Wi.3.1" and "Wi.3.2" displaying 2 X chromosome signals

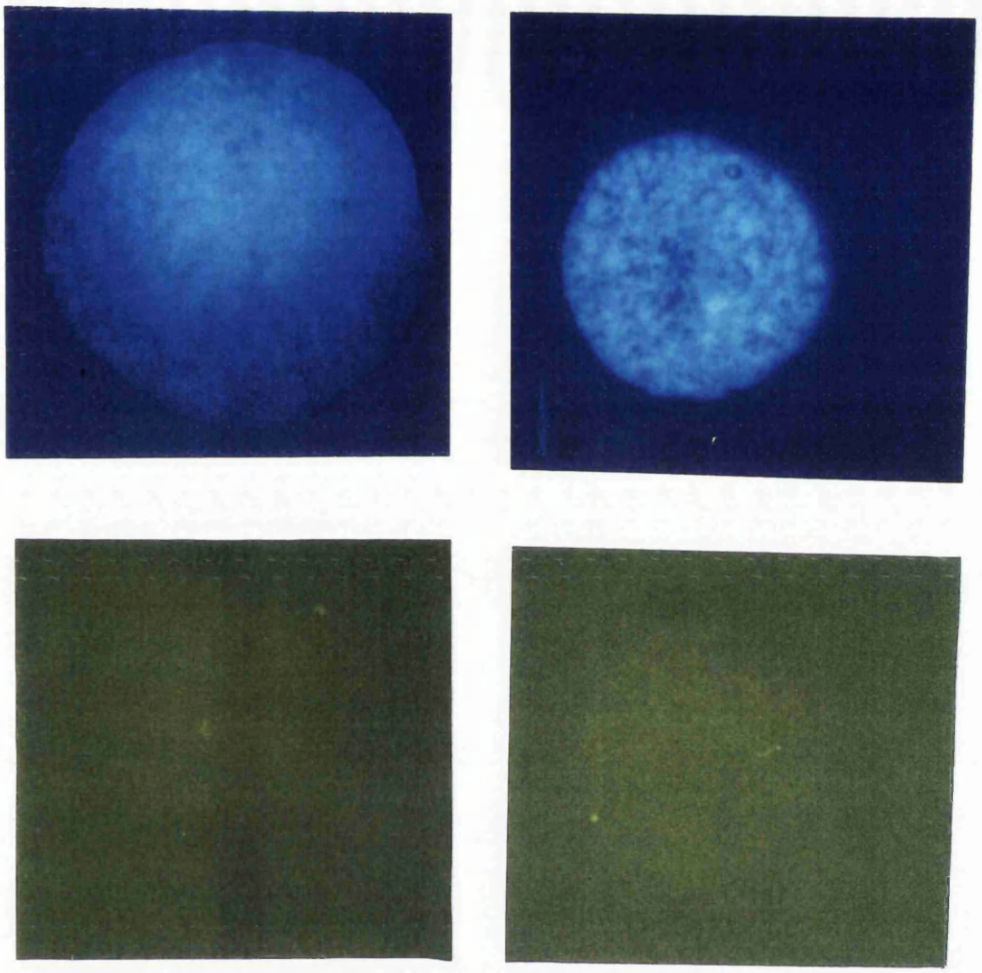
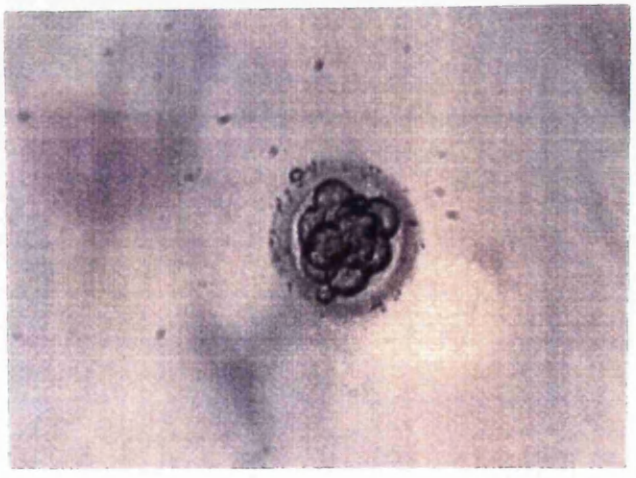


Fig. 3.43:- The remainder of embryo Wi.3 prior to transfer.



Mrs Po. is a carrier of Fragile X

Eight day-2 embryos were chosen for suitable, namely numbers 4, 5, 6, 7, 8, 14, 15 and 16.

Table 3.13a:- Mrs Po.embryo biopsy details

Embryo name	No. of cells on day-2 and state of embryo	No. of cells biopsied	State of biopsied cells on day-3	Spreading comment
Po.4	4+fragments	1	Still 1 cell	Spread on 1 slide (cell lysed).
Po.5	5 (good)	1	Cell divided into 2	Each spread on separate slides
Po.6	8 (good)	2	Still 2 cells	Each spread on separate slides
Po.7	4 (uneven)	1	Still 1 cell	Spread on 1 slide
Po.8	8 (good)	2	Still 2 cells	Each spread on separate slides
Po.14	8 (good)	2	Still 2 cells	Each spread on separate slides
Po.15	6+fragment	1	Uneven division	Each spread on separate slides
Po.16	4+fragments	1	Cell divided into 2	Each spread on separate slides

With the exception of the cell that lysed, both biopsy and spreading procedures were not problematic.

Hence there were 14 slides.

Table 3.13b:- FISH sexing results on the embryos of Mrs Po.

Cell name	Comment prior to FISH	FISH result and comment		Sex of embryo
Po.4.1	No clear nucleus	Enormous nucleus,1 X,1 Y	-	M
Po.5.1	Large nucleus	Disintegrating,1X,1Y	]_	M
Po.5.2	Large nucleus	1X,1Y	]	
Po.6.1	Large nucleus	Unable to locate nucleus	]_	?
Po.6.2	Large nucleus	No signal	]	
Po.7.1	Can't locate nucleus	Still can't find	-	?
Po.8.1	Some cytoplasmic debris	Broken nucleus,no signal	]_	F
Po.8.2	Clean nucleus	2 X signals,no Y	]	
Po.14.1	Cytoplasmic debris	1 X,1 Y signals	]_	M
Po.14.2	Clean nucleus	1 X,1 Y signals	]	
Po.15.1	Some cytoplasmic debris	2 X,no Y signals	]_	F
Po.15.2	Tiny nucleus	2 X,no Y signals	]	
Po.16.1	Clean nucleus	Unable to relocate nucleus	]_	M
Po.16.2	Clean nucleus	1 X,1 Y (signal split)	]	

The remaining cells of Embryos 8 and 15 were transferred into the uterus of Mrs Po.

Mrs. Po. showed no signs of pregnancy

Mrs Wa. is a carrier of Lesch-Nyhan syndrome

Two day-2 embryos were suitable for biopsy namely numbers 5 and 8.

Table 3.14a:- Mrs Wa. embryo biopsy details.

Embryo name	No. of cells on day-2 and state of embryo	No. of cells biopsied	State of biopsied cells on day-3	Spreading comment
Wa.5	4 (uneven)	1	Cell divided into 2	Each spread on separate slides
Wa.8	4 (uneven)	1	Cell divided into 2	Each spread on separate slides

Both biopsy and spreading procedures were not problematic.

Hence there were 4 slides.

Table 3.14b:- FISH sexing results on the embryos of Mrs Wa.

Cell name	Comment prior to FISH	FISH result and comment		Sex of embryo
Wa.5.1	Large,clean nucleus	1 X,1 Y signals	]_	M
Wa.5.2	Clean nucleus	1 X,1 Y signals	]	
Wa.8.1	Clean but Disintegrating	5+ Xsignals,no Y	]_	?(F)?
Wa.8.2	Not sure of location	Damaged nucleus,no signal	]	

Hence no embryos were transferred

Four embryos were suitable for biopsy namely numbers 1, 7, 8 and 9.

Table 3.15a:- Mrs Ro. embryo biopsy details

Embryo name	No. of cells on day-2 and state of embryo	No. of cells biopsied	State of biopsied cells on day-3	Spreading comment
Ro 1	4 (uneven)	1	Cell divided into 2	Both fixed on separate slides
Ro.7	4 (uneven)	1	Cell divided into 2	Both fixed on separate slides
Ro.6	6 (uneven)	1	Cell divided into 2	Both fixed on separate slides
Ro.10	4+fragments	1	One cell	Fixed on one slide

All embryos were reported as "difficult to biopsy" and none of the cells seemed to have clear nuclei. The spreading procedure was not problematic.

Hence there were 7 slides.

Table 3.15b:- FISH sexing results on the embryos of Mrs Ro.

Cell name	Comment prior to FISH	FISH result and comment		Sex of embryo
Ro.1.1	Foreign body visible	Body fluorescent,no signal	]_	M
Ro.1.2	Some cytoplasmic debris	1Y, 1X signal	]	
Ro.7.1	Unsure of location	Can't find nucleus	]_	F
Ro 7.2	2 clean nuclei	Both 2 X signals	]	
Ro.8.1	Can't find nucleus	Still can't find	]_	?
Ro.8.2	Nucleus cevered in debris	Autofluorescent debris	]	
Ro.10.1	Clean nucleus	1X, 1Y+2 small red signals	-	M

The remaining cells of embryo 7 were transferred into the uterus of Mrs Ro.

Mrs. Ro. showed no signs of pregnancy.

Mrs Ha. is a carrier of X-linked mental retardation.

Two embryos were suitable for biopsy namely numbers 2 and 8.

Table 3.16a:- Mrs Ha. embryo biopsy details.

Embryo name	No. of cells on day-2 and state of embryo	No. of cells biopsied	State of biopsied cells on day-3	Spreading comment
Ha.2	8 (fragment)	1	4 cells/fragments	Fixed on 4 separate slides
Ha.8	8 (fragment)	1	1 cell & 1 fragment	Fixed on to 2 slides

During the biopsy procedure cells were not very clear so in some cases, cell-size fragments/chunks were removed. In the spreading procedure, this meant that fragments were fixed in the hope that they contained nuclei.

Hence there were 6 slides.

Table 3.16b:- FISH sexing results on the embryos of Mrs Ha.

Cell name	Comment prior to FISH	FISH result and comment		Sex of embryo
Ha.2.1	Can't find nucleus	Still can't find	]	
Ha.2.2	Can't find nucleus	Still can't find	]_	?
Ha.2.3	Can't find nucleus	Still can't find	]	
Ha.2.4	2 small nuclei+1 fragment?	No signal	]	
Ha.8.1	2 clean nuclei	Both 2 X signals	]_	F
Ha.8.2	Can't find nucleus	Still can't find	]	

The remaining cells of embryo 8 were transferred into the uterus of Mrs Ha.

Mrs. Ha. showed no signs of pregnancy

Mrs Hd. is the sister of Mrs Ha. and also carries X-linked mental retardation.

Six embryos were suitable for biopsy namely numbers 8, 9, 11, 12, 16 and 20.

Table 3.17a:- Mrs Hd embryo biopsy details

Embryo name	No. of cells on day-2 and state of embryo	No. of cells biopsied	State of biopsied cells on day-3	Spreading comment
Hd.8	4 (fragment)	1	Still 1 cell (misshapen)	Fixed on to 1 slide
Hd.9	4 (good)	1	Cell divided into 2	Each fixed on to a separate slide
Hd.11	4 (fragment)	1	Cell divided into 2	Each fixed on to a separate slide
Hd.12	4 (fragment)	1	Still 1 cell, ? binucleate	Fixed on 1 slide
Hd.16	4 (good)	1	Divided into 2 cells	Each fixed on to a separate slide
Hd. 20	4 (good)	1	Still 1 cell, ? binucleate	Fixed on 1 slide

Biopsy and spreading procedures were both not problematic.

There were hence 9 slides.

Table 3.17b:- FISH sexing results on the embryos of Mrs Hd.

Cell name	Comment prior to FISH	FISH result and comment		Sex of embryo
Hd.8.1	Large nucleus	Disintegrating, no signal	-	?
Hd.9.1	Clean nucleus	1 X,No Y signals	]_	F
Hd.9.2	Large,clean nucleus	2 X,No Y signals	]	
Hd.11.1	Can't find nucleus	2 X,No Y signals	]_	F
Hd.11.2	Small,some cytoplasm	2 X,No Y signals	]	
Hd.12.1	Can't locate	Disintegrating,1 X,1 Y	-	M
Hd.16.1	Small,some cytoplasm	1 X,1 Y signals	]_	M
Hd.16.2	2 nuclei,some cytoplasm	Both 1 X,1 Y signals	]	
Hd.20.1	Disintegrating+cytoplasm	2nuclei,both 1 X,1 Y	-	M

The remaining cells of embryos 9 and 11 were transferred into the uterus of Mrs Hd.

Her hormone levels indicated that the embryo had implanted though the pregnancy did not continue further - a so-called "biochemical pregnancy."

Fig. 3.44:- Male embryo nucleus, (Po 14.2) DAPI (blue) counterstain, FITC (green) X chromosome signal, TRITC (red) Y chromosome signal

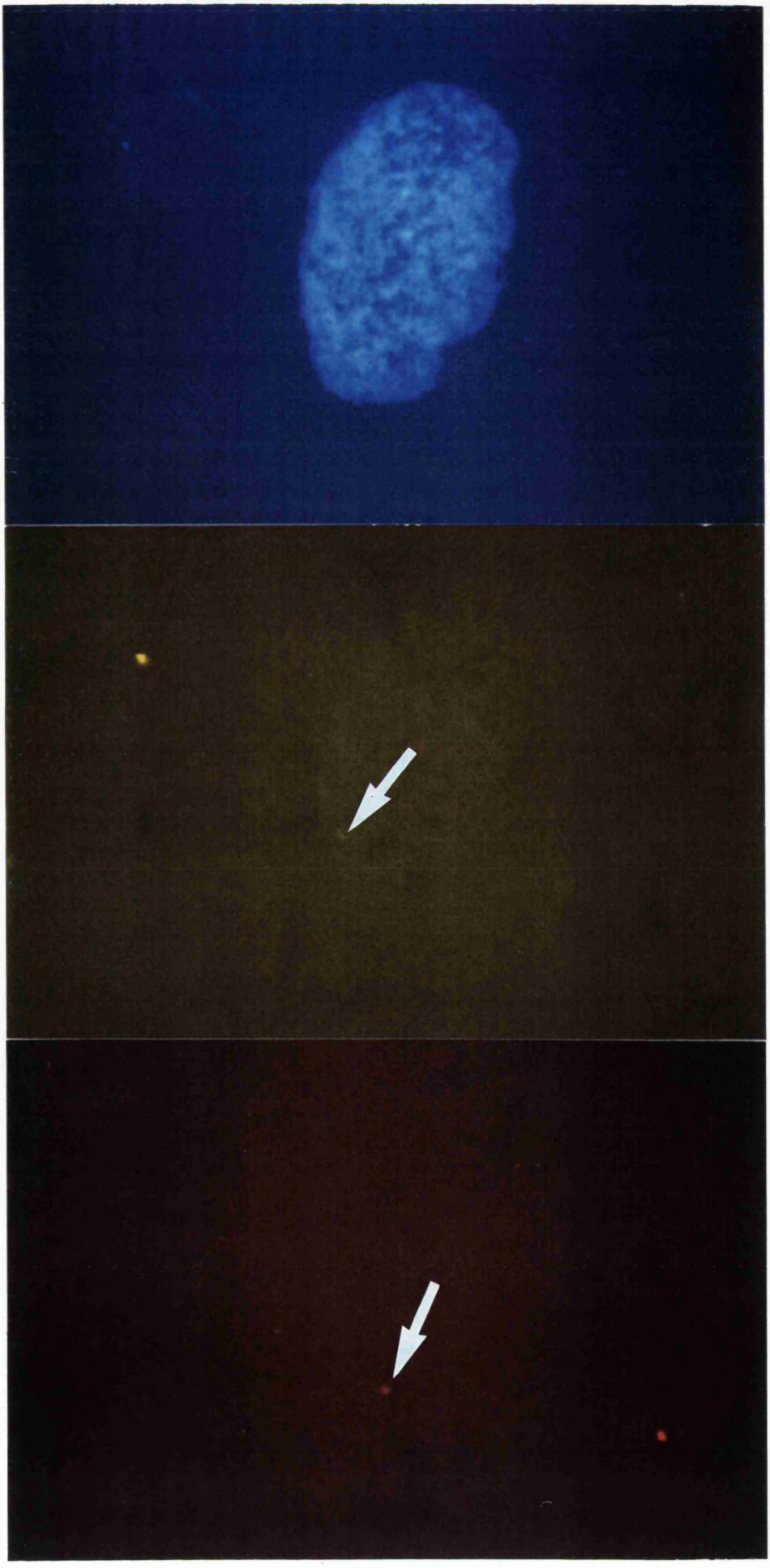
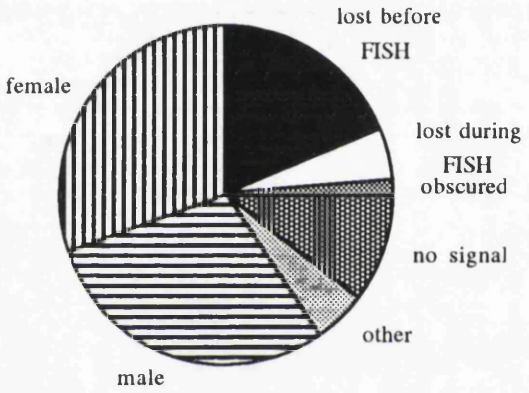


Table 3.18:- Summary of patient results

	No. of slides	Fate of slides a	b	c	d	e	f	g	No. of embryos	Totals F	M	?	Number transferred	PREGNANCY?
Wi	9	4	0	0	0	0	2	3	5	1	2	2	1	Biochemical
Po	14	1	2	0	2	0	3	6	8	2	4	2	2	None
Wa	4	0	0	0	1	1	0	2	2	0	1	1	0	N/A
Ro	7	1	1	1	1	0	2	1	4	1	2	1	1	None
Ha	6	4	0	0	1	0	1	0	2	1	0	1	1	None
Hd	9	0	0	0	1	1	3	4	6	2	3	1	2	Biochemical
	49	10	3	1	6	2	10	17	27	7	12	8	7 (5 patients)	2 biochemical

a. could not locate cell before or after FISH; b. could locate before FISH but not after; c. signal obscured by autofluorescent material; d. no signal; e. other; f. positive result - female; g. positive result - male

Fig. 3.45:- Efficiency of FISH sexing on single cells (from data in Tables 3.11 and 3.18)

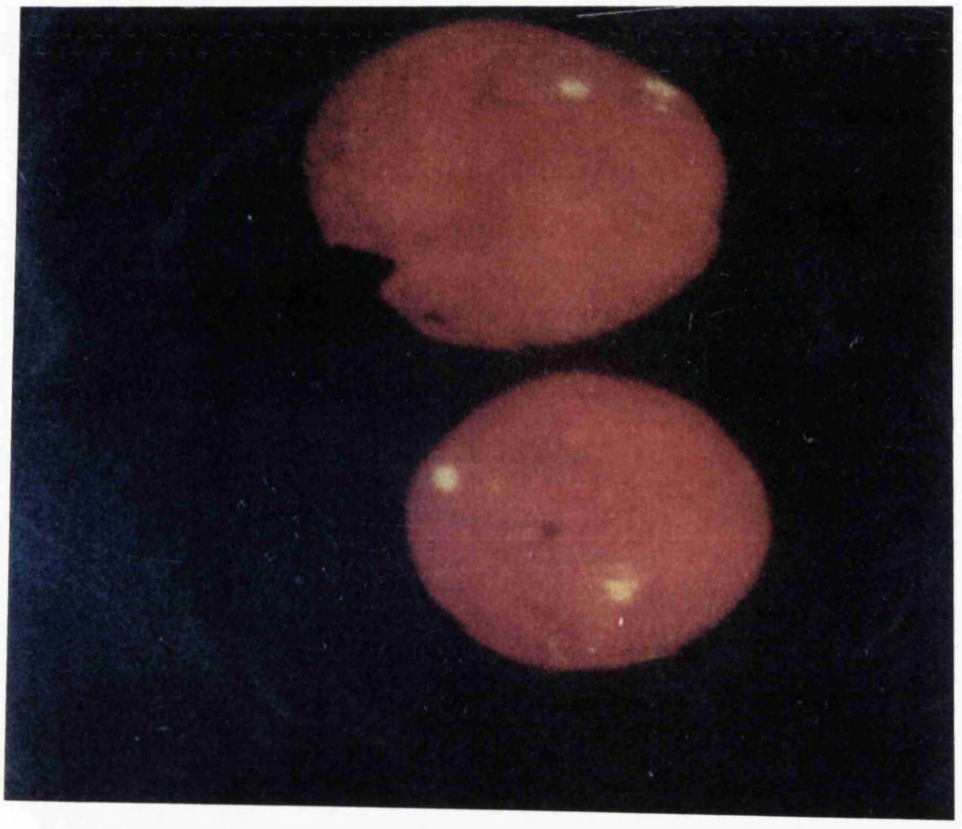


PRELIMINARY RESULTS FOR FUTURE APPLICATIONS

ALPHOID PROBE ON EMBRYONIC NUCLEI

Trisomy 18 is the second most common human autosomal trisomy resulting in live-born infants. The probe p18 was hybridididised to two embyonic nuclei thus indicating the feasibility of this approach for screening for trisomy as well as sex.

Fig. 3.46:- Chromosome 18 probe on 2 interphase nuclei of preimplantation embryos.



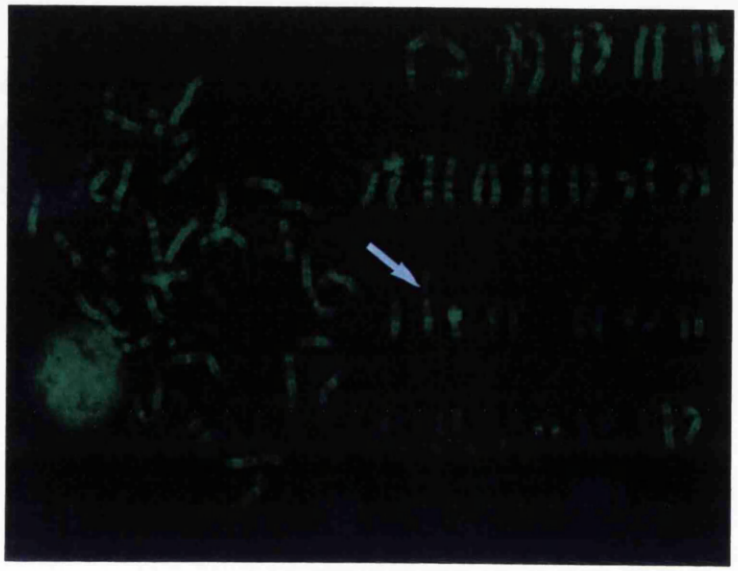
SCREENING TRANSLOCATION PATIENTS PRIOR TO PID

In this study, four patients (namely Mrs Te, Mrs Sp and Mrs Si) known to carry Robertsonian translocations and hence at high risk of transmiting trisomy have been screened. Firstly classical cytogenetics (Q- and/or G- banding) was performed and secondly FISH was used (biotin labelled probes p13/21 and αXT detected with FITC) on normal lymphocyte material with a view to possible preimplantation diagnosis affected offspring.

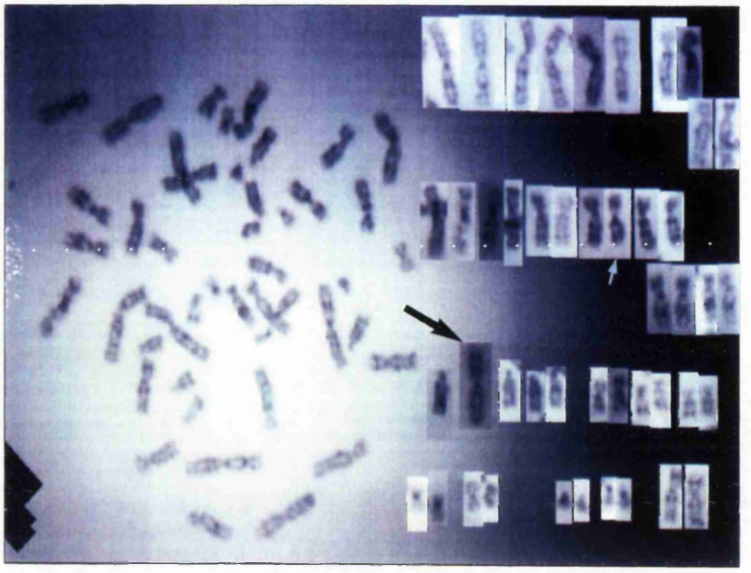
Mrs Te and Mrs were found to have 13-14 Robertsonian translocations and hence are at high risk of transmiting Patau's syndome (trisomy 13). Careful G-band analysis revealed that Mrs Te also had a pericentric inversion involving the centromere of chromosome 9. This abnormality is not thought to be significant i.e. it does not put her at further risk of giving birth to abnormal offspring. Mrs Sp was found to have a 14-21 Robertsonian translocation and hence is at high risk of transmiting Down's syndrome (trisomy 21).

Fig. 3.47:- Karyotypes of translocation patients

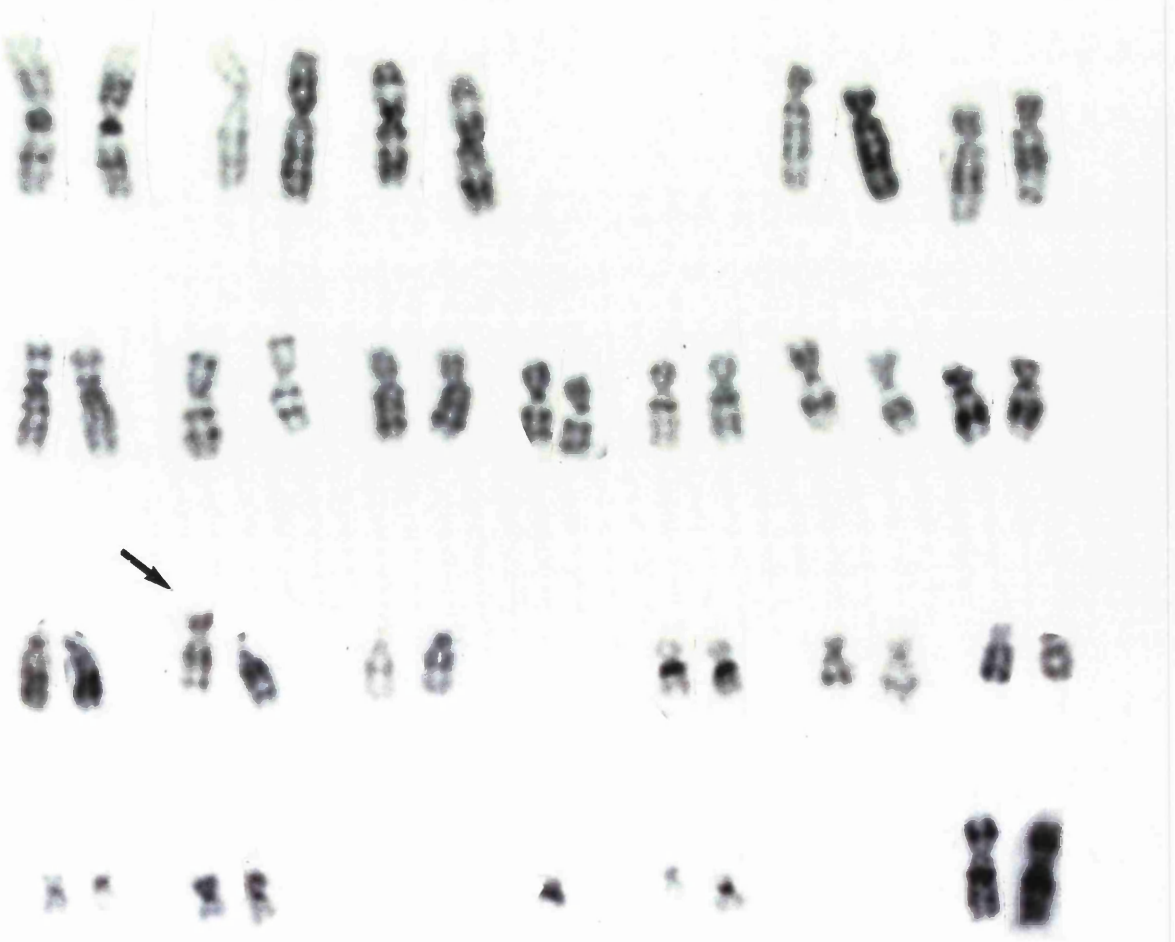
a) Mrs Te
i) Q-banding confirming 13-14 Robertsonian translocation (arrowed).



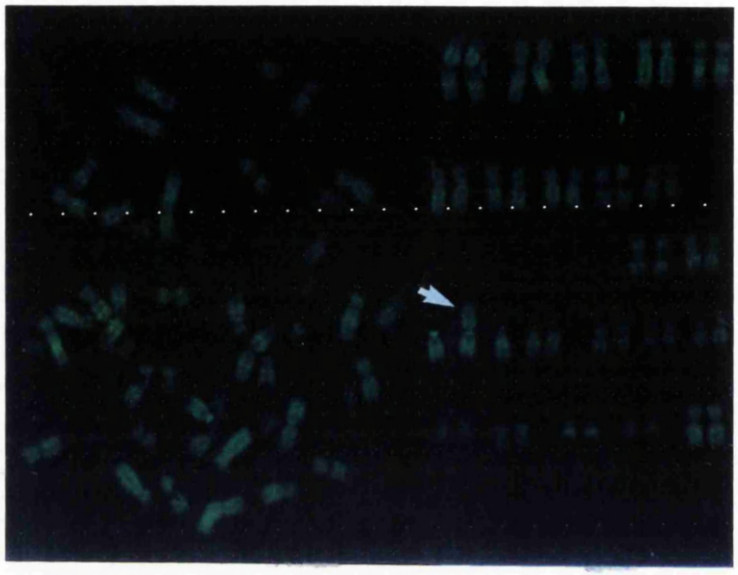
ii) G-banding revealing 13-14 translocation (large black arrow) and inverted chromosome 9 (small white arrow)

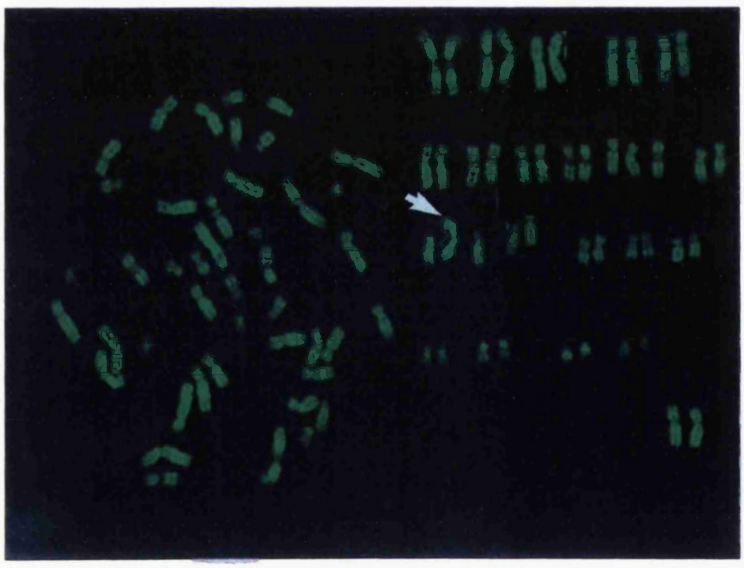


b) Mrs Sp - G-banding revealing 14-22 Robertsonian translocation



c) Mrs Si - Q-banding revealing 13-14 Robertsonian translocation.

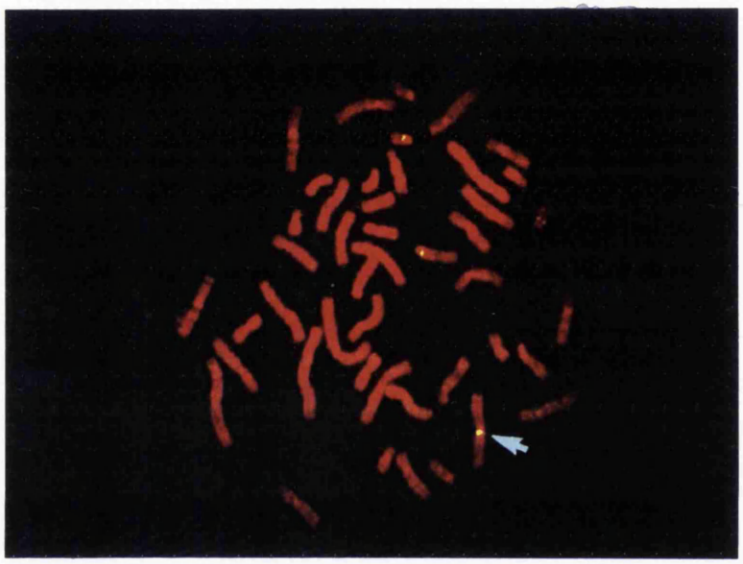




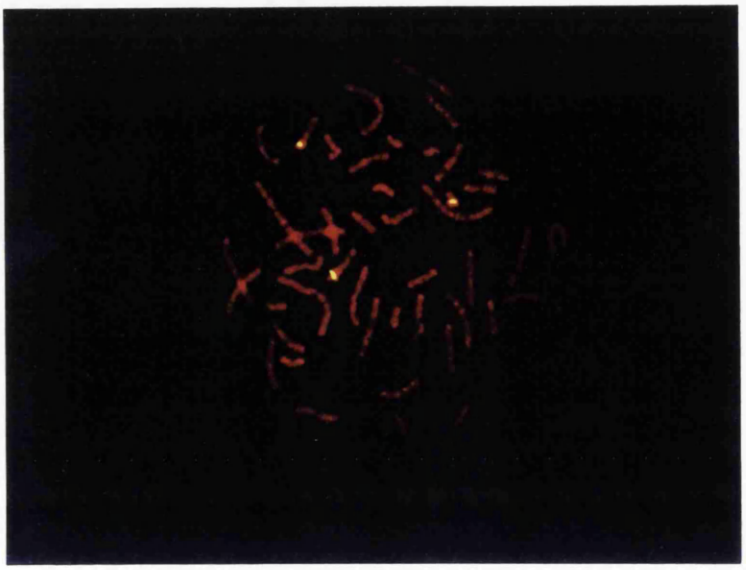
FISH studies on these patients revealed that, in the cases of Mrs Te and Mrs Si, both probes illuminated the translocation chromosome. In the case of Mrs Sp however,the 13/21 probe did not light up the translocation chromosome.

Fig. 3.48:- Chromosome 13/21 probe on translocation patients

a) Mrs Te (translocation chromosome arrowed). Probe (p13/21) hybridises to translocation chromosome and 3 normal chromosomes.



b) Mrs Sp (translocation chromosome not lit up). Probe (p13/21) hybridises to normal 13s and 21 only.



c) Mrs Si (translocation chromosome arrowed). Probe (p13/21) hybridises to translocation chromosome and 3 normal chromosomes.

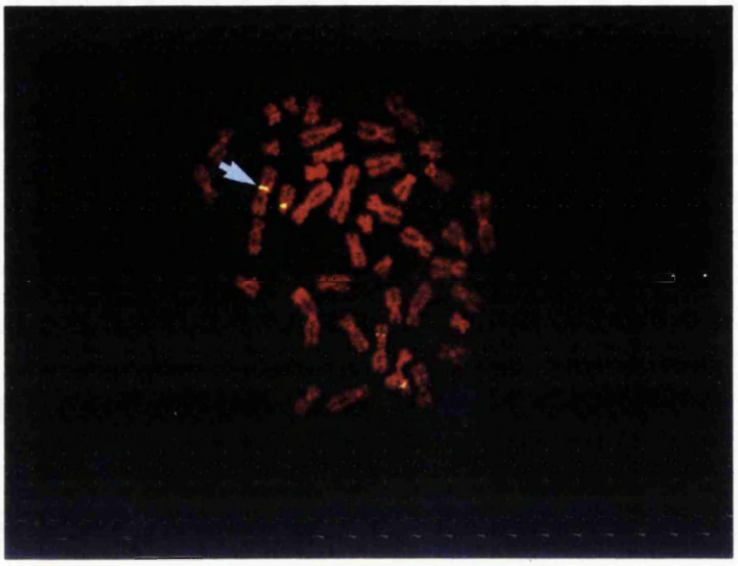
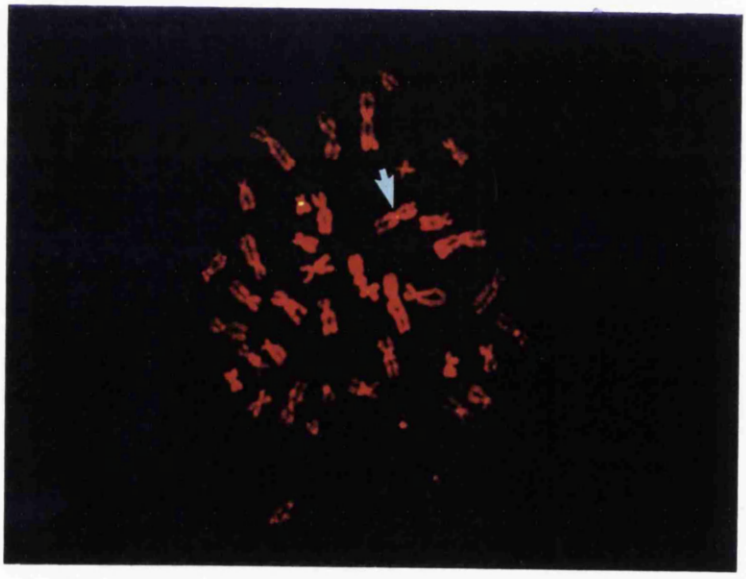


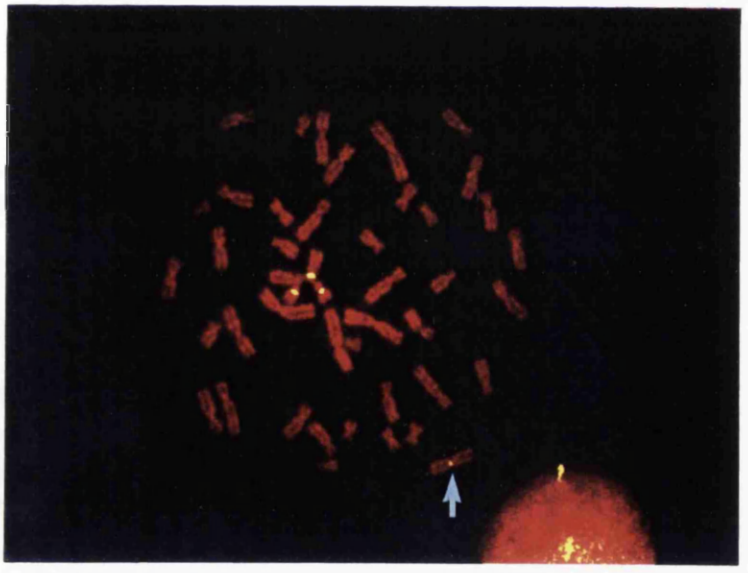
Fig. 3.49:- Chromosome 14/22 probe on translocation patients

a) Mrs Te (translocation chromosome arrowed). Probe (αXT) hybridises to translocation chromosome and three normal chromosomes.



b) Mrs Sp (translocation chromosome arrowed). Probe (αXT) hybridises to translocation chromosome and three normal chromosomes.

c) Mrs Si (translocation chromosome arrowed). Probe (αXT) hybridises to translocation chromosome and three normal chromosomes.



The results in section 5 have illustrated how FISH can be a powerful tool in the field of preimplantation diagnosis. Sexing of the preimplantation embryo has progressed from the research stage to a clinical trial. Diagnosis of chromosomal abnormalities remains a possibility which has not yet been put into clinical practice.

SECTION 6: TECHNICAL ASPECTS OF FISH

REPETITIVE PROBES

RELATIVE SUCCESSES OF PROBES USED

From the data given in figures 3 to 13, it is apparent that the alphoid and classical satellite repetitive probes were an efficient vehicle for analysing chromosome copy number in the interphase nucleus. It was also apparent that when the signal was large, the number of interphases displaying only one signal when they should be displaying 2 increased. This is obvious when comparing figures 3 (for the chromosome 1 probe) and 12 (for the chromosome 18 probe). PUC1.77 hybridises to the whole heterochromatic region of chromosome 1 which is considerably larger than the centromeric region of chromosome 18 to which the probe in figure 12 hybridises. Hence when the signal is larger, according to the criteria for scoring interphase signals, the likelihood of two signals overlapping and being scored as one is greater. This was also apparent when comparing parallel experiments identical except for the fact that one signal was amplified using the "Pinkel sandwich" and one was not. Preparations with amplified signals tended to give a greater proportion of nuclei exhibiting one signal only. However, often an experiment performed without Pinkel amplification lead to the preparation showing no signal at all. This could be rectified by removing the coverslip and then going through an amplification step however this proved to be detrimental to chromosome morphology and was avoided when not absolutely necessary.

When the signal was very tiny, the number of interphases not exhibiting a signal increased. This is apparent when comparing figures 10 (for the chromosome 16 probe) and 12 (for the chromosome 18 probe). The signal seen on the chromosome 16 centromere was considerably smaller than the one on the chromosome 18 centromere,

thus was presumably too small to see in a proportion of nuclei, and hence scored in the "no signal" column.

A problem often encountered using these probes was aspecific hybridisation to the centromeres of other chromosomes. This was often the case when hybridisation conditions were not optimal and was usually due to the formamide concentration in the hybridisation mix or washes, or due to the formamide not being properly deionised

The chromosomal assignment of mini-satellite probes λMS8 and λMS32 to chromosomes 5 and 1 respectively (Royle *et al.*, 1988) was confirmed by FISH. The two probes did not exhibit strong enough signals to be visible at interphase. Why this should be the case when the target sequence for the probe should be sufficiently large to produce signals at interphase is not clear at present. It is possible, since experiments on these probes were performed in the earlier stages of this study and not continued later, that more recent improvements in the technique may allow these probes to be visualised in the interphase nucleus.

COMPARISON WITH ESTABLISHED DATA

The probes shown in section 2 of the results are all ones used and discussed in subsequent sections hence alphoid probes available for other chromosomes are not included. Comparison with the data from the University of Leiden is given in the following table. Differences in specificity under optimal conditions (i.e how much the probe hybridises to other chromosomes) and utility (i.e. how useful the probe is thought to be in genetic research) are compared.

Table 3.18

PROBE	CHR.	LEIDEN RESULTS		THIS STUDY RESULTS	
		SPECIFICITY	UTILITY	SPECIFICITY	UTILITY
PUC1.77	1	very good	good	good	good
pSE16	16	medium	low	very good	medium
p17H8	17	very good	good	very good	good
p18	18	good	good	very good	very good
p13/21	13/21	low	medium	low	medium
pBamX7	X	good	very good	good	very good
pHY2.1	Y	good	good	very good	very good

Differences in specificity are generally very similar with the exception of the chromosome 16 probe. In this study the probe proved to be very specific, though the signals were a little weak. Why the Leiden group should not find it so is unclear. There is general agreement as to the utility of these probes.

TOTAL GENOMIC PROBES

Total human placental DNA used for the screening of somatic cell hybrids was the most reliable of all probes. Aspecific background hybridisation was never seen and signals were usually bright and compact. There appeared to be no difference in signal irrespective of the nick-translation methods used to label the probe. Cotl DNA was also used as a genomic probe. Signal intensity was not improved when comparing it with the use of human placental DNA if the probe was labelled using a kit. However when the probe was labelled from first principles (see materials and methods) cotl DNA tended to give brighter signals than human placental DNA. In some cases, cotl DNA also hybridised dimly to the centromeres of the hamster chromosomes. Recent experimentation has indicated that greater stringency post-hybridisation washes (i.e. as for a cosmid) largely alleviates this problem. Hybrid DNA as a probe on to human chromosomes (reverse chromosome painting) was technically more difficult. First of all, as explained in the introduction, CISS (competitive *in-situ* suppression) needs to be used and furthermore, the proportion of human DNA in comparison to rodent background is usually very small.

Two methods of CISS are described in the materials and methods section. The first involves use of human placental DNA, the second cotl DNA. Despite a number of attempts, the method employing human placental DNA did not work in this study for reverse chromosome painting. Cotl DNA did however yield acceptable results.

SINGLE PROBES REQUIRING CISS

Most of the investigations in this study were performed using individual phage and cosmid clones. These clones are of sufficient insert size that they require CISS prior to FISH due to interspersed repeat elements in the insert. Of the two methods of CISS which are described in the materials and methods section, both produced adequate results. Initially, results using cot1 DNA were very sporadic. The cause of this was found to be the fact that it was originally being precipitated in the same way as human placental DNA. When the precipitation time and temperature were altered to 2 hours and -70°C respectively, results became much more reliable. Use of sonicated human placental DNA often yielded equally good preparations however results varied depending on the batch of human placental DNA used. Despite standardising all methods for sonicating the DNA, the concentration of DNA and size of fragments varied enormously from batch to batch. Furthermore batches needed to be made up regularly because of the amounts used. Hence commercially available cot1 DNA is now used exclusively in this laboratory.

TROUBLE-SHOOTING GUIDE

When developing a technique in the laboratory many practical difficulties and unclear results are obviously encountered. This study was no exception. Hence a guide to problems frequently encountered in FISH and advice on how to rectify them based on the results obtained in this study is given in table 3.19:

Table 3.19

PROBLEM	POSSIBLE SYMPTON	ACTION TO TAKE
No signal	Slide too old	Use a fresher slide
	Cell suspension too old	Use only suspension kept at -20°C
	Probe label not incorporated	Always assay label incorporation
		Clean DNA of salt (e.g. phenol/ chroroform)
	DNA not suitable for labelling	Ensure DNA is free from RNA and digests
	Too much CISS (if applicable)	Decrease DNA or annealing time
	DNAse denatured	Make up fresh DNAse stocks
	Proteinase K denatured	Make up fresh do not re-freeze
	Proteinase K concn. too low	Increase concentration
	DNA not properly denatured	Ensure denaturing oven reaches 80°C
		Ensure hybridisation mix is made properly
	Wash stringency too high	Reduce formamide concentration in wash
		Decrease temperature of wash
	Fluorescent material denatured	Use fresh aliquot of fluorescent material
	Signal not amplified enough	Include another round of amplification
Aspecific hybridisation	Stringency too low	Increase formamide concentration in hybridisation mix and post-hybridisation washes
		Increase temperature of post-hybridisation washes
		Include a 3x5min 0.1xSSC 60°C wash
	Formamide not deionised	Make up new hybridisation mix with freshly deionised formamide, ensure deionising resin is fresh and does not break-up in the deionising reaction
Background fluorescence	Probe not properly purified	Re-label probe with new purification column
	Foreign bodies in cell suspension	Use fresh suspension
	Foreign bodies in fluorescent mix	Filter blocking reagent
		Centrifuge fluorescent materials before use
	Foreign bodies in a solution	Millipore all solutions
Poor morphology	Slides too fresh	Age slides 56°C for 2 hours or keep overnight
	Proteinase K concn. too high	Lower proteinase K concentration
	Acid in formaldehyde	Store formaldehyde with bicarbonate in it
		Check final formaldehyde solution is at pH7
Preparation fading	Anti-fade medium too old	Prepare fresh anti-fade medium
	Signal too weak	Include another amplification step

FURTHER TECHNICAL ASPECTS

SLIDE PREPARATION AND STORAGE

The method for slide preparation described in the materials and methods section is now the one being used routinely in the laboratory. Experimentation has revealed that it is essential for metaphases and interphases to be clean of surrounding cytoplasm. It is

hence desirable to examine slides under phase-contrast illumination before FISH. Surrounding cytoplasmic debris leads to weak or no signals in the final analysis.

The age of slides was also a factor in FISH efficiency. Slides left on the bench for over two weeks consistently yielded no result. Slides could be stored for up to a month dessicated at 4°C or a year or more dessicated at -20°C without loss in signal intensity.

CHROMOSOME BANDING PROBLEMS

In order to assign a clone to a particular chromosome band, a reproducible banding method needs to be available. Initially in this study, pre-lipsol banding was used (as in figure 2 of the results). When assigning λMS32 signals to the cell line LIM1215, pre-G-banding was used (figure 33 of the results). Lipsol banding however was a very temperamental technique and G-banding often led to the chromosomes being unfit for subsequent FISH. Additionally, both approaches were very labour intensive and time consuming. Hence a method of fluorescent post-banding was desirable. Experimentation revealed that storing the slide at 4°C then baking at 56°C (as described in materials and methods) led to some weak Q- and R- banding patterns with DAPI and propidium iodide respectively. For very accurate band assignment however, it is becoming apparent that this approach can be unsatisfactory because of the weak bands it produces.

A number of fluorescent post-banding methods have been described (see introduction). There are two currently under investigation in this laboratory. The first (Cherif *et al.*, 1990) involves incorporation of Bromodeoxyuridine in the lymphocyte culture leading to a clear R-band pattern in the final analysis with propidium iodide. The second (Berube and Gagne., 1990) involves simultaneous hybridisation of alu-PCR amplified human DNA (yielding an R-band pattern) with different probe label and fluorochrome.

FLUORESCENT MICROSCOPY AND PHOTOGRAPHY

Fluorescent photography required long camera exposure times. It was hence found that films with higher ASA values were most preferable. For taking merely prints, the "Kodak Ektachrome ASA 1000" was very useful. However, in this study, it was found the most effective, and indeed the cheapest, strategy was to use a fast transparency film ("Scotch 640T" was found to be the best) and take prints from that if necessary.

COMMAND FILE ON THE CONFOCAL MICROSCOPE FOR THE SORTING OF CHROMOSOMES

Also in this study, a programme for the sorting of chromosomes on the confocal microscope (using a command file made up of existing functions) was developed for the rapid karyotyping of G- and Q- banded preparations in these and other patients. This programme is presented in the following table.

Table 3.20:- Command file on the confocal microscope for karyotyping.

```
FOR n=1 TO 999
FROM
COPYTO
INPUT x
PRINT "TYPE <ENTER> TO CONTINUE OR <CTRL/U> TO ABORT"
NEXT n
```

DISCUSSION

THE FISH TECHNIQUE

FISH IN COMPARISON TO OTHER ISH TECHNIQUES

The superiority of the fluorescent approach, in most cases, over isotopic and enzymatic means is documented in the literature (e.g. Pinkel *et al.*, 1986). FISH has proven, in this study, to be quicker and less laborious than the other two methods used (Figs 3.1-3.2). Use of the tritium based approach required an autoradiographic exposure time of around two weeks before results could be analysed. Experimental failure after this time could prove very disheartening. The alkaline phosphatase based approach was much quicker (taking around 30 hours to complete) but was a temperamental technique and only worked using the most reliable of probes. FISH could be performed within 24 hours and was as reliable if not more so than the radioactive method. Regarding safety, the hazards of handling radioactive isotopes are well known hence the non-radioactive methods were favoured in this respect. Figures 3.1 and 3.2 clearly illustrate the superior specificity of FISH. With the repetitive probe "pHY2.1" there is obviously the least background hybridisation, the signal is sharper and more aesthetically pleasing to the eye. In the case of the single-copy probe "pPGDH4", fewer metaphases needed to be counted (29 in comparison to 65) and furthermore the hybridisation peak is much more specific using FISH. Despite several attempts, this probe did not give a readable signal using the alkaline phosphatase based technique. The possibility of simultaneous detection of two or more probes on the same preparation further made FISH the most attractive of the three techniques. One disadvantage of FISH in comparison with isotopic ISH however, was that FISH did not work with some very small single-copy probes (~1kb) which had previously worked using tritium. Cherif *et al* (1989) have achieved FISH using 1kb probes however reports such as these are rare with the fluorescent yet commonplace with the

isotopic approach. This hence suggests that FISH is not quite as sensitive as radioactive ISH.

APPLICATIONS OF REPETITIVE PROBES

In the light of the results obtained in this study and from the literature (as reviewed in the introduction), it is apparent that chromosome specific repetitive probes have many uses. In this study, a quick, efficient approach to screening prenatal samples for Edward's syndrome (Trisomy 18) has been developed. Such an approach can be performed in six to seven hours. Use of the alphoid probe specific for chromosomes 13 and 21 on control lymphocyte material with a view to screening out Down's and Patau's syndrome proved less successful. Intensity and size of signal appeared to vary depending on the individual from whom the sample was taken making reading of signals at interphase extremely difficult, this is because proportions of alpha satellite DNA varies between individuals in all chromosomes but more so in the acrocentrics (Willard., 1990). In one case, a patient with a perfectly normal karyotype showed no signal on one chromosome 21. Analysis was further compounded by the fact that more signals per nucleus needed to be analysed because the probe detected two chromosomes rather than one (the alpha satellite DNAs on chromosomes 13 and 21 are very similar) and by the fact that this probe occasionally showed minor binding sites on other acrocentric chromosomes, again due to similarites in the alpha satellite DNA (Willard., 1990). Cremer *et al* (1988) were the first to describe methods of ISH for screening for trisomy 18 using a probe "L1.84" specific for the centromere of chromosome 18. The alphoid probe used in this study generally gives a more reliable and brighter signal than L1.84.

These probes are also useful for clarifying complex karyotypes in cancer preparations and for ascertaining chromosome number in preparations from which metaphases

cannot be obtained (Cremer *et al.*, 1988 a,b; Hopman *et al.*, 1988; Pieters *et al.*, 1990).

The hypervariable probes are potentially useful in clarifying complex karyotypes particularly since they reside close to the telomeres of certain autosomes (Royle *et al.*, 1988). However the ideal situation would be to have a system of generating probes which would light up metaphase and interphase of any region of the genome at will. Klinger *et al* (1991) report the use of cosmid contigs (i.e. contiguous stretches of DNA cloned into cosmid vectors) as probes for FISH. These probes can potentially be made for any region of the genome and, it is claimed, are detecable at both metaphase and interphase.

MERITS OF MULTIPLE LABELLING

Double labelling (i.e visualisation of two separate signals on the same preparation) has been cited by many authors (e.g. Hopman *et al.*, 1986, 1988; Cremer *et al.*, 1988 a,b). The generally preferred method and the one used in this study incorporates biotin and digoxigenin as probe labels (e.g. Lichter *et al.*, 1990). This has proven extremely efficient in this study when using chromosome specific probes and amplification steps are rarely needed. Nederlof *et al* (1989) report the use of a three colour detection system (triple labelling) employing red, green and blue fluorochromes. Such an approach has limited applicability since a fluorescent counterstain (usually DAPI) needs to be used to locate the chromosomes. This counterstain needs to be sufficiently bright to be visible under low power microscopy and hence would obscure one of the signals (in the case of DAPI - the blue one). Klinger *et al* (1991) report a three colour detection system using green, red and infra-red coloured fluorochromes. The latter is not visible with the naked eye but is detectable using certain cameras. In such a strategy, use of a DAPI counterstain is feasible. This, of course, has the drawback that expensive equipment needs to be available.

REVERSE CHROMOSOME PAINTING

Although reverse chromosome painting has been reported in the literature using hybrid DNA as a probe (Kievits *et al.*, 1990) this was not achieved in this study. However when hybrid DNA amplified by human specific "Alu PCR" was used as a probe, signals were obtained. Signals appeared brighter in the pale bands of chromosomes where the Alu sequences are most rich. When applying this technique the mapping of fragment hybrids, fragments derived from Alu-rich regions should theoretically be easily detected. Fragments derived from non Alu-rich regions may not show up. This could theoretically be solved by simultaneous PCR of the LINE sequences which are abundant in the dark chromosome bands. A very different approach to enriching human content in somatic cell hybrids would be to use commercially available "streptavidin conjugated magnetic beads." If hybrid DNA is sonicated, hybridised to biotin labelled rodent DNA then the magnetic beads added, the hamster element of the hybrid could be drawn off with a magnet leaving the human element behind. This DNA could then be purified, labelled and used as a probe. Both the above strategies for enriching the human element of hybrid DNA are currently under experimental investigation in this laboratory.

THE FUTURE OF FISH

A number of areas of research involving FISH technology are rapidly becoming apparent:

Klinger *et al* (1991) have reported simultaneous detection of five different probes each in a different colour. This was achieved by using combinations of colours and instructing a computer to recognise each combination and assign it a different pseudocolour. For instance, with two fluorochromes, FITC and Texas-red:- a computer linked to an image analysis system could assign a Texas-red image a red

pseudocolour, an FITC image a green pseudocolour and an image fluorescing both FITC and Texas-red a blue pseudocolour. Thus from a two colour system, three colour fluorescence can be obtained. Hence with a three colour detection system (red, green and blue or infra-red, red and green), seven colour fluorescence could theoretically be achieved. Seven colour fluorescence would be invaluable in the clarifying of complex karyotypes whether using chromosome specific centromeric probes or chromosome painting. A further application would be the ability to put in order seven cosmids relative to each other.

A future application which is often mentioned in verbal presentations is the possibility of using FISH to map the physical position of genes in the intact interphase nucleus. If a method could be devised of preparing and fixing whole nuclei then hybridising probes (e.g cosmids) to them, then not only could the position of genes at interphase be mapped but also the communication between genes in the nucleus could be investigated. In such an application, a confocal microscope would be an invaluable tool. Trask *et al* (1988) and Mannuelidis (1985) have described methods of preparing whole nuclei preparations and performing FISH on them. They used total genomic probes on somatic cell hybrid nuclei and chromosome-specific satellite probes to reveal specific chromosomal domains in the interphase nucleus. Both groups are reportedly proceeding to the use of cosmid clones.

Another possible direction of investigation is to combine FISH and PCR technology. A small oligonucleotide could be annealed to a chromosome (as in PCR) then a string of nucleotides "zipped" on after it (as in PCR). This approach is referred to as "primed *in-situ* DNA synthesis or "PRINS." Gosden *et al* (1991) have described the use of this approach incorporating a biotinylated dUTP in the nucleotide mix followed by detection of it with fluorescent avidin. They detected human satellite sequences and *Alu* sequences and also telomere specific sequences in *Tetrahymena* and *Trypanosoma.* If these nucleotides were labelled with fluorescein dUTP then this could be a very

quick approach to perform FISH and could be particularly useful in diagnostic applications when speed is of the essence.

Meltzer *et al* (1992) have described the rapid (24 hour) generation of region-specific FISH probes and applying them to identify chromosomal rearrangements. The strategy they presented was to microdissect chromosomal regions and amplify them *in-vitro* by PCR. PCR products were then labelled with biotin and used as probes back on to metaphase preparations. Using such an approach, it is theoretically possible to generate probes (for FISH or other purposes) for any region of the genome and thus identify most cytogenetically visible chromosomal reaarangements unequivocally.

TUBEROUS SCLEROSIS

IRRADIATION-FUSION HYBRID GENE MAPPING STRATEGY

Use of irradiation hybrids appears to be effective as a gene mapping strategy however, it is thought that its true potential has yet to be evaluated (HGM11; Cox *et al.*, 1991; J. Fitzgibbon., personal communication). In this study the hybrids tested were ones generated using high doses of irradiation (~45 krads) which appeared to generate human fragments in the range of 0-5 megabases on a hamster background. Thus small areas of the genome can be studied using such hybrids, the whole region cloned into vectors (e.g. cosmids) and individual clones picked in the search for the disease locus.

FISH SCREENING OF CHROMOSOME 9q FRAGMENT HYBRIDS

In this study, FISH has been demonstrated to be an efficient method of distinguishing human from hamster DNA in somatic cell hybrid metaphases. Earlier methods (e.g the G11 technique) colour human and rodent DNA two different shades of pink/purple and hence are only applicable for distinguishing between large pieces. Furthermore the technique is a temperamental one (L. West., personal communication).

This study further demonstrates that FISH can be used as a rapid method of screening a panel of irradiation-fusion hybrids for human material. In this particular panel however, a number of apparent discrepancies between FISH and molecular data should be pointed out. For instance FISH data showed hybrid 12C to have two fragments integrated into hamster chromosomes however the molecular studies indicated that there were at least three (fig 3.23 and table 3.2). This could probably be

explained by two fragments lying close to each other and unresolvable by FISH or one of the fragments being too small for detection. Similarly hybrids 17A and 20A only display one fragment using FISH but two with PCR and Southern blotting. In both cases the fragments are small and "floating" (i.e. not integrated into hamster chromosomes) and present in most, if not all cells analysed hence suggesting that they must contain centromeric material (otherwise they would not be maintained in the cell line). 17A is indeed positive for centromeric markers however 20A is not and both its fragments are derived from 9q34. The most likely explanation for this is that 17A contains a single, small, floating human fragment on it with two pieces (one on 9q34,one centromeric) very close together. 20A however, appears to contain a human piece with two fragments from 9q34 and a small piece of hamster centromere. It is clear therefore that, although FISH is a very efficient method of screening somatic cell hybrids, results, when obtained, are occasionally open to some misinterpretation.

SENSITIVITY OF THIS APPROACH

The question "what is the smallest size size fragment which can be detected by FISH?" often arises. Hybrid 17B was shown by FISH to have a very tiny fragment which, although present in 100% of metaphases examined, was only just visible. Had it been any smaller, it is doubtful whether it could be identified with confidence. Indeed, in more than one case, when the FISH technique was not working optimally, it was not detected. Molecular evidence (J. Fitzgibbon and K. Woodward., personal communication) indicates that this fragment is around 500kb-1Mb in size. Results in this study therefore suggest that 500kb is approaching the lower limit of sensitivity of this approach. It is possible that further refinements of the technique perhaps involving the use of cot1 DNA could reduce this lower limit.

REVERSE CHROMOSOME PAINTING

It is clear that the ideal way to screen irradiation-fusion hybrids is to employ "reverse chromosome painting" i.e. using hybrid DNA as a probe on to human chromosomes as this would alleviate the problem of two close fragments on the hybrid metaphase appearing as one if the two fragments were from spatially different places in the human genome. Furthermore the chromosomal origin of these fragments would be instantly identfiable. This study however highlights the practical difficulties associated with using this approach. In summary therefore it would be advisable to use human DNA on to hybrid metaphases as a general screening tool and then to only use reverse chromosome painting on hybrids of particular interest.

MAPPING OF DERIVED CLONES

RELATIVE SUCCESSES OF PHAGE AND COSMID CLONES

This study demonstrates how FISH can be used as a tool to map clones isolated from somatic cell hybrids. Two types of probe were used namely phage and cosmid clones. Because of the relative insert size (15+ kb for phage, 40+ kb for cosmid) and relative vector : insert ratio (~3:1 for phage ~1:5 for cosmids), cosmid signals were brighter and appeared more frequently. For instance many cosmids produced double signals on both homologues in over 80% of metaphases examined whereas phages had double signals in only one homologue in around 30% of metaphases examined. In addition, many cosmid signals were visible in the interphase nucleus where the phage signals were not. Why two of the phage clones in this study (table 3.4) did not work, when they were isolated in the same fashion and were of approximately the same size as the others, is not clear. It is possible that the majority of sequence in these particular clones was repetitive as opposed to unique sequence and was hence competed out by CISS.

FUTURE APPLICATIONS

In any gene mapping strategy, FISH is an essential element. This particular strategy in search of the TSC1 locus is no exception. For instance clones could be mapped to the original metaphases of the hybrids to ensure that they do indeed derive from that hybrid. Given the merits of FISH multiple labelling and the fact that cosmid signals can be visualised in interphase nuclei, clones liked to TSC1 could be ordered with respect to one another. Indeed Harris *et al.*(1991) have used dual FISH to place the clones linked to TSC1 in the order cen-ALAD-ORM-D9S16-AK1-SPTAN-ASS-ABL-D9S10-DBH-tel. FISH is also useful in characterising breakpoints of translocation patients, this has also been achieved by Harris *et al* (1991) mapping a breakpoint in 9q34 in a patient with TS.

Currently, cosmid libraries are being made in this department from hybrids 20A and 17B in order to saturate the interval spanned by the hybrids with cloned DNA. Experimentation involving double labelling is being undertaken to order cosmid clones picked from these libraries. It is hoped that a) one of these hybrids will contain the disease locus and b) that eventually one of the clones picked from the libraries made from the hybrids will contain either the TSC1 locus or that of torsion dystonia (DYT1) or nail-patella syndrome (NPS1) and hence complete the gene cloning strategy.

COLORECTAL CANCER

TERMINAL CHROMOSOME 1q

PRESENCE IN CANCER CELL LINES

Both colon cancer cell lines studied exhibited trisomy for a section of chromosome 1q (1q32-qter). In the case of LIM1215, it was found to be the only karyotypic abnormality (Jenkyn *et al* and our own observations) and hence possibly responsible for the immortal phenotype. This study provided molecular confirmation that the extra material was derived from 1q32-qter. In the line AA/C1/SB10 karyotype analysis suggested that there might be a whole extra chromosome 1q however dual FISH revealed that the centromere of the chromosome in question was derived from chromosome 18 and not chromosome 1 and hence only terminal 1q (probably again 1q32-qter) was present.

ROLE IN CELLULAR IMMORTALISATION

Chromosome 1 abnormalities are frequent in human cancer (Povey and Parrington., 1986), colorectal cancer is no exception. Reichmann *et al* (1984) reported regions of consistent duplication of chromosome 1q were q24-qter in patients with colon cancer. Studies on the cell line PC/AA (from which AA/C1/SB10 was derived) have also implicated chromosome 1q in cellular immortalisation. PC/AA is an immortal, non-tumorigenic adenoma line derived from a patient with FPC and a normal diploid karyotype. Two derivatives from it each had an isochromosome 1q and C-band polymorphisms revealed that each Iso1q was derived from a different homologue (Paraskeva *et al.*, 1988, 1989).

Loss of regions of chromosome 1p has also been implicated in tumour formation (e.g. Atkin., 1986; Leister., 1990). It has been suggested that tumorgenicity arises from

interaction between genes which suppress malignancy on chromosome 1p and others which express the immortal phenotype (Benedict *et al.*, 1984). It is likely therefore that the latter may reside on chromosome 1q.

PLACE IN COLORECTAL CARCINOGENESIS

Fearon and Voglestein (1990) proposed a model for colorectal tumour progression involving chromosomes 5, 12, 17 and 18. This model does not however, include chromosome 1. Paraskeva *et al* (1989) suggest that there is no general relationship between acquisition of *in-vitro* immortality and adenoma formation when they are small (<1cm) but go on to hypothesize that acquisition of *in-vitro* immortality could be associated with a late stage in adenoma development.

CHROMOSOME 18q

PRESENCE IN CELL LINE

Karyotypes supplied of AA/C1/SB10 (C. Paraskeva) indicated absence of a normal chromosome 18 the in cell line . This is in contrast to the results of Williams *et al* (1990) detecting 1 normal chromosome 18 in the same cell line. This can be easily explained by the fact that we obtained the line at a later stage from when it was examined by Williams *et al* and further karyotypic changes had obviously occured in the meantime. Paradoxically, FISH results indicated that the line possessed four copies of the centromere of chromosome 18.

ROLE IN COLORECTAL CARCINOGENESIS

Both cytogenetic (Muleris *et al.*, 1990a,b; Reichmann and co-workers., 1980-1986) and molecular studies (reviewed in Fearon and Voglestein., 1990) implicate the loss of chromosome 18 in colorectal carcinogenesis, specifically in the adenoma to carcinoma sequence. The karyotype of AA/C1/SB10 (Fig 3.34) supports this in this study where

no normal chromosome 18 was found and also in the studies of Williams *et al* (1990) where a single chromosome 18 was identified. It is interesting therefore that FISH evidence indicated the presence of four chromosome 18 centromeres. The presence of multiple chromosomes in the line is not unusual in his line for instance six chromosomes 9 can be consistently identified. Therefore attention turned to the likely common region of loss on chromosome 18 in colorectal carcinogenesis .

SIGNIFICANCE OF THE DCC GENE

Fearon *et al* (1990) identified a candidate gene for the common region of loss on 18q and named it "DCC" (deleted in colorectal cancer). Three clones were isolated from it. Figure 3.35 shows the mapping of a combination of these three clones (Josh 4.4, Sam1.1 and p15-65) to chromosome 18q21 thereby confirming the assignment of Fearon *et al.* This probe cocktail gave very weak signals on normal human chromosomes however, and were hence assumed to be an unsuitable FISH probe for investigating the presence of the DCC gene in the cell line AA/C1/SB10. Thus a human genomic cosmid library was screened using p15-65 (as this was the one clone which contained no repetitive sequences). Of all the cosmid clones which were picked from this screen, none hybridised to 18q21. One can only speculate as to the reason for this but technical flaws and/or poor quality of the DNA of the p15-65 probe seem the most likely possibilities. Two of these clones gave very strong signals on terminal 14q. A known gene in the region is alpha-1-antitrypsin, these clones are currently being tested for homology to this locus. Research is currently being carried out a) to try and improve the intensity and reliability of the signal when using the three cDNAs as probes; and b) to obtain a cosmid probe from the DCC region either by using p15-56 or one of the other two probes. If either could be achieved then experimentation in this field could continue and the presence of DCC in this line evaluated. As AA/C1/SB10 is thought to represent an *in-vitro* model for the adenoma-carcinoma sequence, one would assume that the line should contain only one copy of DCC or no copies at all since deletion of DCC is thought to be an event in the progression from

adenoma to carcinoma. From banding homology the most likely site of DCC hybridisation would be the section of chromosome 18 which is translocated on to chromosome 1. Earlier passages of this line (Williams *et al.*, 1990) show that this translocation chromosome is present along with a normal chromosome 18. The karyotype shown in fig 3.35 (later passage) shows that there is no normal 18 but that the 18/1 translocation chromosone is still present. Hence the most likely scenarios are that a) at the passage level studied by Williams *et al* the line contains two copies of DCC and the later passage used in this study only one; or b) the earlier passage contains one copy and the later one none. This still awaits further elucidation.

CHROMOSOME 5q

FISH AS A TOOL FOR SCREENING COMPLEX REARRANGEMENTS

The merits of FISH as a tool for the ordering of cosmids and subsequent screening of chromosomal rearrangements are well documented (reviewed in Trask., 1991). In this study, it was hoped that it might be possible to order all the available cosmids around APC and go on to define the breakpoints of families with rearranged chromosomes 5 in molecular terms. In practice however few of the chromosome 5 cosmids gave signals on 5q despite other cosmids working satisfactorily in the laboratory. Cosmids ECB27 and L5.79 did give very good signals on chromosome 5 however and were mapped to normal chromosomes and to the chromosomes of the proband of the family studied. The proband has an interstitial deletion on chromosome 5 (5q22-23.2 - see appendix 5), this causes mental retardation and FPC. The two cosmids which worked on the chromosomes of this individual (namely ECB27 and L5.79) helped to define the deletion in molecular terms. L5.79 was deleted on one chromosome 5 whereas ECB27 was not, thus indicating that (since ECB27 is the most proximal) the proximal breakpoint of the deletion lies between these two clones. Molecular analysis has shown that the deletion encompasses the loci MCC, APC, EF5.44, and YN5.48, and

by comparison with published data (Kinzler *et al.*, 1991), it is thought to be 2-5 Mb in size (Cross *et al.*, 1992). The many studies on the APC locus (see introduction) have suggested that it lies on the junction of 5q21-22 or in 5q21. However, as the proximal breakpoint of the deletion appears to be in the middle of 5q22 in this patient, this thus suggests that APC may be, in fact, located in the distal half of 5q22.

FUTURE APPLICATIONS

Currently more families with rearrangements on chromosome 5 are being studied and more cosmids (and YACs) are becoming available on and around the APC region. Research is in progress in this laboratory therefore into defining, in molecular terms, the cytogenetic breakpoints of these rearrangements, and into mapping the APC and MCC genes in precise cytogenetic terms.

FURTHER EVIDENCE FOR FISH AS A GENE MAPPING TOOL

Figure 3.37 shows the mapping of clones isolated from expressed sequences on chromosome 5 expressed in normal colonic mucosa (see materials and methods for details). This is an example of the application of FISH in a very different gene mapping strategy to the one described for chromosome 9. The phage clone λ5.3 has an insert of 2-3 kb (S. Leigh., personal communication) and was successfully mapped by FISH with CISS. This was the only one of a panel of 10 of these clones however which gave paired signals on chromosome 5. This perhaps indicates that 2-3 kb is the lower limit for the sensitivity of FISH with CISS. It is not clear why none of the other clones could be successfully mapped in this way, it may be that that particular clone contained few repetitive elements when compared with the others. Cosmid "cos5.3iv" was isolated by screening a human genomic cosmid library with λ5.3. It is not clear why the two clones mapped to different bands of the same chromosome region. Cosmid "cos5.5iv" was made from a phage clone λ5.5 with which FISH mapping was unsuccessful. It was found to reside on 5q31. It is apparent therefore that FISH can be used in more than one type of gene mapping strategy.

PREIMPLANTATION DIAGNOSIS

SINGLE LABELLING

SUCCESS OF EARLY WORK

This study was the first to perform and evaluate FISH for X and Y chromosome specific probes on human embryonic material (Griffin *et al.*, 1991). With the exceptions of Pieters *et al* (1990) (performing FISH with a chromosome 1 probe on a single human embryo) and Grifo *et al* (1990) (reportedly achieving FISH and detecting an XYY nucleus in a single human embryo), it is the first to report FISH of any kind on human embryos.

Since independent confirmation of embryo sex was not achieved, an accurate assessment of hybridisation efficiency could not be made. However, if one determines sex on the basis of number of hybridisation signals in the majority of nuclei, the efficiencies with both probes (pHY2.1 and pBamX7) using FISH appeared to be higher in male embryonic nuclei than previously réported using other ISH methods with probe pHY2.1 (Jones *et al.*, 1987; West *et al.*, 1987, 1988; Penketh *et al.*, 1989). For example 85% of these nuclei in this study gave positive signals compared with 66% using an enzymatic based biotin detection system (Penketh *et al.*, 1989).

Hybridisation with probe pBamX7 had not been previously evaluated using any form of ISH on human embryos. It is interesting that there was a high incidence of (18%) of nuclei displaying two (or more) signals in interphase nuclei classified as male and four signal in a nucleus designated as female. All of these embryos had more than 10 nuclei on day-5 post-fertilisation. In most cases, the nuclei with twice the number of expected signals were appreciably larger than those of surrounding cells. This can be easily explained because of the occurrence of tetraploidy. One male nucleus with two

signals however was not larger than the remainder. Chimaeric origin or gain of an X-chromosome are possible explanations for this. Tetraploid nuclei with two Y chromosome signals have been already observed in human embryos (West *et al.*, 1987, 1988), and it has been suggested that their occurrence in early preimplantation embryos may be a culture induced phenomenon (West., 1990).

AUTOFLUORESCENT FOREIGN BODIES

In a number of cases, unexpected fluorescent "signals" were seen. These were more yellow in colour than the true signal, could be visualised using both green and red fluorescent filters and could be filtered out using a fluorescein filter block equipped with a selective barrier filter. Their appearance was hence attributed to foreign material on the slide and was disregarded in the analysis.

THE NECCESSITY OF EFFICIENT SPREADING

From these results, it is apparent that hybridisation is more efficient with small groups of cells (intended to model cells biopsied from the embryo) and earlier embryos where less cytoplasm remained around the nucleus after cytogenetic preparation (tables 3.6-3.7). It therefore was essential to develop methods of embryo spreading which will lead to a minimum of residual cytoplasm surrounding the nucleus before FISH preimplantation sexing was put into clinical practice.

DRAWBACKS OF SINGLE LABELLING

Single FISH employing either probe has its disadvantages. Use of the Y chromosome specific probe "pHY2.1" has an efficiency of around 85%. Hence if two cells were biopsied (leaving six to be transferred), the probability of misdiagnosis would be more than 1%. Because of the frequent occurrence of twice the expected number of signals using the X probe alone, tetraploid male cells and female cells would be indistinguishable, again leaving the probability of misdiagnosis greater than 1%. It is clear therefore that use of either probe alone is not an ideal approach to the sexing of

human embryonic material. The problems associated with each of these probes alone are however alleviated when X and Y probe are hybridised and detected simultaneously on the same nucleus.

DOUBLE LABELLING

EARLY ATTEMPTS

Initially, double labelling was attempted using pHY2.1 (digoxigenin-fluorescein) and pBamX7 (biotin-Texas red), results are summarised in table 3.8. It is clear that this approach is not an adequate one for the sexing of human embryonic material. Problems arose when trying to reconcile the hybridisation conditions of the two probes. Under high stringency conditions pHY2.1 gave very weak signals however under low stringency conditions pBamX7 showed minor hybridisation signals on chromosomes 11 and 17. A compromise of conditions hence had to be devised. This was acceptable on lymphocyte material but, as results indicate, not on embryos. Eight out of 12 (67%) displayed no signal in the nucleus. It also became apparent that many of these type of nuclei were very large and had a "cobweb-like" appearance. It was these which were least likely to produce a signal. Furthermore, as with single labelling, it was the nuclei which could be seen as clean of cytoplasm before FISH that produced the more analysable signals in the final analysis.

INTRODUCTION OF cY98

Experimentation revealed that the probe cY98 required similar hybridisation conditions to that of pBamX7. It was hence decided to continue dual labelling using all three probes under high stringency conditions. pBamX7 was supplied labelled with biotin and was detected using Texas-red, pHY2.1 and cY98 were hence labelled with digoxigenin and detected using FITC. This combination was chosen (since biotin was the most reliable of the two labels and FITC the most reliable of the two

fluorochromes) thus signals were of a similar intensity. Table 3.9 clearly indicates the improvement in efficiency brought about by the introduction of probe cY98. Positive diagnosis of sex could be made in 78% of cases when using cY98 compared with 33% when not. Hybridisation efficiency was greater than than with other ISH techniques (Jones *et al.*, 1987; West *et al.*, 1987,1988; Penketh *et al.*,1989; Grifo *et al.*, 1990; Griffin *et al.*, 1991). This approach was an improvement on single labelling as the possibility of misdiagnosing a male as female was virtually eliminated since two clear X and no Y signals needed to be seen before the embryo was determined as female (and thus suitable for transfer). Failure to obtain a result was generally attributed to three factors: a) inability to relocate the cell at the end of the procedure (due to cell detachment during the FISH procedure); b) no signal visible in the nucleus (usually related to the size and "cobweb-like" nature of the nucleus); c) the presence of autofluorescent foreign bodies obscuring the signal. Autofluorescent material masquerading as a Y chromosome signal lead to a misdiagnosis in one case. This was discovered as the other cell was clearly female and this led to a closer inspection of the first cell.

DOUBLE LABELLING ON METAPHASES

The most consistent results were obtained on metaphase preparations despite the fact that they were unsuitable for cytogenetic analysis. All preparations were clean of cytoplasmic debris prior to FISH. Results are summarised in table 3.10. Of the two results in the column headed "other" the XO result is thought to be due to loss in preparation of a Y chromosome, the XXY result is thought to be a tetraploid nucleus with two overlying Y signals (the spot was large enough to represent two signals). It is noteworthy that the former would have been classified as a female using Y chromosome single labelling and the latter also as female using X chromosome single labelling. In fact both were male.

REFINEMENT OF THE TECHNIQUE

The biggest disadvantage of FISH sexing as compared with PCR sexing is that FISH methods generally take 24 hours to perform whereas the PCR based sexing strategy takes 5 hours only. In this study, a method of performing FISH sexing within one working day involving double labelling has been developed. Improvement in cytogenetic preparation and the use of freshly prepared slides allowed the protocol to be followed with a low proteinase K concentration, high stringency hybridisation and washes, 90-120min hybridisation time and no signal amplification (see materials and methods). Use of freshly prepared slides mimics the diagnostic situation when slides would normally be prepared and used on the same day. Probe pBamX7 was supplied labelled with biotin and it was decided to use FITC as the detecting fluorochrome since the unamplified signal (although very reliable) was very small and not easily detected with a red fluorochrome. cY98 and pHY2.1 were hence labelled with digoxigenin and detected using TRITC labelled anti-digoxigenin. The whole procedure was performed in six to seven hours. The evidence (Figs 3.15d and 3.16d) clearly indicate that non-amplification of signals greatly increases the efficiency of FISH on control lymphocyte material, rising from 90% to 100% expected number of signals in males and from 83% to 94% in females.

PROSPECTS FOR CLINICAL APPLICATION

Embryo biopsy and sexing by PCR is reported in the literature and five out of eight women became pregnant after 13 treatment cycles (Handyside *et al.*, 1990,1991; Handyside and Delhanty., 1992), two with twins, the other three singletons. Each were sexed by day-three biopsy of one cell (with one exception where two cells were removed), each patient had between 2 and 11 embryos (mean of 5). Of the seven fetuses, one was revealed by CVS to be male. Further experimentation has revealed that using that particular PCR method, 15% of female embryos are identified as male (A. H. Handyside., personal communication). This, of course, is only as efficient as

Table 4.1:- Relative merits of dual FISH and PCR for the sexing of preimplantation embryos

PCR	**Dual FISH**
Rapid (can be performed in 5 hours)	Can be performed in 6-7 hours + spreading time
Technically simpler	Technically more difficult
Contamination can lead to misdignosis	Contamination of foreign bodies does not impede accurate diagnosis
Failure of amplification can lead to misdiagnosis	Failure of hybridisation does not lead to misdiagnosis
Accurate diagnosis probably needs two cells	Diagnosis can be confidently made on one cell
Indirect (visualising a band on a gel)	Directly visualising the cell
Detecting only one sequence	Independant detection of two sex-chromsome sequences
Does not detect sex chromsome aneuploidy	Can detect sex-chromsome aneuploidy
Also applicable for detection of single gene defects	Not applicable for detection of many single gene defects
Not practically applicable for the detection of autosomal aneuploidy	Applicable for the detection of autosomal aneuploidy

using FISH single labelling with pHY2.1 as a probe. Using the refined double labelling approach virtually eliminates the chance of misdiagnosis in control material and can be performed nearly as quickly as PCR. Table 3.11 indicates the applicabilty of this approach on embryonic nuclei. This was attempted on both metaphase and interphase nuclei. All but one of the metaphases could not be located both prior to or after FISH suggesting a problem in the spreading technique. All but one of the interphases gave positive signals. Interestingly all were clear females apart from one XO cell, this was attributed as having two overlying X signals. On the basis of these results it was decided to proceed with clinical application.

PATIENT STRATEGIES

So far, six patients, all carrying sex-linked disorders (TypeII motor neurone disease, Fragile X, Lesch-Nyhan syndrome, X - linked myotubular myopathy and X-linked mental retardation (two patients)), have had preimplantation embryos sexed by FISH. From the patient reports (tables 3.12 - 3.17), it is clear that it is, again, the large "cobweb-like" nuclei which often give no signal. However using the refined method even some of these yield positive results. Table 3.18 summarises all the patient results and figure 3.45 combines results from that and table 3.11 to give a complete breakdown of the efficiency of this technique for any single cell. 59% of all cells therefore gave a positive diagnosis. The most frequent reason for not getting a result (19%) was the inability to locate the cell either before or after FISH. This was not a factor in the developmental stages of this approach as only nuclei which could be seen under phase-contrast illumination prior to FISH were used. Since a small number of nuclei could not be located before but were visible after FISH, it is possible that some of the 19% were lost in preparation. However, because there were only 3 (5%) which were seen before FISH but not after, it is likely that the majority were lost during the spreading stage. In the case of Mrs Ha, of the four cells in the category "could not locate before

or after FISH", three did not seem to have intact nuclei before spreading and one was probably anucleate as its sister cell had two nuclei (tables 3.16 a,b). The second largest cause of failure (10%) was due to no signal on the nucleus. In all but one of these cases this was obviously due to one of two factors namely a) the nucleus being of poor quality or b) cytoplasm around the nucleus prior to FISH. Both of these are directly related to the spreading. In only one case out of 59 (1.6%) was the result obscured by autofluorescent foreign bodies. This is in contrast to 2 cases in 18 (11%) using the older method. Of the three cells in the "other" category, two were XO (probably explained by overlying signals) and one showed many scattered X signals. The latter can be explained by the fact that the nucleus was of very poor quality and the chromatin was spread over a wide area - again a function of the spreading.

Spreading of human embryos in order for them to be suitable for FISH is a very tricky procedure (L.J. Wilton., personal communication) and can only be performed by a skilled embryologist. The majority of embryos supplied for this study were of excellent quality; had they not been FISH would have never been possible on them. The results indicate however, that the efficiency of this approach for preimplantation diagnosis would be improved further if there was any way in which the spreading could be improved further. Spreading of embryos was also compounded by the problem of the cells lysing following biopsy. Experimentation is currently under way into finding methods of minimising ths occurence.

Using the current strategy 70% of embryos have been sexed with confidence (results figure 3.45). Purely by chance, and quite unluckily, only 26% have turned out to be female whereas 44% have been male. Currently only two of these patients have shown hormonal signs of embryo implantation (Mrs Wi and Mrs Hd) and the pregnancies did not progress past this stage. As only five transfers have been performed, two of those with only a single embryo (IVF transfers are more productive when involving two embryos:- A.H. Handyside., personal communication), it is perhaps premature to suggest reasons why there are still no pregnancies using this

approach. The only difference between this biopsy approach and that involving the PCR based diagnoses is that biopsy was performed on day-3 then but on day-2 here. Initial evidence has suggested that there may be a slight reduction in embryo viability between day-2 biopsy and day-3 biopsy (A.H. Handyside., personal communication) however, because of the small sample size, the most likely explanation for lack of pregnancy is simply bad luck.

POSSIBLE EARLY X-INACTIVATION IN HUMANS

Dyer *et al* (1989) report the use of FISH to distinguish between active and inactive X chromosomes in interphase nuclei. They found that when female fibroblasts were grown directly on a microscope slide a) the Barr-Bodies (compact, inactive sex chromosome bodies) could be visualised using Giemsa stain, and b) FISH with probe pBamX7 revealed two types of X signals. The first diffuse and appearing peripherally and centrally with equal frequency, the second compact, appearing mostly peripherally and corresponding to the Barr body.

Figure 3.42 shows a female day-3 blastomere (cell Wi 3.1) with a diffuse and a compact X chromosome signal. This pattern was visualised in a number of the other nuclei also.

X-chromosome inactivation was first reported by Lyon (1961). In the mouse it first occurs in trophectoderm (Gartler and Riggs., 1983) and later in the whole embryo at around the time of neural tube formation (P. Burgoyne., personal communication). This is also assumed to be the case in humans. Because of the paucity of human embryonic material for experimentation however, the exact stage of human X-inactivation has yet to be determined. Ashworth *et al* (1991) report that the difference between XO humans and XO mice (i.e. XO humans usually abort prenatally and live births lead to Turner's phenotype whereas XO mice are relatively normal) is due to

differences in X inactivation. Thus it may be unwise to assume that the time of X-inactivation is comparable in the two species.

Dyer *et al* (1989) hypothesise that the initiating event in X chromosome inactivation is the formation of the Barr body. The question arises therefore:- "does the X chromosome pattern in figure 3.42 represent an active and inactive X and thus is Barr body formation and hence X-inactivation occuring at day-3 or earlier?" The pattern seen, of course, may be due merely to splaying of the chromatin on one of the chromosomes during the spreading procedure. Only further studies on e.g. methylation patterns in human preimplantation embryos will further elucidate this enigma.

SCREENING OF FUTURE PATIENTS

Figures 3.47 - 3.49 show results of early investigations into assessing patients for the possibility of another type of preimplantation diagnosis. The karyotypes confirm that all patients have Robertsonian translocations (incidentally, Mrs Te also has an inverted chromosome 9) they are hence at risk of producing trisomic offspring, indeed Mrs Te has already given birth to one child with trisomy 18. All have been screened with probes recognising chromosomes 13/21 and 14/22 with a view to the possibility of screening affected embryos. Because these probes show varying size signals between the chromosomes and because eight signals would have to be viewed (four for each colour as compared with two for sexing) it is likely that the best strategy would be to reject all embryos carrying the translocation chromosome i.e. those with overlying red and green signals in the interphase nucleus.

In the case of Mrs Sp (Fig 3.48b) the 13/21 probe does not give an adequate signal on the 14-21 translocation chromosome hence further research needs to be carried out before she can be considered for preimplantation diagnosis.

FUTURE PROSPECTS

Figure 3.46 shows the detection of chromosome 18 in two nuclei of a preimplantation embryo. Chromosome specific probes are now available for nearly all the human chromosomes. Carriers of reciprocal translocations are at risk of producing chromosomally abnormal embryos however use of these probes is limited in preimplantation diagnosis. This is because they would only detect a minority of unbalanced meiotic products, i.e. those which result in an abnormal number of centromeres appearing in the embryo. Other imbalances not involving the centromere would have to be detected using cosmids, cosmid contigs or chromosome painting.

Research is currently being carried out in this laboratory into the possibility of improving IVF success rate in general by applying FISH to detect the major trisomies which lead to spontaneous or induced abortion i.e those of chromosomes 16, 18 and 21. The extent to which mosaicism is a factor in this regard also warrants further investigation

Research is also being carried out in this laboratory into the possibility of detecting other chromosome abnormalities in the preimplantation embryo. Lichter and Ward (1990) describe how the use of cosmids can detect most types of gross chromosome abnormality in the interphase nucleus (reviewed in the introduction). Research is ongoing also into methods of preparing analysable metaphases. If good chromosome preparations were available then it is feasible that even the most subtle of chromosome abnormalities could be detected at this stage using FISH with cosmids, cosmid contigs or chromosome painting. One possible barrier to the use of FISH and karyotyping is the fact that when performing day-2 or 3 cleavage stage biopsy, it is desirable to implant the embryo on day-3 otherwise pregnancy rate may be reduced (Handyside and Delhanty., 1992). Current chromosome preparation techniques and FISH

approaches involving cosmids and chromosome painting each take 24 hours to perform. Possible solutions to this problem could involve trophectoderm biopsy or cryopreservation of embryos. Both of these at present however, can lead to some reduction in pregnancy rates (Handyside and Delhanty., 1992). A possible approach to speeding the technique would be by using probes directly labelled with the fluorochrome (Wiegant *et al.,* 1991). Hence lengthy detection procedures could be shortened in this way

It is apparent therefore, that there is still some research to be done before FISH preimplantation diagnosis of chromosomal disorders as well as sex becomes practicable. However the obstacles in the way of this are clearly not unsurmountable ones. Use of cosmid contigs or single cosmids allow all areas of the chromosome complement can be illuminated using FISH. It is hence not beyond the bounds of possibility that diagnosis of any chromosomal abnormality at the preimplantation stage could become a reality before the end of the century.

APPENDIX

APPENDIX1: HUMAN MALE KARYOTYPE AND IDEOGRAM

Fig 5.1:- KARYOTYPE

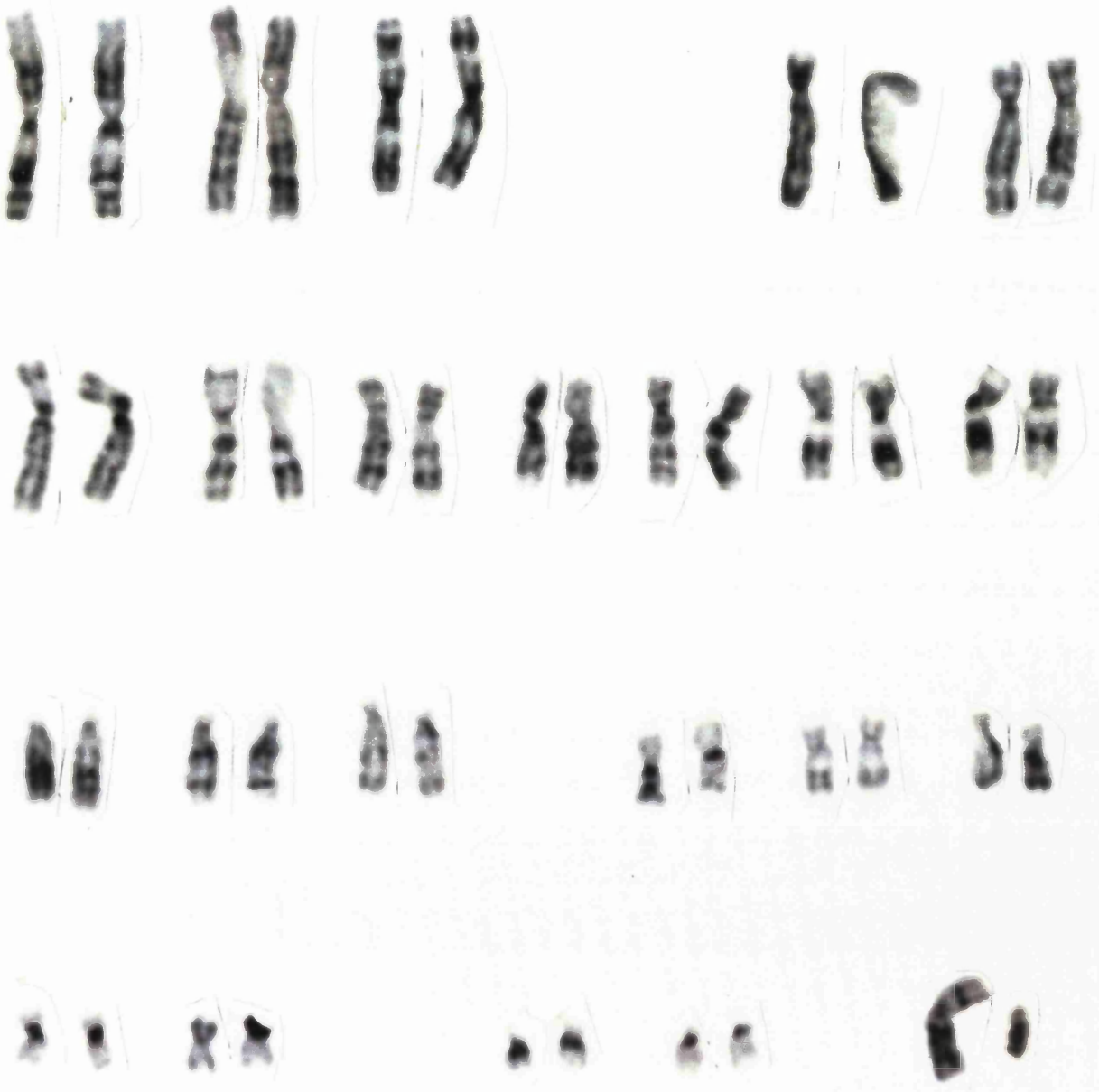
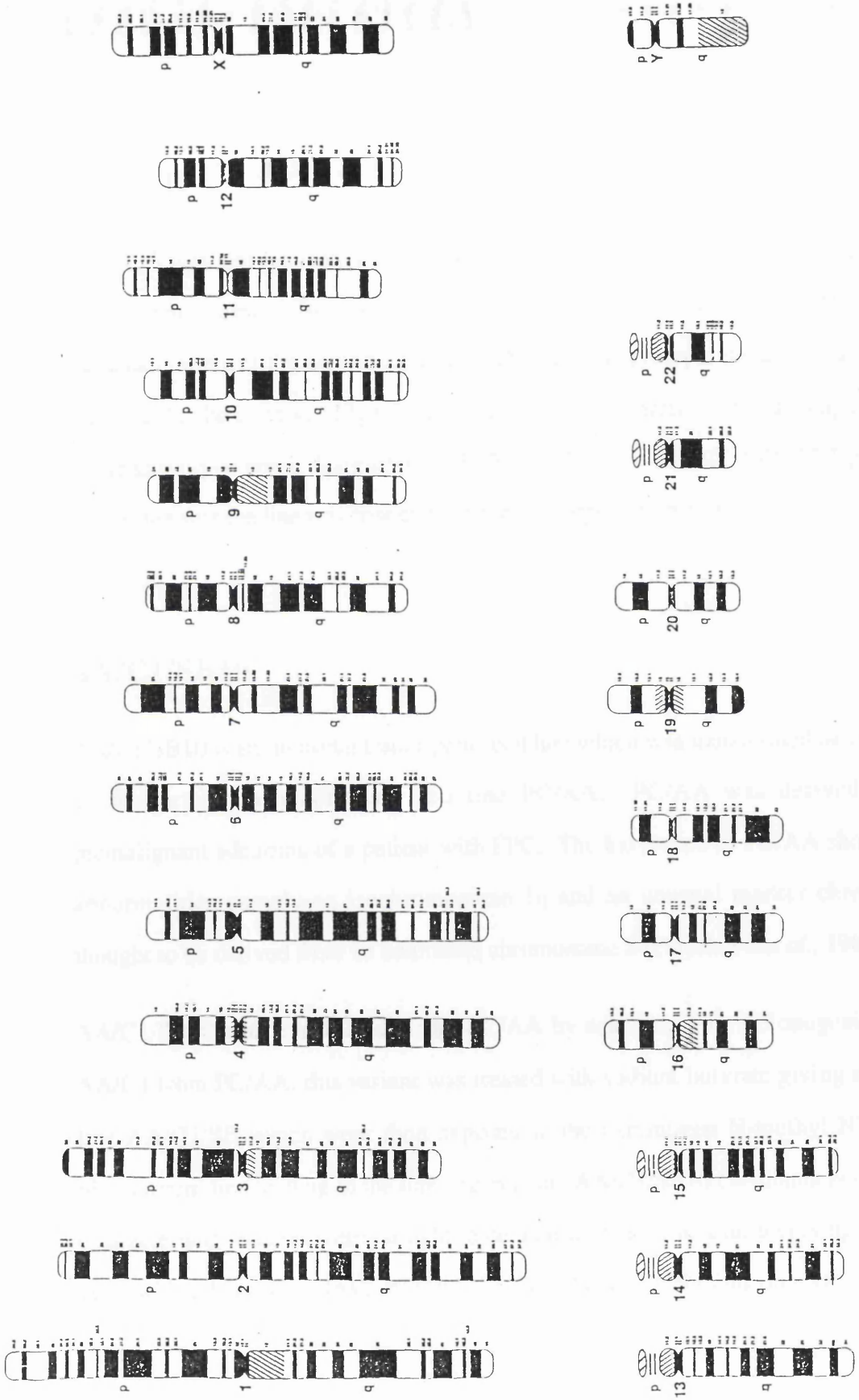


Fig 5.2:- IDEOGRAM:



APPENDIX 2: DERIVATION OF CELL LINES USED IN THIS STUDY

LIM1215

LIM1215 was derived from a patient with inherited, non-polyposis colorectal cancer. The primary tumour from which the line was made was a poorly differentiated adenocarcinoma of the ascending colon. The single karyotypic defect in this cell line was found to be a marker 13p+ (Jenkyn *et al.*, 1987). Careful G-band comparison led to the karyotype being designated as 46 XY, -13, + der (13) t(1:13)(q32.1:p11) thus suggesting that the line was trisomic for chromosome region 1q32-qter.

AA/C1/SB10

AA/C1/SB10 is an immortal tumorigenic cell line which was transformed *in-vitro* from an immortal non-tumorigenic cell line PC/AA. PC/AA was derived from a premalignant adenoma of a patient with FPC. The karyotype of PC/AA showed two abnormalities namely an isochromosome 1q and an unusual marker chromosome thought to be derived from an additional chromosome 8 (Paraskeva *et al.*, 1988).

AA/C1/SB10 was transformed from PC/AA by selecting a rare clonogenic variant AA/C1 from PC/AA, this variant was treated with sodium butyrate giving rise to the line AA/C1/SB which were then exposed to the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine leading to the tumorigenic line AA/C1/SB10 (Williams *et al.*, 1990). Characteristic features were thought to be a pericentric inversion involving 1p32 and 1q23 with a deletion of 1p32-pter and also loss of a single chromosome 18.

This line provides an excellent *in-vitro* model for the adenoma to carcinoma sequence. The deletion of 1p and the extra copy of 1q provide further evidence of the chromosome in tumour progression. Furthermore, the loss of chromosome 18 is consistent with studies on *in-vivo* carcinomas (Fearon *et al.*, 1990).

CJ9q

In this study, the original hybrid irradiated in the tuberous sclerosis section (the "parent hybrid") was named "64063a12" (Jones and Kao., 1984). For reasons of simplicity it was referred to as "CJ9q" ("CJ" being the initials of the patient from which it was originally made).

This hybrid was made by extensive subcloning of an original hybrid which contained only 2-4 chromosomes including 9. Molecular analysis and differential human/hamster chromosome staining (G-11 technique) indicated that this hybrid contained only the single human piece 9cen - 9qter. Hence, irradiation hybrids made from CJ9q were certain to contain a piece or pieces from the long arm of chromosome 9 only (if they contained a human piece at all).

APPENDIX 3:

This section deals with recipes of reagents etc abbreviated in the text of the materials and methods section for the sake of continuity.

PROBE LABELLING TECHNIQUES

TE: 10mM Tris, 1mM EDTA, pH7.6, millipore through 0.2μm.

10x salts: 0.5mM Tris pH7.8, 0.05M $MgCl_2$, 0.01M β-mercaptoethanol, 50μg/ml BSA, store at -20°C.

TNE: 0.2M NaCl, 10mM Tris, pH8.0, 1mM EDTA.

Labelling soln: 7μl 1mM Tris pH7
2.5μl **DTM:** 1M Tris, pH8 - 250μl,
1M $MgCl_2$ - 25μl,
β-mercaptoethanol - 3.5μl,
dNTP's - 6μl,
Make to 1ml with deionised H_2O,
2μl Hepes (Sigma) pH6.6.
Store at -20°C.

BSA: Bovine serum albumin fraction IV (Sigma).

TNT: 0.1M Tris.HCl, 0.15M NaCl, pH7.5, 0.1% Tween 20 detergent (Sigma).

TN: As TNT without Tween 20.

TNB: As TN + 0.5% blocking reagent (Boehringer), dissolve for 3 hours at 60°C, filter twice through 1M filter paper (Whatmann), Store at -20°C.

CYTOGENETIC PREPARATION

GPS: 100ml 200mM glutamine (Imperial), 600mg Penicillin (Sigma) H_2O, 300mg Streptomycin monosulphate (Sigma) in 3ml H_2O, store at -20°C.

FCS: Fetal calf serum (Tissue Culture Services).

PHA: Phytohaemaglutinin (Flow), 1 vial reconstituted in 1ml H_2O, store at 4°C.

MEM Medium: 9ml 10x Minimun essential medium Eagle's (Flow), 10ml FCS, 81ml sterile H_2O, 1ml 1M Hepes, 1ml GPS, Store at 4°C.

Hank's soln: 10x Hank's balanced salt solution witout calcium or magnesium ions (Imperial) dilute 1:9 in sterile H_2O, pH until red with 5.3% Na_2HCO_3.

KHE hypotonic: KCl - 3%, EDTA - 0.2%, Hepes - 4.8%, pH7.4, sterilise and store at 4°C.

CHROMOSOME BANDING

2xSSC: 0.033M Trisodium citrate, 0.33M NaCl, pH7 with 1M citric acid.

pH 6.8 buffer: Buffer tablets pH6.8 (Gurr), 1 tablet in 1 litre H_2O.

Q buffer: 1.78% $Na_2HPO_4.2H_2O$ pH6.5.

NON FLUORESCENT *IN-SITU* HYBRIDISATION

RNAse: RNAse A (Sigma) 100µg/ml in 2xSSC, boil 3min, store at -20°C.

To deionise formamide: 5g Amberlite monobed MB-1 mixed resin (BDH), 100ml formamide (Fluka/BDH), Stir in sealed bottle in the dark for 1 hour, Filter twice through 1M filter paper (Whatmann), Add immediately to hybridisation mix.

Hyb^n mix tritium: 50% deionised formamide

5x **Denhardt's solution:** 50x is: 5g Ficoll,5g polyvynylpyrrolidine,5g BSA,500ml H_2O, store at -20°C.

5x **SSPE:** 20x is: 174g NaCl, 88.2g sodium citrate, 7.4g EDTA, 800ml H_2O, pH7.4, adjust volume to 1 litre, store at 4°C,

Sonicated salmon sperm DNA (Sigma) 5µg/ml,

10% dextran sulphate,

Tritiated probe: Purified insert - 5ng/slide, Plasmid - 10ng/slide, Tandem repeat probe - 50ng/slide.

Store at -20°C.

2xSSC: As above

0.2/0.1xSSC: 2xSSC 1:9/1:19 in distiled H_2O.

PBS: Phosphate buffered saline tablets (Sigma), 1 tablet in 200ml H_2O.

Hyb^n mix <u>biotin:</u> 50% deionised formamide,
20% dextran sulphate,
2xSSC,
0.0001M EDTA pH8,
0.0002M Tris pH7.6,
Sonicated salmon sperm DNA (Sigma) 100µg/ml,
Biotinylated probe 50ng/slide.

Buffer 1: For 500ml:
50ml 1M Tris pH7.6, 10ml 5M NaCl, 0.2g $MgCl_2.6H_2O$,
250µl Triton X 100, make up to volume with water, stir 10min.

Buffer 2: For 150ml:
4.5g BSA, 150ml buffer 1, stir 10min, millipore through 0.2µm.

Buffer 3: For 300ml:
3.63g Tris, 6ml 5M NaCl, 3.04g $MgCl_2.6H_2O$, make up to volume with water, pH9.5.

Buffer 4: For 100ml:
2ml 1M Tris pH7.6, 1ml 0.5M EDTA pH8, make up to volume with water.

Substrate mix: 1ml buffer 3,
From Amersham kit: 4.4µl Nitrobluetetrazolium (NBT),
3.3µl Bromochloroindoline phosphate (BCIP),
10µl Levamisole (aqueous).

FLUORESCENT *IN-SITU* HYBRIDISATION

PBS: Phosphate buffered saline tablets (Sigma), 1 tablet in 200ml H_2O.

RNAse: As above

2xSSC: As above

ProK buffer: 20mM Tris, 2mM $CaCl_2$, pH7.4.

Deionising formamide as above.

SSCT: 0.66M NaCl, 0.066M sodium citrate (4xSSC), 0.05% Tween20 detergent (Sigma).

SSCM: 4xSSC, 5% non-fat dry milk (Marvel), filter through 1M paper (Whatmann).

TNT: 0.1M Tris.HCl pH7.5, 0.15M NaCl, 0.05% Tween20.

TNB: 0.1M Tris.HCl pH7.5, 0.15M NaCl, 0.5% blocking reagent (Boehringer), dissolve for 3 hours at 60°C, filter through 1M paper (Whatmann) twice, store at -20°C.

Anti-fade I: 9 parts:- 2% 1,4-diaza-bicyclo-(2,2,2)-octane (DABCO)(Sigma) in glycerol,
1 part:- 0.2M Tris.HCl pH7.5, 0.02% NaN_3,
Counterstains:- DAPI (4,6-diamidino phenylindole) 0.15µg/ml and/or PI (propidium iodide) 1µg.
Store at -20°C in the dark.

Anti-fade II: 100mg p-phenylenediamine dihydrochloride (Sigma),
10ml PBS,
Adjust to pH8 with 0.5M **carbonate-bicarbonate buffer:**
0.42g Na_2HCO_3,
10ml H_2O,
pH 9 with NaOH.
Millipore through 0.2µm,
Add 90ml glycerol, mix well,
Counterstains:- As for antifade I,
Store at -20°C in the dark.

PROBE PREPARATION

L-Broth: 1% tryptone, 0.5% NaCl, 0.5% yeast extract, in deionised H_2O, autoclave to sterilise.

"Cosmic" broth: For 100ml:
1.2g tryptone, 2.4g yeast extract, 0.5ml glycerol, in deionised H_2O
10ml **KPO_4 buffer:** 12.5% K_2HPO_4,
3.8% KH_2PO_4.

Antibiotics: Ampicillin 200µg/ml,
Kanamycin 25µg/ml.

Agar plates: L-broth + 1.5% bacto-agar (Difco), autoclave,
Add antibiotic when agar is just hand hot,
Pour into sterile petri dishes and put on a flat surface to set.

Solution 1: 50mM glucose, 25mM Tris pH8 , 10mM EDTA pH8.

Solution 2: 1% SDS, 2 pellets NaOH.

Solution 3: For 100ml:
60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml H_2O.

10xTE: 100mM Tris, 10mM EDTA, pH7.4.

APPENDIX 4: PROBES USED IN THIS STUDY - ACKNOWLEDGEMENTS AND REFERENCES

Table 5.1:

PROBE	TYPE OF PROBE	REFERENCE	PROVIDED BY:
pHY2.1	Satellite	Cooke *et al.*,1976	H. Cooke
PGDH4	Single copy cDNA	Klein personal comm.	P. Klein, UCL.
pUC1.77	Satellite	Cooke *et al.*, 1979	J. Wiegant, Leiden.
CY98	Satellite	Wolfe personal comm.	J. Wolfe, UCL.
pBamX7	Alphoid	Waye *et al.*,1985	ONCOR, USA.
pSE16	Alphoid	Werrick *et al.*,1989	ONCOR, USA.
p17	Alphoid	Willard *et al.*,1987	ONCOR, USA.
p18	Alphoid	Willard personal comm.	ONCOR, USA.
p13/21	Alphoid	Willard personal comm.	ONCOR, USA.
p14/22	Alphoid	Kolvraa *et al.*,1991	A. Bak, Aarhus.
λMS32	VNTR	Royle *et al.*,1988	A. Jeffreys,.Leicester.
λMS8	VNTR	Royle *et al.*,1985	A. Jeffreys, Leicester.
9a1	Phage clone	Florian *et al.*,1991	N. Hornigold, UCL.
9a6	Phage clone	Florian *et al.*,1991	N. Hornigold, UCL.
9a26	Phage clone	Florian *et al.*,1991	N. Hornigold, UCL.
9a45	Phage clone	Florian *et al.*,1991	N. Hornigold, UCL.
9a50	Phage clone	Florian *et al.*,1991	N. Hornigold, UCL.
CFF3	Cosmid	Florian personal comm	J. Wolfe, UCL.
CFF40	Cosmid	Florian personal comm	J. Wolfe, UCL.
CFF56	Cosmid	Florian personal comm	J. Wolfe, UCL.
CFF59	Cosmid	Florian personal comm	J. Wolfe, UCL.
CFF97	Cosmid	Florian personal comm	J. Wolfe, UCL.
p15-65	cDNA	Fearon *et al.*,1990	B. Voglestein, Baltimore.
Sam1.1	cDNA	Fearon *et al.*,1990	B. Voglestein, Baltimore.
Josh4.4	cDNA	Fearon *et al.*,1990	B. Voglestein, Baltimore.
λ5.3	Phage clone	Leigh *et al.*,1991	S. Leigh, UCL.
cos5.3	Cosmid	Leigh *et al.*,1991	S. Leigh, UCL.
cos 5.5	Cosmid	Leigh *et al.*,1991	S. Leigh, UCL.
L5.79	Cosmid	Kinzler *et al.*,1991	M. Dunlop, Edinburgh
ECB27	Cosmid	Griffin *et al.*,1991b	B. Cachon, UCL.

APPENDIX 5: PEDIGREE OF FAMILY WITH CHROMOSOME 5 REARRANGEMENT AND CHROMOSOMES 5 OF PROBAND WITH THE DELETION

Fig 5.3:- PEDIGREE

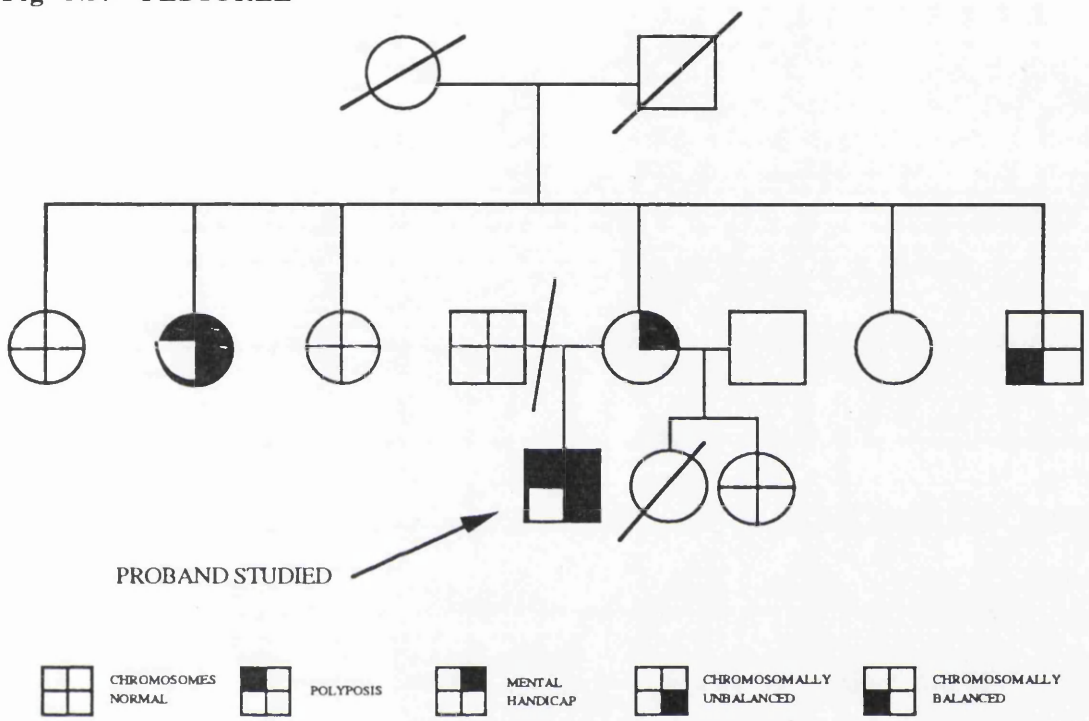
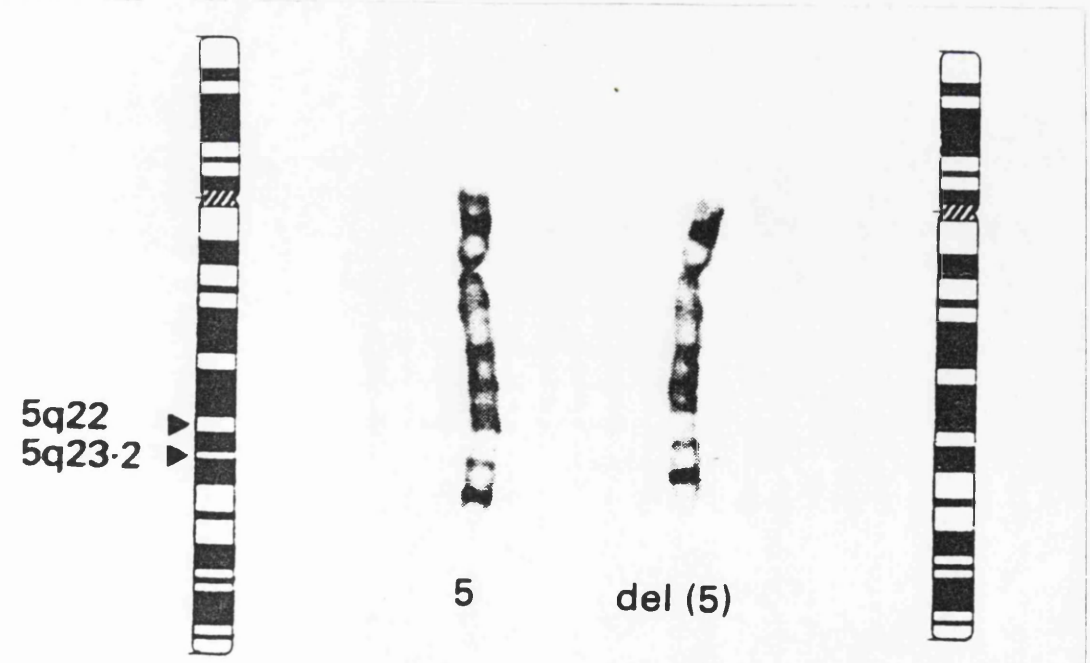


Fig 5.4:- CHROMOSOMES 5:



REFERENCES

Adinofi M & Polani PE (1989).
Prenatal diagnosis of genetic disorders in preimplantation embryos: Invasive and non-invasive approaches.
Hum. Genet. 83: 16-19.

Albertson DG, Sherrington P, Vaudin P (1991).
Mapping nonisotopically labelled DNA probes to human chromosome bands by confocal microscopy.
Genomics 10: 143-150.

Aldred MA, Rees M, Tsioupra K, Leigh SEA, Neale KF, Delhanty JDA (1988).
Familial Polyposis Coli.
Lancet I: 565.

Angell RR, Sumner AT, West JD, Thatcher SS, Glasier AF, Baird DT (1987).
Post-fertilisation polyploidy in human preimplantation embryos fertlilised *in-vitro*.
Hum. Reprod. 2,8: 721-722.

Arnoldus EPJ, Wiegant J, Noordmeer IA, Wessels JW, Beverstock GC, Grosveld GC, van der Ploeg M, Raap AK (1990).
Detection of the Philadelphia chromosome in interphase nuclei.
Cytogenet. Cell Genet. 54: 108-111.

Atkin NB (1985).
Chromosome 1 aberrations in cancer.
Cancer Genet. Cytogenet. 21: 279-285.

Ashworth A, Rastan S, Lovell-Badge R, Kay G (1991).
X-chromosome inactivation may explain difference in viability of XO humans and mice.
Nature 351: 406-408.

Bacchus C & Buselmaier W (1988).
Blastomere karyotyping and transfer of chromosomally selected embryos.
Hum. Genet. 80: 333-336.

Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Presinger AC, Jessup JM, van Tuines R, Ledbetter OH, Barker DF, Nakamura Y, White R, Voglestein B (1989).
Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas.
Science 244: 217-221.

Barlow DH (1988).
IVF, GIFT *et al.*
J. Roy. Soc. Med. 81: 622-624.

Benedict WF, Wiessmann BE, Mark C, Stanbridge EJ (1984).
Tumorgenicity of human HT 1080 fibrosarcoma and normal fibroblast hybrids: Chromosome dosage dependancy.
Cancer Res. 44: 3471-3479.

Benham F, Hart K, Crolla J, Bobrow M, Francavilla M, Goodfellow PN (1989).
A method for generating hybrids containing nonselected fragments of human chromosomes.
Genomics 4: 509-517.

Bentley-Lawrence J, Singer RH, McNeil JA (1990).
Interphase and metaphase resolution of different distances within the human dytrophin gene.
Science 249: 928-932.

Berube D & Gagne R (1990)
Exonuclease digestion of human chromosomes for *in-situ* hybridisation and R-banding.
N.A.R. 18,9: 2831.

Bodmer WF, Bailey CJ, Bodmer J, Bussey HJR, Ellis A, Gorman P, Lucibello FC, Murday VA, Rider SH, Scambler P, Sheer D, Solomon E, Spurr NK (1989).
Localisation of the gene for familial adenomatous coli on chromosome 5.
Nature 328: 614-616.

Bolton VN, Wren ME, Parsons JH (1991).
Pregnancies after *in-vitro* fertilization and transfer of human blastocysts.
Fert. Steril. 55: 830-832.

Bourne HR (1991).
Consider the coiled coil.
Nature 351: 188-190.

Bourneville DM (1880).
Sclerose tubereuse des circonvolutions cerebrales: Idiote et epilepsie hemiplegique.
Arch. Neurol. 1: 81-91.

Brambati B & Lucia T (1990).
Prenatal diagnosis before the 8th gestational week.
Proceedings of the 5th international conference on early fetal diagnosis. Prague.

Britten R & Davidson EH (1971).
Repetitive and non-repetitive sequences and a speculation on the origins of evolutionary novelty.
Quan. Rev. Biol. 46: 111-113.

Brondum-Nielsen K, Biilow S, Tommerup N (1985).
Chromosome studies in familial polyposis coli.
Cancer Genet. Cytogenet. 17,4: 355-357.

Bundey S & Evans K (1969).
Tuberous sclerosis: a genetic study.
J. Neurol. Neurosurg. Psyc. 32: 591-603.

Bussey HJR (1975).
Familial Polyposis Coli.
Johns Hopkins University Press.

Buster JE, Busillo M, Rodi IA, Cohen SW, Hamilton M, Simon JA, Thorneycroft IH, Marshall SR (1985).
Biologic and morphological development of donated human ova recovered by non-surgical uterine lavage.
Am. J. Obstet. Gynecol. 153: 211-217.

Cachon-Gonzalez MB, Delhanty JDA, Burn J, Tsioupra K, Davis MB, Attwood J, Chapman P (1991).
Linkage analysis in adenomatouis polyposis coli: the use of four closely linked DNA probes in 20 U.K. families.
J. Med. Genet. 28: 681-685.

Cancer Research Campaign (1990).
Facts on cancer.
Fact sheets 1990.

Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Goodbout R, Gaille BL, Murphee AL, Strong LC, White RL (1983).
Expression of recessive alleles by chromosomal machanisms in retinoblastoma.
Nature 305: 779-784.

Chiganti RSK, Balazs I, Jhanwar SC, Murty VS, Kodwa PRK, Greschik KH, Stainezer E (1986).
The cellular homologue of the transforming gene of SKV avian retrovirus maps to human chromosome 1q22-q24.
Cytogenet. Cell Genet. 43: 181-186.

Cherif D, Bernard O, Berger R (1989).
Detection of single copy genes by non-isotopic *in-situ* hybridisation on human chromosomes.
Hum. Genet. 81: 358-362.

Cherif D, Julier C, Delattre O, Derre J, Lathrop GM, Berger R (1990).
Simulataneous localisation of cosmids and chromosome R-banding by fluorescence microscopy. Application to regional mapping of human chromosome 11.
P.N.A.S. USA 87: 6639-6643.

Choo KH, Filby G, Earle E, Brown R (1988).
Isolation of human chromosome 21 sequences and their application to *in-situ* hybridisation.
Hum. Genet. 81: 49-53.

Clark RD, Smith M, Pandolfo M, Fausel RE, Bustillo AM (1988).
Tuberous sclerosis in a liveborn infant wit trisomy due to t(11q23; 22q11.2) translocation: is neural cell adhesion molecule a candidate gene for tuberous sclerosis?
Am. J. Hum. Genet. 43 (suppl 3) 44A.

Connor JM, Pirrit LA, Yates JRW, Fryer AE, Fergusson-Smith MA (1987).
Linkage of the Tuberous Sclerosis locus to a DNA polymorhism detected by *v-abl.*
J. Med. Genet. 24: 544-546.

Cooke HJ & Hindley (1979).
Cloning of human satellite III DNA: Different components are on different chromosomes.
N.A.R. 6: 3177.

Cooke HJ, Schmitke J, Gosden JR (1982).
Characterisation of a human Y chromosome repeated sequence and related sequence in higher primates.
Chromosoma 87: 491-502.

Cotton RGH, Rodrigues N, Campbell RD (1988).
Reactivity of cytosine and thymine in single base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations.
P.N.A.S. USA 85: 4397-4401.

Coutelle C, Williams C, Handyside A, Hardy K, Winston R, Williamson R (1989).
Genetic analysis of DNA from single human oocytes: a model for preimplantation diagnosis of cystic fibrosis.
B.M.J. 299: 22-24.

Cox DR, Burmeister M, Price ER, Kim S, Myers RM (1990).
Radiation hybrid mapping: A Somatic cell genetic method for constructing high resolution maps of mammalian chromosomes.
Science 250: 245-250.

Cremer T, Landegent J, Bruckner A, Scholl HP, Shardin M, Hager HD, Devilee P, Pearson P, van der Ploeg M (1986).
Detection of chromosome aberrations in the human interphase nucleus by visualisatin of specific target DNAs with radioactive and non-radioactive *in-situ* hybridisation techniques.
Hum. Genet. 74: 346-352.

Cremer T, Lichter P, Borden J, Ward DC, Mannuelidis L (1988a).
Detection of chromosome aberrations in metaphase and interphase tumour cells by *in-situ* hybridisation using chromosome specific library probes.
Hum. Genet. 80: 235-246.

Cremer T, Tesin D, Hopman AHN, Mannuelidis L (1988b).
Rapid interphase and metaphase assesement of specific chromosomal changes in neuroectodermal tumour cells by *in-situ* hybridisation with chemically modified DNA probes.
Exp. Cell Res. 176: 199-220.

Cross I, Delhanty J, Chapman P, Bowles L, Griffin D, Wolstenholme J, Bradburn M, Brown J, Gunn A, Burn J (1992).
An intrachromosomal insertion causing 5q22 deletion and familial adenomatous polyposis coli in two generations.
J. Med. Genet. 29: 175-179.

Dawson KJ, Rutherford AJ, Winston NJ, Subak-Sharpe R, Winston RML (1988).
Human blastocyst transfer, is it a feasible proposition?
Hum. Reprod. supplement 145: 44-45.

Delattre P, Olochwang S, Law DJ, Melot T, Remvikos Y, Salmon RJ, Sastre X, Validire R, Feinberg AP, Thomas G (1989).
Multiple genetic alterations distinguish distal from proximal colorectal cancer.
Lancet II: 353-356.

Delhanty JDA, Davis MB, Wood J (1983).
Chromosome instability in lymphocytes, fibroblasts and colon eepithelial-like cells from patients with familial polyposis coli.
Cancer Genet. Cytogenet. 8: 27-30.

Devilee P (1988).
Towards a molecular understanding of breast cancer.
Chapter 3: The organisation and localisation of human repetitive DNA pp29-33.
University of Leiden publications.

Devilee P, Kievits T, Waye JS, Pearson PL, Willard HF (1988a).
Chromosome specific alpha satellite DNA: Isolation and mapping of a polymorphic alphoid repeat from human chromosome 10.
Genomics 3: 1-7.

Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AHN, Willard HF, Pearson PL, Cornelisse CJ (1988b).
Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumours using chromosome specific repetitive DNA probes.
Cancer Res. 48: 5825-5930.

Duhamel RC & Johnson DA (1985).
Use of non-fat dry milk to block non-specific nuclear and membrane staining by avidin conjugates.
J. Histochem. Cytochem. 33: 711-714.

Dunlop MG, Steel CM, Wylllie AH, Bird CC, Evans HJ (1989).
Linkage analysis in familial adenomatous polyposis. Order of C11P11 (D5S71) and Pi 227 (D5S37) loci at the apc gene.
Genomics 5: 350-353.

Dyer KA, Confield TK, Gartler SM (1989).
Molecular cytological evidence of active from inactive X-domains in interphase: implications for X-chromosome inactivation.
Cytogenet. Cell Genet. 50: 116-120.

Fan Y-S, Davis LM, Shows TB (1990).
Mapping small DNA sequences by fluorescent *in-situ* hybridisation directly on banded metaphase chromosomes.
P.N.A.S. USA 87: 6223-6227.

Fanning J & Singer M (1987).
The LINE 1 DNA sequence in four mammalian orders predict proteins that conserve homologies to retrovirus proteins.
N.A.R. 15: 2252-2260.

Fashold R, Rott H-D, Lorenz P, Claussen U (1991).
A new gene locus for tuberous sclerosis maps to chromosome 12.
Cytogenet. Cell Genet. 58: 1976.

Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM, Hamilton, SR, Preissenger AC, Thomas G, Kinzler KW, Voglestein B (1990).
Identification of a chromosome 18q gene that is altered in colorectal cancers.
Science 247: 49-56.

Fearon ER & Voglestein B (1990).
A genetic model for colorectal tumorigenesis.
Cell **61**: 759-767.

Finlay CA, Hinds PW, Levine AJ (1989).
The p53 proto-oncogene can act as a suppressor of transformation.
Cell **57**: 1083-1093.

Fitzgibbon J, Nahmias J, Griffin D, Hornigold N, Delhanty J, Povey S, Wolfe J (1991).
A deletion map of distal 9q based on a panel of irradiation hybrids.
Cytogenet. Cell Genet. 83: 1937.

Florian F, Hornigold N, Griffin DK, Delhanty JDA, Sefton L, Abbot C, Jones C, Goodfellow PN, Wolfe J (1991).
The use of irradiation fusion gene transfer (IFGT) hybrids to isolate DNA clones from human chromosome region 9q33-q34.
Som. Cell Mol. Genet. 17,5: 445-453.

Friedl W, Kromer A, Propping P (1991).
Probe KK5.33 (D5S85) improves indirect genotype analysis in familial adenomatous polyposis.
Hum. Genet. 86: 422-423.

Fryer AE, Connor JM, Povey S (1987).
Evidence that the gene for tuberous sclerosis is on chromosome 9.
Lancet I: 659-661.

Fryer AE & Osborne JP (1987).
Tuberous sclerosis - a clinical appraisal.
Ped. Rev. Commun. 1: 239-255.

Fuscoe JC, Collins CC, Pinkel D, Gray JW (1989).
An efficient method for selecting unique sequence clones from DNA libraries and its application to fluorescent staining of human chromosome 21 using *in-situ* hybridisation.
Genomics 5: 100-109.

Gall JG & Pardue ML (1969).
Formation of RNA-DNA hybrid molecules in cytological preparations.
P.N.A.S. USA 63: 378-383.

Gardner RL & Edwards RG (1968).
Control of the sex ratio at full term in the rabbit by transferring sexed blastocysts.
Nature 218: 346-348.

Gardner D & Leese HJ (1986).
Non-invasive measurement of nutrient uptake by single culture preimplantation mouse embryos.
Hum. Reprod. 1: 25-27.

Garson JA, van den Berghe JA, Kemshead JT (1987).
Novel isotopic *in-situ* hybridisation technique detects small (1kb) unique sequences in routinely G-banded human chromosomes: fine mapping of N-myc and βNGF genes.
N.A.R. 15,12: 4761-4770.

Gartler SM & Riggs AD (1983).
Mammalian X-chromosome inactivation.
Ann. Rev. Genet. 17: 155-190.

Goodfellow PJ, Povey S, Nevalinna HA, Goodfellow PN (1990).
Generation of a panel of somatic cell hybrids containing unselected fragments of human chromosome 10 by X-ray irradiation and cell fusion. Application to isolating the MEN2A region in hybrid cells.
Som. Cell Mol. Genet. 16,2: 163-171.

Gosden JR, Mitchell AR, Buckland RA, Clayton RP, Evans HJ (1975).
The localisation of four satellite DNAs on human chromosomes.
Exp. Cell Res. 92: 148-158.

Gosden JR, Gosden CM, Christie S, Cooke HJ, Morsmann JM, Rodeck CH (1984).
The use of sloned Y-chromosome-specific DNA probes for fetal sex determination in first trimester prenatal diagnosis.
Hum. Genet. 66: 347-351.

Gosden J, Hanratty D, Starling J, Fantes J, Mitchell A, Porteous D (1991).
Oligonucleotide-primed in situ DNA synthesis (PRINS): a method for chromosome mapping, banding and investigation of sequence organisation.
Cytogenet. Cell Genet. 57: 100-104.

Goss SJ & Harris H (1975).
New method for mapping genes in human chromosomes.
Nature 255: 680-684.

Griffin DK, Leigh SEA, Delhanty JDA (1990).
Use of fluorescent *in-situ* hybridisation to confirm trisomy of chromosome region 1q32-qter as the sole karyotypic defect in a colon cancer cell line.
Genes, chromosomes and cancer 1: 281-283.

Griffin DK, Handyside AH, Penketh RJA, Winston RML, Delhanty JDA (1991a).
Fluorescent *in-situ* hybridisation to interphase nuclei of human preimplantation embryos with X and Y chromosome specific probes.
Hum. Reprod. 6,1: 101-105.

Griffin DK, Bowles L, Tsioupra K, Barber J, Delhanty JDA (1991b).
Characterisation of adjascent deletions on chromosome 5q.
Cytogenet Cell Genet. 83: 1895

Griffin DK, Wilton LJ, Handyside AH, Winston RML, Delhanty JDA (1992).
Dual fluorescent *in-situ* hybridisation for the simultaneous detection of X and Y chromosome specific probes for the sexing of human preimplantation embryonic nuclei.
Hum. Genet. 89: 18-22.

Grifo JA, Boyle A, Fischer E, Lavy G, DeCherney AH, Ward DC, Sanyal MK (1990)
Preembryo biopsy and analysis of blastomeres by *in-situ* hybridisation.
Am. J. Obs. Gyn. 6,1: 2013-2019.

Groden J, Thliveris A, Samovitz W and 20 others (1991).
Identification and characterisation of the familial adenomatous polyposis gene.
Cell 66: 589-600.

Guyot B, Bezin A, Sole Y, Julien C, Daffos F, Forrestier R (1988).
Prenatal diagnosis with biotinylated chromosome specific probes.
Prenatal Diagnosis 8: 485-493.

Habeebu SSM, Spathas DH, Fergusson-Smith MA.
Non-radioactive *in-situ* hybridisation of DNA probes to chromosomes and nuclei: A comparison of techniques.
Mol. Biol. Med. 7: 423-435.

Handyside AH, Penketh RJA, Winston RML, Pattison JK, Delhanty JDA, Tuddenham EGD (1989).
Biopsy of human preimplantation embryos and sexing by DNA amplification.
Lancet II: 347-349.

Handyside AH, Kontogianni EH, Hardy K, Winston RML (1990).
Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification.
Nature 344: 768-770.

Handyside AH (1991).
Biopsy of human cleavage stage embryos and sexing by DNA amplification.
In: Proceedings of the first symposium on preimplantation genetics.
Ed: Verlinsky and Strom pp 75-83.
Plenum, New York.

Handyside AH & Delhanty JDA (1992).
Cleavage stage biopsy of human embryos and diagnosis of X-linked recessive disease.
In: Preimplantation diagnosis of human genetic disease (ed: Edwards RG).
Cambridge University press.

Hardy K, Hooper MAK, Handyside AH, Rutherford AJ, Winston RML, Leese HJ (1989).
Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos.
Hum. Reprod. 4: 188-191.

Hardy K, Martin KL, Leese HJ, Winston RML, Handyside AH (1990).
Human preimplantation development *in-vitro* is not adversely affected by biopsy at the 8-cell stage.
Hum. Reprod. 5,6: 708-714.

Hardy K, Winston RML, Handyside AH (1991).
Binucleate cells in normally fertlised human preimplantation embryos *in-vitro*: Failure of cytokinesis during early cleavage.
Development in press.

Harris RM, Carter NP, Leversha M, Goudie DR, Sargent CA, Hampson M, Warner JP, Yates JR, Affara NA, Fergusson-Smith MA (1991).
Physical mapping with DNA probes in the region 9q32-34 which show linkage to the tuberous sclerosis locus.
Cytogenet. Cell Genet. 58: 1940.

Hegnauer R (1971).
Planzenstoffe und Pftanzensytematik.
Naturewissenschaften 58: 585-598.

Heiles HBJ, Generch E, Kessler C, Neumann R, Eggers HJ (1988).
In-situ hybridisation with digoxigenin labelled DNA of human papilloma viruses (HPV/18) in HeLa and SiHa cells.
Biotechniques 6: 978-981.

Hermanek P, Karrer K, Sobin LH (1984).
Atlas of colorectal tumours.
Butterworths. pp 21, 32, 62.

Herrera L, Kakati S, Gibas L, Pietrzak E, Sandberg A (1986).
Brief clinical report: Gardner syndrome in a man with an interstitial deletion of 5q.
Am. J. Med. Genet. 25: 473-476.

Herrington CS, Burns J, Graham AK, Evans M, McGee JOD (1989a).
Interphase cytogenetics using biotin and digoxigenenin labelled probes I: Relative sensitivity of both reporter molecules for detection of HPV16 in CaSki cells.
J. Clin. Pathol. 42: 592-600.

Herrington CS, Burns J, Graham AK, Evans M, McGee JOD (1989b).
Interphase cytogenetics using biotin and digoxigenenin labelled probes II: Simulataneous differential detection of human and papilloma virus nucleic acids in individual nuclei.
J. Clin. Pathol. 42: 601-606.

Hockey KA, Mulahy MT, Montgomery P, Levit S (1989).
Deletion of chromosome 5q and familial adenomatous polyposis.
J. Med. Genet. 26: 61-62.

Holding C & Monk M (1989).
Diagnosis of Beta-thallasemia by DNA amplification in single blastomeres from mouse preimplantation embryos.
Lancet II: 532-535.

Holmes DS, Fifer AM, MacKenzie WE, Griffiths MJ, Newton JR (1988).
Direct and short-term culture preparation of chorionic villi, is any one method best?
Prenatal Diagnosis: 8: 501-509.

Hopman AHN, Wiegant J, Raap AK (1986).
Bi-color detection of two target DNAs by non-radioactive *in-situ* hybridisation.
Histochemistry 85: 1-4.

Hopman AHN, Wiegant J, Van Duijn P (1987).
Mercurated nucleic acid probes, a new principle for non-radioactive *in-situ* hybridisation.
Exp. Cell Res. 169: 357-368.

Hopman AHN, Ramaekers FCS, Raap AK, Beck JLM, Devilee P, van der Ploeg M, Voojs GP (1988).
In-situ hybridisation as a tool to study numerical chromosome aberrations in solid bladder tumours.
Histochemistry 89: 307-316.

Jannsen B, Sampson J, Sandkuyl L, Fleury P, Lindhout D, Connor M, Smith M, Haines J, Amos J, Kwiatkowski D, Short P & 10 others (1991).
Mapping studies in tuberous sclerosis: a collaborative effort.
Cytogenet Cell Genet. 83: 1940.

Jauch A, Daumer C, Lichter P, Muken J, Schroeder-Kurth T, Cremer T (1990).
Chromosomal *in-situ* suppression hybridisation of human gonosomes and autosomes and ots use in clinical cytognetics.
Hum. Genet. 85: 145-150.

Jeffreys AJ, Wilson V, Thein SL (1985).
Hypervariable "minisatellite" regions in human DNA.
Nature 314: 67-73.

Jenkyn DJ, Whitehead RH, House AK, Maley MAL (1987).
Single chromosome defect, partial trisomy 1q in a colon cancer cell line.
Cancer Genet. Cytogenet. 27: 357-360.

Johnson GD & de Nogueira-Araujo-Gloria M (1981).
A simple method of reducing the fading of immunofluorescence during microscopy.
J. Immun. Met. 43: 349-350.

Johnson GD, Davidson RS, McNamee KC, Russel G, Goodwin D, Holborrow EL (1982).
Fading of immunofluorescence during microscopy: a study of its phenomenon and its remedy.
J. Immun. Met. 55: 231-242.

Jones C & Kao FT (1984).
Physical mapping of the folylpolyglucamate synthetase gene (FPGS) to 9cen-q34.
Cytogenet. Cell Genet. 37: 499-500.

Jones KW, Singh L, Edwards RG (1987).
The use of probes for the Y chromosome in preimplantation embryo cells.
Hum. Reprod. 2: 439-445.

Joslyn G, Carlson M, Thliveris A and 20 others (1991).
Identification of deletion mutations and three new genes at the familial polyposis locus.
Cell 66: 601-613.

Julien C, Bazin A, Guyot B, Forrestier F, Daffos F (1986).
Rapid prenatal diagnosis of Down's Syndrome with *in-situ* hybridisation of fluorescent DNA probes.
Lancet II: 863-864.

Kandt RS, Pericak-Vance MA, Hung W-Y, Gardner RJM, Nellist M, Phillips K, Warner K (1989).
Absence of linkage of ABO blood group locus to familial tuberous sclerosis.
Exp. Neurol. 104: 223-228.

Kasukawa T, Watanabe T, Ends A (1985).
Cytogenetic and cytokinetic analysis of lymphocytes from patients with hereditary adenomatosis of the colon and rectum.
Cancer Genet. Cytogenet. 16: 73-79.

Khalfan H, Abuknesha R, Randweaver M, Prid RG, Robinson D (1986).
Amino-methyl coumarin acetic acid: A new fluorescent labelling agent for proteins.
Histochem J. 18: 497-499.

Kievits T, Devilee P, Wiegant J, Wapenaar MC, Cornelisse CJ, van Ommen GJB, Pearson PL (1990a).
Direct non-radioactive *in-situ* hybridisation of somatic cell hybrid DNA to human lymphocyte chromosomes.
Cytometry 11: 105-109.

Kievits T, Daueverse JG, Wiegant J, Devilee P, Breuning MH, Cornelisse CJ, van Ommen GJB, Pearson PL (1990b).
Rapid subchromosomal location of cosmids by non-radioactive *in-situ* hyridisation.
Cytogenet. Cell Genet. 53: 134-136.

Kinzler KW, Nilbert MC, Voglestein B, Bryan TM, Levy DB, Smith KJ, Presinger AC, Hamilton SR and 10 others (1991a).
Identification of a gene located at chromosome 5q21 that is mutated in colon cancer.
Science 251: 1366-1370.

Kinzler KW, Nilbert MC, Su L-K, Voglesteinb B, Bryant TM, Levy DB, Smith J, Preisinger AL, Hedge P, McKenzie D, Finnear R & 10 others (1991b).
Identification of FAP locus genes from chromosome 5q21.
Science 253: 661-665.

Klinger KW, Landen G, Dackowski W, Leverone B, Lopez L, Locke P, Ward D, Ried T (1991).
Mapping by five colour fluorescence *in-situ* hybridisation.
Cytogenet. Cell Genet. 83: 2194.

Knudson AG (1971).
Mutation and cancer: A statistical study of retinoblastoma.
P.N.A.S. USA 68: 820-823.

Kola I & Wilton LJ (1991).
Preimplantation embryo biopsy: detection of trisomy in a single cell biopsied from a four cell mouse embryo.
Mol. Reprod. Devt. 29: 16-21

Kolvraa S, Koch J, Gregersen N, Jensen PKA, Jorgensen AL, Petersen KB, Ramunsen K, Bolund L (1991).
Application of fluorescence *in-situ* hybridisation techniques in clinical genetics: use of two alphoid repeat probes detecting the centromeres of chromosomes 13 and 21 or chromosomes 14 and 22 respectively.
Clin. Genet. 39: 278-286.

Koregn JR & Rykowski MC (1988).
Human genome organization: Alu, LINES and the molecular structure of metaphase chromosome bands.
Cell 53: 391-400.

Kozma R & Adinolfi M (1988).
In-situ fluorescence hybridisation of Y translocations: cytogenetic analysis using probes Y190-Y431.
Clin. Genet. 33: 156-161.

Kramer PL, de Leon D, Ozelius BS & 10 others (1990).
Dystonia gene in Askenazi Jewish Population is located on chromosome 9q32-34.
Annals Neurol. 27,2: 114-120.

Landegent JE, Jansen D, Wal N, Baan RA, Hoeijmakers JHJ, van der Ploeg M (1984).
2-acetylaminofluorene modified probes for the indirect hybridocytochemical detection of specific nucleic acid sequences.
Exp. Cell Res. 153: 61-72.

Leigh SEA, Gayther SA, Lawson CM, Griffin DK, Delhanty JDA (1991).
Two cosmid clones containing expressed sequences mapping to 5q31 & 5q35.
Cytogenet. Cell Genet. 83:

Leister I, Weith A, Bruderlei S, Cziepluck C, Kangwangpong D, Sclag P, Schwab M (1990).
Human colorectal cancer: High frequency of deletion at chromosome 1p35.
Cancer Res. 50: 7232-7235.

Leppert M, Dobbs M, Scambler P, O' Connel P, Nakamura Y, Stauffler D, Woodward S, Burt R, Hughes J, Gardner E, Lathrop M, Wasmuth J, lalouel JM, White R (1987).
The gene for familial polyposis coli maps to the long arm of chromosome 5.
Science 238: 1411-1413.

Levin B & Reichmann A (1986).
Chromosomes and large bowel tumours.
Cancer Genet. Cytogenet. 19: 159-162.

Lichter P, Cremer T, Chang-Tang C-J, Watkins PC, Mannuelidis L, Ward DC (1988a).
Rapid detection of human chromosome 21 aberrations by *in-situ* hybridisation.
P.N.A.S. USA 85: 9664-9668.

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

Lichter P & Ward DC.(1990).
Is non-isotopic *in-situ* hybridisation finally coming of age?
Nature 345: 93-95

Lichter P, Jauch A, Cremer T, Ward DC (1990a).
Detection of Down Syndrome by *in-situ* hybridisation with chromosome specific DNA probes.
In: Molecular genetics of chromosome 21 and Down Syndrome (ed: Patterson D & Epstein CJ).
Wiley-Liss Inc. pp 69-78.

Lichter P, Chang Tang C-J, Call K, Hermanson G, Evans GA, Housman D, Ward DC (1990b).
High resolution mapping of human chromosome 11 by *in-situ* hybridisation with cosmid clones.
Science 247: 64-69.

Lilford RJ (1991).
The rise and fall of chorionic villus sampling.
B.M.J. 303: 936-937.

Lyon MF (1961).
Gene action in the X chromosome of the mouse (*Mus-musculus L*).
Nature 190: 372-373.

Malcolm S, Cowell JK, Young BD (1986).
Specialist techniques in research and diagnostic clinical cytogenetics (Ch 7).
In: Human cytogenetic - a practical approach (ed: Rooney D & Czepulkowski)
IRL Press Oxford.

Mannuelidis L (1985).
Individual interphase chromosome domains revealed by in situ hybridisation.
Hum. Genet. 71: 288-293.

Mannuelidis L, Langer-Safer PR, Ward DC (1982).
High resolution mapping of satellite DNA using biotin labelled DNA probes.
J. Cell Biol. 95: 619-625.

Marshall CJ (1991).
Tumour suppressor genes.
Cell 64: 313-326.

McLaren A (1989).
IVF: Regulation or prohibition?
Nature 342: 469-470.

McLaren A (1990).
Research on the human conceptus and its regulation in Britain today.
J. Roy. Soc. Med. 83: 209-213.

Meera-Khan P, Tops CMJ, van der Brock M, Breukel C, Wijnen JT, Oldenburg M & 10 others (1988).
Close linkage of a highly polymorphic marker (D5S37) to familial adenomatous polyposis (FAP) and confirmation of FAP localisation on chromosome 5q21-q22.
Hum. Genet. 79: 183-185.

Meltzer PS, Guan X-Y, Burgess A, Trent JF (1992).
Rapid generation of region specific probes by chromosome microdissection and their application.
Nature Genetics 1: 24-28.

Miller DA, Okamoto E, Erlanger BF, Miller OJ (1982).
Is DNA methylation responsible for mammalian X-chromosome inactivation?
Cytogenet. Cell Genet. 33: 345-349.

Mitchell CJ, Griffin DK, Carritt B (1991).
Assignment of the beta enolase locus ENO3, to 17p13 by fluorescent *in-situ* hybridisation.
Cytogenet. Cell Genet. 83: 2008.

Monk M, Handyside A, Hardy K Whittingham D (1987).
Preimplantation diagnosis of deficiency of hypoxanthine phosphoribisyl transferase in a mouse model for Lesch-Nyhan syndrome.
Lancet II: 423-424.

Moore KL (1988).
The developing human (clinically orientated embryology) fourth edition.
W.B. Saunders company.

Moyzis RK, Torney DC, Meyna J & 10 others(1989).
The distribution of interspersed repetitive DNA sequences.
Genomics 4: 273-289.

MRC working party on the evaluation of chorionic villus sampling (1991).
Medical research council European trial of chorionic villus sampling.
Lancet 337: 1491-1499.

Muleris M, Delatrre O, Olchwang S, Dutrillaux AM, Remvikos Y, Salmon R-J, Thomas G, Dutrillaux B (1990a).
Cytogenetic and molecular appraoches of polyploidization in colorectal adenocarcinomas.
Cancer Genet. Cytogenet. 44: 107-118.

Muleris M, Salmon R-J, Dutrillaux B (1990b).
Cytogenetics of colorectal adenocarcinomas.
Cancer Genet. Cytogenet. 46: 143-146.

Murday V, Cottrel S, Bodmer WF, Scheer D, Jones TA, Varesco L, Frischauf A-M, Thomas HJ, Solomon E, Hockey A, Dunlop MG, Steele CM (1989).
Fine linkage map around the adenomatous polyposis (APC) gene.
Cytogenet. Cell Genet. 5: 1049.

Muto T, Bussey HJR, Morson BC (1977).
The evolution of cancer of the colon and rectum.
Cancer 36: 2251.

Nahmias J, Fitzgibbon J, Hornigold N, Florian F, Griffin D, Delhanty J, Benham F, Povey S, Wolfe J (1991).
Characterisation of a panel of irradiation hybrids from chromosome arm 9q.
Cytogenet. Cell Genet. 83: 1942.

Nakamura Y, Lathrop M, Leppert M, Dobbs M, Wasmuth J, Wolff E & 10 others (1987).
Localisation of the genetic defect in familial adenomatous polyposis within a small region of chromosome 5.
Am. J. Hum. Genet. 43: 638-644.

Nederlof PM, Robinson D, Abuknesha R, Wiegant J, Hopman AHN, Tanke HJ, Raap AK (1989a).
Three colour fluorescence *in-situ* hybridisation for the simulataneous detection of multiple nucleic acid sequences.
Cytometry 10: 20-27.

Nederlof PM, van der Flier S, Raap AK, Tanke HJ, van der Ploeg M, Kernips F, Geradts JPM (1989b).
Detection of chromosome aberrations in interphase tumour nuclei by non-radioactive *in-situ* hybridisation.
Cancer Genet. Cytogenet. 47: 87-98.

Nishisho I, Nakamura Y, Myosho Y & 20 others (1991).
Mutation of chromosome 5q21 genes in FAP and colorectal cancer patients.
Science 253: 665-669.

Northrup H, Beaudet AL, O'Brien WE, Herman GE, Lewis RA, Poolack MS (1987).
Linkage of tuberous sclerosis to ABO blood group.
Lancet I: 804-805.

Okamoto M, Sato C, Kohno Y, Mori T, Iwama T, Tonomura A, Miki Y, Utsunomija J, White R, Miyaki M (1990).
Molecular nature of chromosome 5q loss in colorectal tumours and desmoids from patients with familial adenomatous polyposis.
Hum. Genet. 85: 595-599.

Osborne JP & Fryer AE (1990).
Tuberous sclerosis..... More than just skin deep.
Tuberous sclerosis association of Great Britain.

Paraskeva C (1990).
Cellular senescence, human chromosome 1 and carcinogenesis.
Int. J. Cancer 46: 753-754.

Paraskeva C, Finerty S, Powell S (1988).
Immortalisation of a human colorectal adenoma cell line by continuous *in-vitro* passage: Possible involvement of chromosome 1 in tumour progression.
Int. J. Cancer 41: 908-912 (1988).

Paraskeva C, Harvey A, Finerty S, Powell S (1989).
Possible involvement of chromsome 1 in *in-vitro* immortalisation: Evidence from progression of a human adenoma-derived cell line *in-vitro*.
Int. J. Cancer 43: 747-748.

Penketh RJA & McLaren A (1987).
Prospects for prenatal diagnosis during preimplantation human development.
Ballieres Clin. Obs. Gyn. 1,3: 747-764.

Penketh RJA, Delhanty JDA, van den Bergh JA, Finklestone EM, Handyside AH, Malcolm S, Winston RML (1989).
Rapid sexing of human embryos by non-radioactive *in-situ* hybridisation, potential for preimplantation diagnosis of X-linked diorders.
Prenat. Diag. 9: 489-500.

Pieters MHEC, Geraedts JPM, Meyer H, Dumoulin JCM, Evers JLH, Jongbloed RJE, Nderlof PM, van der Flier S. (1990).
Human gametes and zygotes studied by non-radioactive *in-situ* hybridisation.
Cytogenet. Cell Genet. 53: 15-19.

Pinkel D, Straume T, Gray J (1986).
Cytogenetic analysis using quantitative high sensitivity fluorescence hybridisation.
P.N.A.S. USA 83: 2934-2938.

Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J (1988).
Fluorescence *in-situ* hybridisation with human chromosome specific libraries: Detection of trisomy 21 and translocations of chromosome 4.
P.N.A.S. USA 85: 9138-9142.

Plachot M, Junca A-M, Mandelbaun J, de Grouchy J, Salat-Baroux J, Cohen J (1987).
Chromosome investigations in early life II. Human preimplantation embryos.
Hum. Reprod. 2,1: 29-35.

Povey S & Parrington JM (1986).
Chromosome 1 in relation to human disease.
J. Med. Genet. 23: 107-115.

Povey S, Jannsen B, Attwood J, Burley M, Smith M, Flodman P, Morton NE (1991).
An attempt to map two genes for tuberous sclerosis using novel two-point methods.
Annals NY Acad. Sci. 615: 298-305.

Raap AK, Dirks RW, Jiwa NM, Nederlof PM, vander Ploeg M (1990).
In-situ hybridisation with hapten modified DNA probes.
In: Modern pathology of AIDS and other retroviral infections. (ed: Raez P, Haase AT, Glucman JC).
Basel Karger. pp17-28.

Reed TE & Neel JV (1955).
A genetic study of multiple polyposis of the colon (with an appendix deriving a method of estimating relative fitness).
Am. J. Hum. Genet. 7: 236-263.

Rees M, Leigh SEA, Delhanty JDA, Jass JR (1989).
Chromosome 5 allele loss in familial and sporadic colorectal adenomas.
Br. J. Cancer 59: 361-365.

Reichmann A, Riddell RH, Martin P, Levin B (1980)
Double minutes in human large bowel cancer.
Gastroenterology 79: 334-339.

Reichmann A & Levin B (1981).
Premature chromosome condensation in human large bowel cancer.
Cancer Genet. Cytogenet. 3: 221-225.

Reichmann A, Martin P, Levin B (1981).
Chromosomal banding patterns in human large bowel cancer.
Br. J. Cancer 28: 431-440.

Reichmann A, Levin B, Martin P (1982a).
Human large bowel cancers: Correlation of clinical and histopathological feaures with banded chromosomes.
Int. J. Cancer 29: 625-629.

Reichmann M, Martin P, Levin B (1982b).
Karyotypic findings in a colonic villous adenoma.
Cancer Genet. Cytogenet 7: 51-57.

Reithman HC, Moyzis RK, Meyne J, Burke DT, Olson MV (1989).
Cloning human telomeric DNA fragments into *Sacchoromyces cerevisiae* using a yeast artificial chromosome vector.
P.N.A.S. USA 84: 6240-6244.

Rowley JD, Diaz MO, Espinosa R & 10 others (1990).
Mapping chromosome 11q23 in human acute luekemia with biotinylated probes: Identification of 11q23 translocation breakpoints with a yeast artificial chromosome.
P.N.A.S. USA 87: 9358-9361.

Royle NJ, Clarkson RE, Wong Z, Jeffreys AJ (1988).
Clustering of hypervariable minisatellites in the proterminal regions of human autosomes.
Genomics 3: 352-360.

Rudkin GT & Stollar BD (1977).
High resolution detection of DNA-RNA hybrids *in-situ* by direct immunofluorecence.
Nature 265: 472-473.

Saiki RK, Scharf S, Falsona FA & 10 others (1985).
Enzymatic amplification of β globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.
Science 230: 1350-1354.

Sampson JR, Yates JRW, Pirrit LA, Fleury P, Winship I, Beighton P, Connor JM (1989).
Evidence for genetic heterogenity in tuberous sclerosis.
J. Med. Genet. 26: 511-516.

Sasaki M, Okamoto M, Sato C, Sugis K, Soejirna J, Iwama T, Ikeuchi T, Tonomura A, Miyaki M, Sasazuki T (1989).
Loss of constitutional heterozygosity in colorectal tumours from patints with familial non-polyposis colorectal carcinoma.
Cancer Res. 49: 4402-4406.

Schleuterman DA, Bias WB, Murdoch JL, McKusick VA (1969).
Linkage of the loci for the Nail-Patella Syndrome and adenylate kinase.
Am. J. Hum. Genet. 21: 606-630.

Scmidt CW & Jelinek WR (1982).
The Alu family of dispersed repetitive sequences.
Science 216: 1065-1070.

Singer MF (1982).
SINEs and LINEs: Highly repeated and long interspersed sequences in mammalian genomes.
Cell 28: 433-434.

Smit VTHBM, Wessels JW, Mollervanger P, Schrier PI, Raap AK, Beverstock GC, Corneliss CJ (1991).
Combined GTG-banding and non-radioactive *in-situ* hybridisation improves characterisation of complex karyotypes.
Cytogenet. Cell Genet. 54: 20-23.

Smith M, Haines J, Trofatter J, Dumars K, Pandolfo M, Conneally M (1987).
Linkage studies in tuberous sclerosis.
Am. J. Hum. Genet. 41: A186.

Smith J, Rebello M, Gray C, Rooney D, Leffler F, Coleman D (1990a).
Amniocentesis at 11-14 weeks gestation.
Proceedings of the 5th international symposium on early fetal diagnosis. Prague 1991.

Smith M, Smalley S, Cantor R, Pandolfo M, Gomez MI, Baumann R & 10 others (1990b).
Mapping of a gene determining tuberous sclerosis to human chromosome 11q14-23.
Genomics 6: 105-114.

Solomon E, Voss R, Hall V, Bodmer WF, Jass JR, Jeffreys AJ, Lucibello FC, Patel I, Rider SH (1987).
Chromosome 5 allele loss in human colorectal carcinomas.
Nature 328: 616-619.

Steptoe PC & Edwards RG (1978).
Birth after implantation of a human embryo.
Lancet II: 366.

Sugarbaker JP, Gunderson LL, Wittes RE (1985).
Colorectal cancer.
In: Cancer: principles and practices on oncology (ed: DeVita VT, Hellman S, Rosenberg SA)
Philadelphia J.B. Lippincott. pp 800-803.

Tchen P, Fuchs RPP, Sage E, Leng M (1984).
Chemical modified nucleic acids as immunodetectable probes in hybridisation experiments.
P.N.A.S. USA 81: 3466-3470.

Tkachuk DC, Westbrook CA, Andreelf M, Donlon TA, Clearly ML, Suryanarayan K, Homge M, Redner A, Gray J, Pinkel D (1990).
Detection of bcr-abl fusion in chronic myelogenous leukemia by *in-situ* hybridisation.
Science 250: 559-562.

Trask B, Pinkel D, van den Engh G (1980).
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 BJ (1991).
Fluorescence *in-situ* hybridisation applications in cytogenetics and gene mapping.
T.I.G. 7,5: 149-154.

Trask BJ, van den Engh G, Pinkel D, Mullikin J, Waldman F, van Dekken H, Gray J (1988).
Fluorescence in situ hybridisation to interphase nuclei in suspension allows flow cytometric analysis of chromosome content and microscopic analysis of nuclear organisation.
Hum. Genet. 78: 251-259.

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

Tucker JD, Christensen ML, Carrano AV (1988).
Simultaneous identification of human chromosome material in somatic cell hybrids.
Cytogenet. Cell Genet. 48: 103-106.

Ulinowski Z, Taylor K, Griffin DK, Delhanty J, Wolfe J (1991).
D12S56: a highly polymorphic locus on human chromosome 12q14.
Annals Hum. Genet. 55: 279-282.

Upadhyay M, Fryer A, Foay G, Robinson D, Quarrel O, Roberts A, Harper PS (1990).
Chorionic villus sampling for prenatal diagnosis in Wales using DNA probes. 5years experience.
Prenat. Diag. 10: 593-603.

Van Dekken H, Pizzolo JG, Reuter VE, Melamed MR (1990).
Cytogenetic analysis of human solid tumours by *in-situ* hybridisation with a set of 12 chromosome specific DNA probes.
Cytogenet. Cell Genet. 54: 103-107.

Van der Ploeg M, Raap AK,Tanke H (1990).
University of Leiden. Dept. of cytometry and cytochemistry. Practical Boerhaave course 1990. Hybridocytochemistry (*in-situ* hybridisation).
Not published.

Van Prooijen-Knegt AC, van Hoek JFM, Bauman JGJ, van Duijn P, Wool IG, van der Ploeg M (1982).
In-situ hybridisation of DNA sequences in human metaphase chromosomes by an indirect fluorescent immunocytochemical procedure.
Exp. Cell Res. 141: 397-407.

Vernole P (1990).
Digoxigenim labelled probes can detect single copy genes in human metaphase chromosomes.
Biotechniques 9,2: 200-204.

Viegas Peguinot E, Dutrillaaux B, Magdelena H, Coppey-Moisan M (1989a).
Mapping of single copy DNA sequences on human chromosomes by *in-situ* hybridisation with biotinylated probes: Enhancement of detection by intensified fluorescent digital imaging microscopy.
P.N.A.S. USA 86: 582 586.

Viegas Peguinot E, Zen Lin L, Dutrillaux B, Apiou F, Paulin D (1989b).
Assignment of human desmin gene to band 2q35 by non-radioactive *in-situ* hybridisation.
Hum. Genet. 83: 33-36.

Viegas Peguinot E, Jeanpierre M, Dutrillaux AM, Gerbault-Sereau M, Muleris M, Dutrillaux B (1989c).
Detection of 1q polysomy in interphase nuclei of human solid tumours with a biotinylated probe.
Hum. Genet. 81: 311-314.

Voglestein B, Fearon ER, Hamilton SR, Kern SE, Presinger AL, Leppert M, Nakamura Y, White R, Smits AMM, Bos JL (1988).
Genetic alterations during colorectal tumour development.
New England J. Med. 319: 525-532.

Wada M, Little RD, Abidi F, Porta G, Labella T, Cooper T, Della Valle G, Diloso M, Schlessinger D (1990).
Human Xq24-q28: Approaches to mapping with yeast artificial chromosomes.
Am. J. Hum. Genet. 46: 95-106.

Waye JS & Willard HF (1985).
Chromosome specific alpha satellite DNA: nucleotide sequence analysis of the 2.0 kilobasepair repeat from the human X-chromosome.
N.A.R. 13: 2731-2743.

Weber PC, Ohlendorf DH, Wencloloskin JJ, Salemme FR (1989).
Structural origins of high affinity biotin binding to streptravidin.
Science 243: 85-88.

Werrick R & Willard HF (1989).
Long range organisation of tandem arrays of alpha satellite DNA at the centromeres of human chromosomes: High frequency array-length polymorphism and meiotic stability.
P.N.A.S. USA 86: 9394-9398.

West JD, Gosden JR, Angell RR, Hastie ND, Thatcher SS, Glasier AF, Baird, DT (1987).
Sexing the human pre-embryo by *in-situ* hybridisation.
Lancet I: 1344-1347.

West JD, Gosden JR, Angell RR, West KM, Glasier AF, Thatcher SS, Baird DT (1988).
Sexing whole human pre-embryos by *in-situ* hybridisation to a Y chromosome specific DNA probe.
Hum. Reprod. 3: 1010-1019.

West JD (1989).
The use of DNA probes in preimplantation and prenatal diagnosis.
Mol. Reprod. Devt. 1: 138-145.

West JD, Gosden CM, Gosden JR, West KM, Davidson C, Nicolaides KH (1989).
Sexing the human fetus and identification of polyploid nuclei by DNA-DNA *in-situ* hybridisation in interphase nuclei.
Mol. Reprod. Devt. 1: 129-137.

West JD (1990)
Sexing the human conceptus by *in-situ* hybridisation.
In: *In-situ* hybridisation and the study of development and differentiation. (ed: Harris N & Wilkinson D).

Whittle MJ & Connor JM.
Prenatal daignosis in obstetric practice.
Blackwell scientific publications.

Wiegant J, Reid T, Nederlof PM, van der Ploeg M, Tanke HJ, Raap AK (1991).
In-situ hybridisation with fluoresceinated DNA.
N.A.R. 19,12: 3237-3241.

Willard HF & Waye JS (1985).
Hierarchical order in chromosome-specific human alpha satellite DNA.
T.I.G. 3,7: 192-198.

Willard HF (1990).
Alpha and beta satellite sequences on chromosome 21: The possible role of centomere and chromosome structure in non-disjunction.
In: Molecular genetics of chromosome 21 and Down syndrome (ed: Patterson)
Wiley-Liss Inc. pp 39-52.

Williams AC, Harper SJ, Paraskeva C (1990).
Neoplastic transformation of a human colonic epithelial cell line: *In-vitro* evidence for the adenoma to carcinoma sequence.
Cancer Res. 50: 4724-4730.

Wilton LJ & Trounson AO (1989).
Biopsy of preimplantation mouse embryos: Development of micromanipulated embryos and proliferation of single blastomeres *in-vitro*.
Biol. Reprod. 40: 145-152.

Wilton LJ, Shaw JM, Trounson AO (1989).
Successful single-cell biopsy and cryopreservation of preimplantation mouse embryos.
Fert. Steril. 51,3: 513-517.

Winston RML (1987).
Why a ban on embryo research would be such a tragedy.
B.M.J. 295: 1501-1502.

Witt M, and Erickson RP (1989).
A rapid method for the detection of Y chromosomal DNA from dried blood specimens by the polymerase chain reaction.
Hum. Genet. 82: 271-274.

Wolfe J, Griffin DK, Hornigold N, Delhanty J (1991).
Mapping D9S39 by fluorescent *in-situ* hybridisation and by genetic linkage analysis.
Cytogenet. Cell Genet. 83: 1945.

ABBREVIATIONS:

Am.	-	American
Ann.	-	Annual
Arch.	-	Archives
Biol.	-	Biology
B.M.J.	-	British Medical Journal
Br.	-	British
Clin.	-	Clinical
Cytochem.	-	Cytochemistry
Cytogentet	-	Cytogenetics
Devt.	-	Development
Diag.	-	Diagnosis
Exp.	-	Experimental
Fert.	-	Fertility
Genet.	-	Genetics
Gyn.	-	Gynecology
Hum.	-	Human
Histochem.	-	Histochemistry
Imm.	-	Immunological
Int.	-	International
J.	-	Journal
Med.	-	Medical/Medicine
Met.	-	Methods
Mol.	-	Molecular
N.A.R.	-	Nucleic Acids Research
Neurol.	-	Neurology
Neurosurg.	-	Neurosurgery
Obs.	-	Obstetrics
Pathol.	-	Pathology
P.N.A.S.	-	Proceedings of the National Academy of Science
Ped.	-	Pediatrics
Prenat.	-	Prenatal
Psyc.	-	Psychiatry
Quan.	-	Quantitative
Reprod.	-	Reproduction
Res.	-	Research
Rev.	-	Review
Roy.	-	Royal
Som.	-	Somatic
Soc.	-	Society
Steril.	-	Sterility
T.I.G.	-	Trends in Genetics

PUBLICATIONS ARISING FROM THIS THESIS

Griffin DK, Leigh SEA, Delhanty JDA (1990).
Use of fluorescent in-situ hybridisation to confirm trisomy of chromosome region 1q32-qter as the sole karyotypic defect in a colon cancer cell line.
Genes, chromosomes and cancer 1: 281-283.

Griffin DK, Handyside AH, Penketh RJA, Winston RML, Delhanty JDA (1991).
Fluorescent in-situ hybridisation to interphase nuclei of human preimplantation embryos with X and Y chromosome specific probes.
Hum. Reprod. 6,1: 101-105.

Griffin DK, Bowles L, Tsioupra K, Barber J, Delhanty JDA (1991).
Characterisation of adjascent deletions on chromosome 5q.
Cytogenet. Cell Genet. 83: 1895.

Leigh SEA, Gayther SA, Lawson CM, **Griffin DK**, Delhanty JDA (1991).
Two cosmid clones containing expressed sequences mapping to 5q31 & 5q35.
Cytogenet. Cell Genet. 83: 1899.

Fitzgibbon J, Nahmias J, **Griffin D**, Hornigold N, Delhanty J, Povey S, Wolfe J (1991).
A deletion map of distal 9q based on a panel of irradiation hybrids.
Cytogenet. Cell Genet. 83: 1937.

Nahmias J, Fitzgibbon J, Hornigold N, Florian F, **Griffin D**, Delhanty J, Benham F, Povey S, Wolfe J (1991).
Characterisation of a panel of irradiation hybrids from chromosome arm 9q.
Cytogenet. Cell Genet. 83: 1942.

Wolfe J, **Griffin DK**, Hornigold N, Delhanty J (1991).
Mapping D9S39 by fluorescent in-situ hybridisation and by genetic linkage analysis.
Cytogenet. Cell Genet. 83: 1945.

Mitchell CJ, **Griffin DK**, Carritt B (1991).
Assignment of the beta enolase locus ENO3, to 17p13 by fluorescent in-situ hybridisation.
Cytogenet. Cell Genet. 83: 2008.

Ulinowski Z, Taylor K, **Griffin DK**, Delhanty J, Wolfe J (1991).
D12S56: a highly polymorphic locus on human chromosome 12q14.
Annals Hum. Genet. 55: 279-282.

Florian F, Hornigold N, **Griffin DK**, Delhanty JDA, Sefton L, Abbot C, Jones C, Goodfellow PN, Wolfe J (1991).
The use of irradiation fusion gene transfer (IFGT) hybrids to isolate DNA clones from human chromosome region 9q33-q34.
Som. Cell Mol. Genet. 17,5: 445-453.

Cross I, Delhanty J, Chapman P, Bowles L, **Griffin D**, Wolstenholme J, Bradburn M, Brown J, Gunn A, Burn J (1992).
An intrachromosomal insertion causing 5q22 deletion and familial adenomatous polyposis coli in two generations.
J. Med. Genet. 29: 175-179.

Griffin DK, Wilton LJ, Handyside AH, Winston RML, Delhanty JDA (1992).
Dual fluorescent in-situ hybridisation for the simultaneous detection of X and Y chromosome specific probes for the sexing of human preimplantation embryonic nuclei.
Hum. Genet. 89: 18-22.

GENES, CHROMOSOMES & CANCER 1:281–283 (1990)

Use of Fluorescent In Situ Hybridisation to Confirm Trisomy of Chromosome Region 1q32-qter as the Sole Karyotypic Defect in a Colon Cancer Cell Line

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The sole chromosome defect in a colon cancer cell line derived from a patient with inherited nonpolyposis colorectal cancer was karyotypically designated as 46,XY,−13,+der(13)t(1;13)(q32.1;p11) on the basis of banding homology. We have obtained molecular confirmation that the additional chromosome material is derived from chromosome region 1q32-qter by the use of a highly specific fluorescent in situ hybridisation technique on G-banded chromosomes and also by Southern hybridisation.

INTRODUCTION

The cell line, LIM 1215, derived from a patient with inherited nonpolyposis colorectal cancer, was established from a poorly differentiated adenocarcinoma of the ascending colon (Whitehead et al., 1985). The sole karyotypic abnormality appeared to be the ubiquitous presence of a marker 13p+ chromosome. Careful G band comparison led to the tumour karyotype subsequently being designated as 46,XY, −13, +der(13)t(1;13)(q32.1;p11) (Jenkyn et al., 1987). We have now been able to confirm that the trisomic material is derived from chromosome region 1q32-qter by in situ hybridisation (ISH) of a locus-specific minisatellite probe to metaphase chromosomes from LIM 1215 by using a nonradioactive fluorescent technique. Further confirmation was obtained by using the same probe in Southern hybridisation.

MATERIALS AND METHODS

The hypervariable minisatellite probe λMS32, which recognises the locus D1S8, was previously assigned to chromosome 1, region q42–43, by Royle et al. (1988). Probe DNA was biotinylated by nick translation using the Bethesda Research Laboratories kit.

Chromosomes, prepared by standard methods, were G banded prior to ISH by treatment with 2× SSC (1× SSC is 0.15M sodium chloride and 0.015M trisodium citrate, pH 7.2) and trypsin (Gallimore and Richardson, 1973). Metaphases were then photographed in order that they could be relocated for subsequent analysis after the ISH procedure had been performed.

The following fluorescent ISH technique was adapted and developed from Pinkel et al. (1986) by Tim Kievits and Joop Wiegant of the Department of Human Genetics, University of Leiden. Slides were destained and dehydrated in an alcohol series and then air dried. Pretreatment with RNase (100 μg/ml in 2× SSC) under a coverslip in a moist chamber for one hour at 37°C preceded washing three times in 2× SSC, dehydration in alcohol, and air drying. Washing with proteinase K buffer (20 mM Tris · HCl, 2 mM $CaCl_2$, pH 7.4) was followed by a 7 minute treatment with proteinase K (500 ng/ml) at 37°C. Slides were then washed in paraformaldehyde buffer [0.9% PBS (phosphate-buffered saline) + 50 mM $MgCl_2$], fixed in 4% paraformaldehyde for 10 minutes, washed again in the buffer, dehydrated, and air dried. (Paraformaldehyde in solution is hazardous and should be handled in a fume hood.) Thirty-five microliters of biotinylated probe DNA in hybridisation buffer (50% formamide, 20% dextran sulphate, 50 mg/ml denatured salmon sperm DNA, 1 mM EDTA, 2 mM Tris · HCl in 2× SSC) was applied at a concentration of 30–40 ng/slide and sealed under a coverslip with rubber solution. Denaturation of probe and genomic DNA was done simultaneously in a preheated 80°C oven for 4 minutes. Hybridisation proceeded overnight in a moist chamber at 37°C.

Coverslips were gently removed and post-hybridisation washes were carried out at 42°C as follows: three times for 5 minutes in 50% formamide in 2× SSC and 5 times for 2 minutes in 2× SSC. Preparatory incubation steps were 5 minutes in 4× SSC + 0.05% Tween 20 detergent and 10 minutes in 4× SSC + 5% non-fat dry milk (Mar-

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vel). Biotin detection was facilitated by sequential layers of Avidin-FITC (fluorescein isothiocyanate) conjugate and biotin–anti-avidin D conjugate, both from Vector Laboratories, U.S.A. In each case conjugate was applied at a concentration of 5 μg/ml (in 5% milk in 4× SSC), 100 μl per slide for 20 minutes under a coverslip and washed off with 0.05% Tween 20 in 4× SSC three times for 5 minutes. Three multilayers were applied to obtain maximum signal and minimum background. Final washes were once in 0.05% Tween 20 in 4× SSC and twice in 0.9% PBS (5 minutes each). Slides were dehydrated, air dried, and then mounted and sealed (with nail varnish) under a coverslip in "anti-fade medium." Anti-fade medium: 9 parts glycerol containing 2% 1.4 diazabicyclo-(2.2.2.)-octane (DABCO) and 1 part 0.2 M Tris · HCl + 0.02% sodium azide pH 7.5. DAPI was added to a concentration of 0.5 μg/ml. DABCO is extremely hazardous and should only be handled in a safe fume hood wearing nitrile gloves.

Slides were observed under a Reichert polyvar microscope fitted with ultraviolet fluorescence and filters for DAPI and FITC. Photographed metaphases were relocated and scored for hybridisation by using the FITC filter. Hybridised areas are recognised as bright fluorescent spots. The position of these spots was marked on the original G-banded photograph to give the band location.

LIM1215 was also examined at the D1S8 locus by Southern hybridisation. DNA prepared from LIM1215 cells was digested to completion with AluI, size fractionated by electrophoresis through a 1% agarose gel, and transferred to Gene Screen Plus hybridisation membrane (NEN, DuPont) according to the manufacturer's recommendations. The λMS32 probe was labelled with α-^{32}P dCTP by the random priming method of Feinberg and Vogelstein (1983). Hybridisation and posthybridisation washes were performed as specified (NEN, DuPont). The hybridisation membranes were autoradiographed at −70°C by using Fuji RX-L film.

RESULTS

Twenty cells were analysed, a total of 87 hybridised areas (seen as bright fluorescent spots) were observed, of which 48 (53.1%) were located on chromosomes 1, at region q32-qter and 23 (26.1%) were on the p arm of the 13p+ chromosome (Fig. 1). This gives molecular confirmation of the chromosome 1 origin of the extra material on the marker chromosome 13 and also confirms the assignment of the locus D1S8, recognised by the probe, λMS32, to region 1q42–q43 where the peak

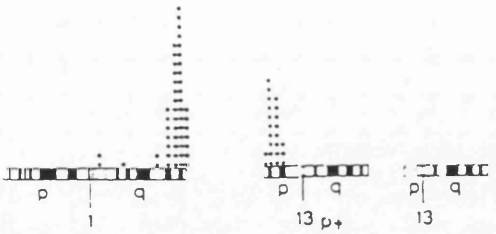

Figure 1. Number and distribution of hybridisation sites of the biotinylated probe λMS32 following in situ hybridisation to metaphases from the cell line LIM 1215. Chromosome pairs 1 and 13 only are shown; 20 cells were analysed.

occurs (Fig. 1). The presence of additional chromosome 1q material was further demonstrated by the relative intensities of the two allelic bands obtained by Southern hybridisation of λMS32 to LIM1215 genomic DNA (Fig. 2). Densitometric measurements reveal that it is the 10.3 kb allele which has been duplicated in the segment translocated to 13p. Unfortunately, normal tissue from the same patient was not available for comparison.

DISCUSSION

We have confirmed by fluorescent ISH to pre-G-banded metaphases that the sole chromosome

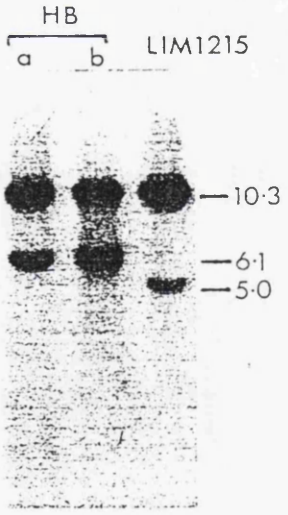

Figure 2. Autoradiograph of Southern blotted DNAs hybridised to λMS32. The sizes of the alleles are given in kb. No normal tissue was available from the patient from whom LIM 1215 was derived. As the probe is hypervariable the hybridisation pattern for each individual is unique. For comparison, samples from patient HB are included. HBb is from normal tissue; HBa is from carcinoma tissue. Short-term culture of the carcinoma revealed a karyotype with duplication of one copy of 1q due to isochromosome formation.

anomaly in the colorectal cancer cell line LIM 1215 is trisomy of chromosome segment 1q32-qter. This accords with previous observations of minimal chromosome changes in some colorectal cancers of right-sided origin (Muleris et al., 1989; Delattre et al., 1989; and our own unpublished observations). Rearrangements of chromosome 1 are frequent in malignant cells, including those of colorectal origin (Mitelman, 1988). Isochromosome 1q formation may be accompanied by loss of material from 1p but this is not universal; regions of consistent duplication have been observed to be 1q24-qter (Reichmann et al., 1984).

Several recent studies implicate human chromosome 1 in the process of cellular immortalisation. PC/AA is an adenoma cell line derived from a familial polyposis patient which originally displayed a normal diploid karyotype; two separate derivatives of this cell line became established in culture and each displayed an isochromosome of 1q, with the origin from different homologues of chromosome 1 (Paraskeva et al., 1988, 1989). Willson et al. (1987) reported a villous-adenoma-derived established cell line with a deletion of the short arm of chromosome 1 and an isochromosome 1q.

Experiments with hybrids between immortal Syrian hamster cells and normal human fibroblasts indicated that each hybrid clone which escaped senescence had lost both copies of human chromosome 1 (Sugawara et al., 1988), again implicating this chromosome in immortalisation. It has been proposed that tumorigenicity results from a balance between genes which suppress malignancy, possibly on 1p, and others which allow expression of the transformed phenotype (Benedict et al., 1984). Perhaps this latter type resides on the terminal region of 1q.

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REFERENCES

Benedict WF, Weissman BE, Mark C, Stanbridge EJ (1984) Tumorigenicity of human HT1080 fibrosarcoma × normal fibroblast hybrids: Chromosome dosage dependency. Cancer Res 44:3471–3479.

Delattre O, Law DJ, Remvikos Y, Sastre X, Feinberg AP, Olschwang S, Melot T, Salmon RJ, Validire P, Thomas G (1989) Multiple genetic alterations in distal and proximal colorectal cancer. Lancet II:353–356.

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13.

Gallimore PH, Richardson CR (1973) An improved banding technique exemplified in the karyotype analysis of two strains of rat. Chromosoma (Berlin) 41:259–263.

Jenkyn DJ, Whitehead RH, House AK, Maley MAL (1987) Single chromosome defect, partial trisomy 1q, in a colon cancer cell line. Cancer Genet Cytogenet 27:357–360.

Mitelman F (1988) Catalog of Chromosome Aberrations in Cancer, Third edition. New York: Alan R. Liss, Inc., 1180 pp.

Muleris M, Salmon RJ, Dutrillaux B (1989) Cytogenetic study of human colorectal carcinomas. First European Workshop on Cytogenetics and Molecular Genetics of Human Solid Tumours. Cancer Genet Cytogenet 38:145–204.

Paraskeva C, Finerty S, Powell SC (1988) Immortalization of a human colorectal adenoma cell line by continuous *in vitro* passage: Possible involvement of chromosome 1 in tumour progression. Int J Cancer 41:908–912.

Paraskeva C, Harvey A, Finerty S, Powell S (1989) Possible involvement of chromosome 1 in *in vitro* immortalization: Evidence from progression of a human adenoma-derived cell line *in vitro*. Int J Cancer 43:743–746.

Pinkel D, Straume T, Gray J (1986) Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938.

Reichmann A, Martin P, Levin B (1984) Chromosomes in human large bowel tumours. A study of chromosome No 1. Cancer Genet Cytogenet 12:295–301.

Royle NJ, Clarkson R, Wong Z, Jeffreys AJ (1988) Clustering of hypervariable minisatellites in the proterminal regions of human autosomes. Genomics 3:352–360.

Sugawara IO, Oshimura M, Koi M, Wiseman R, Barrett JC (1988) Senescence of hybrids between immortal Syrian hamster cells and normal human fibroblasts; correlation of escape from senescence with loss of chromosome 1. In Vitro Cell Dev Biol 24:20A.

Whitehead RH, Macrae FA, St John DJB, Ma J (1985) A colon cancer cell line (LIM 1215) derived from a patient with inherited nonpolyposis colorectal cancer. J Natl Cancer Inst 74:759–765.

Willson JKV, Bittner GN, Oberley TD, Meisner LF, Weese JL (1987) Cell culture of human colon adenomas and carcinomas. Cancer Res 47:2704–2713.

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Fluorescent in-situ hybridization to interphase nuclei of human preimplantation embryos with X and Y chromosome specific probes

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Fluorescent in-situ hybridization (ISH) to interphase nuclei of human preimplantation embryos has been demonstrated with the X and Y chromosome-specific DNA probes, pBamX7 and pHY2.1, respectively. Assigning the sex on the basis of the number of hybridization signals in the majority of nuclei, the efficiencies with both probes to nuclei from male embryos were considerably higher than those previously reported for pHY2.1 detected by isotopic or conventional non-isotopic methods. Only ~15% of nuclei from male embryos failed to hybridize with these probes. With pBamX7, a high incidence (18%) of nuclei with two (or more) signals in embryos classified as males and four signals in a female embryo was observed. In some cases, the double spot nuclei were larger than those with single spots, providing evidence of tetraploidy. The feasibility of using fluorescent ISH for sexing biopsied embryos in couples at risk of X-linked disease and for the preimplantation diagnosis of chromosome abnormalities is discussed.

Key words: human preimplantation embryo/fluorescent in-situ hybridization/X chromosome/Y chromosome/preimplantation diagnosis

Introduction

Over 200 recessive X chromosome-linked diseases, typically only affecting hemizygous boys, have been identified. In many of these, early prenatal diagnosis is possible by chorion villus sampling (CVS) and biochemical or DNA analysis of the cells recovered from the conceptus. In others, for which the molecular basis has not yet been identified, the only alternative is to determine the sex of the fetus by karyotype analysis. If the fetus is affected by the defect or is male, abortion can be offered. However, for some couples at risk of transmitting these defects, selective abortion is unacceptable for moral or religious reasons and others suffer repeated terminations of pregnancy to prevent the birth of an affected child. For these couples, in-vitro fertilization (IVF) and preimplantation diagnosis of the X-linked defect, or alternatively sexing, would offer the opportunity to start a normal pregnancy after transfer of unaffected or female embryos (Penketh and McLaren, 1987).

Following IVF, biopsy of the embryo early on the third day post-insemination at the 8-cell stage allows removal of one or two cells for diagnosis without detriment to the preimplantation development of the biopsied embryo (Hardy *et al.*, 1990) and the sex has been accurately determined by DNA amplification of a Y-specific sequence using the polymerase chain reaction (PCR) (Handyside *et al.*, 1989). This enabled female embryos to be selectively transferred in five couples at risk of sex-linked diseases resulting in two twin and one singleton pregnancies (Handyside *et al.*, 1990). All five fetuses were confirmed to be female by CVS and karyotype analysis and both of the first set of twins to be born are normal females. However, sexing based on the presence or absence of an amplified fragment from a single cell may result in misdiagnosis caused by contamination or more seriously from the inadvertent sampling of anuclear cytoplasmic fragments and is not, in general, applicable to the detection of aneuploidies (Handyside, 1990).

An alternative method of sexing human embryos is by in-situ hybridization (ISH) using Y-specific probes (West *et al.*, 1987, 1988; Jones *et al.*, 1987; Penketh *et al.*, 1989). ISH is also widely applicable to the detection of aneuploidy since chromosome-specific probes are now available for more than half of the autosomes as well as for the X chromosome (Cremer *et al.*, 1986; Pieters *et al.*, 1990). The use of radiolabelled probes (West *et al.*, 1988) requires several days for detection by autoradiography. This would necessitate cryopreservation of biopsied embryos and transfer in a later cycle which is likely to reduce pregnancy rates. The non-isotopic method using biotinylated probes and a streptavidin-linked alkaline phosphatase based detection system is more rapid, requiring <24 h. But the efficiency of hybridization with human embryos was relatively low and it was estimated that at least three cells would be required for a reliable result (Penketh *et al.*, 1989). Fluorescent methods for detecting ISH can also be completed within a day but have the added advantages of increased specificity and sensitivity, and simultaneous detection of several probes is possible using different fluorochromes (Nederlof *et al.*, 1989).

Here, we demonstrate fluorescent ISH to the interphase nuclei of human preimplantation embryos with the X and Y chromosome-specific probes, pBamX7 and pHY2.1, respectively, and make an initial assessement of the feasibility of using this technique for sexing biopsied embryos and for the preimplantation diagnosis of chromosome abnormalities.

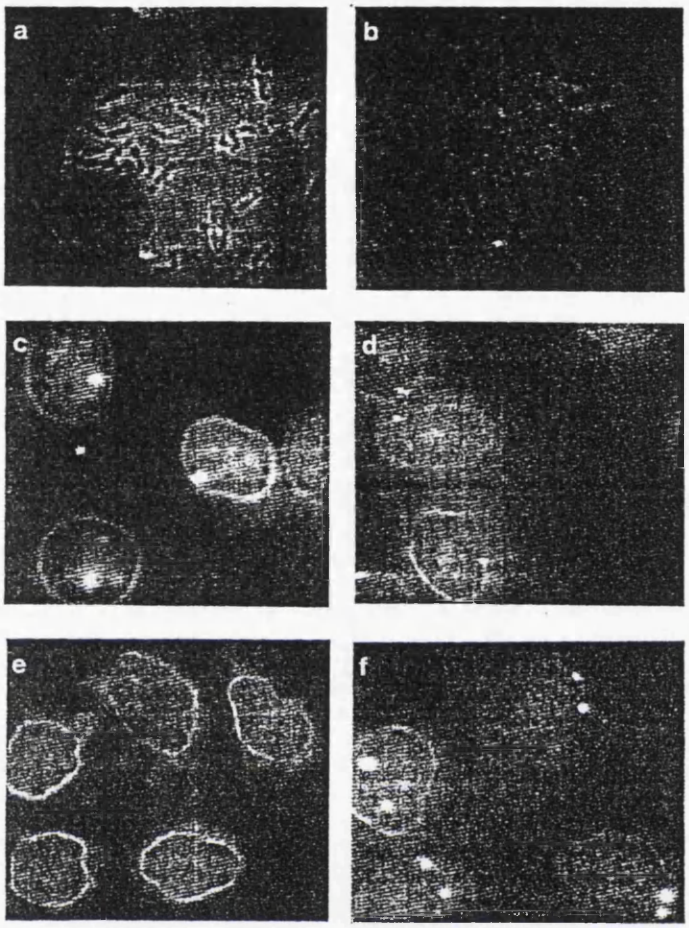

Fig. 1. Fluorescent in-situ hybridization with (1) the probe pHY2.1, specific for the long arm of the Y chromosome: (a) metaphase chromosomes of male lymphocytes (one hybridization signal), (c) male CVS interphase nuclei (one signal) and (e) female CVS interphase nuclei (no signals); and (2) the probe pBamX7, specific for the centromeric region of the X chromosome: (b) metaphase chromosomes of male lymphocytes (one signal), (d) male CVS interphase nuclei (one signal) and (f) female CVS interphase nuclei (two signals).

Materials and methods

Human preimplantation embryos

Human embryos were obtained from patients undergoing in-vitro fertilization (IVF) treatment for infertility as described in detail previously (Hardy *et al.*, 1990). Oocytes were collected, inseminated (day 0) and checked for fertilization 15–18 h later by examining for pronuclei. On day 2, two or three embryos were selected for transfer and after confirming the patients' consent, the surplus embryos were processed for cytogenetic analysis or maintained in culture for up to 3 more days.

Cytogenetic preparation

Cytogenetic preparation of embryos and groups of cells disaggregated from cleavage stages were as described previously (Penketh *et al.*, 1989). Standard cytogenetic preparations of male and female lymphocytes and chorion villus cells were used as controls for hybridization efficiency.

DNA probes

The probe pHY2.1 which recognizes a repeated sequence on the long arm of the Y chromosome (Cooke *et al.*, 1982) was biotinylated by nick translation (BRL) and purified with a Sephadex G50 column. The probe pBamX7, which preferentially recognizes a repeated centromeric sequence on the X chromosome (Willard, 1985), was supplied already labelled (Oncor/Hybaid).

Fluorescent in-situ hybridization

Hybridization conditions were similar to those described by Penketh *et al.* (1989) and the method for the fluorescent detection of the probe has been described in detail elsewhere (Griffin

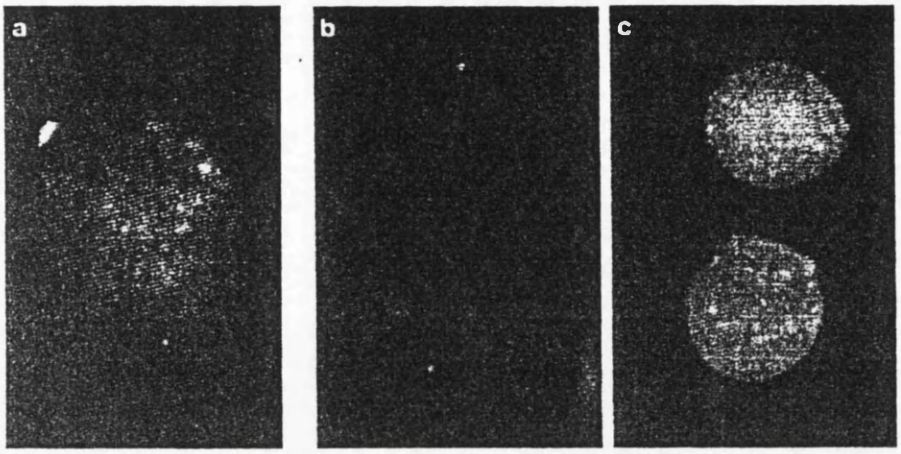

Fig. 2. Fluorescent in-situ hybridization to interphase nuclei from human preimplantation embryos classified as male with (a) pHY2.1 (one hybridization signal in a single nucleus) and with (b,c) pBamX7: (b) hybridization signals (one signal in each of two nuclei), (c) position of DAPI labelled nuclei located under UV illumination.

et al., 1990). Briefly, slides were pretreated with RNase and proteinase K and fixed in 4% paraformaldehyde. Biotinylated probe dissolved in hybridization buffer was applied under a coverslip and sealed. Simultaneous denaturation of probe and genomic DNA preceded overnight incubation.

Post-hybridization washes were followed by probe detection by sequential incubations in avidin-FITC, biotinylated anti-avidin D and avidin-FITC. Slides were then washed in saline and mounted in antifade medium containing either 2% DABCO (1,4 diazabicyclo-[2,2,2] octane) or 0.1% pPD (*p*-phenyldiamine dihydrochloride) with 0.15 μg/ml DAPI (4′,6-diamidino-2-phenylindole) to counterstain the nuclei. Nuclei were examined with a Reichert-Jung Polyvar microscope equipped for fluorescence with UV and fluorescein filter sets. With UV illumination, DAPI-labelled chromosomes or interphase nuclei are visible and their exact position can be located. With the fluorescein filters, only the FITC-labelled probe is visible as a bright yellow/green hybridization signal.

Results

Control cells

Examples of fluorescent ISH with the Y and X-specific probes, pHY2.1 and pBamX7, to metaphase chromosomes of control lymphocyte preparations are shown in Figure 1a and b, respectively. Ninety-five to 100% of the interphase nuclei of these cells, had the expected number of hybridization signals according to the probe employed. With CVS cells, the expected number of fluorescent spots was seen in all 400 nuclei analysed, examples of which are shown in Figure 1 c–f.

Human preimplantation embryos and embryo cells

Nuclei from a total of 11 embryos, three 8-cell embryos and groups of between one and four cells from a further eight cleavage stage embryos, were examined by fluorescent ISH with pHY2.1 (Table I). Six out of 11 (two embryos and four groups of cells) had single hybridization signals in the majority of nuclei and were classified as male (Figure 2a). Four out of 25 nuclei (16%) from these embryos had no signal. The remaining five (one embryo and four groups of cells) had no signal in any of the 15 nuclei examined and were classified as female.

Fluorescent ISH with probe pBamX7 was performed on eight embryos at various stages between days 3 and 5 with six to 19 nuclei per embryo (Table II). With six embryos classified as male, a single hybridization signal was present in the majority of nuclei (Figure 2b,c). Ten out of 68 nuclei (15%) from these embryos had no signal and 12 (18%) had two or more signals. The other two embryos were classified as female on the basis of two signals in the majority of nuclei. Six out of a total of 17 (35%) nuclei had zero or one hybridization signals and one nucleus had four.

Discussion

Fluorescent in-situ hybridization (ISH) to interphase nuclei of human preimplantation embryos has been demonstrated for the X and Y chromosome specific probes, pBamX7 and pHY2.1 (Tables I and II, respectively; Figure 2). Without independent confirmation of the sex of these embryos, it is not possible to assess the efficiency of hybridization accurately. However, assigning the sex on the basis of the number of hybridization signals in the majority of nuclei, the efficiencies with both probes to nuclei from male embryos were considerably higher than those previously reported for pHY2.1 detected by isotopic or conventional non-isotopic methods (Jones *et al.*, 1987; West *et al.*, 1987, 1988; Penketh *et al.*, 1989). For example, only ~15% of nuclei from male embryos failed to hybridize with these probes compared with 34% using streptavidin-linked alkaline phosphatase detection (Penketh *et al.*, 1989).

Hybridization was more consistent with small groups of cells (intended to model cell biopsies) and earlier embryos in which

Table I. Y chromosome-specific fluorescent in-situ hybridization with probe pHY2.1

		No. of nuclei: No. of hybridization signals per nucleus 0	1	Total no. of nuclei scored
Male embryos	a	1	7	8
	b	1	7	8
Male embryo cells	c	1	3	4
	d	1	1	2
	e	–	2	2
	f	–	1	1
Total		4	21	25
Female embryos	g	8	–	8
Female embryo cells	h	2	–	2
	i	2	–	2
	j	2	–	2
	k	1	–	1
Total		15	0	15

Table II. X chromosome-specific fluorescent in-situ hybridization with probe pBamX7

		No. of nuclei: No. of hybridization signals per nucleus 0	1	2	3	4	5+	Total no. of nuclei scored
Male embryos	a	1	5	–	–	–	–	6
	b	2	4	–	–	–	–	6
	c	–	8[a]	2	–	–	–	10
	d	4	10[a]	3	–	–	2[a]	19
	e	2	12	2	–	1	–	17
	f	1	7	2	–	–	–	10
Total		10	46	9	0	1	2	68
Female embryos	g	4	–	5[a]	–	1	–	10
	h	1	1	5	–	–	–	7
Total		5	1	10	0	1	0	17

[a]Includes at least one nucleus in metaphase.

less cytoplasm remained over the nuclei during spreading (Table Ic–f; Table IIc). With improved preparation of nuclei, therefore, it should be possible to improve the efficiency of hybridization further, especially as virtually all control lymphocyte and chorion villus nuclei had the appropriate numbers of hybridization signals (Figure 1). Sexing might then be sufficiently reliable with as few as two cells which can be removed from 8-cell embryos without detriment to preimplantation development (Hardy *et al.*, 1990). If the hybridization time can also be reduced so that female embryos can be transferred on the same day, fluorescent ISH would then be a viable alternative to DNA amplification for couples at risk of sex-linked disease.

Hybridization with the X chromosome-specific probe, pBamX7, has not been previously evaluated with human embryos. An interesting observation with this probe was the high incidence (18%) of nuclei with two (or more) signals in embryos classified as males and four signals in a female embryo all of which had > 10 nuclei on day 5 (Table IIc–f and g). In some cases, the double spot nuclei were larger than those with single spots, providing evidence of tetraploidy. However, in one embryo scored as male, double spot nuclei were no larger than the remainder and this may have had a chimaeric origin or gain of an X chromosome by non-disjunction. Tetraploid nuclei with two Y-specific signals have been observed previously in human blastocysts and cleavage stage embryos (West *et al.*, 1987, 1988) and it has been suggested that their occurrence in early embryos may be a culture-induced phenomenon (West, 1990). For sexing preimplantation embryos, however, a nucleus with two hybridization signals could result from either a tetraploid male cell or a normal diploid female cell. In practice, therefore, either the Y-specific probe or a combination of both probes is likely to be the most reliable for determining embryo sex. With fluorescent ISH this can be accomplished using probes with different haptens and labelled with distinct fluorochromes (Nederlof *et al.*, 1989). However, in our hands, problems arise when trying to reconcile two different optimal hybridization conditions. For instance, the X chromosome probe recognizes minor binding sites on chromosomes 11 and 17 when conditions are not ideal (unpublished observations).

Carriers of reciprocal or Robertsonian translocations are at a high risk of producing chromosomally abnormal embryos. The use of chromosome-specific centromeric probes would detect only a minority of unbalanced meiotic products, those which result from adjacent 2, 3:1 or 4:0 segregations. The major risk is of adjacent 1 disjunction (homologous centromeres separate). To detect this category, fluorescent ISH with chromosome specific library probes, the so called 'chromosome painting' technique (Pinkel *et al.*, 1988), will probably be necessary. Used in conjunction with karyotyping, this method would allow precise analysis of even a poor quality preparation.

In many cases, IVF is offered to women who have had difficulty in conceiving over a considerable period of time. As a result these women fall on average into the category of 'older mothers'. Even from data on recognized conceptions, it is known that at age 35 at least 12% of ovulated oocytes will not have the normal number of haploid chromosomes (Warburton, 1987). It is certain that with the addition of anomalies involving large autosomes and the male contribution that the incidence of chromosome imbalance will be much higher at conception. Previous research has shown that embryos with lethally abnormal chromosome complements are not always detectable morphologically (Angell *et al.*, 1986). Fluorescent ISH with combinations of centromeric probes provides the opportunity to screen embryos for the common trisomies before transfer to the mother's uterus. This could reduce later spontaneous (or therapeutic) abortions and improve prospects for these patients. An increasingly wide range of chromosome-specific alphoid repetitive and genomic library probes are becoming available. Fluorescent ISH to interphase nuclei thus appears to have considerable potential for use in preimplantation diagnosis and screening for chromosome abnormalities.

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Interim Licensing Authority for Human In Vitro Fertilization and Embryology.

References

Angell,R.R., Templeton,A.A. and Aitken,R.J. (1986) Chromosome studies in human in-vitro fertilization. *Hum. Genet.*, 72, 333–339.

Cooke,H.J., Schmidtke,J. and Gosden,J.R. (1982) Characterisation of a human Y chromosome repeated sequence and related sequences in higher primates. *Chromosoma*, 87, 491–502.

Cremer,T., Landegent,J., Brückner,A., Scholl,H.P., Schardin,M., Hager,H.D., Devilee,P., Pearson,P. and van der Ploeg,M. (1986) Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe L 1.84. *Hum. Genet.*, 76, 346–352.

Griffin,D.K., Leigh,S.E.A. and Delhanty,J.D.A. (1990) Use of fluorescent in situ hybridisation to confirm trisomy of chromosome region 1q32-qter as the sole karyotypic defect in a colon cancer cell line. *Genes Chromosomes Cancer*, 1, 281–283.

Handyside,A.H. (1990) Preimplantation diagnosis by DNA amplification. In Chapman,M., Grudzinskas,G., Chard,T. and Maxwell,D. (eds), *The Embryo: Normal and Abnormal Growth and Development.* Springer Verlag, London, in press.

Handyside,A.H., Pattinson,J.K., Penketh,R.J.A., Delhanty,J.D.A., Winston,R.M.L. and Tuddenham,E.G.D. (1989) Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet*, i, 347–349.

Handyside,A.H., Kontogianni,E.H., Hardy,K. and Winston,R.M.L. (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*, 344, 768–770.

Hardy,K., Martin,K.L., Leese,H.J., Winston,R.M.L. and Handyside,A.H. (1990) Human preimplantation development *in vitro* is not adversely affected by biopsy at the 8-cell stage. *Hum. Reprod.*, 5, 708–714.

Jones,K.W., Singh,L. and Edwards,R.G. (1987) The use of probes for the Y chromosome in preimplantation embryo cells. *Hum. Reprod.*, 2, 439–445.

Nederlof,P.M. Robinson,D., Abuknesha,R., Wiegant,J., Hopman,A.H.N., Tanke,H.J. and Raap,A.K. (1989) Three-color fluorescence in situ hybridization for the simultaneous detection of multiple nucleic acid sequences. *Cytometry*, 10, 20–27.

Penketh,R.J.A. and McLaren,A. (1987) Prospects for prenatal diagnosis during preimplantation human development. In Rodeck,C. (ed.), *Clinical Obstetrics and Gynaecology. Vol. 1.* Ballière Tindall, London, pp. 747–764.

Penketh,R.J.A., Delhanty,J.D.A., van den Berghe,J.A., Finkelstone,E.M., Handyside,A.H., Malcolm,S. and Winston,R.M.L. (1989) Rapid sexing of human embryos by non-radioactive in situ hybridization: potential for preimplantation diagnosis of X-linked disorders. *Prenat. Diagn.*, 9, 489–500.

Pieters,M.H.E.C., Geraedts,J.P.M., Meyer,H., Dumoulin,J.C.M., Evers,J.L.H., Jongbloed,R.J., Nederlof,P.M., and van der Flier,S. (1990) Human gametes and zygotes studied by non-radioactive in situ hybridization. *Cytogenet. Cell Genet.*, 53, 15–19.

Pinkel,D., Landegent,J., Collins,C., Fuscoe,J., Segraves,R., Lucas,J. and Gray,J. (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.

Warburton,D. (1987) Reproductive loss: how much is preventable? *N. Engl. J. Med.*, 316, 158–160.

West,J.D. (1990) Sexing the human conceptus by in situ hybridisation. In Harris,N. and Wilkinson,D. (eds), *In situ Hybridisation: Application to Developmental Biology and Medicine:* Society for Experimental Biology Seminar Series, Vol. 40, pp.205–239.

West,J.D., Gosden,J.R., Angell,R.R., Hastie,N.D., Thatcher,S.S., Glasier,A.F. and Baird,D.T. (1987) Sexing the human pre-embryo by DNA–DNA in situ hybridisation. *Lancet*, i, 1345–1347.

West,J.D., Gosden,J.R., Angell,R.R., West,K.M., Glasier,A.F., Thatcher,S.S. and Baird,D.T. (1988) Sexing whole human pre-embryos by in situ hybridisation with a Y-chromosome specific DNA probe. *Hum. Reprod.*, 3, 1010–1019.

Willard,H.F. (1985) Chromosome-specific organization of human alpha satellite DNA. *Am. J. Genet.*, 37, 524–532.

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27224

omplement c6 (c6) on chromosome 5

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C7 and C9 has been mapped to chromosome 5 by ation by Jeremiah et al. (HGM10). RFLP markers re studied to further localize complement C6. The ve been typed on 15 families: D5S10, D5S11, 21, D5S37 and IL3. Using the LINKAGE program ted to be: (D5S19,D5S20)- -D5S21-C6-D5S37. A vas found to IL3 (z=-2 O=0.2). Lod scores and ncies between C6 and other RFLP markers were D5S11 (O=0.1 z= -3.8), D5S19 (O=0.20; z=0.3), .), D5S21 (O=0.20; z=1.8), D5S37 (O=0.40 z=0.3) -9.4) in males and females combined.

mining three more RFLP markers in the region D5S37 to construct a detailed map.

27189

around the spinal muscular atrophy gene on ag yeast artificial chromosomes (YACs).

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8,XXXX (1), 46,XY (2)) were screened for the), D5S112, D5S39, D5S125 and D5S127, using a action (PCR) based strategy. These loci are of ince they are genetically linked to autosomal cular atrophy (SMA) (3,4). The most likely genetic

125,SMA)-D5S112-(D5S127,D5S39)-qter

loci were obtained covering a 2Mb region of DNA. ntains the minisatellite adjacent to D5S127, which us lies within the D5S39 YAC. A microdissection gion was prepared as previously described. Many give clear signals on Southern blots. Two clones, ve clear signals which were localised to the region

P, Lehrach H: Yeast artificial chromosome libraries nserts from mouse and human DNA. Proc Natl Acad (1991)

F, Olson MV: Cloning of large segments of to yeast by means of artificial chromosome vectors. 12 (1987)

ehner T, Castilla LH, Penchaszadeh GK, Wilhelmsen avies KE, Leppert M, Ziter F, Wood D, Dubowitz V, owa-Petrusewicz, Ott J, Munsat T, Gilliam TC: f chronic childhood-onset spinal muscular atrophy to .2-q13.3. Nature 344: 540-541 (1990)

S, Sheth P, Bachelot MF, Burlet P, Marcadet A, , Carriere JP, Fardeau M, Fontan D, Ponsot G, Billette bosa C, Ferriere G, Lanzi G, Ottolini A, Babron MC, A, Clerget-Darpoux F, Lathrop M, Munnich A, r chronic proximal spinal muscular atrophies maps to ature 344: 767-768 (1990)

27208

Characterisation of adjacent deletions on chromosome 5q

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We have reported a familial interstitial deletion of chromosome 5 (del(5)(q22 q23.1)) associated with adenomatous polyposis coli (APC) and mental retardation. Two normal family members had a direct insertion of the deleted segment (dir ins(5)(q31.3 q22 q23.2). DNA analysis of this family showed that D5S81 (detected by the probe YN5.48) and the locus detcted by pEF5.44 are within the deletion wheras D5S37 (Pi 227) lies outside. Fluorescent in situ hybridisation to lymphocyte metaphases using a cosmidcBegrecognising, D5S98 (ECB27),showed that this locus was not deleted either. An adjacent deletion,(del(5)(q15 q22)) was found in a 44 year old woman with cerebral palsy; an apparently identical deletion was present in her phenotypically normal mother.There is no history of colon cancer in the family and DNA analysis shows that D5S81 (YN5.48) is not deleted; other probes have so far proved uninformative. Full characterisation of these two deletions by fluorescent in situ hybridisation with cosmids derived from chromosome 5 will assist in the mapping of the region 5q15-q23.

This work is supported by: Quest Cancer Test.

1 Cross I, Delhanty J, Chapman P, Griffin D, Wolstenholme J, Bradburn M, Brown J, Wood C, Gunn A, Burn J: An intrachromosomal insertion causing 5q22 deletion and familial polyposis coli in two generations. (1991). Am J Hum Genet Submitted: (0)

27049

A linkage map spanning the diastrophic dysplasia locus

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Diastrophic dysplasia (DTD) is an autosomal recessive chondrodysplasia of unknown pathogenesis. We have mapped the DTD locus to the distal long arm of chromosome 5 by using a panel of RFLPs spanning the human genome. Here we report the refined localization of DTD in relation to 16 polymorphic markers on distal 5q. No recombinations occurred with the loci D5S72 and D5S66. One presumptive candidate gene, osteonectin (SPARC) could be excluded on account of 3 recombinations with the DTD locus. Multipoint linkage analysis performed against a fixed order of markers placed DTD between the glucocrticoid receptor (GRL) and SPARC loci. This order was favored by the odds of 33:1 over the next best location of DTD between D5S72 and D5S55. The maximum multipoint lod score was 10.47. The sex averaged distance between the definite flanking markers, GRL and D5S55, is 17.5 cM. Based on previously reported data on the physical localization of markers we conclude that the DTD locus is in 5q31-q34. These findings allow carrier and prenatal diagnosis by RFLP analysis in affected families.

conjugated avidin. The cytogenetic localizations of each probe is shown below. Each of the probes was mapped previously to 5q by genetic linkage analysis. A single probe (KK5.33, D5S85) was sublocalized to 5q14-q21 by this method; however, our FISH results suggest that this probe is in 5q22 and, thus, is somewhat more distal. We have begun to determine the order of the cosmids which map within 5q31 by two-color fluorescence in situ hybridization.

Probe	Locus	Location	Probe	Locus	Location
YN5.132	D5S127	5q13	KK5.19	D5S121	5q31
MC5.100	D5S128	5q13	92.15	D5S179	5q32
KK5.33	D5S85	5q22	92.21	D5S180	5q32
MH5.15	D5S169	5q22	21.26	D5S186	5q32-q33
MC5.71	D5S150	5q23.3-q31.1	N5.36	D5S181	5q33
MC5.95	D5S147	5q31	92.27	D5S193	5q33
YN5.116	D5S151	5q31	EF5.21	D5S195	5q33
KK5.100	D5S155	5q31	YN5.62	D5S168	5q34
E5.21	D5S156	5q31	MH5.3	D5S171	5q34
YN5.32	D5S162	5q31	93.121	D5S198	5q34
MC5.138	D5S166	5q31	MC5.133	D5S189	5q35
EF5.12	D5S178	5q31			

1 Nakamura Y, Lathrop M, Leppert M, Dobbs M, Wasmuth J, Wolff E, Carlson M, Fujimoto E, Krapcho K, Sears T, Woodward S, Hughes J, Burt R, Gardner E, Lalouel J-M, White R: Localization of the genetic defect in familial adenomatous polyposis within a small region of chromosome 5. Am J Hum Genet 43: 638-644 (1988)

2 Kidd K, Bowcock AM, Schmidtke J, Track RK, Ricciuti F, Hutchings G, Bale A, Pearson P, Willard HF: Report of the DNA committee and catalogs of cloned and mapped genes and DNA polymorphisms Cytogenet Cell Genet 51: 622-947 (1989)

3 Williamson R, Bowcock A, Kidd K, Pearson P, Schmidtke J, Chan HS, Chipperfield M, Cooper DN, Hewitt J, Lewitter F, Maidak B, Quitt M, Ricciuti F, Track R: Report of the DNA committee and catalogues of cloned and mapped genes and DNA polymorphisms Cytogenet Cell Genet 55: 457-778 (1990)

27155

Two Cosmid Clones containing expressed sequences mapping to 5q31 & 5q35.

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Two human genomic clones were isolated from a chromosome 5 specific lambda library (ATCC: LAO5NSO1) on the basis that they contained sequences expressed in normal colonic mucosa(1). These were in turn used to screen a total human genomic cosmid DNA library. We report here the identification of two cosmid clones, cos5.3iv (D5S321) and cos5.5iv (D5S322), which have been mapped to 5q35 and 5q31 respectively by fluorescent in situ hybridisation. These localizations are currently being confirmed with a panel of somatic cell hybrods.

This work was funded by The Gloria Miles Cancer Foundation and Biomed Ltd.

1 Wong P, MacDonald I, Tenniswood M: Use of the polymerase chain reaction for the differential cross screening of libraries in phage-lambda-based vectors. Gene 85: 59-65 (1989)

27444

Prenatal diagnosis of spinal muscular atrophy using polymorphic DNA probes of the 5q12-q14 region.

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Hereditary childhood spinal muscular atrophies (SMA) are characterized by degeneration of anterior horn cells of the spinal cord and make up the second most common autosomal recessive disease after cystic fibrosis. The underlying biochemical defects remain unknown. Improvements in medical management over the past decade may reduce the progression of the disease. However, many families wish to have prenatal diagnosis for subsequent pregnacies. Until recently, no test was available to allow diagnosis of SMA in families with a 1-in-4 risk of recurrence. The gene responsible for the three forms of SMA has recently been mapped to chromosome 5q using genetic linkage studies (1,2). Among six markers mapping to this region, five were shown to be linked with the gene locus in SMA families. Two of them (M4 and p105153Ra) are flanking the SMA gene (3). The linked probes are potentially useful for prenatal diagnosis in families with at least one affected child. Here, we report our preliminary experience with prenatal diagnosis of SMA in 9 families with the linked probes in chorionic villus sample and amniotic fluid during the first and second trimesters of pregnancy.

1 Melki J: Nature 344: 767 (1990)

2 Melki J: Lancet 336: 271 (1990)

3 Sheth: Am J Hum Genet in press: (1991)

27453

De novo chromosome 5 translocation associated with adenomatous polyposis coli

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Adenomatous polyposis coli (familial adenomatous polyposis, APC) is a dominantly inherited cancer syndrome characterized by the development of multiple (>100) colonic polyps. Extracolonic features are variable, but most patients show multiple areas of congenital hypertrophy of the retinal pigment epithelium (CHRPEs). Following the report of a mentally retarded patient with APC and an interstitial deletion of chromosome 5 [del(5)(q13q15) or (q15q22)] (1), the APC gene was mapped to chromosome 5 by genetic linkage analysis (2,3). Two further examples of interstitial deletions of 5q in mentally retarded patients with APC (4,5) have refined the localization of the APC gene. Genetic linkage studies have mapped the APC gene close to the anonymous DNA probe YN5.48 (D3S81) (6).

As part of a population based study of familial colon cancer a 39 year old man with APC and mild/moderate mental retardation was ascertained. There was no family history of APC or mental retardation. Further evaluation revealed minor dysmorphic features and multiple (>10) CHRPEs, but no osteomas or cutaneous cysts. Cytogenetic analysis revealed an apparently balanced complex rearrangement involving chromosomes 5, 9, 11, 12 and 16. The breakpoint on chromosome 5 was at 5q21-q22. In situ hybridization demonstrated that the cosmid probe YN5.48 mapped to a position in 5q22 which was close to and telomeric to the translocation breakpoint. Both parents had normal chromosomes and phenotype.

Our findings support the results of genetic linkage studies which localise the APC gene centromeric to YN5.48 (D5S81) (7), and are compatible with translocation disrupting the APC gene.

1 Herrera L, Kakati S, Gibas L, Pietrzak E, Sandberg AA: Gardner syndrome in a man with an interstitial deletion of 5q Am J Med Genet 25: 473-476 (1986)

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d out for the ABO blood group locus,
), ASS (ASSg3) and a number of
hromosome 9, using a large, multiply
DNA markers used were D9S7
35), D9S21 (CR1-P111) and D9S39

ulated using the Mlink programme of
shown in Table 1.

ction

0.05	0.10	0.15	0.20	0.3	0.4
0.32	0.25	0.19	0.14	0.06	0.02
1.03	0.93	0.83	0.72	0.50	0.25
0.29	-0.06	0.05	0.09	0.11	0.17
1.26	1.40	1.38	1.28	0.92	0.40
1.61	1.71	1.66	1.53	1.10	0.50
0.01	-0.01	0.02	0.02	0.02	0.01
0.45	-0.03	0.14	0.20	0.18	0.06
0.17	-0.12	-0.08	-0.06	-0.02	-0.01
2.46	2.44	2.27	2.03	1.42	0.65
0.08	0.13	0.14	0.15	0.13	0.07
0.12	0.16	0.17	0.16	0.13	0.08
0.77	0.91	0.90	0.82	0.55	0.21
0.66	-0.22	-0.04	-0.05	0.08	0.03
0.35	-0.27	-0.20	-0.14	-0.06	-0.02
0.38	-0.27	-0.20	-0.13	-0.05	-0.01
0.23	-0.17	-0.12	-0.09	-0.04	-0.01
0.23	0.20	0.17	0.14	0.07	0.02
0.06	-0.04	-0.03	-0.02	-0.01	-0.01
0.36	0.34	0.31	0.27	0.18	0.09
0.35	0.33	0.30	0.27	0.18	0.09
0.56	0.72	0.74	0.70	0.49	0.19
0.28	0.41	0.41	0.36	0.20	0.06
4.43	3.86	3.30	2.74	1.70	0.70
0.24	-0.20	-0.12	-0.11	-0.05	-0.01
0.82	0.81	0.75	0.65	0.39	0.13
0.27	-0.22	-0.18	-0.13	-0.06	-0.01
1.34	1.22	1.08	0.92	0.56	0.19
0.84	-0.55	-0.38	-0.26	-0.11	-0.03

were identified which give useful
region. These are:

ABO)
GSN)

physical mapping data is consistent

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ent computations in multilocus linkage
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chromosome 9q31 (0)

27294

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27409

A deletion map of distal 9q based on a panel of irradiation hybrids

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We have used an irradiation-fusion technique to generate 39 irradiation fragment hybrids from a somatic cell hybrid containing chromosome 9q as its only human material (Nahmias et al submitted). These hybrids have been characterized using 16 defined loci and 27 new markers derived by Interspersed Repetitive Sequence PCR. Further analysis of 12 hybrids using fluorescent in situ hybridisation (FISH) indicated that several have retained only one or two fragments of human origin. Using these simple hybrids, a contiguous irradiation hybrid map of the distal 9q region has been deduced (Table). It is expected that this working panel can be expanded to cover other 9q regions particularly since the FISH data suggest that hybrids containing many fragments contain different fragments in different cells and that therefore the cell lines can be subcloned to facilitate their use in such a panel. These additional hybrids should provide a valuable tool for high resolution mapping of the long arm of chromosome 9 and allow for the saturation of defined regions with newly derived markers.

TABLE: Deletion map of distal 9q.

MARKERS	ORM	GSN	AK1	ABL	ASS	cFF3	cFF38	D9S10	DBH	D9S17	D9S7	D9S11
HYBRID *:												
6C (>2)	-	-	-	-	-	-	-	+	+	-	-	-
12C (2)	-	+	+	+	-	-	+	+	+	+	+	+
14A (2)	-	-	+	-	-	-	-	-	-	+	+	+
17A (1)	-	-	ND	+	+	-	-	-	-	-	-	-
17B (ND)	-	-	-	-	-	-	-	+	+	-	-	-
19B (2)	-	-	-	-	+	+	+	-	-	-	-	-
20A (1)	-	-	-	-	-	+	+	+	+	+	-	-
21A (1)	-	-	-	-	-	-	-	-	-	-	+	-

Note: cFF3 and cFF38 have been derived by IRS PCR

*The number of human fragments as deduced by FISH is noted in parenthesis

27210

Localization of the catalytic subunit Cgamma of cAMP-dependent protein kinase on human chromosome 9 q13

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Many hormones exert their effects on cellular metabolism and gene expression by regulating cAMP and thereby the activity of the cAMP-dependent protein kinase. Cyclic AMP-dependent protein kinases (PKA) are composed of two regulatory (R) and two catalytic (C) subunits. Gene products of four different regulatory subunits RI alpha, RI beta, RII alpha and RII beta and three different catalytic subunits Calpha, Cbeta and Cgamma have been identified. Whereas the alpha-forms of both R and C subunits are found in all tissues, the beta-forms reveal distinct cell specific expression and regulation. So far Cgamma

27018

Assignment of Two Human alpha 1-3-Galactosyltransferase Genes to Chromosomes 9q33-34 and 12q14-15.

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The enzyme UPD-Gal:Gal beta 1->4GlcNAc alpha 1->3-galactosyltransferase (alpha 1,3-GT) is a Golgi membrane bound enzyme involved in the biosynthesis of the carbohydrate chains of glycoproteins and glycolipids. It catalyzes the reaction: UPD-Gal + Gal beta 1->4GlcNAc-R-->Gal alpha 1->3Gal beta 1->4GlcNac-R +UPD. Enzyme activity is developmentally regulated and differentiation dependent. The enzyme shows an interesting species-specific distribution: It is present in most mammals, but cannot be detected in man, apes and Old World Monkeys. The carbohydrate structure produced by alpha 1,3-GT is immunogenic in man, and most normal, healthy individuals have a significant titer of a natural anti-Gal alpha 1->3Gal antibody in their serum. It has been postulated that aberrant expression of alpha 1,3-GT in man may be linked to autoimmune disorders and the occurence of certain germ cell tumors. We recently isolated two human alpha 1,3-GT homologs, that most likely represent a processed pseudogene (HGT-2) and the inactivated remnant of the once functional source gene (HGT-10), respectively.

Southern blotting analysis of DNA from 18 human-rodent somatic cell hybrids (NIGMS, Camden, NJ) localized HGT-2 to human chromosome 12, and HGT-10 to chromosome 9 (1). The regional assignment of these two genes was accomplished by flurescent in situ hybridization. Human metaphase chromosomes were prepared from peripheral blood lymphocytes of two normal individuals. The phage DNA probes, labeled with biotin-11-dUTP by nick-translation, were used for hybridization at concentration of 25 ng/ml. The chromosomes were stained by chromycin A_3/distamycin A/DAPI technique for fluorescent microscopic analysis. In a total of 50 cells scored for HGT-2, 29 (58%) revealed hybridization signals on both chromosomes 12, in bands q14-15, and 19 (38%) had signals on one chromosome 12q14-15. For HGT-10, 32 of 50 (64%) cells analyzed had signals on both chromosomes 9, at region 9q33-34, and 12 (24%) revealed signals on one chromosome 9q33-34. These results map the HGT-2 to chromosome 12q14-15 and the HGT-10 to chromosome 9q33-34.

1 Joziasse DH, Shaper JH, Jabs EW, Shaper NL: Characterization of an alpha 1->3-galactosyltransferase homologue on human chromosome 12 that is organized as a processed pseudogene. J Biol Chem 266: (in press) (1991)

27414

Characterization of a panel of irradiation hybrids from chromosome arm 9q

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Rodent-human radiation hybrids containing only a defined subset of the human genome are a valuable means for obtaining and mapping human sequences which can serve as polymorphic markers or STSs and thus contribute to the fine mapping and subsequent cloning of genes of interest. We have derived a panel of 39 radiation hybrids by X irradiating human-hamster hybrid 64063a12 (1) which contains only human chromsome arm 9q.

Cytogenetic analysis by fluorescent in situ hybridization (FISH) with total human DNA has been performed on 12 of the hybrids selected because they contained markers from 9q34. While 3 of the hybrids examined exhibit a multitude of fragments, the rest contain only one or two human fragments. This renders them particularly useful for the high resolution mapping of 9q34 derived sequences. Indeed, a characterization of the hybrids with conventional single copy probes (9 genes and 7 random sequences) in conjunction with the in situ hybridization has provided the framework within which new 9q derived sequences can be assigned. Such sequences can in addition be utilised to link "single fragment hybrids" together, where pre-existing markers fail to do so (Fitzgibbon et al abstract submitted).

We have used three approaches, all based on interspersed repetitive sequence (IRS) PCR, to obtain and map single copy sequences from 9q:

1) Alu PCR using primer IV (2):

Primer IV contains sequences from the 3' alu end and is highly human specific. The amplification products of the "parental" 9q hybrid 64063a12 were cloned and used to probe Southern transfers of the alu PCR fingerprints of the panel of radiation hybrids; 9 sequences have been mapped using this approach.

2) Alu PCR using primer 68D:

68D is a 35mer priming from the 5' end of the alu sequence. In contrast to primer IV, which gives a characteristic PCR pattern for each hybrid, 68D lacks species specificity and its amplification products with any of the hybrids appear as a smear on an agarose gel. We performed 68D primer PCR on cosmids of human origin derived from a genomic library of 64063a12. The amplified sequences were subsequently mapped by their hybridization pattern on Southern blots of 68D amplified DNA from the hybrids; 16 cosmids have been mapped by this method.

3) LINE PCR using primer L1Hs (3):

Primer L1Hs is highly human specific. When used alone in the panel of hybrids it gives a small number of amplification products which can then be easily identified by their pattern following a restriction digest. We have mapped 2 such products.

These techniques have enabled us to characterize the hybrids quickly. We have gone on to apply the hybrids to mapping 9q using two different approaches. See the abstracts submitted by Fitzgibbon et al. and Wolfe et al.

1 Jones C, Kao FT: Regional mapping of the folylpolyglutamate synthetase gene (FPGS) to 9cen-9q34 Cytogenet Cell Genet 37: 499-500 (1984)

2 Cotter FE, Hampton GM, Nasipuri S, Bodmer WF, Young BD: Rapid isolation of human chromosome specific DNA probes from a somatic cell hybrid. Genomics 7: 257-263 (1990)

3 Ledbetter SA, Nelson DL, Warren ST, Ledbetter DH: Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence polymerase chain reaction Genomics 6: 475-481 (1990)

27326

Genetic linkage map of chromosome 9q32-q34.

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We have constructed a genetic linkage map spanning a total recombinational distance of theta =0.84 of the long arm of human chromosome 9q32-q34 using 47 Venezuelan reference pedigrees (Haines et al,1991). This map was constructed using 16 distinct polymorphisms, 12 RFLPs and 4(GT)n repeats defining 12 separate loci, including the genes for HXB,GSN,AK1,ABL1,ASS,ABO and DBH. Loci were placed using the MAPMAKER program at the given positions with odds of at least 1000:1 over any other position in the map. The order of the markers in the brackets could not be determined with

27101

A highly polymorphic locus on human

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(CEL), a major component of pancreatic juice, and a variety of dietary esters including he enzyme is particularly important for the nilk triglycerides in newborns and is believed ption of dietary cholesterol throughout life. As ad substrate specificity, CEL has been studied ive names, including carboxyl ester hydrolase, specific lipase and lysophospholipase.

elucidate the role of CEL in the genetic control have determined the chromosomal location of using a CEL cDNA probe (2). Southern blot A from a panel of mouse-human somatic cell e CEL gene resides on chromosome 9. In situ omes allowed assignment to 9q34.3, the most long arm. A chromosome 9 translocation 11-4,(3)) was utilized to confirm that the CEL oint at 9q31-q32. We pinpointed the position e to DNA markers in the region by Southern used are linked in the order centromere-Lamp D9S16, D9S28)-gelsolin-AK1 (4, 5), and the lie between Lamp 92 and MCOA12.

multi-allele polymorphism at the CEL locus, nzymes Taq I, Pvu II and Eco RI. The pattern pervariable region such as a VNTR sequence. nises to be a useful marker for linkage studies ease loci previously mapped to this vicinity (locus DYT1) (6), nail patella syndrome-1 (5) sclerosis (7).

genetic heterogeneity in cholesterol absorption cholesterol levels (8) suggests a role for CEL of cholesterol metabolism. The high degree of EL locus should be useful in addressing this

L: Pancreatic carboxyl ester lipase (cholesterol 185-204 5 Amsterdam (1984)

ong H, Lee G, Leete TH, Ronk M, Shively JE, 3, Ameis D, Schotz MC: cDNA cloning of om human pancreas reveals a unique proline-rich . 32: 267-276 (1991)

S, Sparkes MC, Shulkin JD, Toomey KE, al localization of human gene loci on of somatic cell hybrids containing human um Genet 31: 586-600 (1979)

Y, O'Connell P, Leppert M, Woodward S, A mapped set of genetic markers for human ics 3: 361-366 (1988)

Report of the committee on the genetic omes 9 and 10. Cytogenet Cell Genet 51, 9)

6 Ozelius L, Kramer PL, Moskowitz CB, Kwiatkowski DJ, Brin MF, Bressman SB, Schuback DE, Falk CT, Risch N, de Lion D, Burke RE, Haines J, Gusella JF, Fahn S, Breakefield XO: Human gene for torsion dystonia located on chromosome 9q32-34 Neuron 2: 1427-1434 (1989)

7 Haines JL, Amos J, Atwood J, Bech-Hansen NT, Burley M, Conneally PM, Connor JM, Fahrold R, Fryer A, Kandt RS, Northrup H, Osborne J, Pericak-Vance MA, Povey S, Sampson JR, Short P, Smith M, Speer MC, Trofatter JA, Yates JR: Linkage heterogeneity in tuberous sclerosis Cytogenet Cell Genet 51, HGM10: 1010 (1989)

8 Kesaniemi YA, Miettinen TA: Cholesterol absorption efficiency regulates plasma cholesterol level in the Finnish population. Eur. J. Clin. Invest. 17: 391-395 (1987)

26934

Microsatellite DNA Polymorphisms

Weber JL, Kwitek AE, May PE, Wilkie PJ, Decker RA

See Abstracts for the committee on the genetic constitution of chromosome 1

26979

Mapping D9S39 by fluorescent in situ hybridization and by genetic linkage analysis.

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Probe pFF9.59.1 has been previously described (1). We have now mapped it by linkage analysis using the CEPH panel of families and by fluorescent in situ hybridization to metaphase chromosomes using the parent cosmid cFF9.59 as a probe.

The local linkage map

D9S27_a_(D9S9,D9S8)_b_D9S39_c_ASSP3

which was derived using the program MAP (2) has distances:

	a	b	c
males	3.12	0.00	1.15
females	0.68	7.15	11.02

assuming P(interference) = 0.35

The parent cosmid, cFF9.59, has been localized to 9q13 by fluorescent in situ hybridization using a the technique of reference 3.

1 Florian F, Hornigold N, Sefton L, Goodfellow PN, Abbott CM, Povey S, Wolfe J: The use of irradiated fragment gene transfer (IFGT) hybrids to isolate DNA probes from defined regions of chromosome 9 Cytogenet Cell Genet 51: 999 (1989)

2 Morton NM, Collins A: Standard maps of chromosome 10 Ann Hum Genet 54: 235-251 (1990)

3 Griffin DK, Leigh SEA, Delhanty jda: Use of Fluorescent in situ hybridization to confirm trisomy of chromosome region 1q32-qter as the sole karyotypic defect in a colon cancer cell line. Gen. Chrom. and Canc. 1: 281-283 (1990)

26980

Can irradiation hybrids constructed with a high dose of X-rays be used for mapping?

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Cox et al (1) have described a method of constructing physical maps of a chromosome based on the frequencies with which marker loci cosegregate in a panel of irradiation hybrids. In their experiments, a relatively low dose (8000rads) of X-rays was used to fragment the parent chromosome. A higher dose of X-rays would be expected to fragment the chromosome more frequently which might have the advantage that better resolution might be obtained between closely spaced loci. It will have the disadvantage that the range at which linkage between loci can be detected will be reduced proportionately which means that many more loci will need to be studied. We have attempted

27236

Assignment of the beta enolase locus, ENO3, to 17p13 by flourescent in situ hybridization..

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The alpha, beta and gamma isozymes of enolase are encoded by the loci ENO1, ENO3 and ENO2, respectively. The cDNAs for these enzymes are >80% homolgous at the nucleotide level (1,2). Using an alpha enolase cDNA clone (2) as a probe we have isolated a genomic clone from a cosmid library, and have used this in flourescent in situ hybridization experiments. Flourescent signals were localized on the short arm of chromosome 17, with no detectable flourescence on chromosomes 1 or 12. This indicates that the cosmid originates from the ENO3 locus, previously assigned using somatic cell hybrids to 17p (3). DAPI banding of metaphase spreads indicated that the ENO3 locus can be assigned to the terminal band, 17p13.

1 Cali L, Feo S, Oliva D, Giallongo A: Nucleotide sequence of a cDNA encoding the human muscle-specific enolase (MSE) Nucleic Acids Res 18: 1893 (1990)

2 Giallongo A, Feo S, Moore R, Croce CM, Showe LC: Molecular cloning and nucleotide sequence of a full-length cDNA for human alpha enolase Proc Natl Acad Sci U S A 83: 6741-6745 (1986)

3 Feo S, Oliva D, Barbieri G, Xu WM, Fried M, Giallongo A: The gene for the muscle-specific enolase is on the short arm of chromosme 17. Genomics 6: 192-194 (1990)

27247

Chromosomal localization of the ACT-2 cytokine.

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Act-2 belongs to a superfamily of structurally related proteins some of which are mediators of inflammation. Many of the proteins in this superfamily are small (70 amino acids) and conserve four cysteine residues. They can be divided into two groups on the basis of whether the first two cysteines are adjacent or separated by a single amino acid. Act-2 belongs to the first group. Act-2 is also highly homologous to murine Macrophage Inflammatory Protein 1 alpha and beta. Act-2 was originally identified as an activation cDNA in differential screening of a cDNA library prepared from mRNA from PBL stimulated with PHA plus PMA, and has been shown to be rapidly synthesized in T cells, B cells, and monocytes following appropriate mitogenic stimuli. This gene was assigned to 17q21-q23 using somatic cell hybrid and in situ hybridization analyses. This result is similar to, but slighdy different from, a previous report (Irving et al., 1990).

1 Irving SG, Zipfel PF, Balke J: Two inflammatory mediator cytokine genes are closely linked and variably amplified Nucleic Acids Res 18: 3261-3270 (1990)

Linkage Mapping of Charcot Marie (CMT1a)

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The mutation for CMT1a has been linl D17S58 and D17S71 which are located on chromosome 17 in two Australian pedigr families (1). The Australian families gave inc than 3.0 for D17S58. An additional large fan Australian dataset which also gives a lod sco marker. Fifteen smaller families have failed with either D17S58 or D17S71.

The three large Australian CMT1a famil been tested with D17S61, D17S62, D17 D17S82, 1516, MYH2 and D17S125 and point and multipoint linkage analysis. T location scores will be presented.

The results exclude the centromere and pr a locus for CMT1a and suggests that the lc Magenis deletion site. The precise locus lc data from the CMT collaborative.

1 Vance JM, Nicholson GA, Yamaoka LH, S MC, Hung W-Y, Roses AD, Barker D, Per Charcot-Marie-Tooth Neuropathy Type 1a Neurol 104: 186-189 (1989)

Chromosomal assignments of (endomembrane) proton pump subuni and VPP3/Vpp-3 (58 kDa) in human a

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ATP-driven proton pumps associat vesicles/synaptic vesicles are a group of p cellular processes through acidification These include intracellular targeting of secretory granules, and receptor-ligan mediated endocytosis. Eight to nine molecular masses of 116, 70, 58, 40, 38. been identified from clathrin-coated vesic the 70- and 58-kDa subunits are homo subunits of the mitochondrial ATPase an (2). The 116 kDa subunit shares a small Willebrand factor (vWF) and may funct hydrolysis-proton translocation coupli intramembranous components of the pur

We used a rat cDNA clone for the 116 for the 58 kDa subunits of the vacuolar p chromosomal loci in human and mo consisted of 16 rodent x human hybrid ce XVII, XVIII, XIX, XXI and 31; an contained Chinese hamster or rat x mou EAS, EBS and RTM (4). The 116 kDa locus VPP1 is on human chromosome 11. Hybridization of the 32P labelled

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The Use of Irradiation and Fusion Gene Transfer (IFGT) Hybrids to Isolate DNA Clones from Human Chromosome Region 9q33-q34

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Abstract—*We have generated somatic cell hybrids containing fragments of human chromosome arm 9q by an irradiation and fusion technique. No selection for human material was imposed, but of 23 clones analyzed most contained human DNA sequences and many contained multiple fragments of the human chromosome arm. A hybrid that appears to contain only two small fragments of human DNA from the regions q33 and q34 has been used as a source from which to clone probes specific to those areas of the chromosome.*

INTRODUCTION

The process of cloning a gene from knowledge of its map position is made easier if cloned DNA probes can be quickly obtained from the relevant region of the genome. Several somatic cell genetics methods exist to enrich for clones from a particular region. Human clones isolated from a somatic cell hybrid containing a single human chromosome or from flow-sorted human chromosomes are enriched for a specific target by 20–100 times compared with clones isolated from a whole genome library. If the target gene is linked to a selectable marker, to a cell surface antigen, or to an oncogene, then chromosome-mediated gene transfer offers the opportunity of even greater enrichment of the relevant region (1). This has proved useful in several cases, for instance to provide cloned probes in the vicinities of the cystic fibrosis gene (closely linked to the oncogene *MET*) (2) and the Y-linked testis determining gene (close to the cell surface antigen *MIC2*) (3). In these cases, CMGT hybrids offered approximately a 1000-fold enrichment of clones from the target area. Two considerations may limit the usefulness of this technique for enrichment of a particular region of the genome. The first is that most genes are not linked to convenient selectable markers and the second is that extensive interstitial deletions are frequently found in the fragments transfered (3). The technique of irradiation–fusion gene transfer (IFGT) (4) does not necessarily require the presence of a selectable human marker in the chosen region and there is so far little evidence of rearrangements within small fragments (5). This approach may have some advantages

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and has been used to isolate markers near the Huntington's chorea gene (6) and from the WAGR region of 11p13 (7). Here we report an attempt to enrich for the chromosome 9-linked tuberous sclerosis gene, *TSC1*, by IFGT.

TSC1 has been mapped by genetic linkage analysis to the region 9q33-34 (8). We have rescued random fragments of 9q in somatic cell hybrids by irradiating a hamster hybrid (64063a12) (9), which contains 9q as its only human genetic material. We then fused the lethally irradiated cells to an *HPRT*$^-$ hamster cell line and selected hybrids in HAT medium. This imposed no direct selection for human material. We screened the resulting panel of hybrid clones with markers from all regions of 9q and have used two methods in an attempt to isolate DNA probes from the target region 9q33-34.

MATERIALS AND METHODS

Cell Lines. The cell line 64063a12 is a hamster–human hybrid containing a single copy of human chromosome arm 9q (9). It is able to grow in HAT medium. WG3H is an hypoxanthine phosphoribosyl transferase (HPRT) deficient hamster cell line (4). OXEN is a human lymphoblastoid cell line (49XYYYY) (10), which was used as a human control on Southern blots.

Cell Culture. Attached cell lines were grown in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. HAT medium was made by adding hypoxanthine (10^{-4} M), thymidine (1.6×10^{-6} M), and aminopterin (10^{-5} M) to the basic medium. OXEN was grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Irradiation and Fusion Gene Transfer. Cells (5×10^6 64063a12) were harvested by trypsinization just before they had reached confluence and, after centrifugation at 1500*g* for 2 min, were resuspended in 20 ml serum-free medium. The cells were irradiated at room temperature until they had received 40,000 rad from an industrial X-ray unit (HF320 SR Pentak). An equal number of similarly harvested (but unirradiated) WG3H cells were mixed with the 64063a12 cells and fused with PEG and selected with HAT medium as described (11). Colonies appearing in the experiment were picked into 25 sq cm flasks and grown up. No colonies appeared in either the irradiated 64063a12 or the WG3H control platings. At an early stage in the growth, cells from each line were set aside to be frozen in liquid nitrogen and another aliquot was harvested to prepare an extract for AK1 assay. DNA was prepared when the cells had reached confluence in a 22-cm × 22-cm dish.

Enzyme Analysis. AK1 was assayed as published (12).

Southern Blot Analysis. DNA (10 μg) from each of the hybrids, the parental cell lines, and other controls were digested with the appropriate restriction endonuclease, electrophoresed through an agarose gel (0.6% or 0.8% as appropriate), and transferred to a nylon membrane (Amersham Hybond N). Blots were hybridised as described (13) either with ^{32}P DNA probes obtained by random primed synthesis using the Amersham Multiprime synthesis kit or with ^{32}P RNA probes obtained from RsaI digests of Lorist B recombinant cosmids using the Amersham SP6/T7 paired promoter kit. Where appropriate, probes were competed for 1 h at 68°C with 1 mg sonicated human DNA in 200 μl 5 × SSC before being applied to blots. The probes together with the restriction enzyme(s) used to remove the inserts from their vectors before labeling and the restriction enzyme used to digest the target DNA are shown in Table 1.

Polymerase Chain Reaction. PCR amplifications were carried out in a Hybaid machine in a volume of 50 μl (*ABL* and *ALDOB*) or 100 μl (*D9S15*). The reactions contained 1 μg of human DNA or 2.5 μg of hybrid DNA. *ABL* and *ALDOB* reactions contained 1 unit of Taq polymerase from Promega used according to the manufacturer's specifications (10× PCR buffer supplied;

Table 1.

Probe	Locus	Remove insert with	Digest target with	Reference
HHH 220	D9S18	PstI	TaqI	27
EKZ 130	D9S9	PstI + BamHI	MspI	28
pAS 1	ASSP12	PstI	EcoRI	29
pAS 1	ASSP3	PstI	PstI	29
ALDHI	ALDHI	EcoRI	HindIII	30
MCOA 12	D9S16	BamHI + PstI	PstI	27
ALAD SO2	ALAD	PstI	HindIII	31
pGEM4efod	SPTAN1	EcoRI	HindIII	32
v-abl	ABL	HindIII + BamHI	TaqI	33
pAS1	ASSG	PstI	PstI	29
DBH A4	DBH	EcoRI	EcoRI	34
MCT 136	D9S10	PstI + BamHI	PstI	27
EFD 126.3	D9S7	PstI + BamHI	TaqI	27
MHZ 10	D9S11	PstI	HindIII	27
pBK1.5	LINE	KpnI	HindIII	35

200 μm each dNTP; 25 pmol each oligonucleotide; 50 μl total volume). In addition the *ABL* reaction contained 20% glycerol. The other reactions were carried out in 100 μl total volume and included 50 pmol of primers, 1.5 mM dNTPs, 10% DMSO, 16.7 mM ammonium sulfate, 67 mM Tris HCl, pH 8.8, 10 mM mercaptoethanol, 6.7 mM EDTA, 0.17 mg/ml bovine serum albumin, and 1 unit of Taq polymerase from Anglian Biochemicals. Primers for the *ABL* locus were 5′ GGAGGGTGAAGGGCTTGAAAGGC 3′ and 5′ AGTCCGCCTGCACCAAGACTCCCT 3′, which amplify 278 nucleotides 5′ to exon 1B and for the *ALDOB* locus were as published (14). Primers specific to *D9S15* were kindly made available by Dr. S. Chamberlain (15). The reactions were incubated at 95°C for 5 min before the addition of enzyme and were then incubated at 94°C for 10 sec, 50°C for 30 sec, and 72°C for 30 sec for 30 cycles (*ABL* and *ALDOB*) or for 95°C for 30 sec, 52°C for 30 sec, 70°C for 30 sec for 30 cycles (*D9S15*).

Cosmid and Lambda Library Constructions. A cosmid library was constructed from 94063a12 in the vector Lorist B according to the method of Little (16). Colonies (5×10^4) of the unamplified library were plated at low density onto three 22-cm × 22-cm Hybond N membranes. Replica filters were screened with a ^{32}P-labeled probe obtained by random primed synthesis from human DNA. After washing in 40 mM sodium phosphate pH 7.2, 1% SDS at 65°C for 30 min, and autoradiography, colonies giving duplicate signals were picked and rescreened with human and hamster probes. The 168 clones gave strong signals with the human probe and weak or no signals with the hamster probe.

A lambda library of 7.5×10^5 clones was constructed from one of the IFGT hybrids, WG64 9A, in the vector EMBL3a according to the method of Maniatis et al. (17). The library was amplified and then 4×10^5 clones were screened with human DNA as above. There were approximately 110 duplicate signals, 70 of which were picked and plaque purified.

Fluorescent in Situ Hybridization. In situ hybridization conditions were as published (18). Modifications to take into account the nature of the probe DNA and to obtain a replication chromosome banding pattern in the final analysis were applied as follows when mapping phage clones. Biotinylation of whole-phage DNA (vector plus insert) (BRL kit) preceded purification in a Sephadex G50 column. In order to compete out repetitive elements in the insert DNA, the probe was ethanol-precipitated with 50-fold excesses of

fragmented salmon sperm DNA and yeast tRNA and a 500-fold excess of sonicated human placental DNA. After freeze drying, the pellet was dissolved in hybridization buffer [50% deionized formamide, 10% dextran sulfate, 2× SSC plus 50 mM sodium phosphate, pH 7.0 (SSCP)]. The solution was heated to 70°C for 5 min to denature the DNA and then allowed to reanneal at 37°C for 2½ h.

Extended chromosomes were prepared from phytohemaglutinin-treated male lymphocytes (18). Slides were stored at 4°C in a desiccator for two weeks then heated for 4 h at 56°C. Prehybridization treatment continued as described (19) with the following exceptions: Proteinase K was used at a concentration of 10 ng/ml and paraformaldehyde at a concentration of 1% in 0.9% NaCl, 1% $MgCl_2$. To denature chromosomal DNA, slides were heated at 80°C for 3 min in 70% deionized formamide SSCP then plunged into 70% ice-cold ethanol (2× 5 min). Ethanol dehydration (90% 5 min, 100% 5 min) preceded air drying. All the above steps were essential to obtain chromosome bands in the final analysis.

Preannealed probe (10 μl) was applied at a concentration of 10 ng/μl to the pretreated slides under a clean cover slip that was then sealed with rubber solution. Hybridization proceeded overnight at 37°C in a moist chamber. Coverslips were removed gently prior to the following posthybridization washes: 50% formamide, 2× SSC, 45°C, 3× 5 min; 0.1× SSC, 60°C, 3× 5 min; 4× SSC, 0.05% Tween 20 detergent, at room temperature, 5 min. FITC biotin detection was achieved as described (18) (multilayers of FITC avidin, biotinylated anti-avidin, and a final layer of FITC avidin), and slides were mounted wet in anti-fade medium [nine parts glycerol, one part *p*-phenyldiamine dihydrochloride (Sigma) 10 mg/ml, pH 8 in PBS] containing fluorescent counterstains (0.15 μg/ml DAPI and 1 μg/ml propidium iodide).

Analysis was performed on an MRC 600 confocal microscope (Biorad) equipped for simultaneous propidium iodide and FITC detection (filter sets A1 and A2) and with a Nikon Optiphot fluorescent microscope equipped for detection of DAPI and FITC fluorescence.

We achieved chromosomal band assignment in two ways: first by comparison with the fluorescent banding pattern on the chromosome—DAPI gives a replication G band pattern, propidium iodide a faint R band pattern; second by measurement of the distance from the p terminus of the chromosome to the signal expressed as a percentage of the total chromosome length. This was possible using software supplied with the confocal microscope. These values were then compared with standard ideograms.

RESULTS

Twenty-three colonies were picked after the irradiation and fusion gene transfer protocol. The series of hybrids was given the name WG64 followed by a number. Cell lines originating from colonies picked from the same dish were named with the same number but with a different letter suffix. Later analysis showed that most of these pairs were, if not truly independent, then at least different. Southern blots of the hybrid DNAs were hybridized to 13 probes revealing 14 loci on 9q and one on 9p. In addition, three loci (*D9S15*, *ALDOB*, and *ABL* exon 1B) were tested by PCR and one (*AK1*) by enzyme assay. The results are shown in Table 2. Four hybrids were negative with all single locus probes tested and are not shown in the table. However, when tested with the probe pBK1.5, which recognizes the LINE 1 repeated sequence element and which hybridizes to many loci on 9q, one of the four gave a small number of bands and the remaining three gave a weak signal in the position of the major repeat band, indicating that some human DNA was present. The majority of the hybrids contained multiple fragments of

Table 2.

Locus	Hybrid																		
	1A	1C	2A	2B	3A	3C	4A	4B	4C	5A	5B	6A	6B	8A	8B	9A	9C	10C	10D
D9S18																			
D9S48	::		::	::	::	::	::		::	::	::	::			::	::	■	■	::
D9S9	■	■			■	■		■			■						■		
D9S15	::	■	::	::	::	::	::	::		::	::	::			::	::			::
ASSP12		■		■	■	■	■						■					■	
ASSP3		■		■	■	■	■		■				■	■	■		■	■	
ALDH1	::	■	::	::	::	::	::	::		::	::	::	■		::	::			::
ALDB					■			::			■								
D9S16	■	■			■	■	■					■							
ALAD	■				■	■	■					■						■	
SPTAN1	::		::	■		::	::			■			::		::			::	::
AK1	■	■		■		■		■								■		■	
ABLex1B				::	::	■		::			::					::			
ABL				■		■											( )		
ASS			■	■		■		■											
DBH				■		■	■												■
D9S10				■			■									■			
D9S7		■		■	■	■	■												
D9S11				■	■	■			■				■						

"■ = locus present; no symbol = locus absent; :: = not tested; ( ) = very weak signal. The order of loci is a best guess based on the data of HGM10 (20,36) and on reference 37. The relative order of *ASS* and *ABL* is uncertain. The order shown is that most usually given, but the alternative order would reduce the number of apparent fragments in hybrid 4B to two.

human DNA. The probes are shown in Table 2 in the order in which they are believed to occur on the chromosome arm (20), and it can be seen that the number of fragments observed ranged from one to five. This must underestimate the true number because not all DNA fragments will contain one of the assayed markers. The frequency with which markers were retained varies from 47.8% (ASSP3) to 4.3% (ABL) in a way that seems to be position independent.

The best map position for the tuberous sclerosis gene *TSC1* is close to *ABL* (21) but a wider range both proximal and distal is by no means ruled out. We wished to use the hybrids as a source from which to clone DNA markers that could be used to refine the *TSC1* map position. Unfortunately, none of the hybrids is perfect as a source of probes from the desired interval. Consequently, we decided initially to see whether a subset of the hybrids could be used as a screen of cosmid clones derived from 64063a12 (which contained the whole of 9q) to find clones originating from the *AK1* region (which is close to *ABL* and within the "target zone" for *TSC1*). We chose hybrids WG64 1A, WG64 2B, and WG64 10C, which contained in common only *AK1*, out of the markers tested, and we screened them on Southern blots (together with human, hamster, and 64063a12 controls) using RNA or DNA probes derived from 23 randomly chosen cosmids. Only one clone, cFF9.59, fulfilled our screening requirements by hybridizing to all three hybrids. However, when we mapped an RFLP derived from this clone, it was discovered to originate from the proximal end of 9q whereas *AK1*, which we had anticipated to map close to the cosmid locus is much more distal at 9q34. Clearly the three hybrids used for the initial screening of cFF9.59 contained in common more than just the region of 9q containing *AK1*.

The second approach taken was to construct and screen a library from hybrid

WG64 9A. This hybrid contained in the initial screen only two markers, *D9S10* and *AK1*, which previously have been assigned to 9q34 and to 9q34.1-34.2, respectively (20). Both markers are in the target zone for *TSC1*, but they cannot be present on the same fragment of human DNA in WG64 9A because loci, including the very well mapped loci *ABL* and *ASS*, are missing from the interval between them. We constructed a genomic library in the lambda vector EMBL3a and isolated human clones from the library as described in Materials and Methods. From 4×10^5 clones, we obtained 110 human signals which, assuming that the cell line is approximately diploid (see Figure 2), means that WG64 9A contains approximately 1.25 Mb of human DNA [$110/(4 \times 10^5) \times (2.3 \times 10^9 \times 2)$]. The first five clones were localized by in situ fluorescence microscopy. Biotinylated probes were prepared from all five, but only three gave clean hybridizations; the other two gave a very high background despite careful prehybridization with unlabeled human DNA. Figure 1 shows the results obtained with the successful probes. Two gave good paired signals in 9q34.1, and the third gave a signal in 9q31. A second aliquot of cells was grown up and its chromosomes were examined by in situ fluorescence using human DNA as the probe. It contained three hybridizing regions, one small and two tiny (Fig. 2). This is consistent with the data of Table 2 and with the above results.

DISCUSSION

A gene for tuberous sclerosis has been mapped by genetic linkage analysis to 9q34 (8), but determining its exact position has proved to be very difficult for a number of reasons. These include its highly variable expressivity and extremely high mutation

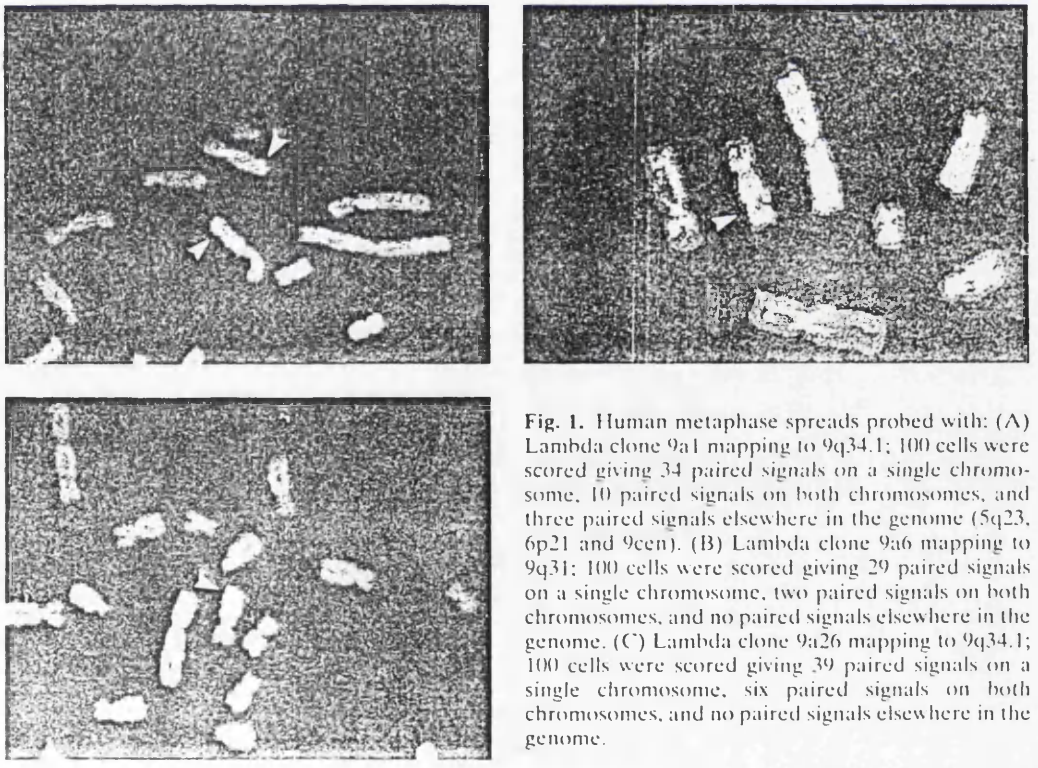

Fig. 1. Human metaphase spreads probed with: (A) Lambda clone 9a1 mapping to 9q34.1; 100 cells were scored giving 34 paired signals on a single chromosome, 10 paired signals on both chromosomes, and three paired signals elsewhere in the genome (5q23, 6p21 and 9cen). (B) Lambda clone 9a6 mapping to 9q31; 100 cells were scored giving 29 paired signals on a single chromosome, two paired signals on both chromosomes, and no paired signals elsewhere in the genome. (C) Lambda clone 9a26 mapping to 9q34.1; 100 cells were scored giving 39 paired signals on a single chromosome, six paired signals on both chromosomes, and no paired signals elsewhere in the genome.

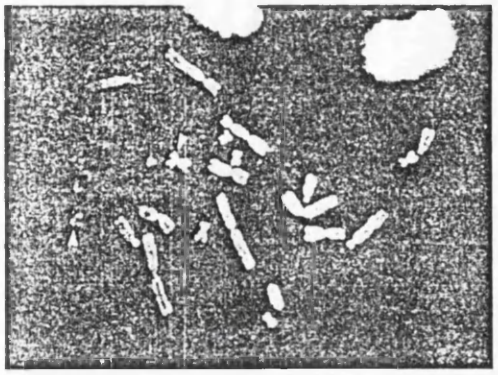

Fig. 2. Metaphase of hybrid WG64 9A probed with total human DNA. One larger fragment on one chromosome and two tiny fragments on a second chromosome are visible (arrowed). The centromeric tiny fragment appears as a single signal because the chromosome arms are close together, whereas the more distal tiny fragment is paired because the arms are splayed out.

rate. However, the primary problem is that a second mutant gene with apparently identical phenotype is also present in the population and is located on chromosome 11 (22). If genetic recombinants are found in a family between *TSC* and chromosome 9-linked markers, it is difficult to distinguish between a genuine recombination event on chromosome 9 involving *TSC1* and the possibility that that family is really segregating the chromosome 11-linked *TSC2*. We anticipate that closely linked highly polymorphic markers will clarify the genetics and lead to a closer localization of *TSC1*.

A number of strategies are possible to develop such markers. We have chosen to search first for a source of closely linked probes and later to try to find polymorphisms. Our panel of hybrids includes three that may be of value in developing probes from the 9q34 region. Of the markers tested, WG64 2A has been shown to contain only *ASS*, WG64 10D to contain only *DBH*, and WG64 9A to contain only *AK1* and *D9S10*. Furthermore, by measuring the frequency with which human clones were recovered from a genomic library made from WG64 9A, we were able to estimate the total amount of human DNA in its genome to be only 1.25 Mb. In this paper we have shown that this hybrid is an efficient source of probes from a subregion of 9q34 and from the neighboring region 9q31. In subsequent experiments we will examine them for polymorphisms.

We also tried to use a subset of the hybrids as a screen for clones derived from a particular region, the vicinity of *AK1*. However, this approach was not efficient because of unsuspected overlaps between human DNA contents of the hybrids chosen for the analysis. This technique would certainly benefit from typing the hybrids with additional probes. Then the pattern of human DNA fragments within each hybrid could be better judged. Furthermore, in retrospect, the screening criterion was too stringent. It would be preferable to chose hybrids that overlapped for two adjacent markers rather than for a single gene. This would increase the minimum area of overlap from a single gene to an interval and would increase the likelihood of finding a probe in the region.

Radiation hybrids have two possible uses. Their use as a cloning resource we have discussed in this paper. A second use is as a tool for mapping a chromosome by studying the frequencies with which markers are jointly retained—the closer two markers are on the chromosome, the more frequently they will both be retained in the same hybrid. Recent papers by Cox et al. (23) and by Falk (24) have demonstrated the utility of this approach using a panel of hybrids derived from chromosome 21. Cox et al. used a dose of only 8000 rads when irradiating the chromosome 21 parent hybrid, and consequently their hybrids contained on average fewer but larger fragments of the chromosome compared to the results that we obtained using a much larger radiation dose. For mapping, the low dose is probably preferable; however, for creating a cloning resource, the larger dose seems better. Several of our panel of hybrids contain only small regions of the target chromosome suitable for library construction and probe

isolation. Even some of those hybrids that contain multiple fragments of human DNA might be reduced to just a single fragment by subcloning when the fragments are carried on different chromosomes.

We have discussed the hybrids in the context of cloning *TSC1*, but there are two additional interesting uncloned genes in 9q34. Closely linked to *AK1* is *NPS1* (nail-patella syndrome 1) (25), which was part of the first three-locus human linkage group. Hybrid WG64 9A might provide a starting point for efforts to clone this gene. Also in 9q34 is the gene *DYT1*, torsion dystonia, which is closely linked to *ASS* (26). Fluorescence in situ hybridization experiments (not shown) have revealed the presence of one small and two tiny fragments of human DNA in the genome of hybrid WG64 2A (which contains only marker *ASS*). This cell line might be a good starting point for cloning *DYT1*. All hybrids are available for collaboration.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Porteus, D. (1987). *Trends Genet.* **3**:177–182.
2. Estiville, X., Farrall, M., Scambler, P.J., Bell, G.M., Hawley, K.M.F., Lench, N.J., Bates, G.P., Kruyer, H.C., Frederick, P.A., Stanier, P., Watson, E.K., Williamson, R. and Wainwright, B.J. (1987). *Nature* **326**:840–845.
3. Pritchard, C.A., and Goodfellow, P.N. (1986). *EMBO J.* **5**:979–985.
4. Goss, S.J., and Harris, H. (1975). *Nature* **255**:680–684.
5. Benham, F., Hart, K., Crolla, J., Bobrow, M., Francavilla, M. and Goodfellow, P.N. (1989). *Genomics* **4**:509–517.
6. Pritchard, C.A., Casher, D., Uglum, E., Cox, D.R., and Myers, R.M. (1989). *Genomics* **4**:408–418.
7. Rose, E.A., Glaser, T., Jones, C., Smith, C.L., Lewis, W.H., Call, K.M., Minden, M., Champagne, E., Bonetta, L., Yeger, H., and Houseman, D. (1990). *Cell* **60**:495–508.
8. Fryer, A.E., Chalmers, A., Connor, J.M., Fraser, I., Povey, S., Yates, A.D., Yates, J.R.W., and Osborne, J.P. (1987). *Lancet* **1**:659–660.
9. Jones, C., and Kao, F.T. (1984). *Cytogenet. Cell Genet.* **37**: 499–500.
10. Bishop, C.E., Guellen, G., Geldworth, D., Voss, R., Fellous, M., and Weissenbach, J. (1982). *Nature* **303**:831–832.
11. Goodfellow, P.N., Pritchard, C.A., and Banting, G.S. (1988). In *Genome Analysis: A Practical Approach*, (ed.) Davies, K.E. (IRL Press, Oxford), pp. 1–17.
12. Harris, H., and Hopkinson, D.A. (1979). *Handbook of Enzyme Electrophoresis in Human Genetics* (North Holland Press, Amsterdam).
13. Church, G.M., and Gilbert, W. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81**:1991–1995.
14. Abbott, C., and Povey, S. (1991). *Genomics* **9**:73–77.
15. Wallis, J., Williamson, R., and Chamberlain, S. (1990). *Hum. Genet.* **85**:98–100.
16. Little, P.F.R. (1987). In *DNA Cloning, Vol. III*, (ed.) Glover, D.M. (IRL Press, Oxford), pp. 19–42.
17. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).
18. Griffin, D.K., Leigh, S.E.A., and Delhanty, J.D.A., (1990). *Genes Chromosomes Cancer* **1**:281–283.
19. Wheater, R.F., and Roberts, S.H. *J. Med. Genet.* (1987). **24**:113–115.
20. Smith, M., and Simpson, N.E. (1989). *Cytogenet. Cell Genet.* **51**:202–225.
21. Povey, S., Attwood, J., Janssen, L.A.J., Burley, M., Smith, M., Flodman, P., Morton, N.E., Edwards, J.H., Sampson, J.R., Yates, J.R.W., Haines, J.L., Amos, J., Short, M.P., Sandkuyl, L.A., Halley, D.J.J., Fryer, A.E., Bech-Hansen, T., Mueller, R., Al-Ghazali, L., Super, M., and Osbourne, J. (1991). *Ann. N.Y. Acad. Sci.* **615**:298–305.
22. Smith, M., Smalley, S., Cantor, R., Pandolfo, M., Gomez, M.I., Baumann, R., Flodman, P., Yoshiyama, K., Nakamura, Y., Julier, C., Dumars, K., Haines, J., Trofatter, J., Spence, M.A., Weeks, D., and Conneally, M. (1990). *Genomics* **6**:105–114.

23. Cox, D.R., Burmeister, M., Price, E.R., Kim, S., and Myers, R.M. (1990). *Science* **250**:245–250.
24. Falk, C.T. (1991). *Genomics* **9**:120–123.
25. Schleutermann, D.A., Bias, W.B., Murdoch, J.L., and McKusick, V.A. (1969). *Am. J. Hum. Genet.* **21**:606–630.
26. Kramer, P.L., de Leon, D., Ozelius, L., Risch, N., Bressman, S.B., Brin, M.F., Schuback, D.E., Burke, R.E., Kwiatkowski, D.J., Shale, H., Gusella, J.F., Breakefield, X.O., and Fahn, S. (1990). *Ann. Neurol.* **27**:114–120.
27. Lathrop, G.M., Nakamura, Y., O'Connell, P., Leppert, M., Woodward, S., Lalouel, J.-M., and White, R. (1988). *Genomics* **3**:361–366.
28. Kumlin-Wolff, E., Nakamura, Y., Myers, R., Eldridge, J.R., O'Connell, P., Leppert, M., Lathrop, G.M., Lalouel, J-M., and White, R. (1987). *Nucleic Acids Res.* **15**:10611.
29. Daiger, S.P., Hoffman, N.S., Wildin, R.S., and Su, T.-S. (1984). *Am. J. Hum. Genet.* **36**:736–749.
30. Hsu, L.C., Tani, K., Fujiyoshi, T., Kurachi, K., and Yoshida, A. (1985). *Proc. Natl. Acad. Sci. U.S.A.* **82**:3771–3775.
31. Wetmur, J.G., Bishop, D.F., Cantelmo, C., and Desnick, R.J. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**:7703–7707.
32. McMahon, A.P., Giebelhaus, D.H., Champion, J.E., Bailes, J.A., Lacey, S., Carritt, B., Henchman, S.K., and Moon, R.T. (1987). *Differentiation* **34**:68–78.
33. Wang, J.Y.T., and Baltimore, D. (1983). *Mol. Cell. Biol.* **3**:773–779.
34. Lamouroux, A., Vigny, A., Faucon Biguet, N., Darmon, M.C., Franck, R., Henry, J.-P., and Mallet, J. (1987). *EMBO J.* **6**:3931–3937.
35. Shafit-Zagardo, B., Maio, J.J., and Brown, F.L. (1982). *Nucleic Acids Res.* **10**:3175–3193.
36. Lalley, P.A., Davisson, M.T., Graves, J.A.M., O'Brien, S.J., Womack, J.E., Roderick, T.H., Creau-Goldberg, N., Hillyard, A.L., Doolittle, D.P., and Rogers, J.A. (1989). *Cytogenet. Cell Genet.* **51**:503–532.
37. Wilkes, D., Shaw, J., Anand, R., Riley, J., Winter, P., Wallis, J., Driesel, A.G., Williamson, R., and Chamberlain, S. (1991). *Genomics* **9**:90–95.

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D12S56: a highly polymorphic locus on human chromosome 12q14

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SUMMARY

A CA repeat, subcloned from a cosmid, has been assigned to chromosome 12 by use of a panel of somatic cell hybrids. The assignment was confirmed by fluorescent *in situ* hybridization and the site further localized to 12q14. The repeat is highly polymorphic and should be useful for genetic mapping.

As part of a study to examine Y chromosome polymorphism, we have isolated clones containing CA repeats from a library of human cosmid clones derived from the mouse–human somatic cell hybrid 3E7 (Marcus *et al.* 1976). This somatic cell line contains a rearranged human Y chromosome as its only overt human genetic contribution. However, we have observed that a small percentage of clones in the library are autosomal in origin. Clones containing CA repeats were isolated by screening dot blots of 1767 human cosmids with the oligonucleotide $(GT)_{15}$ (hybridized in 0·5 M sodium phosphate, pH 7·2, 7% SDS, 1 mM-EDTA at 45 °C and washed in 40 mM sodium phosphate, pH 7·2, 1% SDS at 45 °C). Positive clones were digested with *Sau*3AI and subcloned into *Bam*HI cleaved M13mp9. Plaque lifts were screened under the above conditions with the $(GT)_{15}$ oligonucleotide and positives were sequenced. Oligonucleotide primers flanking the CA repeats were designed and used to amplify human male and female DNA. Y-chromosome derived, male-specific clones will be described elsewhere. However, one of the first three PCR products, derived from cosmid LOR 2.27.6, proved not to be male specific. The sequence spanning the CA repeat is shown in Fig. 1 in which the regions corresponding to our PCR primers are underlined. Amplification of cosmid DNA gave the expected product of 138 nucleotides. We performed PCR amplification on DNA from a panel of somatic cell hybrids (Table 1). No chromosome gave complete concordance with LOR 2.27.6 but chromosome 12

```
1                                                              50
GATCACGTGA GGTCAGGAGT TCGTGAGTAG CCTGGCCAGC ATGGTGAAAC
51                                                             100
CCCATCTCTA CTAAAAATAC ACACACACAC ACACACACAC ACACACACAC
101                                                            150
ACACACACAC ACACAATTAG CCAGGCATGG TGGCTTACGC CTGTAATCCC
151                                                            200
AGCTACTAGG GAGGCTGAGA CAGGAGAATT GCTGGAACCA GGAGGCAGAG
201                                233
GCTGCAGTGA GCTGAGATGC GCACAGCATC CAG
```

Fig. 1. The sequence spanning the CA repeat in cosmid LOR 2.27.6. The positions corresponding to the oligonucleotides used for PCR are underlined.

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Table 1. *Results of hybrid cell DNA analysis*

Hybrid	Ref.	*D12S56*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
DUR 4R3	1	+	−	−	+	−	+	−	−	/	−	−	+	+	+	+	−	−	+	+	−	/	+	+	−	/
PCT BA1·8	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	/
853	2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
TW1N 19D12	1	+	+	−	+	+	−	+	−	+	−	−	−	+	−	+	−	+	+	+	−	+	+	+	−	/
TW1N 19F9	1	+	+	P	+	+	−	+	−	+	+	−	−	+	/	+	−	−	+	+	−	+	+	+	−	/
FG10	1	−	−	+	−	−	+	/	−	+	/	+	−	−	−	−	+	−	−	+	−	−	+	−	+	/
FST 9/7	3	+	−	−	+	+	−	+	−	+	/	+	−	+	+	+	+	−	−	+	−	+	−	+	+	/
FST 9/10	1	+	−	−	+	+	−	+	−	+	/	+	−	+	+	+	+	−	−	+	−	+	−	+	+	/
MOG 2C2	1	+	+	−	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	/
MOG 2E5	1	+	+	−	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	−	−	+	+	+	/
HORP9.5	1	+	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−	−	−	+	+	/
SIF 1SP5	1	−	−	+	−	−	−	+	+	−	−	+	−	−	−	+	+	−	−	−	−	+	−	−	+	/
HORL411B6	1	−	+	−	+	−	−	−	−	−	−	−	+	−	+	−	+	−	−	+	−	−	−	+	+	/
1αA9602+	1	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	+	/
Concordant																										
+/+			4	1	7	6	4	5	2	6	2	5	3	8	4	8	4	3	5	7	1	5	6	8	6	0
−/−			4	3	4	5	4	3	4	4	4	3	4	5	4	4	2	5	4	3	5	4	4	4	2	0
Discordant																										
+/−			5	8	2	3	5	4	7	2	5	4	6	1	4	1	5	6	4	2	8	3	3	1	3	0
−/+			1	2	1	0	1	1	1	1	0	2	1	0	1	1	3	0	1	2	0	1	1	1	3	1

+, present; −, absent; /, not tested or equivocal result; p, short arm only of chromosome.
References: 1, Wong *et al.* (1987); 2, Burke *et al.* (1985); 3, Abbott *et al.* (1990).

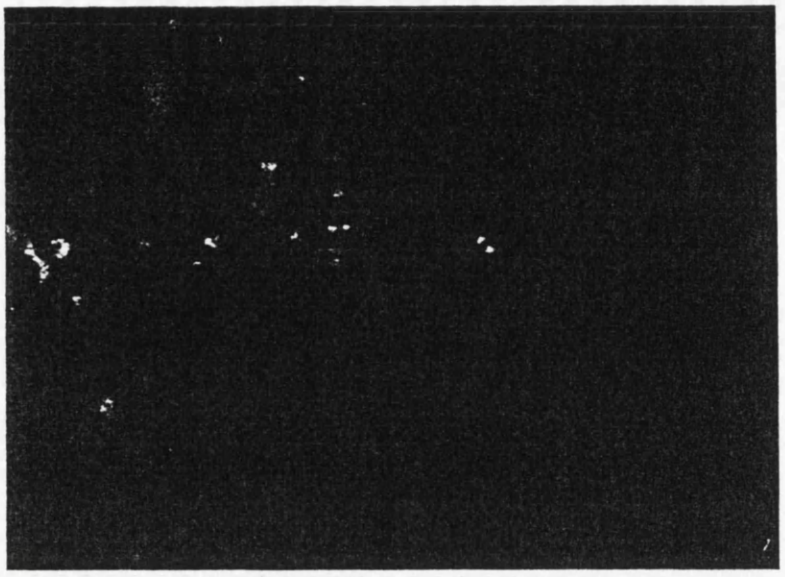

Fig. 2. Fluorescent *in situ* hybridization of LOR 2.27.6 to human metaphase chromosomes. A double paired signal at 12q14 was observed in 80% of metaphases.

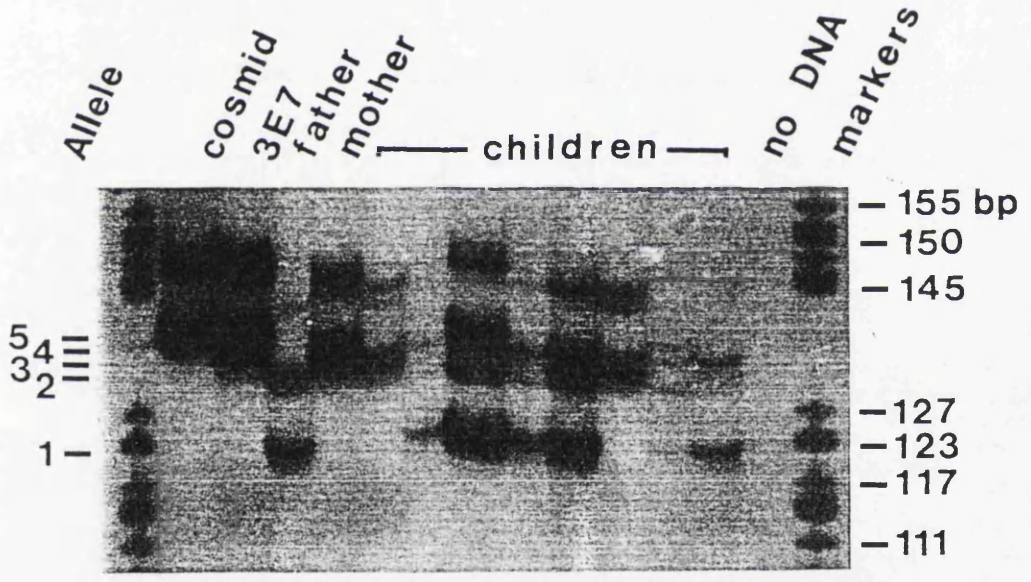

Fig. 3. PCR amplifications of cosmid LOR 2.27.6, hybrid 3E7 and a family consisting of father, mother and eight children. 100 μl reactions contained from 100 ng to 1 μg DNA, 1 μM each primer, 100 μM dATP, dGTP, dTTP, 6 μM dCTP, 0·6 μCi [α-^{32}P]dCTP (3000 Ci/mmol), 50 mM-KCl, 1·6 mM-$MgCl_2$, 10 mM Tris-HCl pH 8·4, 1 unit *Taq* DNA polymerase from Cetus. They were incubated for (1) 10 min at 95 °C (before the addition of polymerase); (2) 30 cycles of 94 °C, 45 s; 59 °C, 30 s; 72 °C, 30 s; (3) 10 min at 72 °C. 8 μl of each completed reaction was loaded onto a 6% polyacrylamide gel (21 cm × 40 cm × 0·4 mm) and electrophoresed at 50 W for 1·5 h in Tris borate EDTA buffer. The marker lane contains a *Hinf*I digest of bacteriophage lambda DNA end-labelled with Klenow and [α-^{35}S]dATP. In addition to the expected amplification product(s) in each lane are 'ghost' bands which migrate as though about 10 bp longer. Fainter ghosts were also visible higher up the gel. Cosmid LOR 2.27.6 and hybrid 3E7 both contain allele 5. Father = 1, 2; mother = 3, 4; children are 2, 3; 1, 3; 1, 4; 1, 3; 1, 3; 2, 3; 1, 4; 1, 3 respectively.

gave only a single instance of discordance. It is possible that the exceptional hybrid, MOG 2C2, which was positive despite apparently possessing no chromosome 12, may contain this chromosome in a very small minority of cells. We refined the localization to 12q14 by fluorescent *in situ* hybridization of the cosmid to metaphase chromosomes (Fig. 2) using standard techniques (Lichter *et al.* 1990).

PCR was performed on DNA from a number of individuals using conditions designed to incorporate [α-^{32}P]dCTP and the products were size fractionated on non-denaturing polyacrylamide gels. After autoradiography it was apparent that the size of the products is polymorphic with amplification products ranging from approximately 122 to 140 nucleotides. Seven different alleles were identified in 40 random individuals (mostly of Caucasian origin). The polymorphism segregated in a Mendelian fashion in one large two-generation family tested (Fig. 3).

Autosomal recessive vitamin D dependency type 1 (*VDD1*) has been mapped to 12q14 by linkage to three markers from this region, all of which were conventional RFLPs (Labuda *et al.* 1989). *D12S56*, which is likely to be more informative, might prove to be useful in the analysis of this disorder.

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REFERENCES

ABBOTT, C., PIAGGIO, G., AMMENDOLA, R., SOLOMON, E., POVEY, S., GOUNARI, F., DE SIMONE, V. & CORTESE, R. (1990). Mapping of the gene TCF2 coding for the transcription factor LFB3 to human chromosome 17 by polymerase chain reaction. *Genomics* **8**, 165–167.

BURKE, R. D., MA, P. & SMITH, K. D. (1985). Characterization and evolution of a single-copy sequence from the human Y chromosome. *Molec. Cell. Biol.* **5**, 576–581.

LABUDA, M., MORGAN, K. & GLORIEUX, F. H. (1989). Autosomal recessive, vitamin D dependency type I (VDD1) mapped to chromosome 12q by linkage analysis. *Cytogenet. Cell Genet.* **51**, 1027–1028.

LICHTER, P., CHIEM-JU, C. T., CALL, K., HERMANSON, G., EVANS, G. A., HOUSMAN, D. & WARD, D. C. (1990). High resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. *Science* **247**, 64–69.

MARCUS, M., TRANTRAWALI, R., DEV, J. G., MILLER, D. A. & MILLER, O. J. (1976). Human–mouse cell hybrid with human multiple Y chromosomes. *Nature* **262**, 65–67.

WONG, Z., WILSON, V., PATEL, I., POVEY, S. & JEFFREYS, A. (1987). Characterization of a panel of highly variable minisatellites cloned from human DNA. *Ann. Hum. Genet.* **51**, 290–298.

An intrachromosomal insertion causing 5q22 deletion and familial adenomatous polyposis coli in two generations

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Abstract

We report familial adenomatous polyposis coli (FAPC) with epidermoid cysts, osteomata, and areas of congenital hypertrophy of the retinal pigment epithelium (CHRPEs) in a male patient and his maternal aunt, both of whom suffered a mild to moderate degree of mental handicap. Both had an interstitial deletion of the long arm of chromosome 5 (del(5)(q22q23.2)). Two other normal family members had the underlying direct insertion of chromosome 5 (dir ins(5)(q31.3q22q23.2)). Molecular genetic and fluorescent hybridisation studies have shown that loci *D5S37* and *D5S98* are outside the deletion whereas loci detected by probes EF5.44 and YN5.48 are lost. As expected, the molecular analyses indicate loss of one allele at the MCC and APC loci. The APC gene is located within band 5q22. Familial direct insertions should be considered as a cause of recurrent microdeletion syndromes.

In 1986, Herrera *et al*[1] reported a mentally retarded male with Gardner's syndrome and an interstitial deletion of the long arm of chromosome 5 (del(5)(q13q15) or (q15q22)). In 1989 an apparently identical deletion of 5q was reported in two brothers with mild to moderate mental retardation, minor dysmorphic features, and FAPC.[2] In the intervening period, linkage of the APC gene to probes mapping in the region 5q21–q22 was shown[3,4] and more recently the APC gene itself has been identified.[5,6] To date, no evidence of genetic heterogeneity in this condition has been reported and the loss of heterozygosity for chromosome 5 in sporadic cancers has raised the possibility of a major role for this gene in colonic cancers.[7,8] We report a family in which FAPC has presented in two generations in handicapped subjects as a result of a familial direct insertion. This unique family contributes to our knowledge of the microdeletion syndrome in the vicinity of the polyposis gene, refines the chromosomal localisation of that locus, and illustrates a little recognised mechanism for recurrent deletion.

Material and methods

Chromosome analysis was performed on preparations from cell cultures of peripheral blood with G banding using trypsin and Leishman's stain. In situ hybridisation was carried out using biotinylated cosmid probes C Beg-1, obtained by screening a human genomic library with the probe ECB27,[9] which recognises the locus *D5S98*, and cL5.79.[5] Detection was by a fluorescein avidin conjugate and fluorescence microscopy.[10]

High molecular weight DNA for restriction analysis was prepared from fresh or frozen blood by standard methods.[11] Informative probe DNA clones used were as follows: pi227

Figure 1 The proband aged 25 years. Note the epidermoid cyst below his right eye.

Figure 2 The colon removed from the proband at colectomy showing the large adenoma in the ascending colon.

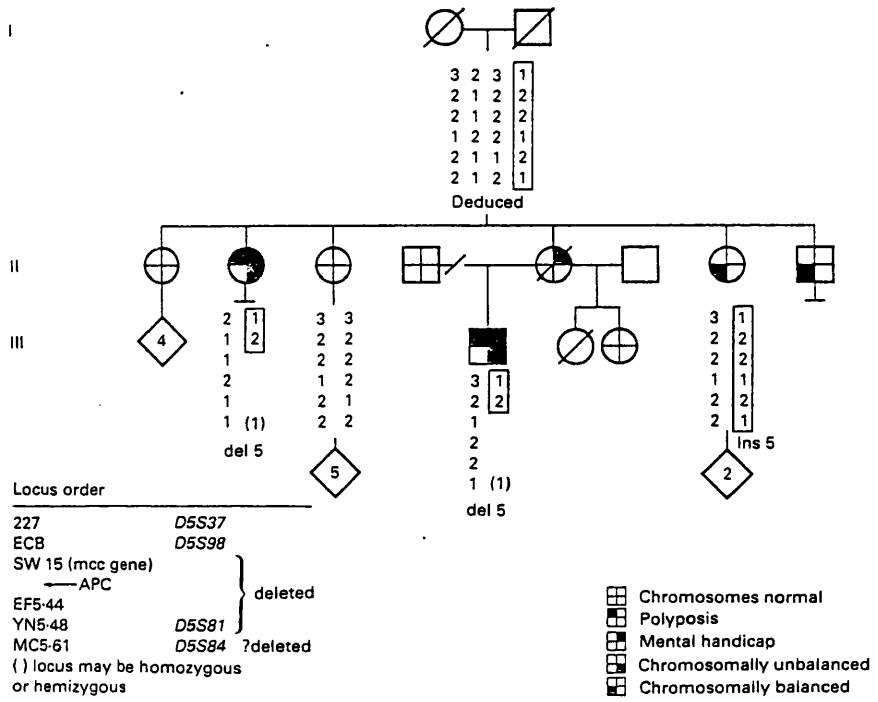

Figure 3 The pedigree of the family, together with a diagrammatic representation of the molecular genetic analysis. Probes distal to ECB27 (D5S98) up to, and possibly including, MC5.61 (D5S84) are deleted. The genotypes for the APC intragenic polymorphism are not shown but both affected subjects showed molecular genetic evidence of allele loss.

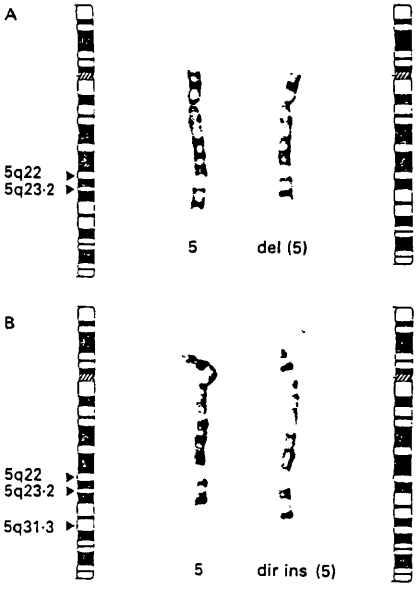

Figure 4 (A) Normal chromosome 5 (left) and del(5)(q22q23.2). (B) Normal chromosome 5 (left) and dir ins(5)(q31.3q22q23.2).

(*D5S37*)[12] as a 900 bp *Hin*dIII/*Eco*RI fragment in piAN7, ECB27 (*D5S98*)[9] as a 2.8 kb *Sal*I fragment in phage lambda, SW15 genomic subclone pL5.71–3 (for the MCC gene)[13] as a 3 kb *Hin*dIII fragment in pUC18, EF5–44[14] as a 1.9 *Eco*RI/*Hin*dIII fragment in pUC18, YN5.48 (*D5S81*)[14] as a 2.4 kb *Taq*I fragment cloned into the *Acc*I site of pUC18, MC5.61 (*D5S84*)[15] as a 3 kb *Taq*I fragment in pUC18 and FB54D, a 2.3 kb fragment of cDNA from the APC gene[5] which recognises an *Msp*I polymorphism.

Five to ten micrograms of purified DNA was digested with the appropriate restriction endonuclease: *Taq*I for the probe MC5.61, *Bcl*I for pi227, *Bgl*II for ECB27, and *Msp*I for SW15, EF5.44, YN5.48, and FB54D. The DNA was then fractionated in 0.6 to 1.5% agarose gel and transferred to Hybond-N Plus nylon filters (Amersham) as recommended by the manufacturers. Before prehybridisation the DNA was cross linked to the membrane by exposing it to 254 nm UV light for three minutes. Prehybridisation was carried out in 0.5% SDS, 10% dextran sulphate, and 0.8 mol/l NaCl for 15 minutes to several hours at 65°C. Probes were radiolabelled with α-^{32}P-dCTP(3000 Ci/mmol^{-1}) by the random hexanucleotide primer method to a high specific activity,[16] and hybridised to the filter at 65°C overnight in the presence of 10 μg l^{-1} salmon sperm DNA. Filters were washed to a stringency of 2 x SSC, 1% SDS and autoradiographed at −70°C using Fuji RX-L *x* ray film.

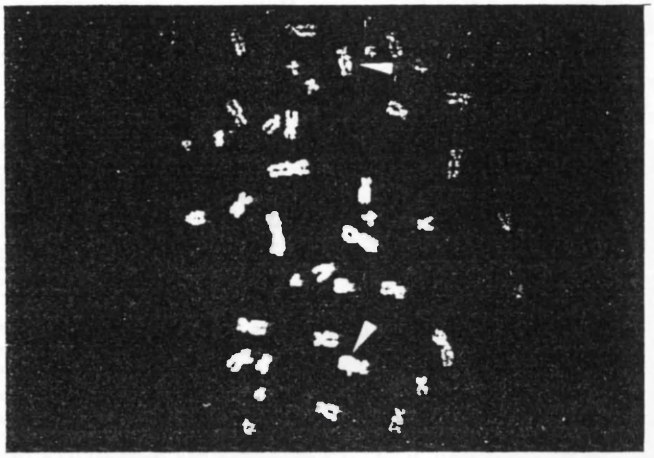

Figure 5 Metaphase from the proband after in situ hybridisation with C Beg-1, showing locus D5S98 to be present on both the normal and the deleted 5.

Case report

A 25 year old male with mild mental handicap presented with iron deficiency anaemia and positive occult faecal blood. Upper gastrointestinal endoscopy showed two severely dysplastic duodenal adenomata. Sigmoidoscopy was normal, but multiple adenomatous polyps were found at colonoscopy, predominantly on the right side of the colon. Clinical assessment suggested a particular impairment of expressive speech. He had a long midface (fig 1), a receding hairline, multiple epidermoid cysts, bilateral areas of congenital hypertrophy of the retinal pigment epithelium (CHRPEs), and multiple mandibular osteomata. Full thickness resection of the duodenal adenomata was performed followed six months later by colectomy and ileorectal anastomosis. The colon contained more than 100 adenomata with one large lesion in the ascending colon (fig 2). There were no visible lesions in the descending colon though microadenomata were found at histological examination.

Shortly afterwards, the proband's 54 year old mentally retarded maternal aunt presented with abdominal pain. She had no significant dysmorphic features or lipomas but had a spastic gait and severe obstructive airways disease. Gastroscopy was normal but colonoscopy again showed multiple colonic polyps. An abdominal ultrasound examination found bilateral adrenal masses; however, owing to the patient's frail state, no further invasive investigation was performed. Indirect fundoscopy showed multiple bilateral CHRPEs.

The proband's mentally retarded mother died in early adulthood from subarachnoid haemorrhage and only a limited necropsy was performed. His father is untraceable. All other living members of the family (fig 3) are of normal intelligence and showed no evidence of FAPC. Of possible interest is the observation that all family members carrying the balanced insertion suffer from dyslexia.

LABORATORY RESULTS

The proband showed an abnormal 46,XY, del(5)(q22q23.2) karyotype (fig 4A) as did his mentally retarded aunt. A maternal aunt and a maternal uncle, both of whom were mentally normal with no evidence of FAPC, showed an intrachromosomal insertion of chromosome 5: dir ins(5)(q31.3q22q23.2) (fig 4B). The aunt had no evidence of CHRPEs on eye examination. All other members of the family so far investigated showed apparently normal karyotypes.

In situ hybridisation showed locus *D5S98* to be present on both the normal and the deleted 5 in chromosome preparations from the proband (fig 5). Southern blot analysis of DNA extracted from blood of the retarded aunt with FAPC indicated heterozygosity at the *D5S98* locus, confirming that this locus is outside the deleted segment. The cosmid probe cL5.79 hybridised to all four chromatids in over 90% of control metaphases, but was consistently seen on one chromosome only in the proband's cells. This indicates deletion of the locus recognised by this probe.

Southern analysis of DNA extracted from blood of the proband and three of his aunts (fig 3) using seven informative DNA probes allowed molecular definition of the deletion. Probe data made possible deduction of parental haplotypes for the aunts. Comparison of the deleted and inserted chromosomes showed that loci *D5S37* and *D5S98* are outside the deletion whereas loci detected by probes SW15, EF5.44, and YN5.48 (*D5S81*) are deleted (fig 3). The results are compatible with *D5S84* also lying within the deletion but are not conclusive. The smaller allele alone from the *Msp*I polymorphism detected by the probe FB54D was present in the proband, whereas only the larger band was present in the affected aunt. Since they have the deleted chromosome in common these results provide evidence of allele loss at the APC locus.

Discussion

From the pedigree, it can be inferred that the proband's mother carried the familial abnormality of chromosome 5 in one form or another, and the fact that she was mentally handicapped suggests that she probably carried the deletion (del(5)(q22q23.2)). The proband's maternal grandparents were described as being of normal intelligence; it can be inferred that one of them carried the balanced insertion, dir ins(5)(q31.3q22q23.2). The interstitial deletion observed in members of this family affected by FAPC could have been produced by a single crossover event between the inserted chromosome 5 and the normal chromosome 5 in meiotic prophase. For complete meiotic pairing of the inserted 5 with its normal homologue, a double insertion loop must form (fig 6). A single crossover within the insertion loop for the region 5q23.2–5q31.3

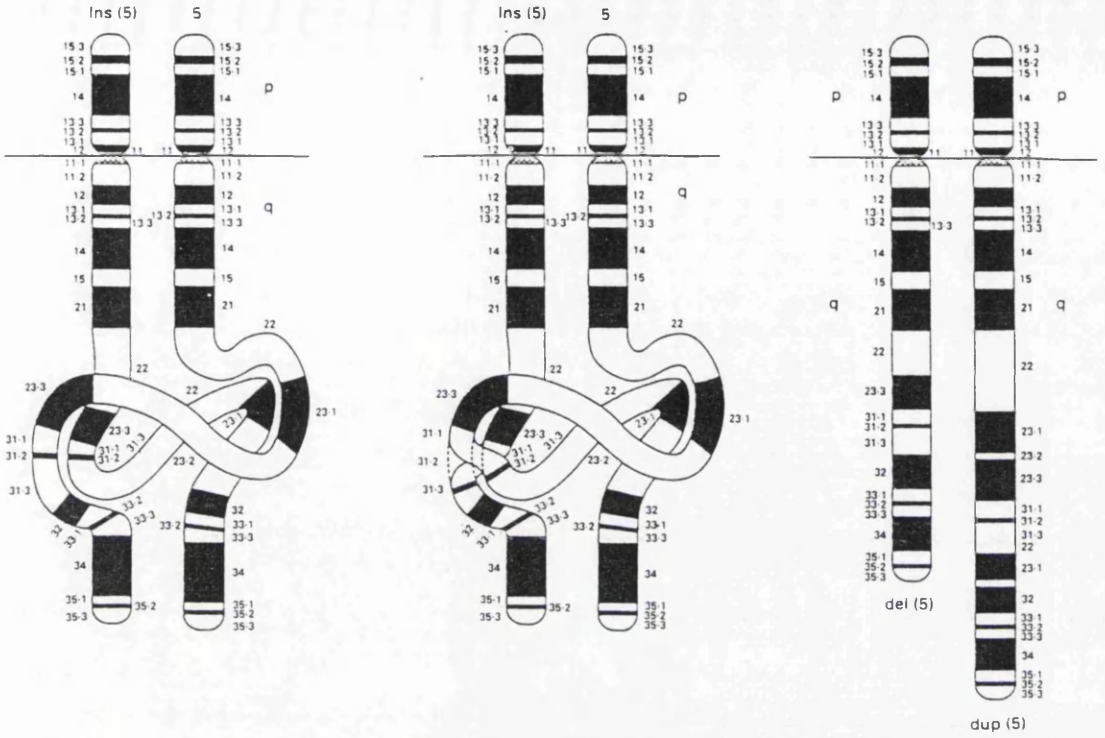

Figure 6 Diagram to show the normal chromosome 5 and the dir ins(5) forming a double insertion loop at meiosis. A single crossover within the region 5q23.3–5q31.3 gives rise to gametes carrying rec del(5) or rec dup(5).

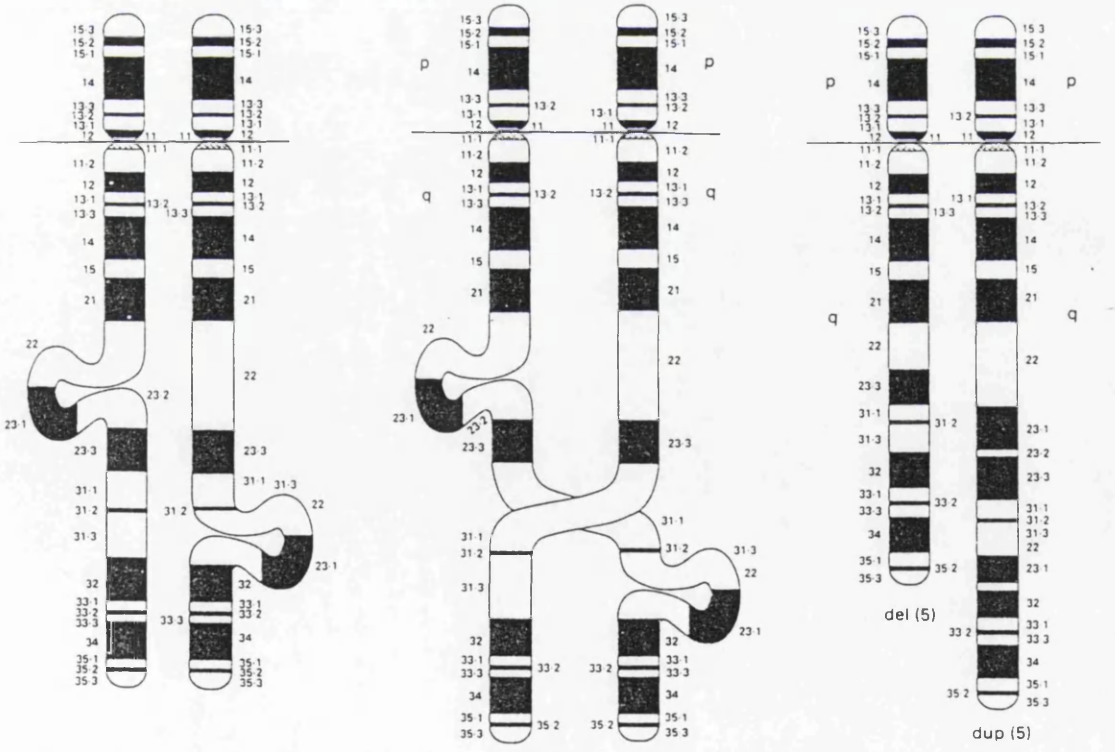

Figure 7 Diagram to show the normal chromosome 5 and the dir ins(5) pairing at meiosis with the inserted region (5q22–q23.2) forming unpaired loops. A single crossover between the loops gives rise to gametes carrying rec del(5) or rec dup(5).

would then result in one daughter chromosome with the deletion (rec del(5)(q22q23.2) dir ins(5)(q31.3q23.2)) and one daughter chromosome with a duplication of the same region (rec dup (5)(q22q23.2) dir ins(5)(q31.3q22q23.2)).

Alternatively, meiotic pairing of the chromosome 5 homologues in subjects with the insertion may occur as in fig 7, with the inserted segment (5q22–5q23.2) forming unpaired loops. Again a single crossover between the two unpaired loops would produce one deleted daughter chromosome (rec del(5)(q22q23.2)dir ins(5)(q31.3q22q23.2)) and one duplicated daughter chromosome (rec dup(5)(q22q23.2)dir ins (5)(q31.3q22q23.2)).

A recent review of cases of intrachromosomal insertions[17] suggested that duplication recombinants from insertions are more frequently encountered than deletion recombinants such as have occurred in this family. The proband did not display overt dysmorphic features though his long midface and receding hairline resembled the features of the brothers described by Hockey *et al.*[2]

The fact that in situ hybridisation showed ECB27 to be present on both the normal and deleted chromosome 5 in the proband placed the proximal breakpoint of the deletion between that marker and cL5.79 which is deleted.

Southern analysis showed that the deletion encompassed loci telomeric to cL5.79 detected by the probes SW15 (MCC gene), FB54D (APC gene), EF5.44, and YN5.48. Using information available from published molecular genetic studies,[5] our results suggest that the deletion is from 2 to 5 Mb in size.

Previous linkage mapping studies[4 15] have suggested that the FAPC gene is located in band 5q22 close to the junction with band 5q21, whereas the most recent publications state that the locus is in 5q21 without presenting further data. The proximal breakpoint of the deletions in this family appears to be at approximately the middle of the band 5q22, indicating that the gene for FAPC is located in the distal half of band 5q22.

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1 Herrera L, Kakati S, Gibas L, Pietrzak E, Sandberg AA. Gardner syndrome in a man with an interstitial deletion of 5q. *Am J Med Genet* 1986;25:473–6.
2 Hockey KA, Mulcahy MT, Montgomery P, Levitt S. Deletion of chromosome 5q and familial adenomatous polyposis. *J Med Genet* 1989;26:61–8.
3 Bodmer W, Bailey C, Bodmer J, *et al.* Localisation of the genes for familial adenomatous polyposis on chromosome 5. *Nature* 1987;328:614–6.
4 Meera Khan P, Tops CMJ, vd Breukel C, *et al.* Close linkage of a highly polymorphic marker (D5S37) to familial adenomatous polyposis (FAP) and confirmation of FAP localisation on chromosome 5q21–q22. *Hum Genet* 1988;79:183–5.
5 Kinzler KW, Nilbert MC, Su L-K, *et al.* Identification of FAP locus genes from chromosome 5q21. *Science* 1991;253:661–5.
6 Groden J, Thliveris A, Samowitz W, *et al.* Identification and characterisation of the familial adenomatous polyposis gene. *Cell* 1991;66:589–600.
7 Solomon E, Voss R, Hall V, *et al.* Chromosome 5 allele loss in human colorectal carcinomas. *Nature* 1987;328:616–9.
8 Vogelstein B, Fearon ER, Hamilton SR, *et al.* Genetic alterations during colorectal tumor development. *N Engl J Med* 1988;319:524–32.
9 Varesco L, Thomas HJW, Cottrell S, *et al.* CpG island clones from a deletion encompassing the gene for adenomatous polyposis. *Proc Natl Acad Sci* 1989;86:10118–22.
10 Lichter P, Tang C-JC, Call K, *et al.* High resolution mapping of human chromosome 11 by in situ hybridisation with cosmid clones. *Science* 1990;247:64–9.
11 Maniatis T, Frisch EF, Sambrook J. *Molecular cloning: a laboratory manual.* New York: Cold Spring Harbour Laboratory, 1982.
12 Stewart GD, Bruns GAP, Wasmuth JJ. An anonymous DNA segment (Pi227) maps to the long arm of human chromosome 5 and identifies a BstXI polymorphism (D5S37). *Nucleic Acids Res* 1987;15:3939.
13 Kinzler KW, Nilbert MC, Vogelstein B, *et al.* Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science* 1991;251:1366–70.
14 Dunlop MG, Nakamura Y, Wyllie AH, *et al.* Genetic linkage map of six polymorphic DNA markers around the gene for familial adenomatous polyposis on chromosome 5. *Am J Hum Genet* 1990;47:982–7.
15 Nakamura Y, Lathrop M, Leppert M, *et al.* Localization of the genetic defect in familial adenomatous polyposis within a small region of chromosome 5. *Am J Hum Genet* 1988;43:638–44.
16 Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983;132:6–13.
17 Vekemans M, Morichon-Delvalez N. Duplication of the long arm of chromosome 13 secondary to a recombination in a maternal intrachromosomal insertion (shift). *Prenat Diagn* 1990;10:787–94.

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Human Genetics

Dual fluorescent in situ hybridisation for simultaneous detection of X and Y chromosome-specific probes for the sexing of human preimplantation embryonic nuclei

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Summary. Dual fluorescent in situ hybridisation has been used for the simultaneous detection of X and Y chromosome-specific probes in single cleavage nuclei from disaggregated 4- to 7-cell human embryos. Based on the presence of a Y signal or 2 X signals in the absence of a Y, 89% of poor quality metaphases and 72% of interphase nuclei could be classified as male or female. With further refinements, this technique will offer a credible alternative to the polymerase chain reaction for the diagnosis of sex in human preimplantation embryos in families segregating for X-linked genetic disease.

Introduction

The application of molecular genetic techniques has resulted in considerable success with regard to prenatal diagnosis of single gene defects. However, for the majority of sex-linked recessive conditions, the molecular basis is unknown and selective abortion of all male fetuses remains the only option. An alternative for these families is offered by the diagnosis of sex in preimplantation embryos. In vitro fertilisation (IVF) allows several embryos to be biopsied and screened simultaneously for genetic defects prior to selective transfer. Cleavage stage embryo biopsy and removal of one or two cells at the 8-cell stage does not adversely affect preimplantion development (Hardy et al. 1990). Handyside and colleagues (1989) have reported the accurate sexing of embryos from single cells biopsied at this stage using the polymerase chain reaction (PCR) to amplify a repeated sequence specific for the long arm of the Y chromosome. Using this approach, pregnancies have been established and female births have resulted following transfer of embryos identified as females (Handyside et al. 1990 and unpublished observations). However, 1 out of 7 fetuses was shown by chorion villus sampling and karyotyping to be male, and the pregnancy was terminated at 11 weeks of gestation.

Sexing of the basis of the presence or absence of an amplified DNA fragment could lead to misdiagnosis for a number of reasons. For example, because of the sensitivity of PCR, females could be diagnosed as males, if contamination by male DNA had occurred. More seriously, males could be diagnosed as females by inadvertent sampling of anuclear cytoplasmic fragments or simply by the failure of amplification from single cells (Handyside 1991; Kontogianni et al. 1991).

An alternative to the use of PCR is in situ hybridisation (ISH) of X or Y chromosome-specific probes to fixed nuclei. The earliest approach involved the use of radiolabelled Y-specific probes for sexing whole fixed human embryos (West et al. 1988); a later improvement was the application of a streptavidin-linked alkaline phosphatase system to detect biotinylated probes (Penketh et al. 1989). Recently, we have described the use of a fluorescent technique that combines both speed and high sensitivity with either an X or Y chromosome-specific probe (Griffin et al. 1991).

Our experience with these probes led to the realisation that the use of either probe alone could lead to misdiagnosis because of the failure of hybridisation and the incidence of polyploid cells (Griffin et al. 1991). In this paper, we demonstrate the simultaneous detection of X and Y specific-sequences using dual fluorescent in situ hybridisation (FISH) to the nuclei of single blastomeres disaggregated from early cleavage stage human embryos. The technique is successful in both metaphase and interphase nuclei and provides a basis on which to proceed to clinical application.

Materials and methods

Fertilised human embryos undergoing regular cleavage were obtained following IVF. All embryos were surplus to the patients' needs and were donated with informed consent. A total of 48 single blas-

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tomeres that had been disaggregated from twenty two 4- to 7-cell human embryos on day 2 post-insemination were used in these experiments.

To arrest blastomeres in metaphase, intact embryos were incubated for 14–18 h in Earle's balanced salt solution (Gibco) containing 27 m*M* sodium bicarbonate, 0.4 m*M* pyruvic acid supplemented with 10% heat-inactivated maternal serum and 1.25 μ*M* colchicine (Sigma, Poole, UK), in an atmosphere of 5% CO_2 in air at 37°C.

Embryos were briefly washed in acid Tyrode's solution to remove the zonae pellucidae and were separated into individual blastomeres by mechanical dispersion through a flame-polished pipette. To verify that cells were arrested in metaphase, the nuclei were examined by fluorescence microscopy after labelling for 10 min in medium M2 (Quinn et al. 1982) containing 0.01 mg/ml of the polynucleotide-specific fluorochrome bisbenzimide Hoechst 33342 (Kontogianni et al. 1991). This enabled blastomeres with metaphase chromosomes to be distinguished from those containing interphase nuclei.

The cytogenetic preparation of blastomeres arrested in metaphase was carried out according to Kola and Wilton (1991). Briefly, cells were incubated in 0.6% sodium citrate and then transferred in a minimal volume of this solution to a clean microscope slide. Cells were fixed by addition of several drops of 3:1 methanol:acetic acid followed by a single drop of 70% acetic acid. To confirm the location of the cell, the slides were stained in 10% Giemsa in phosphate buffered saline for 30 min and the chromosomes were examined using bright field or phase contrast microscopy. Interphase nuclei for FISH were obtained using the same techniques except that the embroys were not incubated in colchicine.

DNA probes

Probe pHY2.1 (Cooke et al. 1982) recognises a 2.45-kb repeat on Yq, and probe cY98 (J. Wolfe, personal communication) also recognises a 3.4-kb repeat on Yq. Both were nick-translated with digoxigenin-11-dUTP (BRL kit) and purified in a Sephadex G50 column.

Probe pBam X7 (Willard 1985) preferentially recognises an alphoid tandem repeat on the centromere of the X chromosome and was supplied in a biotinylated form (ONCOR, Gaithersburg, USA).

Fluorescent in situ hybridisation

The FISH technique was adapted from Pinkel et al. (1986) and Heiles et al. (1988) by Dr. Joop Wiegant and colleagues at the University of Leiden. Further adaptations were made in this study. Prior to hybridisation, slides were washed in 0.9% NaCl (10 min), dehydrated, (5 min each in 70%, 90%, 100% ethanol) and air-dried. To remove the excess cytoplasm associated with cleavage nuclei, RNase (100 μg/ml in 2 × SSC, 37°C, 1 h) and proteinase K (500 ng/ml in TC: 20 m*M* TRIS-HCl, 2 m*M* $CaCl_2$, pH 7.4; 37°C, 7 min) treatments were applied followed by their respective washes (2 × 5 min in 2 × SSC, room temperature, and 5 min in TC, 37°C after RNase; 5 min in 0.9% NaCl, 1% $MgCl_2$, room temperature, after proteinase K). Preparations were then re-fixed in 1% formaldehyde, 0.9% NaCl, 1% $MgCl_2$ (10 min, room temperature), washed in 0.9% NaCl, dehydrated and air-dried again.

The probe mix dissolved in 62.5% formamide, 2 × SSC, in 50 m*M* phosphate buffer was applied and sealed under a coverslip. For each hybridisation, a volume of 10 μl was used containing 1 ng/μl biotin-labelled pBamX7, and 2 ng/μl each digoxigenin labelled pHY2.1 and CY98. Probe and chromosomal DNA denaturation was achieved simultaneously by heating at 80°C for 3 min followed by hybridisation overnight at 37°C. Post-hybridisation washes were as follows: 3 × 5 min in 62.5% formamide in 2 × SSC at 37°C, 2 × 5 min in 2 × SSC, room temperature, and 5 min in 4 × SSC with 0.05% Tween 20 (Sigma) (SSCT), room temperature.

A four-step procedure facilitated probe detection and each step was followed by 3 × 5 min washes at room temperature. Step 1: 4 × SSC containing 5% non fat dry milk (Marvel) (SSCM), 20 min, room temperature (pre-incubation step). Texas red Avidin D (Vector, Peter-borough, UK) (1:200) in SSCM, 20 min, room temperature. Washes: 5 min in SSCT; 2 × 5 min in 0.1 *M* TRIS-HCl, 0.15 *M* NaCl, pH 7.4, 0.05% Tween 20 (TNT). Step 2: Biotinylated anti-avidin D (Vector) (1:100) and mouse anti-digoxin antibody (Sigma) (1:1000) in 0.1 *M* TRIS-HCl, 0.15 *M* NaCl, pH 7.4, 0.5% Boehringer, Lewes, UK, blocking reagent (TNB), for 30 min at 37°C. Washes: 3 × 5 min in TNT. Step 3: Texas red Avidin D (1:200) and fluorescein isothyocyanate labelled (FITC) rabbit anti-mouse antibody (Sigma) (1:1000) in TNB, 30 min at 37°C. Washes: 3 × 5 min in TNT. Step 4: FITC goat anti-rabbit antibody (Boehringer) (1:1000) in TNB. Washes: 5 min in TNT, 2 × 5 min in 0.9% NaCl.

Finally, slides were mounted in anti-fade medium (0.1% p-phenyl diamine dihydrochloride, 90% glycerol), containing 0.15 μg/ml DAPI (4,6 diamidino-phenyl indole) DNA counterstain; they were examined using a Reichert Jung polyvar fluorescence microscope. Blue DAPI counterstain was detected by the U1 filter set; filter B1 detected the green FITC (Y chromosome) signal and filter G2 detected the Texas red (X chromosome) signal. Scoring of embryonic nuclei was carried out in the absence of knowledge of the source of each cell. Preparations were photographed using Scotch 640T film. Metaphases and interphases from human lymphocytes were used as control material.

Results

Following FISH with X- and Y-specific probes, diploid nuclei exhibit two spots, both red in females, one green and one red in males. In control metaphases, probe hybridisation is visible as red fluorescence at the centromere of the X chromosome and green on the long arm of the Y chromosome. In lymphocyte controls, 87%–89% of interphases and 100% of metaphases gave the expected number and type of signals. Embryonic nuclei were classified as male by the presence of a Y signal or female if 2 X signals were present in the absence of a Y. A high percentage of single embryonic nuclei were arrested at metaphase, but the quality of the chromosome preparations obtained did not allow exact counting or analysis. Despite this, 25 out of 28 metaphases (89%) could be classified as male or female using FISH (Fig. 1). All 12 embryos therefore could be sexed, and there was no case in which single cells from the same embryo gave conflicting results (Table 1).

Eighteen interphase nuclei were available from 11 embryos; 13 (72%) gave a clear result (Fig. 2, Table 2). Two embryos (nos. 1 and 4), from which only a single cell was available, could not be sexed as the hybridisation result was obscured by autofluorescent foreign bodies, a factor that also led to an initial misdiagnosis in one cell from a third embryo (no. 10). The remaining 8 embryos could be sexed according to our criteria.

Discussion

We have described a rapid and effective method of sexing human embryos by means of dual FISH. As expected from control data, efficiency was considerably higher in metaphases than in interphases; however, even in the latter, hybridisation efficiency is greater than with other ISH procedures (Jones et al. 1987; West et al. 1987, 1988; Penketh et al. 1989; Grifo et al. 1990). Our earlier

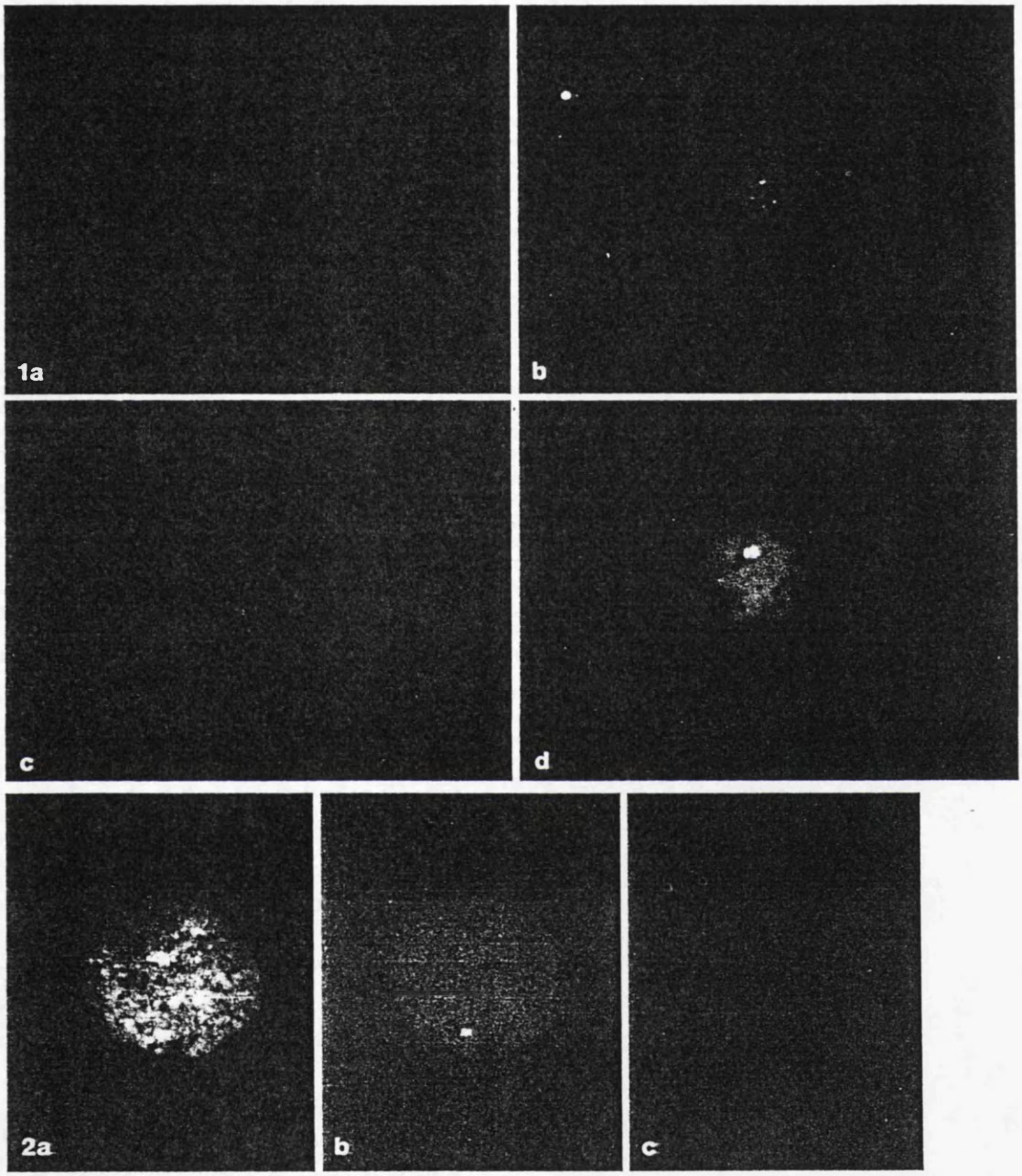

Fig. 1a–d. Dual FISH to single embryonic metaphase nuclei from two embryos. **a, b** First embryo. **a** Two X signals detected by Texas Red. **b** Absence of a Y signal after FITC detection: embryo classified as female. **c, d** Second embryo. **c** A single X signal detected by Texas Red. **d** A signal Y signal detected by FITC: embryo classified as male

Fig. 2a–c. Dual FISH to a single embryonic interphase nucleus, classified as male. **a** Blue DAPI counterstain for total DNA. **b** Y signal detected by FITC. **c** X signal detected by Texas Red

Table 1. Results of fluorescent in situ hybridisation with X- and Y-specific probes to metaphase nuclei

Material	No. of cells	Signal			No result	Sex
		XX	XY	Other		
M lymphocytes	20	0	20	0	0	M
F lymphocytes	20	20	0	0	0	F
Embryo						
1[a]	1	1	0	0	0	F
2	4	3	0	0	1[b]	F
3	3	0	3	0	0	M
4	1	1	0	0	0	F
5	3	3	0	0	0	F
6	3	0	2	0	1[c]	M
7	4	0	3	XXY	0	M
8	1	0	1	0	0	M
9	2	2	0	0	0	F
10	2	0	1	X0	0	M
11	3	0	3	0	0	M
12	1	1	0	0	0	F

[a] Fortuitous metaphase in embryo 1 of Table 2
[b] Cell lost in procedure
[c] No hybridisation signal

Table 2. Results of fluorescent in situ hybridisation with X- and Y-specific probes to interphase nuclei

Material	No. of cells	Signal			No result	Sex
		XX	XY	Other		
M lymphocytes	100	0	89	4:Y0 1:XXY	6[b]	M
F lymphocytes	100	87	0	6:X0 1:XXXX	6[b]	F
Embryo						
1	1	0	0	0	1[c]	?
2	2	2	0	0	0	F
3	1	0	1	0	0	M
4	1	0	0	0	1[c]	?
5	2	0	2	0	0	M
6	2	0	1	0	1[a]	M
7	2	0	2	0	0	M
8	2	0	1	0	1[b]	M
9	1	0	0	1:Y0	0	M
10	2	1	1[d]	0	0	?
11	2	0	2	0	0	M

[a] Cell lost in procedure
[b] No hybridisation signal
[c] Result obscured by autofluorescent debris
[d] Misdiagnosis because of presence of autofluorescent debris

approach (Griffin et al. 1991) involved FISH using either probe pHY2-1 or pBamX7 with a single detection system. Each had drawbacks: misdiagnosis of male embryonic cells as female was possible either because of failure of hybridisation with the Y-specific probe or as a result of the presence of a tetraploid cell when the X-specific probe was used. The male lymphocyte in Table 2 with XXY signals is an example of this; use of the X-probe alone would have led to a classification of female. With the dual system, the possibility of misdiagnosing a male as a female is virtually eliminated as two distinct X chromosome signals with no Y signal need to be seen before the embryo is determined to be female and therefore suitable for transfer. For example, the XO cell of embryo 10 (Table 1) was not given a definite classification. Previous studies with the probe pHY2.1 have shown that, with both Southern and ISH, the male-specific sequence can be detected in all males examined, even those lacking the entire fluorsecent region of the Y chromosome (Gosden et al. 1984).

Initially, the different hybridisation conditions required for each of the probes pHY2-1 and pBam X7 appeared to present a barrier to the application of a dual fluorescent technique; the latter probe recognises minor binding sites when conditions are sub-optimal (Griffin et al. 1991). Introduction of the probe CY98, requiring similar hybridisation conditions to those of pBam X 7, alleviated this problem to a large extent. The most consistent results were obtained with metaphases, despite the fact that these were totally unsuitable for cytogenetic analysis. The two inconsistent results in Table 1 are thought to be attributable to the loss of a Y chromosome (embryo 10) and tetraploidy (embryo 7; the Y spot was large enough to represent two overlying signals). The detection of a single spot in 5% of lymphocyte interphases is most probably the result of overlying signals. Only one embryo gave a conflicting result initially (embryo 10, Table 2). The cell originally classified as male was later re-assessed in the light of a clear XX result in the other cell. It was then realised that autofluorescent debris had masqueraded as a Y signal.

Failure to obtain a result was generally attributed to three factors: namely, the inability to relocate the cell at the end of the procedure, presumably because of cell detachment: the failure of hybridisation or detection (early cleavage cells are often enormous with extremely diffuse chromatin); or the presence of autofluorescent foreign bodies obscuring the signal. Further research has suggested that improvements in the cytogenetic preparation and the use of freshly prepared slides, requiring a lower proteinase K concentration and no signal amplification, can minimise these difficulties. This mimics the diagnostic situation when slides would normally be prepared and used on the same day.

So far, the major disadvantage of all reported ISH procedures over the PCR approach is that, even with non-radioactive techniques, ISH takes 24 h, whereas with PCR, a diagnosis can be made within one working day enabling preimplantation diagnosis without the need for cryopreservation of the embryo. Recent preliminary experiments on control material have however demonstrated that a dual FISH strategy with very high efficiency can be performed in 6 h, making this approach a credible alternative to PCR for the diagnosis of sex in human preimplantation embryos in families segregating for X-linked genetic disease. Additionally, the availability of centromere-specific probes for most of the auto-

somes would allow the use of FISH for screening for autosomal trisomies (Cremer et al. 1986).

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References

Cooke HJ, Schmidtke J, Gosden JR (1982) Characterisation of a human Y chromosome repeated sequence and related sequences in higher primates. Chromsoma 87:491–502

Cremer T, Landegent J, Brückner A, Scholl HP, Schardin M, Hager HD, Devillee P, Pearson P, Ploeg M van der (1986) Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe L 1.84. Hum Genet 76:346–352

Gosden JR, Gosden CM, Christie S, Cooke HJ, Morsman JM, Rodeck CH (1984) The use of cloned Y chromosome-specific DNA probes for fetal sex determination in first trimester prenatal diagnosis. Hum Genet 66:347–351

Griffin DK, Handyside AH, Penketh RJA, Wintson RML, Delhanty JDA (1991) Fluorescent in-situ hybridisation to interphase nuclei of human preimplantation embryos with X and Y chromosome specific probes. Hum Reprod 6:101–105

Grifo JA, Boyle A, Fischer E, Lavy G, DeCherney AH, Ward DC, Sanyal MK (1990) Pre-embryo biopsy and analysis of blastomeres by in situ hybridisation. Am J Obstet Gynecol 163: 2013–2019

Handyside AH (1991) Preimplantation diagnosis by DNA amplification. In: Chapman M, Grudzinskas G, Chard T, Maxwell D (eds) The embryo: normal development and growth. Springer London Berlin Heidelberg New York, pp 81–90

Handyside AH, Pattinson JK, Penketh RJA, Delhanty JDA, Winston RML, Tuddenham EGD (1989) Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet I:347–349

Handyside AH, Kontogianni EH, Hardy K, Winston RML (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 344:768–770

Hardy K, Martin KL, Leese HJ, Winston RML, Handyside AH (1990) Human preimplantation development in vitro is not adversely affected by biopsy at the 8-cell stage. Hum Reprod 5: 708–714

Heiles HBJ, Genersch E, Kessler C, Neumann R, Eggers HJ (1988) In situ hybridisation with digoxigenin labelled DNA of human papilloma viruses (HPV 16/18) in HeLa and SiHa cells. Biotechniques 6:978–981

Jones KW, Singh L, Edwards RG (1987) The use of probes for the Y chromosomes in preimplantation embryo cells. Hum Reprod 2:439–445

Kola I, Wilton LJ (1991) Preimplantation embryo biopsy: detection of trisomy in a single cell biopsied from a four-cell mouse embryo. Mol Reprod Dev 29:16–21

Kontogianni EH, Hardy K, Handyside AH (1991) Co-amplification of X- and Y-specific sequences for sexing preimplantation human embryos. In: Verlinsky Y, Strom C (eds) Proceedings of the First Symposium on Preimplantation Genetics. Plenum Press, New York, pp 139–145

Penketh RJA, Delhanty JDA, Berghe JA van den, Finkelstone EM, Handyside AH, Malcolm S, Winston RML (1989) Rapid sexing of human embryos by non-radioactive in situ hybridization: potential for preimplantation diagnosis of X-linked disorders. Prenat Diagn 9:489–500

Pinkel D, Straume T, Gray JM (1986) Cytogenetic analysis using quantitative high sensitivity, fluorescent hybridization. Proc Natl Acad Sci USA 83:2934–2938

Quinn P, Barro C, Whittingham DG (1982) Preservation of hamster oocytes to assay the fertilising capacity of human spermatozoa. J Reprod Fertil 66:161–168

West JD, Gosden JR, Angell RR, Hastie ND, Thatcher SS, Glasier AF, Baird DT (1987) Sexing the human pre-embryo by DNA-DNA in situ hybridisation. Lancet I:1345–1347

West JD, Gosden JR, Angell RR, West KM, Glasier AF, Thatcher SS, Baird DT (1988) Sexing whole human pre-embryos by in situ hybridisation with a Y-chromosome specific DNA probe. Hum Reprod 3:1010–1019

Willard HF (1985) Chromosome-specific organization of human alpha satellite DNA. Am J Hum Genet 37:524–532