OPTIMIZATION AND APPLICATION OF CHROMOSOME IN SITU SUPPRESSION HYBRIDIZATION

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DECLARATION

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

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THANK YOU. MAY GOD BLESS YOU.

KHAS BUAT SAYANG, ALONG, ABANG DAN ADIK

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LIST OF ABBREVIATIONS

AAF	-	Acetylaminofluorene
ATCC	-	American Type Cell Collection
BCIP	-	5-Bromo-4-chloro-3-indolyphoshate
Bio-11-dUTP	-	biotinylated deoxyuracil 5′-triphosphate
BrdU	-	5-bromodeoxyuridine
BRL	-	Bethesda Research Laboratories
CISS	-	Chromosomal in situ suppression
CLSM		confocal laser scanning microscope
DAPI	-	4,6-diamino-2-phenyl-indole
dATP	-	deoxyadnosine 5′-triphosphaste
dCTP	-	deoxycytidine 5′-triphosphate
dGTP	-	deoxyguanosine 5′-triphosphate
dTTP	-	deoxythumidine 5′-triphosphate
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylene Diamine Tetra-acetic acid
e.g	-	exempli gratia (for example)
et al.	-	et alia (and others)
FITC	-	fluorescein isothiocyanate
gm	-	gram
G-banding	-	Giemsa banding
ISCN	-	International System for Chromosome Nomenclature
ISH	-	In situ hybridization
kb	-	kilobase
mg	-	milligram
ml	-	microlitre
NBT	-	Nitroblue Tetrazolium Chloride
ng	-	nanogram
NOR	-	Nucleolar Organiser Region
OD	-	Optical Density
p	-	short arm of the chromosome
PBS	-	Phosphate-buffered saline
PHA	-	phytohaemagglutinin
PNB	-	phosphate nonidet-P40 buffer
PNM	-	phosphate nonidet-P40 non-fat milk
q	-	long arm of the chromosome
Q-banding	-	Quanacrine banding
R-banding	-	Reverse banding
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
rpm	-	revolutions per minute
SA-AP	-	Streptavidin-alkaline phosphatase conjugate
SDS	-	Sodium Ddodecyl Sulphate
SSC	-	Standard Saline Citrate
Tris	-	Tris (hydroxymethyl) aminoethane
TRITC	-	Tetramethyl-rhodamine-isothiocyanate
UV	-	Ultra Violet
ug	-	microgram
ul	-	microlitre
v/v	-	volume per volume
w/v	-	weight per volume

SUMMARY

The overall aim of this project was to develop the technique of chromosomal in situ suppression (CISS) hybridization using whole chromosome specific libraries (chromosome painting) and to apply it to the investigation of diagnostic problems in clinical cytogenetics.

Initially to gain experience with non-isotopic in situ hybridization, repetitive target probes DYS59 (GMY10) and DYS58 (GMGY7) were used. This provided experience in labelling of probe with biotin, hybridization and detection conditions (alkaline phosphatase detection) and analysis of results. The technique was reliable and sensitive and was applied to map the gene for angiotensinogen to 1q42. In the later part of this initial work, a fluorescence detection technique using fluoresceinated avidin and goat biotinylated anti-avidin was applied to confirm an isochromosomes Yp and Yq using DYS59 (GMGY10) and DYS58 (GMGY7) probes.

The study then progressed into the development of the chromosome painting technique. Difficulties were encountered in preparing the working library probe from the chromosome 21 specific library and a major part of the work involved solving these problems. The libraries were found to be less concentrated than indicated by the supplier. Consequently, the amplification and purification following established protocols failed to produce a concentrated DNA library in the phage. However, a good yield of the DNA library was achieved by using trypticase in the culture

media and high purity agarose as the top agar during the amplification. Labelling of the library by nick-translation and random priming did not achieve decoration of the whole chromosome 21 but direct labelling of Biotin-11-dUTP by polymerase chain reaction (PCR) amplification was found to be efficient and overcame the problem of non-homogenous painting of the target chromosome. This direct labelling approach had difficulties in the cleaning and concentration of the PCR product. These were overcome by cleaning with Sephadex G-50 column chromatography and freeze drying of eluate.

Once homogeneous painting had been achieved the probe was applied for chromosome painting. Many problems and parameters for the optimum working conditions were identified in this part of study. These are either independent or/and related to various conditions involved during all stages of the technique. The maximum final concentration of the DNA mixture per slide was 10ug/10ul and increasing the ratio of the probe and/or the unlabelled DNA did not improve either the quality of suppression or the hybridization signal. Addition of human cot 1-DNA in 1 to 4 ratio with total human DNA gave better suppression. Denaturation of labelled probe and competitor DNA mixture was optimum at 75°C for 8 minutes and for optimum preannealling, the mixture was prehybridized for a minimum of 60 minutes at 37°C. Slides were only treated with RNase when necessary and not with Proteinase K as the latter tended to wash the cells off the slide. Denaturation

of the slides was carried out at 70-75°C in 70% formamide/2XSSC for a maximum of 8 minutes. At temperature of 80°C the chromosome morphology was found to be distorted.

Hybridization when carried out at 37°C for 15 to 20 hours showed good hybridization with chromosome morphology undisturbed. Hybridization at 42-45°C showed crystallization and heavy background deposits. Post-hybridization washing in three changes of 50% formamide/2XSSC at 45°C was found to be optimal in producing a clean background. In between detection washing using 0.1M sodium phosphate buffer with 0.1% Nonidet P-40 carried out at room temperature is sufficient to remove excess stain as compared to other washing buffers such as 2XSSC or 4XSSC containing Triton-X or Tween 20. Detection was carried out at room temperature for 15-20 minutes and any slide dried during this stage produced high autofluorescence of fluoresceinated avidin which was difficult to remove by washing. A single amplification cycle was sufficient to enhance the decoration of chromosome 21.

Prebanding of slides prior to hybridization did not affect the target chromosomes, however, incomplete destaining did hinder probe penetration and interfere with counterstaining. It was found that refixing of slides (either new or old slides) in methanol:acetic acid (3:1) before denaturation tended to improve the hybridization result as well as reducing background signal.

In general, the technical difficulties were related to either probe preparation, poor hybridization, non-homogeneous painting or high background but with modifications of the parameters as detailed above the method was shown to be reliable and reproducible.

Chromosomes obtained from phytohaemagglutinin (PHA) stimulated blood cultures were used during the initial phase. Subsequently, painting was successfully performed on cytogenetically normal metaphase and prometaphase samples of cultured amniocytes, lymphoblastoid cell lines, chorionic villus samples (CVS) and bone marrow preparations. The results showed that all normal chromosome 21s in all types of preparation except direct chorionic villus sample (CVS) were intensely painted and distinctly recognisable. However, results with interphase nuclei were not encouraging. The signals produced were not consistent enough to produce as reliable results. Twelve cases with cytogenetic abnormalities involving the chromosome 21 were investigated using chromosome painting. These results proved that chromosome painting can be used for rapid identification of individual chromosomes and is complementary and confirmatory to conventional karyotyping and as such is predicted to have a future routine diagnostic role in clinical cytogenetics in additions to its research applications.

CHAPTER 1

INTRODUCTION

1.1 PROGRESS IN CLINICAL CYTOGENETICS

Human cytogenetics has progressed rapidly over the past two decades due mainly to constant improvements of cell culturing and staining techniques that have enhanced the identification of individual chromosomes and of particular regions or bands. Historically, human cytogenetics came of age during 1956 to 1960 after Tjio and Levan showed that the normal human chromosome number was 46 rather than 48 (reviewed by Therman, 1986). At that time, chromosome analysis was mainly undertaken either on cultured fibroblasts or bone marrow specimens, and it was only after the introduction of the short term phytohaemagluttin (PHA) stimulated culture by Moorhead in 1960 that it could be carried out on a larger scale (reviewed by Therman 1986). In the early days chromosomes were stained uniformly either by Fuelgen, orcein or Giemsa. Identification of individual chromosome was difficult, and was based on the overall size of the chromosome and the position of the primary constriction or centromere. Thus the first abnormalities to be detected were those that involved gain or loss of entire chromosomes, for example trisomy 21 (Down's syndrome) and 45,X (Turner's syndrome), and structural abnormalities that resulted in a marked change in size or arm ratio such as partial deletion of

chromosome 5p (Cri du chat syndrome) (reviewed by Therman, 1985; Jonasson, 1986; Verma and Babu, 1988).

The introduction of chromosome banding in the late 1960s and early 1970s was a major development in human cytogenetics as it allowed a more precise identification of individual chromosomes and their bands or regions. In 1971, at the fourth International Congress in Human Cytogenetics which was held in Paris, the human chromosomes were classified into seven distinguishable groups based on descending order of size and the position of the centromere. The short arm was designated as "p" and the long as "q". A band was defined as that part of a chromosome that is clearly distinguishable from adjacent segments by appearing darker or lighter with one or more banding techniques; Q-bands by fluorescence, G-bands by trypsin using Giemsa (GTG) and R-bands by fluorescence using acridine orange (RFA) and R-bands by heat using Giemsa (RHG) (ISCN, 1978).

During this era the choice of the banding technique depended on the types of abnormalities being investigated. G-banding developed by Seabright in 1973 was used routinely to investigate abnormalities involving G-positive bands and the demonstration of structural and functional composition of individual chromosomes (Verma et al. 1983). Identification of abnormalities involving the heteromorphisms associated with chromosomes 3, 4, 13, 15, 22 and the distal region of Yq was performed with fluorescence banding (Q-banding) using quinacrine mustard.

R-banding where the pattern is the reverse of G- and Q-banding was used to identify the telomeric regions which are generally pale with the other methods. The selective staining techniques which are useful are C-banding (constitutive heterochromatin) which allows identification of aberrations involving centromeric regions especially of chromosomes 1, 9, 16, and also Yq, and NOR-banding (nucleolus organising region) which can discriminate translocations involving the nucleolar regions of acrocentric chromosomes 13, 14, 15, 21, and 22 (reviewed by Therman, 1986; Watt et al. 1986; Verma and Babu, 1988).

Another important development in cytogenetics technique was the production of elongated chromosomes in late prophase and early metaphase by synchronizing the T-lymphocytes in culture. Synchronization is carried out by blocking the DNA synthesis with methotrexate, releasing with thymidine rich medium and allowing the cells to continue into mitosis before harvesting. Chromosomes obtained by synchronized culture produce up to 1000 bands per haploid set which is more than the routine phytohaemagglutinin stimulated cultures which normally produce up to 600 bands. This allowed a more detailed study of the banding pattern and it became possible to identify minute deletions, or additions of chromosome material and to locate the breakpoints more precisely in structural abnormalities (Yunis, 1976; Yunis 1981).

The developments in synchronised culture to obtain prophase and prometaphase chromosomes paved the way for

further sub-classification of the bands. In 1980, at a second meeting held in Paris a cytogenetics nomenclature was devised for high-resolution banding entitled " An International System for Human Cytogenetics Nomenclature-High Resolution Banding" (ISCN, 1981.). It provided schematic ideograms of chromosomes corresponding to approximately 400, 550, and 580 bands per haploid set, numbered from the centromere outward along each chromosome arm. The terminology for most common chromosomal abnormalities such deletions, inversions, and reciprocal translocations was described in detail and symbols and abbreviations were standardized for ease of reporting and communication among cytogeneticists (ISCN, 1985).

Equipped with a battery of banding techniques and improved methods of culturing blood and other tissues, the role of cytogenetics in clinical medicine progressed rapidly. Many more chromosomal syndromes were characterised and many structural abnormalities detected and prenatal diagnosis using either amniocentesis or chorionic villus sampling was developed. The application of various banding techniques also resulted in the description of specific structural chromosomal changes in certain types of cancer (Sandberg, 1986; Verma and Babu, 1989). In spite of these achievements there are still limitations of chromosome banding as summarised in **Table 1.** The development of in situ hybridization techniques has a potential for addressing at least some of these limitations.

Table 1. Major limitations of banding analysis in clinical cytogenetics (Pinkel et al. 1986b)

1. Requires preparation of high quality metaphases.

2. Time consuming work and requires skilled observer for interpretation of banding patterns.

3. Prenatal diagnosis of aberrations is slow and laborious because of cell culture and scoring process.

4. Detection of terminal translocations is very difficult.

5. Most banding techniques especially C-banding, NOR and BrdU staining are labour intensive.

6. Tumour cytogenetics is limited by the difficulty of preparing high quality metaphase spreads from tumour cells.

7. Banding is not useful for investigating the location of chromosomes in interphase nuclei.

Outline of gene structure

Genetic information is stored in the double helix of DNA. Each strand of the helix is a chain of nucleotide each comprising deoxyribose sugar and phosphate group which form the strand's backbone. The orientation of the phosphate group defines the 5′ and 3 ′ ends of the molecules. The purine bases adenine (A) and guanine (G) and pyrimidine bases cytosine (C) and thymine (T) are attached to the deoxyribose units. The complementarity of the bases (A always pair with T and G with C) is fundamental to DNA replication during which the two strands separate and each acts as a template for the synthesis of a new strand. One strand of DNA acts as a template for mRNA synthesis, a process that occurs by pairing of specific bases as it does in DNA synthesis.

The coding sequence of a gene is not continuous but is interrupted by varying numbers and lengths of intervening non-coding sequences. The sequences are called exons and the intervening sequences introns. In addition to the introns, there are non-coding regions of DNA at both 5′ and 3′ of genes. Both coding and non-coding DNA sequences in a gene are initially transcribed into mRNA. The structure of RNA is similar to that of the DNA, except that the sugar backbone is composed of ribose and uracil (U) replaces thymine as one of the bases.

It is estimated 50,000-100,000 pairs of functional genes exist in human. More than 80% of this total genome consists of non-coding DNA and 30-40% are high repetitive sequences that occur interspersed throughout the genome or as cluster regions. These highly repetitive sequences include Alu, L1 and Kpn1. Alu and L1 account for one third of the major repetitive sequence in human DNA.

Under physiologic conditions, the two strands of DNA are held together in a stable configuration. At high temperature above 90°C and at extreme acidic (less than pH 3.0) or alkaline (more than pH 9.0) conditions, the hydrogen bonds between the two break and the DNA becomes single stranded (denatures). When the temperature falls below the melting point those complementary strands reassociate (reanneal).

The kinetics of nucleic acid reassociation in solution are dependent on the total concentration of nucleic acid (Co, in moles of nucleotide per litre) and the time of renaturation (t, in seconds) which are expressed as Cot values. As in the case of Tm (temperature of melting point at which the strands are half dissociated) the Cot of a DNA is usually expressed at $Cot_{1/2}$ (Jones 1973). This property is important in the kinetics of hybridization as it influences the stability and efficiency of hybridization duplexes (Raap et al. 1986). Under defined conditions which include the temperature, ionic strength and the pH of the solution the highly repetitive DNA is completely reannealled at Cot value between 1×10^{-1} and 5×10^{-1}. Thus

at human DNA concentration of 1.0mg/ml (corresponding to 3 X 10^{-3} moles of nucleotide) the fast fraction will denature in approximately 10 second (Lichter et al. 1988a).

1

1.2 THEORETICAL BACKGROUND OF DNA HYBRIDIZATION

Human genomic DNA is double-stranded and composed of two complementary strands with specific base pairing of adenosine [A] and thymine [T] and guanine [G] and cytosine [C]. DNA hybridization makes use of this base pairing to detect and identify specific DNA sequences. Nucleic acid hybridization is based on the fact that two complementary, single-stranded nucleic acids will recognise one another and bind (hybridize) on the basis of hydrogen bonding and the hydrophobic base-stacking interaction of complementary base pairs (Gall and Pardue, 1969; John et al. 1969).

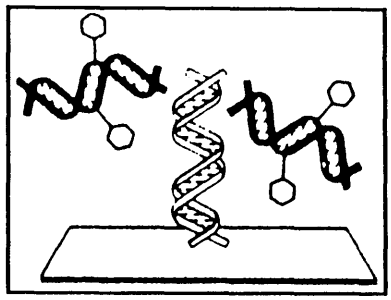
When double-stranded DNA is heated, the complementary strands separate (denature) to form single strands. Under appropriate conditions, the separated complementary DNA strands then recognise one another and bind by means of hydrogen bonds to form a duplex of double-stranded DNA molecules. The reference point of this hybrid stability is the melting temperature (Tm) at which 50% of the population of duplex molecules dissociate (or melt) into single strands. Other major conditions that affect hybrid stability of this hybridization or renaturation process include: ionic strength of the mixture, the percentage of guanine/cytosine (G/C) base pairs in the probe, the probe length, the percentage of complementary bases between probe and target, the concentration of duplex destabilising agents such as formamide, and the concentration of the probes in the reaction (Jones, 1974; Donlon, 1986; Naylor

et al., 1987; Wetmur et al. 1986; Tecott et al., 1987). Any DNA strands which are not complementary will fail to hybridize with each other but will not affect the hybridization process of other complementary strands (Naylor et al. 1987).

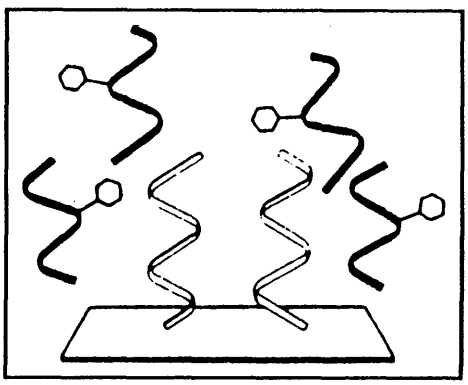
In general, DNA hybridization with a DNA probe (a cloned segment of DNA that is specific for and complementary to the DNA target in the specimen) takes place in three stages; first, denaturation of the double-stranded DNA probe and target DNA (e.g chromosome DNA), second, the hybridization of the single-stranded probe with the complementary target DNA and third, detection of the hybridized duplexes of DNA probe and DNA the specimen **(Fig.1)**.

1.3 GENERAL DEVELOPMENT OF IN SITU HYBRIDIZATION

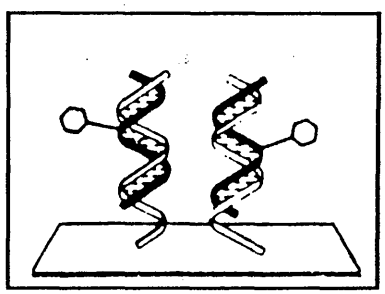
In situ hybridization is the hybridization of nucleic acid sequences, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), to complementary targets within structurally intact cells or tissues. This technique which was an extension of membrane filter hybridization (Gillespie and Spiegelman, 1965) was first described in 1969 by two independent groups Gall and Pardue (1969) and John et al. (1969). Hybridisation was performed by incubating radioactively labelled ribosomal RNA together with intact oocytes of *Xenopus laevis* and autoradiography



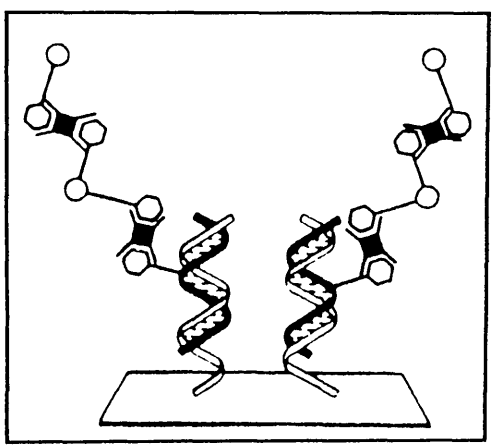
1. DNA probe added to specimen



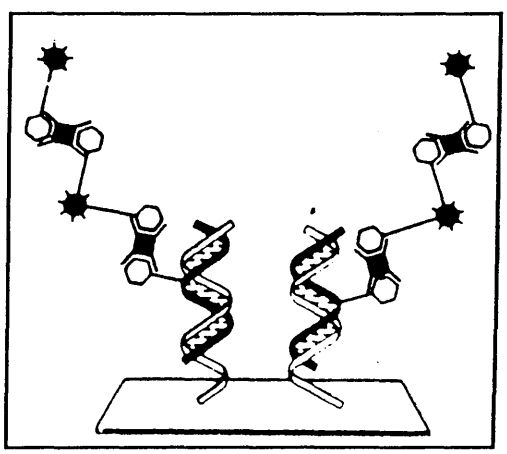
2. Binding of detection complex to hybridized DNA probe



3. Localised production of detection complex



4. Binding of detection to hybridized DNA probe



5. Localised production of coloured precipitate

Figure 1. Schematic representation of the principle of DNA hybridization between DNA probe and the DNA in the specimen. If there is no DNA complementary to the DNA probe, no probe will hybridize (taken from ENZO, USA).

was used to visualise the sites of hybrids. John et al. (1969) concluded that the in situ hybridization technique was specific and sensitive for demonstrating cytological distribution of DNA sequences. Following this so called 'marriage of molecular biology and cytology' as described by Hsu (1979), extensive discussions on the complexity of hybridization (Szabo 1977), and the advantages and applications of in situ technology were published. (Gall and Pardue, 1971; Hennig, 1973; Jones, 1974; Price and Hirshon, 1975; Steffensen, 1977). Within a short period the technique was significantly improved and the sensitivity and utility of in situ hybridization was enhanced (Szabo et al., 1982). The sensitivity of in situ hybridization depends on the retention of target DNA which is accessible to the probe, the length of the probe and the effect of the hybridization conditions (Naylor et al., 1987; Tecott et al., 1987; Hofler et al., 1989).

The technique was later applied extensively for localization of viral DNA sequences and cellular RNA, and chromosomal mapping (Gerhard et al., 1981; Malcolm et al., 1981; Harpers and Saunders, 1981; Barton et al., 1982; Donlon et al., 1986). Since then with the improvement of the technique and the advent of recombinant DNA technology for the production of probes, in situ hybridization has become an established chromosomal mapping technique. Originally the probes for in situ hybridization were radioactively labelled and this required autoradiography for visualization of the hybrids (Gall and Pardue, 1969).

Although the technique was highly sensitive and provided direct visualization, it suffered several disadvantages. The radioisotopes used (^{32}P, ^{3}H, ^{35}S, ^{14}C and ^{125}I) were relatively expensive, involved a radiation risk to the scientists and were difficult to handle. Some of these radioisotopes had limited half-lifes (Donlon, 1986). The procedure of in situ hybridization and the long exposure time for signal detection by autoradiography required 2 to 4 weeks to complete. These disadvantages limited the use of isotopic in situ hybridization as a routine diagnostic tool in cytogenetic laboratories and alternative labelling and detection technique were sought (Singer et al., 1982; Manuelidis et al., 1982; Brigati et al., 1983).

1.4 DEVELOPMENT OF NON-ISOTOPIC IN SITU HYBRIDIZATION

The objective was to replace isotopic probe labelling and autoradiographic detection with non-radioactive chemicals that could improve the stability of labelled probes and yet be sensitive, rapid, economical and safe to use in practice. There are two main types of non-isotopic in situ hybridization method. In the direct method the reporter molecules are bound to the nucleic acid probe so that hybrids formed can be visualised microscopically after in situ hybridization. In the indirect method the probe contains an element (a hapten) that renders it detectable by affinity cytochemistry (Leary et al., 1983; Hopman et

al., 1986a; Viscidi et al., 1989; Hopman et al., 1986b; Raap et al., 1989). Several haptens for labelling DNA probes have been introduced including: enzymatic incorporation of biotin (Langer et al., 1981), chemical modification using 2-acetyl-aminofluorene (AAF) (Landergent et al., 1984; Tchen et al., 1984), labelling with mercuric ion (Bauman et al., 1984; Bauman, 1985), dinitrophenyl groups (Shroyer and Nakane, 1983), mercury and sulphydril-hapten ligands (Hopman et al., 1986a; Hopman et al., 1986b; Hopman et al., 1987), chemical sulphonation (Marimoto et al., 1987), enzymatic incorporation of alkaline phosphate bromodeoxyuridine (Niedobitek et al., 1988), and labelling with digoxigenin (Herrington et al., 1989a; Herrington et al., 1989b). Detection of hybrids is carried out immunologically by binding of the haptens with specific antibodies. These antibodies are cross-linked to either enzymes or fluorescent dyes. The most popular enzymes are alkaline phosphatase and horseradish peroxidase, and the commonly used fluorochromes are fluorescein-isothiocynate (FITC), Texas Red, rhodamine (TRITC) and aminomethylcoumarin acetic acid (AMCA). Visualization of the hybrids is carried out by light microscopy {reflection contrast (Landergent, 1985a), phase contrast (Garson et al., 1987)} or fluorescence microscopy (Pinkel et al., 1986).

Development of non-radioactive labelling and immunodetection allowed the completion of in situ hybridization experiments within two days. The technique

also eliminated the problems of safety and disposal of radioactive material. However, the non-radioactive technique appeared to be less sensitive than the radioactive method and its use was initially restricted to the detection of high copy number or relatively large (20-40 kilobases) single copy sequences (Landergent et al., 1985b; Landergent et al., 1986; Landergent et al., 1987). Further refinements of labelling and detection systems improved the sensitivity (Garson et al., 1987) and following the successful localization of small single copy genes (Landergent et al., 1985b; Burns et al., 1985; Garson et al., 1987) there was a widespread interest in using in situ hybridization for the mapping of unique sequences in man and other species.

Of the different non-radioactive haptenization methods described, biotinylation has been the most studied. Biotin has been a highly successful non-radioactive label of DNA and can be enzymatically incorporated into DNA probes either by nick-translation (Rigby et al., 1977; Langer et al., 1981) or by random-priming (Feinberg and Vogelstein, 1984). The detection of biotin can be easily accomplished with anti-biotin antibodies or with streptavidin. The advantages and disadvantages of biotinylated probes are summarised in **Table 2.** Since the initial documentation of biotin labelling by nick-translation (Rigby et al., 1977, Langer et al., 1981) as an alternative to radioisotopic labelling other methods of biotinylation have also been reported. Chollet et al. (1985) chemically labelled DNA

Table 2. Advantages and disadvantages of biotin-labelling (taken from Hofler, 1987)

Advantages	Disadvantages
Stability of probes	Difficult incorporation of biotin-triphosphates
No radioactivity	Decreased hybridization efficiency
Inexpensive	Low sensitivity of signal detection
Rapid detection	
High resolution	
Localization at electron microscopic level	

probes with biotin at the 5′ terminus and this proved to be simple and efficient. Forster et al. (1985) described the use of photobiotin for labelling DNA with protein but this method gives a higher non-specific background as compared to nick-translation. Enzymatic synthesis of oligonucleotide probes with biotin for labelling using DNA polymerase I (Klenow fragment) in the primer extension reaction was described by Murasugi et al. (1986). Although other markers such as mercury, 2-acety-aminofluorene (AAF) sulphonate and bromodeoxyuridine (BrdU) were shown to be comparably sensitive, their wide usage was discouraged by safety measures. Biotin also gained in popularity because the immunocytochemical detection can be readily established and the reagents are commercially available.

Another non-radioactive reporter for situ hybridization is digoxigenin. This is a derivative of the cardiac glycoside digoxin and was found to be equivalent to biotin in terms of sensitivity (Herrington et al., 1989a). Use of synthetic oligodeoxynucleotide probes to complement the nick-translated probe has also been described but was found to be little different in efficiency (Taneja et al., 1984).

1.4.1 Basic outline of non-isotopic in situ hybridization

The non-isotopic in situ hybridization procedure differs little from the isotopic procedure. Many

laboratories have developed their own non-isotopic protocols but the key parameters are basically the same. Apart from the probe labelling the basic non-isotopic procedures include the preparation of the target materials, denaturation of the target materials and the probes, hybridization and detection of sites of hybridisation. These stages are interdependent and therefore, many factors have to be considered. The general procedure of non-isotopic in situ hybridization is summarised in **Table 3.**

Good quality chromosome spreads are required for hybridization. These can be obtained from phytohaemagglutinin (PHA) stimulated lymphocyte cultures that are fixed in methanol:acetic acid (3:1) as in conventional cytogenetic procedures. These target chromosomes are then pretreated with ribonuclease (RNase 1) to digest any cellular RNA that can interfere and compete in the hybridization process (Ploeg et al. 1990) Both the chromosomes on the slide and the labelled probes are denatured at 70°C to 80°C to render them single-stranded before allowing hybridization. Hybridization of the probe to the target chromosomes is performed at 37°C to 45°C for 12 to 24 hours according to the nature of the probes and their sizes. Proper control of the temperature and the salt concentration of the reaction will allow only specific complementary sequences to form hybrids. Detection of the hybrid sequences is then carried out directly or indirectly using immunocytochemical or enzymatic reactions. The signal can be visualised either with phase contrast microscopy,

Table 3. Schematic representation of non-isotopic in situ hybridization procedure to metaphase chromosomes

1. Preparation of metaphase chromosomes.

2. Probe labelling with haptens by nick-translation or random-priming.

3. Pre-hybridization.
   a. RNase treatment of chromosomes on slides.
   b. Denaturation of chromosomes on slides.
   c. Denaturation of probes.

4. Hybridization.
   Overnight incubation at 37°C to 42°C.

5. Post-hybridization.
   a. Washing of slides.
   b. Detection of formed hybrids either by enzymatic reaction or by fluorescein.

6. Microscopy.
   Phase contrast or fluorescence microscopy

fluorescence microscopy, interference reflection microscope or confocal laser microscope depending on the final detection product.

1.4.2 Key parameters in the efficiency of non-isotopic in situ hybridization

The key parameters of non-isotopic in situ hybridization can be categorised as five main components: (a) the fixation of target materials to preserve the morphology and the DNA structure, (b) denaturation of probes and (c) hybridization of labelled known DNA sequences or probes to the target material, (d) detection and visualization of the hybrids and (e) other variables related to hybridization and detection, for example the type of probe.

1.4.3 Fixation of target materials (e.g chromosomes)

Many fixatives such as methanol, acetic acid, paraformaldehyde and ethanol are used individually or in combination to ensure proper fixation of chromosomes. Proper fixation will preserve the morphology, prevent the destruction of the structure and allow easy permeability of the probes. Bouin's fluid (i.e methanol:acetic acid) has proved to be effective in maintaining the morphology and

yielding a good signal in in situ hybridization (Hofler, 1989; Larsson and Hougaard, 1990).

Treatment of slides with RNase will reduce the cellular RNA and other non-specific binding of non-target sequences and slides are also pretreated by digestion with Proteinase K, pepsin, pronase or/and dilute hydrochloric acid to enhance probe permeability (Burns et al., 1985; Raap et al., 1986; Raap et al.,1989). Larsson and Houggard (1990) emphasized the interdependence of fixation and the permeabilization of the probes but excessive enzymatic digestion should be avoided as it may lead to poor morphology and a decrease in the hybridization signal. Fan et al. (1990) noted a significant loss of chromosomes and distortion of morphology after treatment with Proteinase K. Loss of target sequences can also be encountered at subsequent stages of the procedures. 70 to 75% of the DNA tends to be lost during fixation and denaturation (Raap et al., 1986) and this directly affects the strength of the final hybridization signal.

1.4.4 Denaturation of probes and chromosomes

Both the probe and the metaphase chromosomes must be rendered single-stranded for maximum renaturation of DNA-DNA hybrids between the probe and target sequences. This can be achieved by treating both the probe and the chromosomes simultaneously or separately by heating, or by

exposure to acidic or alkaline conditions (Jones et al., 1973). Probes are usually denatured by heating at 70-80°C for 5 to 10 minutes but the chromosomes are generally denatured by treating in 0.05-0.1N sodium hydroxide or 0.05-0.1N hydrochloric acid. Raap et al., (1986) showed that all of these treatments resulted in complete strand separation but 40% of DNA was lost by either heat or aqueous alkaline treatment and 20% was lost by acid denaturation. However, ethanolic alkaline denaturation retained the DNA better and the morphology of the chromosome was superior. When probes and chromosomes are to be denatured simultaneously heat treatment is the method of choice (Raap et al. 1986).

1.4.5 Hybridization of the probes and target sequences

The size of the probe, the probe concentration, hybridization temperature and the morphology of the target sequences contribute to the hybridization efficiency.

The probe length will determine the permeability and stability of the hybrids. Langer and Singer (1985) found that probes less than 1000 base pairs had adequate penetration and that longer probes resulted in a slower hybridization rate (Tecott et al., 1987). Cox et al., (1984) found a linear relationship between the optimum probe concentration and the signal-to-noise ratio. Less probe resulted in a decreased signal but a higher probe

concentration adversely increased the background. The optimum concentration, was difficult to predict and required empiric determination.

The temperature of hybridization directly influences the hybridization rate (Britten et al. 1988). The temperature will determine the dissociation of the DNA strands before hybridization takes place and also maintains the melting temperature (Tm) of the DNA for hybrid stability. An increase or decrease in the temperature will affect the hybridization efficiency and the non-specific hybridization of poorly matched strands (Tecott et al., 1987). The kinetics of in situ hybridization are also influenced by the concentration of salt and formamide in the hybridization medium. Formamide, decreases the melting temperature (Tm) of the hybrids (Wetmur et al., 1986; Larsson and Houggard, 1990). The salt concentration (eg. dextran sulphate, sodium chloride and sodium citrate) decrease the solubility of the bases and can be adjusted to render hybrids more stable (Gerhard et al., 1981; Tecott et al., 1987). Post-hybridization washes are employed to remove nonspecific binding of labelled probes to non-complementary sequences. The stringency can be manipulated by varying the salt concentration, formamide concentration and the temperature of those washes (Malcolm et al., 1986; Angerer et al., 1987).

1.4.6 Detection of the hybridized probe

Visualisation of hybridized sequences depends on the type of label used. Probes that were coupled with fluorochromes such as fluorescein or rhodamine before hybridization can be directly detected with fluorescent microscopy (Bauman et al., 1980; Bauman et al., 1981) as can probes that have been coated with colloidal gold using electron microscopy (Narayanswami et al., 1990). Direct detection is commonly used with RNA probes especially for tissue localisation of expression (Bauman et al., 1984).

The indirect immunocytochemical method involving enzymatic reactions and the addition of fluorochromes is widely used. The basis of this immunocytochemical reaction is the linking of the hapten to the hapten-specific antibody and reporter molecules. Mostly straightforward immunocytochemical procedures are used and when high sensitivity is required, further amplification of signal can be achieved by immunocytochemical amplification (Bauman et al., 1981; Prooijen-Knegt et al., 1982; Pinkel et al., 1986a; Raap et al., 1989).

The most commonly used enzymes are peroxidase and alkaline phosphatase. Leary et al., (1983) detected biotinylated probes by reaction with streptavidin and alkaline phosphatase with bromo-chloroindolylphosphate (BCIP) and nitroblue tetrazolium (NBT) to precipitate the colour. This dark blue coloured water-insoluble precipitate adhered to the sites of hybridization and could be

visualised with phase contrast microscopy. The technique has proved to be sensitive and the colour is stable (Garson et al., 1987; Bhatt et al., 1988). This technique also gained popularity because the reagents are commercially available.

Another approach to indirect detection is the fluorescence technique. The hybridized sequences are linked to fluorochromes conjugated to the antibodies specific to the hapten. The common fluorochromes used are fluorescein isothiocynate (FITC), tetramethylrhodamine (TRITC), Texas Red and recently amino-methyl coumarin acetic acid (AMCA) (Khalfan et al., 1986). Although fluorescence detection suffers the disadvantage of photobleaching (fading) under intense illumination, the technique has several advantages over the enzymatic alkaline phosphatase detection. Different fluorochromes can be used for simultaneous labelling and in situ hybridization of several probes (Raap et al., 1989; Nederlof et al., 1990). Fluorescence microscopy is highly sensitive and as the image is self luminous contrast is not a problem (Bradbury, 1989). Furthermore the technique is convenient and rapid with an intense signal and high spatial resolution (Pinkel et al., 1986a; Pinkel et al. 1986b; Albertson et al., 1988; Trask and Pinkel, 1990) and the signal can be easily amplified and used in high sensitivity image analysis (Alberston et al., 1988; Nederlof et al., 1990).

For the digoxigenin labelled probe, detection is performed by the reaction of anti-digoxigenin antibody and

alkaline phosphatase. The location of antibody-antigen conjugate is visualised by an enzyme-linked colour reaction of bromo-chloroindolylophosphate (BCIP) and nitroblue tetrazolium (NBT) (Hoeltke et al., 1990,). This detection procedure is sensitive and reliable (Herrington et al., 1989; Morris et al., 1990) and mostly used on tissue sections because of the specificity of digoxigenin which is absent in tissue thus producing no background signal.

For the mercurated and aminoacetylfluorene (AAF) probes detection is carried out by tagging the trinitrophenyl sulphhydryl onto the antibodies or the fluorochromes (Hopman et al., 1986a; Hopman et al. 1986b). These detection systems have the disadvantages of relatively poor signal, damage of chromosome morphology, toxicity of mercury and cyanide, and carcinogenicity of aminoacetylfluorene (Herrington et al., 1989).

1.4.7 Other variables that are related to hybridization efficiency

The stages in the non-isotopic procedures are interdependent and other variables that are related directly or indirectly to the individual stages can affect the hybridization efficiency. Significant parameters are the ageing of the slides, pre-treatment of the slides, chemical composition and pH of the hybridization medium, hybridization temperature and the different stringencies of

post-hybridization washes.

Old slides of more than six months sometimes show degradation of DNA especially when improperly stored and this can be avoided by keeping the slides under vacuum or at -70°C (Hofler, 1987). Banding of slides before hybridization will sometimes affect the hybridization efficiency as trypsinization tends to remove the DNA.

The hybridization solution that is commonly used contains 50% deionised formamide in 0.5M sodium chloride and 0.5M sodium citrate (2XSSC). Addition of 10% dextran sulphate increases the rate of hybridization and inclusion of other chemicals such as ethylene-diamine-tetra-acetic acid (EDTA), Ficoll and bovine serum albumin decreases non-specific binding to proteins (Tecott et al., 1987). The hybridization medium needs to maintain a neutral pH for a maximum rate of hybridization (Larsson and Houggard, 1990).

Post-hybridization washing at various stringencies removes the non-specific binding together with mismatched and non-hybridised probe. The washing buffer commonly includes detergents; Tween 20, Triton X, and Nonidet-40 (Garson et al., 1987; Pinkel et al., 1988). These detergents which ~~acts~~ ~~bleaching agents~~ help to reduce non-specific binding during the detection process. Non-specific binding during detection is also reduced by the presence of non-fat milk or bovine serum albumin in the diluent buffer (Lichter et al., 1988a; Pinkel et al., 1988; Trask et al., 1990).

1.5 DEVELOPMENT OF FLUORESCENCE IN SITU HYBRIDIZATION

In situ hybridization using fluorescence detection was first described by Landergent et al. (1983) to visualise DNA cloned in a cosmid by aminoacetylfluorene (AAF). Alberston (1984) showed that the double layer fluorescent (tetramethyl rhodamine) labelled anti-goat IgG could detect the site of hybridization of the ribosomal genes in *Caenorhabditis elegans*. The approach, however, only become popular after Pinkel et al. (1986a) developed a more straightforward and sensitive technique. After complementary hybridization of the probes and the target sequences, the slides are incubated with conjugated fluorochromes to stain the sites of the hybridized sequences. The signal can then be amplified with its specific antibodies (**Figure 2**). The common fluorochromes are fluorescein isothiocynate (FITC), rhodamine and Texas Red. For contrast of the signal the slides are counterstained with propidium iodide or DAPI (4.6-diamidino-2-phenylindole). To minimise the signal fading, the stained slides are mounted in glycerol containing antifade agent either DABCO [1-4-diazobicyclo (2-2-2) octane] (Johnson et al. 1982), or para-phenylalanine (Lengauer et al., 1991)

Fluorescence in situ hybridization is sensitive and the localisation of the signal is precise. Alberston et al., (1988) claimed that the fluorescence in situ hybridization method has several advantages over bright

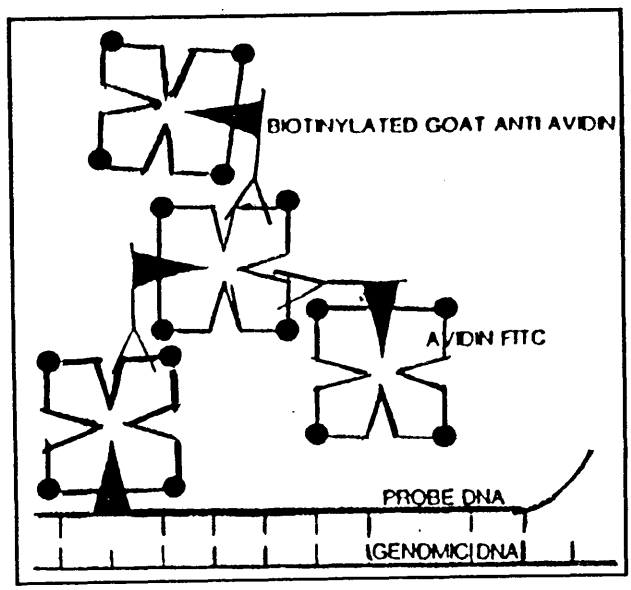


Figure 2. Schematic representation of fluorescence signal amplification using double layer antibody technique. The probe was labelled with biotin-dUTP and hybridized to cells or metaphase. FITC-labelled avidin was then added, followed by biotinylated goat anti-avidin and again FITC-labelled avidin. Further layers of biotinylated goat anti-avidin and FITC-avidin can be used to increase the signal (Kozma et al. 1987).

field or light microscopic technique in that chromosome bands may be obtained without pretreatment of chromosome spreads, it is possible to resolve hybridization signal to individual chromatids and signals can be photographically or electronically recorded. The double hybridization signals obtained on both sister chromatids offer a major advantage for gene mapping, as fewer slides need to be scored and statistical analysis as used in radioactive and light microscopy detection is obviated (Alberston et al., 1988; Cherif et al., 1989).

1.6 CHROMOSOMAL IN SITU SUPPRESSION (CISS) HYBRIDIZATION WITH WHOLE CHROMOSOME SPECIFIC LIBRARY OR CHROMOSOME PAINTING

Continuing development of fluorescence in situ hybridization technique led to a new technique described as chromosome painting (Pinkel et al., 1988) or chromosomal in situ suppression (CISS) hybridization (Lichter et al., 1988a). The technique used a labelled whole chromosome specific library to hybridise to the specific chromosome of interest after being prehybridized or pre-annealled with genomic DNA to suppress the repetitive and non-specific sequences. As a result the whole chromosome of interest could be painted or decorated. The technique had the potential of being a useful addition to conventional karyotyping (banding) techniques.

This technique involves several consecutive stages (**Table 4**). At the initial stage the sequences shared with other chromosomes that are contained in the chromosome specific library have to be blocked or depleted. This is achieved by denaturing both the labelled library and the unlabelled human genomic DNA and allowing them to preanneal (or prehybridize) at 37°C. This is followed by subsequent hybridization with the target chromosomes and finally the hybrids formed are detected. Although the method appeared promising, several factors needed to be optimised to ensure that the procedure was sensitive and reproducible (Pinkel et al. 1988; Lichter et al.1988a).

Table 4. Schematic representation of stages involved in chromosomal in situ suppression (CISS) hybridization

1. Denaturation of labelled probe (library) and competitor DNA (unlabelled genomic DNA) at 75°C fo 5-8 minutes.
   -Preannealling the hybridization mixture at 37°C for 60 minutes.

2. Denaturation of target material (e.g chromosomes or cells) at 75-80°C for 5 minutes.

3. Hybridization of hybridization mixture (probe and competitor DNA) and target material (e.g chromosomes) at 37°C for 16-20 hours.

4. Detection of hybrids formed using FITC-avidin and biotinylated goat anti-avidin amplification.

5. Microscopic analysis.

AIMS OF THE PROJECT

The overall aim of this project was to optimise the technique of chromosomal in situ suppression hybridization (CISS) using whole chromosome specific libraries (chromosome painting) and to apply it to diagnostic problems in clinical cytogenetics.

The following strategies were adopted:

1. Familiarization with existing non-isotopic in situ hybridization (alkaline phosphatase detection).

2. Application of fluorescence in situ hybridization using repetitive sequences.

3. Preparation of the DNA probe from a whole chromosome 21 specific library constructed in Charon 21A.

4. Optimization of chromosome painting or chromosomal in situ suppression hybridization using whole chromosome 21 specific library.

5. Assessment of the applicability of chromosomal in situ suppression (CISS) hybridization on normal blood lymphocytes, amniocytes, chorionic villus samples (CVS), lymphoblastoid cell lines and bone marrow cultures.

6. Assessment of the applicability of chromosomal in situ suppression (CISS) hybridization in diagnostic problems in clinical cytogenetics.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General

Plastic tubes (13ml) and micropipetting tips were supplied by Sarstedt. Plastic universal (20ml) were Sterilin types 128B and 129C respectively. Eppendorf tubes (1.5ml and 1.0ml) and plastic petri dishes/plates (Nunc 140mm).

Glassware and tips for micropipetting were sterilized in an autoclave (Denly, UK) at 121lb/in^2 for 10 minutes. All micropipettes (10ul, 200ul and 1000ul) were Gilson "Pipetman" type and all tips for micropipetting were sterilized by autoclaving.

All aqueous solutions were prepared in water deionized by a Millipore "Milli RQ" water purification system. If necessary, solutions were sterilized by autoclaving as above.

Preparation of basic reagents and buffers was as described by Maniatis et al. (1982) and Sambrook et al. (1989) unless stated in the text. All chemicals used in this study were obtained from Sigma (UK) and BDH (UK) unless special supplier is mentioned.

Hand gloves (Glaxo UK) were used at all times to avoid biological and chemical contamination.

The microscope glass slides and coverslips were supplied by Chance Propper Ltd, UK.

The glass slides were cleaned by first soaking in Decon (Decon Lab. Ltd, UK) overnight and then rinsed thoroughly in running tap water for 3 hours and kept in 70% alcohol. The coverslips were soaked in 70% alcohol and dried by wiping with Kimwipes tissue (Kimberly-Clark).

2.1.2 Solutions

L B (Lurias-Bertoni) Lambda medium

NaCl	5g
Typtone (Difco Bacto)	10g
Yeast Extract (Difco Bacto)	5g

Distilled water to 1 Litre.

Sterilized by autoclaving at 121°C 15 minutes. Cool to approximately 50°C and added the following sterile solutions to 500 ml of the medium ;

1M $CaCl_2$	5ml
IM $MgSo_4.H_2O$	2.5ml
50% maltose	5ml

Agarose plate

Tryptone (Difco Bacto)	10g
Yeast extract (Difco Bacto)	5g
NaCl	10g
Distilled water to 1 Litre	
Adjusted to pH 7.2	
Agarose (Medium Type lll Sigma)	15g

Sterilized by autoclaving at 121°C for 15 minutes. Poured on petri dish plate.

Top Agarose

Trypticase (BBL)	10g
NaCl	5g
Agarose (Medium Type lll, Sigma)	6.5g
Agarose (Medium Type lll, Sigma)	6.5g

Aliquoted 100ml each and sterilized at 121°C for 15 minutes. (Liquified by boiling for 30 minutes before used).

S M buffer

0.1M NaCl

50mM HCl Tris HCl pH 8.0

0.01% gelatine (w/v)

10mM $MgSO_4$

20XSSC (Saline sodium citrate) -1 Litre

NaCl	175.3g
NaCitrate	88.2g

Dissolved in 800ml of H_2O. Adjusted to pH 7.0 with 1M HCl. The final volume adjusted to 1 Litre with distilled water. Sterilized by autoclaving.

Deionised Formamide

50ml of Formamide (Fluka, Switzerland) with 5g ion exchange resin (Bio-Rad Ag 501-x8;-20-50 mesh) added, stirred at room temperature with a magnetic stirrer for 24 hours in Vacuum Cupboard (Morgany Grundy UK), filtered twice through Whatman No 1 filter paper and was stored at -20°C.

Phenol

Phenol was distilled using Quickfit distillation apparatus. 0.1% 8-hydroxyquinoline was added to give a yellow colour which, if lost, colour darkens when oxidised.

Equilibrated to pH 7.5 and stored at 4°C until required.

Phenol-chloroform

50% phenol

50% chloroform

Kept at 4°C until required.

1% Ethidium bromide

Ethidium bromide 1g

Distilled H_2O 100ml

Stirred to dissolve dye. Container wrapped in aluminiumfoil to protect from direct light and stored at 4°C.

7.5M Ammonium acetate

Dissolved 557.5 ammonium acetate in 800ml of distilled water and volume adjusted to 1 litre. Dispensed in aliquout and sterilized.

5M NaCl

292.2g NaCl dissolved in distilled water and volume adjusted to 1 litre. Aliquoted in 100ml and sterilized by autoclaving.

1M Tris HCl pH 8.0

121.1g of Tris base dissolved in distilled water. Adjusted to pH 8.0 by adding 42ml concentrated HCl. Adjusted to 1 litre with water. Dispensed into aliquots and sterilized by autoclaving.

0.5M EDTA (ethyl diamine tetra-acetate)

To 800ml of distilled water in beaker added 186.1g of NaEDTA. Mixed well and adjusted to pH 8.0 with 10N NaOH. Volume adjusted with distilled water to 1 litre.

Dispensed into 100ml bottle and sterilized by autoclaving.

T E buffer

10mM Tris HCl pH 8.0
20mM NaEDTA pH 8.0
Sterilized by autoclaving.

Electrophoresis buffer

40mM Tris-acetate pH 8.0
20mM Potassium or Sodium acetate
1mM NaEDTA
Sterilized by autoclaving.

1 kb Ladder

DNA stock (BRL)	25ul
Distilled H_2O	725ul
Loading mix	100ul

2mM Biotin-11-dUTP

0.5mg of (5(N-Biotinyl-Aminocaproyl)-3-Aminoally)-2′ Deoxyuridine 5′-triphosphate-(Biotin-11-dUTP) Ammonium salt. FW-862.7 (B-7645 Sigma UK) dissolved in 290ul of 1M Tris HCL pH 8.0. Stored at -20°C.

Equilibrium buffer

10mM Tris pH 7.5 0.5 ml of 2M stock
1mM EDTA pH 8.0 200ul of 0.5M stock
Make to 100ml with distilled water.
Sterilized with 0.22um (ACRODISC) disposable filter (Millipore UK).

Hybridization buffer for fluorescence in situ hybridization (for unique sequence probe only)

1gm of dextran sulphate was added to a 20ml universal container containing 5.5ml deionized formamide and 0.5ml sterilized 20XSSC. The solution was heated at 70°C for 5 hours to dissolve the dextran sulphate, then

adjusted to pH 7.0 and the volume made to 7ml. The buffer was aliquoted in 1ml Eppendorf tube and kept at -20°C.

2.2 SOLUTIONS FOR CHROMOSOMAL IN SITU SUPRESSION HYBRIDIZATION (CISS)

Hybridization buffer

1gm of dextran sulphate was added to a 20ml universal container (Sterilin) containing 5ml deionized formamide and 1ml sterilzed 20XSSC. The solution was heated at 70°C for 5 hours to dissolve the dextran sulphate. The solution was adjusted to pH 7.0 when cooled and the volume made to 7ml.

The buffer was aliquoted in 1ml Eppendorf tube and kept at -20°C.

Denaturation buffer

70% formamide (v/v), 2XSSC.
Adjusted to pH 7.0. Stored at 4°C in between use and discarded when it became cloudy.

Washing solution

50% formamide (v/v) 2XSSC.
Adjusted to pH 7.0. Kept at 4°C in between use and discarded when it became cloudy.

0.1M Na_2HPO_4

35.8gm in 1000ml distilled water. Stirred to dissolve and kept at 4°C.

0.1M NaH_2PO_4

7.8gm in 500ml. distilled water. Stirred to dissolve and kept at 4°C.

Phosphate-Nonidet-P40 buffer (PN buffer)

500 ml of 0.1M Na_2HPO_4 was titrated to pH 8.0 with 0.1M NaH_2PO_4. 500ul of Nonidet-P40 (Sigma) was then added to make a 0.1% concentration.

5% Non-fat milk diluent (PNM buffer)
(Modification of Mukherjee et al. 1990)

5gm of non-fat milk (Marvel UK) was dissolved in 100ml of PN buffer (as above) and 50ul of 1% sodium azide was added as preservative. The solution was shaken vigorously for 10 minutes and left at 37°C in waterbath for 48 hours. It was then centrifuged at 3000 rpm for 20 minutes and the supernatant collected and stored at 4°C.

Total human DNA (competitor DNA)

Human placental DNA (Sigma) was dissolved in purified water (Millipore) to make 10mg/ml concentration. When completely dissolved, it was placed on ice and then sonicated at high power for 4 bursts of 15 seconds each.

The solution was boiled for 10 minutes, chilled and kept at 4°C. The fragment size was checked by gel agarose electrophoresis (500bp) on average.

Salmon sperm DNA (Carrier DNA)

10mg/ml of 500bp size was prepared as described for the total human DNA (competitor DNA) above.

Fluorescein isothiocyanate (FITC)-Avidin (Vector Lab. UK. A201. Conc. 1.0 mg/ml)

(Alternative 1)

For detection, 5.0ul of the above stock was diluted in 995ul of 5% Phosphate Nonidet-Milk buffer (5ug/ml) immediately before used. 200-250ul of this solution was applied directly on each slide.

(Altenative 2)

This detection solution can also be prepared by diluting 200ul of FITC-avidin (above stock) in 40ml Phosphate Nonidet-Milk buffer and kept in 50ml coplin jar. It should be covered with aluminium foil to avoid direct exposure to light. Keep the solution at 4°C and can be used several times for up to 2 months. Bring the solution to 20°C before placing the slides.

Biotinylated goat anti-avidin (Vector Lab UK. BA-0300 Con. 0.5mg/ml).

Alternative 1

For detection, 10ul of the above stock was diluted in 990ul of 5% Phosphate Nonidet-milk buffer (5ug/ml) immediately before used. 200-250ul of this solution can be applied directly on each slide.

Alternative 2

This solution can also be prepared by diluting 400ul of biotinylated goat anti-avidin (above stock) in 40ml Phosphate Nonidet-Milk buffer and kept in 50ml coplin jar. It should be covered with aluminium foil to avoid direct exposure to light. Keep the solution at 4°C and can be used several times up to 2 months. Bring the solution to 20°C before placing the slides.

Antifade medium-[(DABCO)(1-4-Diazobicyclo (2-2-2)octane)]

Glycerol	90ml
0.2M Tris HCl	10ml
$NaNi_3$	0.02g
Adjusted to pH 8.0	
DABCO	2.3g

Dissolved by warming in 70°C waterbath for 2 to 3 hours. Aliquoted into tubes and kept at 4°C.

Propidium Iodide (2ug/ml)

To 1ml of DABCO antifade medium added 2ul of 1ug/ml of propidium iodide. Mixed and kept at 4°C in box to avoid direct exposure to light.

2.3 NON-ISOTOPIC IN SITU HYBRIDIZATION.

2.3.1. In situ hybridization using alkaline phosphatase detection (taken from Kalaitsidaki 1990)

The method for nick translation and detection is used routinely in the department and was essentially developed by Garson et al.(1987) and adapted from the BRL protocol for chromosomal preparations. The DNA probe is labelled

with biotin-11-dUTP using a standard nick-translation protocol as in BRL Nick Translation System (Cat. No. 8160SB) (Life Sci. U.K). Following hybridization of the biotinylated probe the chromosomal preparations are incubated with alkaline phosphatase conjugate (SA-AP) which binds to the biotinylated-alkaline probe/target hybrid. BCIP and NBT are used as the chromogenic substrate for the enzyme alkaline phosphatase. The colour reaction is initiated by the cleavage of the phosphate group from the BCIP by alkaline phosphatase. This reaction yields a blue colour and produces a proton which reduces NBT to yield a purple insoluble precipitate.

2.3.2 Biotin labelling of probe by nick-translation

The probe used was GMGY10 (DYS59), a 4.5kb insert cloned at a Hind III site of pUC19 isolated in the department from the Lawrence Livermore flow-sorted Y chromosome library (Affara et al. 1986). The probe was labelled by nick-translation with biotin-11-dUTP (Sigma, U.K.) using the BRL Nick Translation System (Cat No. 8160SB) for 50 reactions.

In an Eppendorf centrifuge tube the following were added sequentially and mixed : 5ul BRL Solution A4 (0.2mM nucleotide C,G and A in 500 mM Tris-Hcl (pH 7.8), 50mM magnesium chloride, 100mM 2-mercapethanol, 100ug/ml bovine serum albumin), 1ug DNA to be labelled, 2.5ul 0.4mM Biotin-

11-dUTP, BRL Solution E (distilled water) to make final volume 45ul. To this 5ul BRL Solution C (0.4 units/ul DNA polymerase I, 40pg/ul DNA PolI/DNAse I, 50mM Tris-Hcl (pH 7.5), 5mM magnesium acetate, 1mM 2-mercapethanol, 0.1mM phenylmethylsulphonyl fluoride, 50% glycerol, 100ug/ul bovine serum albumin). The reaction was mixed gently and spun briefly and was incubated at 15°C for 90 minutes. The reaction was stopped by adding 5ul BRL Solution D (300mM disodium EDTA, pH 8.0).

To precipitate the labelled DNA and to remove the unincorporated nucleotides the following were added to the reaction: 4.6ul 3M sodium acetate (pH 5.2) (filtered and sterilized), 1ul 20mg/ml glycogen (Life Sci. U.K) and 122ul iced ethanol. After vortexing it was then centrifuged for 30 minutes at 12,000 rpm. The supernatant was aspirated and the pellet was vacuum dried. The labelled DNA was dissolved in 10ul of TE buffer to give a concentration of 100ng/ul. After fully dissolved it was kept at -20°C until used.

2.3.3 In situ hybridization and probe detection

Chromosome metaphase spreads were prepared on glass slides by standard cytogenetics methods. Chromosomes were stained by Lipsol method. After treating with 1% Lipsol 10-15 seconds the slide was rinsed with normal saline followed by staining with 1:4 Leishman pH6.8 for 2 minutes 30 seconds. The slide was then rinsed in phosphate buffer pH

6.8 for differentiation.

After staining the slide was mounted in phosphate buffer, blotted to dry and the well-banded metaphases were photographed using Kodak Imagecapture AHU film. The vernier scale of individual metaphase was documented.

Slides were soaked in buffer to remove the coverslip and destained in an ascending ethanol series (50%,70%,90% and 100%) and air dried at room temperature and kept at 4°C prior to use.

Prior to in situ hybridization the slides were treated with 150ul RNase (100ug/ml) for 1 hour at 37°C in moist conditions. After washing off with phosphate buffered saline the slides were washed in a fresh ascending alcohol series (50%,70%, 90% and100%) and air dried at room temperature or in vacuum hood.

20ng of biotinylated GMGY10 probe in 10ul hybridization buffer (50% deionised formamide, 10% dextran sulphate, 2XSSC, 0.1mM EDTA, 0.05mM Tris-HCl, pH 7.5 and 100ug/ml denatured sonicated salmon sperm DNA) was placed on the $2cm^2$ marked area of the slide and covered with 24x24mm coverslip. The coverslip was sealed with cow gum and the chromosomal DNA and probe denatured simultaneously in a hot air oven at 80°C for 10 minutes. The slides were incubated in a 42°C waterbath overnight.

The cow gum was removed and the slide was placed in 2XSSC to remove the coverslip. This was followed by washing in 2XSSC for 30 minutes, 0.1XSSC at 42°C for 30 minutes,

2XSSC for 15 minutes and finally for 15 minutes in **Buffer 2** (3% bovine serum albumin in 0.1M Tris-HCl, pH7.5, 0.1M NaCl, 2mM $MgCl_2$, 0.05% Triton X-100) at room temperature.

For the detection of the hybridized probes the slide was incubated with 100ul of streptavidin alkaline phosphatase conjugate (1mg/ml) for 20 minutes at 37°C in a humid chamber. (The conjugate was prepared by diluting 1ul of streptavidin alkaline phosphatase (SA-AP) BRL BluGENE Kit cat. no. 8279SA in 99ul of filtered Buffer 2). The slides were flushed with **Buffer 1** (0.1M Tris-HCl, pH7.5, 0.1M NaCl, 2mM $MgCl_2$, 0.05% Triton X-100) and then washed 2 times, 5 minutes per wash, at room temperature in Buffer 1 followed by one washing for 10 minutes with **Buffer 3** (0.1M Tris-HCl, pH9.5. 0.1M NaCl, 50mM $MgCl_2$) at room temperature. The slides were then incubated with 150ul chromogenic substrate solution for 1 hour in a humidified condition in subdued light at room temperature. The substrate was prepared by adding 4.4ul Nitroblue tetrazolium (NBT), (BRL BluGENE Kit), 3.3ul Bromochloroindolylphosphate (BCIP), (BluGENE Kit), 10ul 10mM Levamisole (Sigma, U.K) to 1ml filtered **Buffer 3.** (The Levamisole helps to inhibit any unbound or endogenous alkaline phosphatase). The colour development was terminated by flushing the slide with **Buffer 3** and then placed in Stop Buffer (20mM Tris-HCl, pH7.5, 5mM EDTA) for 5 to 10 minutes at room temperature. The slides were air-dried and mounted with Glycergel (Dako., U.K). Each metaphase was relocated and viewed using phase contrast illumination to locate the signal. Each

signal was marked on the each pre-hybridized photograph. Only those signals associated with the chromosome were recorded. Scoring of cells and analysis of results was performed using statistical expression of chi-square (X^2) and as a probability (p) value (see Results **Table 5**). A value of $p< 0.001$ was considered a very highly significant.

2.3.4 Fluorescence in situ hybridization using unique sequence probe (Pinkel et al. 1986a)

The metaphase chromosomes were prepared according to the standard conventional cytogenetic technique and were aged by leaving at room temperature for 24 hours or by overnight incubation at 60°C

The slides were treated with RNase (100ug/ml) at 37°C for 1 hour, washed in phosphate buffered saline pH 7.0 (Sigma, U.K) and dehydrated in ascending alcohol series (50%, 70%, 90%, and 100%) and dried at room temperature.

The chromosomes on the slides were denatured by immersing the slides in 70% Formamide/2X SSC, pH7.0 at 80°C for 5 minutes and dehydrated in iced cold ethanol series (50%.,70%, 90%, and 100%) and dried at room temperature. 10 ul of hybridization mixture containing 40ng/ml biotinylated probe was also denatured at 80°C for 8 minutes and then applied immediately to the slides,(no pre-annealling required),then covered with 24x24mm coverslip and sealed with cow gum. Hybridization was carried out at

37°C for 16 to 18 hours. [The hybridization mixture was prepared by diluting 7ul of hybridization buffer (70% Formamide/2XSSC/10% dextran sulphate, pH 7.0), 2ul of probes (2ug/ml), (prepared by nick-translation reaction using BRL kit with Biotin-11 dUTP), and 1ul of sonicated salmon sperm DNA (1mg/ml)].

After peeling off the cow-gum, the slides were washed in three changes of 50% Formamide/2XSSC pH 7.0 at 45°C. 200ul of FITC-avidin (5ug/ml in 5% non-fat milk in phosphate buffer) was applied on the slide and incubated for 20 minutes at room temperature and covered with aluminium foil. After draining off the liquid, the slides were then washed in three changes of phosphate Nonidet-P40 buffer (0.1M NaH_2PO_4/ 0.1M Na_2HPO_4 pH8.0/ 0.1% Nonidet-P40) for 5 minutes each by gentle agitation on the shaker. Amplification of the probe fluorescence was carried out by incubating with 200ul biotinylated goat anti-avidin (5ug/ml in 5% non-fat milk in phosphate buffer) and incubated at room temperature for 20 minutes under aluminium foil. After draining off excess fluid, the slides were then washed in three changes of phosphate buffer pH8.0 for 5 minutes each. It was followed by a second layer of FITC-avidin by applying 200ul of FITC-avidin and incubated for 20 minutes at room temperature covered with aluminium foil. The slides were later washed in three changes of phosphate buffer pH8.0 for 5 minutes each and mounted with DABCO antifade solution containing 2ug/ml propidium iodide. (For further amplification the slides were further incubated with

biotinylated goat anti-avidin and FITC-avidin as above).

The slides were examined under fluorescence microscope (Zeiss Axioplan) with excitation wavelength 450-490 nm and photographed with Kodakchrome slide film ASA 400 using automatic exposure.

2.4 PREPARATION OF DNA PROBES

2.4.1 DNA Library

Throughout this study the whole chromosome 21 specific library, LL21SNO2 was used (purchased from ATCC, American Type Culture Collection, USA). This library was prepared by flow-sorting from a human diploid fibroblast cell line. After isolating the chromosome 21 DNA it was digested with Hind III restriction enzyme and then cloned into bacteriophage vector Charon 21A. The average insert size is 4.0kb. For amplication this phage library requires LE 392 as the bacterial host cells.

2.4.2 Preparation of host cells LE 392

The strain of bacteria used for host cells was LE 392. A wire loop was used to plate the glycerol stock onto the LB-agar (Lurias-Bertoni) plate. The plate was incubated at 37°C overnight.

A single colony was picked from the plate and transferred into 100ml of L-B broth (see general section) in a 250ml conical flask. 0.2% maltose, 10M magnesium sulphate, and 1M Calcium chloride were added to the culture medium to aid the absorption of bacteriophage lambda to the host bacteria. The culture was incubated by shaking overnight at 37°C in an orbital shaker.

The cells were spun down in plastic universals at 3,500 Rpm (IEC DPR 6000-Damon) for 15 minutes at 4°C. The pellet was washed twice in sterile SM. After the final wash the pellet was resuspended in SM. The concentration of the suspension was checked at OD 600 using Perkin Elmer 600 Spectrophotometer. The cell suspension was finally adjusted to an OD 600 of 0.8 and stored at 4°C until used for library titration and amplification.

2.4.3 Titration of ATCC LL21SN02 library (plate lysis)

Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the ATCC library stock to be titrated were prepared in SM buffer in a final volume of 10ul in 1.5ml sterile Eppendorf tubes. 1ul of each of the dilutions was transferred into a 10ml Sarstedt tube containing 100ul of LE 392 host cells. The suspensions were thoroughly mixed and incubated at 37°C in waterbath for 30 minutes to allow phage to adsorb. 3ml 0.5% top agarose cooled to 50°C was added to each tube, mixed and poured on the agarose plate. The plates were left

half-covered to set and cool. They were then inverted and incubated at 37°C overnight.

The plaques formed were evidence by the presence in the plate of a round clear spots of bacterial lysis. The number of plaques in each dilution plate was counted. The titer per ml of the ATCC library stock was then calculated by multiplying the dilution factor and the number of plaques obtained from each dilution.

Calculation (example):

in a 10^{-2} dilution : 120 plaques counted

120 (plaque) X 10 (0.1ml of 1ml) X 10^{-2} =1.2 X 10^{4} phages/ml.

The library used (the third shipment, see results) in this study was found to have 1.2 X 10^{7} pfu/ml.

2.4.4 Preparation of high titre library working stock

Immediate amplification of the supplied library was made to avoid shortage of the library in the later part of the project.

10 ul of the supplied library was amplified using the plate lysate as in the following amplification and purification procedure.

With this amount 100% lysis on the plate was obtained. 3 ml of SM was added to the plate and left to shake gently in cold room to remove the phage. The supernatant was collected into 2 Eppendorf tubes (approximately 1.5ml each) and 2.5ul of chloroform was added to each tube. After gentle mixing the tubes were left at room temperature for 5 minutes. The tubes were centrifuged at 10,000 RPM for 10 minutes and the supernatant collected into new tubes and kept at 4°C until further use.

This stock were then titrated as above and was found to be 11 X 10^{10} pfu/ml.

This new working stock was used for DNA extraction and amplification.

2.4.5 Amplification and purification of the library.

The amount of the working stock library was used for further purification. 0.5 ul of this stock which represented 7.80 X 10^3 pfu/ml for complete coverage of phage as stated by the supplier.

This representative amount was added to a blood culture tube containing 100ul LE 392 cells. The tubes were incubated at 37°C for 30 minutes in the water bath to allow attachment of the phage. 3ml of the top agar, made up of 0.5% agarose cooled to 55°C were added to each tube, mixed by inversion and poured on the agarose plate. The plates were set to cool for 10 minutes and then incubated

overnight at 37°C. The representative amount of phage library showed more than 80% lysis.

After incubation, the phage were removed by adding 3ml of SM to each plate. The plates were sealed to avoid spillage and evaporation and were left overnight shaking gently in the cold room.

The following day the plates were tilted and the supernatant drained to one side for 5-10 minutes. The supernatant from each plate was completely aspirated and transferred into 2 Eppendorf tubes. Each tube contained an approximately 1.5ml of suspension. To each tube were added 120ul of 5M NaCl, 2ul of RNase (10mg/ml) and 2ul DNase (10mg/ml) respectively. The tubes were vortexed to mix the contents and placed at 37°C in waterbath for 60 minutes followed by centrifugation at 10,000 rpm for 10 minutes to precipitate the bacteria and agar debris. The supernatant was aspirated and transferred into Eppendorf tubes each containing 100mg PEG 8000 (polyethylene glycol). The suspension was vortexed and left to mix (Spiralmix 10, Denley UK) overnight in cold room.

On the third day the suspension was centrifuged at high speed for 30 minutes. The supernatant was discarded and the pellet was dissolved in 300ul of TE buffer. The suspension was left at room temperature for 30 minutes with occasional mixing. The two tubes were then combined making a total of 600ul suspension.

The suspension was phenol-extracted twice, by adding equal volume of phenol to each tube. After adding 600ul of

phenol, the suspension was vortexed until milky and spun for 3 minutes at high speed. The aqueous layer was aspirated and again extracted with phenol. It was then extracted with phenol/chloroform and finally with chloroform. The aqueous layer was transferred into a new tube to be precipitated by ethanol.

To all of the suspensions, a double volume of cold ethanol and half volume of 7.5M ammonium acetate was added. After thorough mixing it was left at -20°C overnight. The suspensions were spun at high speed (10,000 rpm) for 30 minutes. After discarding the supernatant the pellet was washed in 70% cold ethanol, spun at 10,000 rpm for 30 seconds and then dried under vacuum for 15 minutes. The pellet was finally resuspended in 30ul T.E and run directly in an ethidium bromide gel to check the purity. Normally each plate yielded 5-7ug of DNA.

2.4.6 Restriction enzyme digest of purified DNA library

The digestion reaction was carried out using 20ul of resuspended purified DNA, 2ul of 0.1M spermidine, 2ul of Hind lll restriction enzyme (BRL, Life Sci. UK), and 4ul of digestion buffer (BRL, Life Sci. UK). The mixture was vortexed and spun briefly to concentrate. The digestion reaction was carried out overnight at 37°C.

2ul of loading mix was added and electrophoresis was carried out on 0.8% agarose (Sea Kem GTG, FMC) at 100 mA

for 2 hours as in the section below to analyse the digest.

2.4.7 Gel electrophoresis

Electrophoresis of the purified DNA was carried out on 0.8% agarose (Sea Kem GTG, FMC). 8mg of agarose was dissolved in 100 ml electrophoresis buffer and melted in a microwave oven for 5 minutes. When cooled to 60°C, 2ul of 1% ethidium bromide was added before pouring the agarose onto the slab. Loading of the samples was accompanied by loading of 1 kb marker (BRL 5615) for identification of size fragments on the gel. LKB Bromma/2197 constant power supply pack was used typically at 200mA/ for 60 minutes for the 10cmx13cm gels used. The DNA was visualized on a UV transilluminator (Foto Dyne Incorp, UK) and photographed using a Poloroid CU-5 hand camera fitted with a red filter and containing black and white Poloroid type 667 film.

2.4.8 Estimation of DNA concentration

10ul of purified DNA was added to 990ul of deionised water in 1.5ml Eppendorf tube. The suspension was transferred into a quartz tube and the optical density was measured at 260nm in a UV/VIS spectrophotometer (PU 8820-Philips UK). Since 1 OD260 unit corresponds to 50ug of DNA and the DNA was diluted by 100, the OD260 reading of the

DNA was multiplied by 5000 to give the concentration in ug/ml.

2.4.9 Direct biotin labelling using the PCR amplification

This technique is a modification of J.Fantas and S.Boyle, Edinburgh, (personal communication), which is based on that described by Saiki et al. (1988) and Lo et al. (1988).

All the dNTPs, 10XPCR buffer and Taq polymerase were obtained from Perkin-Elmer Cetus. The Biotin-11-dUTP (Sigma) was prepared in the laboratory. Forward and reverse primers were obtained from Oswell DNA Service, Edinburgh. The procedure described was for one PCR amplification of 100ul volume including target DNA and Taq polymerase.

To the tube was added 2ul of 10mM dATP, (Sodium 2'-Deoxyadenosine 5'-Triphosphate), 2ul of 10mM dCTP (Sodium 2'-Deoxycytidine 5'-Triphosphate) 2ul of dGTP (Sodium 2'-Deoxyguanosine 5'-Triphosphate) each at a final concentration of 100uM, followed by 5ul of 2mM Biotin-11-dUTP (final concentratiom 100uM), 10ul of 10XPCR buffer (500mM KCL/ 100mM Tris-HCL (pH 8.3)/ 15mM MgCl2/ 0.1% gelatin), 1.5ul of forward primer 459H (5'-CCT TTA AAA AAG TCG TTT CTG CA -3') and 1.8ul of reverse primer 460H (5'-TCA CTT CGA AAG AGT TAG TTC -3'). Both primers were at a final concentration of 0.3mM. 71.2ul of deionized water was added to make to a total volume of 100ul.

The mixture was vortexed thoroughly and spun briefly at high speed to concentrate the fluid in the bottom of the tube, then placed on UV transilluminator (FotoDyne Incorp.UK) for 15 minutes to denature any DNA contaminants present.

2ul of purified DNA library was added to the mixture, vortexed and spun briefly at high speed to concentrate. The mixture was placed at 96°C in the heating block (Dri-Block DB-1, Techne, UK) for 10 minutes for predenaturation.

The mixture was left at room temperature for 5 minutes before adding 0.5ul of Taq polymerase (final concentration of 2.5units/100ul). It was then vortexed thoroughly and spun briefly to concentrate. 80ul of mineral oil was layered on the mixture to prevent any evaporation during the amplification. Bubble formation in the mixture should be avoided to prevent oxidation of enzymes in the solution.

All the the reactions were performed on the PCR Thermal Programmable Block (PHC-1-Techne, ~~Perkin-Elmer~~) for 30 cycles amplification; denaturation at 94°C for 1 minute, primer annealing at 42°C for 1.5 minutes and polymerisation at 72°C for 3.5 minutes followed by a 7 minutes final extension at 60°C.

2.4.10 Checking of PCR product

Initially, the amount of the purified library DNA to be added to the PCR mixture was determined with at least 5 separate volumes (1ul, 2ul, 3ul, 4ul, 5ul) to check the optimum working concentration for every new batch of purified DNA.

After the PCR amplification was completed the product was checked by visualization using agarose gel electrophoresis. To 20ul of the reaction mix was added 3ul of loading buffer and the mixture was loaded into 0.8% agarose gel (Sea Kem GTG-FMC). Electrophoresis was then carried out at 100mA for 1 hour. The gel was placed on a U.V transilluminator (Foto Dyne Inc) to assess the size and yield of the PCR products.

2.4.11 Cleaning of PCR product

Unincorporated nucleotides were separated by Pharmacia Nick Columns (Pharmacia UK). The recovered eluate was freeze dried overnight and dissolved in TE buffer.

The prepacked disposable columns containing Sephadex-50 (Nick columns) were obtained from Pharmacia UK. and the equilibrium buffer prepared in the department.

The storage liquid was poured off by removing the cap and the column was rinsed according to the manufacturer's instruction. It was equilibrated with 4ml of equilibrium

buffer twice. The column was clamped to the laboratory stand and the bottom cap was removed to allow the equilibrium buffer to enter the gel bed freely. After removing the mineral oil on the top layer of the PCR reaction tube, the whole mixture (100ul) was placed onto the column. 400ul of equilibrium buffer was added to the column after the PCR mixture had entered the gel bed. The equilibrium buffer was allowed to elute and discarded. An Eppendorf tube was placed at the bottom of the column after adding another 400ul of equilibrium buffer and the eluted purified sample was collected.

The 400ul of the eluted sample was placed in liquid nitrogen for 10 minutes to freeze. A number of holes were made on the cover of an Eppendorf tube before placing it in the freeze dryer chamber (Harris UK). The sample was freeze dried overnight.

The dried sample was resuspended in 50ul of Tris buffer (pH 8.0) and the concentration was checked by dot-blot hybridization using the Blue Gene Kits (BRL. Life Sci. UK).

2.4.12 Estimation of biotinylated polymerase chain reaction (PCR) product using dot blot hybridization

The method is based on the immunological detection of biotin which couple to alkaline phosphatase and conjugation

of colour substrate BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitroblue tetrazolium) giving rise to a deep blue colour adhering to the membrane. This protocol is simplified from the BRL, BluGENE Nonradioactive Nucleic Acid Detection System, (Cat.no. 8279SA).

2 ul of biotinylated PCR product was dropped on the 5mmx10mm nitrocellulose membrane filter (Hybond) dried at 80°C for 3 hours and kept in the dark until used. The control biotinylated DNA was diluted with the dilution buffer (supplied in the kit) at a concentration of 5pg, 10pg, 20pg respectively, and 2ul of each dilution was dropped on a similar size membrane nitrocellulose membrane filter and dried at 80°C for 3 hours. These membrane filters were washed briefly twice for 5 minutes per wash, with 50 ml of **Buffer 1** (0.1M Tris-CL; 0.15M NaCl; pH 7.5) by constant shaking and then incubated in Buffer 2 (0.1M Tris-Cl; 0.15 NaCl;3% Bovine serum albumin fraction V; pH 7.5) at 60°C waterbath for 60 minutes. After discarding **Buffer 2**, the membrane were then incubated in 10 ml diluted conjugated streptavidin alkaline phosphatase (0.01mg/ml) (10 ul SA-AP steptavidin alkaline phosphatase in 10 ml **Buffer 1**) for 10 minutes in the dark at room temperature. The unbound conjugates were removed by washing the membranes twice, 15 minutes per wash, with 200 ml **Buffer 1.** Finally the membranes were transferred into 10 ml freshly prepared chromogenic substrate solution. The substrate was prepared by diluting 35 ul BCIP (5-bromo-4-chloro-3-indolylphosphate and 45 ul NBT (nitroblue tetrazolium) in

10 ml **Buffer 1**) and incubated for 1-3 hours in subdued light at room temperature for colour development. Colour developments were then terminated by washing the membrane filters twice in stop buffer (0.01M Tris-Cl; 1mM EDTA pH 8.5), 10 minutes per wash, and dried briefly for 10 minutes in 80°C oven. These membrane filters were wrapped with a Saran film. The results were documented by photocopying the membranes. The concentration of the biotinylated PCR was determined by comparing the colour with control biotinylated DNA.

2.5 CHROMOSOMAL IN SITU SUPPRESSION HYBRIDIZATION USING WHOLE CHROMOSOME 21 SPECIFIC LIBRARY. (Modifications of Pinkel et al.(1988) and P.Lichter et al.(1988a)).

The procedure required three working days after the slide preparation and the probe labelling. On the first day, the labelled probe was ethanol-precipitated with the competitor and carrier DNA. The slide was refixed and if required Lipsol banded. The denaturation of probe/competitor/carrier DNA mixture, preannealing and application to slide and hybridization incubation and denaturation of target metaphases on the slides were carried out on the second day. The following day included the immunocytochemical detection, counterstaining and mounting, and microscopy.

Day 1

a) Slide preparation

The slides were scanned under low power phase contrast microscope and areas of good metaphases were marked. The slides were then refixed in methanol-acetic acid (3:1) for 60 minutes and left to dry at room temperature before keeping in a closed coplin jar.

(Optional: when pre banding was required, the slides were Lipsol banded according to Boyd et al. (1989) and destained in two changes of 100% methanol. These destained slides have to be treated with RNase 100ug/ml at 37°C for 60 minutes and cleaned with ascending series of alcohol(50%, 70%, 90%, 100%) prior to denaturation on Day 2).

b) Ethanol-precipitation of DNA

The following volume was applied for a single slide preparation only. 2ul of total human DNA (1ug/ul) and 2ul of human cot-1-DNA (0.5ug/ul) as competitor DNA, 3ul of salmon sperm DNA (1ug/ul) as carrier DNA and 10ul of biotin labelled probe (2ng/ul) were combined in an Eppendorf tube. The total DNA for a single slide should not exceed 10ug (see result and discussion). 60ul of filtered cold absolute ethanol was added, vortexed thoroughly to mix and

spun briefly to concentrate before keeping at -20°C overnight.

Day 2

a) Denaturation of hybridization mixture

The hybridization mix was centrifuged at 10000 RPM (IEC Centra 4X International Equipment) for 30 minutes. The supernatant was discarded and the tube was placed upside down on Kleenex tissue for 20 minutes to dry the pellet. 12ul of hybridization buffer (50% Formamide/10% dextran sulphate/2XSSC, pH 7.0) was pipeted into the test tube to resuspend the pellet. The suspension was vortexed occasionally for 10 minutes to ensure the DNA was completely homogeneous and spun briefly to concentrate the mixture at the bottom of the tube.

The hybridization mixture was placed in a 75°C waterbath to denature the DNA. After 8 minutes denaturation, the tube was immediately transferred into ~~an~~ ice and then left in the 37°C waterbath to preanneal for 60-90 minutes.

a) Denaturation of target material (chromosomes)

Slide denaturation was carried out separately during the preannealling time. The metaphases on the slides were denatured by immersing the slide in 70% Formamide/2XSSC

preheated to 75°C. After 8 minutes denaturation, the slides were transferred immediately to wash sequentially in ascending series of cold-ethanol 50%, 70%, 90%, 100% for at least 3 minutes each. The slides were left dried in a vacuum cupboard until further application of the hybridization mixture.

After 60-90 minutes of the preannealing, 10ul of the hybridization mixture was placed on the pre-marked area of each slide. The 24x24mm coverslip was placed on each slide carefully avoiding any air bubble formation. The coverslips were sealed with cow gum. Slides were incubated in moist oven or waterbath at 37°C for 16-20 hours for hybridization.

Day 3

a) Post hybridization washing

The cow gum was carefully peeled and the slides were placed in three changes of 50% Formamide/2XSSC warmed to 45°C washing. The coverslip will be automatically removed after 3-5 minutes in the first coplin jar. The slides were further washed in another two changes of 50% Formamide/2xSSC at 45°C for 5 minutes each then finally transferred into a plastic sandwich box containing phosphated-nonidet buffer (0.1% Nonidet P-40 in NaHPO4-pH 8.0) and left to wash by constant shaking on a shaker (R100 Rotatest Shaker-Luckham) until further treatment.

b) Immunocytochemical detection

The fluoresceinated avidin (FITC) and biotinylated goat anti-avidin (Vector Laboratory-UK) were diluted in 5% non-fat milk (Marvel-UK) prepared in phosphate nonidet-40 buffer (see the above non-fat milk preparation) 5.0ul of FITC was diluted in 995ul of non-fat milk diluent. 10ul of biotinylated goat anti-avidin was diluted in 990ul of the similar diluent to a final concentration of 5ug/ml.

All incubations were carried out in subdued light by covering the slides with the aluminium foil. The phosphate-nonidet buffer was used as washing solution. The washes were performed in a sandwich box with constant shaking.

The surrounding part of the marked area of the slide was briefly dried with Kimwipes tissue and the slides were placed in the tray. To each slide, 200ul of FITC diluent was immediately applied on the marked area. The tray was covered with aluminium foil. Incubation was carried out at room temperature for 20 minutes. Slides were taken and placed in a sandwich box containing the washing buffer and were then washed 3 times for 5 minutes each at room temperature. Washing was by gentle shaking on the automatic shaker (R100 Rotatest, Luckham, UK).

After the final wash the slides were again wiped briefly surrounding the marked area and placed on the tray. 200ul of biotinylated goat anti-avidin diluent was then applied to each slide and incubated for a similar duration at room temperature. After the similar washes and

treatments, another 200ul of the FITC diluent was applied for the second layer of detection and was further incubated at room temperature for 20 minutes. The slides were finally washed in another three changes of washing buffer and left in the buffer until being counterstained and mounted.

Alternatively the slides were stained by dipping in the coplin jar for 20 minutes each. This had an advantage that the slide did not dry even the timing exceeded the limit. However, the stains should be warmed up to room temperature before staining.

c) Counterstaining and mounting

The slides were dried briefly by wiping the surrounding area and placed face down on a 22x32mm coverslip with 10ul antifade medium (1,4-diazobicyclo-(2,2,2 acetone)) (DABCO-Sigma UK) containing 2ug/ml propidium iodide (Sigma UK) as a counterstain. The filter paper was used to blot excess antifade. Nail vanish was applied around the edge of the coverslip to seal.

2.5.1 Microphotography

All observations were made by epi-fluorescence using a Zeiss Axioplan microscope equiped with fluorescence filter combination 487709, BP450-490 FT510. A decorated target chromosome 21 appeared yellow and the blocked

chromosomes red colour under the microscope.

The photographs were taken with Kodak Ektachrome 400 (colour slide film) using automatic mode or 30-60 seconds exposure time with manual mode and printed on Kodak or Fujicolor paper. Printing had to be done commercially and thus the quality of the photograph could not be monitored.

CHAPTER 3

RESULTS

3.1 NON-ISOTOPIC IN SITU HYBRIDIZATION USING ALKALINE PHOSPHATASE DETECTION

The initial stage of the study was to become familiar with the non-isotopic in situ hybridization technique using alkaline phosphatase detection system and phase contrast microscopy according to the method of Garson et al. (1987) which was in routine use in the department. Probe DYS59 (GMGY10) a highly repetitive probe assigned to the short arm of the Y chromosome was used as a model (**Figure 3**). With this technique the chromosomes were prebanded and photographed. The optimal hybridization temperature was found to be between 37-42°C and the hybridization was performed at 42°C for 12 to 14 hours. Before hybridization both the probe and chromosome DNA have to be denatured at 75°C to 80°C to make them single stranded. With the alkaline phosphatase detection system the hybridization signals were visualised using phase contrast microscopy. Final interpretation of results involved statistical analysis of scored signals as in **Table 5.**

Subsequently, the technique was successfully used to localise the angiotensinogen gene to 1q42-43 using the probe peAHB. This probe is a pBR322-derived pECE plasmid containing a 2.5-kb cDNA sequence. Following hybridization to karyotypically normal male metaphase chromosomes, a

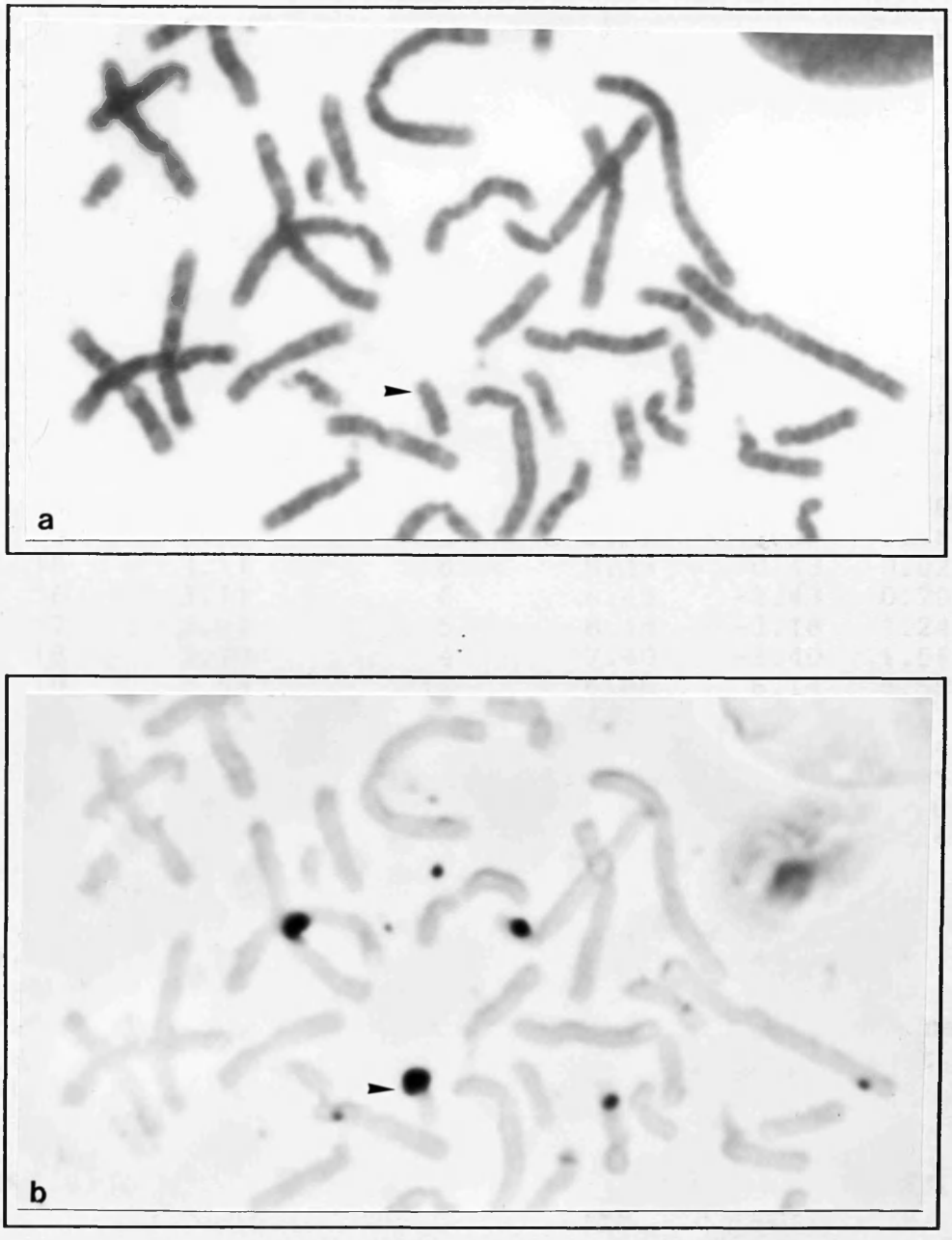


Figure 3. Photomicrographs of non-isotopic in situ hybridization with GMG Y10 probe using alkaline phosphatase detection. Partial metaphase of (a) pre- and (b) post hybridization with hybridization sites on Y indicated by arrowheads.

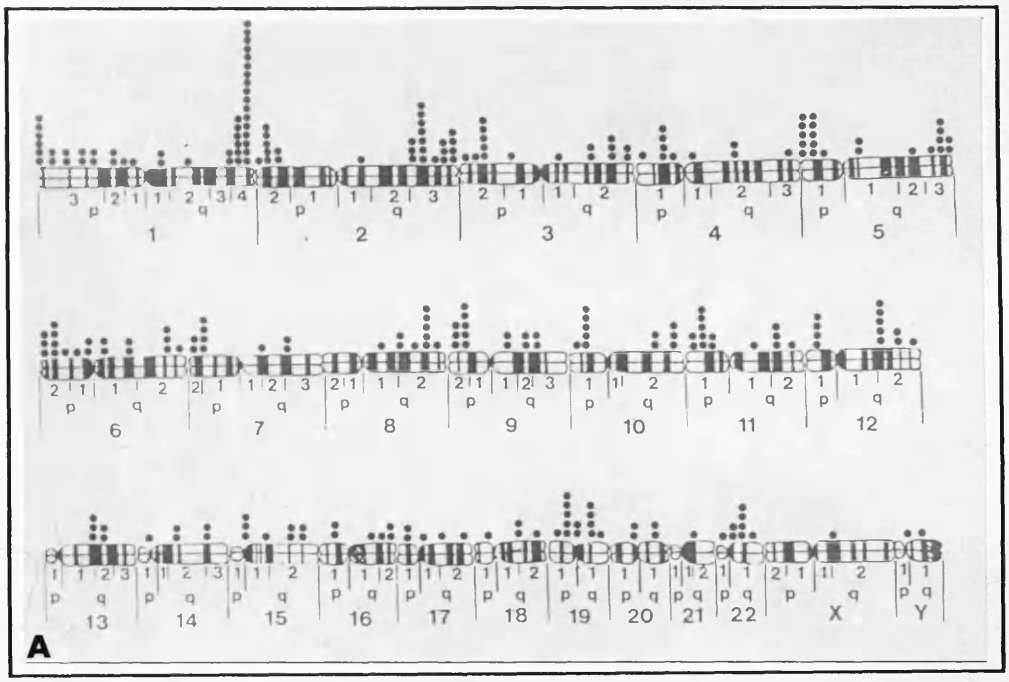
Chromosome	Relative Length	Observed O	Expected E*	O-E	$\frac{(O-E)^2}{E}$
1	8.47	46	22.95	23.05	23.15
2	7.76	27	21.00	6.00	1.17
3	6.56	16	17.78	-1.78	0.18
4	6.13	10	16.61	-6.61	2.63
5	5.58	21	15.12	5.58	2.29
6	5.56	19	15.31	3.69	0.89
7	5.00	9	13.55	-4.55	1.53
8	4.77	10	12.92	-2.92	0.66
9	4.73	14	12.81	1.19	0.11
10	4.35	11	11.78	5.78	0.05
11	4.35	14	11.78	2.22	0.42
12	4.16	12	11.27	0.73	0.04
13	3.59	5	9.73	-4.73	2.30
14	3.28	5	8.89	-3.89	1.70
15	3.11	8	8.43	0.43	0.02
16	3.11	6	8.43	-2.43	0.70
17	3.02	5	8.18	-3.18	1.24
18	2.73	4	7.40	-3.40	1.56
19	2.58	13	6.86	6.14	5.50
20	2.31	4	6.26	-2.26	0.81
21	1.90	1	5.51	-4.15	3.34
22	1.69	8	4.58	3.42	2.55
X	5.14	1	6.96	-5.96	5.10
Y	2.09	2	2.83	-0.83	0.24

Table 5. Statistical analysis of signal distribution of biotinylated peAHB probe in normal male blood. A total number of 271 signals counted from 70 metaphases examined. Chi-square for the chromosome 1 is highly significant at $p<0.005$.

$$\text{Expected (E)}^* = \frac{\text{Relative length X Total signal counted}}{100}$$

total of 271 hybridization signals were recorded in a total of 70 metaphases. Of the total signal counted, 17% (46/271) was located on chromosome 1, 50% (23/46) of which was located between 1q42 and 1q43 (**Figures 4A-C**). These hybridization signals observed at 1q42-43 were statistically highly significant ($p<0.005$) thus, allowing regional assignment (Isa et al., 1990).

In the later part of this initial work, a fluorescence detection technique using fluoresceinated avidin and biotinylated goat anti-avidin (Vector Lab. UK) was applied to detect the hybridization of highly repetitive probes DYS58 (GMGY7) and DYS59 (GMGY10). Instead of detecting the signals with the alkaline phosphatase reaction, these signals were observed under fluorescence microscopy. As compared to the alkaline phosphatase detection the results showed that majority of the signals were fine and discrete and located on both chromatids of the Y chromosome (**Figures 5 and 6**). These results showed that fluorescence detection is highly sensitive, requires fewer metaphases for mapping and obviates the need for statistical analysis.



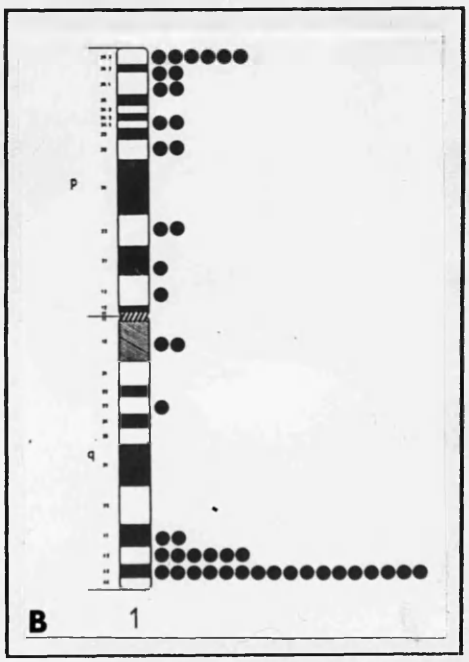


Figure 4. (A) A diagrammatic representation of distribution of 271 signals in 70 normal male metaphases after in situ hybridization with the human angiotensinogen (peAHB) using alkaline phosphatase detection method. (B) A diagrammatic representation of chromosome 1 showing precise distribution of signals. 50% (23/46) signals located between 1q42 and 1q43.

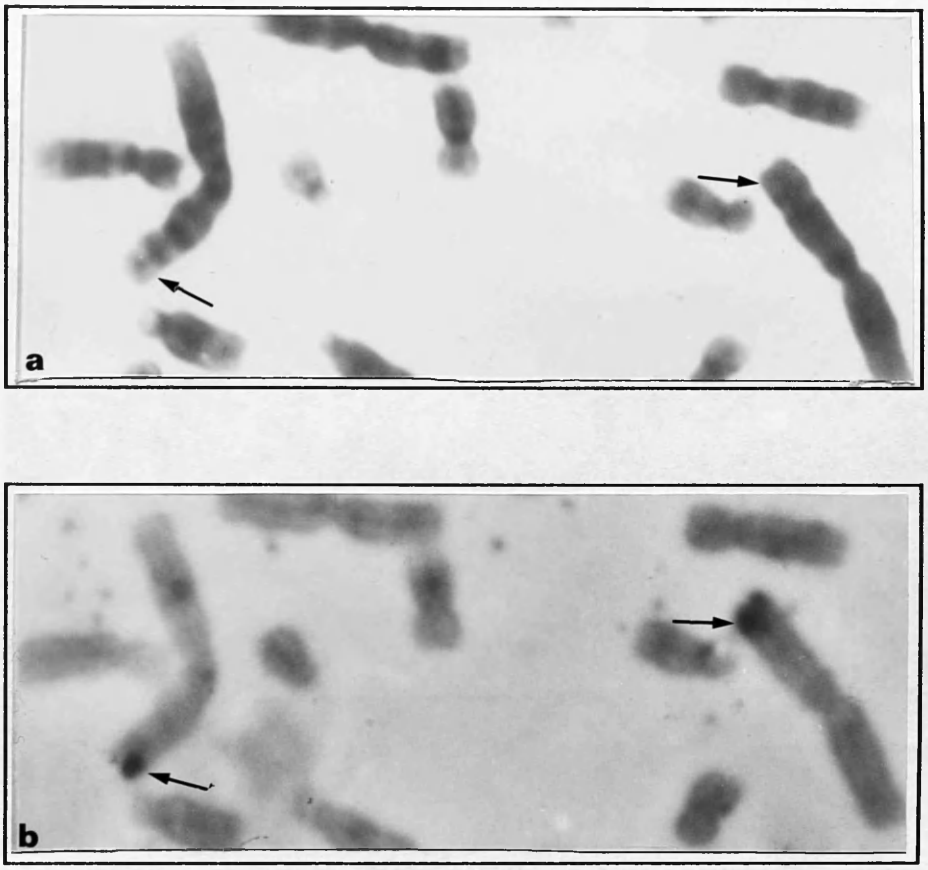


Figure 4C. Partial metaphases of pre (a) and (b) post hybridization sites of human angiotensinogen gene. Arrows showing the site and signal over 1q42-q43.

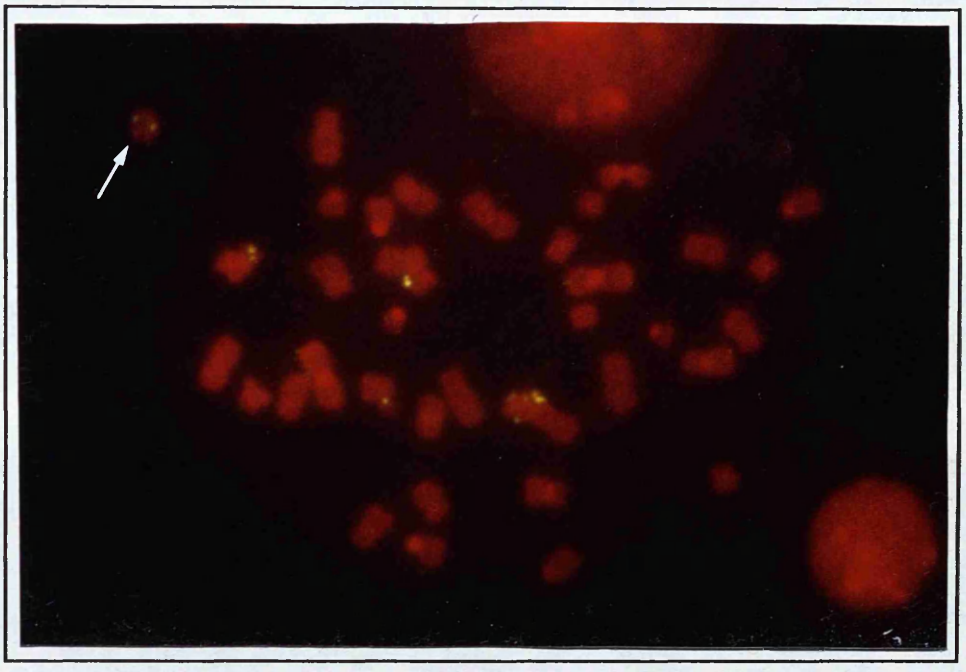


Figure 5. Photomicrograph of fluorescence in situ hybridization using GMG Y10. A partial metaphase chromosomes after fluorescence in situ hybridization. 12 of the 25 metaphases observed showed two adjacent signals located on the chromosome Y. Arrow shows chromosome Y with two fine signals.

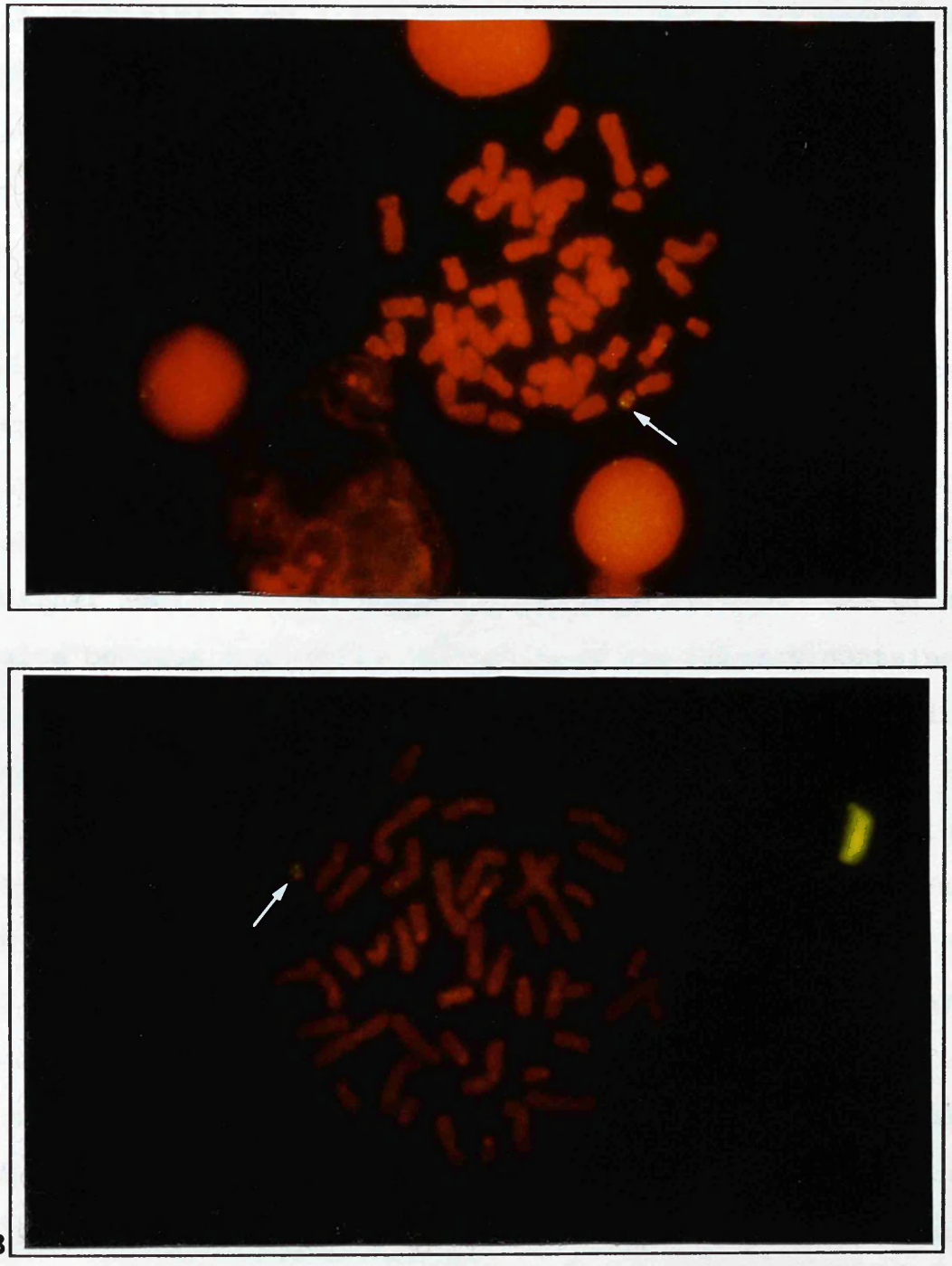


Figure 6. Photomicrographs of (A) Fluorescence in situ hybridization of a case of isochromosome Yp using GMG Y7 and (B) Fluorescence in situ hybridization of a case of isochromosome Yq using GMG Y10. Arrows show the signal on the isochromosmes Yp ~~and Yq~~ respectively.

3.2 PREPARATION OF A DNA PROBE FROM A WHOLE CHROMOSOME 21 SPECIFIC LIBRARY (ATCC LL21SN02)

3.2.1 Titration of the DNA library

Before commencing the amplification, the number of bacteriophage containing the flow-sorted sequences representing the library was checked. The titre of the first shipment was 2.5 X 10^4 pfu/ml which was almost 90% less than the stated concentration (2.0 X 10^7 pfu/ml). Further amplification and purification therefore, was of no value because the remaining volume of the library contained insufficient number of phage needed to represent the entire length of the chromosome.

On the second shipment, the titre was higher than the first shipment but the concentration was 6.5 X 10^6 pfu/ml which was almost 30% less than the stated concentration (2.0 X 10^7 pfu/ml). However, amplification of the library bacteriophage lambda DNA was attempted by carrying out a mini preparation and a large scale purification following the described protocol by Maniatis et al. (1982); Sambrook (1989) and P.Lichter (personal communication). Plaque lysis of the mini preparation on the agar culture was tiny as compared to the control phage. However, the purification of the bacteriophage lambda DNA was continued. Both amplifications failed to produce any DNA when checked by gel electrophoresis. Consequently, the standard techniques described by Boulnois et al. (1987), Arrand et al. (1988)

and the Qiagen Kit purification (Qiagen. UK) were also adopted but failed to produce the required bacteriophage lambda DNA. No modification of these methods was attempted since it was concluded that the failure was due to the poor condition of the library on arrival.

3.2.2 Amplification and purification of the library

On the third shipment of the library the titration was also carried out as described by the ATCC but with modifications of the agar content. As an alternative, amplification and purification of this third sample was also performed with the modifications of the protocols suggested by J. Fantes and S. Boyle, MRC Edinburgh (personal communication) as described by Dumanski et al. (1988).

The titre was found to be better (12 X 10^6 pfu/ml) although still only 70% of that shown by the ATCC. The plaque lysis was found to be slightly larger and more prominent than that obtained with amplification. The volume used, to represent the entire chromosome was shown to give 80-90% lysis on the plate. This major improvement was due to the content of the agar base and top agarose used in the amplification culture. The Bacto agar (Difco. UK) was replaced by agarose (Sigma Type ll Medium EEO. UK) for the agar base and the top agarose. For the top agarose itself the tryptone and the yeast extract were replaced by

trypticase, a pancreatic digest of casein (BBL-119921, Becton Dickinson, UK).

Following the successful amplification, purification and extraction were performed on plate lysate (mini preparation). The total amount of the bacteriophage lambda DNA obtained from the pool averaged 60-80ug/ml. The undigested purified DNA showed a single band on gel electrophoresis (**Figure 7**). No discrete band or individual fragments were observed when digested with the Hind III restriction enzyme but an area of diffuse fluorescence was observed, demonstrating a variety of DNA fragments released from the vector (**Figure 7, Lane C**). The whole vector and human DNA insert was then labelled with Biotin 11-dUTP. Unfortunately, the nick-translated DNA failed to produce a homogeneous signal (decoration) with the chromosome 21 (**Figure 10**).

3.2.3 Direct labelling of probe using polymerase chain reaction (PCR) and amplification

The DNA (both the whole vector and insert) was labelled directly with Biotin-11-dUTP using the polymerase chain reaction (PCR) as described by Saiki et al. (1988) and as suggested by J.Fantes and S.Boyle, MRC Human Genetics Unit Edinburgh (personal communication). The volume of the DNA solution used in the reactions is dependent on the concentration of the DNA and varies for

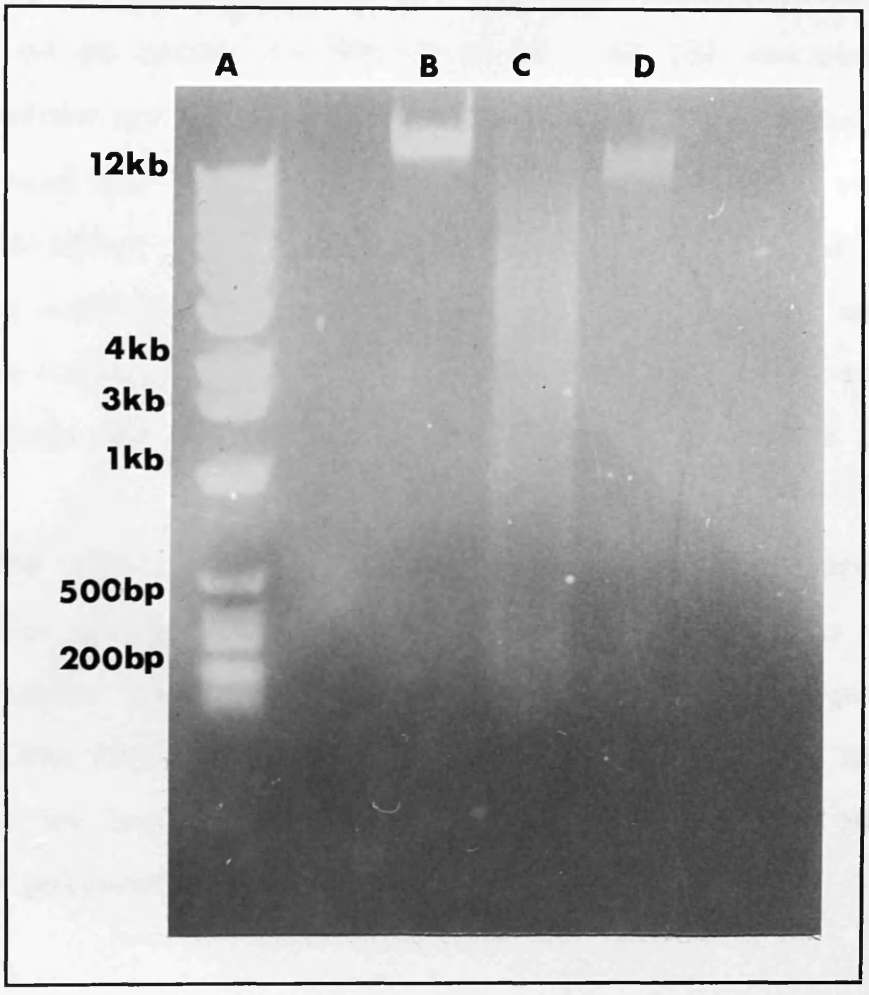


Figure 7. Gel electrophoresis of purified whole chromosome 21 specific library constructed in bacteriophage Charon 21A. **Lane A**, 1Kb ladder.
Lane B, Undigested DNA probe. **Lane C**, Hind lll digested DNA probe. **Lane D**, Different batch of undigested DNA probe.

the different purified batches. It was found that a purified DNA concentration of 50-60ug/ml required 2ul to 3ul (approximately 100ng) for one PCR tube reaction, and for a DNA of concentration 60 to 80ug/ml 1ul was required. Thus before continuing the large scale labelling the volume to be used was tested (1ul up to 5ul) for each PCR reaction to find which gave the maximum result. The yield of PCR product amplified often differed from one batch to another. Optimum direct biotin labelling using PCR reaction is shown by diffuse DNA fluorescence on gel electrophoresis (**Figure 8**).

The PCR labelling showed a significant improvement when the dNTPs/primer/biotin-11-dUTP mixture was placed under ultra-violet (UV) light for 10-15 minutes prior to adding the DNA and also heating the ingredients at 96°C for 10 minutes for initial denaturation prior to the addition of Taq polymerase.

3.2.4 Cleaning the PCR product

Several methods of cleaning the PCR product were investigated including direct ethanol precipitation, Gene Cleaning (Bio1, USA) and Sephadex G-50 column cleaning (Pharmacia, UK). It was found that ethanol precipitation failed to remove excess dNTPs while the gene cleaning tended to contain some glass milk which interfered with the subsequent hybridization. Cleaning by Pharmacia Nick-column

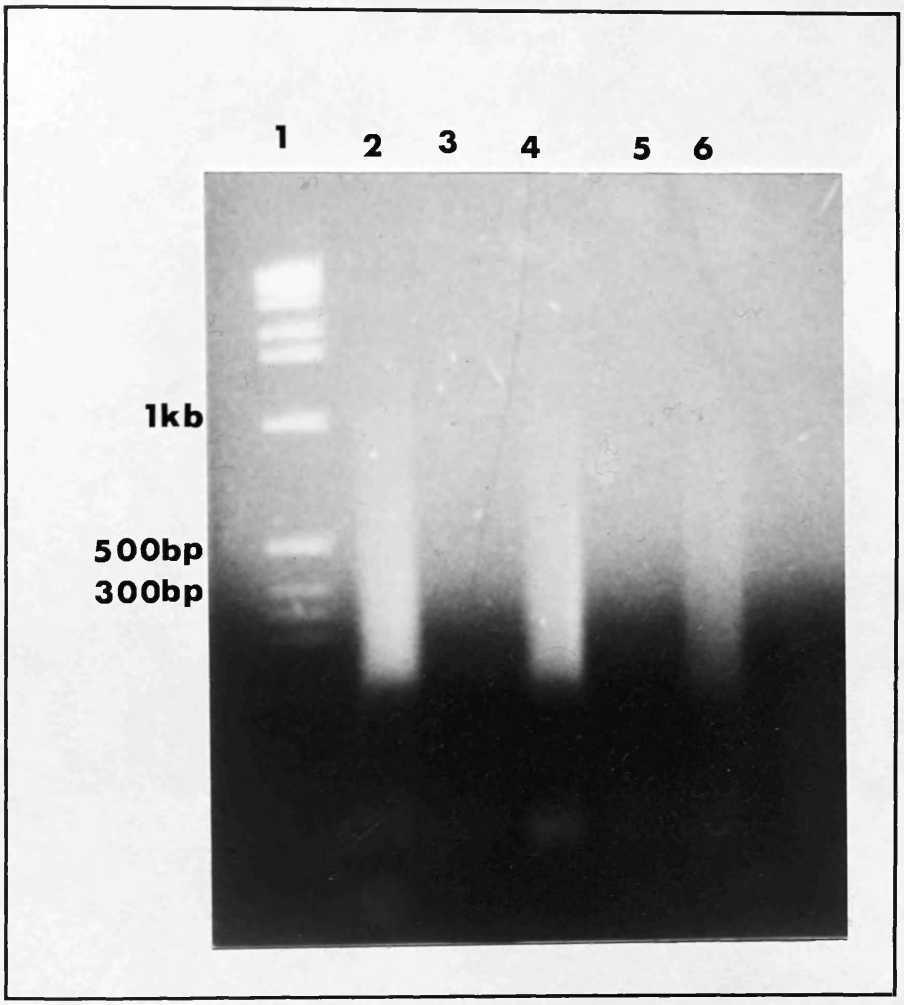


Figure 8. Gel electrophoresis of direct biotin labelling of whole chromosome 21 specific library with polymerase chain reaction. **Lane 2**, **4**,and **6** showing a smear extending less than 500bp. **Lane 1** is a 1 kb ladder. No DNA in lane **3** and **5**.

chromatography was better but resulted in a high dilution of the DNA in the final suspension. Freeze drying after liquid nitrogen freezing of the 400ul elution allowed the biotin labelled DNA to be concentrated and easily measured by dot-blot hybridization (**Figure 9**). With this approach the labelled DNA was found to be clean and highly purified.

3.3 CHROMOSOMAL IN SITU SUPPRESSION HYBRIDIZATION USING WHOLE CHROMOSOME 21 SPECIFIC LIBRARY

In developing the technique which was based on those of Pinkel et al. (1988) and Lichter et al. (1988) variable conditions and parameters were noted. While trying to make our own probe from the composite library, we obtained the chromosome 21 Bluescribe plasmid, pBS21 library from Prof. J.Gray, Lawrence Livermore and began working on the chromosome method. The pBS21 library was labelled with biotin-11-dUTP by nick-translation. Results showed that chromosome 21 was stained lightly and signal was seen as speckles over the chromosome 21 as in **Figure 10.** This result led to modifications of various related factors as shown in the following results. The later work was carried out using purified whole chromosome 21 specific library probe (ATCC LL21SNO2).

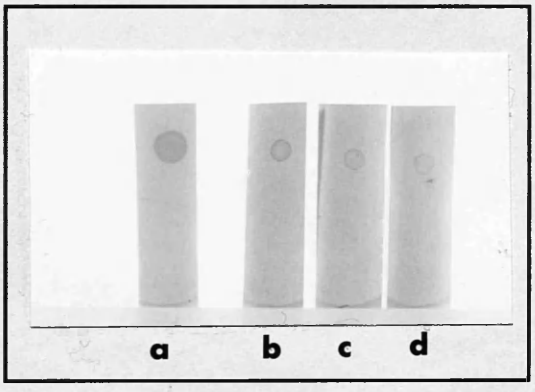


Figure 9. Photograph of dot-blot hybridization to check the concentration of direct biotinylation of whole chromosome 21 specific library (ATCC LL21SNO2) using Polymerase Chain Reaction (PCR).
(a) biotinylated probe. (b) standard 20pg. (c) standard 10pg. (d) standard 5pg.

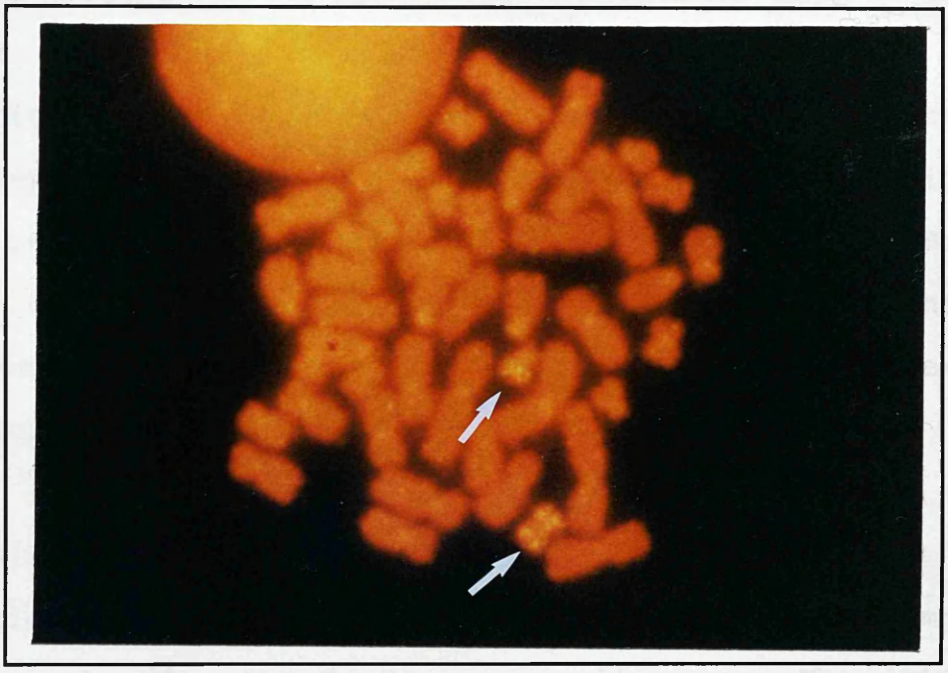


Figure 10. Photomicrograph of CISS hybridization on metaphase chromosomes of a case of isochromosome 21 using whole chromosome specific library constructed in Bluescribe plasmid (pBS21) obtained from Prof.J.Gray, Lawrence Livermore. The arrows show that both the normal and isochromosome 21 have a speckled appearance and are not fully decorated.

3.3.1 Probe mixture

Probe handling prior to hybridization is critical. Both the competitor (total human placental) DNA and carrier (salmon sperm) DNA were sonicated to fragments of less than 500bp (**Figure 11**) for optimal hybridization. Each of these DNAs was applied individually to the slide and the hybridization procedure was performed to check their different effect and as the reference for the signal (decoration) interpretation later (**Figures 12 A-C**). Both, when counterstained with propidium iodide (1ug/ml) showed a homogeneously orange-red colour. A similar orange-red colour was also observed with untreated chromosomes.

A total of 10ul of the hybridization mixture (i.e total human competitor DNA, salmon sperm DNA, probe and hybridization buffer) in a final concentration of 1mg/ml was used under the 24x24mm coverslip area. The optimal final concentration of the DNA mixture per slide was 10ug/10ul. The amount of labelled probe used was between 20 to 50ng/ul per reaction. This amount of probe used in the ratio to the competitor DNA of between 80 to 150 was optimal for the specific chromosome 21 library. This ratio varied for different batches of the labelled probes. **Figures 13** and **14** show results of chromosomal in situ suppression hybridization showed poor suppression where the target chromosome 21 cannot be delineated.

Individual concentrations of carrier DNA, competitor DNA and probe were varied extensively in the final mixture

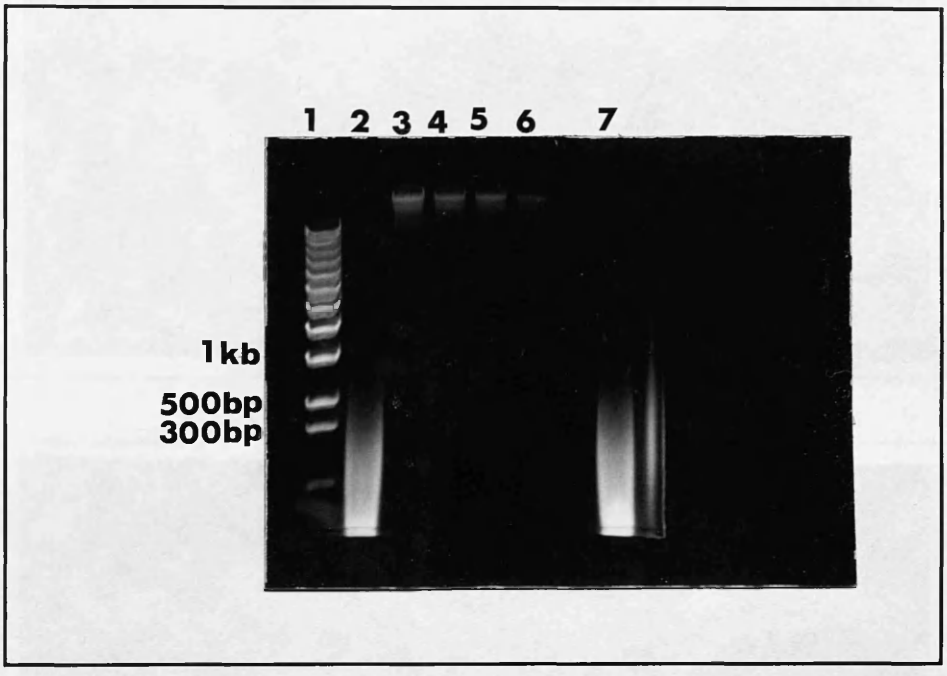


Figure 11. Photograph of gel electrophoresis of sonicated total human and salmon sperm DNA. **Lane 2**, Human placental DNA. **Lane 7**, Salmon sperm DNA. Both are concentrated below 500 bp. **Lane 3-6** are the undigested DNA library.

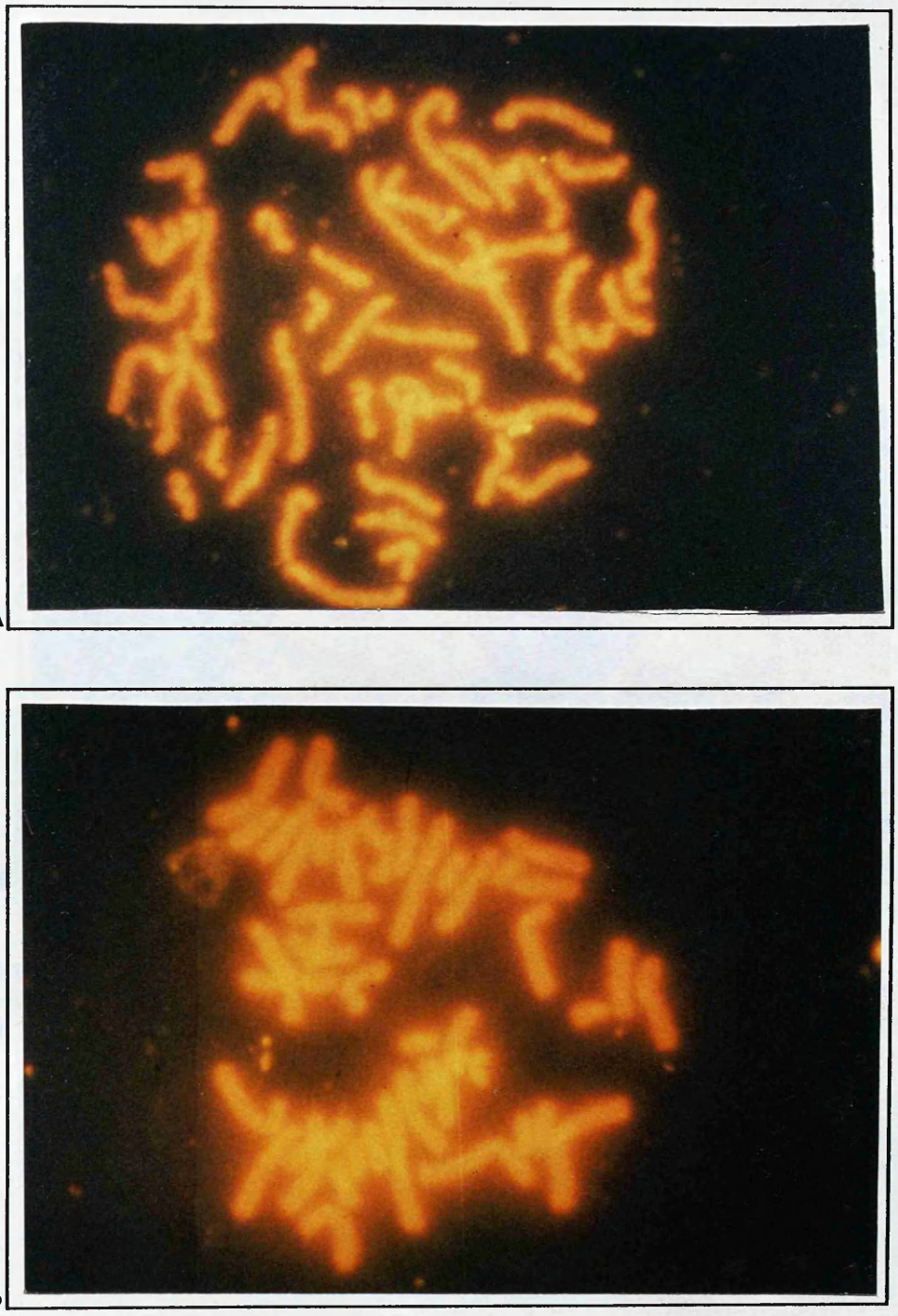
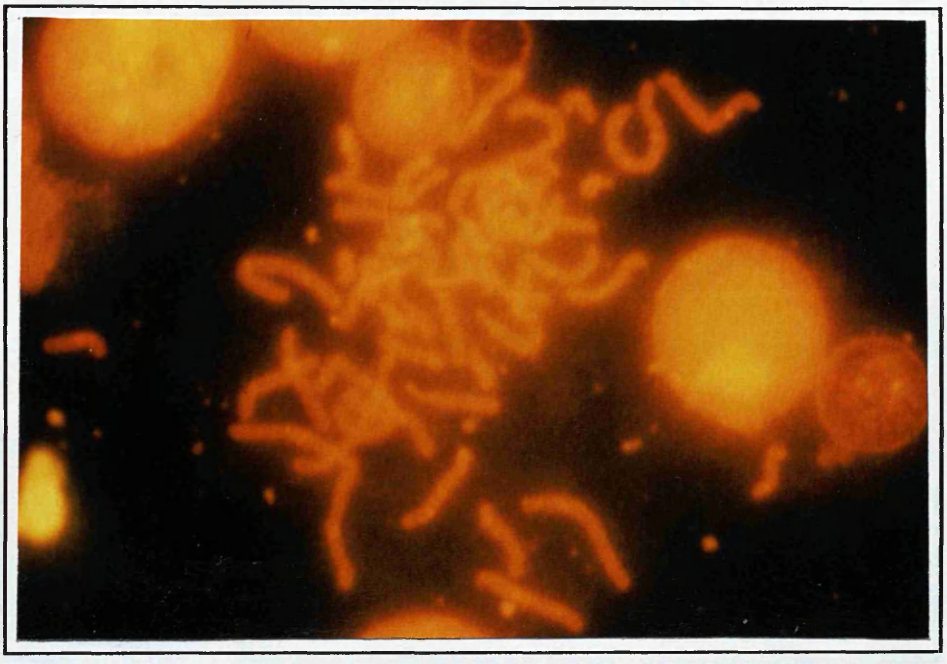


Figure 12. Photomicrographs of in situ hybridization with (A) unlabelled salmon sperm DNA only (B) unlabelled total human placental DNA only. These hybridizations did not contain the library DNA (probe). The hybridization was performed at 37°C for a minimum of 14 hours after denaturation at 75°C for 8 minutes and preannealled for 60 minutes at 37°C. These results were used as standard control for comparison of hybridization signal (decoration) for further assessment of the specific DNA concentration to be used in the experiments.



C

Figure 12 (C). Photomicrograph of CISS hybridization with unlabelled total human (placental) DNA, salmon sperm DNA and with unlabelled whole chromosome 21 specific library.

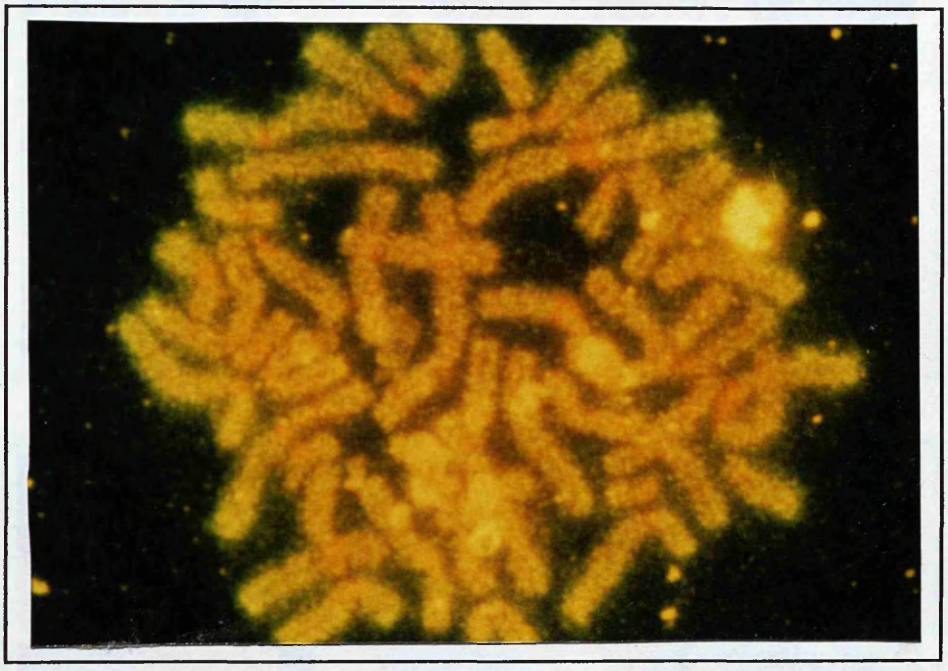


Figure 13. Photomicrograph of a poor CISS hybridization of the whole metaphase chromosomes. The hybridization was carried out with biotinylated whole chromosome 21 specific library, salmon sperm DNA and total human DNA. As a result of poor suppression the target chromosomes 21 cannot be delineated.

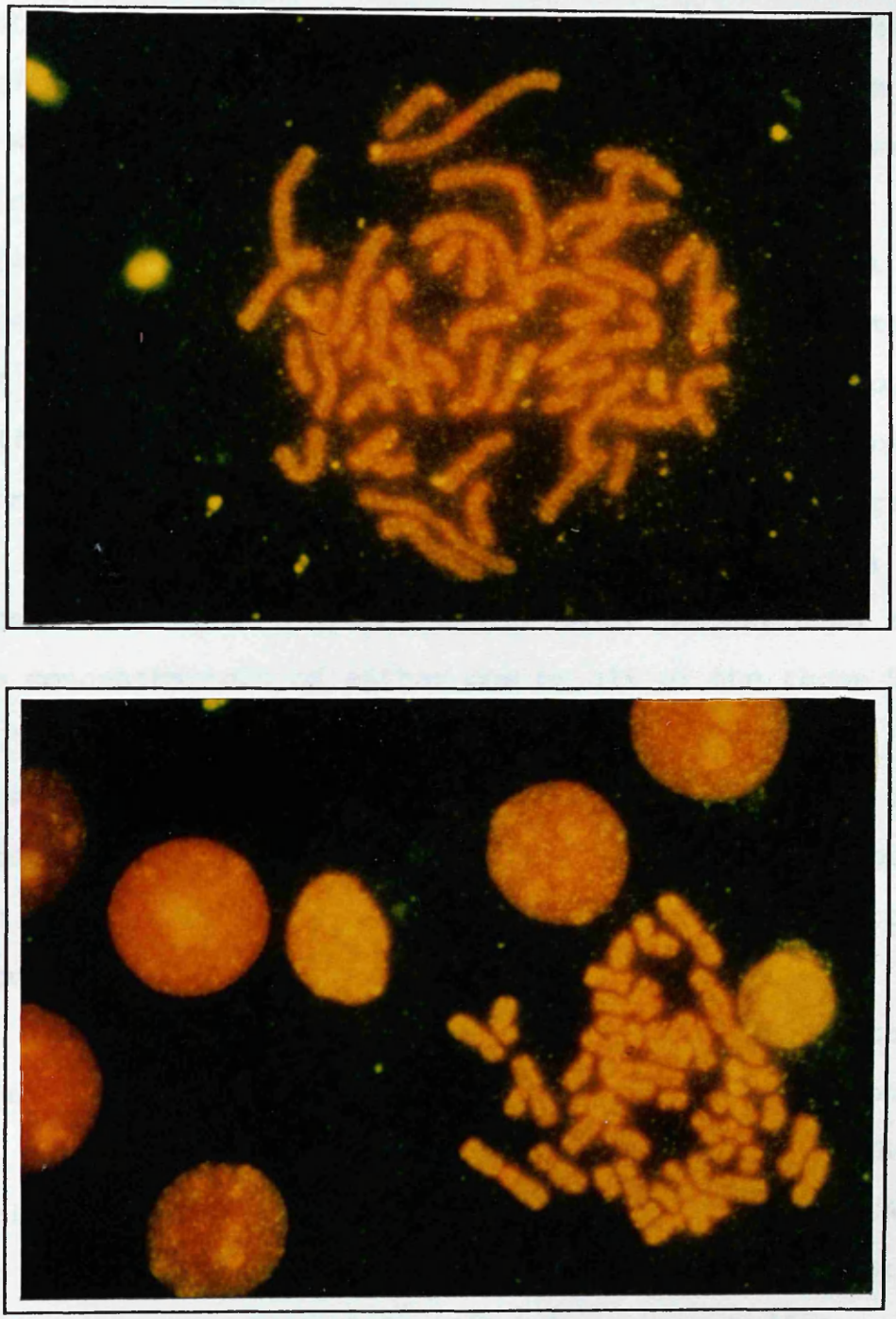


Figure 14. Photomicrographs of CISS hybridization with biotinylated whole chromosome 21 specific library showing low suppression of other chromosomes. (A) Poor contrast of target chromosomes 21. (B) No hybridization signal observed on the interphase nuclei.

to check their effectiveness. Probe concentrations between 10ng/ul to 100ng/ul and 1.0ug/ul to 8.0ug/ul of total human DNA were tried. It was shown that excess probe, carrier or competitor DNA did not improve the suppression or the hybridization signal. Increased total human DNA as competitor DNA failed to enhance the contrast. Instead, this caused difficulty in dissolving the DNA homogeneously in the hybridization mixture. This failure led to uneven distribution of hybridization mixture under the coverslip and thus resulted in uneven final hybridization on the target metaphases (**Figure 15**). It also demonstrated that high concentrations of either one or all of the three DNAs in the hybridization mixture greatly encouraged the accumulation of autofluorescence artefacts and background noise that could affect the result. PCR biotinylated probe when used directly without cleaning caused high background (**Figure 16**).

Human cot-1 DNA was also used to replace the total human DNA as the competitor. No significant improvement in the suppression was noted when using human cot-1 DNA by itself (**Figure 17**). However, adding the human cot-1 DNA in 1 to 4 ratio with the total human DNA resulted in better suppression and was used for all subsequent studies.

To concentrate the small amount of probe with the other DNAs, they were mixed and ethanol-precipitated at -20°C overnight prior to suspending in the hybridization buffer. This additional treatment helped to increase the concentration of all DNAs prior to denaturation. This step

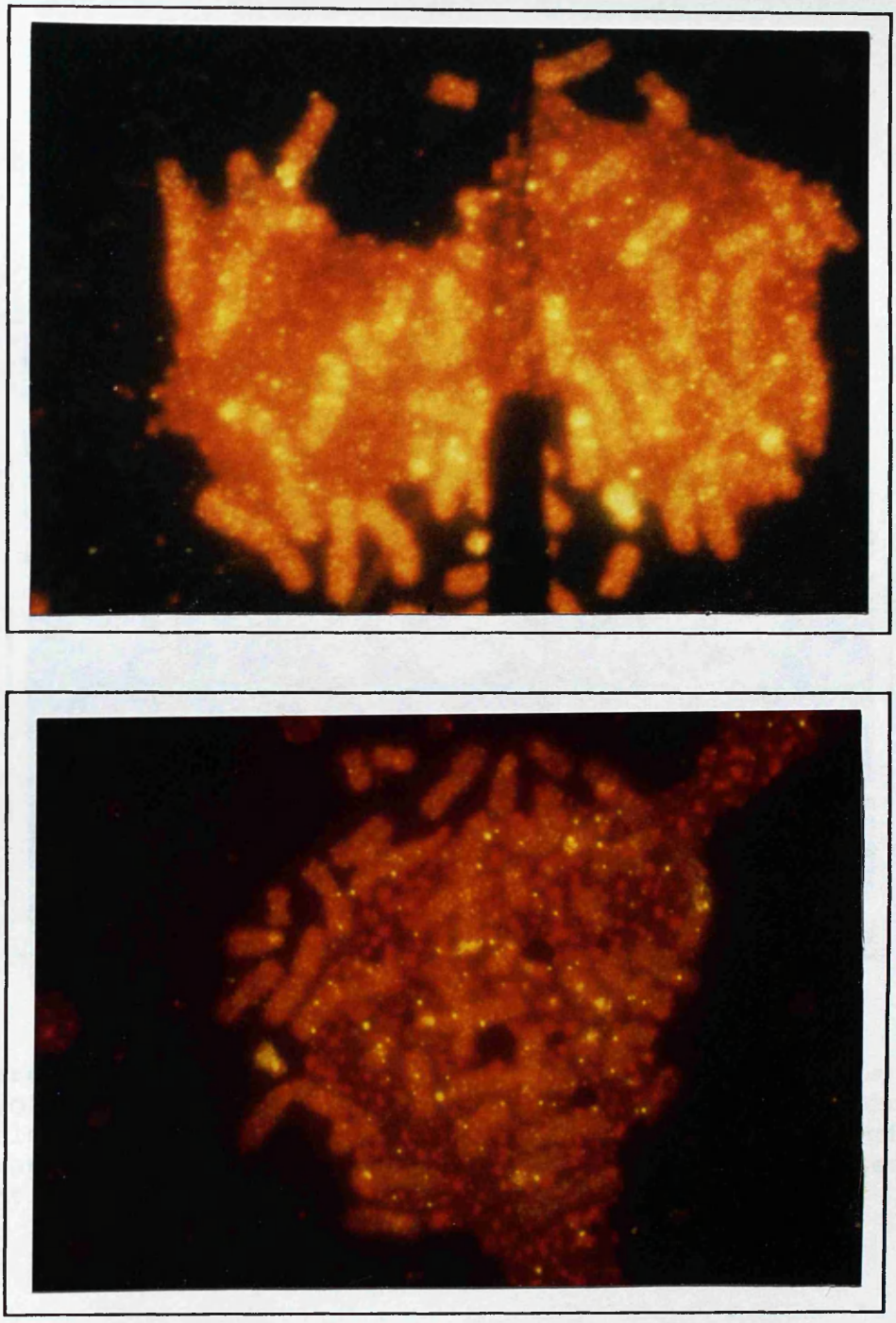


Figure 15. Photomicrogaphs of uneven distribution of DNA and deposits over the metaphase chromosomes observed after CISS hybridization. The DNA mix did not properly dissolve in the hybridization buffer before denaturation and\or hybridization. This will intefere with the hybridization reaction.

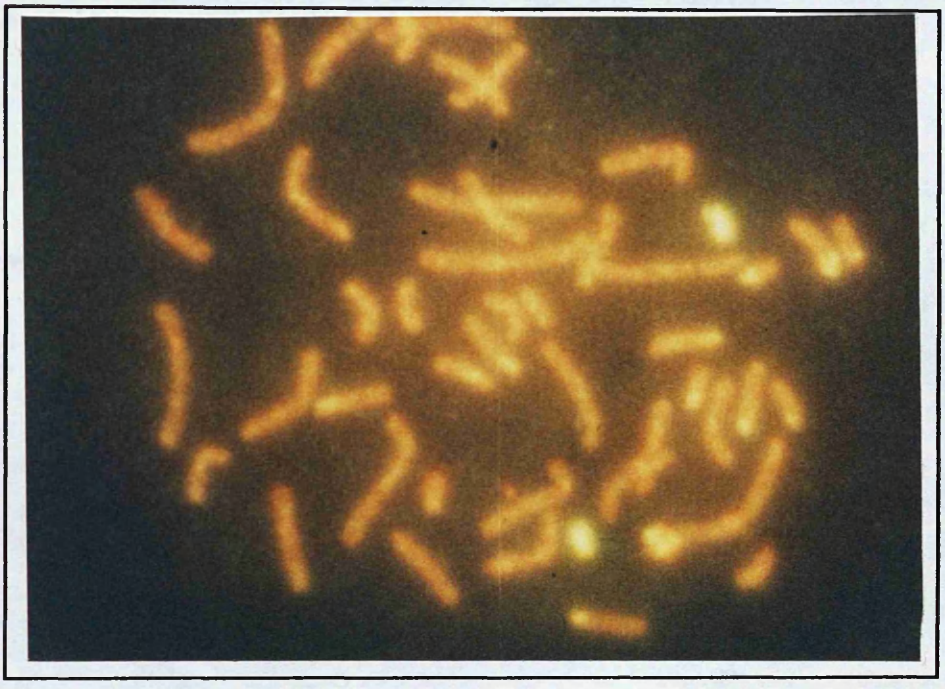


Figure 16. Photomicrograph of CISS hybridization of karyotypically normal metaphase chromosomes using direct (uncleaned) PCR biotinylated whole chromosome 21 specific library probe. The background was dirty and caused a blurring effect.

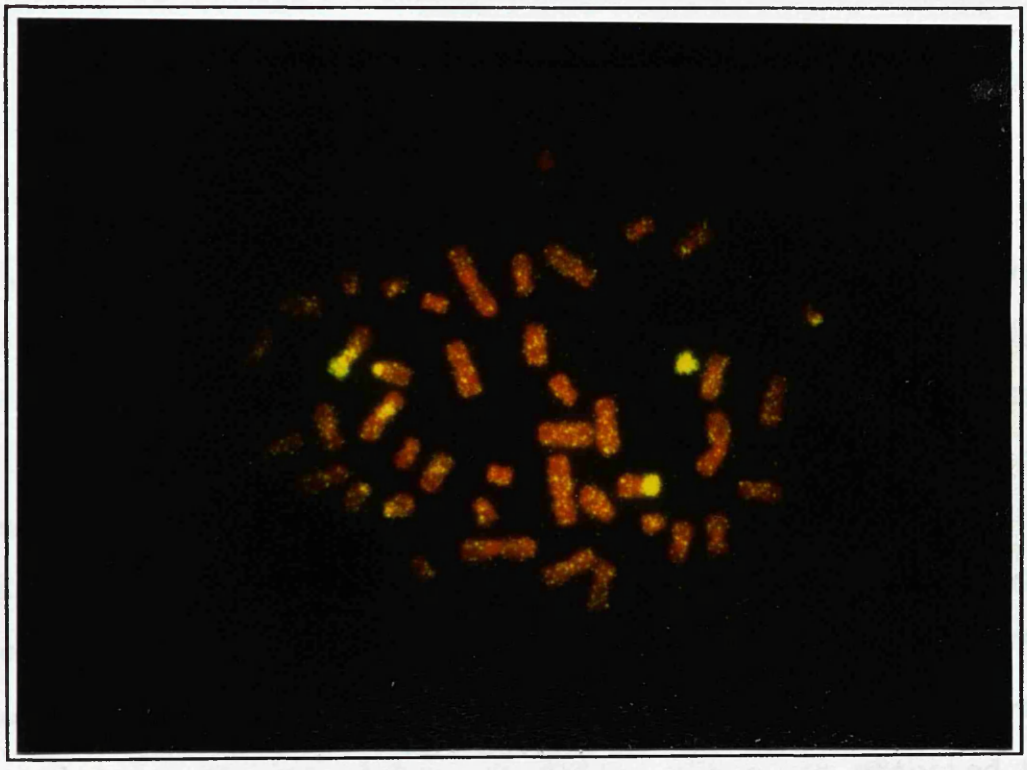


Figure 17. Photograph of partial metaphase from a case of t(14;21) after the CISS hybridization using whole chromosome 21 specific library using 20ng/ul oflabelled chromosome 21 library and only human cot-1-DNA as competitor DNA (5ug/ul) and hybridized at 37°C for 15 hours. Although other chromosomes were not completely suppressed the chromosomes 21 were intensely stained and easily distinguished showing good contrast.

also eliminated the unpurified fragments. The background was cleaner than without pre-precipitation and homogeneous high intensity decoration on chromosome 21 was achieved.

3.3.2 Hybridization buffer

Hybridization buffer prepared according to Garson et al. (1987), which contained 0.1mm EDTA and Tris salt was found to produce high residual artefacts. In this study, only 10% dextran sulphate was added to 50% formamide/2X SSC without any other salts or salmon sperm DNA. However, the formamide has to be deionized for 3-4 hours and with a mixed-bed resin. The dextran sulphate and the 20XSSC should be autoclaved and filtered. The mixture was left at 37°C for 48 hours to ensure that the dextran sulphate dissolved completely. The pH of hybridization buffer was adjusted to pH 7.0 and filtered before aliquoting and storing at -20°C. Any aliquot of hybridization buffer kept at -20°C that showed deposits was discarded after 6 months.

3.3.3 Treatment of slide prior to hybridization

When freshly prepared slides with good quality metaphases were treated with RNase prior to denaturation no improvement in probe penetration was observed. However, metaphases covered with protein membranes and slides that

were also more than one month old showed an improvement with RNase treatment. Proteinase K treatment of the slides was also tried but even at low concentration (0.2-0.6ug/ml) tended to wash the cells off the slides. This step was omitted in subsequent experiments.

In the study it was found that refixing dried slides by placing in methanol-acetic acid (3:1) fixative tended to improve the hybridization result and reduced 'dirty' background.

3.3.4 Denaturation of chromosomes and DNA hybridization mixture

Denaturation of chromosomes was performed in 70% formamide/2XSSC at 70-75°C for a maximum of 8 minutes followed by dehydration in ascending concentration of ice-cold ethanol. Longer times of denaturation up to 10 minutes were found to distort the chromosome morphology and a similar effect was observed if the temperature was raised to 80°C or if the denaturation time was shortened to 5 minutes.

Hybridization mixture (containing labelled probe, salmon sperm DNA, total human DNA and hybridization buffer) was denatured at 75°C for 8 minutes and immediately transferred to ice for quick cooling before placing in a 37°C waterbath for preannealling. Results showed that hybridization mixture preannealled for minimum of 60

minutes produced better hybridization than that preannealled for less than an hour.

3.3.5 Hybridization

Hybridization when carried out at 37°C in a humid chamber for 16 to 20 hours showed optimum probe penetration and hybridization. Poor hybridization was observed when slides were incubated for periods of 10, 12 and 14 hours. At 42°C to 45°C crystallisation and heavy deposits were noticed that were due to drying and evaporation of the hybridization mixture (**Figure 18**). Incubation time of less than 15 hours at same temperature did not improve the situation. Excellent undistorted chromosome morphology was observed at 37°C incubation.

Post-hybridization washing performed in three changes of 50% formamide/2XSSC at 45°C for 5 minutes was found to be optimal. To avoid the hazardous effect of formamide, washing at different stringencies; 4XSSC, 2X SSC and 0.1XSSC at 60°C were tried but all these were shown to be less efficient than with 50% formamide/2X SSC. Washing with 0.1XSSC at 60°C distorted the chromosome morphology.

After washing with 50% formamide/2XSCC to remove the unhybridized sequences the slide was washed once in the phosphate buffer. Although this step helped to equilibrate the slide to the detection conditions, it was found more satisfactory not to pre-incubate the slide with non-fat

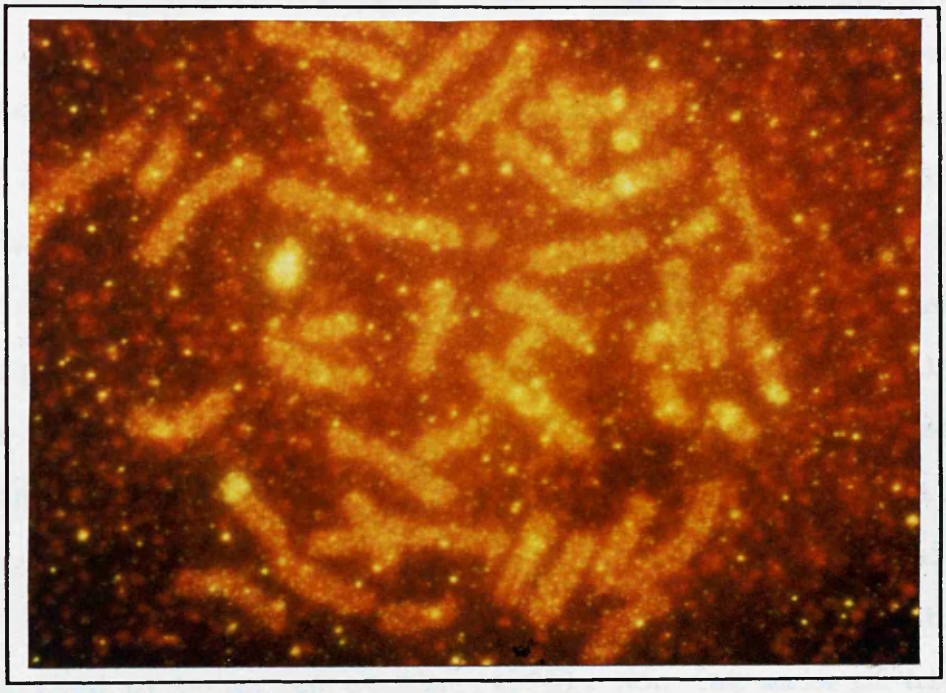


Figure 18. Photomicrograph showing heavy deposits and crystallization over the metaphase chromosomes probably due to the effect of drying during the hybridization. A total of 8ug/ul of DNA per slide was used and was hybridized at 42°C for 15 hours.

milk solution as this tended to promote drying even when performed at room temperature for a short time.

For in-between detection washes, 0.1M Sodium phosphate buffer pH 8.0 containing 0.1% Nonidet 40 produced cleaner background than using 4XSSC containing 0.1% Tween 20. Both the Nonidet-P40 and Tween 20 act as cleaning detergents. However, the phosphate buffer turned cloudy when warmed to 45°C. The phosphate Nonidet-P40 buffer also delayed the drying process on the slide during incubation with fluoresceinated avidin (FITC) and biotinylated goat anti-avidin mixture. The non-fat milk also dissolved easily in this buffer. This is also an advantage over the use of bovine serum albumin (BSA) in the buffer because it is easily dissolved and cheaper and can be obtained locally.

Non-fat milk diluent was prepared with modifications described by Mukerjee et al. (1990). Non-fat milk of higher than 5% concentration was found to dry rapidly on the slides and caused a 'film' over the preparation that prevented proper fluoresceinated avidin (FITC) labelling later (**Figure 19**). No further investigation of the use of non-fat milk below 5% concentration was made.

Freshly prepared milk, even when centrifuged to sediment undissolved material, was prone to precipitation of milk particles (**Figure 19**). This could be avoided by dissolving the milk powder at 37°C overnight. 0.02% sodium azide was added to the milk diluent to avoid bacterial contamination and it was found that this suspension could be kept at 4°C for up to 2 months without deterioration.

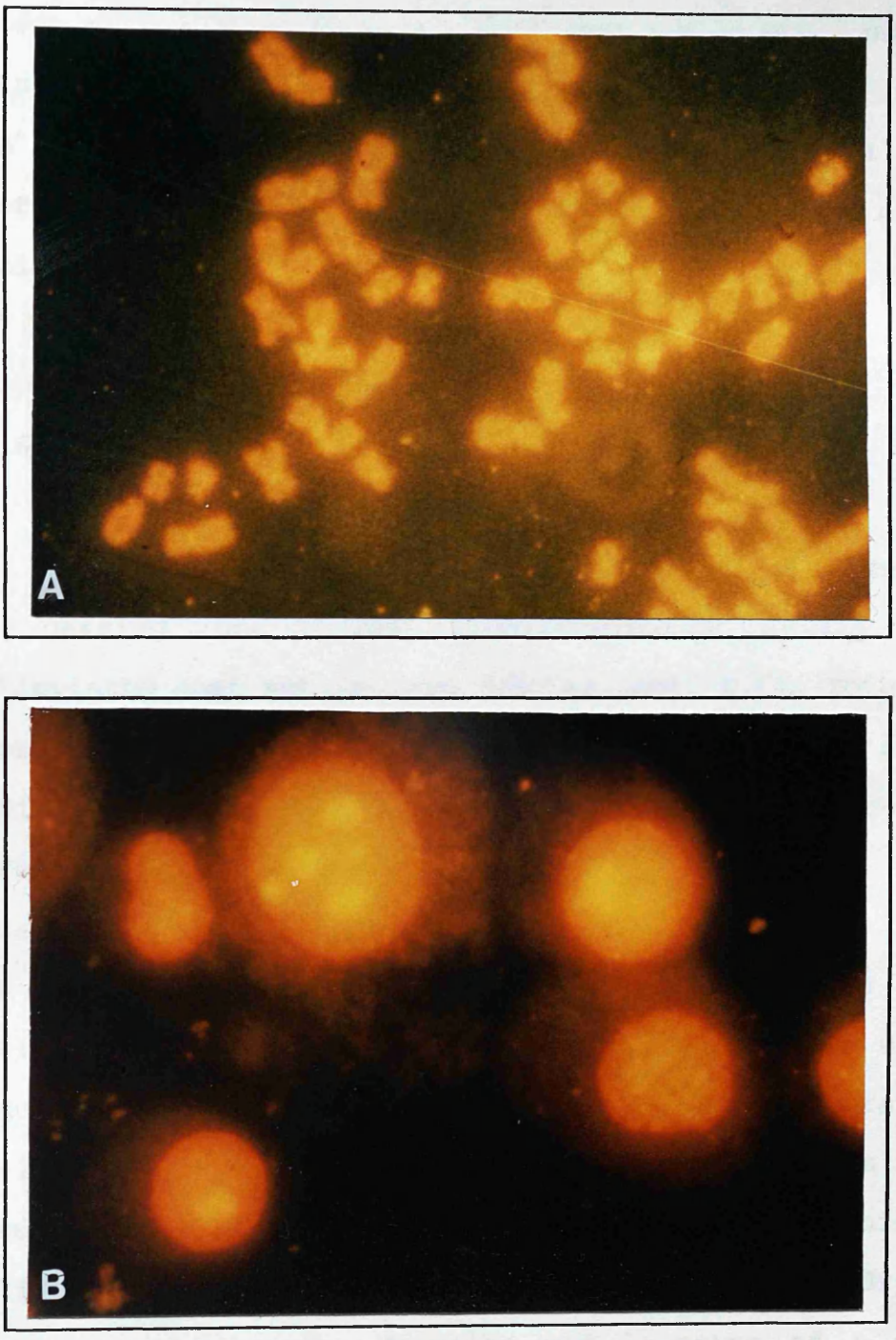


Figure 19. Photomicrographs of (A) metaphase chromosomes and (B) nuclei covered with the non-fat milk film and precipitates. The non-fat milk was dissolved in phosphate nonidet P-40 buffer pH 8.0 and used immediately (fresh) for diluting the Fluorescein avidin and biotinylated goat anti-avidin.

Non-fat milk diluent that has developed a sour smell due to bacterial contamination should be discarded as a " milk curd" will be formed that will interfere with the fluoresceinated avidin (FITC) and biotinylated goat anti-avidin.

3.3.6 Detection of hybridized sequences

In this study, detection of hybridization sequences was carried out using fluoresceinated avidin and biotinylated goat anti-avidin (Vector Lab). U.K). Both the fluoresceinated avidin (FITC) and biotinylated goat anti-avidin were diluted to 5ug/ml in phosphate buffer containing 5% non-fat milk. 200ul of the diluent were placed on each slide to cover the marked area of 24x24mm. Both the FITC-avidin reaction and the amplification with biotinylated goat anti-avidin were carried out under subdued light either for 20 minutes at room temperature (23-25°C) or at 37°C for 20 minutes and/or 30 minutes in a humid chamber. During the detection stage it was highly critical to avoid drying of the slides. Even slight drying and evaporation caused deposits and layering of a milk "film" that were difficult to remove by washing. The effect of drying was to produce high autofluorescence of labelled fluoresceinated-avidin (FITC) all over the slides covering the metaphases and obscuring the decorated chromosomes (**Figure 20**). Those slides that were incubated at 37°C in a

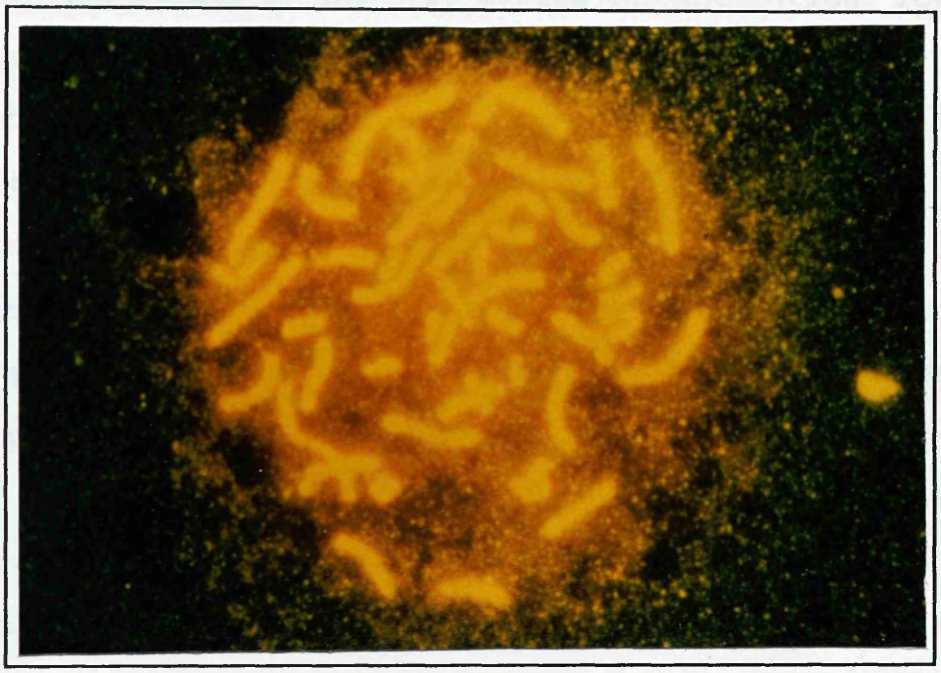


Figure 20. Photomicrograph of high autofluorescence over the metaphase chromosomes. This slide dried during the detection (incubation) with fluoresceinated avidin.

humid chamber for 20 or 30 minutes tended to dry out easily. Clean background and uniform true decoration can be achieved when slides are incubated for 20 minutes at room temperature. To minimise drying when incubating at room temperature a wet tissue or cotton wool could be placed beside the slides on the tray during the incubation. Toward the end of this study, the detection was carried out by incubating the slides in a coplin jar containing fluoresceinated-avidin (FITC) and goat anti-avidin. No obvious difference in the background was observed but this can certainly help to avoid drying of detection reagents on the slides. The diluted reagents can be used several times for up to one month if stored at 4°C.

One cycle of signal amplification with biotinylated goat anti-avidin was sufficient to enhance the decoration of the chromosome 21. Instead of enhancing the decoration, further cycles of amplification caused high FITC background fluorescence.

During the FITC-avidin reaction and also during the amplification cycle slides had to be placed on a level base. This ensured uniform flooding of the detection reagents over the whole marked area as uneven distribution could cause later misinterpretation.

The storage of fluoresceinated avidin and biotinylated anti-avidin was also critical. As the reagents should not be exposed directly to strong light they were aliquoted separately into small volumes and stored at 4°C or -20°C. Aliquots kept for more than 3 months at either 4°C or -20°C

lost their immunofluorescent intensity and were also prone to produce deposits. Any aliquot approaching this duration of storage needed to be checked carefully for deposits. Old fluoresceinated-avidin (FITC) tended to form deposits on the slides thus covering the whole metaphase chromosomes (**Figure 21**).

3.3.7 Counterstaining and use of antifade medium

Two antifading agents were investigated during this study. They were DABCO (1-4-diazobicyclo-(2,2,2)-octane) described by Johnson and Nogueira, (1981); Johnson et al. (1982) and para-phenylendiamine (Lengauer et al. 1991). The counterstains used were propidium iodide and DAPI (4,'6-amidino-2-phenylindole) and each was dissolved separately in the antifade solution and applied directly to the slides. Both these anti-fade agents were shown to prevent the fading of the fluorescence colour for up to 10 minutes when exposed at high power under the microscope. These agents are best kept at 4°C. However, the para-phenylendiamine deteriorated (turned brownish) after 2 months storage at 4°C or -20°C.

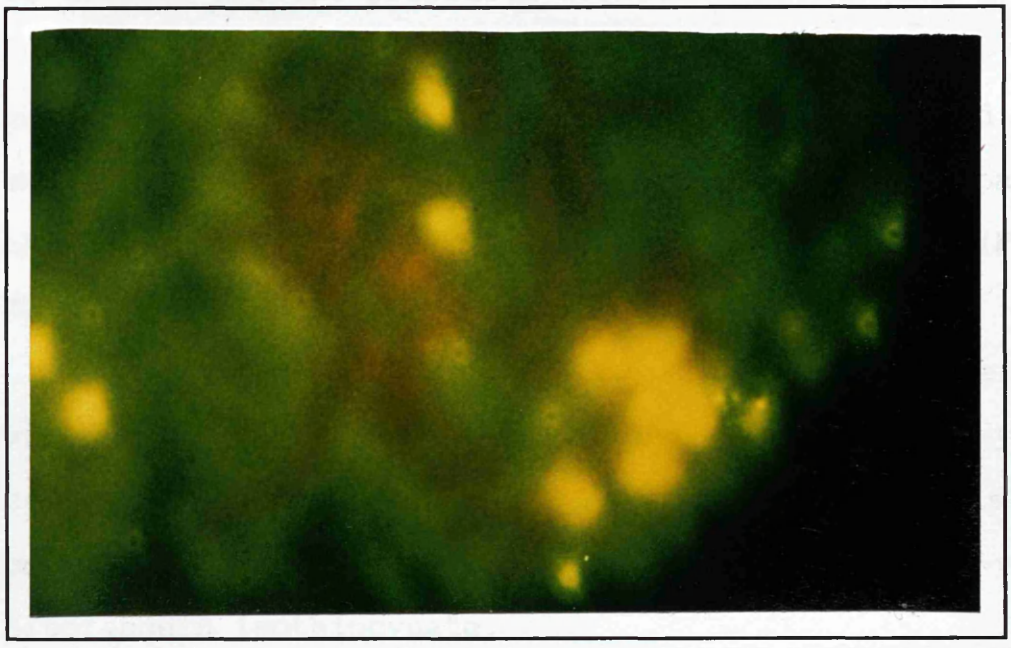


Figure 21. Photomicrograph of metaphase chromosomes covered with fluoresceinated avidin deposits. This batch of FITC aliquot was kept at -20°C for over 14 weeks.

3.3.8 Microscopy

The microscopic analysis was performed with a Zeiss Axioplan using Epi-fluorescence filters 4887702 and BP 390-420 for DAPI counterstained chromosomes and Filter 487709 BP 450-490 for the propidium iodide.

No distinct banding pattern was observed when chromosomes were counterstained with DAPI and viewed under ultra violet filter 4887702 BP 390-420nm although painted chromosomes did show a different colour intensity (**Figure 22**).

Slides counterstained with propidium iodide when viewed with Filter 487709 BP 450-490 showed green-yellow colouration of the hybridized probe. This is the combination of the red propidium iodide and green-yellow fluorescein isothiocynate.

Figure 23 shows that the decorated chromosomes 21 are yellow in colour. The colour is homogeneously decorating the entire chromosome 21 except the pter region. This signal or decorating can be distinguished without ambiguity to identify the target chromosomes on the metaphases. Other non-targeted chromosomes were sufficiently suppressed and almost completely stained red. The pter region of chromosome 13 stained more intensely than the other chromosomes (**Figure 24**). Although it also stained yellowish it can be easily distinguished from the true decorated chromosome 21 by the lack of intensity it displays.

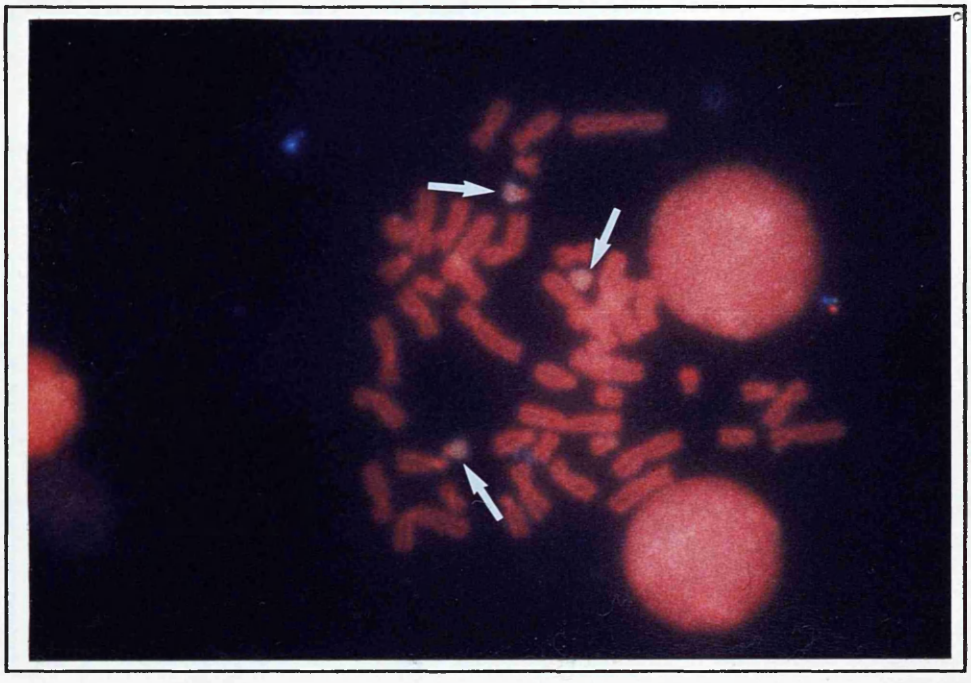


Figure 22. Photomicrograph of CISS hybridization of metaphase chromosomes of trisomy 21 counterstained simultaneously with DAPI and propidium iodide and viewed under UV filter BP390-420. No distinct banding pattern observed but the three chromosome 21 (arrows) show different colour intensity.

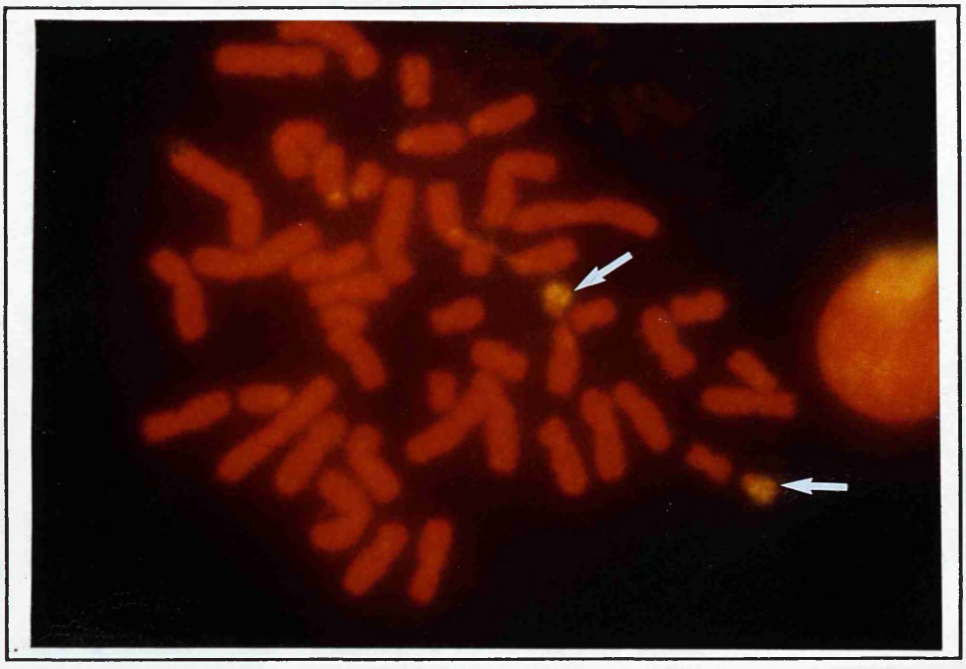


Figure 23. Photomicrograph of CISS hybridization of metaphase chromosomes of a karyotypically normal blood sample using whole chromosome 21 specific library. Arrows showing two decorated chromosomes 21. The pter region of these chromosomes are not stained.

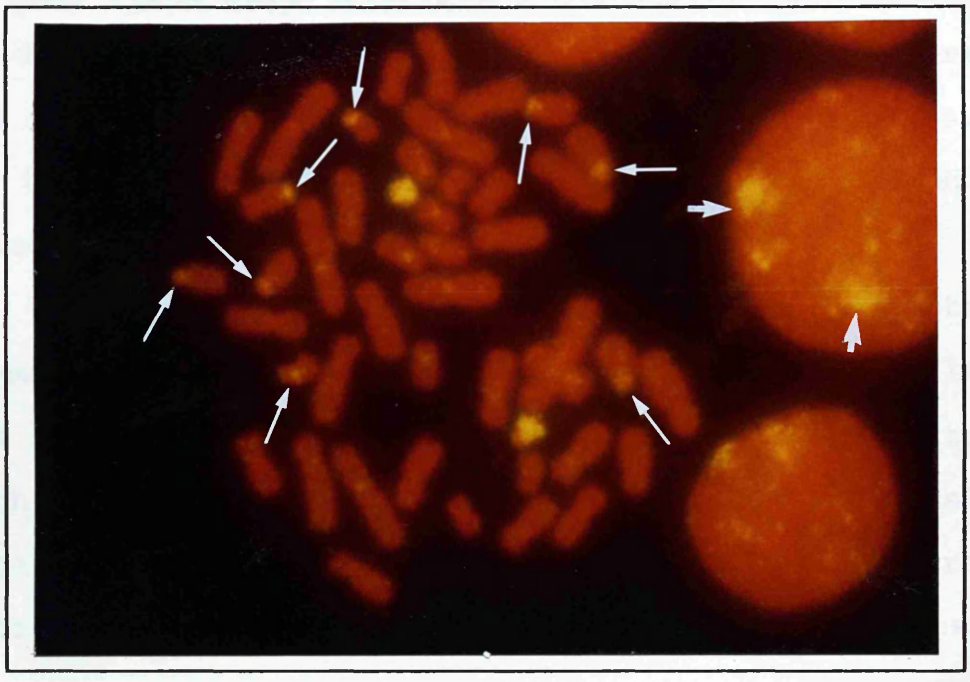


Figure 24. Photomicrograph of CISS hybridization of a karyotypically normal metaphase chromosomes using whole chromosome 21 specific library. Apart from the two chromosomes 21 that are clearly decorated, signal is also observed at the short arm of chromosomes 13, 14, 15 and 22 (arrow). Two brightly clear yellow signals are also observed on the interphase cells (arrowhead).

For the photomicrography different exposure times were tested with the above filter combinations. The optimum exposure time for the photography was found to be 30-60 seconds using Kodakcolor Ektachrome ASA 400. Longer exposure time tended to capture excessive autofluorescence.

All films were developed commercially but mounted in the department. The quality of the results was generally poor when printed as compared to the projected images. An ASA 1000 colour print film was tried but there was no difference in the quality obtained.

One finding noted in this study was the inconsistency of general background colour of the slides. Some slides were found to produce clean dark background and some to be slightly greenish. This might depend on the method of slide preparation of the samples as this greenish background condition was commonly noted on the bone marrow slides.

3.3.9 Other related parameters and conditions

Chromosomal in situ suppression hybridization still proved to be possible on blood slides of 2 years old that had been stored at room temperature (**Figure 25**). Pre-treatment with RNase on these slides prior to denaturation was found to be important to remove any endogenous materials. The results showed no difference from freshly prepared slides.

Although the process (decoration) is highly specific

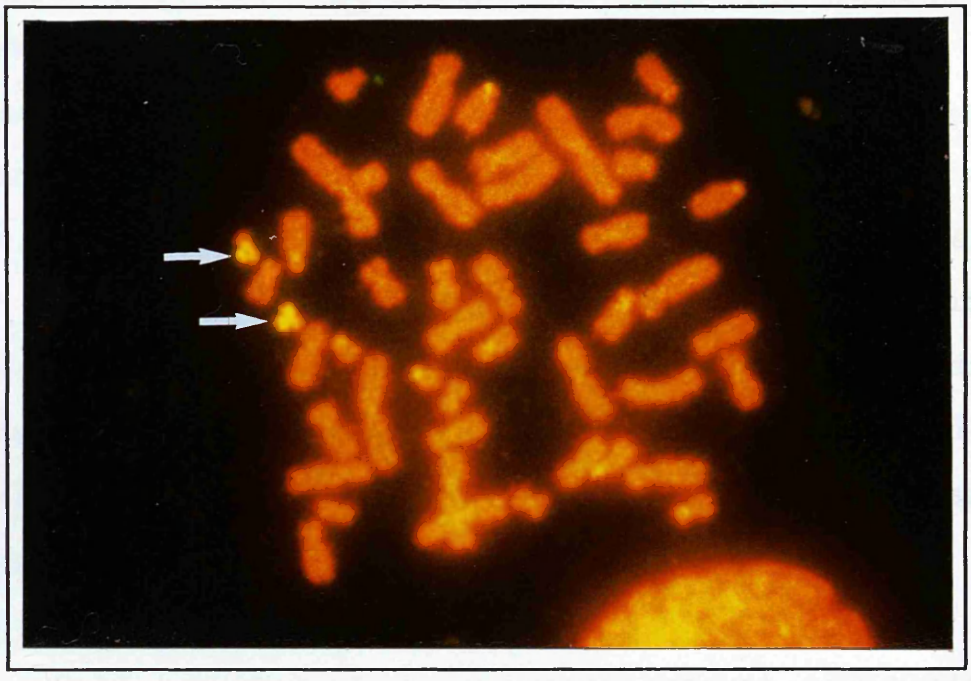


Figure 25. Photomicrograph of CISS hybridization of metaphase chromosomes on an old slide from karyotypically normal blood sample. The slide has been kept in box at room temperature for nearly 2 years. No decrease in hybridization efficiency noted. It was treated with RNase at 37°C for an hour followed by washing in phosphate buffered saline and dehydrated with ethanol series 50%, 70%, 80%, 90%, and 100% prior to denaturation and hybridization.

to the individual chromosome, identification of other chromosomes is difficult when relying only on the size and arm ratio when counterstaining with propidium iodide, or the indifferent banding pattern produced using DAPI. Identification of other chromosomes could be important when investigating a translocation, and this was attempted by pre-banding the chromosomes prior to hybridization. The slides were banded using Lipsol and Leishman treatment without trypsinization. The slides were destained with either ascending concentration of ethanol or two changes of 100% methanol. Both were shown to destain the slides efficiently but the ethanol treated slides showed a dirty background. However, neither method completely removed the stain. The result showed that it did not affect the target chromosomes (**Figure 26**). However, in some slides the background was less clean due to some diffuse stain not completely removed. Thus, an excess amount might hinder probe penetration during hybridization and interfere with the counterstaining.

3.3.10 Application on normal tissue samples

The optimised chromosomal in situ suppression hybridization (chromosome painting) was used on normal PHA-stimulated lymphocytes cultures, lymphoblastoid cell lines, amniocytes, bone marrow cell cultures and chorionic villus samples. **Figures 22-26** clearly demonstrate two chromosomes

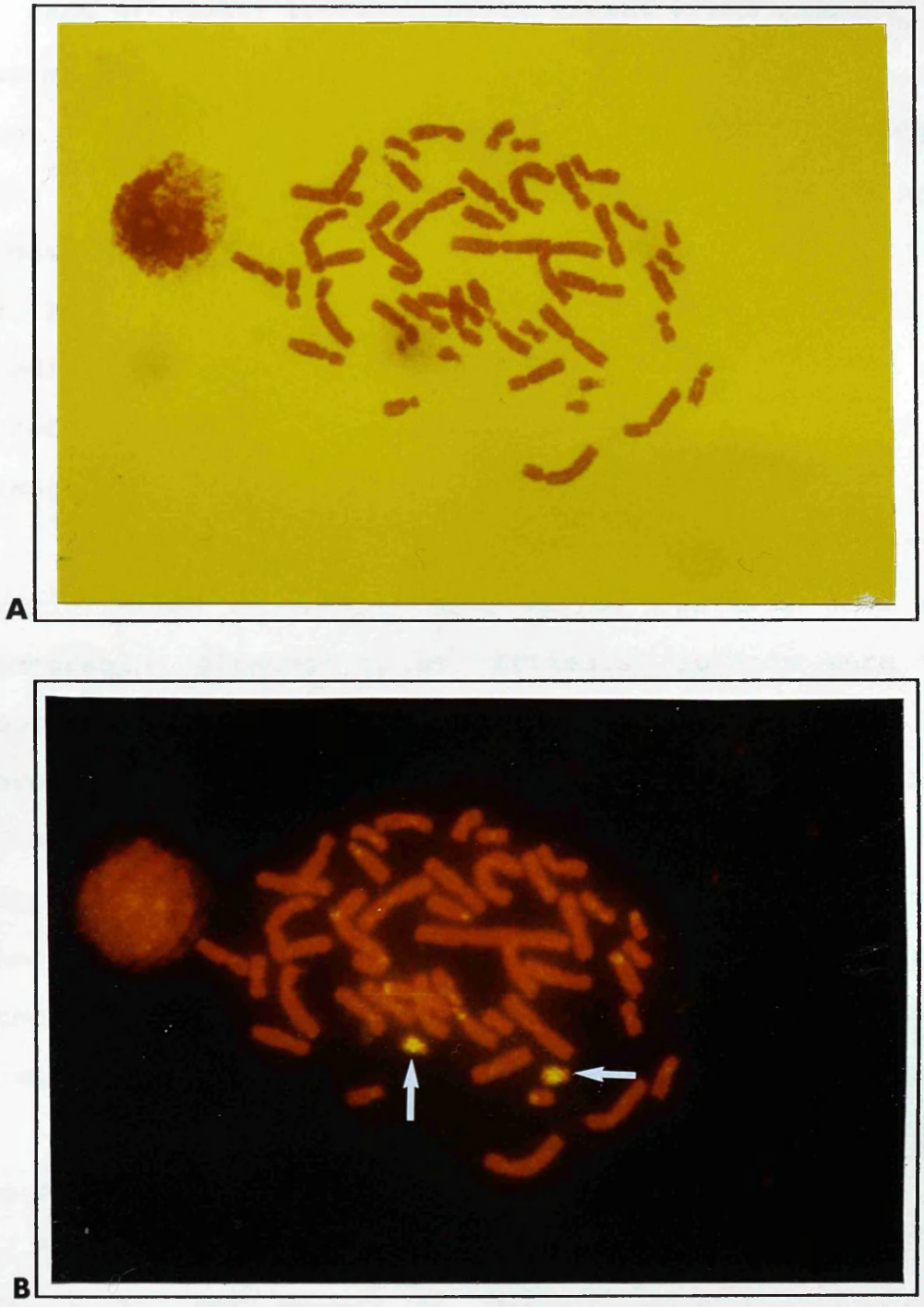


Figure 26. Photomicrographs of (A) Giemsa banded metaphase chromosomes prior to the CISS hybridization and (B) after CISS hybridization with whole chromosome 21 specific library. The slide was destained in two changes of methanol twice for 10 minutes each. Arrows show the chromosome 21 that are clearly delineated. The background was clean and there was no left-over stain.

21 from different karyotypically normal blood samples. The chromosomes 21 of the lymphoblastoid cell lines were also shown to be painted similarly to those of the blood (**Figure 27**). The pter regions were also not stained and a similar cross-hybridization noted on the chromosome 13, 14, 15 and 22. However, the chromosomes 21 are intensely stained and readily identifiable. A satisfactory result was also obtained with amniocytes that had been kept at 4°C in the fixative for more than 2 years (**Figure 28**).

The results of chromosomal in situ suppression hybridization on direct bone marrow cultures were also encouraging although these particular spreads were very poor and hardly produced good Giemsa banding. The chromosomes 21 were clearly stained and easily distinguished (**Figure 29**). However, chromosomal in situ suppression hybridization on 10 slides of a direct preparation of chorionic villus sample (CVS) was not as encouraging as other samples (**Figure 30**). The chromosomes 21 in the CVS samples did not stain as in the blood or other samples although the concentration of the probe was increased even to 80ng/ul.

Results obtained on interphase nuclei were as shown in **Figure 31.** The number of signals or spots counted on different cells differed widely even from those on the same slide. There were inconsistencies in the number of spots although the signal was distinct. In this photomicrograph (**Figure 31**) only 1 cell showed two signals out of five cells observed. Secondly, the majority of the signals

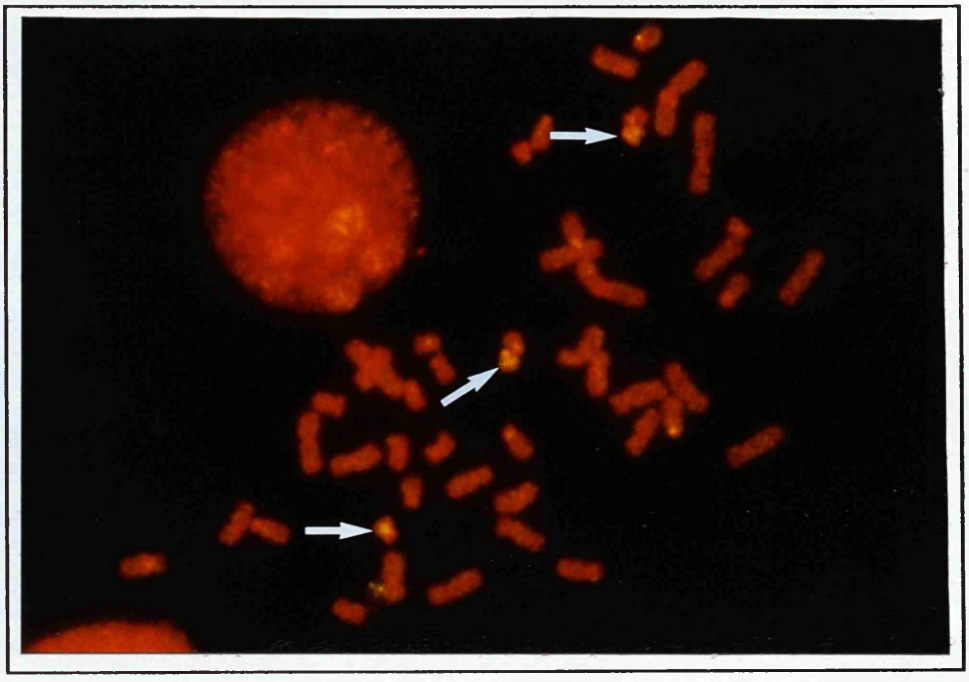


Figure 27. Photomicrograph of CISS hybridization on metaphase chromosomes of trisomy 21 obtained from a lymphoblastoid cell line. Three chromosomes 21 are clearly stained.

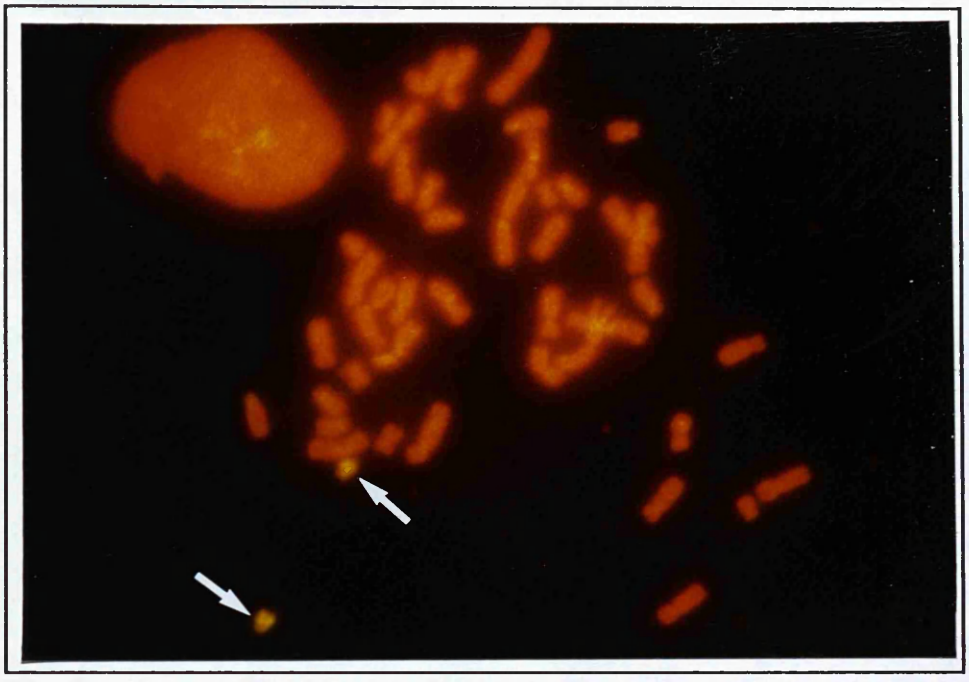


Figure 28. Photomicrograph of metaphase chromosomes of normal amniocytes after CISS hybridization with whole chromosome 21 specific library. The spread was prepared from methanol:acetic acid (3:1) suspension kept at 4°C for 2 years.

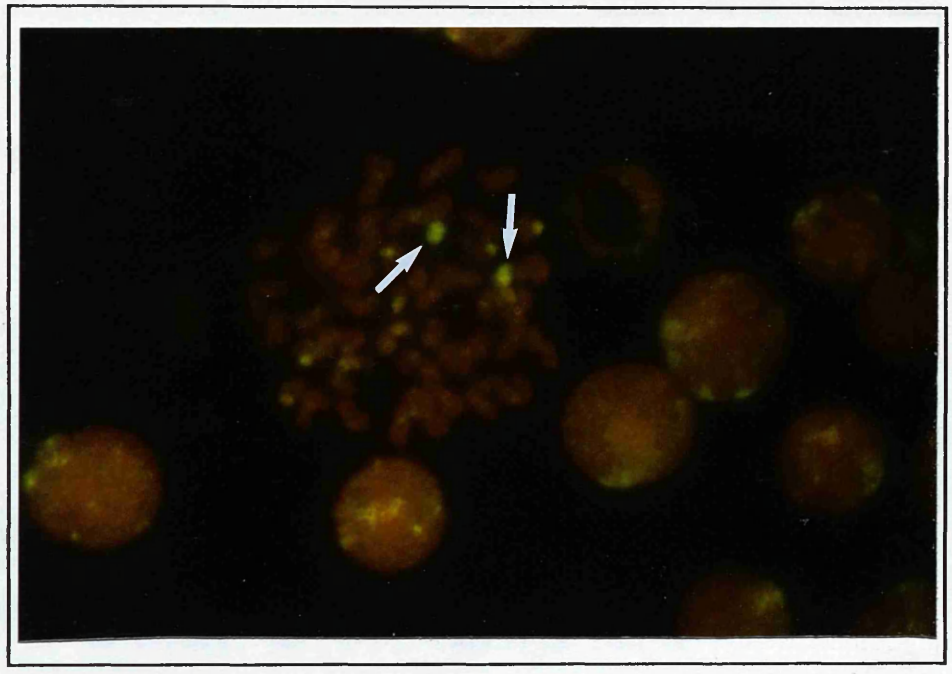


Figure 29. Photomicrograph of metaphase chromosomes from a karyotypically normal bone marrow after CISS hybridization with chromosome 21 specific library. Arrows show two chromosomes 21 that are clearly decorated.

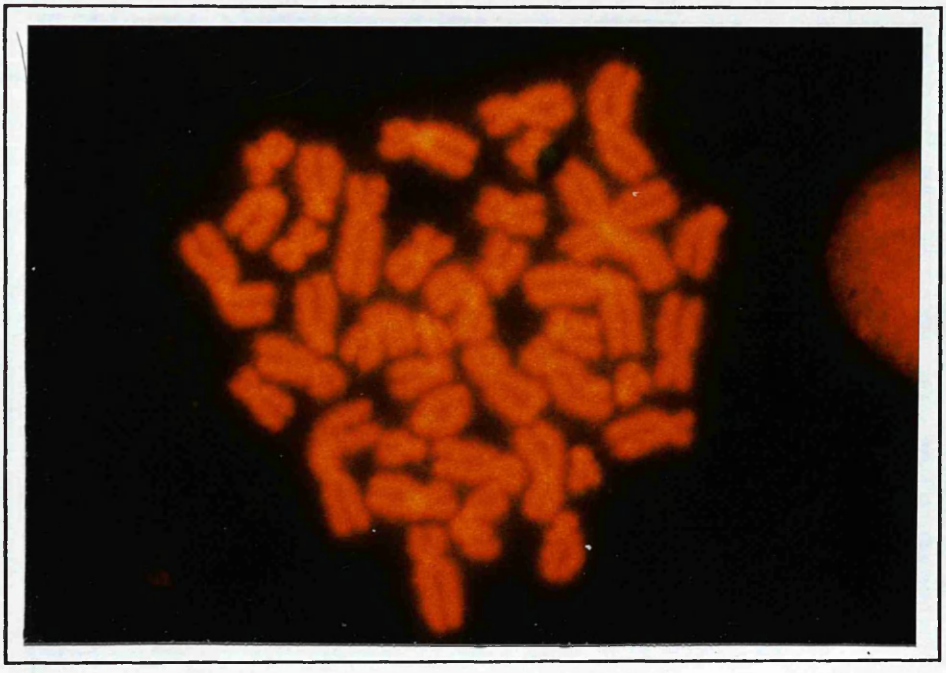


Figure 30. Photomicrograph of metaphase chromosomes prepared from chorionic villus sample after CISS hybridization with whole chromosome 21 specific library. The chromosome 21 was not clearly stained as compared to the blood sample although the concentration of the probe was increased to 80ng/ul.

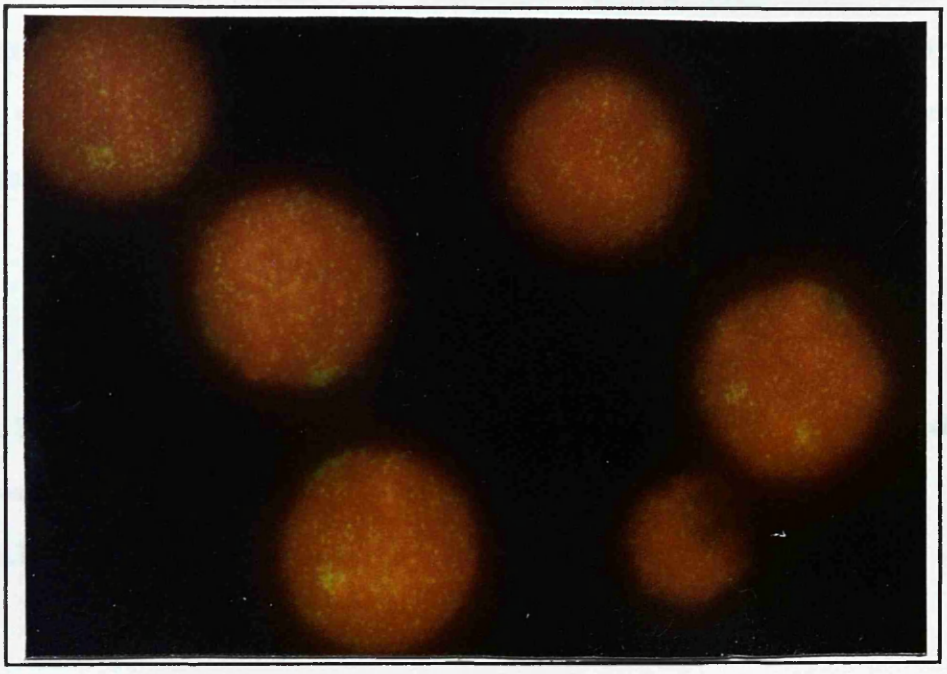


Figure 31. Photomicrograph of interphase nuclei from a normal male after CISS hybridization with whole chromosome 21 specific library. The number of hybridization signals (spots) are inconsistent. One out of five cells shows two distinct signals and three cells show only 1 signal.

observed were less compact and diffused and thus did not allow accurate definition of the signal to be counted.

Results of chromosomal in situ suppression hybridization with whole chromosome 21 specific library on prometaphase chromosomes showed that the chromosomes 21 were also decorated but at a slightly lower intensity. Although these chromosomes were thin and long the staining was clear and could be easily distinguished as shown in **Figure 32.**

3.3.11 Clinical application of chromosome using whole chromosome 21 specific library, case descriptions and results

In the course of the study, the chromosome 21 specific library was used extensively on regular trisomy 21 cases as in **Figure 33** to test the parameters in developing the technique. After the technique was shown to be reproducible it was then applied to the cases that were collected throughout the study to demonstrate its clinical applications.

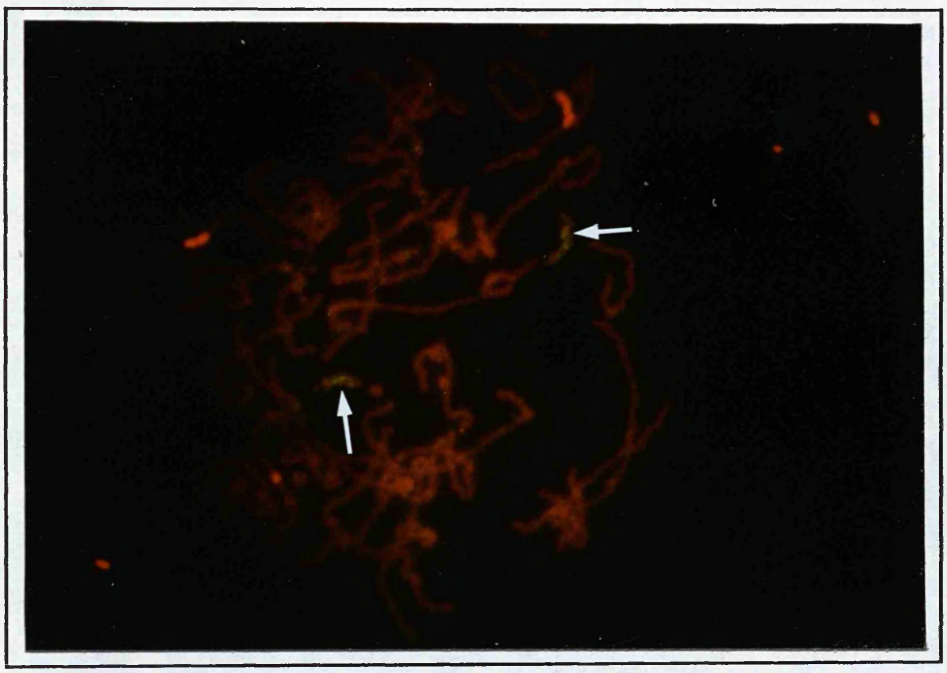


Figure 32. Photomicrograph of CISS hybridization of prometaphase chromosomes with whole chromosome 21 specific library. Two chromosomes 21 were stained and easily identified (arrows).

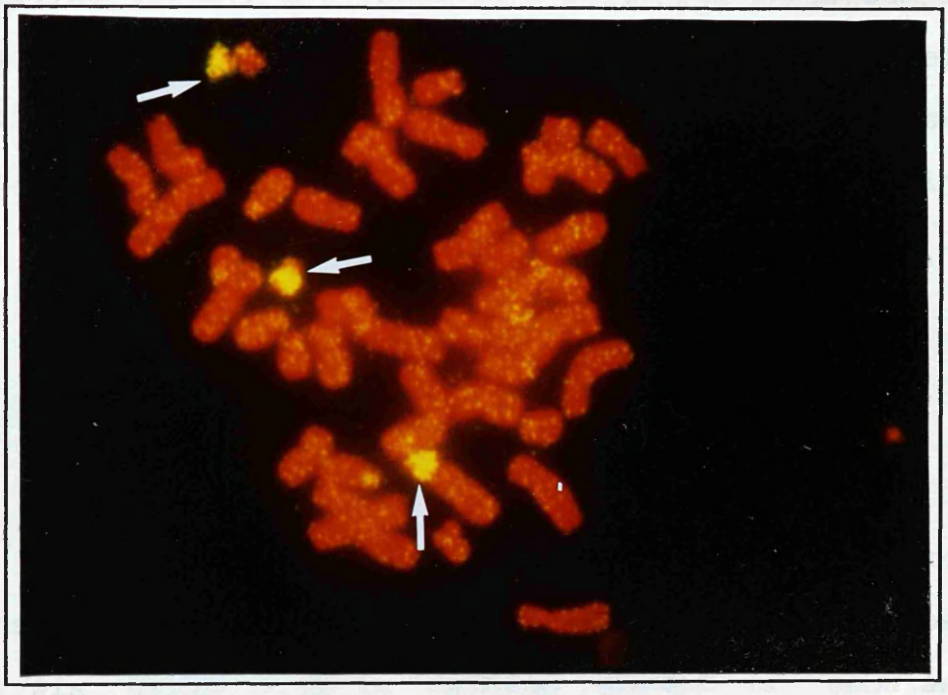


Figure 33. Photomicrograph of a regular trisomy 21 after CISS hybridization with whole chromosome 21 specific library. Arrows show three 21 chromosomes that are clearly delineated.

Case 1

Down's syndrome was suspected on a newborn and chromosome analysis by Giemsa banding reveals an additional chromosome 21 due to a Robertsonian translocation with karyotype 46, XY,-21,t(21q;21q). Following chromosomal in situ supperssion hybridization two fluorescently labelled chromosomes were delineated, a normal 21 and one consisting of the long arms of two chromosome 21 as shown in **Figure 34.** .

Case 2

This patient is a clinically normal carrier of a translocation between chromosomes 2 and 21. Chromosome analysis using trypsin banding showed an unbalanced karyotype. Every cell has one chromosome 21 missing while having extra material present on the short arm of chromosome 2 karyotype 45,XX,-2,-21,+der(2)t(2;21)(p25;q21)mat. At one time, however, it had been thought this translocation might be insertional.

Results of chromosomal in situ suppression hybridization confirmed the interpretation of a reciprocal rather than an insertional translocation, since no unlabelled material was visible beyond the brightly stained chromosome 21 material on the short arm of the translocated 2 (**Figure 35**). This family member was lacking the small

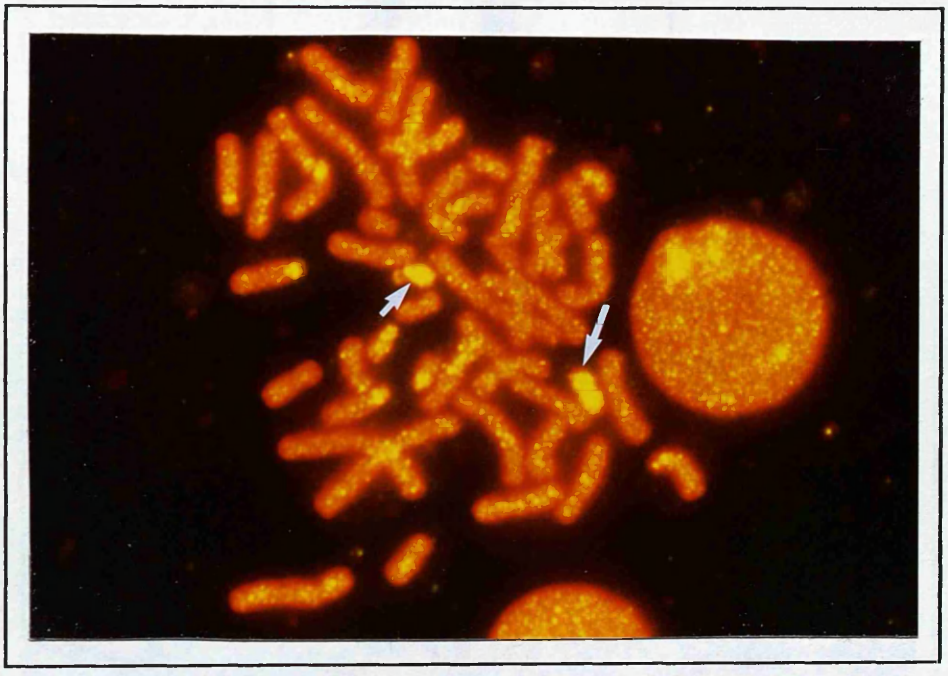


Figure 34. Photomicrograph of CISS hybridization of a case of isochromosome 21 using whole chromosome 21 specific library. Two fluorescently stained chromosomes are clearly delineated. The larger is the isochromosome 21 (arrow) and smaller the normal chromosome 21 (arrowhead).

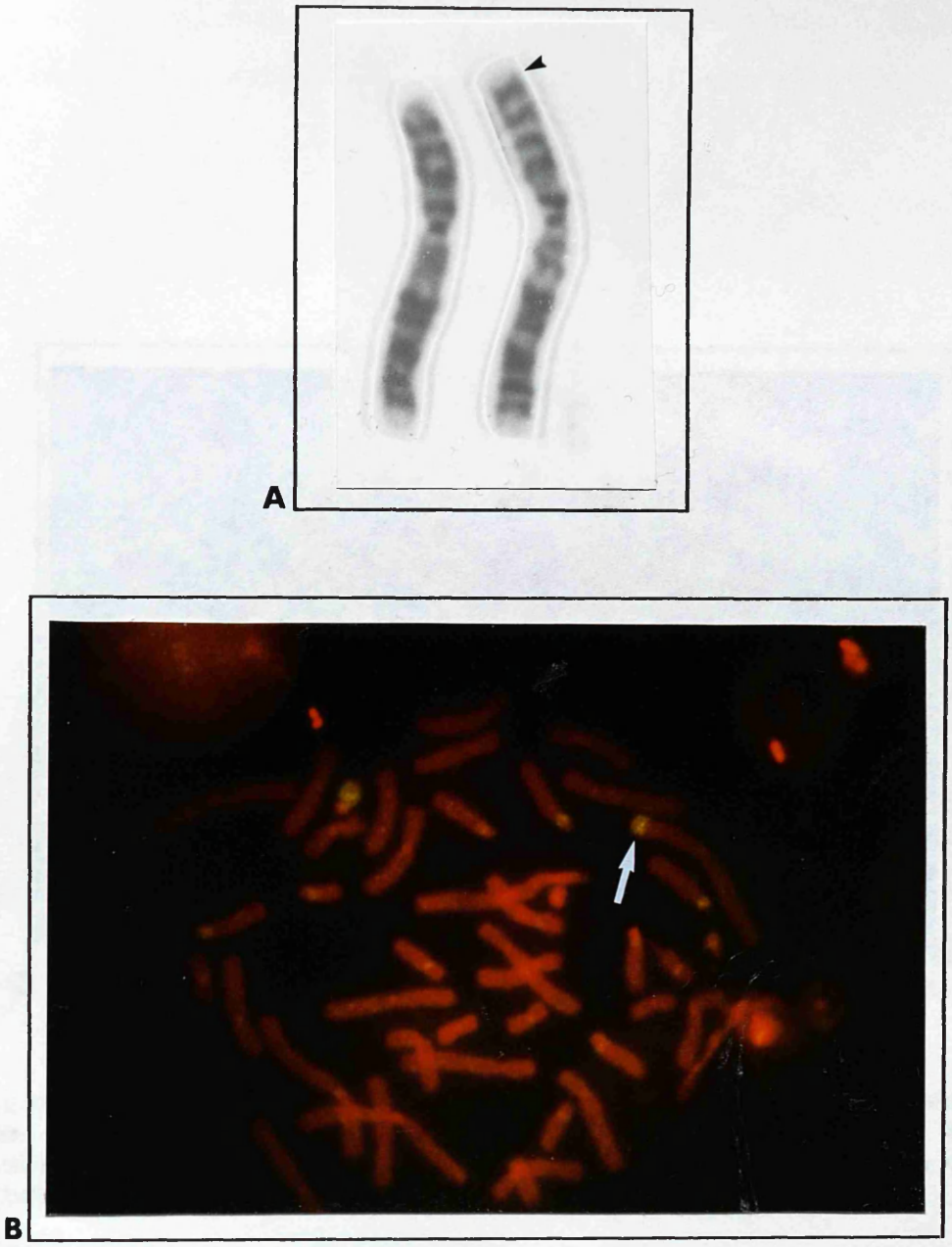
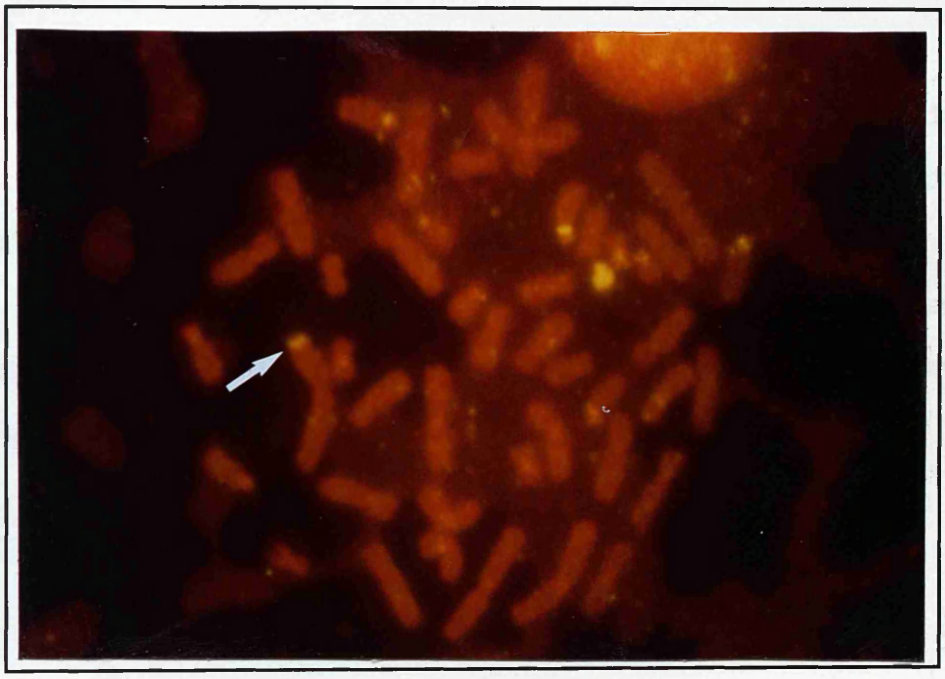


Figure 35. Photomicrographs of metaphase chromosomes of case 2 karyotyped 46,XX,-2,+der(2). (A) A partial Giemsa banded metaphase with extra material (black arrow) observed on the short arm of chromosome 2.
(B) Metaphase chromosomes following CISS-hybridization with whole chromosome 21 library. Only one normal chromosome 21 observed. A white arrow shows fluorescently stained material derived from chromosome 21 translocated onto the distal tip of the short arm of chromosome 2.



C

Figure 35(C). Photomicrograph of metaphase chromosomes of the same case showing the chromosomes covered with cell membrane and exogenous material. Result still showed a good hybridization signals (arrow).

derivative 21 and so it was not possible to demonstrate the presence of unstained material from chromosome 2 on the derivative 21.

Case 3

A newborn with clinical diagnosis of Down's syndrome and chromosome analysis showed an unbalanced karyotype 46,XY,t(14,21).

Results of chromosomal in situ suppression hybridization confirmed a Robertsonian translocation between chromosome 14 and 21 (**Figure 36**).

Case 4

A female patient with severe mental handicap and dysmorphic features who on cytogenetic analysis by G banding had two cell lines: 21 out of 25 cells showed an apparently normal female karyotype but in the remaining four cells there was an additional small marker chromosome of unknown origin. This marker was a ring chromosome of approximately half the size of a chromosome 21 and containing one G band positive area. C banding and AgNor's yielded no further information. Parental chromosomes were normal.

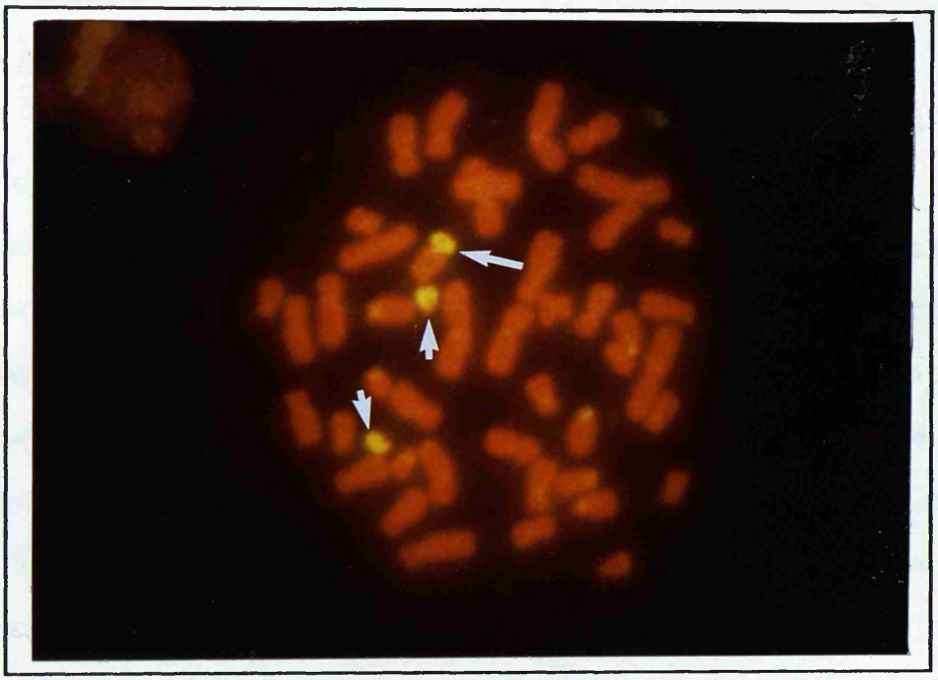


Figure 36. Photomicrograph of CISS hybridization on metaphase chromosomes of case 3, t(14:21). Two normal chromosomes 21 (arrowhead) and a translocated chromosome 21 (arrow) on chromosome 14. The human cot-1 DNA was used with total human DNA as competitor DNA. Better suppression was obtained as compared to using human cot-1 DNA alone (refer **Fig.17**).

Chromosomal in situ suppression hybridization using the 21 specific library eliminated chromosome 21 as the source of marker chromosome (**Figure 37**).

Case 5

A newborn with a clinical suspicion of Down's syndrome had chromosome analysis that showed 46 chromosomes with two normal chromosomes 21 but a small amount of additional material on the short arm of one X chromosome. Parental chromosomes were normal. The extra material on the patient's chromosome X consisted of a small G positive band distal to which was a small pale terminal region that could be interpreted as being derived from chromosome 21, karyotype 46 ,X ,der(X), t(X;21)(p22.33;q22.1).

The situation was resolved by using chromosomal in situ suppression hybridization that showed unambiguously that the material was derived from chromosome 21. Most of the metaphases observed showed distinct signals on the two chromatids of the short arm of one X chromosome (**Figure 38**).

Case 6

A 31 year old man with moderate mental, handicap and dysmorphic features had chromosome analysis using trypsin

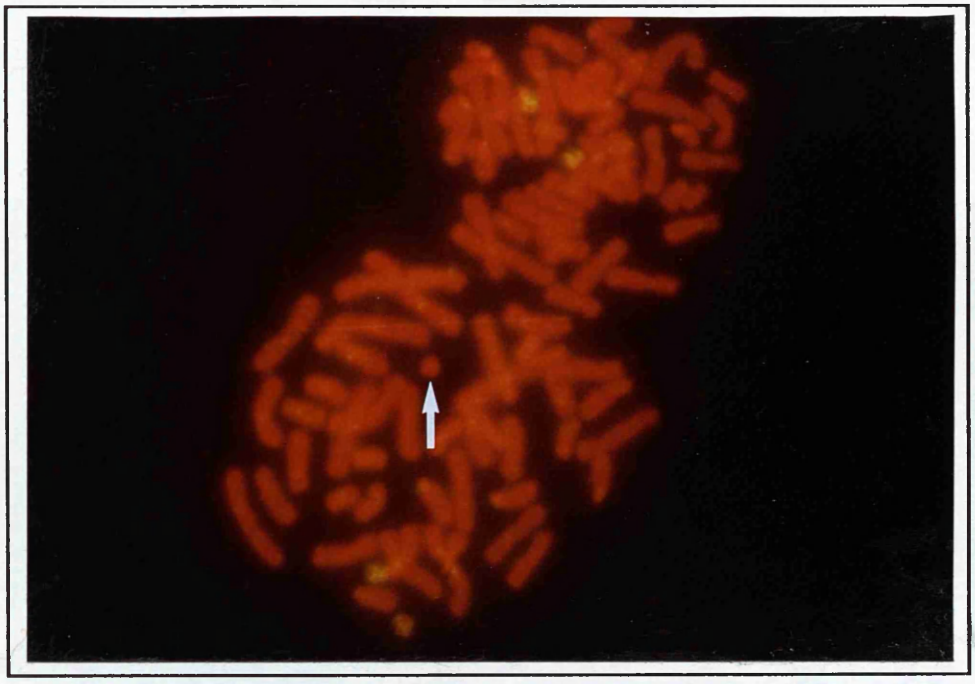


Figure 37. Photomicrograph of CISS hybridization of case 4 to identify origin of the marker chromosome. Result demonstrated that the marker (arrow) did not hybridize with whole chromosome 21 specific library indicating that it is not a derivative of chromosome 21.

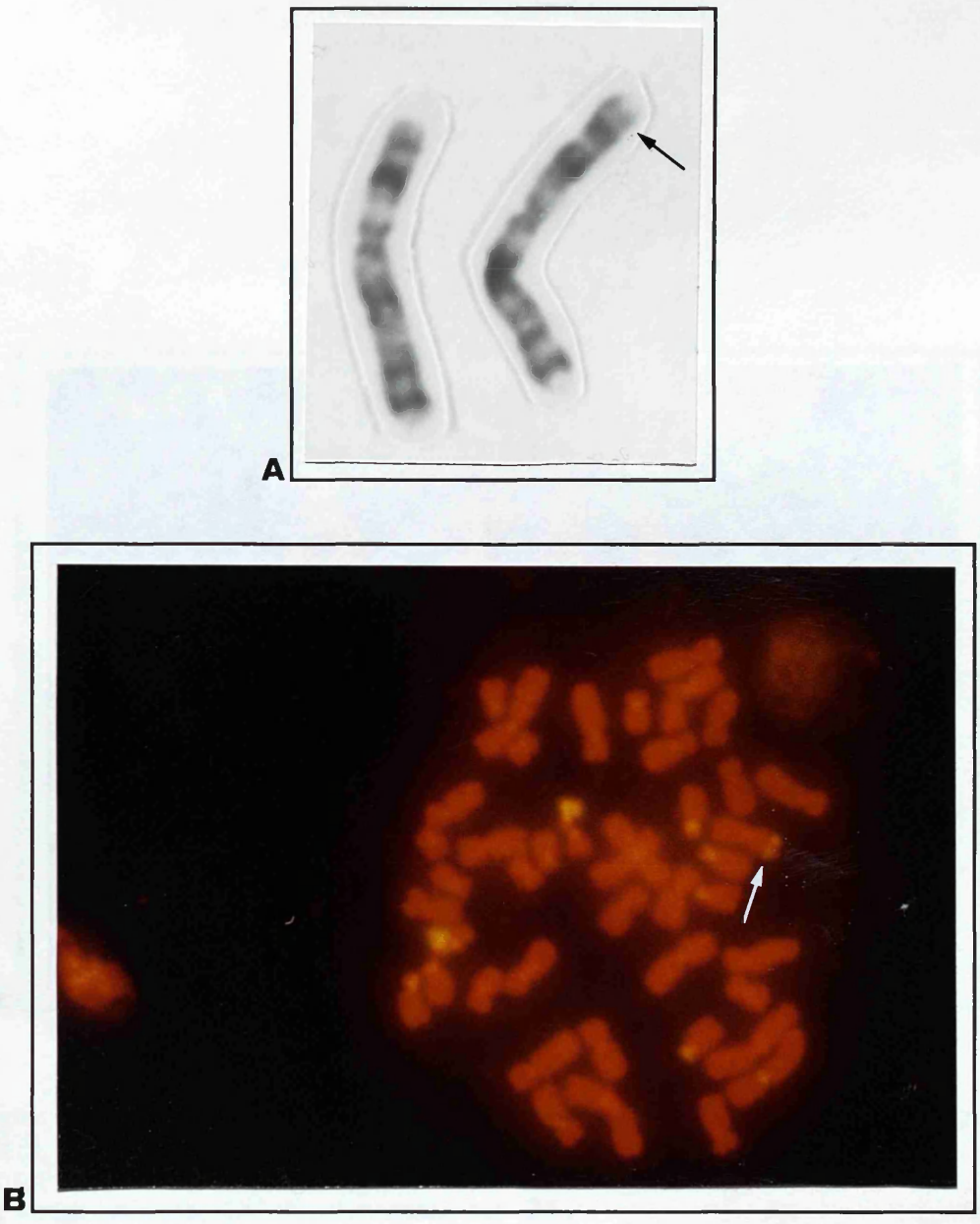
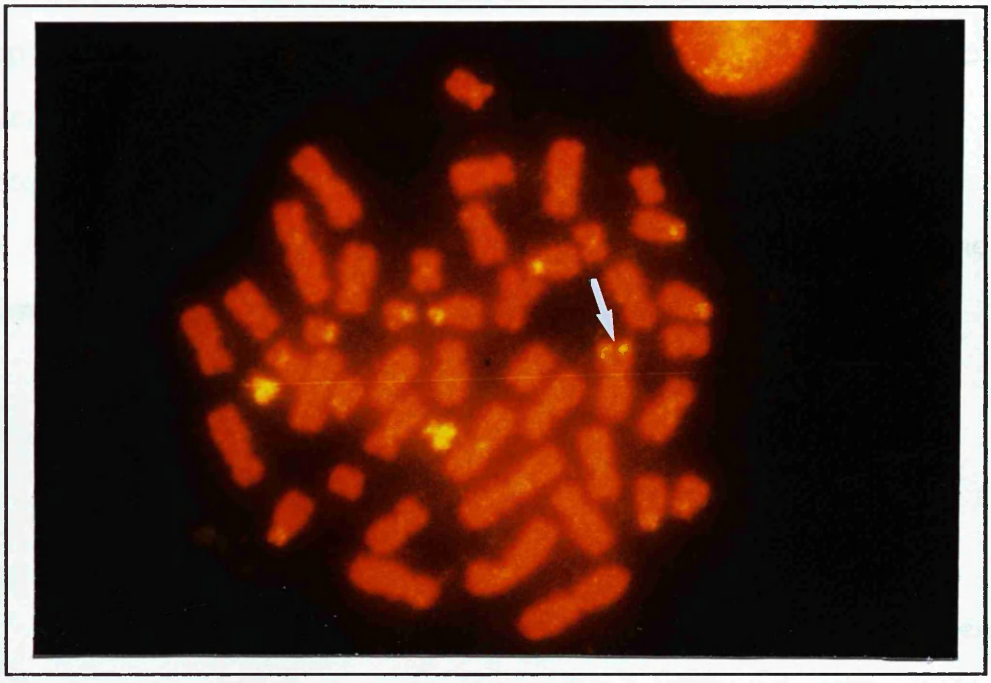


Figure 38. Photomicrographs of metaphase chromosomes of case 5, karyotype 46,X,der(X),t(X;21). (A) A partial metaphase after G-banding showing additional material on the short arm of X chromosome (arrowhead).
(B) Following CISS hybridization of metaphase chromosomes of the same case. Two chromosomes 21 are clearly decorated. The arrows indicate a translocated chromosome 21 material on the terminal region of short arm of X chromosome.



C

Figure 38 (C). The same case showing two distinct signals located on the chromatid of short arm of chromosome X (ar arrow). These results confirm unambigously that the derivative is from chromosome 21.

banding which revealed a mosaic karyotype 46, XY/47 ,XY,+ ring/48,XY,+ 2 rings. The ring chromosomes were small approximately half the size of a 21 with one (C-banding positive) centromere. There were no cytogenetic features to aid identification.

Out of 72 cells examined after chromosomal in situ suppression hybridization with whole chromosome 21 specific library, 27 cells were found to contain 1 or 2 ring chromosomes. None of these was stained thus confirming that the ring chromosome was not derived from chromosome 21 (**Figure 39**).

Case 7

Cytogenetic analysis of this case was undertaken in 1975 shortly after birth. Karyotype was 46, XX with two normal chromosomes 21 but with additional material present on the terminal region of the long arm of one chromosome 10. This consisted of one strong G positive band and one small one followed by a pale telomeric region, and could have been derived from part of the long arm of chromosome 21. Parental chromosomes were normal.

The results of chromosomal in situ suppression hybridization, however, ruled out chromosome 21 as the donor chromosome (**Figure 40**).

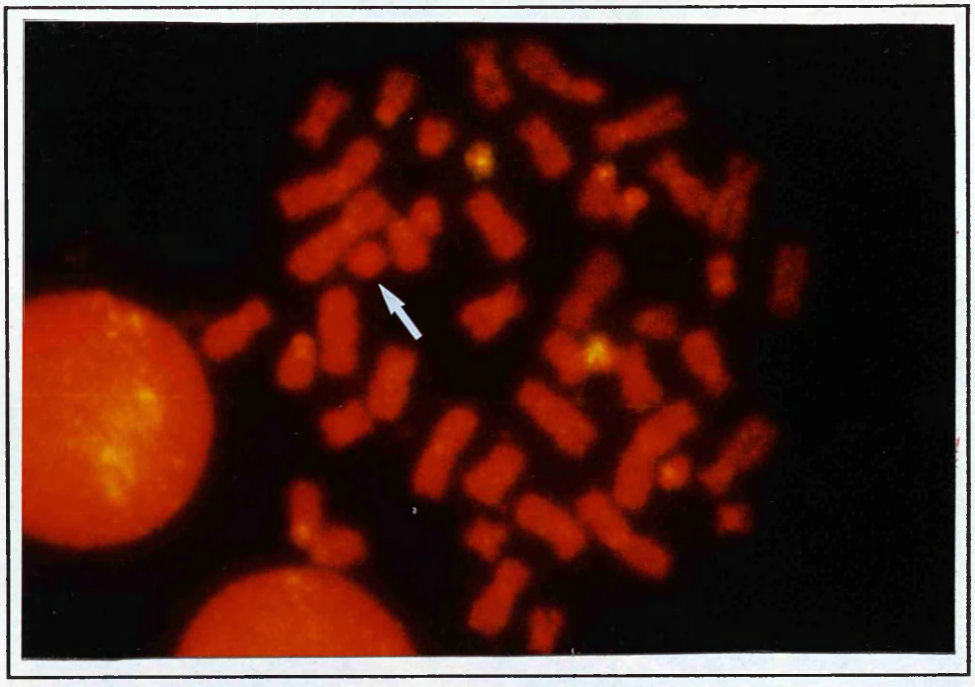
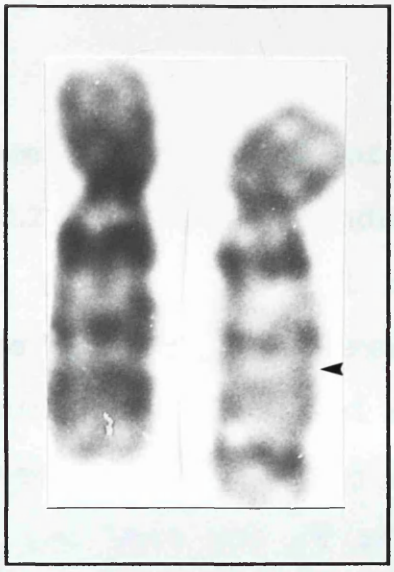
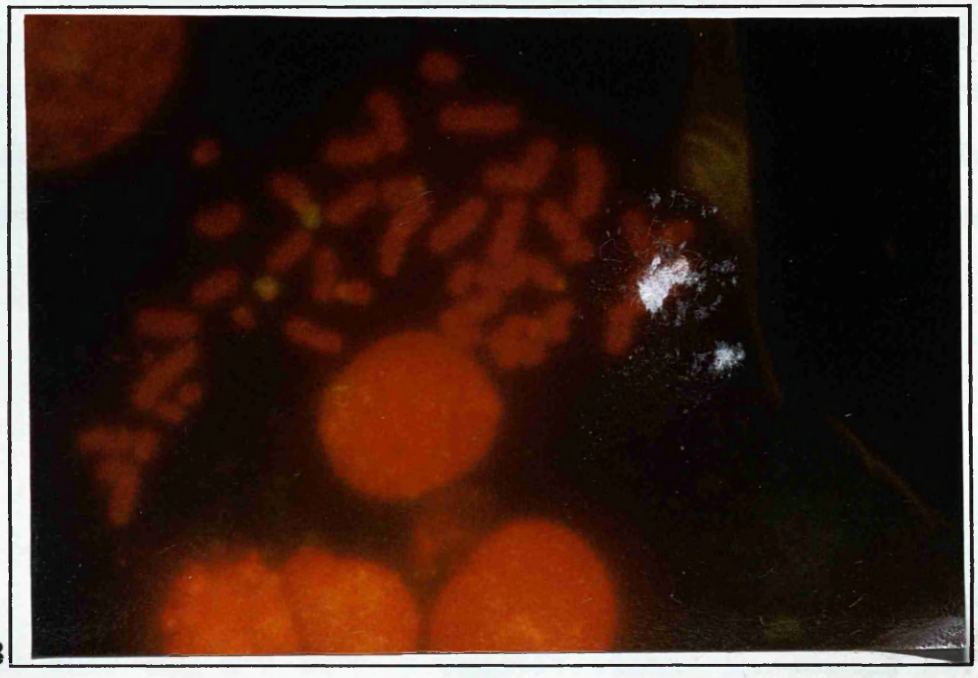


Figure 39. Photomicrograph of CISS hybridization of metaphase chromosomes of a male patient with mosaic karyotype 46,XY/47XY,+ring/48,XY,+2 rings. The result shows that this fragment did not hybridize with whole chromosome 21 specific library (arrow), this confirming that it was not derived from chromosome 21.



A



B

Figure 40. Photomicrographs of (A) partial G-banded and (B) CISS hybridization of metaphase chromosomes of case 7 using whole chromosome 21 specific library. No chromosome 21 derivative observed on the other chromosomes. This rules out that the extra material on long arm of chromosome 10 derived from chromosome 21.

Case 8

A specimen from a bone marrow of a case of 21 month old baby with ALL(L2) and Downs' syndrome. The chromosome analysis of direct bone marrow cell cultures revealed a 47,XX,+21 karyotype in most cells examined. Four out of thirteen cells examined had additional abnormalities in the form of loss of material from the long arm of chromosome 6, extra material on the long arm of chromosome 5 and one additional G group chromosome.

Results of the chromosomal in situ suppression hybridization performed on a different slide showed a tetrasomy of chromosome 21 in one of the metaphases examined and trisomy on in the other metaphases (**Figure 41**).

Case 9

A specimen from a direct bone marrow cell culture of a 64 year old man with a constitutional karyotype t(7;10)(q22.3;p15) who was diagnosed as AML(M4). Chromosome counts ranged from 43 to 46 (M=45). In particular all cells had a balanced translocation between the long arm of chromosome 7 and the short arm of chromosome 10. Other abnormalities included -1,-4,-5,-11,-17,-21, and markers including rings. Features common to most cells included the loss of one homologue of chromosome 1, 4, 5, 11, 8, 11, 17 and 21 and

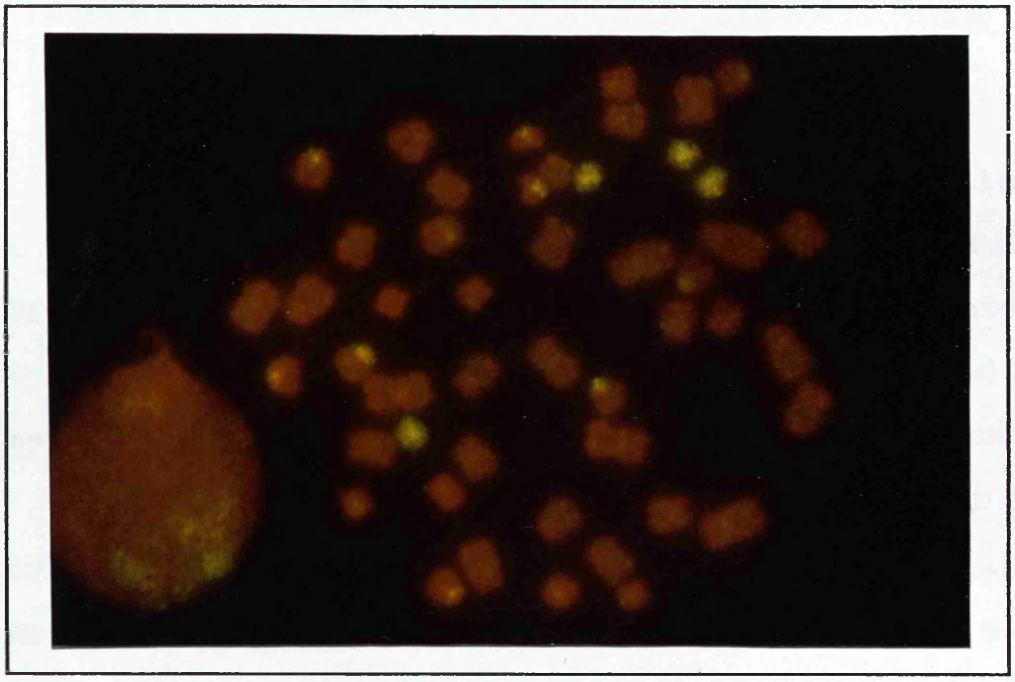


Figure 41. Photomicrograph of CISS hybridization of metaphase chromosomes from a direct bone marrow culture of case 8. This results shows a tetrasomy of chromosome 21 in one of the metaphases examined.

the addition of several common marker chromosomes.

Results of the chromosomal in situ suppression hybridization show that none of the markers was derived from chromosome 21 material. All three metaphases examined showed the presence of only one chromosome 21 (**Figure 42**).

Case 10

A specimen from a direct bone marrow cell culture of a 90 year old woman with chronic myeloid leukaemia at diagnosis. All the 16 cells analysed showed the karyotype 46XX,t(8;21)(q22:q22) del 9(q12-q31) which favoured acute myeloid leukaemia rather than chronic myeloid leukaemia.

Results of chromosomal in situ suppression hybridization with the whole chromosome 21 specific library demonstrated that the material on chromosome 8 was derived from chromosome 21 (**Figure 43**).

Case 11

A specimen from direct bone marrow cell cultures from a 3 year old boy. Most cells examined revealed apparently normal karyotype but six of the cells examined had a clonal abnormality. These cells, with poor chromosome morphology had 45 or 46 chromosomes and at least one common marker chromosome.

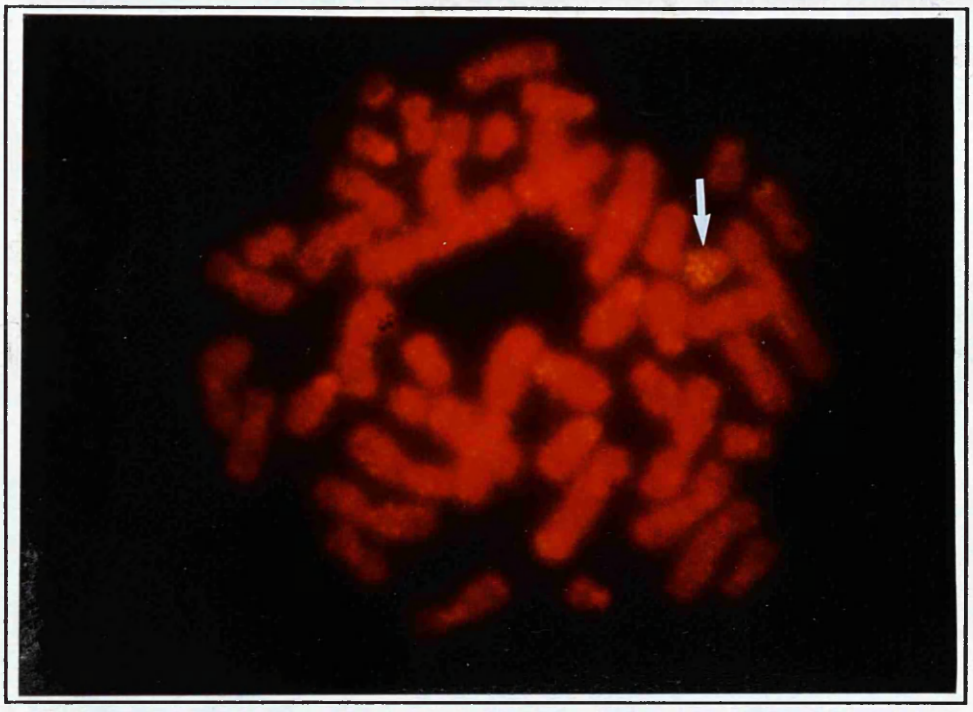


Figure 42. Photomicrograph of metaphase chromosomes from a direct bone marrow cell culture of case 9 following CISS hybridization. Result shows that only one chromosome 21 is observed and none of the markers were stained suggesting that they were not derived from 21 material.

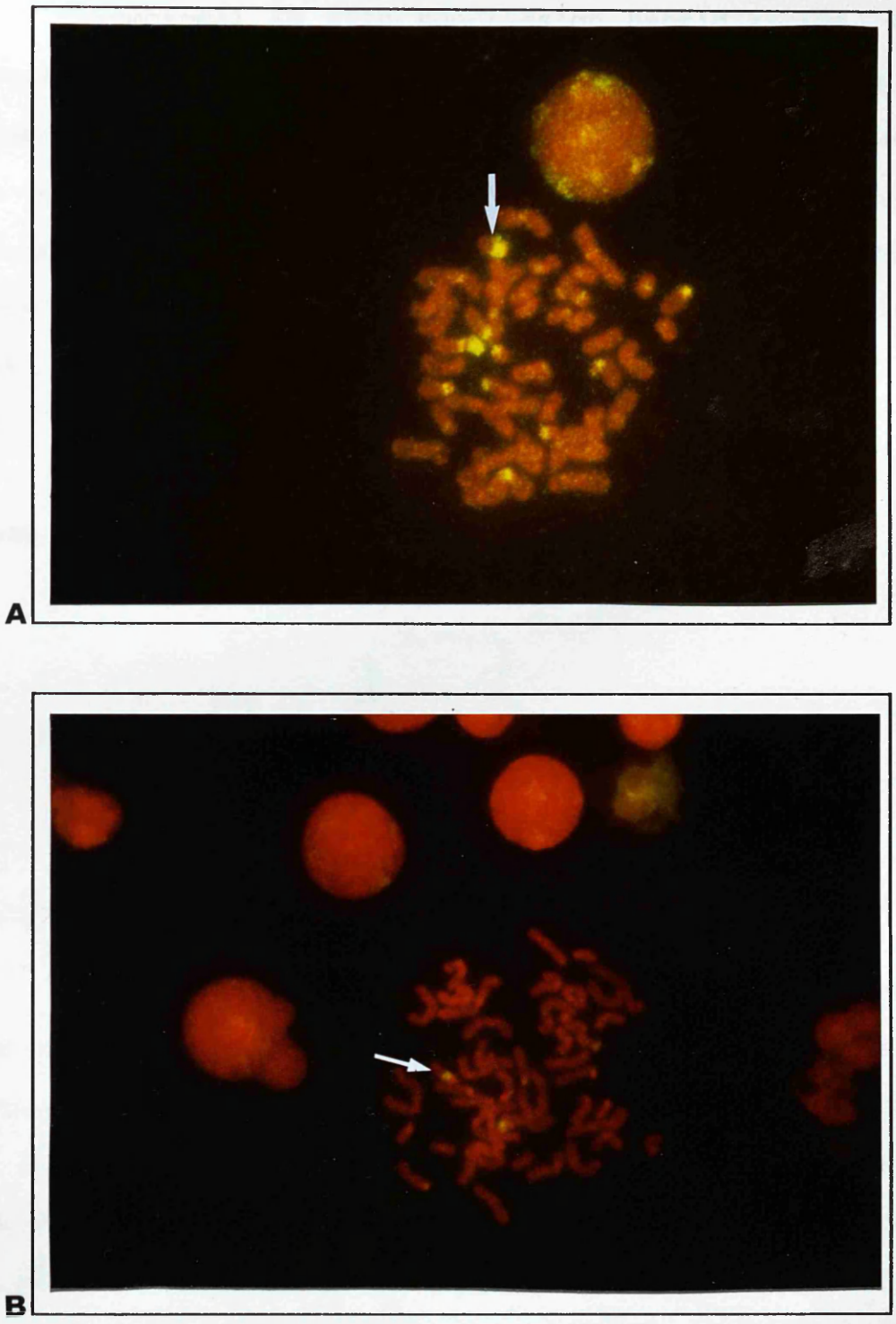
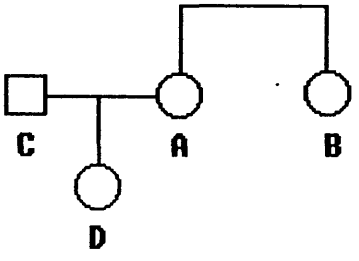


Figure 43 A and B. Photomicrographs of CISS hybridization with whole chromosome 21 specific library on metaphase chromosomes of direct bone marrow culture of case 10. The arrows show the translocated t(8;21).

Chromosomal in situ suppression hybridization with whole chromosome 21 specific library were performed on two destained slides originally stained with Leishman. Results in two metaphases located showed a translocated chromosome 21 and one normal chromosome 21 (**Figure 44**). Metaphase spreads on a second slide showed two chromosomes 21 (**Figure 45**).

Case 12



The proband had developmental delay and the mother's sister was also mentally handicapped. Another laboratory reported a possible small deletion of 21q in both proband, **B**, **D** and her mother. Cytogenetic analysis in our laboratory showed the mother to carry a balanced reciprocal translocation t(13;21)(q12.3;q22.11). Both **D** and **B** had the unbalanced form 46,XX,-21,der(13)t(13;21)(q12.3;q22.11)mat. and were thus monosomic for the region 21pter-q22.11, and trisomic for the region 13pter-q12.3.

These results were confirmed by chromosomal in situ suppression hybridization where in the mother the two

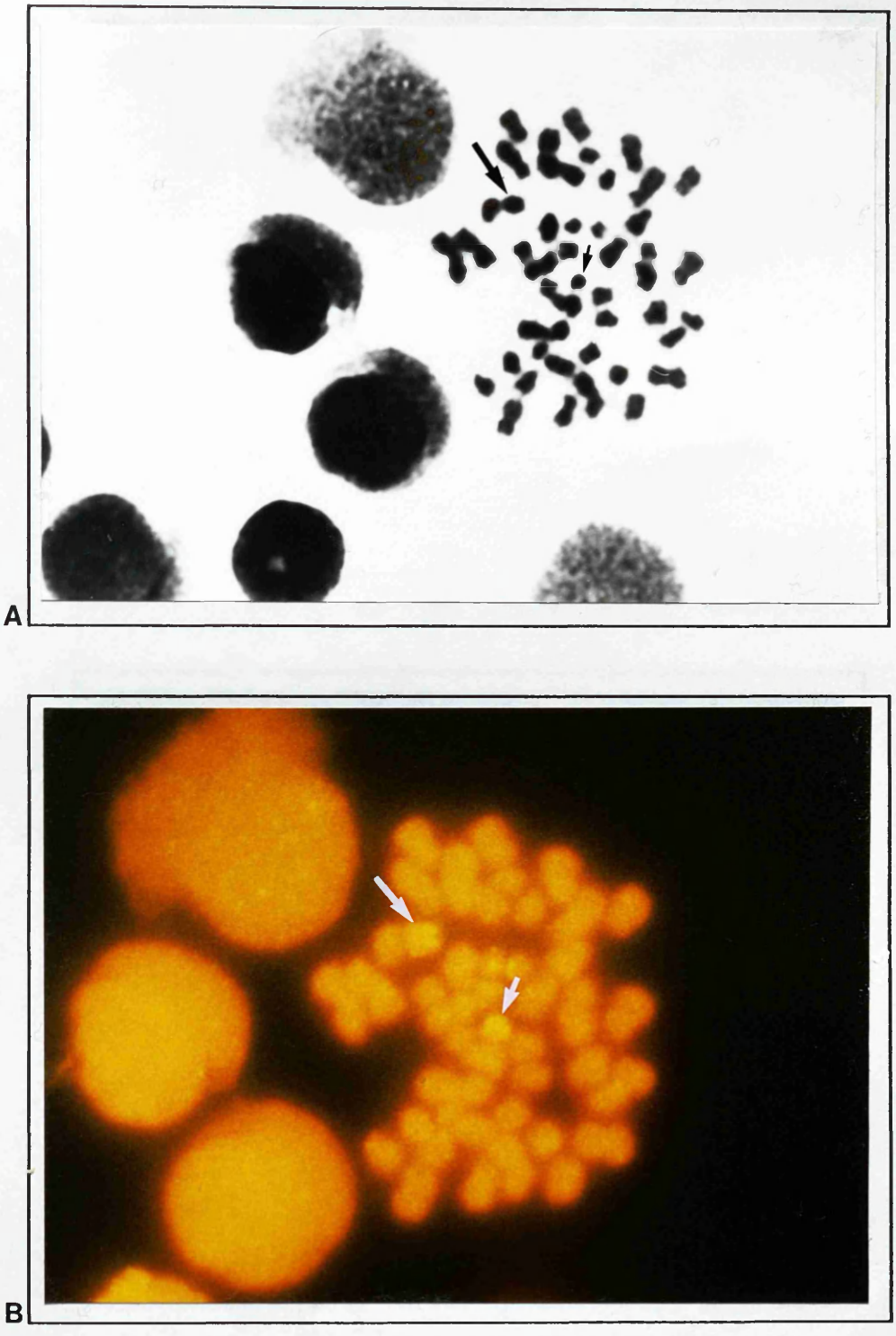


Figure 44. Photomicrographs of metaphase chromosomes of direct bone marrow cultures of case 11 (A) block staining with Leishman stain and (B) after CISS hybridization with whole chromosome 21 specific library. The arrow shows a translocated chromosome 21 marker in the abnormal cell line. Normal chromosome 21 shown by arrowhead.

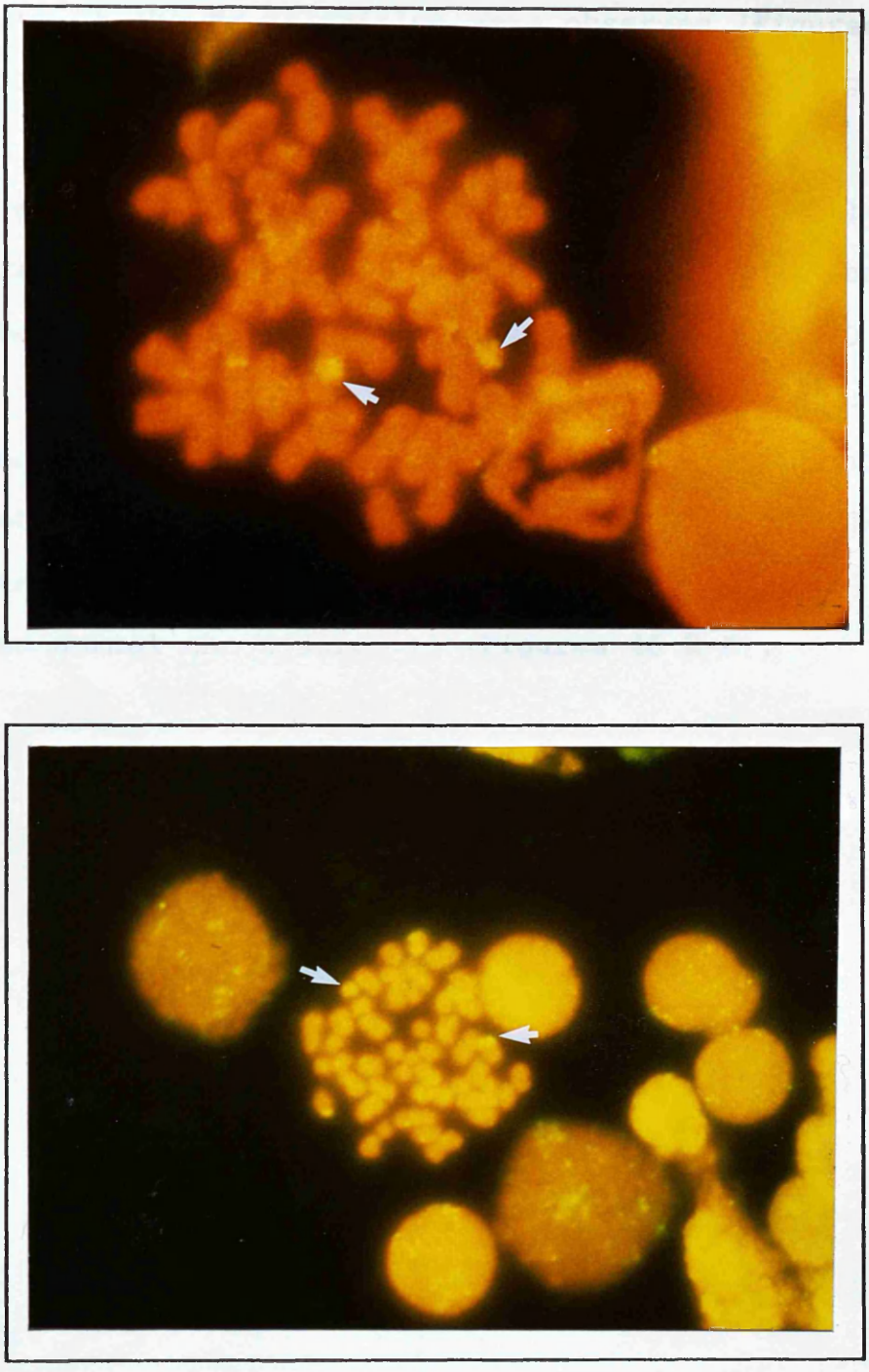


Figure 45. Photomicrographs of metaphase chromosomes of direct bone marrow cultures of the case 11 (as in **Fig. 44**) showing an example of the normal cell line with only two 21 chromosomes present.

products of the translocation were observed (**Figures 46-A and B**). The first product, der(13) is seen as a G group sized acrocentric chromosome (**Figure 46-C**) in which the short arm, centromeric region and proximal part of the long arm are not painted, but the distal part of the long arm is. The second product, der 21, is observed as a D group sized acrocentric where the short arm, centromere and proximal long arm are painted, and the distal long arm, consisting of chromosome 13 material is not. The two affected individuals have the derivative chromosome 13 with the two normal chromosomes 13 (**Figures 46 D-E**).

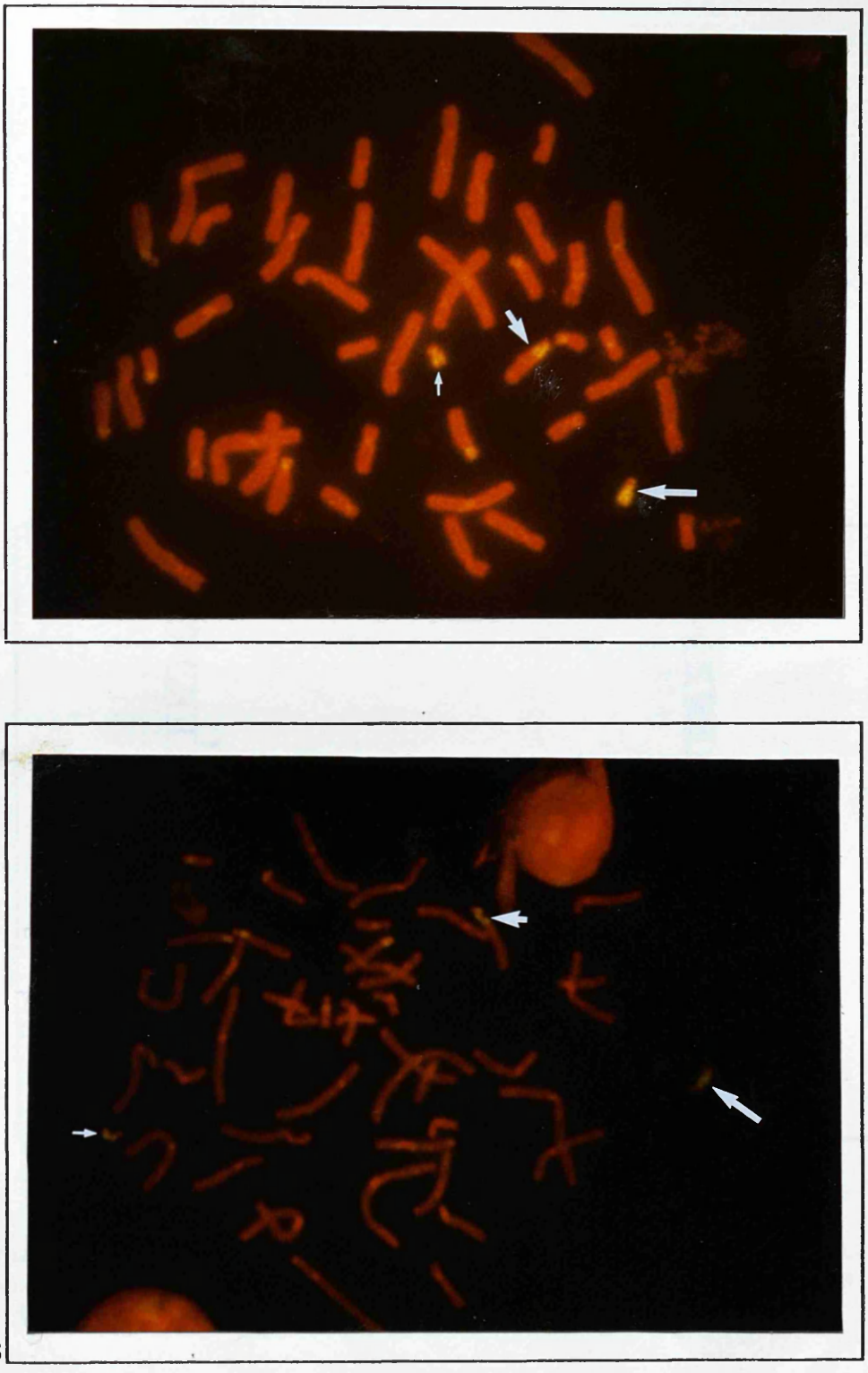


Figure 46 A and B. Photomicrographs of chromosomal in situ suppression hybridization of partial metaphase chromosomes of mother of case 12 using whole chromosome 21 specific library. Both A and B are from the mother showing the derivative 21 (large arrowhead), derivative 13 (small arrowhead) and normal chromosome 21 (arrow).

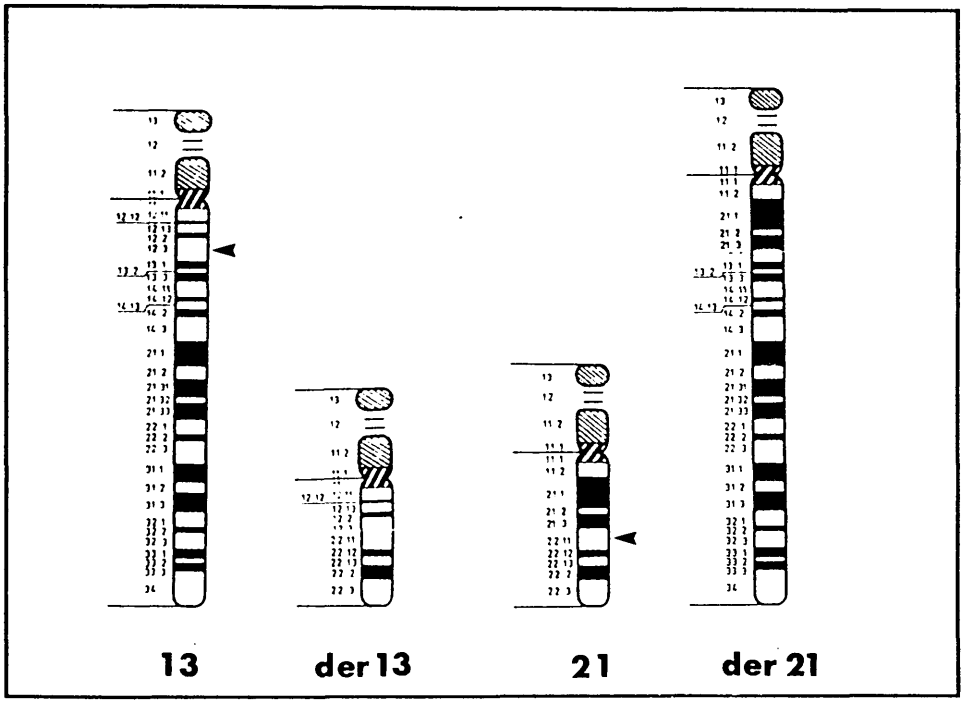


Figure 46 C. A diagramatic representation of balanced translocation involving chromosomes 13 and 21. Arrows show the breakpoints at 13q12.3 and 21q22.

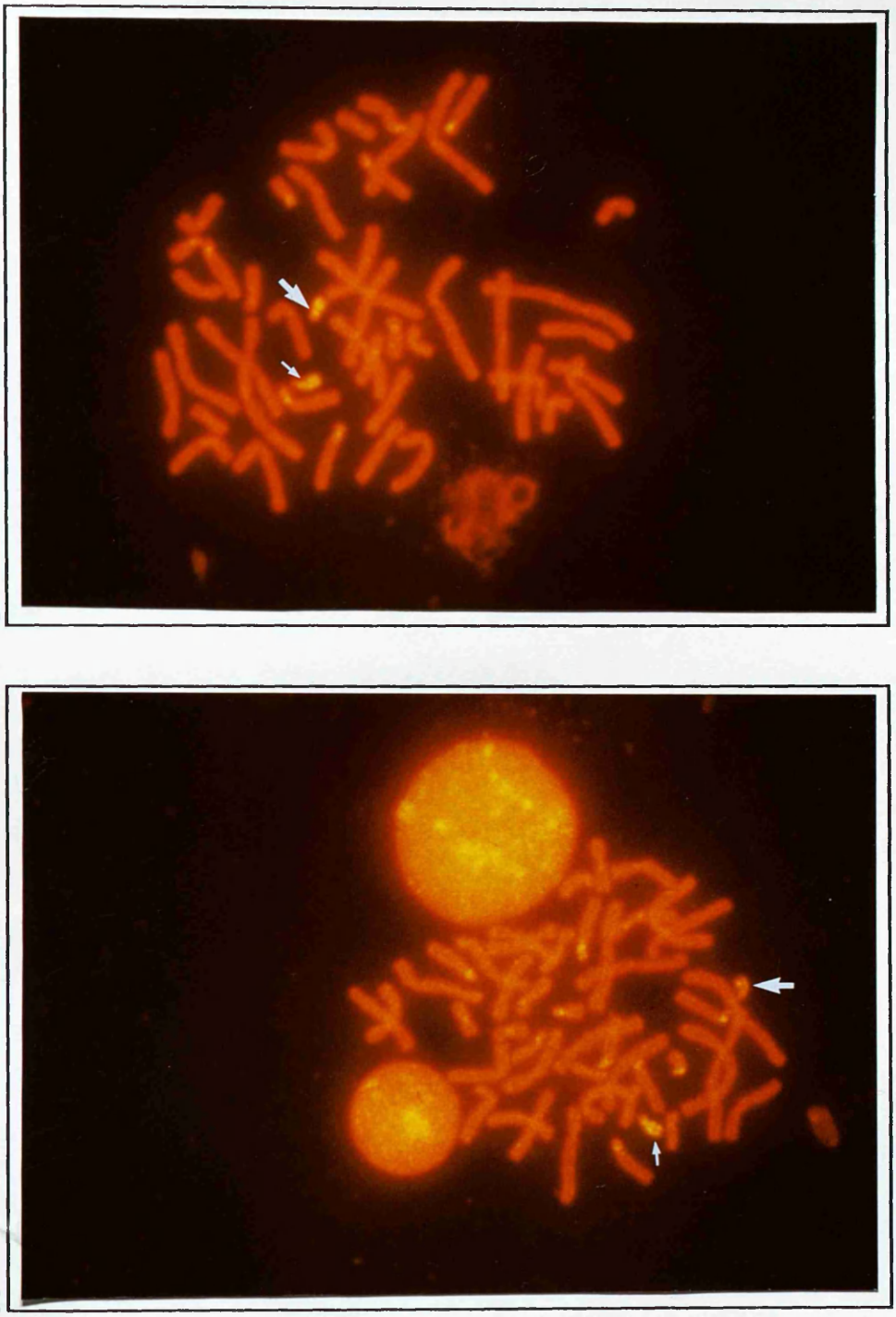


Figure 46 D and E. Photomicrographs of chromosomal in situ suppression hybridization of partial metaphase chromosomes of proband in case 12 showing the derivative 13 (large arrowhead) and normal 21 (small arrowhead).

CHAPTER 4

DISCUSSION

4.1 METHODOLOGICAL DIFFICULTIES

Chromosomal in situ suppression hybridization using whole chromosome specific library or chromosome painting was successfully developed and applied in the project but there were numerous methodological difficulties to overcome. These difficulties were encountered in two main areas. First, in probe preparation in particular low phage titre and probe labelling, and secondly in the actual chromosomal in situ suppression hybridization painting technique that included prehybridization treatment of target chromosomes, ratio of labelled probes to competitive human DNA, denaturation of chromosome and composite DNA, general hybridization, detection and microscopy.

4.1.1 Purification and extraction of phage particles containing human DNA inserts (library)

For the first two shipments of the library there was no success in growth and purification although the technique of Maniatis et al. (1982), as was used similarly by Pinkel et al. (1988) and by Lichter et al. (1988a) was employed. The earlier failure encouraged us to explore other techniques. With the modifications of culture and

purification techniques described in Material and Methods, an increase of 30% in the phage titre was obtained with the third batch of the library. Both the base and top agar (Difco, UK) were replaced with agarose (Sigma, UK). The top agarose contained only trypticase that contains no potent phage inhibitor and thus improved the phage growth (Becton Dickson, U.K). One advantage of the plate lysate method over the liquid culture was the more straightforward isolation of the phage particles and DNA extraction. The procedure is fast and simple and is suitable for routine use. Techniques based on equilibrium sedimentation (Maniatis et al. 1982; Sambrook et al., 1989) that include dialysis steps and removal of caesium chloride (CsCl) are complex and time consuming (Gonzalez and Marquez, 1990). The modified purification avoids the use of caesium chloride and ultra centrifugation. This procedure is rapid, cost-effective and applicable as a routine procedure.

Using the described method (Material and Methods) it was advisable to amplify the library and use this as a stock. The amplified stock which contained 2ul/ml chrloroform was kept at 4°C for more than a year without decrease in phage the titre.

4.1.2 Labelling of probe by direct incorporation of biotin using polymerase chain reaction (PCR)

The probe was initially labelled with Biotin 11-DUTP by nick translation (Rigby et al., 1977; Langer et al.,

1981; Garson et al., 1987) using the Blue Gene Kits that Pinkel et al. (1988) and Lichter et al. (1988a) had used. Unfortunately, the result showed poor staining (non-homogeneous or non-uniform decoration with patches only) of the chromosomes 21 and 'dirty ' background signal. According to Weir et al. (1990), this might be due to a low concentration of vector-containing insert being labelled and/or non-homogeneous length and a variable labelling pattern. However, this problem was overcome with the labelling of probe by direct incorporation of biotin using the polymerase chain reaction (PCR).

Although the original average insert size of the library was 4kb., after PCR the majority of labelled fragments were less than 400bp measured by gel electrophoresis. However, these fragment sizes were within an empiric probe length that is believed to be important for the stability of the hybrid (Tecott et al., 1987; Kuo et al., 1991). This proved that the direct biotin labelling using PCR amplification enriched the library sequences, increased the concentration of insert DNA library and the probe length was also cut for the optimum size. It is claimed that nick-translation labelling produced probes of 500bp on average (BRL< Life Sci.,U.K).

Cleaning of the PCR product was essential as dNTPs, primers and other impurities interfered with the final concentration biotin labelled DNA in the hybridization buffer. Removal of these products also encouraged better probe penetration. Direct use of uncleaned PCR product was

also shown to produce painting signal but suffered heavy background signals. Cleaning of biotin labelled PCR product by Sephadex G-50 chromatography was shown to eliminate such impurities. Those cleaned by ethanol/ ammonium sulphate failed to give homogenous decoration probably due to loss ofsome sequences during cleaning.

Labelling the library using PCR technology as in this project had many advantages. Purification of large amounts of the chromosome specific library proved to be time-consuming. Tedious bacteriophage lambda amplification and the biochemical purification time can be reduced considerably. Saiki et al. (1988) showed how using appropriate primers PCR was an effective and efficient way to isolate the insert of bacteriophages and /or plasmids thus eliminating the vector and insert isolation procedures. It also selectively amplifies the target sequences of interest (Lo et al., 1988; Vosberg et al., 1989) and produces a selective enrichment of the DNA sequences by a factor of 10^6 after 30 cycles of amplification (Saiki et al.1988). The direct labelling of biotin with PCR amplifications helps to minimize the time and labour of handling of the DNA (probe). Labelling by PCR can be performed automatically and economically and is suitable for routine use.

With the slight modification of the protocol PCR in the future will support probe handling and complement the labelling as shown in the project. Using PCR technology Lengauer et al. (1990) successfully generated the probes

from hybrid cell lines with Alu and L1 primers for specific delineation of human chromosomes. Recently, Tarleton and Schwarz, (1991) reported using the PCR to maintain DNA probe inventories in clinical and diagnostic laboratories. Baldini and Ward (1991) also demonstrated the use of biotin labelled Alu-PCR product generated by Alu primers to produce a chromosome banding that is almost identical to the conventional R-banding pattern. Weir et al. (1991) demonstrated the highly specific and excellent signal intensity of PCR-generated probe DNA of human chromosome 8 to identify the heterochromatin in interphase nuclei of a chronic myeloid leukaemia (CML) patient with trisomy 8.

One disadvantage of PCR is its high sensitivity to reaction condition reactions. For example, an unsuitable annealing temperature can result in the amplification and labelling of sequences other than those of the target sequence. This might give a signal equivalent to one with specific sequences that could not be completely suppressed by competitor DNA. The size of the PCR labelled sequences varies according to the original size of insert and they are not constant sized fragments as by nick translation (an average of 500 base pairs). In this project the PCR product on gel electrophoresis showed size variation with most were around 400 base pairs. Shorter fragment size can be easily achieved when required by sonication but this might remove or dislodge the biotin.

Another common disadvantage of the polymerase chain reaction (PCR) is that even minute contamination with

extraneous sequences may lead to inadvertent addition of target sequences in the assay mixture. Such situations are difficult to verify. Inconsistent results can also be caused by variations of pH, temperature, salt concentration, dNTP concentration, primer concentration, and the amount of DNA and Taq polymerase useds. During the course of the study it frequently failed to produce a high yield of product for a different batch even with similar conditions. Hence, the amount of DNA to use in the mixture needs to be checked when using newly purified DNA.

4.1.3 Optimization of chromosomal in situ suppression hybridization with chromosome 21 specific library

Two protocols as described by Pinkel et al.(1988) and Lichter et al. (1988a) were used as the basis of the method. Both of these procedures follow similar basic steps. There were some differences however, such as the probe to competitor DNA ratio, slide handling, hybridization and detection time, washing buffer, and time and temperature of washing. These factors were investigated extensively and were found to be helpful to the optimization of the procedure for the clinical application in the department. Difficulties were encountered at every stage and are discussed accordingly

4.1.4 Importance of proper treatment of slide

Fresh slides prepared for chromosomal in situ suppression hybridization within two weeks were kept in a slide box at room temperature. Those to be used later than 30 days were kept at 4°C. This was to prevent any denaturation of DNA and bacterial or fungal contamination. Results showed that they could be used successfully up to 2 years. According to Pinkel et al. (1988) slides were kept at -70°C in liquid nitrogen to age and to prevent further deterioration.

RNase pretreatment was not found to affect either the level of background or the probe penetration when slides were freshly made (less than 14 days old). Treatment with RNase was found to remove some endogenous materials on older slides (more than 3 weeks old) and this was especially so in those kept at room temperature for a period for up to 2 years. However, in the case of prebanded slides which had been destained, pretreatment with RNase helped to ensure removal excess stain that could hinder the hybridization efficiency.

Treatment of slides with Proteinase K before hybridization has been proposed as a means to decrease background when fluorescent detection method is used (Raap et al. 1986). It was also claimed to improve the hybridization and probe penetration (Cremer et al. 1988a; Cremer et al. 1988b; Chan and McGee, 1989). In this project it was shown that the cells and chromosomes were removed by

this treatment. This treatment also tended to distort the cell morphology even at a concentration of 0.2ug/ml at 37°C for 5 minutes. Zhang et al. (1990) similarly found that the treatment was harmful to the cells and chromosomes.

All fresh slides (less than 14 days old) were treated by placing them in methanol-acetic acid (3:1) for one hour followed by drying in vacuum cupboard prior to denaturation. These slides were not enzymatically treated either with RNase or Proteinase K and showed considerably reduced background.

Destaining of G-banded chromosomes was carried out by immersing in two changes of 100% methanol for 10 minutes each and this did not severely damage the chromosome morphology. After destaining these slides also showed more satisfactory painting (signal) when they had been stored at 4°C until used. When G-banded metaphases were destained by ascending concentration of ethanol, poor hybridization was observed and there were additional background deposits, probably due to the precipitation of degenerate cellular materials. Metaphases where the stain was not completely removed showed low colour intensity of the painted chromosome caused by an overlap of red propidium iodide colour on the bluish stain.

4.1.5 Proportional composition of labelled probes and competitive (unlabelled human) DNA

Since the library contained both unique and repetitive sequences blocking of these repetitive sequences was necessary. The repetitive sequences that are shared by all other chromosomes could be selectively prehybridized with unlabelled genomic DNA (normally total human placental DNA). The depletion of these shared sequences thereby increased the contrast of unique sequences.

The amount of unlabelled genomic DNA to use for maximum blocking could be deduced from the ratio of unlabelled competitor DNA to the final probe concentration as formulated by Pinkel et al. (1988). This ratio defined as "Q" can vary from 1 to 5 and can also specify prehybridization and hybridization time. The "Q" also varies for individual chromosomes (**Table 6**). Hulten et al. (1991), recently outlined these variations and described differences of prehybridization and hybridization times for each specific library in order to obtain minimal cross-hybridization.

In this project the final concentration of the total DNA (including chromosome 21 specific library as probe, competitor DNA, and carrier DNA) in the hybridization buffer was 1mg/ml. The carrier DNA (sheared salmon sperm DNA) was used to make up the final concentration. The amount of each DNA added was calculated in appropriate ratio according to Pinkel et al. (1988). (Kuo, Lawrence

Chromosome #	Genomic DNA content	Blocking DNA Q(1)	Q(5)
1	0.0856	0.134ug	0.67ug
2	0.0838	0.136ug	0.68ug
3	0.0698	0.164ug	0.82ug
4	0.0662	0.173ug	0.86ug
5	0.0636	0.18ug	0.90ug
6	0.0594	0.195ug	0.97ug
7	0.0552	0.21ug	1.03ug
8	0.0504	0.227ug	1.13ug
9	0.0476	0.24ug	1.20ug
10	0.0470	0.243ug	1.22ug
11	0.0468	0.244ug	1.22ug
12	0.0462	0.25ug	1.25ug
13	0.0376	0.304ug	1.52ug
14	0.0356	0.32ug	1.62ug
15	0.0346	0.33ug	1.65ug
16	0.0322	0.356ug	1.78ug
17	0.0294	0.39ug	1.96ug
18	0.0280	0.408ug	2.04ug
19	0.0218	0.524ug	2.62ug
20	0.0230	0.497ug	2.49ug
21	0.0164	0.70ug	3.5ug
22	0.0184	0.63ug	3.17ug
X	0.0528	0.216ug	1.08ug
Y	0.0194	0.59ug	2.95ug

Table 6. Concentration of human placental DNA required to achieve a blocking ratio **Q(1)** and **Q(5)** with each 20ng biotinylated Bluescribe plasmid library used. (taken from Kuo, Lawrence Livermore, personal communication)

$$Q(1) = \frac{(4/7)\ (\text{Probe conc.})(\text{Vol. of probe used})}{\text{Genomic DNA content}}$$

Livermore, USA, personal communication). The ratio of the chromosome 21 specific DNA to competitor used in this project varied from 1:150 to 1:80. This variation also might be due to less accurate measurement of the biotin labelled PCR product. With the chromosome 21 specific library constructed in Bluescribe plasmid, Hulten et al. (1991) used a ratio of 1:25 for probe and competitor DNA. Increased concentration of the blocking (competitor) DNA or the probe itself did not improve the painting. Instead, an increased amount of total DNA above 10ug/10ul in the hybridization mixture was hard to dissolve homogenously. Undissolved or not fully resuspended DNA caused uneven distribution of the mixture and also poor hybridization of the probe.

According to Lichter et al. (1988a) addition of human cot-1 DNA as competitor DNA with the total human placental DNA ensured additional suppression of Alu and Kpn sequences that might not be sufficiently suppressed by total human placental DNA alone. Using human cot-1 DNA alone as blocking DNA did not improve the painting. Instead, in this project, the human cot-1 DNA was added with the probe, carrier DNA and total human placenta DNA. They were mixed accordingly and ethanol precipitated at -20°C overnight or at -70°C for one hour before being resuspended in hybridization mixture. Ethanol precipitation of combined salmon sperm, total human DNA, human cot-1 DNA and biotin labelled probe improved the painting (signal) as well as reducing the background. This step was essential to

concentrate the small amount of target probes and to remove other impurities present in the mixture. Kievits et al. (1990) reported vacuum drying of the probe and competitor DNA prior to dissolving in the hybridization buffer as an alternative.

4.1.6 Denaturation of hybridization mixture

The pH of the hybridization buffer was critical and adjusted to exactly pH 7.0 and no carrier DNA added. The Denhardts solution and/or EDTA and bovine albumin were not added to the hybridization buffer as these chemicals were sometimes hard to dissolve and caused some deposits. Similar dirty background deposits were reported by Fans et al. (1990) when these chemicals were added to the hybridization buffer. Hybridization buffer stored at -20° could maintained its efficiency for 6 to 8 months.

Before denaturation the hybridization mixture needed to be checked for complete and homogeneous resuspension of the DNA in the buffer as presence of even small clumps could affect the denaturation and preannealling and the final distribution of mixture on the slide.

Denaturation of hybridization mixture was performed at 75°C for 5 minutes or for a slightly longer time up to 8 minutes to ensure optimal denaturation of DNA. It was important to stop the denaturation immediately by transferring the hybridization mixture onto the ice prior

to preannealling. Preannealing for 60 minutes at 37°C was found to be optimal and a similar prehybridization time has also been reported by Hulten et al. (1991). However, Lichter et al. (1990b) used a prehybridization time of 30 minutes with cosmid probes. Longer prehybridization time may however, be required when the ratio of probe to competitor DNA is higher (Pinkel et al. 1988, Kievits et al. 1990).

4.1.7 Distortion of chromosome morphology during denaturation

Denaturation of chromosomes tends to distort their morphology. Lomholt et al. (1990 showed that denaturation of chromosomes in 70% formamide/2XSSC at pH 9.0 to pH 9.5 caused poorly conserved morphology. Denaturation of target chromosomes performed at 75°C to 80°C for 5 to 8 minutes in 70% formamide/2XSSC showed no morphological changes in the present study. After denaturation the slides were immediately transferred into cold-ethanol series to stop the denaturation and for dehydration. Slides denatured at 80°C for more than 5 minutes gave distorted cell morphology. Distortion of chromosome morphology was also observed when the denaturing solution was not adjusted to pH 7.0. All denatured slides were used immediately after 10-30 minutes of drying under vacuum. Those left overnight at 4°C failed to give satisfactory signal.

4.1.8 High background deposits in relation to hybridization temperature and time.

Hybridization was achieved by incubating the slides for 16 to 20 hours at 37°C and this resulted in maximum probe hybridization showing intense decoration of chromosome 21 with a clean background. Hybridization incubation at 40-45°C showed high background deposits despite increased washing stringency. This might be due to drying of slides during the incubation with subsequent non-specific binding.

4.1.9 Removal of un-hybridized and non-specific binding sequences

Post-hybridization and in between detection washes were ~~was~~ essential to remove un-hybridized and non-specific binding of DNA or stains. Different types of post hybridization washes were tried as described in the results. One major aim was to minimize the use of formamide in the procedure because of its teratogenic property. It was found that post-hybridization washing in 50% formamide/2XSSC at 45°C was essential to remove excessive non-binding sequences and to maintain chromosome morphology. The formamide was used to maintain the stringency. During post-hybridization washing it was important not to remove the coverslips by force as this

could distort or peel off the chromosomes from the slides.

In-between washes were carried out using the phosphate buffer as described by Pinkel et al. (1988). This phosphate buffer pH 8.0 containing 1% Nonidet P-40 (octylphenol-ethylene oxide) a petroleum by-product as non-ionic detergent is preferred to buffer containing Triton X and/or Tween 20 (polyoxethylene-20 sorbitan) for use in between detection washes. This washing solution washed and reduced the non-specific binding of fluorescein and antibody effectively and produced a clean background.

4.1.10 Effect of non-fat milk solution

5% non-fat milk dissolved in the phosphate-nonidet P-40 buffer pH 8.0 showed satisfactory results as a blocking agent. This was helpful in decreasing the background. Non-fat milk was better than bovine albumin because the latter was hard to dissolve in the buffer. Furthermore non-fat milk is cheaper and could be easily obtained.

4.1.11 Unnecessary stain deposits observed during detection of hybrids formed

Throughout the project the detection of the hybrids was carried out using fluorescence microscopy with the FITC excitation filter available in the department. According to Pinkel et al.(1986a), Pinkel et al. (1988), Lawrence et

al. (1988) this technique was shown to be highly sensitive, convenient, rapid and to produce good spatial resolution. It was decided to chose to concentrate on fluorescent technique because it appeared to be the method of choice for future multiple labelling and detection using different fluorochromes as described by Nederlorf et al. (1989a) and Nederlof et al. (1990).

Following the hybridization visualisation of the hybridized DNA library could be accomplished in a minimum of three steps. First the fluoresceinated labelled avidin was bound to the biotin of the hybridized DNA library. In the second step the biotinylated anti-avidin then bound to the avidin. This binding provided amplification of the fluorescence signal and the third step introduced additional labelled fluorescein-avidin in the hybridized sites. The result of the staining of those hybridized area was observed by fluorescence microscopy. An intense yellow-green fluorescent staining on the chromosome is indicative of a positive signal decoration. When counterstained with propidium iodide the area not hybridized by the biotinylated DNA probe stained orange-red.

Although the fluorescence detection was highly specific it was shown that the fluorescence detection could be affected by temperature, light, presence of excess fluorochromes and antibody, storage life and counterstaining. A common problem during the detection stage were the drying of stain on the slide during incubation. This resulted in a high background and the

formation of milk film on the slides. A similar result was also noticed even if the detection was carried out for 10 minutes at 37°C in a humid chamber as performed by Lichter et al. (1988a).

Two or more amplifications of the detection by FITC did not improve the decorated signal but instead increased the autofluoresence background. This could be due to increased non-specific binding of the fluoresceinated avidin to extrageneous and cellular materials.

4.1.12 Fading of fluorescent signal and microphotography of results

The fading of signals is a major disadvantage. However, with the use of DABCO as an anti-fading agent fading during the microscopic analysis could be effectively reduced but the signal could not be kept permanently as in the alkaline phosphatase detection system. Thus, the results had to be photographed for further analysis and storage.

Unambiguous yellow-green decorated hybridization staining of chromosome 21 could be easily visualized using a standard Zeiss Axioplan with fluorescence filter 487709. All results in this project were photographed using colour slide film. These slides when viewed with the projector were excellent. Unfortunately when printed on photographic paper the spatial resolution deteriorated.

This problem has now been overcome by storage of the signal with computerised image capture or with the latest confocal laser scanning microscope (CLSM) analysis (Bauman et al., 1989; Emmerich et al., 1989; Lichter et al. 1990b; Trask et al., 1991; Alberston et al., 1991; Ward et al. 1991). Signal decoration can be stored in digital form using digital imaging microscopy. Laser scanning microscopy facilitated not only the collection (White at al. 1987; Shotton, 1989) but also enhanced the analysis of hybridization signal to noise ratio (Lichter et al., 1990b; Albertson et al., 1991).

4.1.13 Inherent effect on destained slides

In slides destained ~~slides~~ after G-banding poor hybridization was noticed. Area surrounding the chromosomes tends to be covered with diffuse left-over stain. Destaining of slides in ascending concentrations of ethanol as compared with 100% methanol showed a heavy background. Thus, the banding resolution has to be compensated equally to the destaining process to avoid poor hybridization. According to Lichter et al. (1990b) conventional Giemsa banding before hybridization lowers hybridization efficiency and residual stain tends to interfere with the fluorescence staining. Hence, when carrying out prehybridization staining the complete removal of stain before further hybridization must be ensured. Recently,

Klever et al. (1991) has published a reliable protocol of GTG-banding and removal of the stain prior in situ suppression hybridization and suggested that proper fixation after destaining was decisive for optimal result.

4.1.14 Deterioration of FITC-avidin and biotinylated goat anti-avidin

During the study a very low signal was occasionally experienced although all of the hybridization conditions were unaltered. The low signal intensity was not as result of poor hybridization but at the stage of the detection due to defective fluorescein-avidin (FITC) and biotinylated goat anti-avidin for labelling and amplification. Defective fluorescein-avidin (FITC) and biotinylated goat anti-avidin usually showed heavy autofluorescence deposits covering entire metaphase and was especially deposited on the cell membrane. Both FITC-avidin and anti-avidin aliquots kept at -20°C worked efficiently up to a maximum of 12 weeks. However, according to Sinclair (St. Mary's Hospital, Manchester, personal communication) no deterioration of fluorescein avidin and/or biotinylated goat anti-avidin efficiency was observed when kept at 4°C for up to 18 months.

4.1.15 High background signals

Background signals were observed as intense greenish autofluorescent signal and non-homogeneous red or yellowish coloured stained material spreading all over the slides. These severely affected the signal intensity and caused difficulty in the identification of small discrete fragments or small translocations. Factors which commonly contributed to this condition were probe size which was too long, high concentration of DNA in the hybridization mixture, FITC-avidin particles and drying of slides during hybridization and/or staining. Other factors noticed to produce high or poor background were a high DNA concentration in the hybridization buffer and presence of heavy deposits of disintegrated and non-cellular materials on the slide, salts and non-fat milk particles.

Although addition of more probe increased the signal intensity, in excess it either hybridized to non-target area or caused deposits on the cellular materials. This also happened with increased concentration of carrier DNA or competitor DNA or both.

Salts and non-milk particles deposited throughout the stages also contributed to heavy background signals. The salt deposits were probably due to undissolved dextran sulphate in the hybridization buffer and sodium phosphate in the preparation of the phosphate buffer solution.

Undissolved heavy non-fat milk particles forming a layer of film on the slides could be overcome by using low

fat milk concentrations and by avoiding drying of the slides.

4.1.16 Uneven distribution of probe mixture on the slide

During the study it was found that at times only a portion of the slides showed hybridization. This was believed to be caused by uneven distribution of hybridization mixture and/or air bubbles under the coverslips. Presence of air bubbles around the side of coverslips allowed diffusion of sealant into the hybridization mixture. It will then glue the coverslips not only to the slides but to the cells that will be peeled off together with the coverslip.

4.1.17 Cross-hybridization and variation of colour intensity

Throughout the study exceptionally bright fluorescent staining on the terminal short arm of D group chromosomes was noticed. The signal could not be eliminated despite adjusting the experimental factors. However, through experience this could be easily differentiated from the true painting of the chromosome 21 specific signal. Similar appearance were also reported by Pinkel et al.(1988) and Kuo et al. (1991) and was postulated that it was due to

highly repetitive sequences of the nucleolar organising region (NOR).

In some experiments it was shown that the colour intensity of the decorated chromosomes varied and the cause of this problem could not be identified. Bartsh et al.(1991) suggested that the different shades of colour on the majority of the chromosomes are sometimes not due to the cross-hybridization, but to differences in the intensity of the counterstain.

4.2 CLINICAL APPLICATIONS

The optimised technique of chromosomal in situ suppression hybridization proved to be highly sensitive and overall protocols are simple and the results were reproducible. It was applicable on cultured blood cells and amniocytes, lymphoblastoid cell lines, cultured preparations from chorionic villus samples, bone marrow cells and even on cells from old fixed suspensions and old slides.

In this project chromosomal in situ suppression hybridization using chromosome 21 specific library was successfully applied to twelve patients with known or suspected aberrations of chromosome 21. These included several cases with constitutive translocations, or small additional chromosomes, and several patients with leukaemia who had acquired abnmormalities. The results proved that

the technique could be utilised for rapid diagnosis which was complementary and confirmatory to conventional cytogenetic techniques. Even when the spreads were poor and the chromosomes were contracted the painted chromosomes 21 were clearly visible and the presence of the third copy of 21 instantly recognisable. The speed of identifying the painted chromosomes or chromosomal regions was rapid and could be easily quantitated without bias or ambiguity. The technique was also shown to complement the analysis of prometaphase chromosomes that are often difficult to band and analyse. Similar applications have also been published recently by Kuo et al. (1991) who used the whole chromosome 21, 18 and 13 Bluescribe plasmid libraries to obtain rapid prenatal diagnosis using cultured amniocytes and analysing both metaphase chromosomes and interphase nuclei. Jauch et al. (1990) also applied chromosomal in situ suppression hybridization on amniotic fluid cells to determine the origin of unknown ring chromosome using whole chromosome Y specific library and to analyse long and stretch chromosomes.

Chromosomal in situ suppression is also useful in resolving problems of interpretation of a structural chromosome rearrangement as in cases of t(2:21)(**Case 1**) and t(X;21)(**Case 5**). This aspect was also illustrated by the work of Callen et al. (1990b) who reassessed two cases of apparent deletions of chromosome 16p showing one to be an insertional translocation and the other a reciprocal translocation.

Identifying the origin of small markers chromosomes is often difficult by conventional cytogenetic methods and is another area where chromosomal in situ suppression hybridization has been shown to be of value. Until recently, marker chromosomes were identified by non-isotopic situ hybridization using single copy sequences or alphoid repetitive probes derived from subregions and/or centromeric region of specific chromosomes (Crolla et al., 1988; Callen et al., 1990a; Kolvraa et al. 1991). However, this approach sometimes hampered by low sensitivity (Kolvraa et al. 1990). Such problems could be overcome by with chromosomal in situ suppression hybridization using whole chromosome specific library. With the application of multiple hybridization which is using two or more different libraries simultaneously in one hybridization and multiple detection as described by Nederlof et al. (1989a) and Nederlof et al. (1990) it can also identify both the specific origin and derivative simultaneously.

4.2.1 Interphase cytogenetics

In their original paper describing the chromosomal in situ suppression hybridization method Lichter et al. (1988a) and Pinkel et al. (1988) claimed that rapid detection of aneuploidy would be possible from interphase nuclei. In their hands this 'interphase cytogenetics' proved rapid and reliable on interphase nuclei from blood

lymphocytes (Lichter et al. 1988a; Pinkel et al., 1988; Eastmond and Pinkel, 1989; Emmerich et al. 1989; Lichter et al. 1990c). It has also been successfully applied to leukaemic cells (Dekken and Bauman, 1988, Nederlof et al., 1989b; Speleman et al., 1991), and solid tumours cells (Cremer et al., 1988b; Hopman et al., 1989; Dekken et al., 1990; Anastasi et al., 1991; Arnoldus et al. 1991) and amniotic fluid cells (Jauch et al., 1990).

However, results obtained in this project were disappointing from the point of view that in none of the tissues studied (PHA-stimulated lymphocyte culture, lymphoblastoid cell lines, bone marrow and both cultured and direct preparations from chorionic villus sample) could examination of the interphase nuclei provide a reliable estimation of the number of chromosomes 21 in the cells. The number of domains (spots) observed on the interphase nuclei varied considerably. Several attempts to score the domains after the chromosomal in situ suppression hybridization showed results which were inconsistent. Failure to observe domains in these experiments (which were mainly designed to allow observation of painted metaphase chromosomes) might be due to stage of the cell cycle of interphase nuclei, poor penetration of probe into nucleus in interphase compared with metaphase chromosomes (in the experiment slides were not treated with Proteinase K) or the probe length used might be slightly longer and not suitable for interphase nuclei. Fuscoe et al, (1989) also suggested that this failure was due to the chromosomes arms

in the interphase nuclei were far less condensed then the centromeres, thus the spots become less distinct and making the scoring less accurate. Further investigations of parameters for working with interphase nuclei, is currently being undertaken by another colleague.

Other approaches in interphase cytogenetics are the use of 'cocktail' of several probes specific for a chromosome region, and the use of repetitive probes specific for the chromosome in question. Lichter et al. (1990c) claimed that in interphase nuclei the use of a pool of cosmid probes derived chromosome 21 carried advantages since it did not contain heavy cross-hybridization sites, gave smaller and more discrete signal and finally is specific to the telomeric band of chromosome 21.

This interphase cytogenetics has been shown to be a suitable method of studying chromosome rearrangements that occur in response to physical and chemical mutagen such as ionizing radiation or radon gas (Eastmond and Pinkel, 1990; Cremer et. 1990). Kibbelaar et al. (1991) has also applied the technique using an alphoid repetitive DNA (D8Z2) in the detection of trisomy 8 in 10 patients with haematological disorders and in monitoring the disease. Recently, Kuo et al. (1991) published results showing that they could reliably distinguish between individuals of normal karyotype and those trisomic for chromosome 21 on interphase nuclei.

Fluorescence in situ hybridization of interphase nuclei in suspension could allowed cytometric analysis of

chromosome and microscopic analysis of nuclear organization (Trask et al, 1988). Recently, they have demonstrated that two colour fluorescence hybridization used on interphase nuclei is a powerful method of ordering loci less than 1Mb apart (Trask et al. 1990; Trask, 1991).

4.2.2 Application in haematologic neoplasia and other malignancies

Application of the technique to bone marrow samples offered significant advantages to cytogenetic analysis in haematologic malignancies when used in parallel with routine karyotyping.

In this project, chromosomal in situ suppression hybridization has been applied to confirm t(8:21) translocation on bone marrow culture of an acute myeloid leukaemia patient as well as confirming chromosome 21 markers and numerical aberrations in patients with other types of leukaemia. With a particular chromosome specific library the technique can be as helpful as in situ hybridization using single copy and alphoid repeats probes (Alitalo et al. (1989), 1990; Anastasi et al., 1990; Kolluri et al., 1990; Poddighe et al., 1991) in the classification of haematologic neoplasia, assessing the specific oncogene activation that include translocation, amplification and mutation, and prognostic implication of tumours. Arnoldus et al. (1990) successfully applied the

technique to detect the Philadelphia chromosome in interphase nuclei thus avoiding the necessity for good metaphase spreads. Many malignancies show highly complex chromosomal rearrangements that are impossible to interpret by classical cytogenetic banding methods. In these circumstances chromosomal in situ suppression hybridization can be a very valuable tool to help understand the composition of the 'markers' which might in turn lead to better understanding of the disease (Anastasi, 1991a). The technique has also been extended for assessing the progress of the bone marrow transplantations especially in the detection of residual cells in blood and bone marrow (Anastasi et al., 1991b)

CONCLUSION

Chromosomal in situ suppression (CISS) hybridization or chromosome painting is relatively simple, reliable, rapid and reproducible and shows considerable potential for application as an additional technique in clinical cytogenetics. The methodology could still be improved to attain higher specificity. Almost all the libraries have now been reconstructed in Bluesribe plasmids (Prof. Joe Gray, Lawrence Livermore, personal communication) which will simplify the production of the probes.

Technically, simultaneous double and multiple probes

hybridization using different labelling is now possible (Nederlof et al., 1990; Arnoldus et al., 1990; Tkachuk et al., 1990). This will extend the utility the method in many ways and but most of all in more precise and rapid identification as in prenatal diagnosis particularly in prenatal screening for common trisomies where probes for chromosomes 21, 18, and 13 can be used simultaneously, and in the study of the complex rearrangement found in malignancies (Jauch et al. 1990).

In addition, the technique will become a powerful research tool in human genome research. Using the confocal laser scanning microscope (CLSM) will improve the resolution of the fluorescent signal and capture and store images before fading, two features which overcome the main the disadvantages of using standard fluorescence microscope. This system will allow quantitation and manipulation for the three-dimensional structural (3-D) analysis for further study of cells and chromosome rearrangement (Bauman et al., 1989;Emmerich et al., 1989; Alberston et al., 1990; Lichter et al., 1990b; Alberston et al., 1991; Ward et al.,1991). Combination of compatible probes and storing the high quality images may allow probe ordering on the target chromosomes to be used for chromosome in situ suppression banding. Chromosome in situ suppression hybridization with human whole chromosome specific libraries has also been used in analysis of chromosomal homologies in comparative evolutionary studies of homidians, lower primates and prosimidians (Wieberg et

al., 1990). Recently, this technique has been applied in the study of meiosis (Goldman et al. 1991).

With these improvements and further refinements of 'interphase cytogenetics' techniques it will be possible to overcome the inherent difficulties of poor and insufficient number of metaphase spreads in cytogenetic analysis of malignancies and solid tumours. Hence, this technique will be accepted and become more friendly for non-molecular cytogeneticists and it will become a powerful technique in routine clinical cytogenetics.

BIBLIOGRAPHY

AFFARA, N. A., FLORENTIN,L., MORRISON, N., KWOK, K., MITCHELL, M., COOKE, A., JAMIESON, D., MEREDITH, L., BOYD, E., FERGUSON-SMITH, M.A. (1986). Regional assignment of Y-linked DNA probes by deletion mapping and their homology with X-chromosome and autosomal sequences. **Nucelic Acids Research**, 14:5353-5373.

ALBERTSON, D. G. (1984). Localization of the ribosomal genes in *Caenorhabditis elegans* chromosomes by in situ hybridization using biotin-labeled probes. **EMBO Journal**, 3:1227-1234.

ALBERTSON, D.G., FISHPOOL, R., SHERRINGTON, P., NACHEVA, E. AND MILSTEIN, C. (1988). Sensitive and high resolution in situ hybridization to human chromosome using biotin labelled probes: assignment of the human thymocyte CD1 antigen genes to chromosome 1. **EMBO Journal**, 7:2801-2805.

ALBERTSON, D. G., SHERINGTON, P., AND VAUDIN, M. (1991) Mapping nonisotopically labeled DNA probes to human chromosome bands by confocal microscopy. **Genomics**, 10: 143-150.

ALITALO, T., WILLARD, H. F. DELA CHAPELLE, A. (1989). Determination of the breakpoint of 1;7 translocations in myelodyoplastic syndrome by in situ hybridization using chromosome-specific alpha satellite DNA from human chromosome 1 and 7. **Cytogenetics Cell Genetics**, 50:49-53.

ANASTASI, J., LEBEAU, M. M., VARDIMAN, J.W., WESTBROOK, C.A. (1990). Detection of numerical chromosomal abnormalities in neoplastic hematopoietic cells by in situ hybridization with a chromosome-specific probe. **American Journal of Pathology,** 136:131-139.

ANASTASI, J. (1991a). Interphase cytogenetic analysis in the diagnosis and study of neoplastic disoders. **American Journal of Clinical Pathology**, 95(Suppl.1):S22-S28.

ANASTASI, J., VARDIMAN, J. W., PATEL, M. (1991). Direct correlation of cytogenetic findings with cells morphology by in situ hybridization: analysis of suspicious cells in the bone marrow of two patients completing therapy by hperdiploid acute lymphoid leukemia. **Blood**, 76:251a.

ANGERER, L. M., COX, K. H., STOLER. M. H., ANGERER, R. C. (1987). In situ hybridization with RNA probes: An annotated recipe. In In Situ Hybridization: Applications to neurobiology. Valentino,V.L., Eberwine,J.,H., Barchas,J. D.,(Eds), pp42-70, Oxford University Press. London.

ARNOLDUS, E.P.J., PETERS, A.C.B., BOTS, G.T.A.M., RAAP, A.K., PLOEG, M. (1989) Somatic pairing of chromosome 1 centromeres in interphase nuclei of human cerebellum. **Human Genetics.** 83:231-234.

ARNOLDUS, E. P. J., WIEGANT,J. NOORDEMEER, I. A., WESSELS, J. W., BEVERSTOCK, G. C., GROSSVELD, G. C., PLOEG, M., RAAP, A. K. (1990). Detection of the Philadelphia chromosome in interphase nuclei. **Cytogenetics Cell Genetics,** 54: 108-111.

ARNOLDUS,E. P. J., NOORDERMEER, A. A., PETERS, B. A. C., VOORMOLEN, J. H. C., BOTS, G. T. A. M., RAAP, A. K., PLOEG, M. (1991). Interphase cytogenetics of brain tumors. **Genes, Chromosomes and Cancer**, 3:101-107.

ARRAND, J. (1988). Preparation of nucleis acid probes. In Nucleic acid hybridization: a practical approach. James, B. D., and Higgins, S. J., (Eds), pp17-44, IRL Press, Oxford.

BALDINI, A., AND WARD, D. C. (1991) In situ hybridization banding of human chromosome with Alu-PCR products: A simultaneous karyorype for gene mapping studies. **Genomics**, 9:770-774.

BARTON, P., MALCOLM, S., MURPHY, C., FERGUSON-SMITH, M.A. (1982). Localisation of the humna alpha-globin gene cluster to the short arm of chromosome 16 (16p12-16pter) by in situ hybridization. **Journal of Molecular Biology**, 156:269-278.

BARTSCH, O., AND SCHWINGER, E. (1991). A simplified protocol for fluorescence in situ hybridization with repetitive DNA probes and its use in clinical cytogenetics. **Clinical Genetics**, 40:47-56.

BAUMAN, J. G. J.,WIEGENT J. BORST P., VAN DUIJN P. (1980). A new method for fluorescence microscopical localization of specific DNA. **Experimental Cell Research**, 138:485-490.

BAUMAN, J. G. J., WIEGANT J., VAN DUIJN, P. (1981). Cytochemical hybridization with fluorochrome-labelled RNA. III. Increased sensitivity by the use of anti-fluorescein antibodies. **Histochemistry**, 73:181-193.

BAUMAN, J. G. J., PLOEG, M., and VAN DUIJIN, P. (1984). Fluorescent hybridocytochemical procedures: DNA.RNA hybridization in situ. In Investigative microtechniques in medicine and microbiology. Chayen, J. and Bitensky, G. (Eds), pp41-48. Marcel Dekker, New York.

BAUMAN, J. G..J. (1985). Fluorescence microscopical hydrochemistry. **Acta Histochemistry**, XXX1S:9-18.

BAUMAN, J. G. J., BAYER, J. A., DEKKEN, H. (1990). Fluorescent in situ-hybridization to detect cellular RNA by flow cytometry and confocal microscopy. **Journal of Microscopy,** 157:73-81.

BHATT, B., BURNS, J., FLANERRY, D., MCGEE, J. O'D. (1988). Direct visualization of single copy genes on banded chromosomes by nonisotopic in situ hybridization. **Nucleic Acid Research,** 16:3951- 3961.

BOULNOIS G.J. (1987). Gene Cloning and Analysis: A Laboratory Guide. Blackwell Scientific Publication, London.

BOYD, E., THERIAULT, A., GODDARD, S. P., KALAITSIDAKI, M., SPATHAWS, D. H., CONNOR, J. M. (1989). Chromosomal assignment of a glutamic acid transfer RNA gene to 1p36. **Human Genetics**, 81:153-156.

BRADBURY. S. (1989). Photomicrography. In In situ hybridization; principle and practice. Polak, J. M. and McGee, J. O'D. (Eds), pp217-237, Oxford University Press, New York.

BRIGATI, D. J., MYERSON, D., LEARY, J. J., SPALHOLZ, B., TRAVIS, S., FONG, C. K. Y., HSIUNG, G. D., and WARD, D. C.(1982). Detection of viral genomes in cultured cells and paraffin-embedded tissues sections using biotin-labeled hybridization probes. **Virology**, 126:32-50.

BRITTEN, R. J., DAVIDSON, E. H. (1988). Hybridization strategy. In Nucleic acid hybridization: a practical approach. James, B. D. and Higgins, S. J., (Eds), pp3-14, IRL Press, Oxford.

BURNS, J., CHAN, V. T. W., JONASSON, J. H., FLEMING, K. A., TAYLOR, S., and MCGEE, J. O'D. (1985). Sensitive system for visualizing biotinylated DNA probes hybridized in situ; rapid sex determination of intact cells. Journal of Clinical Pathology, 38:1085-92.

CALLEN, D. F., RINGERBERGS, M. L., FOWLER, J. C. S., FREEMANTLE, C. J., HAAN, E. A. (1990a). Small marker chromosomes in man: origin from pericentric heterochromatin of chromosomes 1, 9, 16. **Journal of Medical Genetics**, 27:155-159.

CALLEN, D.F., BAKER, E., EYRE, H. J., CHERNOS, T .E., BELL, J. A., SUTHERLAND, G. R. (1990b) Reassessment of two apparent deletions of chromosome 16p to an ins(11;16) and a t(1;16) by chromosome painting. **Annales Genetique,** 33:219-221.

CHAN, V. T. W., AND MCGEE, J. O'D (1989). Non-radioactive probes: preparation, characterization and detection. In In situ hybridization: principles and practice. Polak, J. M, and Mcgee, J. O'D.(eds), pp59-70. Oxford University Press, New York.

CROLLA, J. A.,AND LLERENA, J. C. (1988). A mosaic 45,X/46,X,r(?) karyotype investigated with X and Y centromere-specific probes using a non-autoradiographic in situ hybridization technique. **Human Genetics**, 81:81-84.

CHERIF, D., BERNARD, O., BERGEN, R. (1989). Detection of single copy-genes by nonisotopic in situ hybridization on human chromosomes. **Human Genetics**, 8:358-362.

CHOLLET, A., KAWASHIMA, E. M. (1985). Biotin labelled synthetic oligodeoxyribonucleotides: synthesis and uses as hybridization probes. **Nucleic Acid Research**, 15:1529-1541.

COGHLAN, J. P., ALDRED, P., HARALAMBRIDIS, J., NIALL, H. D., PENSCHOW, J. D.,AND TREGEAR, G. W. (1985). Hybridization histochemistry. **Analytical Biochemistry,** 149:1-28.

COX, K. H., DELEON, D. V., ANGERER, L. M., ANGERER, R. C. (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. **Developmental Biology**, 101:485-503.

CREMER, A. F. M., JANSEN IN DE WAL, N., WIEGENT, J., DIRKS, R. W., WEISBEEK, P., PLOEG, M., LANDEGENT, J. E. (1987). Non-radioactive in situ hybridization. A comparison of several immunocytochemical detection systems using reflection-contrast microscopy and electron microscopy. **Histochemistry**, 86:609-615.

CREMER, T., LICHTER, P., BORDEN, J., WARD, D. C., and MANUELIDIS, L. (1988a). Detection of chromosome aberrations in metaphase and interphase tumour cells by in situ hybridization using chromosome-specific library probes. **Human Genetics**, 80:235-46.

CREMER, T., TESSIN, D., HOPMAN, A. H. N., and MANUELIDIS, L. (1988b). Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumour cells by in situ hybridization with chemically modified DNA probes. **Experimental Cell Research**, 76:199-206.

CREMER, T., POPP, S., EMMERICH, P., LICHTER,P., and CREMER, C.(1990). Rapid metaphase and interphase detcion of radiation-induced chromosome aberratiosn in human lymphocyte by chromosomal suppression in situ hybridization. (1990), **Cytometry**, 11:110-118.

DEKKEN, H., PINKEL, D., MULLIKAN, J., AND GRAY, J. (1988). Enzymatic production of single-stranded DNA as a target of fluorescence in situ hybridization. **Chromosoma**, 97:1-5.

DEKKEN, H., AND BAUMAN, J. G. J. (1988). A new application of in situ hybridization: detection of numerical and structural chromosome aberrations with a combination centromeric-telomeric DNA probe. **Cytogenetics Cell Genetics**, 480:188-189.

DEKKEN, H., PIZZOLA, J. G. REUTER, V. E, and MELAMED, M. R. (1990). Cytogenetic analysis of human solid tumours by in situ hybridization with a set of 12 chromosome-specific DNA probes. **Cytogenetics Cell Genetics**, 54:103-107.

DEVILEE, P., KIEVITS, T., WAYE, J. S., PEARSON, P. L. AND WILLIARD, H. F. (1988). Chromosome-specific alpha satellite DNA: Isolation and mapping of a polymorphic alphoid repeat from human chromosome 10. **Genomics**, 3:1-7.

DEVILEE, P., THIERRY,, R. F., KIEVITS, T., KOLLURI, R., HOPMAN, A. H. N., WILLARD, H. F., PEARSON, P. L., and CORNILISSE, C. J. (1988). Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumours using chromosome-specific repetitive DNA probes. **Cancer Research**, 48:5825-5830.

DONLON, T. A. (1986). Practical approaches to in situ hybridization. **Karyogram**, 12:3-10.

DUMANSKI, J. P., RUTTLEDGE, M., AND DATTA, S. (1988). Rapid minipreparations of bacteriophage lambda DNA. **Nucleic Acid Research**. 16:9044.

EASTMOND, D. A., AND PINKEL, D. (1990) Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence in situ hybridization with chromosomes-specific DNA probes. **Mutation Research**, 234:301-318.

EMMERICH, P., LOOS, P., JAUCH, A., HOPMAN, A. H. N., WIEGANT, J., HIGGINS, M., WHITE, B. N., VAN DER PLOEG, M., CREMER, C., AND CREMER, T. (1989b). Double in situ hybridization in combination with digital image analysis: A new approach to study interphase chromosome topography. **Experimental Cell Research**, 181:126-40.

FAN, Y. S., DAVIS, L. M., and SHOWS, T. B. (1990). Mapping small DNA sequences by fluorescence in situ hybridization directly on banded metaphase chromosome. **Proceeding of the National Academy of Sciences (USA)**, 87: 6223-6227.

FEINBERG, P., AND VOGELSTEIN, B. (1984). A technique for radiolabelling DNA restriction enzyme fragments to high specific activity. **Analytical Biochemistry**, 137: 266-267.

FORSTER, A. C., MCINNES, J. L., D. C., AND SYMONS, R. H. (1985). Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. **Nucleic Acids Research**, 13:745-61.

FUSCOE, J.C., COLLINS, C.C., PINKRL, D., GRAY, J.W. (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 hybridization. **Genomics**,5:100-109.

GALL, J. G. AND PARDUE, M. L. (1969). Formation and detection of RNA-DNA hybrids molecules in cytological preparations. **Proceeding of the National Academy of Sciences (USA)**, 63, 378-383.

GALL, J. G. AND PARDUE, M. L. (1971). Nucleic acid hybridization in cytological preparations. In Methods in Enzymology, Vol. 21, Grossman, L. and Moldave, K (Eds),pp470-480. Academic Press, New York.

GARSON, J. A., VAN DER BERGHE, J. A., KEMSHEAD, J. A. (1987). Novel non-isotopic in situ hybridization technique detects small (1 Kb) unique sequences of N-myc and beta-NGF genes. **Nucleic Acid Ressearch**, 15:4761-4770.

GERHARD, D. S., KAWASAKI, E. S., BANCROFT, F.C., AND SZABO, P. (1981). Localization of a unique gene by direct hybridization in situ. **Proceeding of the National Academy of Science (USA)**, 78:3755-3759.

GILLESPIE, D. AND SPIELGMAN, S. (1965). A quantitative assay for DNA-RNA hybrids with immobilised on a membrane. **Journal of Molecular Biology**, 12:829-842.

GOLDMAN, A. S. H. AND HULTEN, M. A. (1991). Meiotic studies by fluoresence in situ hybridzation. In **Clinical Cytogenetics Bulletin** Vol. 2 No. 4. 1991.

GONZALEZ, A. AND GOMEZ-MARQUEZ, J. (1990). Purification of bacteriophage DNA by gel filtration chromoatography. **Genetics Analysis Technical Application**, 7:2-4.

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

HENNIG, W. (1973). Molecular hybridization of DNA and RNA in situ. **International Review of Cytology**, 36:1-44.

HERRINGTON, C. S., BURNS, J., GRAHAM, A. K., BHATT, B., and MCGEE, J. O'D. (1989a). Interphase cytogenetics using biotin and digoxigenin-labelled probes I: relative sensitivity of both reporter molecules for HPV 16 detection in CaSki cells. **Journal of Clinical Pathology,** 42:592-600.

HERRINGTON C. S., BURNS, J., BHATT, B., GRAHAM, A. K., and MCGEE, J. O'D. (1989b). Interphase cytogenetics using biotin and digoxigenin-labelled probes II: simultaneous differential detection of human and HPV nucleic acids in individual nuclei. **Journal Clinical Pathology,** 42:601-606.

HOELTKE, H. J., SEIBL, R., BURG, J., MUEHLEGGER, K., KESSLER, C. (1990). Non-radioactive labelling and detection of nucleic acids: ll. Optimization of the digoxigenin. **Biology Chemistry Hoppe-Seyler**, 371:929-938.

HOFLER, H. (1987). What's new in "In Situ Hybridization". **Pathology Research Practice**, 182:424-430.

HOFLER, H. (1989). Principles of in situ hybridization. **In In situ hybridization; principles and practice.** Polak, J. M. and McGee, J. O'D (eds) pp15-30, Oxford University Press, New York.

HOPMAN, A. H. N., WIEGANT, J., TESSER, G. I., DUIJN, P. (1986a). A non-radioactive in situ hybridization method based on mercurated nucleic acid probes and sulfhydryl-hapten ligands. **Nucleic Acids Research.** 14:6471-6488.

HOPMAN, A. H. N., WIEGANT, J., and VAN DUIJN, P. (1986b). A new hybridocytochemical method based on mercurated nucleic acid probes and sulfhydryl-hapten ligands. I. Stability of the mercury-sulfhydryl bond and influence of the ligand structure on the immunochemical detection of the hapten. **Histochemistry**, 84:169-178.

HOPMAN, A. H. N., WIEGANT, J., DUIJN, P. (1987). Mercurated nucleic acid probes, a new principle for non-radioactive in situ hybridization. **Experimental Cell Research**, 169:357-368.

HOPMAN, A. H. N., WIEGANT, J., RAAP, A. K., LANDEGENT, J. E., PLOEG, M., DUIJN, P. (1986c). Bi-color detection of two target DNAs by non-radioactive in situ hybridization. **Histochemistry**, 85:1-4.

HOPMAN, A. H. N., RAMAEKERS, F. C. S., RAAP, A. K., BECK, J. L. M., , P., PLOEG, M., AND VOOIJS, G. P. (1988). In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumours. **Histochemistry**, 89:307-16.

HOPMAN, A. H. N., RAAP, A. K., LANDEGENT, J. E., WIEGANT, J., BOERMAN, R. H., AND PLOEG, M. (1988d). Non-radioactive in situ hybridization. In Molecular neuroanatomy. Leeuwen, F. W., Buijs, R.M., Pool, C. W and Pach, O.(Eds), pp43-68. Elsevier, Amsterdam.

HOPMAN, A. H. N., PODDIGHE, P., SMEETS, W., MOESKER, O., BECK, J., VOOIJS, G. P., and RAMAEKERS, F. C. S. (1989). Detection of chromosome #1 and #18 aberrations in bladder cancer by in situ hybridization. A comparison with flow cytometric data. **American Journal of Pathology**, 135:1105-1117.

HSU, T. C. (1979). In situ hybridization: A marriage between molecular biology and cytology. In Human and Mammalian Cytogenetics pp108-115, Springer-Verlag, New York.

HULTEN, M. A., GOULD, C. P., GOLDMAN, A. S. H., WALTERS, J. J. (1991). Chromosome in situ suppression hybridization in clinical cytogenetics. **Journal of Medical Genetics**, 28:577-582.

ISA, M. N., BOYD, E., MORRISON, N., HARRAP, S., CLAUSER, E., AND CONNOR, J. M. (1990). Assignment of the human angiotensinogen gene to chromosome 1q42-43 by nonisotopic in situ hybridization. **Genomics**, 8:598-600.

ISCN (1978): An International System for Human Cytogenetics Nomenclature :Birth Defects. Original Articles Series, Vol 14, No 8. New York, The National Foundation.

ISCN (1981): An International System for Human Cytogenetic Nomenclature-High Resolution Banding: Birth Defects. Original Article Series, Vol 17. New York, March of Dimes Birth Defects Foundation.

ISCN (1985): An International System for Human Cytogenetics Nomenclature: Birth Defects. Original Articles Series, Vol 21, No 1. New York, The National Foundation, 1985.

JAUCH, A., DAUMER, C., LICHTER, P., MURKEN, J., SCHROEDER-DURTH, T., CREMER, T. (1990). Chromosomal in situ suppression hybridization of human gonosomes and autosomes and its use in clinical cytogenetics. **Human Genetics,** 85:145-150.

JOHN, H.A, BIRNSTIEL, M. L. AND JONES, K. W. (1969). RNA-DNA hybrids at the Cytological Level. **Nature**, 223, 582-587.

JOHNSON, G. D. AND ARAIJO, G. M. C. N. (1981). A simple method of reducing the fading of immunofluorescence during microscopy. **Journal of Immumunological Methods**, 43:349-350.

JOHNSON, G. D., DAVIDSON, R. S.,MACNAMEE, K. C., RUSSELLL, G., GOODWIN, D., and HOLBROW, E. J. (1982) Fading of immunofluorescence during microscopy: a study of the phenemenon and its remedy. **Journal Immunological Methods**, 55:231-242.

JONASSON, J. A. (1986). Analysis and interpretation of human chromosome preparation. In human cytogenetics: a practical approach. Rooney, D. E. and Czepulkowski, B. H. (Eds),pp85-134. IRL Press, Oxford.

JONES, K. W. (1974). The method of in situ hybridization. In New Techniques in Biophysics and Cells Biology. Pain R. H and Smith, B. J. (Eds), Vol. 1, pp.29-66. John Wiley and Sons, London.

Kalaitsidaki, M (1990), In situ hybridization for human and comparative gene mapping. **PH.D Thesis** submitted to University of Glasgow, Scotland.

KHALFAN, H., ABUKNESHA, R., RAND-WEAVER, R., PRICE, R. G., ROBINSON, D. (1986). Aminomethyl coumarin acetic acid: a new fluorescent labelling agent for proteins. **Journal of Histochemistry**, 18:497-499.

KIBBELAAR, R. E., KAMP, H., DREEF, E. J. WESSELS, J. W., BEVERSTOCK, G. C., RAAP, A. K., FIBBE, W. E., OTTOLANDER, G. T., KLUIN, P., M. (1991). Detection of trisomy 8 in haematological disorders by in situ hybridization. **Cancer Genetics Cytogentics,** 56:136-139.

KIEVITS, T., , P., WIEGNAT, J., WAPENAAR, M. C.,CORNELISSE, C., OMMEN, G. J. B., and PEARSON, P. L.(1990). Direct non-radioactive in situ hybridization of omatic cell hybrids DNA to human lymphocytes chromosomes. **Cytometry**, 11:105-109.

KLAVER, M., GROND-GINSBACH, C., SCHERTHAN, H., SCHROEDER-KURTH, T. M. (1991) Chromosomal in situ suppression hybridization after Giemsa banding. **Human Genetics**, 86:484-486.

KOLLURI, R. V., MANUELIDIS, L., CREMER, T., SAIT, S, GEZER, S, RAZA, A. (1990). Detection of monosomy 7 in interphase cells of patients with myeloid disorders. **American Journal of Hematology**, 33:117-122.

KOLVRAA, S., KOCH, J., GREGERSEN, N., JENSEN, P. K. A., JORGENSEN, K., PETERSEN, B., RASMUSSEN, K. and BOLUND, L. (1991). Application of fluorescence in situ hybridization technique in clinical genetics: use of two alphoid repeat probes detecting the centromeres of chromosomes 13and 21 or chromosomes 14 and 22 respectively. **Clinical Genetics**, 39:278-286.

KOZMA, R. AND ADOLFI, M. (1987). In situ hybridization and the detection of biotinylated DNA probes. **Molecular Biology Medicine**, 4:357-364.

KUO, W. L., HISAKO, T., SEGRAVES, R., PINKEL, D., GOLBUS, M., S. (1991). Detection of aneuploidy involving chromosomes 13, 18, 21, by fluorescence in situ hybridization (FISH) to interphase and metaphase amniocytes. **American Journal of Human Genetics**, 49:112-119.

LANDERGENT J. E., JANSEN IN DE WAL, N., BAAN, R. A., HOEIJMAKERS, J. H. J., PLOEG, M. (1984). 2-etylaminofluorene-modified probes for the indirect hyridocytochemical detection of specific nucleic acid equences. **Experimental Cell Reseaerch**, 153:61-72.

LANDERGENT J. E., JANSEN in de WAL, N., PLOEM, J. S., and PLOEG, M., (1985). Sensitive detection of hybridocytochemical results by means of reflection-contrast microscopy. **Journal Histochemistry Cytochemistry**, 33:1241-1246.

LANDERGENT, J. E., JANSEN IN DE WAL, N., OMMEN, G. J. B., BAAS, F., DE VIJLDER J. J. M., DUIJN, P., PLOEG, M., (1985b). Chromosomal localization of a unique gene by non-autoradiographic in situ hybridization. **Nature**, 317: 175-177.

LANDERGENT, J. E., JANSEN IN DE WAL, N., FISSER-GROEN, Y.M., BAKKER, E., PLOEG, M., PEARSON, P.L. (1986). Fine mapping of the Huntington disease linked D4S10 locus by non-radioactive in situ hybridization. **Human Genetics**, 73:353-357.

LANDERGENT, J. E., JANSEN IN DE WAL, N., DIRKS, R. W., BAAS, F., PLOEG, M. (1987). Use of whole cosmid cloned genomics sequences for chromosomal localization by non-radioactive in situ hybridization. **Human Genetics**, 77:366-30.

LANGER, P. R., WALDROP, A. A., WARD, D. C. (1981). Enzymatic synthesis of biotin-labeled polyynucleotides: novel nucleic acid affinity probes. **Proceeding of the National Academy of Sciences (USA)**, 78:6633-6637.

LARSSON, L. I., and HOUGAARD. (1990). Optimization of non-radioactive in situ hybridization: Image analysis of varying pretreatment, hybridization and probe labelling conditions. **Histochemistry**, 93:347-354.

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

LEARY, J. L., BRIGATI, D. J., WARD, D. C., (1983). Rapid and sensitive colorimetric method for visualizing biotin-labelled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. **Proceeding of the National Academy of Sciences (USA)** 80:4045-4049.

LENGAUER, C., RIETHMAN, H., AND CREMER, T. (1990) Painting of human chromosomes with probes generated from hybrid cell lines by PCR with Alu and L1 primers. **Human Genetics**, 86:1-6

LICHTER, P., CREMER, T., BORDEN, J., MANUELIDIS, L., AND WARD, D. C. (1988a). Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. **Human Genetics**, 80:223-234.

LICHTER, P., CREMER, T., CHANG TANG, C. J., WATKINS, P. C., MANUELIDIS, L., and WARD, D. C. (1988b). Rapid detection of human chromosome 21 aberrations by in situ hybridization. **Proceeding of the National Academy of Sciences (USA)**, 85:9664-9668.

LICHTER, O., LEDBETTER, S.A., LEDBETTER, D.H., WARD, D.C. (1990a). Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosome in hybrid cell lines. **Proceeding of the National Academy of Sciences (USA)**, 87:6634-6638.

LICHTER, P., TANG, C, C., CALL, K., HERMANSON., G., EVANS, G, A., HOUSMAN, D., WARD, D. C. (1990b) High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. **Science**, 247:64-69.

LICHTER, P., JAUCH, A., CREMER, T., and WARD, D. C. (1990c). Detection of Down's syndrome by in situ hybridization with chromosome 21 specific DNA probes. **In Proceedings of the Sixth Annual National Down's Syndrome Society Symposium.** Patterson, D. and Epstein, C. J.(Eds), pp69-78, New York.

LO, Y. M. D., MEHAL, W. Z. and FLEMING, K. A. (1988). Rapid isolation of vectro-free biotinylated probes using the polymerase chain reaction. **Nucleic Acids Research**, 16:8719.

LOMHOLT, B., SORENSEN, P. D., SIMONSEN, H., FREDERIKSEN, S. (1989). Optimization of in situ hybridization to human metaphase chromosomes. **Analytical Biochemistry**, 182:25-31.

MALCOLM, S., BARTON, P., MURPHY, C., and FERGUSON-SMITH, M.A. (1981). Chromosomal localisation of a single copy gene by in situ hybridization of human beta globin genes on the short arm of chromosome 11. **Annals Human Genetics**, 45:135-141.

MALCOLM S. CONELL, J. K., YOUNG, B.D. (1986). Specialised techniques in research and diagnostic clinical cytogeneticss. **In Human cytogenetics: a practical approach**. Rooney, D. E. and Czepulkowski, B. H. (Eds), pp197-226. IRL Press, Oxford.

MANIATIS, T., FRITITSCH, E. F., SAMBROOK, J., (1982). Molecular cloning; a laboratory manual. Cold spring Harbor Laboratory.

MANUELIDIS, L. LANGER-SAFER, P. R. and WARD, D. C. (1982). High-resolution mapping of satellite DNA using biotin-labelled DNA probes. **Journal of Cell Biology**, 95: 619-625.

MARIMOTO, H., MONDEN., SHIMANO, T., HIGASHIYAMA, M., TOMITA, N., MUROTANI, M., MATSUURA, N., OKUDA, H., and MORI, T. (1987). Use of sulphonated probe for in situ hybridization detection of amylase mRNA in formalin-fixed paraffin sections of human pancreas and submaxillary glang. **Laboratory Investigation**, 57:737-741.

MASUMOTO, H., SUGIMOTO, K., AND OKAZAKI, T. (1989). Alphoid satellite DNA is tightly associated with centromere antigens in human chromosomes throughout the cell cycle. **Experimental Cell Research**, 181:181-196.

MCGEE, J. O'D., BURNS, J., AND FLEMING, K. A. (1987). New molecular techniques in pathological diagnosis visualization of genes and mRNA in human tissue. In Investigational techniques in oncology, Bleehen, N. M. (Ed),pp21-34. Springer-Verlag, London.

MORRIS, R. G., ARENDS, M. J. BISHOP, P. E., SIZER, K. DUVALL, E., BIRD, C. C. (1990) Sensivity of digoxigenin and biotin labelled probes for detection of human papilloma virus by in situ hybridization. **Journal of Clinical Pathology**, 43:800-805.

MUKHERJEE, A. B., and PARSA,N. Z. (1990). Detection of sex chromosomal constitution and chromosomal origin of drumsticks, drumstick-like structures, and other nuclear bodies ion human blood cells at interphaase by fluorescence in situ hybridization. **Chromosoma**, 99:432-435.

MURASUGI, A., and WALLACE, R.B.(1984). Biotin-labeled oligonucleotides: Enzymatic synthesis and use as hybridization probes, **DNA**, 3:269-277.

NARAYANSWAMI, S., and HAMKALO, B. A. (1990). High-resolution mapping of Xenopus laevis and ribosomal RNA genes by electron microscopy in situ hybridization. **Cytometry**, 11:144-152.

NAYLOR, S. L., MCGILL, J. R., and ZABEL, B. U. (1987). In situ hybridization of metaphase and prometaphase chromosomes. In Methods Enzymology, 151:279-292.

NEDERLOF, P. M., ROBINSON, D., ABUKNESHA, R., WIEGANT, J., HOPMAN, A. H. N., TANKE, H. J. and RAAP, A. K. (1989a). Three-color-fluorescence in situ hybridization for the simultaneous detection of multiple nucleic acid sequences. **Cytometry**, 10:20-8.

NEDERLOF, P. M., VAN DER FLIER, S., RAAP, A. K., TANKE, H. J., PLOEG, M., KORNIPS, F., and GERAEDTS, J. P. M. (1989b). Detection of chromosome aberrations in interphase tumour nuclei by non-radioactive in situ hybridization. **Cancer Genetics Cytogenetics**, 42:87-98

NEDERLOF, P., FLIER, S., WIEGANT, J., RAAP, A. K., TANKE, H. I., PLOEM, J. S., PLOEG, M. (1990). Multiple-fluorescence in situ hybridization. **Cytometry**, 11:126-131.

NIEDOBITEK, G., FINN, T., HERBST, H., BORNHOFT, G., GEDES, J., and STEIN, H. (1988). Detection of viral DNA by in situ hybridization using bromodeoxyuridine-labeled DNA probe. **American Journal Pathology**, 131:1-4.

PARDUE, M. L., GALL, J. G., (1969). Molecular hybridization of radioactive DNA to the DNA of cytological preparations. **Proceeding of the National Academy of Science**, 64:600-604.

PEQUIGNOT, E.V., JEANPIERRE, M., DUTRILLAUX, A. M., SEUREAU, M. G., MULERIS, M., DUTRILLAUX, B.(1989). Detection of 1q polysomy in interphase nuclei of human solid tumours with a biotinylated probe. **Human Genetics,** 81:311-314.

PINKEL, D., GRAY, J. W., TRASK, B., VAN DEN ENGH, G., FUSCOE, J., VAN DEKKEN, H. (1986a). Cytogenetic analysis by in situ hybridization with fluorescently labeled nucleic acid probes. **Cold Spring Harbor Symposium Quanterly Biology**, 51:151-157.

PINKEL, D., STRAUME, T. and GRAY, J. (1986b). Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. **Proceeding of the National Academy of Sciences (USA)**, 83:2934-2938.

PINKEL, D., LANDERGENT, 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 chromosomes 4. **Proceeding of the National Academy of Sciences (USA)**, 85:9138-9142.

PLOEG, M. AND RAAP, A. K. (1988). In situ hybridization: An overview. In New frontiers in cytology. Goerttler, K., Feichter, G. E., Witte. (Eds), pp13-21. Springer-Verlag, New York.

PLOEG, M., RAAP, A, K., TANKE, H. (1990). Hybridocytometry (In Situ Hybridization). Practical Boerhaave Course, Leiden.

PODDIGHE, P. J., MOESKER, O., SMEETS, D., AWWAD, B. H., RAMAEKERS,F. C. S., and HOPMAN, H. N. (1991). Interphase cytogenetics of haematological cancers: Comparison of calssical karyotyping and in situ hybridization using a panel of eleven chromosome specific DNA probes. **Cancer Research**, 51:1956-1967.

PRICE, P. M. AND HIRSCHORN. (1975). In situ hybridization for gene mapping. **Cytogenetics and Cell Genetics**, 14:395-401.

PROOIJEN-KNEGT, A. C., HOEK, J. F. M., BAUMAN, J. G. J., DUIJN, P., WOOL, I. G., AND PLOEG, M. (1982). In situ hybridization of DNA sequences in human metaphase chromosomes visualized by an indirect fluorescent immunocytochemical procedures. **Experimental Cell Research**, 141:397-407.

RAAP, A. K., MARIJNEN, J. G. J., VROLIJK, and VAN DER PLOEG, M. (1986). Denaturation, renaturation, and loss of DNA during in situ hybridization procedures. **Cytometry**, 7:235-242.

RAAP, A. K., HOPMAN, A. H. N., and VAN DER PLOEG, M. (1989). Hapten labeling of nucleic acid probe for DNA in situ hybridization. In Techniques in immunocytochemistry. Bullock, G. R (Ed), Vol. 4. Springer-Verlag. New York.

RIGBY, P. W. J., DIEKMAN, M., RHODES, C., and BERG, P., (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase 1. **Journal of Molecular Biology**, 113:237-241.

SAIKI, R. K., GELFLAND, D.H., STOFFEL. S., SCHARF, S.J., HIGUCHI,R., HORN,G.T., MULLIS, K.B., and ERLICH, H. A (1988). Primer-directed enzymatic amplification of DNA witha thermostable DNA polymerase. **Science**, 239,487-91.

SAMBROOK, J., FRITSH, E. F., MANIATIS, T.(1989) Molecular cloning: laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor,New York.

SANDBERG, A. A. (1986). The chromosomes in human leukemia. **Seminar Hematology.** 23:201-217.

SHOTTON, D. M. (1989). Confocal scanning microsopy and its applications for biological specimens. **Journal of Cell Science**, 94:175-206.

SHROYER, K. P., and NAKANE, P. K. J. (1983). Use of DNP-labeled cDNA for in situ hybridization. **Journal Cell Biology**, 97:377a.

SINGER, M. (1982). Highly repeated sequences in mammalian genomes. **International Review of Cytology**, 76:67-112.

SINGER, R. H., LAWRENCE, J. B., LANGEVIN, G. L., RASHTCHIAN, R. N., VILLNAVE, C. A., CREMER, T., TESIN, D., MANUELIDIS, L., AND WARD, D. C. (1987). Double labelling in situ hybridization using non-isotopic and isotopic detection. **Acta Histochemistry Cytochemistry,** 20:589-99.

SPELEMAN, F., MANGELSCHOTS, K., VERCRUYSSEN, M., DAL CIN, P., AVENTIN, A., OFFNER, F., LAUREYS, G., VAN DEN BERGHE, H., LEROY, J. (1991). Analysis of whole-arm translocations in malignant blood cells by non-isotopic in situ hybridization. **Cytogenetics Cell Genetics**, 56: 14-17.

SPIEGELMAN, G. B., HABER, J. E., HALVORSON, H. O., (1973). Kinetics of ribonucleic acid-deoxyribonucleic acid membrane filter hybridization. **Biochemistry**, 12:1234-1242.

STEFFENSEN, D. M., CHU, E. H. Y., SPEERT, D. P., WALL, P. M., MEILINGER, K., and KELCH, R. P. (1977) (Partial trisomy of the long arm of human chromosome 1 as demonstrated by in situ hybridization. **Human Genetics**, 36:25-33.

SZABO, P., YU, L. AND PRENSKY, W (1977). Kinetic aspects of in situ hybridization in relation to the problem of gene localisation. In Human cytogenetics, Sparkes, R. S., Comings, D. E., and Fox, C.F. (eds), Vol. 7, pp283-294. Academic Press, New York.

SZABO, P. AND WARD, D. C. (1982). What's new with hybridization in situ? **Trends in Biochemical Science**, 7: 425-427.

TANEJA, K., and SINGER, R. H. (1987). Use of oligonucleotide probes for quantitative in situ hybridization to actin mRNA. **Analytical Biochemistry**, 166:389-398.

TARLETON, D. C. AND SCHWARZ (1991). Using the polymerase chain reaction to maintain DNA probe inventories in clinical and diagnostic laboratories. **Clinical Genetics**, 39:121-124.

TCHEN, P., FUCHS, R. P. P., SAGE, E., LENG, M. (1984) Chemically modified nucleic acid as immunodetectable probes in hybridization experiments. **Proceeding of the National Academy of Science (USA)**, 81:3466-70.

TECOTT, L. H., EBERWINE, J. H., BARCHAAS J. D., and VALENTINO, K. L. (1987). Methodological consideration in the utilization of in situ hybridization. **In In situ hybridization: application to neurobiology.** Valentino,K. L., Eberwine,J. H., Barchas, J. D. (Eds),pp3-24. Oxford University Press, New York.

THERMAN E. (1986). Human Chromosomes: Structure, Behavior, Effects. 2nd. Ed. Springer-Verlag. New York.

TKACHUK, D., WESTBROOK, C. ANDREEFF, M., DONLON, T. A., CLEARY, M. L., SURYANAARAYAN, K., HOMGE, M. REDNER, A., GRAY, J., PINKEL, D. (1990). Detection of bcr-abl fusion in chronic myelogeneous leukamia by in situ hybridization. **Science**, 250:559-562.

TRASK, B., VAN DEN ENGH, G., LANDEGENT, J., JANSEN IN DE WAL, N., PLOEG, M. (1985). Detection of DNA sequences in nuclei in suspension by in situ hybridization and dual beam flow cytometry. **Science**, 230:1401-1403.

TRASK, B., VAN DEN ENGH, G., PINKEL, D., MULLIKIN, J., WALDMAN, F., DEKKEN, H., GRAY, J. (1988). Fluorescence in situ hybridization to interphase cell nuclei in suspension allows cytometric analysis of chromosome content and microscopic analysis of nuclear organization. **Human Genetics**, 78:251-259.

TRASK, B. AND PINKEL, D. (1990). Fluorescence in situ hybridization with DNA probes. **Methods in Cell Biology**, 33:383-400.

TRASK, B. (1991). Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. **Trends in Genetics**, 7:149-154.

VERMA, R.S., AND DOSIK, H. (1983). Chromosome banding: Techniques and Prospects. **Laboratory Management**, (March). 42-47.

VERMA, R. S., AND BABU, A. (1989). Human chromosomes: manual of basic technique. Pergamon Press, New York.

VISCIDI, R. P., CONNELLY, C. J., YOKLKEN, R. H., (1986). Novel chemical method for the preparation of nucleic acids for non-isotopic hybridization. **Journal Clinical Microbiology**, 23:311-317.

WARD, D. C., LICHTER, P., BOYLE, A., BALDINI, A., MENNINGER, J., BALLARD, S., W. (1990). Gene mapping by fluorescent in situ hybridization and digital imaging microscopy. In Etiology of human disease at the DNA level. Lindsten, J and Pettersson, U.(Eds), pp291-305. Nobel Symposium 80, Raven Press, New York.

WATT, J. L. AND STEPHEN, G. S. (1986). Lymphocytes culture for chromosome analysis. In Human cytogenetics: a practical approach. Rooney, D. E. and Czepulkowski, B. H.(Eds), pp39-56. IRL Press, Oxford.

WIENBERG, J., JAUCH, A., STANYON, R., CREMER, T. (1990). Molecular cytotaxonomy of primates by chromosomal in situ suppression hybridization. **Genomics**, 8:347-350.

WEIR, H. G., KLEINE, H. D., and GRAY, J. (1991) Labeling of the centromeric region on human chromosome 8 by in situ hybridization. **Human Genetics**, 87:489-494.

WETMUR, J. G. (1975). Acceleration of DNA renaturation rates. **Biopolymers**, 14:2517-2254.

WHITE, J. G., AMOS, W. B., FORDHAM. (1987). An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. **The Journal of Cell Biology**, 105:41-48.

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

YUNIS, J. J. (1981). New chromosome banding techniques in the study of human neoplasia. **Human Pathology**, 12:540:549.

ZHANG, F. R., HEILIG, R., THOMAS, G., AND AURIAS, A. (1990). A one-step efficient and specific non-radioactive non-fluorescent method for in situ hybridization of abnded chromosomes. **Chromosoma**, 99:436-439.

APPENDIX

APPENDIX 1

PROTOCOL FOR CHROMOSOME PAINTING OR CHROMOSOMAL IN SITU SUPPRESSION HYBRIDIZATION USING WHOLE CHROMOSOME 21 SPECIFIC LIBRARY

Notes:

a. All reagents are prepared as in Material and Methods.

b. The library was purchased from American Type Cell Culture (ATCC) Maryland .U.S.A.

A. PROBE LABELLING BY POLYMERASE CHAIN REACTION (PCR) (Direct Incorporation of Biotin-11 dUTP)

Add sequentially the following to a PCR tube:

10mM dATP	2ul
10mM dCTP	2ul
10mM dGTP	2ul
10mM dTTP	1ul
2mM Biotin 11-dUTP	5ul
10XPCR buffer	10ul
Primer A	1.5ul
Primer B	1.4ul
Distilled water	74.6ul

Vortex and spin briefly.

Place the tube under the ultra-violet light for 15 minutes.
Add 2.0ul of purified probe. Mix and spin briefly.
Place the tube at 94°C for 10 minutes.
Add 0.5ul Taq polymerase (Perkin Elmer-Cetus).
Mix and spin briefly. Layer with 80ul mineral oil.

Reaction conditions for 30 cycles.

94°C	1 min.
50°C	1.5 mins.
72°C	3.5 mins

End extension time at 68°C for 7 mins

Notes:

a. The amount of the DNA (library) varies considerably on different batches even at a similar concentration.

b. Avoid the presence of bubbles in the reaction.

Cleaning of PCR product

Use Pharmacia Nick Columns and follow the instruction for use as enclosed in the package. Elute purified sample can be freeze-dried and reconstituted with TE buffer.

B. CHROMOSOME PAINTING

Day 1 Precipitation of DNA

Mix the following in the microcentrifuge tube.

Biotinylated probe (2ng/ul)	10ul
Sonicated unlabelled human DNA (1mg/ml0)	2ul
Cot-1 Human DNA (0.5mg/ml)	2ul
Sonicated salmon sperm DNA (1mg/ml)	2ul
Iced-cold absolute ethanol	50ul

Mix and centrifuge briefly for 2-3 minutes

Keep at -20°C overnight

Day 2 Denaturation and Hybridization

A. Probe denaturation

Centrifuge the DNA mixture for 30 minutes at 10,000rpm.

Aspirate and discard the ethanol.

Add 10ul of hybridization buffer.

Dissolve the pellet thoroughly.

Centrifuge briefly.

Denature the hybridization mixture at 75°C in a waterbath for 8 minutes.

Transfer the tube immediately to ice and then to a 37°C waterbath and incubate for 60 minutes.

B. Slide denaturation

(All metaphase chromosomes are prepared according to standard cytogenetic procedures).
Select the desired area of the slides for hybridization.
Denature the slides in denaturing solution (70% Formamide/2XSSC, pH7.0) at 75°C for 5 minutes.
Transfer slide into series of iced-cold ethanol concentration of 70%, 80%, 90%, 100% for 3 minutes each and dry at room temperature.

Notes: Prior to denaturation

a. Old slides (more than 1 month old) should be treated with RNase to remove unnecessary endogenous and non-endogenous material and to ensure good hybridization efficiency.
b. Fresh slides can be re-fixed in acid-methanol (1:3) solution for 30-60 minutes and dry at room temperature.

B. Hybridization

After 60 minutes incubation of the hybridization mixture then apply them completely (10ul) onto the denatured slides.
Cover with 24x24 mm coverslips.
Seal with cow gum.

Place slides on the tray and incubate the slides in a waterbath at 37°C for 16 hours or more.

Day 3 Detection and microscopy

A. Detection

Gently peel the cow gum.
Place the slides into wash solution (50% Formamide, 2XSSC at 45°C to remove the coverslips.
Transfer into a second and third washing solution to wash for 3-5 minutes each.
Wipe the slides except the marked area and place 200ul of FITC- avidin (5ug/ml in phosphate non-fat milk buffer) and incubate at room temperature for 20 minutes under aluminium foil.
Wash the slides in three changes of phosphate-nonidet P-40 buffer pH8.0 at room temperature for 5 minutes each.
Wipe slides as before and place 200ul of biotinylated goat anti- avidin (5ug/ml in phosphate non-fat milk buffer) and incubate at room temperature for 20 minutes under aluminium foil.
Wash off the slide with three changes of phosphate-nonidet P-40 buffer (PNB) at room temperature for 5 minutes each.
Wipe slide as before and place 200 ul of FITC avidin (5ug/ml in PNM) and incubate at room temperature for 20 minutes.

Wash the slide in three changes of phosphate-nonidet P-40 buffer (PNB) at room temperature for 5 minutes each.
Apply 10ul of anti-fade (DABCO) containing propidium iodide (2ug/ml) on the marked area.
Place a 24x35mm coverslip, blot excess fluid and seal with the nail vanish.

Notes:

a. Slide should not be left drying at any time during the detection.

b. Detection can also be performed by dipping slide in the coplin jar. The stain can be reused up, to 2 months when kept at 4°C after used.

B. Microphotography

Examine the result using fluorescent microscope using FITC specific band filter (Zeiss Axioplan filter 448709).
For photography used Kodakchrome ASA 400 slide film.

APPENDIX 11

PROTOCOL FOR NICK-TRANSLATION OF UNIQUE SEQUENCE PROBE WITH BIOTIN-11-dUTP

Notes: All reagents were supplied by BRL Nick-Translation Kits 86505B (Life Sci. U.K)

A. Mix in the eppendorf centrifuge tube:

1ug of DNA (calculate the volume to use)

5ul of nucleotide solution containing 0.2mM dATP, dCTP, dGTP.

2.5ul 0.4mM Biotin 11-dUTP (Sigma, U.K)

X ul of H_2O to make final volume of 45ul

5ul of 0.4 units/ul DNA polymerase I

Mix well and incubate at 15°C for 1.5-2 hours.

B. Stop reaction by adding 5ul of 300mM sodium EDTA (pH 8.0) and to precipitate add:

4.6ul 3NaAc pH 5.2

1.0ul 20mg/ml glycogen (Boehringer Mannheim)

122ul filtered iced 100% ethanol

Vortex to mix. Spin pellet at 10,000rpm for 30 minutes.

Aspirate supernatant and vacuum dry the pellet for 30 minutes and dissolved in 10ul TE buffer to give a concentration of 100ng/ul. Store at -20°C.

LIST OF PUBLICATIONS AND POSTER PRESENTATIONS

Publications and poster presentations

1. ISA, M. N., BOYD, E., MORRISSON, N., HARRAP, S., CLAUSER, E., CONNOR, J. M.(1989). Regional chromosomal localisation of the human angiotensinogen gene to 1q42-4.43 band. The American Journal of Human Genetics, 45:49 (Suppl. 1).

2. ISA, M. N., BOYD, E., MORRISSON, N., HARRAP, S., CLAUSER, E., CONNOR, J. M.(1990). Regional chromosomal localisation of the human angiotensinogen gene to 1q42-4.43 band. European Society of Human Genetics Symposium.

3. ISA, M. N., BOYD, E., MORRISSON, N., HARRAP, S., CLAUSER, E., CONNOR, J. M.(1990). Assignment of the human angiotensinogen gene to 1q42-4.43 by nonisotopic in situ hybridization. Genomics, 8:598-600.

4. ISA, M. N. (1991). Problems in chromosome painting. Northern Meeting of Assoc. of Clinical Cytogenetics. Edingburgh.

5. ISA, M. N., BOYD. E., CONNOR, J. M. (1991). Chromosome painting: Suppression or Depression! Assoc. of Clinical Cytogeneticists Meeting. Sheffield, U.K.

6. ISA, M, N., BOYD, E., TOLMIE, J., CONNOR, J. M. (1991). Assessment of chromosome 21 aberrations by chromosome in situ suppression hybridization with chromosome 21 specific library. Assoc. of Clinical Cytogenetics Bulletin Vol.2 No. 4 pp38.

7. ISA, M. N., BOYD, E., CONNOR, J. M. (1991). Detection of chromosome aberrations with chromosome specific library. Proceeding of the 8th International Congress of Human Genetics, October, 1991. Waashington, D.C.

8. ISA, M, N., TURNER, T., BOYD, E., TOLMIE, J., CONNOR, J. M. (1991). The confirmation of the chromosome 21 material on the X chromosome by chromosome in situ suppression hybridization with biotin labelled by PCR amplification. (In preparation).

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BRIEF REPORT

Assignment of the Human Angiotensin Gene to Chromosome 1q42–q43 by Nonisotopic *in Situ* Hybridization

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Angiotensin is a glycoprotein that provides the substrate for the enzyme renin in the essential first step of the biochemical cascades that result in the formation of angiotensin II. Angiotensin II has important effects on vascular tone and fluid and electrolyte homeostasis that may be important for the control of blood pressure. The human angiotensin gene has been cloned recently and was assigned to chromosome band 1q4 by Gaillard-Sanchez *et al.* (1990), using an isotopic *in situ* hybridization technique. Our aim was to confirm this localization.

In this experiment, chromosome spreads were obtained from phytohemagglutinin-stimulated lymphocyte cultures from three males with normal karyotypes. The probe was peAHB, a pBR322-derived pECE plasmid (Ellis *et al.*, 1986) containing a 2.5-kb cDNA sequence corresponding to the gene for human angiotensin (Kageyama *et al.*, 1984) inserted at *Hin*dIII–*Xba*I sites. It was labeled with biotin–11-dUTP (Sigma) and used at a concentration of 5 ng/μl for *in situ* hybridization according to the method described by Garson *et al.* (1987). The distribution of

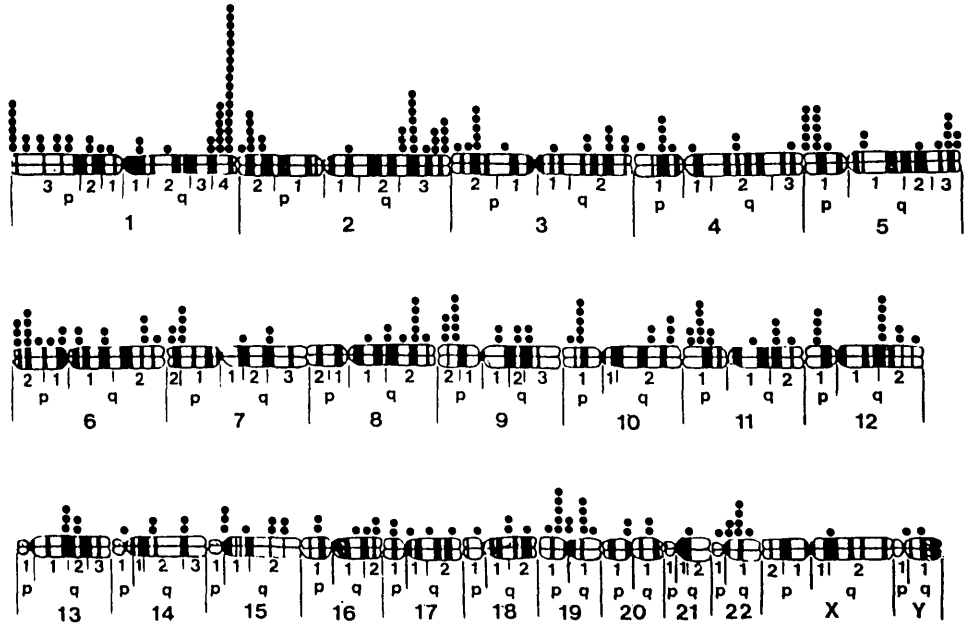

FIG. 1. A diagrammatic representation of the distribution of 271 signals in 70 normal male metaphases.

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hybridization over the studied metaphases was analyzed statistically using the χ^2 test.

Following hybridization, the positions of 271 hybridization signals were recorded in a total of 70 metaphases (Fig. 1). Of the total signal count, 17% (46/271) was on chromosome 1, 50% (23/46) of which was located between 1q42 and 1q43 with 28% (13/46) on 1q42 and 22% (10/46) on 1q43. The hybridization observed at 1q42–43 was found to be highly significant ($P < 0.005$). With this nonisotopic *in situ* hybridization technique some signals appear as a relatively large dark area covering more than one band rather than a distinct spot. In preparation of the signal idiogram (Fig. 1) each dot has therefore been placed at the chromosome band that lies beneath the center of a hybridization signal. The dots representing signals present on 1q42 and 1q43, respectively, have been placed in single rows opposite these bands due to limited space. Figure 2 represents distal chromosome 1q showing more precise placement of signal dots over 1q42–43, and Fig. 3 shows partial metaphase spreads.

Using isotopic *in situ* hybridization Gaillard-Sanchez *et al.* (1990) recently assigned angiotensin to 1q4 and found a hybridization peak at 1q42. Our results confirm this assignment and suggest that the location of angiotensin may be in the region of the border between 1q42 and q43.

This observation shows that angiotensin gene is located close to the enzyme renin (REN), which has been localized to the long arm of chromosome 1 (McGill *et al.*, 1987), although there is some doubt about its specific site (Nakai *et al.*, 1988; Cohen-Haguenauer, *et al.*, 1989). This is of interest because their roles are interdependent in the regulatory system for the control of blood pressure.

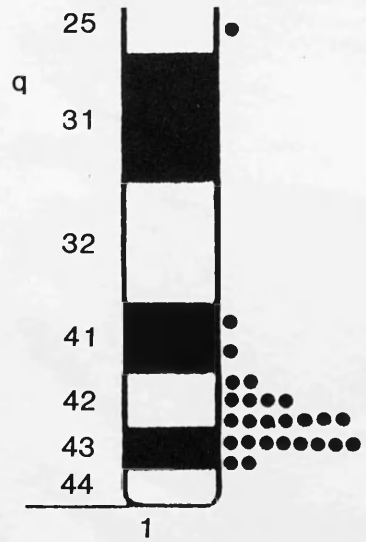

FIG. 2. A diagrammatic representation of distal chromosome 1q showing the precise distribution of signals over 1q42 and 1q43.

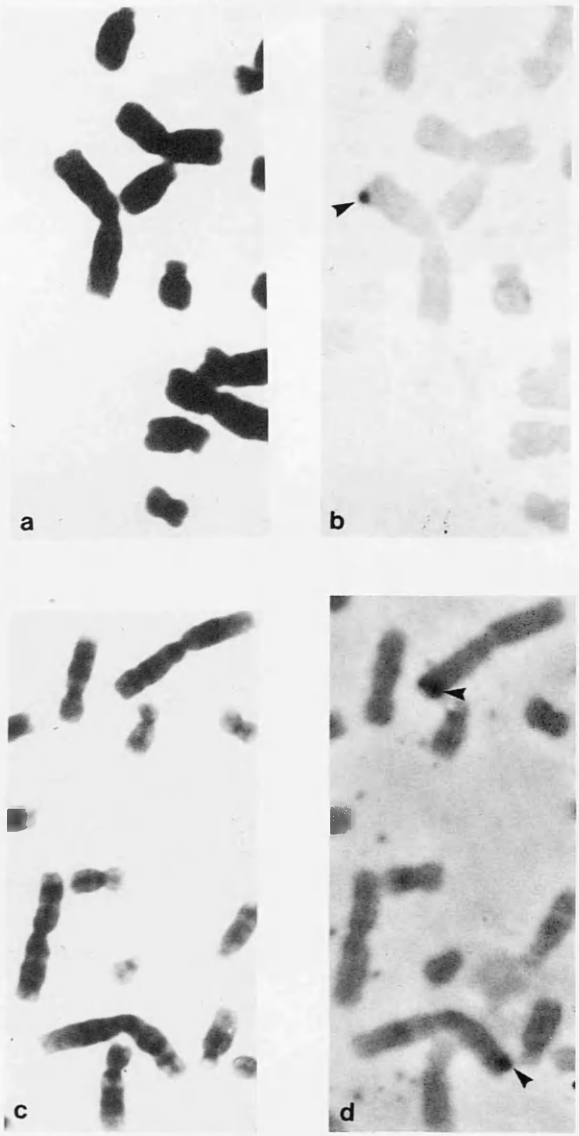

FIG. 3. Partial metaphases pre- (**a** and **c**) and posthybridization (**b** and **d**) with hybridization sites on chromosome 1 indicated by arrowheads.

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REFERENCES

1. Boyd, E., Theriault, A., Goddard, J., Kalaitsidaki, M., Spathas, D. H., and Connor, J. M. (1989). *Hum. Genet.* **81:** 153–156.
2. Cohen-Haguenauer, O., Soubrier, F., Van Cong, N., Serero, S., Turleau, C., Gross, M. S., Jegou, C., Corvol, P., and Frezal, J. (1989). *Ann. Genet.* **32:** 16–20.
3. Ellis, L., Clauser, E., Morgan, D. O., Eddery, M., Roth, R. A., and Rutter, W. J. (1986). *Cell* **45:** 721–723.
4. Gaillard-Sanchez, I., Mattei, M. G., Clauser, E., and Corvol, P. (1990). *Hum. Genet.* **84:** 341–343.
5. Garson, J. A., van den Bergh, J. A., and Kemshead, J. T. (1987). *Nucleic Acids Res.* **15:** 4761–4770.
6. Kageyama, R., Ohkubo, H., and Naknishi, S. (1984). *Biochemistry* **23:** 3603–3609.
7. McGill, J. R., Chirgwin, C. M., and McCombes, J. L. (1987). *Cytogenet. Cell Genet.* **45:** 55–57.
8. Nakai, H., Inoque, S., Miyazaki, H., Murakami, K., and Tada, K. (1988). *Cytogenet. Cell Genet.* **47:** 90–91.