IN SITU HYBRIDISATION FOR HUMAN AND COMPARATIVE GENE MAPPING

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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.

Marianna Kalaitsidaki

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Γεωργιου Φραγκακη-Καψαλιανου

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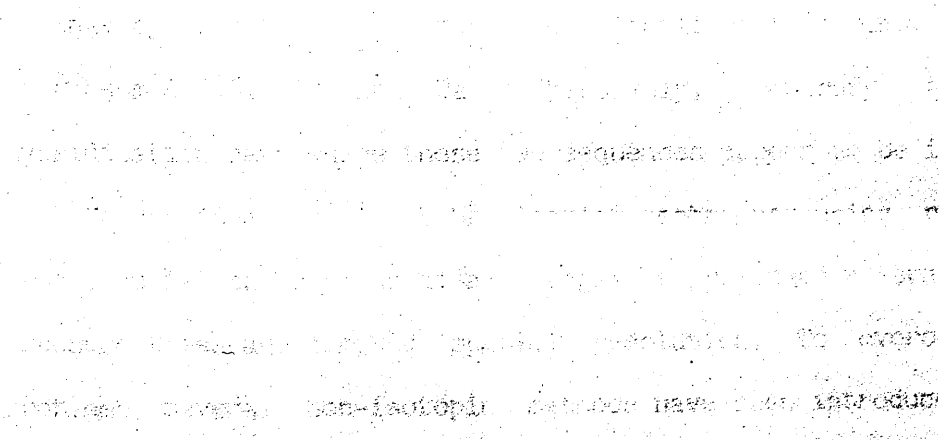
LIST OF ABBREVIATIONS

AAF	-	Acetylaminofluorene
ABC	-	Avidin-biotinylated horseradish peroxidase
Amp	-	Ampicillin
BCIP	-	5-Bromo-4-chloro-3-indolylphosphate p-Toluidine Salt
Bkm	-	banded krait minor satellite
Bio-11-dUTP	-	biotinylated deoxyuracil 5´-triphosphate
BrdU	-	5-bromodeoxyuridine
BRL	-	Bethesda Research Laboratories
BSA	-	Bovine Serum Albumin
cDNA	-	Complementary DNA
Ci	-	Curie
$C_o t$	-	product of original concentration of nucleic acid and time
DAB	-	diaminobenzidine
DAPI	-	4,6-diamino-2-phenyl-indole
DA	-	distamycin A
dATP	-	deoxyadenosine 5´-triphosphate
dCTP	-	deoxycytidine 5´-triphosphate
dGTP	-	deoxyguanosine 5´-triphosphate
dpm	-	disintegrations per minute
dTTP	-	deoxythymidine 5´-triphosphate
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylene Diamine Tetra-acetic Acid
e.g.	-	exempli gratia (for example)
Eno-1	-	enolase 1
et al.	-	et alia (and others)
FITC	-	fluorescein isothiocyanate
g	-	gram
G-banding		Giemsa banding
GGO	-	Gorilla gorilla
HEPES	-	N-2-Hydroxyethylpiperazine-N´-2-ethanesulfonic Acid
HGM	-	Human Gene Mapping Workshop
HSA	-	Homo sapiens
ISCN	-	International System for Chromosome Nomenclature
ISH	-	In situ hybridisation
kb	-	kilobase
g	-	microgram
mg	-	milligram
ml	-	microlitre
NBT	-	Nitroblue Tetrazolium Chloride
N-Aco-AAF	-	N-acetoxy-2-acetylaminofluorene
ng	-	nanogram
NOR	-	Nucleolar Organiser Region
O.D.	-	Optical Density
p	-	short arm of a chromosome
Pep-C	-	peptidase C
PBS	-	Phosphate-buffered saline
PHA	-	phytohaemagglutinin
PGM-1	-	phosphoglucomutase 1
PPY	-	Pongo pygmaeus (orangutan)
PTR	-	Pan troglodytes (Chimpanzee)

LIST OF ABBREVIATIONS (continued)

q	-	long arm of a chromosome
Q-banding	-	Quinacrine banding
R-banding	-	Reverse banding
rRNA	-	ribosomal RNA
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
rpm	-	revolutions per minute
SA-AP	-	Streptavidin-alkaline phosphatase conjugate
snRNA	-	Small nuclear RNA
SSC	-	Standard saline citrate
TCA	-	Trichloroacetic acid
TDF	-	Testis determining factor
T_m	-	melting temperature
Tris	-	Tris (hydroxymethyl) aminomethane
TRITC	-	Tetramethyl-rhodamine-isothiocyanate
tRNA	-	transfer RNA
U.V.	-	Ultraviolet
v/v	-	volume per volume
w/v	-	weight per volume
X-gal	-	5-Bromo-4-chloro-3-indoyl-beta-D-galactopyranoside
ZFY	-	Y-encoded zinc finger protein
ZFX	-	X-encoded zinc finger protein



SUMMARY

The overall aim of this project was to develop the technique of in situ hybridisation (ISH) for the localisation of cloned DNA sequences on the Y and other chromosomes in man and other primates using both ^{3}H- and biotin labelling.

Using tritium labelling two problems were encountered and circumvented, high non-specific labelling and loss of chromosome morphology following hybridisation. Following this, seven anonymous DNA sequences cloned in plasmid vectors were localised to specific chromosome sites in the human: GMGY10 (DYS59) and GMGY7 (DYS58) to the short of the Y chromosome (Yp), P2F2 (DY25) to chromosome X band q21 and Yp, pY3.4 (DYZ1) to chromosome Y distal band Yq12, GMGY3 (DYS13) to Yp and chromosome 9 region p23-pter, GMGY4b (DYS51) to the pericentromeric region of chromosomes Y, 15, 21, 22, JG73 to pericentromeric region of chromosomes 19, 21, 22 , Y and (14, 15, and 20) and finally JG51 (D21S89) to the pericentromeric region of chromosomes 13, 14, 15, 20, 21, and 22. Under conditions of high stringency the last two probes were localised to chromosome 21, and chromosomes 13, 14 and 22 respectively, contrary to filter hybridisation data where these two sequences appear to be identical.

Conventional in situ hybridisation techniques using radioactive probes suffer the serious disadvantages of prolonged autoradiographic exposure times and limited spatial resolution. To overcome these problems several non-isotopic methods have been introduced using a variety of different immunogenic, fluorescent and enzymatic labels. The possibility of using biotin-labelling for gene mapping was explored during this project using a recently described technique

that employs a streptavidin-alkaline phosphatase detection system. The method was shown to be simple, reliable, rapid and sufficiently sensitive to detect single copy DNA sequences, as was indicated by the localisation of a 3.2kb DNA sequence, p72 (D21S92), to chromosome 21 proximal band q21. Results were obtained in 24 hours as compared with 1 week autoradiographic exposure for repetitive probe and 3 weeks for single copy probe. Another probe, GMGXY8 (DXYS34) was mapped to chromosome Yp.

All probes mentioned above had been regionally mapped by Southern analysis using somatic cell hybrids. ISH not only confirmed but also extended the previous findings by indicating autosomal homologies for probes GMGY3, GMGY4b and regions of extended homology for JG73 and JG51. The homology between short arm of chromosomes Y and 9 revealed for probe GMGY3 is of special interest in view of the fact that GMGY3 resides in the sex-determining region of the Y chromosome. This finding coupled with observations that 9p monosomy is sometimes associated with anomalous sex differentiation could suggest a functional homology between Yp and distal 9p.

ISH was also used to determine the chromosomal location of Y-specific sequences in eleven patients with paradoxical sex chromosome complements previously shown to possess such sequences. Eight XX males were studied with ^{3}H-labelled GMGY10 and/or GMGY7 and in all cases it could be clearly demonstrated that these Y-specific sequences were located on distal short arm of the X chromosome (band Xp22.3) as predicted by the X-Y interchange hypothesis (Ferguson-Smith, 1966). The ninth XX male was investigated with probe P2F2 and although signal was recorded on the short arm of the X chromosome it could not be shown conclusively that this was due to an

X-Y interchange. Using GMGY10 or GMGY7 probes in two apparent XO males with an additional minute chromosomal fragment present in their peripheral blood lymphocytes it was demonstrated that the fragment had originated from a Y chromosome thus explaining the male phenotype of these individuals.

Apart from its value in gene mapping studies, ISH has an enormous potential as a tool in clinical diagnosis. This was demonstrated in the present study by the confirmation of a suspected Yp:15q translocation in amniotic fluid cells and in paternal lymphocytes with a chromosome 15 polymorphism by using ^{3}H-labelled probe pY3.4.

The last part of the project involved comparative mapping in the gorilla (Gorilla gorilla), chimpanzee (Pan troglodytes) and orangutan (Pongo pygmaeus) of a human transfer RNA gene for glutamic acid ($tRNA^{Glu}$) and probes GMGY10 and GMGY7.

The $tRNA^{Glu}$ gene was localised to distal long arm of chromosome 1 (band 1p36 according to ISCN nomenclature) in all three species, providing further evidence for homology between distal long arm of the ape chromosome 1 and distal short arm of human chromosome 1 where this gene has been previously localised. Interestingly, two secondary sites of hybridisation were observed above and below the centromere on chromosome 1 in the chimpanzee and orangutan but none in the gorilla. These secondary sites may represent pseudogenes or related sequences as has been suggested for the secondary site observed in the human (chromosome 1q21-q22). Their variable distribution in man and the great apes demonstrate that qualitative differences do exist on chromosome 1 of the four species despite the remarkable similarity in the banding pattern.

Probe GMGY10 was previously shown to be Y-specific in the great apes while probe GMGY7 was shown to be Y-specific in the chimpanzee zand orangutan. Using biotinylated in situ hybridisation GMGY10 was localised to the short arm of the orangutan Y chromosome, proximal long arm of the gorilla Y chromosome and the Y chromosome of the chimpanzee. In the latter, localisation to a specific region was not possible due to the extremely small size of this chromosome and the great intensity of the hybridisation signal. The above finding in the gorilla supports recent evidence that changes have occurred in the euchromatic part of the Y chromosome during the course of evolution between man and great apes. This is contrary to reports based on banding studies which describe a basic homology for this chromosome in all four species. Studies with GMGY7 were only performed in the orangutan where it was localised to the short arm of the Y chromosome. The finding that both GMGY10 and GMGY7 map to the same region in man and orangutan are against the suggestion that the orangutan Y differs from the human by a pericentric inversion in the region p11.2-q11.23. Although cytogenetic investigation of the great ape karyotypes was not an objective of this study, a diagrammatic representation of the primate Y chromosomes showing G-bands is proposed as previously reported diagrams did not represent the G-banded patterns observed here.

The present study has demonstrated the value of in situ hybridisation in human and comparative gene mapping by localising nine DNA sequences in man and three DNA sequences in the great apes. The biotinylated technique used was preferable to tritium labelling in terms of speed, cost, safety and topological resolution. In view of these advantages it is predicted that biotinylated and in general

non-isotopic in situ hybridisation will completely replace conventional radioactive procedures in gene mapping studies and molecular diagnosis.

CHAPTER ONE

INTRODUCTION

1.1 In situ hybridisation

1.1.1 Historical perspectives

Nucleic acid hybridisation, the formation of a duplex between two complementary nucleotide sequences, is the basis for a range of techniques now in widespread use in modern biology. This process was first described by Marmur et al., (1961) and Doty et al., (1961). The double stranded molecules of DNA will denature and reassociate under controlled reaction conditions. Reassociation in the presence of other molecules of DNA or RNA will result in the formation of hybrid molecules. Control of the stringency of the reaction conditions will ensure that only well matched hybrids result. These reactions can take place either in solution or with the DNA immobilised in some material such as nitrocellulose filters (Gillespie and Spiegelman, 1965). In 1969, the filter hybridisation method was modified independently by Gall and Pardue (1969) and John et al., (1969), using DNA of chromosomes and cell preparations on glass slides as the immobilised phase in order to produce hybrids "in situ". They used this method, in situ hybridisation, to study the amplified ribosomal DNA in the oocytes of Xenopus laevis (Gall and Pardue 1969; John et al., 1969). Cells fixed on a microscope slide were denatured and hybridised with highly radioactive RNA and incubated under conditions which allowed the formation of RNA-DNA hybrids. The slides were then covered with an autoradiographic emulsion and after exposure and development, silver grains appeared in specific regions of the nuclei that presumably contain the DNA sequences complementary to the probe RNA. Thus, historically, in situ hybridisation referred to the visualisation of heteroduplex or hybrid RNA/DNA molecules on cytological preparations. The current usage of this term has expanded

to also include DNA/DNA and RNA/RNA homoduplex molecules on metaphase spreads, intact cells, interphase nuclei, or tissue sections.

In its original form the method could only be applied to the localisation of repeated DNA sequences with easily purified RNA or DNA on both polytene and diploid chromosomes of many species. The advent of DNA recombinant technology together with improvements in the efficiency of hybridisation and in the quality of chromosome banding have made the technique of in situ hybridisation sufficiently sensitive to permit the localisation of single copy sequences. Since the first localisation of a single copy gene in 1981 by three groups independently (Gerhard et al., (1981); Harper and Saunders (1981); Malcolm et al., (1981) several localisations of single copy genes and anonymous DNA sequences have been reported and in situ hybridisation became a classical method for mapping DNA or RNA sequences in cytological preparations.

Until the 1980s the sites of hybridisation have been routinely detected with autoradiography. The serious disadvantage of prolonged autoradiographic exposures and limited spatial resolution due to the track of the decay particle through the autoradiographic emulsion made it desirable to have alternative, but equally sensitive methods for detecting or localising specific nucleic acid sequences in biological specimens. Several methods have been developed to replace routinely used radioactive labels in order to allow non-autoradiographic detection of the hybrids. In these methods, labels are either coupled directly to the probe (direct methods) or introduced via specific antibodies with hapten modified probes (indirect methods). A number of such haptens has been described i.e. the acetylaminofluorene, mercury and biotin modification. The

non-radioactive methods were however less sensitive than the radioactive, and although they were applied successfully to the localisation of highly or moderately repeated DNA sequences, little hope was entertained for the localisation of single copy genes. Nevertheless, the first localisation of a single copy gene was reported in 1985 (Landegent et al., 1985b; Burns et al., 1985b; 1985c), non-radioactive procedures became increasingly popular and much effort is being put to enhance their sensitivity at the hybridisation, detection and microscopy level.

In the following sections the different steps of DNA-DNA hybridisation to metaphase spreads, the variables affecting the rate of hybridisation, the different non-radioactive methods with emphasis to the biotinylated approach, and finally the applications and limitations of the technique will be presented. This will be followed by an introduction to the karyotypes and gene mapping studies in the great apes, (Gorilla gorilla, Pan troglodytes and Pongo pygmaeus) with emphasis on chromosomes 1 and Y.

1.1.2 In situ hybridisation: "A marriage between molecular biology and cytology"

After the discovery that RNA molecules with sequences complementary to those of DNA can anneal with the template to form DNA-RNA hybrid molecules, biologists began to consider the possibility of DNA-RNA hybridisation in cytological preparations. If a particular species of RNA was made radioactive and if such RNA molecules formed hybrids with complementary DNA in the cell nuclei and chromosomes, the cytological localisation of this DNA (or genes)

could be identified after autoradiography. In principle, this procedure should be quite feasible; in practice, a number of technical problems had to be solved. Hsu (1979) gave a personal account of how he and his colleagues had to abandon such a project after encountering numerous technical problems. The DNA molecules embedded in chromatin containing a variety of protein molecules. Although in solution one may denature the DNA and immobilise the single stranded molecules, in the chromosomes DNA must be denatured and must be held in this state or there is no hybridisation. Finally the RNA molecule used as a probe had to be extremely radioactive because of the limited efficiency of the autoradiographic technique. Credit must go to Joseph Gall and Mary Lou Pardue and the group of Jones for perfecting the in situ hybridisation procedure that achieved as Hsu put it "a marriage between molecular biology and cytology" by relating microscopic topological information with gene activity at the DNA level.

In situ hybridisation was pioneered independently by two groups (Gall & Pardue 1969; John, Birnstiel & Jones, 1969); it involves the annealing of radioactive polynucleotide probes to chromosomal or cellular preparations whose DNA (or RNA) has been denatured to facilitate hybridisation with the exogenous probe followed by detection of the hybridised site by autoradiography.

In the original investigation, radioactive 18S and 28S rRNA was hybridised with Xenopus oocytes and the sites of hybrid formation detected by autoradiography. Silver grains were visible over the nucleolar cap and nucleoli of the oocytes in the light microscope. No hybrids were detected if the preperations were treated with deoxyribonucleases or if excess non-radioactive rRNA was added to

compete with the labelled probe. These observations confirmed that true hybrid formation was being observed.

Consequently, both groups applied the technique successfully to chromosomes. Mouse satellite DNA, which was then the most thoroughly studied fraction of repetitive DNA, was localised to the centromeres of the mouse chromosomes (Jones, 1970), more specifically to the centromeric heterochromatin of mouse chromosomes except the Y (Pardue and Gall, 1970). The autoradiographs were exposed for several days when hybridisation was done with complementary RNA and for several months when hybridisation was done with radioactive DNA. The method had the advantage that it allowed direct visualisation of the site of hybrid formation in the cells and on the chromosomes themselves. The method has since been modified and used by many groups to locate the sites of various molecules of DNA and RNA. It should be noted that Pardue and Gall also noticed that Giemsa staining after denaturation procedures produced bands over centromeric heterochromatin. This was the first demonstration of C-banding and was the forerunner of G-bands.

1.2 The different steps of in situ hybridisation

1.2.1 Fixation of material on slides

The procedure relies heavily on the quality of the specimen (tissue sections, whole cells, metaphase chromosomes) which should be prepared in such manner that the target DNA is maximally exposed to the denaturing environment and accessible to the labelled DNA or RNA probe without substantial loss of material. The removal of basic proteins from cell preparations seems to be essential to the in situ method (Gall & Pardue, 1969). However, in order to see the

chromosomes at the end of the procedure and to keep them on the slide, many proteins must be retained. To preserve morphology the biological specimen has to be fixed. Some fixatives, such as those containing formaldehyde, appear to interfere with the denaturation of DNA and should be avoided. Excessive acid treatments may depurinate the DNA and so reduce the level of hybridisation. The most commonly used fixative is 3:1 methanol/acetic acid. Studies on methanol/acetic acid fixed chromosomes with acridine orange (Comings et al., 1973) which stains double-stranded DNA green and single-stranded DNA red, showed that air-dried chromosomes were stained green (double-stranded DNA) whereas flame drying or storage for prolonged times resulted in red staining of chromosomes (single stranded DNA /denatured). Kurnit (1974) using S1 nuclease digestion of DNA isolated from methanol/acetic-fixed chromosomes found that less than 20% of the DNA is single stranded. This in situ denaturation may be caused by the fixative (Raap et al., 1986).

Regarding the age of chromosomal preparations to be used for in situ hybridisation, controversial reports exist. Although quantitative data have not been presented, it has been recommended on the basis of experience, to hybridise slides that have been freshly prepared, as old slides give low grain counts possibly due to DNA degradation and loss during hybridisation or protein denaturation that occurs with age and must also have an effect (Wimper & Steffensen, 1974). Pinkel et al., (1986) made a similar observation for fluorescence biotinylated hybridisation. The hybridisation signal was most intense when the specimens were fresh, although the chromosomes appeared "fluffy" after hybridisation, and decreased with increased storage time in air, possibly due to reduced accessibility

of the hybridisation reagents, although the chromosomes remained compact. A reasonable compromise was reached after about a week, while after several months, hybridisation was visible only on chromosome surfaces. Degradation of nuclear and chromosomal DNA during ageing has been recently reported (Mezzanote et al., 1988). However, chromosomal preparations stored for a year (Burns et al., 1985) or even 5 years (Kozma & Adinolfi, 1988) have been used successfully for biotinylated hybridisation.

1.2.2 Pretreatment of material on slides

RNase treatment serves to remove endogenous RNA but careful washing is required after the RNase treatment to ensure that no enzyme remains to degrade the hybridisation probe. Proteinase K treatment has been used to increase accessibility of the exogenous nucleic acid to the chromosomal DNA by digesting protein surrounding the target DNAs, after the method of Brahic and Haase (1978). In non-isotopic methods where an enzyme is used as the final label, the endogenous enzyme activity may have to be inactivated. For peroxidase, this is done by treating with 1% H202 in methanol. For alkaline phosphatase, levamisol may be added to the enzyme staining medium. Triton X-100 or sodium dodecyl sulphate incubations may be used when it is suspected that lipid membrane components have not been extracted by other procedures such as fixation, dehydration, embedding and endogenous enzyme inactivation procedures.

1.2.3 Choice of probe and labelling

Recombinant DNA technology now provides the opportunity to obtain either DNA or RNA probes of any desired sequence. For

hybridisation to DNA in cytological preparations double- and single-stranded probes both DNA and RNA have been used successfully (Malcolm et al., 1977).

Choice of radioactive isotopes involves a compromise between high specific activity and good resolution. The distance an emitted particle will travel through the photographic emulsion depends on the initial particle energy (Rogers, 1967).

Tritium has a specific activity of 50-85 Ci/mmol with a half life of 12.3 years. It emits beta particles, with a maximum particle energy of 18 keV and an average energy of 9-12 keV, which travel less than 1μ m through the autoradiographic emulsion ensuring that the silver grains remain closely associated with the site of the radioactive molecule. A disadvantage of using a tritiated probe is the long exposure time required for detection of unique sequences. Iodine with its high specific activity (1500 Ci/mmol) due to a relatively short half-life of 60 days has also been used. ^{125}I decays by internal conversion and a variety of particles are emitted with a wide range of energies. Seventy seven percent of electrons emitted have a particle energy of less than 4 keV, thus good resolution is obtainable with this isotope at the expense of a higher background (Buckle and Craig, 1986).

^{35}S has a half-life of 87.2 days and emits beta particles with a high energy (50 keV average). Although high specific activities are obtainable with ^{35}S-labelled probes, such high initial energies make this isotope unsuitable for work requiring high resolution.
Radioactive nucleotides can be introduced into double-stranded DNA by nick translation with E coli polymerase I (Rigby et al., 1977), in vitro transcription by E coli polymerase (Gall and Pardue, 1971), in

vitro transcription with SP6 RNA polymerase (Melton et al., 1984) or the random primer DNA synthesis (Feinberg and Vogelstein, 1983).

One technical difficulty in relation to single copy sequence localisation had been the inability to prepare probes of sufficiently high specific activity to obtain an interpretable signal in a reasonable amount of time. Malcolm et al., (1977) compared three types of probes specific for ribosomal genes for their usefulness in hybridisation in situ, a recombinant plasmid labelled by nick translation, complementary DNA and complementary RNA. Although the plasmid was labeled to only one-fifth the specific activity of the complementary DNA it gave a 10-fold increase in in situ hybridisation efficiency after a much shorter exposure time. This amplification in the hybridisation signal was further documented for unique sequences cloned in a plasmid or phage vector and made radioactive by nick-translation (Gerhard et al., 1981); Nick translation of DNA results in randomly cleaved molecules of variable length, depending upon the degree of nicking. Upon denaturation, partially complementary DNA fragments are generated which can then reanneal to each other by their remaining single stranded regions, forming extensive DNA network (Bolton et al., 1965). These networks may form in solution and then hybridise to the chromosomal DNA by one of many sequence-specific single stranded regions and/or may be built up directly on the chromosomal target sequence. Either phenomenon, results in the depositing of many probe molecules at the specific chromosomal site. The optimal size of probe molecule for network formation was determined to be 500-1000 bp (Alwine et al., 1979) and this is more important for signal detection than the specific activity of the probe.

Non-isotopic methods of labelling nucleic acids are described in section 1.4.

1.2.4 Denaturation of chromosomal DNA

Denaturation of the chromosomal DNA is a necessary precondition for in situ hybridisation. This can be achieved by a variety of techniques including heat, alkali, acid or certain organic compounds such as formamide. In general such treatments may lead to loss of morphology and a compromise has to be found between hybridisation signal and chromosome morphology. It is important to choose methods that will cause the minimum of cytological disruption and thereby help in localisation of the hybrid to specific regions within the cells or on the chromosomes.

Heat alone is effective but tends to destroy the chromosomes. A good compromise involves the use of formamide. The melting temperature of DNA is reduced 0.72°C per 1% of formamide in the solution (McConaughy et al., 1969). In a 50% solution, the reduction in melting point is 36°C. HCl denaturation tends to be less damaging to chromosome morphology than NaOH, formamide or heat but hybridisation is less efficient (Jones, 1973).

Singh et al., (1976) compared the effect of 0.2N HCl, 0.007N NaOH, 90% formamide and heat and found that heat denaturation in 0.1xSSC at 100°C gave significantly higher grain counts regardless of DNA base composition, HCl denaturation discriminated markedly against GC rich DNA and finally, chromosome morphology was best preserved with HCl and heat denaturation.

Raap et al., (1986) studied alkaline, acid and thermal

denaturation procedures (0.07N NaOH, 0.42N HCl, and 70% formamide, 2xSSC, 70°C) in hybridisation in situ with acetylaminofluorene modified mouse satellite DNA for their ability to separate the DNA strands of nuclear DNA and for the DNA losses they induce. The results obtained with acridine orange staining and microfluorometry, showed that all denaturations studied led to almost complete strand separation. Quantitative DNA staining and cytometry indicated that with heat and alkaline denaturation about 40% of the DNA is lost. Acid denaturation leads to about 20% DNA loss. For the alkaline denaturation the DNA retention could be improved to a 20% DNA loss by adding 70% ethanol to the denaturation medium. These DNA losses were in reasonable agreement with data published earlier for metaphase chromosomes. DNA loss due to 0.07N NaOH treatment from chromosomes was found to be 16 to 80% (Comings et al., 1973). Hubbell et al., (1976) compared alkaline and formamide denaturation (0.07N NaOH and 90% formamide at 65°C or 37°C) in relation to DNA loss and efficiency of in situ hybridisation in guinea pig chromosomes and found that alkaline and formamide at 65°C denaturation, resulted in losses similar to these reported by Comings et al (1973) while little or no DNA loss occurred with formamide at 37°C. This latter condition however, did not fully denature the DNA.

1.2.5 Hybridisation

Henning (1973) pointed out that precise hybrid formation is the keystone of any successful in situ technique. This reaction critically depends upon completeness of denaturation of the cytological DNA, addition of the probe under conditions that do not permit premature reannealing and selection of hybridisation

conditions so that optimal specificity of the reaction is obtained. Experiments have been carried out showing that hybrid molecules can be extracted from cells after in situ hybridisation. These hybrid molecules were not observed under experimental conditions in which the denaturation step was omitted (John et al., 1969). Henning (1973) found that DNA-[^{3}H] RNA hybrid molecules could also be extracted by sodium dodecyl sulphate-pronase treatment of Drosophila salivary gland chromosomes hybridised with cRNA complementary to a high density satellite DNA band. These experiments confirmed the existence of RNA-DNA hybrids in situ.

Hybridisation may be carried out in varying amounts of SSC, usually between 2xSSC and 6xSSC or the formamide method of McConaughy et al., (1969). Hybrid formation in the presence of formamide is considerably slower than the formation rate in salt solutions. The reaction occurs at a faster rate when formamide is combined with a salt solution (McConaughy et al., 1969). Increasing formamide concentration decreased the renaturation rate linearly by 1.1% per percent formamide for sodium chloride concentrations of 0.035M-0.88M (Hutton 1977). This produced an optimal hybridisation rate in 50% formamide/salt solution. The concentration of the probe, the duration of hybridisation and the temperature at which the procedure is carried out, all vary the amount of hybrid formed.

The inclusion of high molecular weight polymers in the hybridisation buffer such as dextran sulphate which effectively concentrates the nucleic acid in solution, accelerate the rate of reassociation. For plasmid probes another advantageous effect of the increased hybridisation rates obtained with dextran sulphate, is that due to the presence of vector sequences, large networks of probe DNA

can be formed at the site of hybridisation. It should be noted that such a phenomenon will also occur in the absence of dextran sulphate but at a lower rate. 10% Dextran sulphate has been shown to accelerate the rate of hybridisation of randomly cleaved double-stranded DNA probes to immobilised nucleic acids 10-fold (Wahl et al., 1979). Gerhard et al., (1981) reported a similar effect of 10% dextran sulphate in in situ hybridisation (rate increased 20- to 30-fold). The model they proposed for the formation of probe networks during in situ hybridisation is presented in Figure 1. Networks of probe molecules can form in solution and then hybridise to a homologous sequence in chromosomal DNA (pathway I) or networks can be built onto a free single-stranded tail of a probe molecule that has hybridised to a homologous sequence in chromosomal DNA (pathway II). The hybridisation steps that actually occur most likely involve a combination of both pathways. Harper & Saunders (1981) reported that nick translated DNA hybridised at a concentration of 5μ g/ml with 5 days exposure and at 1μg/ml with 22 days exposure resulted in similar frequencies of labelling indicating that aggregation of probe is indeed occurring.

Specificity of hybrid formation can be tested by competition experiments with unlabelled nucleic acids complementary to the probe used. It should be noted that during hybridisation a relatively large loss of DNA occurs (20% according to Raap et al., 1986).

When complementary DNA strands are in approximate alignment and in (partial) register, a situation probably encountered after in situ DNA denaturation, very rapid renaturation will occur (Wetmur & Davidson, 1968; Manning, 1976). Thus, during in situ hybridisation with double stranded probes, the renaturation of probe in solution

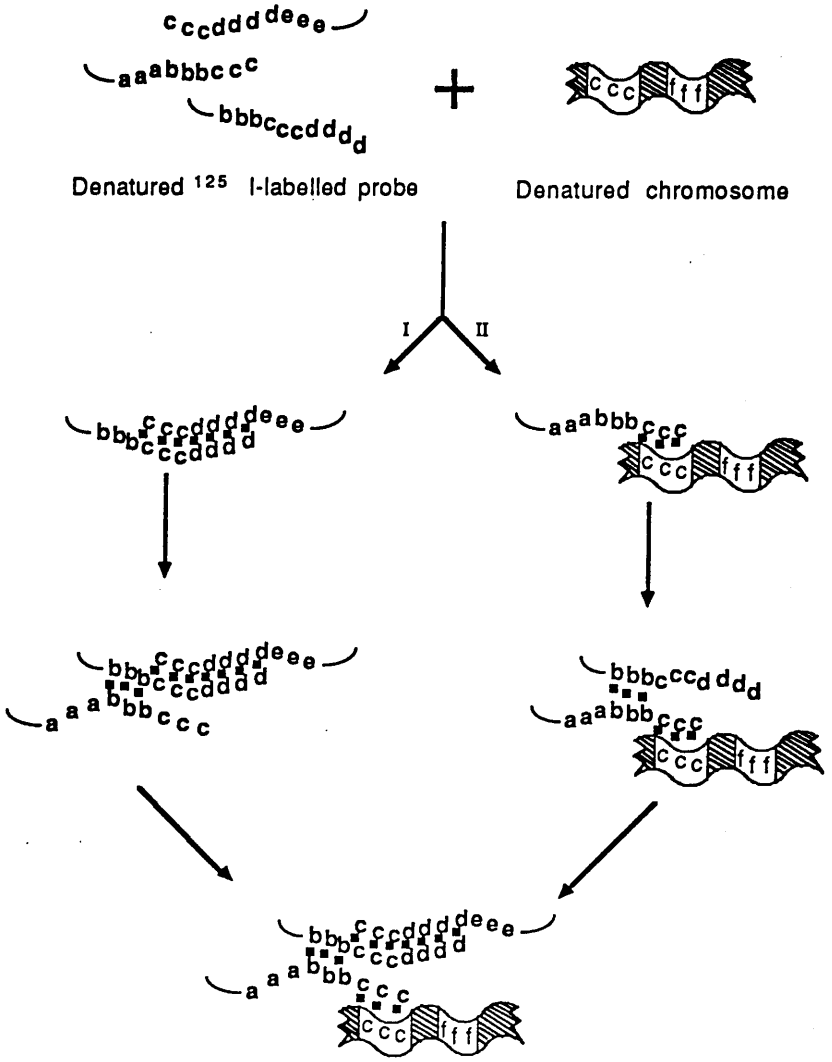


Figure 1. Model for the formation of probe networks during in situ hybridisation. Probe strands that were originally in a double-stranded molecule can, after random shearing and denaturation, hybridise to each other and form networks. When the annealing is carried out in the presence of denatured chromosomal DNA, the probe networks can form and hybridise to homologous chromosomal sites. This may occur either by the formation of probe networks in solution followed by hybridisation to chromosomal DNA (pathway I) or by formation of a probe network on the single stranded tail of a probe molecule that has hybridised to a homologous sequence in chromosomal DNA (pathway II).
(taken from Gerhard et al., 1981)

and in situ renaturation of target DNA sequences can compete with in situ hybridisation. The renaturation of probe in solution can be avoided if single-stranded probes are used, but the formation of probe-networks which, as mentioned above contribute to signal amplification, will also be prevented. Regarding in situ renaturation, Steffensen et al., (1977) reported that when slides for in situ hybridisation are cooled rapidly immediately after denaturation, dehydrated in alcohol and air-dried, the chromosomes do not seem to subject to DNA-DNA reannealing. This was confirmed by two methods using Drosophila chromosomes. Firstly, after autoradiography, salivary glands were still completely red using acridine orange (as mentioned before, double-stranded DNA complexed with acridine orange fluoresces green). The second method showed that there was no loss of satellite DNA when the slides were pre-incubated for 12 hours in 50% formamide in 2xSSC at 35°C before the ^{3}H-cRNA was added. Szabo et al., (1977a) also concluded on the basis of prehybridisation experiments that DNA-DNA renaturation is not a competing reaction within the time-scale of in situ hybridisation.

In contrast, Comings et al., (1973) observed that both repetitious and non-repetitious DNA rapidly renature in situ. Similarly, Raap et al., (1986) demonstrated that a rapid renaturation of a considerable fraction of DNA does occur. They suggested that this competing effect was missed in Szabo et al., (1977a) quantitative hybridisation experiments because of the extreme rapidity of (part of) the in situ DNA renaturation. The extent of renaturation was found to be dependent on the type of denaturation used, estimated to be 35% with ethanolic alkaline denaturation.

With DNA.DNA in situ hybridisation, the in situ renaturation of

target DNA sequences cannot be prevented as a consequence of the similar thermal stability of the in situ hybrids and in situ renatured sequences. However, there is a lot of circumstantial evidence that repeated sequences are preferentially renatured (Stockert & Lisanti, 1972) since they have the greater chance of being in (partial) register. In situ renaturation of DNA targets can be prevented by the use of RNA probes. Since DNA-RNA hybrids are thermally more stable than DNA-DNA hybrids, hybridisation conditions can be designed at which DNA-DNA hybrid formation is prevented, whereas DNA-RNA hybrid formation is not.

Dorsey-Stuart and Porter (1978) combined the denaturation and reannealing processes by denaturing the chromosomal DNA in the presence of the exogenous probe in a solution of 50% formamide at 70°C thus eliminating the possibility of premature reannealing of the chromosomal DNA.

1.2.6 Post-hybridisation washes and detection

During hybridisation, duplexes will be formed between the perfectly matched sequences and between less homologous sequences. The latter are less stable than the perfectly matched hybrids and can be disassociated by performing washings of various stringencies. The stringency of the washings can be manipulated by varying formamide concentration, salt concentration and temperature. Often a wash in 50% formamide/2 x SSC suffices. The extent to which non-perfectly matched hybrids occurs can be manipulated to some extent by varying the stringency of the hybridisation reaction.

Once hybridisation has taken place the detection of chromosomal sites depends on the specific activity of the probe, the length of

chromosomal DNA at the hybridisation site, the number of copies of sequence at the chromosomal site and the length of the autoradiographic exposure. Slides are dipped in a photographic emulsion to produce a thin film. After exposing the film for a week or more these autoradiographs are developed and silver grains are seen where radioactive label is present. This label should correspond to the site of the gene sequence being studied.

For non-autoradiographic detection see section 1.4.

1.2.7 Identification of chromosomes

Correct identification of the chromosomes is important when using in situ hybridisation to map genes and there are a variety of banding methods that can be used before or after the hybridisation. Chromosome banding before in situ hybridisation has been recommended to avoid bias in metaphase selection but has the disadvantage of possible loss of chromosomal DNA resulting from the banding procedure. If banding prior hybridisation is employed, after autoradiography the pre-photographed cells are relocated and dual karyotypes are constructed. Both G- and Q-banding have been used for this purpose. One disadvantage in using Q-banding is that the fluorescence of the cells fades rapidly making documentation of results difficult. Methods using G-banding have the advantage of producing permanent bands. Such bands can be produced using the detergent lipsol prior to hybridisation (Elliot 1980). Zabel et al., (1983) described a method that utilizes the incorporation of the base-analog 5-bromodeoxyuridine (BrdU) to produce replication bands. Because BrdU-substituted DNA is more prone to degradation than native DNA (D'Andrea et al., 1983) this technique is not recommended by

other investigators (Donlon 1986).

A different approach involves banding after hybridisation. Methods have been described involving quinacrine staining that allow simultaneous observations of the banding patterns and the silver grains (Sawin et al., 1978; Lawrie and Gosden, 1979). Methods for simultaneous observations of G-bands and hybridisation grains have also been reported (Chandler & Yunis, 1978). These methods have the advantage of reducing time on photography necessary in the prebanding methods.

Cytogenetic resolution can be increased further using methods for synchronising cell divisions in culture. Addition of aminopterin increases the number of cells in early stages of mitosis. Prometaphase chromosomes can be twice as long and have four times the number of bands of metaphase chromosomes. This will allow more accurate assignment of gene sequences to specific chromosome regions (Yunis et al., 1978).

The assignment of silver grains to chromosomal sites is a tedious and time consuming process. The usual scoring procedures require a subjective judgment as to whether a particular grain is associated with a particular chromosome. Hence, either they are susceptible to bias or they omit a good deal of data through the use of an arbitrary criterion such as grain-chromatid contact. The use of computers in the analysis of grain distribution has been reported (Warburton et al., 1978) to eliminate subjectivity, increase resolution and simplify data analysis.

1.3 Variables affecting the rate of hybridisation and/or the stability of hybrids

A number of factors namely temperature, concentration of salt, fragment length, base-pairing mismatch, base composition can affect the rate of hybridisation and/or the stability of the formed hybrids.

Results derived from procedures using solution hybridisation have shown that the maximum rate of hybridisation of DNA occurs at 25°C below the melting temperature (T_m) of the duplexes. Melting temperature of a DNA duplex or an RNA-DNA hybrid is the temperature at which the strands are half dissociated or denatured. Depending on the concentration of salt present hybridisation may effectively cease at temperatures well below the optimum. DNA denatures in 0.1-0.2 M Na+ at 90-100°C. This would imply for in situ hybridisation that the chromosomal preparations have to be hybridised at 65-75°C for prolonged periods of time which may lead to deterioration of morphology. A compromise have been found in the use of formamide (McConaughy et al., 1969) as mentioned previously (section 1.2.4). Formamide destabilises DNA duplexes more than the DNA-RNA hybrids (Bishop 1972 ; Birnstiel et al., 1972). However the rate of renaturation decreases in the presence of formamide.

Salt concentration affects the rate of reassociation markedly. Salt concentration is also an important factor in the stability of the formed duplex. Below 0.1 M NaCl, a 2-fold increase in concentration increases the rate by 5- to 10-fold. The rate continues to rise with salt concentration but becomes constant when the concentration exceeds 1.2 M NaCl. The T_m of a duplex changes about 16°C for each factor of 10 in salt concentration in the lower concentration range (0.01-0.1 M) but the effect is reduced at high

concentrations (1M).Divalent cations, which are often present as impurities in solutions, strongly stabilize duplex DNA at low concentrations thus use of EDTA as a chelator may be required.

The rate of renaturation of DNA in solution is proportional to the square root of the fragment length. Therefore maximum hybridisation rates are obtained with intact probes. However, with in situ hybridisation, reduction of the fragment length is essential for the probe to diffuse into the chromosomes. The fragment length also influences thermal stability. In the pH range 5 to 9 the rate of renaturation is fairly independent of pH. 20-50mM phosphate pH 6.5-7.5 buffers are frequently used.

The higher the concentration of probe the higher the reannealing rate. The maximum concentration of probe used is (practically) determined by the signal-to-noise ratios obtainable or necessary.

1. 4 Non-isotopic methods of in situ hybridisation

In hybridisation procedures in which radioactive nucleic acids and autoradiography are used, the limiting factors are autoradiography, which necessitates long exposure times with relatively low resolving power resulting from the scattered occurrence of silver grains in an emulsion around a radioactive point source (Prensky et al., 1973). This limits the precision with which the target sequences can be localised within chromosomes or cells. These reasons together with the personnel safety and isotope disposal problems made a number of investigators to explore alternative approaches to polynucleotide labelling in order to allow nonautoradiographic detection of the hybrids with immunological or affinity reagents.

These methods differ in the way the alternative markers, such as fluorochromes and cytochemically detectable enzymes, are introduced at the target sequences. Basically, they can be classified as direct and indirect methods. In the former the label is coupled directly to the hybridisation probe so that formed hybrids can be visualised microscopically immediately after the in situ hybridisation procedure. It is essential that the probe-label bond survives the hybridisation and washing conditions and most importantly does not interfere with the hybridisation reaction. The terminal fluorochrome labelling procedure of RNA probes as developed by Bauman et al., (1980, 1981, 1984) and the direct enzyme labelling procedure of nucleic acids described by Renz and Kurz (1984) fulfil these criteria.

In the indirect methods the probe contains an element introduced chemically or enzymically, that renders it detectable by affinity cytochemistry hence the term indirect. Again, its presence should not or only in a limited way interfere with the actual hybridisation reaction and the stability of the resulting hybrid and should be accessible to antibodies. The site of hybridisation is rendered immunologically detectable, either by generating antiDNA-RNA antibodies (Rudkin and Stollar, 1977; van Prooijen-Knegt et al., 1982;) or by hapten modification of the probe. A number of such hapten modifications has been described i.e. the acetylaminofluorene, mercury and biotin modifications. The main advantages of these new procedures over autoradiography are the speed of visualization and the precision of localisation.

Rudkin and Stollar (1977) described a technique for the detection of RNA-DNA hybrids by indirect immunofluorescence. The

chromosomal preparations following hybridisation were exposed to an antiserum raised in rabbits against poly(rA)poly(dT) complexed with methylated bovine serum albumin-rabbit anti-RNA-DNA-hybrid serum. The slides were then exposed to an rhodamine-labelled goat IgG fraction of anti-rabbit-IgG and photographed using a UV microscope.

Bauman et al., (1980) described an alternative method of that of Rudkin and Stollar, in which a fluorescence marker, the hydrazine derivative of TRITC (tetramethyl-rhodamine-isothiocyanate) is attached directly to cRNA. The thio-semicarbazide is reacted with the dialdehyde produced at the 3' terminus of the RNA by periodate oxidation. In this way the rhodamine molecule is linked to the RNA via a morphiolo derivative (Hansske and Cramer 1979). The label added no specific binding properties to the RNA and did not interfere with its hybridisation properties and had no influence on the specificity and stability of the hybrids (Bauman et al., 1981). Hybridisation was performed at low temperatures because at higher temperatures the fluorochrome RNA bond breaks. This method was used to visualise kinetoplast DNA (kDNA), adenovirus 5 (Ad-5) in human tissue culture cells and the genes for 5S ribosomal RNA (rRNA) in the polytene chromosomes of Drosophila hydei. Quantification of the RNA hybridised was possible by fluorescence measurements with a microphotometer directly on the object or by scanning densitometry after quantitative photography. Disadvantage of this (direct) method is that only RNA probes can be used.

van Prooijen-Knegt et al.,(1982) modified the method of Bauman et al., (1980) in order to achieve lower non-specific background fluorescence. Antisera specific for RNA.DNA hybrids were raised in rabbits by injecting complexes of poly(rA)poly(dT) and methylated

bovine serum albumin. Nonspecific fluorescence was rarely confusing since was usually distinct from metaphase chromosomes as determined with the aid of the DAPI staining pattern. The feasibility of this method was demonstrated in visualisation of the 18S and 28S ribosomal cistrons in human metaphase chromosomes with higher topological resolution and a lower background than in most autoradiogaraphic procedures. The amount of RNA hybridised can be quantified by microfluorometry or quantitative photography followed by scanning densitometry of the photographic negatives (van der Ploeg et al., 1977). Disadvantage of this anti-RNA-DNA procedure is that only one type of nucleic acid can be used because the localisation of a DNA target requires the use of RNA probes and vice versa.

1.4.1 Acetylaminofluorene labelling

The use of acetylaminofluorene modified nucleic acid probes in in situ hybridisation was first described by Landegent et al., (1984) and Tchen et al., (1984). The hapten acetylaminofluorene (AAF) is introduced chemically in the nucleic acid probe by reaction with a chemically activated form of AAF, N-acetoxy-2-acetylaminofluorene (N-Aco-AAF). The main site of covalent binding is the C-8 position of the guanine residues (Kriek et al., 1967). Both DNA and RNA, double and single stranded, can be labeled with AAF. The immunocytochemical detection of the hybridised probes is achieved by means of antibodies directed against AAF-modified guanosine (G-AAF) and a fluorochrome labelled second antibody. For each percent of AAF modification the thermal stability of duplex DNA is reduced by 1.1^{o}C (Fuchs & Daune 1973) indicating that mismatching is introduced by AAF binding. A degree of modification of 5% is considered sufficient for

immunocytochemical detection, since at higher modification steric factors impede further antibody binding. This procedure was tested on two model systems: mouse satellite DNA in interphase nuclei and chromosomes, and kinetoplast DNA in Crithidia fasciculata. An application of clinical importance was the detection of human cytomegalovirus in infected human lung fibroblasts. The advantages of the method are the simple rapid and reproducible labelling procedure, the feasibility of labelling both double stranded and single stranded probes (DNA as well as RNA) and the rapid and sensitive detection of hybrids. The disadvantage is the carcinogenicity of N-AcO-AAF which requires careful experimentation during the modification procedure.

Landegent et al., (1985a) demonstrated that visualisation of nonautoradiographic in situ hybridisation results by means of reflection-contrast microscopy allowed a more sensitive detection of the hybridised probe than by absorption and fluorescence microscopy. Using AAF-modified probes and reflection contrast microscopy the chromosomal localisation of the human thyroglobulin gene by using a large (22.3kb) probe was reported (Landegent et al, 1985b) as well as the localisation of a unique DNA fragment closely linked to Huntington's disease, to chromosome 4p16 (Landegent et al., 1986).

1.4.2 Mercuration of nucleic acids

The covalent mercuration of polynucleotides was described about 15 years ago by Ward and coworkers (Dale et al., 1975). The idea of using the mercury atom in the probe as an anchor point for hapten introduction after in situ hybridisation by using hapten carrying mercaptans originated from van Duijn and coworkers (Hopman et al.,

1985a, 1985b, 1986, 1987). The hapten is introduced after the hybridisation and in this respect it differs from the AAF, biotin and other procedures. The covalent mercuration is achieved by a single incubation of the probe (DNA or RNA, single and double stranded) with mercury(II)acetate at elevated temperatures (Dale et al., 1973). This incubation leads to a slow covalent binding of Hg to the C-5 position of the pyrimidines Uracil and Cytosine, and rapid complexing of Hg at the nitrogen atoms of the bases. The complexed Hg is efficiently removed by cyanide ions (Dale et al., 1973) except the Hg at the C-5 position which by acting as a counterion, protects the probe from inter- and intramolecular crosslinking and demercuration. The presence of the Hg atom on C-5 of Uracil and Cytosine does not affect the hybridisation characteristics, in the presence of slight excess of cyanide or other effective counterion (Hopman et al., 1986). After hybridisation the hapten is introduced by reaction with a ligand that carries a sulfydryl residue on one terminus to assure binding to the Hg and on the other terminus a hapten (Hopman et al., 1986). A variety of haptens can be applied eg trinitrophenyl, biotinyl and fluorescyl groups.

1.4.3 Biotin-labelling of nucleic acids

The highly specific and tenacious interaction ($K_{dis}=10^{-15}$) between biotin, a small water-soluble vitamin, and avidin, a 68,000-dalton glycoprotein from egg white, has been used as the foundation for constructing sensitive methods for the visual localisation of specific proteins, lipids, or carbohydrates on or within cells. When avidin is coupled to appropriate indicator molecules (fluorescent dyes, electron dense proteins, enzymes or

antibodies), minute quantities of biotin can be detected. Investigators tried to extend this general methodology to the detection of polynucleotide sequences.

Davidson and associates chemically crosslinked biotin to RNA, via cytochrome c or polyamine bridges, and used these RNA-biotin complexes as probes for in situ hybridisation (Manning et al., 1975). The sites of hybridisation were visualised in the electron microscope through the binding of avidin-ferritin or avidin-methacrylate spheres. Although this approach to the detection of polynucleotide sequences was successful in the specialised cases examined, a simpler and more general procedure for preparing biotin-substituted nucleic acids was desirable. This was achieved by Ward and associates who in 1981 reported the synthesis of biotinylated nucleotides, notably dUTP analogs that contained a biotin molecule biotin attached covalently to the C-5 position of the pyrimidine ring through an alylamine linker arm (Langer et al., 1981). These nucleotides derivatives served as substrates for a variety of DNA and RNA polymerases in vitro and could be introduced in nucleic acids by nick translation. Biotin-substitute polynucleotides were shown to have denaturation and reassociation characteristics similar to those of biotin-free polynucleotide controls, thus they could be used effectively in standard hybridisation protocols. The thermal stability of biotinylated DNA was reduced about $1^{o}C$ for each % modification (Langer et al., 1981), indicating that some destabilization of duplexes is introduced by the biotin modification.

Drosophila polytene chromosomes were used as a test system for establishing whether biotinylated probes could be detected after in situ hybridisation, by biotin specific immunological or affinity

reagents (Langer-Safer et al., 1982). Several cloned Drosophila sequences between 8 and 22 kb were biotinylated by nick translation. After in situ hybridisation the biotin molecules in the probe served as antigens which bound affinity purified rabbit antibiotin antibodies. The site of hybridisation was then detected either by using fluorescein-labelled goat anti-rabbit IgG or histochemically, by using an anti-rabbit IgG antibody conjugated to horseradish peroxidase. The catalytic activity of the peroxidase enzyme converts diaminobenzidine into highly insoluble brown precipitates which can be detected by microscopy. The initial intention to use avidin conjugated to various indicator molecules for the detection procedure, was abandoned in favour of affinity-purified antibiotin antibodies, because of reports that avidin binds nonspecifically to DNA and chromatin (Heggeness, 1977). Although the immunofluorescent detection revealed hybridisation loci with speed and precision, the bleaching of the fluorescent signal within minutes after exposure to a high-intensity light source and its fading during storage made the immunoperoxidase detection superior, in that it provided a permanent record and it allowed detailed cytogenetic analysis when combined with Giemsa staining. It also allowed maximization of the signal-to-noise ratio by visually monitoring the reaction by light microscopy as it occurred. The described immunological technique had four advantages over autoradiographic procedures: speed, high resolving power, less background noise and the chemical stability of biotinylated probes which gave reproducible results for many months.

This methodology was applied successfully to the localisation of specific DNA sequences in mammalian metaphase chromosomes (Hutchison et al., 1982; Manuelidis et al., 1982). Brigati et al. (1983)

extended the methodology to the detection of viral DNA and RNA sequences in infected cell cultures and viral DNA in clinical tissue specimens. Increasing the length of the linker arm of the biotinylated nucleotide over that initially reported, improved the ability of both the antibody and avidin to interact with the biotinylated hybridisation probes (Brigati et al., 1983). Brigati et al., (1983) synthesized nucleotides in which the biotin molecule was attached to the C-5 position of the pyrimidine ring through linker arms that were 11, or 16 atoms long respectively which were designated as Bio-11-dUTP and Bio-16-dUTP respectively (see Figure 2). Biotinylated probes were used over periods as long as 18 months with high reproducibility. Brigati et al., (1983), favoured histochemical detection, using avidin-biotinylated horseradish peroxidase (ABC) complexes (Hsu et al., 1981). Streptavidin, a biotin-binding protein from Streptomyces avidini gave no detectable background as opposed to avidin. The melting temperature of biotinylated DNA duplexes was reported to be slightly lower than that of biotin-free DNA. The observation that biotin-labelled polynucleotides exhibited less nonspecific binding to tissues and chromosomes than did comparable radiolabelled probes allowed the use of high concentrations of biotinylated DNA probe, making it possible to decrease markedly the hybridisation times required to achieve any desired $C_o t$ value.

Biotinylated nucleotides became commercially available and many investigators adopted this method for non-radioactive in situ hybridisation studies. Manuelidis et al., (1984) studied the chromosomal and nuclear distribution of a HindIII 1.9kb human DNA repeat but failed to obtain signal with single-copy probes (e.g, a

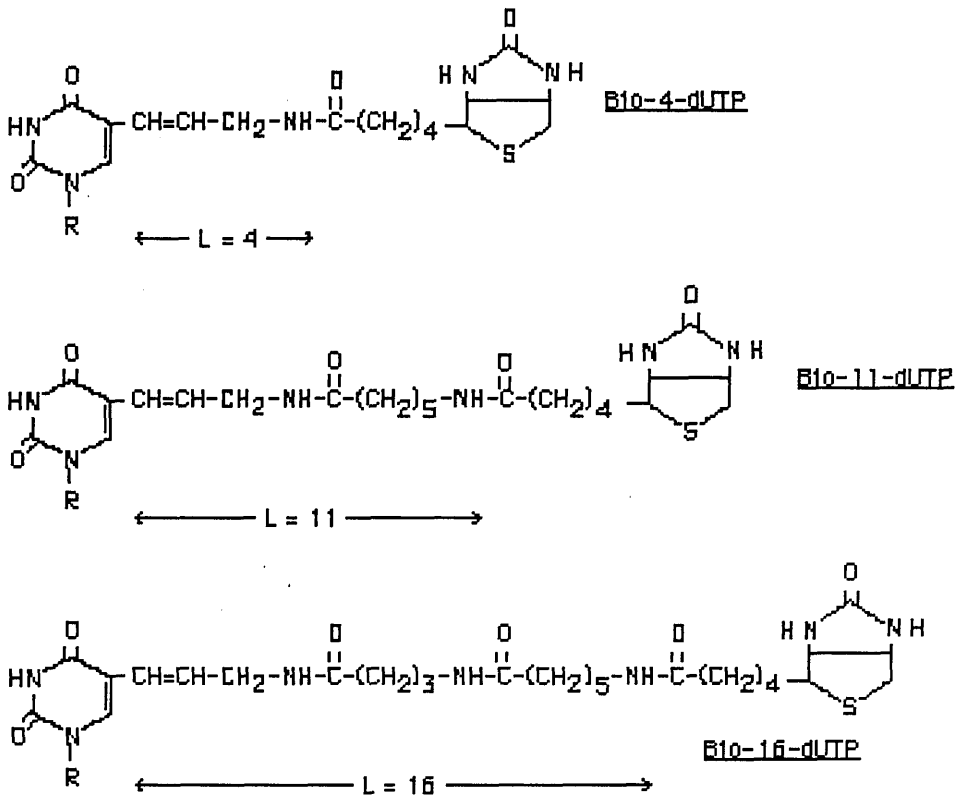


Figure 2. Structures of Bio-4-dUTP, Bio-11-dUTP and Bio-16-dUTP. R= deoxyribose 5'triphosphate (taken from Brigati et al., 1983).

1.5kb globin sequence). Burns et al., (1985) used a biotinylated Y-specific DNA probe for rapid sex determination of free and cultured amniotic cells, chorionic villus biopsies, peripheral white blood cells, sperm solid tissues and results could be obtained within 24 hours. The DAB/hydrogen peroxide signal was amplified by silver amplification, by modifying a method described previously by Gallyas et al., (1982). This amplification was based on the ability of DAB complexes to bind gold salts which when converted to gold sulphide, precipitate metallic silver and allowed the localisation of the autosomal copies (present in less than 100 copies) of the Y-specific sequence pY2.1, the H-ras-1 gene and finally the alphafetoprotein gene, a reputed single copy gene (Burns et al., 1985). Ambros et al., (1986) reported the detection of a 17kb unique sequence in plant chromosomes using biotin labelling. This study demonstrated that biotinylated probes in combination with reflection-contrast microscopy were superior to tritium-labelled probes. Pinkel et al., (1986) described a high-sensitivity fluorescence hybridisation that permitted the visualisation of two chromosomal domains in most interphase nuclei of XYY amniocytes using a 0.8kb Y-specific probe, and the identification of interspecies translocations in human-hamster hybrid cell lines when human genomic DNA was used as a probe.

1.5. Kinetics of in situ hybridisation

The kinetics of the in situ hybridisation can be followed by determining the mean number of grains as a function of time of hybridisation or the concentration of the probe used. The kinetics of the in situ hybridisation reaction have been studied by successful

quantitation of in situ hybridisation of ^{125}I-labeled 5S and 18+ 28S ribosomal RNA on polytene chromosomes by Szabo et al., (1975; 1977a; 1977b) and on diploid cells by Coté et al., (1980).

The rate equation for the in situ hybridisation reaction could be complicated by DNA-DNA renaturation during RNA-hybridisation. That was not found to be the case at the 5SRNA site, as preincubation of the slide under hybridisation conditions before addition of the radioactive 5SRNA did not change significantly the amount of the in situ hybrid formed, suggesting that DNA-DNA renaturation is not a competing reaction with the formation of RNA-DNA hybrid within the time-scale used for in situ hybridisation (Szabo et al., 1977a).

In an in situ hybridisation experiment, the RNA is in a large excess over the complementary DNA. Thus, the in situ hybridisation reaction should follow pseudo first-order kinetics. Kinetic data from 50 slides showed a reasonable fit of the expected rate equation but the rate was found to be five times slower than filter hybridisation for 5SRNA and three times slower for 18+28S rRNA. Birnstiel et al., (1972) found that the rate of filter hybridisation is inversely proportional to the complexity of the RNA when the rate is measured at the optimal temperature. The dependence of rate on the complexity of the RNA was approximately the same for in situ and filter hybridisation, a higher complexity giving a slower rate. The data taken together showed that the kinetics of the in situ hybridisation reaction are very similar to the DNA-RNA hybridisation reaction with immobilised DNA. The presence of proteins on the slide does not seem to greatly influence the type of reaction or the rate of reaction. Szabo et al., (1977a) also measured the thermal stability of the RNA-DNA in situ hybrid as expressed by its T_m value, the temperature

(in ^{o}C) at the midpoint of the transition where the hybrid is thermally dissociated, after completion of the hybridisation procedure through the final washes in 2xSSC. The value of T_m was similar to the one expected for a filter hybrid in the same buffer (McConaughy et al., 1969). Also as expected, if the hybrid was heated at a lower ionic strength the value of T_m is lowered by the expected amount. Thus it is likely that the in situ hybrid is a true DNA-RNA hybrid. The presence of unremoved proteins on the chromosomes did not significantly affect the thermal denaturation properties of the in situ DNA-RNA hybrid, therefore thermal stability may be used to characterize in situ hybrids as it is used for other RNA-DNA hybrids.

Similar studies were performed on human fibroblasts and leukocyte cells as a model for human diploid cell types by Coté et al., (1980) in order to determine whether the parameters of the in situ hybridisation reaction are the same for diploid chromosomes as they are for polytene chromosomes. In addition, in order to determine whether the degree of condensation affects the in situ hybridisation reaction, metaphase and interphase chromosomes were compared. The in situ kinetics could be fitted with a single first-order rate constant as with filter hybridisation but with 10 times slower rate. The kinetics appeared to be the same for fibroblasts and interphase leukocytes. When interphase nuclei and metaphase chromosomes were compared a somewhat faster rate of hybridisation was observed for the highly compact metaphase chromosomes. This study showed that characteristics of the in situ hybridisation of two different RNAs to diploid chromosomes were very similar to those found on Drosophila polytene chromosomes (Szabo et al., 1977a).

1.6 Efficiency of in situ hybridisation

It is possible in theory to use the method of in situ hybridisation to locate any gene provided that a suitable DNA sequence is available. For isotopic in situ hybridisation, the detection of the hybrids depends upon the ability to anneal sufficient radioactive probe at the site to be detected by autoradiography. Several factors contribute to the success of such an experiment including autoradiographic efficiency, hybridisation efficiency, cistron redundancy and specific activity of the probe.

Autoradiographic efficiency is the ability of the emulsion to detect energy released by radioactive disintegrations which appear as silver grains following development. The ideal situation is a one-to-one correspondence of radioactive disintegration to silver grains within the emulsion. The efficiency varies depending upon the energy of the particle emitted. For tritium, the autoradiographic efficiency has been estimated to be only 10%, i.e. only one out of every ten particles emitted is in fact detected by the emulsion. Radioactive iodine can be detected with 20% efficiency (Prensky et al., 1973; Prensky, 1976). However, due to the higher energy from decay of ^{125}I poorer resolution is achieved. Grains from decay of tritium will lie a maximum distance of 0.5μm from the source whereas in the case of ^{125}I this may be up to 16μm. In general the thinner the emulsion and the shorter the exposure time the better the point resolution. (for review of autoradiography techniques see Rogers 1967). The sensitivity of the emulsion is assessed by the measurement of number of grains per emergent electron. Ilford K2 emulsion produces grains of 0.21μ m diameter, is less sensitive than other emulsions but it does not accumulate background so rapidly as the

more sensitive emulsions. The number of grains per unit area must be controlled by adjusting either the exposure time or the concentration of the isotope administered so that the possibility of coincidence (one or more disintegrations resulting in a single observable silver grain) is small.

Hybridisation efficiency is the proportion of complementary sites available to which the probe actually binds. Szabo et al., (1977a) reported that only 5-10% of the chromosomal DNA is available to form hybrids in situ. This hybridisation efficiency is a function of how the slides were prepared and the conditions of annealing, but it was found approximately constant with a given procedure for both 5S RNA and 18+28S RNA over a different cell types (salivary glands, adult testis and adult ovaries). The low efficiency is likely to be due to the hybridisation procedure used. A four-fold increase was observed in the efficiency when chromosomes were not denatured but hybridised in 80% formamide/4xSSC at 55^{o}C, but extensive cytological damage was also seen. Thus it was concluded that the in situ hybridisation procedure is a compromise between cytological quality and hybridisation efficiency (Szabo et al., 1977a). The fact that not every possible specific hybridisation site reacts, can at least be partially explained by loss of chromosomal DNA during preparation and hybridisation. In a different study, in both leukocyte and fibroblast cell types and for metaphase and interphase nuclei, hybridisation efficiency was found to range from 5% to 15% for both 5S and 28S RNA (Coté et al., 1980). Thus neither the type of DNA sequence nor the degree of condensation of the chromatin is responsible for the low efficiency observed. Contrary to this is the observation that the efficiency of hybridisation to nuclei was four times that for

metaphase chromosomes (Henderson et al., 1974) possibly due to the chromatin being less condensed and therefore more accessible in the nucleus than in the chromosomes.

If the number of gene copies and specific activity and complexity of the probe are known, then the number of autoradiographic grains appearing over a site for a given exposure can be predicted. When nick translated DNA sequences cloned in a vector were used as probes, the observed hybridisation efficiency was markedly increased to 70% (Malcolm et al., 1977) due to the formation of probe-networks. The presence of 10% of dextran sulphate in the hybridisation solution was also reported to result in signal ten times more than expected in the absence of probe network formation (Gerhard et al., 1981).

1.7 Sensitivity of in situ hybridisation

The detection limits of in situ hybridisation were initially determined through quantitative model studies in which 5SRNA and rRNA sequences were hybridised to Drosophila polytene chromosomes (Szabo et al., 1977a) or on human diploid chromosomes (Coté et al., 1980). One can calculate that conventional in situ hybridisation and probe labelling methods would give only 0.12-0.6 silver grains per site of hybridisation for a unique gene of about 103 base pairs in a 60-day autoradiographic exposure time. This result would be obtained using probes having specific activities of $1\text{-}5 \times 10^8$ dpm/μ g (^{125}I-labelled probes). Thus the technique was limited to detection of repetitive gene families.

By using (cloned) nucleic acid probes of high specific activity made radioactive by nick translation ($1\text{-}6 \times 10^7$ ^{3}H dpm/μg) and fairly

short exposure times of 1 to 4 weeks, it was possible, by statistical analysis of 10 to 100 metaphase plates, to assign unique DNA sequences of 1.0 to 15.0 kb to specific chromosomal locations. The current sensitivity of radioactive in situ hybridisation is detection of target sequences as short as 0.5kb (Jhanwar et al., 1983) a sensitivity not yet obtained with non-autoradiographic procedures. Manuelidis & Ward (1984) reported that 20kb of target sequence was required at a single locus to generate a visible signal with the biotin-specific detection reagents employed, even with heavy atom intensification. They failed to obtain any signal with single-copy gene probes (e.g a 1.5kb globin sequence). Pinkel et al., (1986) also reported detection in the vicinity of 20kb. Unique and low copy number sequences were visualised but required extensive experimentation and sophisticated microscopy (Albertson et al., 1985; Ambros et al., 1986; Landegent et al., 1985b, 1986). Gold/silver amplification procedures of DAB products may bring single copy gene detection into the realm of bright field microscopy (Burns et al., 1985).

1.8 Applications

Although in situ hybridisation has been used for the detection of mRNA sequences in cells and tissue sections, diagnosis of viral sequences in pathological tissues, the identification of species specific chromosomes in somatic cell hybrids, cytogenetics and cancer genetics, this section is concerned only with its use in gene mapping.

1.8.1 Gene mapping by in situ hybridisation

Since first described in 1969, in situ hybridisation has been used primarily for the localisation of DNA sequences, initially to highly or moderately repeated sequences on both polytenes and diploid chromosomes. The DNA coding for 18S+28S ribosomal RNA (rRNA) has been localised in the nucleolus of the interphase nucleus or the nucleolus organising regions of metaphase chromosomes in Xenopus (Gall and Pardue, 1969; John et al., 1969), Drosophila (Pardue et al., 1970; Alonso 1973) and man (Henderson et al., 1972, 1973). The DNA coding for 5S rRNA has been localised in one band on polytene chromosomes of D. melanogaster (Wimber and Steffensen 1970; Szabo et al., 1977). tRNA genes were assigned to specific loci in Drosophil a first by Steffensen and Wimber (1971, 1972) satellite DNA of mouse (Pardue and Gall 1969; Jones 1970); Drosophil a (Hennig et al., 1970; Gall et al., 1971) and man (Jones and Corneo 1971; Saunders 1974).

The first factor that limited the application of hybridisation in situ for the localisation of unique sequences, was the need for high purity nucleic acid probes. In the case of relatively abundant nucleic acids (rRNA, 5SRNA, satellite DNA) sufficient quantities of highly purified probes could be isolated by conventional biochemical means. Small amounts of contaminating RNA species could produce a detectable signal over their gene sites that could be stronger than the signal generated from the major probe RNA species e.g. rRNA. Genes coding for specific proteins such as the histone genes (Pardue et al., 1971) and genes activated by heat shock (McKenzie et al., 1975; Spradling et al., 1975) were localised using the corresponding messenger RNA. Fortunately, the advent of recombinant DNA technology has essentially eliminated the problem of contaminating nucleic acid

species; pure probes for analysing any gene or genomic site of interest can now be prepared in large quantities.

The second limiting factor was the strength of the signal necessary for detection of the site of chromosomal location. Polytene chromosomes as found in Drosophila salivary glands, were ideal objects for initial in situ hybridisation experiments, because the genome in polytene chromosomes can be present in up to $1024(2^{10})$ copies and the DNA is topologically separated in hundreds of bands. It was clear however that for convenient and routine localisation of unique genes some enhancement of the signal produced by a single hybrid was required. Early claims for the detection of unique genes on diploid chromosomes were contested, primarily because the probes used in these studies had relatively low specific activities and should not have been able to produce a detectable signal at the gene site. Price et al., (1972) claimed the localisation by in situ hybridisation of the rabbit haemoglobin mRNA genes for globin on chromosomes 2 and 4 or 5, but this was heavily criticised by Bishop and Jones (1972) and Prensky and Holmquist (1973) on technical grounds. Henderson et al., (1978) concluded from analysing in situ hybridisation data from haemoglobin mRNA to mouse chromosomes that although the correct chromosomal sites could be identified, other sites were labelled as well so that the technology could not be relied upon to localise a gene of unknown location. Moreover, Deisseroth et al., (1978) localised the beta globin gene to chromosome 11 by solution hybridisation and somatic cell genetics.

However, it was shown that when unique sequences were cloned in a plasmid or a phage vector and used as a probe for in situ hybridisation significant hybridisation occurred at specific sites

(Malcolm et al., 1977). The signal was amplified through the formation of probe networks. If the hybridisation reaction was carried out in the presence of 10% dextran sulphate, the rate of probe network formation was accelerated. This was first demonstrated for the human alpha-globin gene (Gerhard et al., 1981; Barton et al., 1982), beta-globin gene (Malcolm et al., 1981) and human insulin gene (Harper et al., 1981) whose chromosome assignments were already known but which had not been regionally mapped, for a single copy anonymous DNA segment (D1S1) (Harper and Saunders, 1981) and later for the previously unmapped kappa light chain immunoglobulin genes which was localised to the short arm of chromosome 2 (Malcolm et al., 1982).

This final proof prompted further activity and in situ hybridisation became a classic method for gene mapping. Several unique genes were mapped to chromosomal loci using fairly short autoradiographic exposures between 5 and 22 days. Extremely high specific activities were not required as it was established that probe size for optimum network formation was more important than the specific activity of the probe. The localisation was still dependent on careful microscopy and the analysis of adequate numbers of chromosomes. The use of chromosome rearrangements and in situ hybridisation allows more precise gene localisation (Donlon et al., 1984; Magenis et al., 1985). In situ hybridisation is the best method to identify homologies between extensive chromosome regions (for example, Devine et al., 1985) thus providing additional insight into chromosomal organization.

1.8.2 Interphase nuclei topological investigations

Over the last few years a considerable body of evidence has been

obtained which indicates that the DNA of individual chromosome occupies focal territories or spattially cohesive domains within mammalian interphase nuclei (Hens et al., 1983; Schardin et al., 1985; Manuelidis 1985; Pinkel et al., 1986).

Studies of the arrangement of chromosomes in interphase are not only of academic interest but can also provide decisive information on the origin of frequent chromosome aberrations (such as translocations and aneuploidies) since it can be argued that chromosomes located close to each other in the interphase nuclei will be involved more frequently in reciprocal translocations than chromosomes situated at a greater distance. The value of in situ hybridisation with cloned DNA probes for the analysis of interphase arrangement of the chromosomes has been demonstrated (Rappold et al., 1984). Furthermore, the detection of chromosome aberrations in interphase nuclei has been reported (Cremer et al., 1986; Pinkel et al., 1986) an approach for which the term "interphase cytogenetics" has been coined (Cremer et al., 1986).

1.8.3 Comparative gene mapping

Comparative mapping studies with in situ hybridisation can confirm and extend homologies inferred from banding studies at the DNA level. Examples of these studies are the localisation of satellite DNA in the great apes (Mitchell et al., 1977), 18S and 28S ribosomal RNA in the great apes (Henderson et al., 1974), the 5S RNA in the great apes (Steffensen et al., 1974) and the Bkm sequences in human DNA and in mice (Jones and Singh, 1981).

1.9 Comparative gene mapping in the great apes

1.9.1 The karyotypes of the great apes

The evolution of man is a subject of considerable interest and as new methods have been devised for comparing physical and biological properties of species, these have been applied to a comparison of the great apes with man. In the late 1950's and early 1960's interest in the karyotypes of primates was aroused by the application of cytogenetic techniques developed primarily for investigating the human karyotype (Chu and Bender, 1962; Chiarelli, 1962; Hamerton et al., 1963). However the first correct counting of the chromosome number of the chimpanzee predated that of the human by approximately 15 years (Yeager et al., 1940). These early studies established that the chromosome number of the chimpanzee, gorilla and orangutan was 48 and not 46 as in man, and on the basis of size and shape parameters, attempted to compare individual human chromosomes to their supposed human counterparts. The general conclusion was that the chimpanzee karyotype most closely resembled that of man, and that the orangutan, although possessing the same chromosome number as the chimpanzee and the gorilla, appeared to have a very different karyotype.

The development of chromosome banding techniques in the early seventies allowed a detailed re-investigation of the relationship between the human karyotype and that of other primates which resulted in extensive banding information on the karyotypes of the great apes (Chiarelli & Lin 1972; de Grouchy et al., 1972; Turleau et al., 1972 and 1975; Bobrow & Madan, 1973; Dutrillaux et al., 1973; Egozcue

et al., 1973a, b; Khudr et al., 1973; Lejeune et al., 1973; Lin et al., 1973; Pearson, 1973; Turleau & de Grouchy, 1973; Warburton et al, 1973; Miller et al., 1974; Dutrillaux et al., 1975; Seuánez et al., 1976a, b; Seuánez 1979). These studies revealed a general homology of chromosomal bands in the four species. The chromosomes which are most similar and show the least differences are 1,5, 6, 13, 14, 15, 19, 20, 21 and 22; the X chromosome is essentially identical in the four species.

When chromosomes of different morphology are compared, it is sometimes possible to derive a chromosome of one species from a chromosome of another by assuming that a chromosome rearrangement has occurred. This assumption is made whenever a postulated rearrangement produces a morphologically similar chromosome, with a similar G- or R-banding pattern. Presumptive breakpoints in chromosomes are identified in relation to bands, and the direction in which this event has occurred can sometimes be deduced on the principle of parsimony, which assumes that a network of descent can be explained by the fewest number of changes. The kinds of chromosome rearrangements which have presumably occurred within the Hominidae are the pericentric inversion, the paracentric inversion and the telomeric fusion. In these lines, banding studies have suggested a common ancestor for chimpanzee, gorilla and man and counterparts for each chromosome of the human complement in the gorilla, chimpanzee and orangutan. Since different authors were using different numbering systems a standard numbering system based on chromosome morphology was presented in the Paris Conference (1971) Supplement (1975)(see table 1). Using high resolution banding Yunis and Prakash (1982) were able to account for every nonheterochromatic band of the great apes

Homo sapiens (HSA)	Pan troglodytes (PTR)	Gorilla gorilla (GGO)	Pongo pygmaeus (PPY)
1	1	1	1
2	12,13	12,11	12,11
3	2	2	2
4	3	3	3
5	4	4	4
6	5	5	5
7	6	6	10
8	7	7	6
9	11	13	13
10	8	8	7
11	9	9	8
12	10	10	9
13	14	14	14
14	15	18	15
15	16	15	16
16	18	17	18
17	19	19	19**
18	17	16	17
19	20	20	20
21	22	22	22
22	23	23	23
X	X	X	X
Y	Y	Y	Y

** This banding homology has not been completely resolved

Table 1: Proposed chromosome homologies of great apes and man, taken from ISCN (1985)

and man not surprisingly, since more than 50 genes were shown by then to be located on homologous chromosomes and chromosome bands of the four species.

Studies of DNA reassociation kinetics, protein structure and antigenicity, histocompatibility antigens and blood groups all indicate that chimpanzee, gorilla and man share a substantial common ancestry and that orangutan diverged earlier from this lineage. Furthermore, almost total homology of single copy DNA and amino acid sequence of proteins has been found in man and chimpanzee suggesting a very close evolutionary relationship between the two. These findings conflict with the view based primarily on anatomic and behavioural data that man diverged separately from the evolutionary line leading to the great apes. The Yunis and Prakash analysis supported the molecular evidence that the great apes and man belong to the Hominidae family which separates into the Ponginae (orangutan) and Homininae (gorilla, chimpanzee and man) subfamilies.

1.9.2 Comparative mapping

The rate at which comparative mapping data is accumulating has increased dramatically during the past few years. The first comparative report at the Baltimore Conference (1975) contained data on three primate species and the mouse. The report at HGM 9 (1987) contained information on eleven primates, the mouse, ten other eutherian mammals, marsupials and monotremes and lower vertebrate such as fish. By the end of HGM 9.5, 66 genes were mapped in the chimpanzee, 41 in the gorilla and 35 in the orangutan (see Table 2).

Comparative gene mapping studies have demonstrated that despite Robertsonian fusions, inversions, translocations and gene

Species	Haploid number	Known linkage and syntenic groups	Number of mapped genes
Human	23	24	(1479)
Chimpanzee	24	(20)	66 (65)
Gorilla	24	(22)	(41)
Orangutan	24	(15)	(35)

Table 2: Mammalian gene maps. This information was primarily compiled from proceedings of the HGM Workshops and genetic Maps, 1987 (S.J. O'Brien, ed). Number in parentheses give data available at the end of HGM 9 (1987). Numbers outside parentheses in column three, indicate the number of assignments since HGM9. For the purposes of this table, a mapped gene is defined as any locus for which a linkage group or chromosomal assignment has been determined. Also included are loci that have been shown to be independent of all other linkage or chromosomal groups based on studies of a single panel of somatic cell hybrids. To be included in this table it is not necessary for the homologous gene to have been mapped in the human (taken from Lalley et al 1988, HGM 9.5)

duplications that took place during the gradual evolutionary divergence of mammalian karyotypes a number of ancestral linkages have been conserved in widely divergent mammalian species during evolution. Annealing studies can confirm and extend banding studies.

Comparative gene mapping studies have not only showed that homologous chromosomes carry the same genes but they have also confirmed or indicated that chromosomal rearrangements have taken place during evolution. For example Magenis et al., (1987a) using in situ hybridisation localised the genes for alpha-fetoprotein (AFP) and albumin (ALB) which are located in the same region of the proximal long arm of chromosome 4 in the human, to chimpanzee proximal short arm of chromosome 3 (p13-p12) showing that the synteny of ALB and AFP has been maintained and that there has been an inversion of chromosome 3 (homologous to human chromosome 4) in the divergence process between human and chimpanzee. Such an inversion was postulated to have taken place from banding studies (Dutrillaux 1979; Yunis and Prakash, 1982).

Further studies of which linkage groups have been conserved or disrupted over a wide range of species are important not only for academic reasons e.g. understanding the evolution of the mammalian genome, but also for developing animal models for human genetic disorders, and extrapolating biomedical data from laboratory animal to man (Lalley & McKusick, 1985).

1.9. 3 Chromosome 1

Chromosome 1 in man has a metacentric homologue in the great apes whose arms show similar G-band patterns to the human chromosome 1. Turleau & de Grouchy (1973) were the first to note that the

secondary constriction region of human chromosome 1 is not present in the chimpanzee and this was later definitely confirmed by de Grouchy et al., (1973) and Bobrow & Madan (1973). In the great apes in which the secondary constriction is not present, the arm ratio is therefore the reverse of that in man. Thus the short arm of their chromosome 1 is homologous to HSA 1q, and the long arm of their chromosome 1 is homologous to HSA 1p. G-band or R-band homologies between chromosome arms are very evident between man and the great apes (see Figure 3). Bobrow & Madan (1973) noticed that the G-11 staining region which is present in the human 1q, is present in the arm of the chimpanzee corresponding to 1p and this finding made them postulate that this region has possibly undergone a small pericentric inversion. Egozcue et al., (1973b) also postulated the occurrence of a pericentic inversion after comparing human and chimpanzee chromosomes. However Turleau & de Grouchy (1973) using a more refined R-banding technique and the trypsin banding reported that except for the absence of the paracentric constriction in the chimpanzee the otherwise perfect homology of banding in the long and short arms excludes the existence of a pericentric inversion. Dutrillaux (1979) mentioned a paracentric and a pericentric inversion to have taken place from the Pongidae (great apes) to man and the aquirement of heterochromatic material adjacent to the centromere of chromosome 1 in man. Yunis and Prakash (1982) using high resolution G-banded chromosomes from late prophase (1000 bands per haploid set) suggested that a small pericentric inversion has taken place in the human chromosome 1. They also found that chromosome 1 of the orangutan and chimpanzee are identical and that a rearrangement has occurred in the distal end of the long arm of the gorilla chromosome 1.

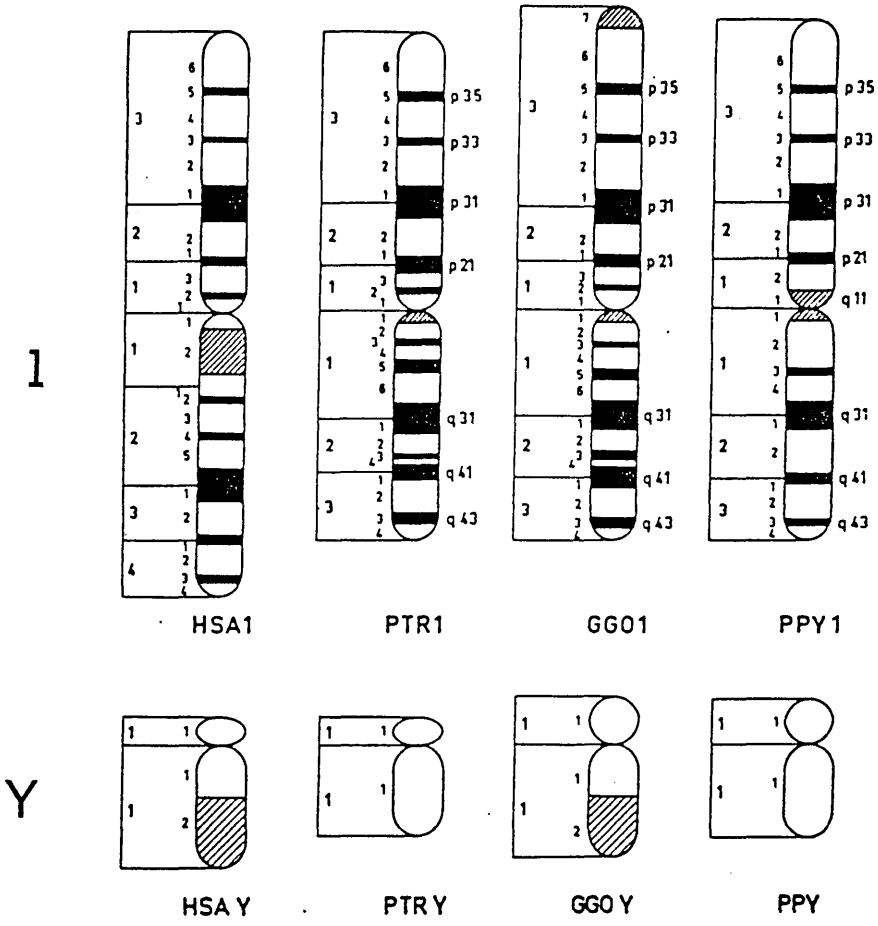


Figure 3. Chromosomes 1 and Y of man and the great apes. Diagrammatic representation of chromosomes 1 and Y of Homo sapiens, Pan troglodytes, Gorilla gorilla, and Pongo pygmaeus, arranged from left to right as observed with the G-staining technique. Open areas show negative or pale staining G bands, solid areas show positive G bands while crosshatched areas depict variable bands. The ape chromosome 1 has been inverted to show the similarities in banding pattern with human chromosome 1.

[taken from ISCN (1985)]

Comparative gene mapping studies have confirmed the remarkable similarity between the human and great ape chromosome 1. Finaz et al., (1977) showed by cellular hybridisation that in the great apes chromosome 1 carries the same genes for ENO-1 and PGM-1 (on 1p in man) and Pep-C (on 1q in man). From these data as well as findings in two more distantly related species, the baboon and the African green monkey, chromosome 1 appears to be a remarkable "paleochromosome" that has maintained its banding pattern and has carried the same genes for some 50 million years (Finaz et al., 1977). Moreover, the 5S rDNA genes which in man are located at the terminal region of the long arm of chromosome 1 (Steffensen et al., 1974; Atwood et al., 1975; Fennel et al., 1979) are placed at the terminal region of the short arm of chromosome 1 in the great apes, thus the same site as in man (Henderson et al., 1976).

1.9.4 The Y chromosome

The Y chromosome of the homonids shows more differences from species to species than almost any other chromosome (Miller, 1977; see figure 3). The Y of the gorilla is longer than the human and has a brightly fluorescing heterochromatic region at the end of the long arm. The gorilla is the only mammal other than the human known to have this bright fluorescing region on the Y. Interestingly Seuanez (1979) failed to observe any size variation of this region, contrary to the situation observed in man. The chimpanzee Y chromosome is a very small submetacentric, with a DA/DAPI positive heterochromatic band at the end of the very small short arm. This region shows a certain interindividual variability in size (Schmid, 1985). The entire short arm stains heterochromatically with Giemsa-11 (Bobrow &

Madan, 1973). The Y of the orangutan shows a large C-band positive paracentromeric region and a smaller one at the end of the long arm. Nothing is known about possible variability of this region (Seuánez (1979).

In earlier banding studies the Y chromosome was believed to differ in each species and no homology could be found. Yunis and Prakash (1982) reported that when finely banded chromosomes are used and only the noncentromeric and nontelomeric heterochromatic regions of the Y chromosome are considered, a basic homology is observed. They also suggested that the orangutan chromosome Y may differ from the human, by a pericentric inversion (p11.2-q11.23). Recent evidence from replication studies however suggested that structural alterations have occurred in both the euchromatic and heterochromatic parts of the Y chromosome which is assumed to have undergone rapid evolution within higher primates (Weber et al., 1986). The advent of recombinant DNA technology started a new phase of investigation in which theories of karyotype homologies can be rigorously investigated at the molecular level. Numerous single-copy sequences which share homologies between the human X and Y chromosomes were shown to be present only on the X chromosome of the great apes (Page et al., 1984). Furthermore, repeated sequences on the human Y are not present on the Y chromosome of the great apes (Kunkel & Smith, 1982; Wolfe et al., 1985).

1.10 Aims of the current project

The overall aim of this project was to develop the technique of in situ hybridisation to map cloned DNA sequences to specific chromosome regions using both tritium and biotin labelling. Specifically the aims were as follows:

1. To optimise the conditions for ^{3}H-labelling in order to localise cloned DNA sequences to specific chromosome regions.
2. Explore the possibility of using biotin-labelling for non-isotopic in situ hybridisation and use this technique for the localisation of cloned DNA sequences to specific chromosome regions.
3. Determine the chromosomal location of Y-specific sequences in 46,XX males and two 45,X/46,X +min males that were previously shown to have such sequences present in their genome.
4. Assess the applicability of in situ hybridisation in clinical diagnosis.
5. Perform comparative mapping studies in the great apes, gorilla (Gorilla gorilla), chimpanzee (Pan troglodytes) and orangutan (Pongo pygmaeus).

CHAPTER TWO

MATERIALS & METHODS

2.1 Materials

2.1.1 General

All aqueous solutions were prepared in water deionised by a Millipore "Milli-RO 15" water purification system. If required, solutions were sterilised in a pressure cooker at 15lbs/in^2 for 15-20 minutes. Solutions for the biotinylated in situ hybridisation were millipored if required, with ACRODISC sterile disposable filters, 0.2µm manufactured by Gelman Sciences.

Glassware, plastics and tips for micropipetting were sterilised in a Sybron/Drayton Castle industrial autoclave for 20 minutes and dried in an 80^{o}C oven for 1-2 hours prior to use. Glassware for in situ hybridisation were tissue culture washed.

Eppendorf tubes (1.5ml and 0.5ml) were supplied by Sarstedt, as were all tips for micropipetting. Micropipettes were Gilson "Pipetman" type.

Microscope glass slides and microscope glass cover slips were supplied by Chance propper LTD; slides were soaked in Decon overnight, then rinsed thoroughly in running tab water. They were stored in 70% ethanol until required, rinsed thoroughly in tap water and finally in distilled water before use. 22x64mm cover slips were kept in 70% ethanol; 22x32mm and 22x22mm ones were kept in HCl and rinsed in distilled water prior to use.

2.1.2 SOLUTIONS

Calf Serum + Glutamine (CSg) culture medium

10ml of Nutrient Mixture F-10 (HAM) with L-glutamine (Gibco)

10ml Newborn Calf Serum

0.4ml Sodium bicarbonate (Gibco)

2ml phytohaemagglutinin (M Form; Gibco)

3ml Heparin (Leo Laboratories Limited)

1ml penicillin-streptomycin (Gibco)

Foetal Calf Serum or foetal Bovine (FB) culture medium

10ml of Nutrient Mixture F-10 (HAM)

10ml Foetal Calf Serum (Gibco)

0.4ml Sodium bicarbonate (Gibco)

2ml phytohaemagglutinin (M Form; Gibco)

3ml Heparin (Leo Laboratories Limited)

1ml penicillin-streptomycin (Gibco)

L-broth

10g Bacto-tryptone

5g Bacto-yeast extract

10g NaCl

0.1% Maltose

10mM $MgSO_4$

Ampicillin

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

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

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

Electrophoresis buffer (E buffer)

40 mM Tris-acetate pH 8.0

20 mM Sodium or potassium acetate

1 mM Na_2EDTA

Standard Saline Citrate (20xSSC)

Sodium chloride 175g

Tri-sodium citrate 88g

Dilute to 1 litre with distilled water

TE

10 mM Tris.HCl pH8.0

1 mM Na EDTA pH8.0

Deionised formamide

Formamide was deionised by stirring at room temperature for 4-5 hours with approximately 5g of Bio-Rad mixed bed resin (20-50 mesh). It was filtered twice trough Whatman Number one filter paper, aliquotted and stored at -20°C in eppendorf tubes covered with aluminium foil.

Phenol

Phenol was distilled and stored at 4°C until required.

8-hydroxyquinoline was added to give a yellow colour, which if lost, would indicate oxidisation of the phenol.

Trichloroacetic acid (TCA) solution

10% (w/v) TCA in water. Due to the corrosive nature of this reagent extra care was taken during its preparation.

Hybridisation buffer

50% (v/v) formamide 0.6 M NaCl, 5mM Hepes , 1mM EDTA, pH7.6

10% dextran sulphate

Denaturation buffer

60% (v/v) formamide 10^{-4}M EDTA, 5mM Hepes, pH 7)

RNase

Pancreatic Ribonuclease A (Sigma) was dissolved in 2xSSC at a concentration of 100µg/ml. It was boiled for 3 minutes to remove any contaminating deoxyribonucleases, cooled down at room temperature, aliquotted and stored at -20°C.

Carrier DNA

Salmon testis DNA was dissolved in deionised water (10mg/ml). When completely dissolved, it was sonicated at high power until solution was no longer viscous. This was done on ice with short bursts of sonication. The solution was then boiled for 10 minutes, chilled on ice and kept at -20°C.

Solutions for biotinylated in situ hybridisation

Hybridisation buffer stored at -20°C (10 ml)

50% formamide	5 ml de-ionised formamide
10% dextran SO_4	2 ml 50% Dextran sulphate
2 x SSC	1 ml 20 xSSC
0.1 mM EDTA	2 µl 0.5 M EDTA
0.05 mM Tris ph 7.6	5 µl 1 M Tris pH 7.6
100 µg/ml denatured salmon sperm DNA	200 µl 5 mg/ml denatured salmon sperm DNA
	2 ml H_2O

Buffer 1 (B^O1, 500 mls)

50 ml 1 M Tris pH 7.6

10 ml 5M NaCl

0.2 g $MgCl_2 6H_2O$

250 µl Triton X 100 (Sigma)

Stir for 10 minutes

Buffer 2 (B^O2, 100 mls)

2.25 bovine serum albumin (Fraction V)

75 ml Buffer 1

dissolved at room temperature without agitation.

Buffer 3 (B^O3, 300 mls)

3.63 g Tris base

6 ml 5 M NaCl

3.04 g $MgCl_2 6H_2O$

(pH to 9.5 with concentrated HCl)

Stop buffer (100 mls)

2 ml 1M Tris pH 7.6

1 ml 0.5 M EDTA pH 8

97 ml H_2O

Stock solutions

50% Dextran SO4

2.5 g dextran sulphate in 3 ml H_2O, requires time to "wet" and frequent mixing. Make up to 5 ml.

5 M NaCl

146.1 g in 350 ml H_2O, stir (will take time as near saturation point). Make up to 500 mls.

1 M Tris

60.55 g add 400 ml H_2O, stir and pH with concentrated HCl (approximately 30 ml for pH 7.6)

0.5 M EDTA

18.61 g to 80 ml H2O, stir and pH with solid NaOH (approximately 2 g for pH 8.0). Solution will not dissolve until pH 8. Make up to 100 ml.

2.1.3 Cell lines used in this project

46,XX males: Lymphoblastoid lines from RH, JM, AG, TA, JT, GA, WB, DR, and KS were established and maintained in the Duncan Guthrie Institute of Medical Genetics, Yorkhill Hospital. The clinical features of these cases were described by Ferguson-Smith & Affara (1988).

45,X males: RW was a 45,X/46,X+mar lymphoblastoid line established in this department. The clinical features of this case are described in Ferguson-Smith et al.,(1987).

15p+ polymorphism: Following two raised serum AFP values, a patient was referred for amniocentesis. The amniotic fluid AFP results were normal. However, chromosome analysis of cultured amniotic cells revealed that the foetus has a 46,XX karyotype with additional material on the short arm of one homologue of chromosome 15 (designated 15p+). Analysis of the chromosomes of the father revealed a 46,XY karyotype with a similar polymorphism to that found in the amniotic fluid

Great ape lines:

CP81: male orangutan, skin, normal. Line received from the American Type Culture Collection (ATCC), no: CRL 6303. Duncan Guthrie Institute number (DGI) 882543.

The following cell lines were received from the Galton Laboratory, Department of Genetics and Biometry, University College London , a gift from Dr Joy Delhanty.

GLPT1: skin, normal male chimpanzee (DGI No 872508)

GLPP1: skin, normal male orangutan (DGI No 872444)

GLPP3: skin, normal male orangutan (DGI No 872445)

GLGG1: skin, normal male gorilla (DGI No 872509)

GLGG2: skin, normal female gorilla (DGI No 872510)

2.1.4 DNA sequences used in this project

The following thirteen DNA sequences were used in this project (see also table 3):

phrS28-1: A 7kb insert containing the genes for 28S ribosomal RNA cloned at an EcoR1 site of plasmid pAT153; obtained from the Beatson Institute, Glasgow.

pY3.4: A 3.4kb fragment cloned at an HaeIII site of pUC19 isolated by Lau et al., (1984).

GMGY10: A 4.5kb insert cloned at a HindIII site of pUC19 isolated in this department from the Lawrence Livermore flow-sorted Y chromosome library by Kenny Kwok (Affara et al., 1986a).

GMGY7: A 3.5kb insert cloned at a HindIII site of pUC19 isolated in this department from the above library by Kenny Kwok (Affara et al., 1986a).

P2F2: A 1.7kb insert cloned at an EcoR1/HindIII site of pBR328 isolated from a chromosome 19-specific library by L Meredith and characterised in this department (Affara et al, 1986a).

GMGY3: A 1.3kb insert cloned at an HindIII/PvuII site of pUC19 isolated in this department by Kenny Kwok from a Y-specific library, constructed from a hamster human hybrid line containing the Y chromosome as the only identifiable human chromosome and characterised by Affara et al., (1986a).

GMGY4b: A 1.8kb insert cloned at a HindIII site of pUC19 isolated in this department from the flow-sorted Y chromosome library and characterised by Mike Mitchell (Affara et al., 1986a).

JG51: A 4.7kb insert isolated from the Lawrence Livermore flow-sorted chromosome 21 library in this department by Galt (1988), cloned in pUC19 at a HindIII site.

JG73: A 3kb insert cloned in pUC19 at a HindIII site isolated in this department from the above library by Galt (1988).

p72: A 3.2kb insert isolated in this department from the Lawrence Livermore flow-sorted chromosome 21 library by Galt (1988) cloned at a HindIII site of pUC19.

GMGXY8: A 3kb insert cloned at a HindIII/BglI site of pUC19, isolated from the Lawrence Livermore flow-sorted Y chromosome library and characterised in this department by Kenny Kwok (Affara et al., 1987)

PLB4: A 2.4kb insert containing a human transfer RNA gene for glutamic acid cloned at a HindIII site of pAT153, isolated by Goddard et al., (1983) from a human fetal liver genomic library and localised to chromosome 1p36 by Boyd et al., (1989)

U1: A 3.4 kb insert containing the 5′ flanking region of a human U1 small RNA gene localised to chromosome 1p36 by Lund et al., (1983).

2.2.1 Preparation of chromosomes

2.2.1 Preparation from peripheral lymphocytes

Samples of 5ml of venous blood were removed with a sterile syringe then transferred to a sterile Pularin brand Heparin B.P. bottle containing 100 units of dried heparin (Evans Medical Limited). The bottle was rolled gently between the hands to mix the blood and heparin to prevent clotting. If the blood sample was donated from a friend it was used right away. Otherwise it had been stored at $4^{o}C$. Experience however showed that blood stored for prolonged periods did not yield many mitoses.

probes	HGM name	insert	vector	Reference
phr28S-1	-	7.0kb	pAT153	
pY3.4	DYZ1	3.4kb	pUC19	Lau et al (1984)
GMGY10	DYS59	4.5kb	pUC19	Affara et al (1986a)
GMGY7	DYS58	3.5kb	pUC19	Affara et al (1986a)
p2F(2)	DYS25	1.7kb	pBR328	Affara et al (1986a)
GMGY3	DYS13	1.3kb	pUC19	Affara et al (1986a)
GMGY4b	DYS51	1.2kb	pUC19	Affara et al (1986a)
JG73	-	3.0kb	pUC19	Galt (1988)
JG51	D21S89	4.7kb	pUC19	Galt (1988)
p72	D21S92	3.2kb	pUC19	Galt (1988)
PLB4	TRE	2.4kb	pAT153	Goddard et al (1983)
GMGXY8	DXYS34	3.0kb	pUC19	Affara et al (1987)
U1	RNU1	3.4kb	pUC19	Lund (1983)

Table 3: DNA sequences used in this project

-: a HGM name is not available

Chromosome spreads were prepared from phytohaemagglutinin (PHA) stimulated blood cultures after Moorhead et al., (1960) as follows: A 0.5ml sample of blood was dispensed into sterile bottles containing 4.5ml tissue culture medium with 0.1ml phytohaemagglutinin (M-form; Gibco). Cultures were incubated at 37°C in an incubator for 72 hours. Then, 0.2 ml of an 80μg/ml solution of colcemid (CIBA) was added and incubation continued for 1 hour 15 min. The incubation time was later reduced to 55 minutes. Cell cultures were transferred to centrifuge tubes and centrifuged at 800 rpm for 5 minutes. The supernatant was "pipetted off" and the cell pellets were gently resuspended in 5-10ml of hypotonic solution (0.075 M KCl) prewarmed to 37°C. The cells were incubated at room temperature for 10 minutes. This time was later increased to 15 minutes. Following centrifugation at 800 rpm for 10 minutes the supernatant was removed and the cells were resuspended in 5-10ml of freshly prepared cold 3:1 methanol:glacial acetic acid.

Cells were spun for 5 min at 800 rpm , the supernatant was replaced with 10ml of fresh fixative, the tubes were sealed with parafilm and were left in -20°C freezer for a minimum of 20 minutes to improve spreading. The cells were then centrifuged at 800rpm for 5 minutes and fresh fixative was added twice more. Following the last centrifugation the cells were resuspended in several drops of fresh cold fixative to make a cloudy suspension. One drop of cell suspension was dropped from a height of 0.5-1 metre onto a wet glass microscope slide, cleaned as described in section 2.1.1. Slides were air-dried overnight at room temperature and were stored in boxes at room temperature until they were banded.

2.2.2 Preparation of chromosomes from lymphoblastoid cell lines

Established lymphoblastoid cell lines transformed with Epstein-Barr virus, were grown in HAMs F10 (Gibco) + 10% fetal calf serum + 10% new born fetal calf serum + 1% penicillin/streptomycin (Gibco). Cell lines were grown until a healthy dividing population was obtained with around $5x10^6$ cells per 10 ml of culture medium. With a 10ml pipette 10 ml of cell/media mixture was removed from culture growing in a 260 ml bottle and were placed in a small 60 ml bottle. Ten ml of fresh 10% FCS media was added. This was left for 24 hours in a 37°C CO_2 incubator. Colcemid was added (0.1ml in 2ml of media) and the cultures were placed back into the CO_2 incubator for 20-30 minutes. The cultures were shaken well, then transferred in centrifuge tubes and were spun at 1000rpm for 5 minutes. The supernatant was poured off and the cell pellet was resuspended in KCl slowly, drop by drop and incubated for 12 minutes. Three drops of fresh fixative were added and the cultures were centrifuged for 5 min at 1000rpm. The cells were resuspended in 10ml of fixative and were left at 4°C overnight as this should improve spreading. Following centrifugation, fresh fixative was added and this was repeated three times. One drop of cell suspension was dropped on wet glass microscope slides and were air-dried at room temperature. Chromosomal preparations were stored in boxes at room temperature.

2.2.3 Preparation of chromosomes from skin fibroblasts

Fibroblast cells were grown in culture bottles in the same medium as for lymphoblastoid lines, until the monolayer was confluent. The cells were then trypsinised and harvested using standard methods and one drop preparations were obtained. Slides were

stored at $4^{o}C$.

The quality of the chromosomal preparations is critical for in situ hybridisation. Splashes should be made such that nuclei and chromosomes are well separated from one another and surrounded by a minimum possible amount of cytoplasm and tissue.

Chromosome morphology is the relative contrast, integrity and overall quality of the chromosomes. Donlon (1986) described four different categories of chromosome morphology. Category I chromosomes have low contrast, a fuzzy appearance and sometimes have separate chromatids. Category II are grey and of medium contrast, the chromatids are together and the borders of the chromosomes are sharp. Category III chromosomes are similar to category II, except that they are dark and of very high contrast. Category IV chromosomes are hyaline, or "glassy" and appear as balloons. The most successful preparations for in situ hybridisation will be those from Categories II and III. Chromosomes from the last category will not even survive the denaturation step and may even appear as "ghosts" at the end of the procedure, similar to over-denatured C-banded chromosomes.

To ensure that the chromosomal preparations met the above criteria, they were carefully examined under phase microscopy before being used for an in situ experiment. That was especially important with the biotinylated method where the hybridisation signal is visualized under phase microscopy and can be obscured by debris or dust which under phase appear as dark objects.

2.2.4 Lipsol banding prior to hybridisation

The chromosome banding method used in this project for chromosome identification was G-banding before in situ hybridisation

using lipsol (Elliot 1980). Freshly prepared chromosome preparations were banded by treatment for 8-15 sec in a 1% aqueous solution of the detergent Lipsol (LIP Equipment Services Ltd). Slides were then washed thoroughly with isotonic saline solution and stained for $2\frac{1}{2}$ minutes using filtered Leishmanns stain (1+4 in pH 6.8 phosphate buffer). They were rinsed in buffer and air-dried. Complete, well spread, well banded metaphase spreads were located under the microscope and photographed under phosphate buffer (pH 6.8). A coverslip was used to prevent oil getting into contact with the slide, and the vernier readings were noted for relocation of the cells after hybridisation. Immersion oil was wiped off and the coverslip was removed by rinsing in phosphate buffer. Slides were destained through an alcohol series, then dried, and stored at $4^{o}C$ until they were used for hybridisation. The area of slide with photographed metaphases was marked with a diamond pen.

Although ageing of chromosomal preparations is recommended by some investigators before they are used for in situ hybridisation, such a step was not included in this project. For the biotinylated method, slides were successfully used for hybridisation straight after destaining without being stored.

The lipsol-banding method produces a satisfactory G-banding pattern which allowed precise identification of individual chromosomes. Unfortunately, the method does not always work every time with every chromosomal preparation for unknown reasons. Experience showed that fresh chromosomal preparations, 1-2 days old, band best with as little treatment in lipsol as 6-8 seconds. After a week, the time in lipsol needs to be increased to 15-30 seconds. Chromosomal preparations older than this do not band well even at

increased time in lipsol. Finally some chromosomal preparations simply do not band and if this is the case, chromosomal preparations should be made from a different blood sample.

2.2.5 Mild treatment in trypsin

Trypsin-banding which is routinely used for chromosome analysis should be avoided for in situ hybridisation chromosomal preparations as the enzyme treatment can remove much of the DNA from the chromosomes. Garson et al., (1987) however reported the successful use of a mild trypsinisation method for purposes of in situ hybridisation. This method was used in this project to band chromosomal preparations from the great apes at times that lipsol-banding would not work. The method is as follows:

Slides bearing metaphase spreads were incubated for 15-120 seconds at $37^{o}C$ in phosphate buffered saline pH 7.4 (PBS) containing $1x10^{-7}$% w/v trypsin. Following a further 1 hour incubation in PBS at $60^{o}C$, G-banding was completed by staining the slides for 8 minutes with 2% Giemsa (Gurr) in pH 6.8 phosphate buffer (Gurr). G-banded metaphases were located and photographed under pH 6.8 buffer. Giemsa stain was removed with ethanol, air-dried and stored at $4^{o}C$.

For this technique, the age of slides is immaterial.

2.2.6 Storage of slides

Slides to be used for in situ hybridisation were stored at $4^{o}C$ in a dessicator as recommended by Gosden et al (1975a). There is considerable controversy over the question of storage of slides before in situ hybridisation. Some investigators reported to have used archive material up to 5 year old, but in this project, slides

which were left at room temperature up to two years did not give positive result (see section 3.5.2)

Throughout the hybridisation procedure slides were handled with care and moved gently from one solution to another in a glass slide rack wherever possible, in order to prevent loss of material from the surface of the slide.

2.3 Preparation of DNA probes

Plamid DNA for the probes used had to be propagated either from glycerol stocks or by transforming bacterial cells and then extracting the plasmid DNA.

2.3.1 Preparation of competent cells and bacterial transformation

It is necessary to use so-called competent cells for the transformation of E.coli strains which are prepared by treatment of a culture in the logarithmic phase of growth with $CaCl_2$ at 0°C.

The strain of bacteria used for transformation was E.coli JM83. An aliquot of 10µl from a glycerol stock of these bacteria was inoculated into 5ml of prewarmed (37°C) L-broth in a 30ml sterile universal. The culture was incubated with shaking overnight at 37°C. From the overnight culture 1ml was inoculated into 100ml of prewarmed L-broth and was grown to an $O.D._{600}$ 0.6. This was reached in approximately 2 hours and represents about $6x10^8$ cells/ml. The cells were spun down in chilled 30ml plastic universals in an IEC Centra-7S benchtop centrifuge at 3,500 rpm for 10 minutes at 4°C. The supernatant was poured off and each of the pellets was gently resuspended in ½ volume (50ml)of sterilised 50mM $CaCl_2$ pre-chilled on

ice. The cells were left on ice for 20 minutes, were gently spun down and were resuspended in 1/15 of the original volume volume cold 50 mM $CaCl_2$.

These competent cells were maintained at 4°C for 12-24 hours. During this period the transformation efficiency increases fourfold to sixfold.

2.3.2 Transformation by the Calcium Chloride procedure as adopted by Galt (1988)

Flat bottomed sterile bijoux bottles were chilled on ice before use. Keeping the bijoux on ice, plasmid DNA in 100µl of TE was mixed with 200µl of competent cells and the mixture was kept on ice for 25 minutes, heat-shocked at 42°C for 2-3 minutes and kept at room temperature for 10 minutes. Prewarmed L-broth (0.7ml) was added to the above and incubated with shaking for 1 hour at 37°C in the orbital shaker. About 100µl of this mixture was spread on an Amp/X-Gal plate. The plates were left to incubate overnight and then stored at 4°C until colonies were picked.

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

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

2.3.3 Recombinant selection

The bacterial host strain JM83 lacks part of the beta-galactosidase gene called lac Z and cannot synthesize this enzyme. This defect is complemented however, by the presence of the lac Z′gene on the pUC series of vectors. Cloning into such plasmids involves insertional inactivation of the lac Z′gene and recombinants are distinguished by their inability to synthesize beta-galactosidase. This is assayed during transformation by adding a lactose analogue, X-gal, which is broken down by beta-galactosidase to give a product which is blue in colour. If X-gal is added to the agar along with ampicillin then non-recombinant colonies, the cell of which will synthesize beta-galactosidase, will be coloured blue. Recombinants with a disrupted lac Z′ gene are unable to synthesizebeta-galactosidase will therefore be white.

2.3.4 Rapid, small-scale plasmid preparation by the boiling method (Holmes & Quigley, 1981)

5 ml of L-broth (made 50-100 µg/ml in ampicillin if appropriate) inoculated with 20µl glycerol stock or with single (white) bacterial colony were incubated overnight in sterile Universals (Sterilin) within a shaking incubator at 37°C.

1.5ml of this culture was transferred to an eppendorf tube and centrifuged for ten minutes at room temperature. The remainder of the overnight culture was stored at 4°C. The medium was removed by aspiration and the bacterial pellet was resuspended in 700µl of cold STET buffer (50mM Tris pH8/ 50mM EDTA, 8% sucrose, 5% Triton X-100). To this, 50µl of freshly prepared cold solution of lysozyme (10mg/ml) was added and the tubes were left on ice for 5 minutes.

They were then placed in boiling water for exactly 60 seconds and spun at full speed in the microcentrifuge for 20 minutes. The supernatant was transferred to a fresh Eppendorf tube and extracted with an equal volume of buffered saturated phenol/chloroform until no interface could be seen. To the aqueous layer, sodium acetate (final concentration 0.2M) and isopropanol (0.6ml) were added. Following centrifugation, the tubes were inverted and left to dry on paper towels for 30 minutes. The DNA pellet was resuspended in 40µl in TE. Half of this was digested in a 40µl reaction volume using the appropriate buffer and 1µl of the desired restriction enzyme to check whether the recombinant of interest had been extracted. The DNA fragments in the restriction digest were analysed by gel electrophoresis.

2.3.5 Large scale preparation of plasmid DNA

To prepare a large amount of plasmid DNA the method described above was simply scaled up. About 300ml of culture was routinely used. One extra step was added to this method by adding preboiled RNase to a concentration of 25 µl/ml and incubating the mixture at 37°C for 60 minutes. This was followed by phenol extraction and ethanol precipitation. DNA pellet was dissolved in 200 µl of TE. 10 µl of this was digested as above to check that the recombinant of interest had been extracted.

2.3.6 Restriction enzyme digest

The digestion reaction was carried out in a 30µl volume which contained 4µl of 10x core buffer (BRL), 2µl of 0.1M spermidine, 2µl of the appropriate volume of sterile distilled water, depending on

the concentration of the DNA sample. Spermidine was used to improve the digestion of the DNA samples (see Bouche 1981). Reactions were terminated by the addition of stop mix (50mM EDTA, 50% glycerol, 20% Ficoll and 50µg/ml Orange G). Digested DNA samples were immediately loaded onto agarose gels, or sometimes stored at -20°C until further use.

2.3.7 Gel electrophoresis

Electrophoresis was carried out in 0.8% agarose (Sigma) gels in "E buffer". LKB "Bromma/2197 constant power supply" power packs were used typically at 200 mA/3-4 hours for the 10cmx13cm gels used. Following electrophoresis, gels were stained for ten minutes in "E buffer" containing ethidium bromide (Sigma; final concentration 1µg/ml). The DNA was visualised on a UV transilluminator (UVP inc) and photographed using a Polaroid CU-5 hand camera fitted with a red filter and a black and white Polaroid type 667 film.

2.3.8 Estimation of DNA concentration

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

2.4 Preparation of radioactively labelled probes

Probes were labelled with deoxy 1',2',5-^{3}H cytidine 5-triphosphate, ammonium salt (50 Ci/mmol; Amersham international) either by nick translation or random oligonucleotide primed ("oligo"-) labelling. ^{3}H-dCTP was supplied in ethanol:water solution (1:1) and it was lyophilized prior to every reaction in a Speed vac centrifuge for 2 hours. For every reaction 50µl (approximately 50µCi) were used. On two occasions (see 2.4.4) two radioactive nucleotides, ^{3}H-dCTP and ^{3}H-dATP, were used.

2.4.1 Nick translation (from Rigby et al., 1977)

In most cases DNA probes were labelled by nick translation using the Amersham nick translation kit, N5000. The reaction was performed in a 100µl volume. In the eppendorf tube that contained the lyophilized tritiated nucleotide, 1µg of DNA was combined with 50µl of ^{3}H-dCTP lyophilized, 20µl Amersham reaction mix and 10µl of Amersham enzyme solution. The reaction was incubated at 14^{o}C for 2 hours and was terminated by adding 10µl of 0.5 M EDTA.

Labelled DNA was separated from unincorporated nucleotides by chromatography through a Sephadex G-50 column, equilibrated with 0.4M NaCl/0.1% SDS. Carrier DNA was added to the column to prevent aspecific binding of the probe. The nick-translation mixture was loaded onto the column made in a 2ml disposable syringe blocked by a GFC filter, and 20 fractions were collected in eppendorf tubes after washes with 120µl of 0.4M NaCl/0.1% SDS. A 5µl aliquot of each fraction was counted on an LKB 1215 liquid scintillation counter using disposable plastic vials. A complete set of fractions takes about ½ hour to count. After an initial low start, the counts should

rise and peak as the radioactivity which has been incorporated into the probe DNA comes through. This peak is followed by a drop and finally another rise, representing the unincorporated radionucleotides. Fractions representing first peak were pooled and a 5µl aliquot of these "pooled fractions" was counted on the scintillation counter. This result was compared to the one obtained after the TCA filter test to allow estimation of the incorporated radioactivity.

2.4.2 TCA test

To monitor the percentage of input radioactivity which has been incorporated, a 5µl aliquot of the "pooled fractions" was transferred to a tube containing 20µl of carrier DNA. To this, 2-3 ml of ice-cold 10% trichloroacetic acid (TCA) solution was added, the mixture was mixed well and allowed to stand on ice for 15 minutes. The labelled and carrier DNA co-precipitate.

The precipitated DNA was collected by vacuum filtration on a glass GFC filter. The filter was washed with a further 5 ml 10% TCA solution and it was dried with a hair-drier. Then it was counted by liquid scintillation. To determine percentage incorporation a 5µl sample of the pooled fraction was counted under the same conditions as the dried filter. To calculate the percentage incorporation:

$$\% \text{ incorporated} = \frac{\text{dpm on filter}}{\text{total activity}} \times 100\%$$

If the incorporated radioactivity was at a range of $x10^7$ dpm/µg the pooled fraction was extracted twice with phenol/chloroform to separate DNA from protein. The labelled DNA was recovered by ethanol precipitation. The DNA pellet was allowed to dry by inverting the

eppendorf tube on a towel and it was dissolved in 100µl hybridisation buffer. An 1µl aliquot of this was counted on the scintillation counter; the approximate specific activity of the labelled DNA was calculated in dpm/µg as follows:

$$\text{DNA specific activity} = \text{dpm of sample} \times \frac{100\mu l}{1\mu l}$$

If the specific activity of the labelled DNA is lower than expected it is possible that the DNA to be labelled may contain enzyme inhibitors and thus need further purification. This was done by an additional ammonium acetate precipitation step. If this did not work a new plasmid preparation was grown and this was used in a new nick-translation reaction. If the nick translation reaction proceeds at a slower rate than expected, this may be increased by using a larger volume of enzyme solution. Handling losses may occur which may explain lower incorporations than expected.

2.4.3 Oligo-labelling (Feinberg & Vogelstein 1983)

The random primer, or oligonucleotide labelling technique relies on DNA synthetic extension from hexanucleotide primers which bind randomly to the single-stranded probe. This method was used for the labelling of DNA probe GMGY3.

Oligo reaction mix

Firstly, the following four solutions are made up:

Solution A:	Solution B:
1.25M Tris.HCl pH8.0	1ml Solution A
0.125M $MgCl_2$	18µl beta-mercapto-ethanol

5µl dATP, dGTP, dTTP

Solution C: (each of these dissolved

2M HEPES, pH6.6 in TE at 100mM)

Solution D:

Hexadeoxyribonucleotides (Pharmacia, PL No.2166)

at 90 O.D. units per ml.

Solutions B, C and D are then mixed in the ratio 100:250:150 to give 10x reaction mix. 400ng of DNA in SDW was boiled for 10 min, and then incubated at 37°C for at least another 10 minutes. In another eppendorf tube containing the lyophilized 50µCi of ^{3}H-dCTP, 10µl of reaction mix were added and 2µl of nuclease-free BSA (10mg/ml from Sigma). The two tubes were combined and 1µl of Klenow fragment (Amersham) was added. The reaction was incubated at room temperature overnight and then terminated by adding 50µl of stop mix (20mM NaCl, 20mM Tris.Cl pH7.5, 2mM EDTA, 0.25% SDS). As with nick translation, the DNA was separated from the unincorporated nucleotides on a G-50 Sephadex column, was phenol-extracted, ethanol precipitated and dissolved in hybridisation buffer.

2.4.4 Oligo-labelling using two ^{3}H-dNTPs (as adopted by Donlon 1986)

This method was used for labelling probes JG73 and JG51 (section 3.2.6)

The following solutions were prepared:

Solution O: 1.25 M Tris-HCl, 0.125 M $MgCl_2$ (pH 8.0)

Solution A: 1 ml solution O+ 18µl 2-mercaptoethanol (14.4M) + 5 µl dGTP (dissolved in 3mM Tris-HCl, 0.2 mM EDTA, pH 7.0 at a final

concentration of 100mM). Store at -20°C.

Solution B: 2M HEPES, titrated to pH 6.6 with 4 M NaOH.

Solution C: Hexanucleotides were suspended in TE buffer at 90 O.D. units/ml.

Oligo-labelling Buffer (OLB) was made by mixing solutions A:B:C in a ratio of 367:917:150 and was stored at -20°C.

20µl ^{3}H-dCTP (Amersham, 50µCi/mM) and 20µl ^{3}H-dATP (Amersham) were lyophilized in an eppendorf tube and combined with 10µl of well suspended OLB and 2µl of bovine serum albumin (BSA) and set aside on ice. In a different eppendorf tube, 250ng DNA in 33µl of water was treated with 2µl of DNase I (20ng/ml) at room temperature for 10 minutes. Then it was boiled in water bath and cooled on ice. The two eppendorf tubes were combined, 2 units of DNA polymerase I were added and the reaction was incubated at room temperature for 24 hours. Incorporated ^{3}H-dNTPs were separated as before (section 2.4.2.) Labelled DNA was dissolved in TE and was kept at -20°C.

2.5 Radioactive in situ hybridisation

The method used is that of Malcolm et al., (1982) with modifications.

2.5.1 Chromosome denaturation and in situ hybridisation

Chromosome spreads fixed to glass slides (prepared as described in Section 2.3) were treated with 100 µg/ml solution of RNase in 2xSSC at 37°C for 30 min under a large (22 x 40 mm) coverslip in order to remove any residual cytoplasmic RNA. RNase was removed by extensive washing in 2 x SSC and slides were dehydrated by immersion

for 2 min each in 50%, 70%, 90% and 100% (v/v) ethanol.

Chromosomal DNA was denatured by incubation with 200 µl of 60% (v/v) formamide, 40% 5 mM HEPES, 0.1 mM EDTA, pH 7, at 65°C for 15 minutes. Following denaturation slides were washed in three changes of ice-cold 2 x SSC and dehydrated through an alcohol series as above.

Probe DNA was denatured at 70°C for 5 minutes in hybridisation buffer 10 µl (0.6 M NaCl, 50% v/v formamide, 5 mM HEPES, 1 mM EDTA, pH 7.6) with 1000-fold Salmon Sperm DNA as a competitor was placed on ice for 5 minutes. Slides were placed on a floating metal tray; 10 µl of the hybridisation mixture was applied onto the marked area of the slides covered with a small (22 x 22 mm) coverslip, sealed with rubber cement (cowgum) to prevent evaporation and incubated in a 43°C water-bath for 18 hours. If conditions of high stringency were desirable, hybridisation was carried out at an elevated temperature (e.g 50°C).

2.5.2 Post-hybridisation washes

Following hybridisation, cowgum was removed and slides were immersed in a coplin-jar with 2 x SSC to gently remove coverslips. Excess probe and was removed by the following washes.
Several washes in 2 x SSC at room temperature; several washes in 0.1 x SSC at room temperature (performed in coplin jars); one hour in 0.1 x SSC at 65°C; several changes on a magnetic stirrer in 2 x SSC at 4°C overnight. This wash was performed in glass staining dish with the slides in a metal rack.

These post-hybridisation washes were later replaced by 3 changes in 50% formamide, 2 x SSC at two degrees higher than the temperature

of the hybridisation after Harper and Saunders (1981).

2.5.3 Autoradiography

Reproducibility is of prime importance when employing the tool of autoradiography. Variability must be eliminated by standardizing as many procedures as possible (the product, technique, darkroom and processing procedures).

The emulsion used was Ilford nuclear emulsion in gel form type K2, size A. The nuclear track emulsion was stored under refrigeration. If stored at room temperature this will result in uncoatability or high background fog. The nuclear track emulsions are expiry dated and should not be used beyond the indicated date because of the possibility of an increase in background fog levels. The emulsion was received in solid form and was liquified before use. In initial studies, all work using the emulsion was done using the Ilford S902 safelight until the slides had been developed and fixed. Later autoradiography was performed in complete darkness except for the stage that blank slides need to be checked.

The emulsion was removed from the cold room and was left at room temperature for 1 hour. It was diluted 1+1 with distilled water and was placed in a water-bath at 45^{o}C for 1 hour with a gentle agitation Caution should be used since too much agitation can lead to the formation of microscopic bubbles in the emulsion and these can be difficult to remove. Blank slides were repeatedly dipped into the emulsion and the coating was closely examined under the safelight. When a satisfactory even coating of emulsion was achieved one was ready to proceed to the coating of the specimen slides. Slides were dipped vertically into the emulsion for 40 seconds then removed

slowly to produce an even coat. The back of the slides were cleaned with tissue and the slides were dried horizontally at room temperature in the dark for approximately 1 hour. Then they were placed in light-tight plastic boxes two at a time, wrapped in aluminium foil, marked clearly and were exposed at 4°C in a container containing dessicant. The drying agent helps to maintain a low humidity condition in the storage box, thereby minimising the effects of latent image fading. The low temperature helps to minimize the effects of chemical interaction between the specimen and the emulsion layer. Exposure time was determined empirically for each experiment. Extra slides were prepared for periodically checking the production of grains.

2.5.4 Processing of Autoradiographs

At the end of the exposure period, to avoid moisture condensation on the surface of the cold slides the slide boxes and their contents were allowed to warm to room temperature for 2-3 hours before unsealing. Then the slides were processed as follows:

Step	Solution	Time	Temp.
1.Develop	Kodak Developer D-19	4 min	15°C
	(1:1) or		
	Kodak Dektol	2 min	15°C
	Developer (1:1)		
2.Stop	Distilled water	30 s	15°C
3.Fix	Ilford Hypam	2 min	15°C
	(1:4)		
4.Wash	Running tap water	30 min	15°C
5.Dry	Air-dry		

Slides were stained for 40 minutes in 5% Giemsa in pH 6.8 phosphate buffer, rinsed in buffer, then air dried. The slides were restained for longer or destained by soaking in buffer as necessary.

2.5.5 Scoring of cells and analysis of results

After each in situ hybridisation experiment the prebanded and photographed metaphase cells were relocated and the silver grains were marked on each photograph. Only silver grains associated with chromosomes were recorded. If hybridisation of the probe DNA to metaphase chromosomes were a random event more grains would be expected on larger chromosomes, and fewer on the smaller ones. Assuming random distribution, the expected number of silver grains on each chromosome can be calculated on the basis of the relative lengths (see Table 4) or the DNA content of each chromosome (Table 5) as follows:.

Expected number of signal on chromosome = (Total number of grains observed in the experiment) x (Relative length of the chromosome) divided by 100

This figure was compared with the observed number of silver grains and tested for statistical significance using the Kolmogorov-Smirnov two sample one tailed test. This is used to test the prediction that the scores of an experimental group will be better than those of the control groups. The method and theory is described by Siegel (1956).The results were expressed as a x^2 (chi squared) value and as a probability (p). The values of p and levels of significance are:

Chromosome Number	Arm Ratio	Relative Length		Total Relative length
		Short Arm	Long Arm	
1	1.13	3.97 ± 0.20	4.50 ± 0.19	8.47
2	1.59	2.99 ± 0.22	4.77 ± 0.31	7.76
3	1.15	3.05 ± 0.18	3.51 ± 0.17	6.56
4	2.45	1.77 ± 0.24	4.36 ± 0.27	6.13
5	2.52	1.58 ± 0.09	4.00 ± 0.24	5.58
6	1.61	2.16 ± 0.20	3.49 ± 0.23	5.65
7	1.56	1.95 ± 0.15	3.05 ± 0.16	5.00
X	1.49	2.06 ± 0.19	3.08 ± 0.25	5.14
8	2.13	1.52 ± 0.16	3.25 ± 0.18	4.77
9	1.88	1.64 ± 0.18	3.09 ± 0.28	4.73
10	2.08	1.41 ± 0.16	2.94 ± 0.28	4.35
11	1.51	1.73 ± 0.20	2.62 ± 0.18	4.35
12	2.12	1.33 ± 0.16	2.83 ± 0.20	4.16
13	5.85	0.52 ± 0.16	3.07 ± 0.21	3.59
14	5.82	0.48 ± 0.11	2.80 ± 0.12	3.28
15	5.39	0.49 ± 0.11	2.62 ± 0.16	3.11
16	1.51	1.24 ± 0.10	1.87 ± 0.14	3.11
17	2.04	0.99 ± 0.12	2.03 ± 0.16	3.02
18	2.60	0.76 ± 0.14	1.97 ± 0.12	2.73
19	1.22	1.16 ± 0.14	1.42 ± 0.10	2.58
20	1.30	1.01 ± 0.14	1.30 ± 0.11	2.31
21	2.82	0.50 ± 0.13	1.41 ± 0.15	1.90
22	2.72	0.45 ± 0.10	1.24 ± 0.14	1.69
Y	2.34	0.63 ± 0.14	1.47 ± 0.31	2.09

Table 4: Relative lengths of human chromosomes.
Taken from Ferguson-Smith (1974)

Chromosome	DNA content	
	Mean	SD
1	4.27	0.079
2	4.16	0.030
3	3.49	0.023
4	3.32	0.015
5	3.17	0.013
6	2.99	0.012
7	2.81	0.011
X	2.69	0.011
8	2.54	0.011
9	2.40	0.082
10	2.36	-
11	2.36	-
12	2.36	-
13	1.89	0.039
14	1.81	0.040
15	1.72	0.052
16	1.61	0.045
17	1.47	0.014
18	1.40	0.028
20	1.16	0.014
19	1.08	0.024
Y	0.96	0.064
22	0.89	0.037
21	0.77	0.030

Table 5: Mean chromosome DNA content determined from ethidium bromide flow karyotypes.
Values expressed as a percentage of the total male diploid DNA content (from Harris et al, 1986)

$p < 0.001$ very highly significant

$0.001 < p < 0.01$ highly significant

$0.01 < p < 0.5$ significant

$0.5 < p$ not significant

2.6 Biotinylated in situ hybridisation

Avidin is a 68,000 molecular weight glycoprotein with an extraordinarily high affinity (10^{15} M^{-1}) for the small molecular weight vitamin, biotin. In addition to this high affinity, the Biotin/Avidin System can be effectively exploited because avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. Streptavidin, a protein produced by Streptomyces avidinii, demonstrates the same high affinity for biotin as egg white avidin, but exhibits far less nonspecific binding at physiological pH.

The method for biotinylated in situ hybridisation used during the course of this project has been developed by Garson et al., (1987) and it is essentially the adaptation of the BRL protocol for biotinylated filter hybridisation to chromosomal preparations (see figure 4). In brief, the DNA probe is labelled with biotin-11-dUTP using a standard nick translation protocol. Following hybridisation of the biotinylated probe the chromosomal preparations are incubated with a streptavidin-alkaline phosphatase Conjugate (SA-AP), which binds to the biotinylated probe/target hybrid. BCIP and NBT are used as the chromogenic substrate for the enzyme alkaline phosphatase. They are used together at a 1:1 molar ratio under alkaline conditions to obtain optimal sensitivity. The colour reaction is initiated by

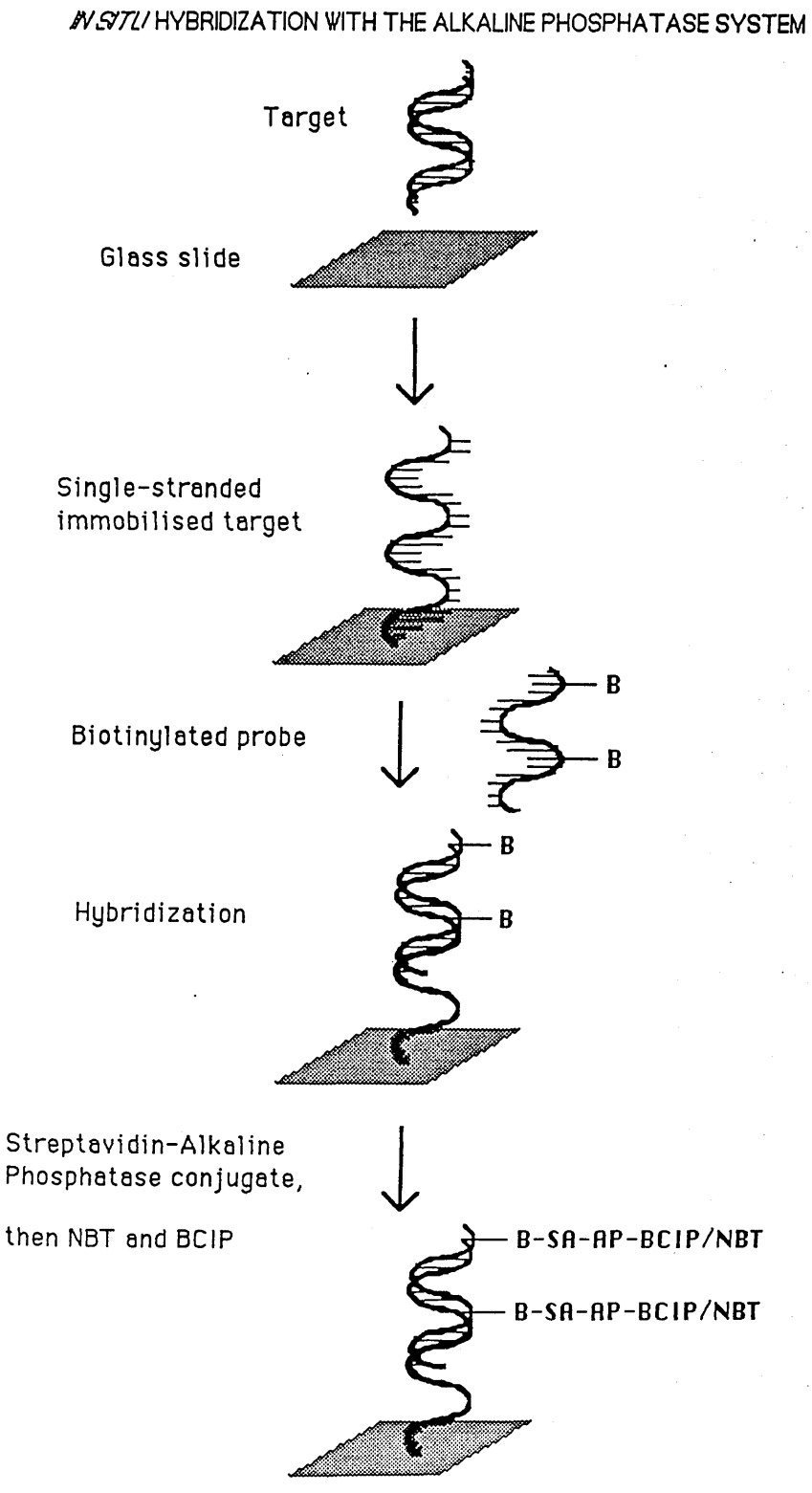


Figure 4. Biotinylated in situ hybridisation and the BRL DNA detection system.
(this diagram was prepared by Dr S Habeebu)

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.

2.6.1 Biotin labelling of DNA by nick translation

Plasmids containing insert were labelled by nick-translation with biotin-11-dUTP (Bethesda Research Laboratories, BRL) using the BRL nick translation kit, catalogue No 8160SB for 50 reactions. The reaction was performed in a 50 µl volume.

In an Eppendorf centrifuge tube the following were mixed: 5 µl of reaction mix]0.2 mM nucleotides C, G, and A in 500 mM tris-HCl (pH 7.8), 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin (BSA) with 1 µg DNA to label, 2.5 µl 0.4 mM Biotin-11-dUTP and H_2O to make final volume 45 µl. To this, 5 µl of enzyme was added, the reaction was mixed gently and was incubated at 14-15°C for 90 minutes. The reaction was stopped with 5 µl of stop buffer (300 mM Na_2EDTA, pH 8.0).

Unincorporated nucleotides were removed by ethanol precipitation as follows: 4.6 µl of 3M NaAc pH 5.2, 1 µl 20mg/ml glycogen and 122 µl iced ethanol were added to the reaction, was vortexed and centrifuged for 30 minutes. The supernatant was aspirated and the eppendorf tube was inverted on a paper towel until the DNA pellet was dry. The DNA pellet was dissolved in 10 µl of TE to give a concentration of 100ng/µl.

Phenol extraction of biotinylated DNA samples is avoided because of extraction into the phenol layer or retention at the phenol water interface (Langer et al., 1981). The degree of dTTP substitution by bio-11-dUTP can be determined by adding a tracer amount of

radioactive dATP and calculating the amount of incorporated radioactive dATP. Biotin labelled probes were stored at -20°C.

2.6.2 In situ hybridisation and probe detection

Slides were dehydrated through an alcohol series and air dried at room temperature. The dehydration procedure effectively removes all traces from Giemsa from the preparations. If traces of stain were present these slides were discarded as documentation of the hybridisation signal proved to be difficult. 100 ng of biotin-labelled probe in 10 µl of hybridisation buffer was placed onto each slide and covered with a small coverslip. The hybridisation buffer contained 50% formamide, 10% dextran sulphate, 2 x SSC, 0.1 mM EDTA, 0.05 mM Tris-Cl, pH 7.5 and 100 µg/ml denatured sonicated salmon sperm DNA. The coverslips were sealed with cowgum and the chromosomal DNA and probe denatured simultaneously in a hot air oven at 78°C for 10 min. The slides were then placed in a 42°C water bath overnight. The coverslips were removed and the slides were washed sequentially in 2 x SSC at room temperature for 15 minutes and finally in Buffer 2 (3% bovine serum albumin in 0.1 M Tris-Cl, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100) at room temperature for 15 minutes.

Biotinylated probes were detected by incubating the slides at room temperature for 20 min with 100µl of alkaline phosphatase conjugated streptavidin (BRL, BluGENE Nonradioactive Nucleic Acid Detection System, cat. no. 8279SA) diluted 1:100 (0.01 mg/ml) in Buffer 2. The slides were then washed 3 times, 5 minutes per wash, at room temperature in Buffer 1 (0.1 M Tris-Cl, pH 7.5, 0.1 M NaCl, 2 mM MgCl2, 0.005% Triton X-100). A final 10 min wash was in Buffer 3 (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) at room temperature. The

slides were incubated with 150 µl of chromogenic substrate solution for 1-3 hours depending of probe copy number in a humidified plastic box in subdued light. The chromogenic substrate was made by adding 4.4 µl of nitroblue tetrazolium (75 mg/ml in 70% dimethylformamide) and 3.3 µl of 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml in dimethylformamide) to 1 ml of Buffer 3. 1 mM levamisole (Sigma) was added to inhibit any endogenous alkaline phosphatase activity (Ponder and Wilkinson, 1981). Colour development was terminated by washing the slides in 20 mM Tris-Cl, pH 7.5, 5 mM EDTA for 5 minutes at room temperature. The slides were mounted in Dako Glycergel and viewed by phase contrast illumination. The preparations do not fade and were stored at room temperature. Scoring of cells and analysis of results was performed as described in Section 2.5.6.

2.7 Photography

Kodak Imagecapture AHU microfilm was used throughout. In complete darkness films were wound onto reels and loaded into light-tight developing tank. Solutions for developing and printing were all used at 20°C. Films were developed in Kodak D163 or Kodak DEKTOL diluted 1 + 3 with tap water for 4½ minutes with agitation every 30 seconds. Developer was poured off and replaced with stop bath (3% glacial acetic acid in distilled water) for 30 seconds with continuous agitation. Fixative was added (Amfix; Kodak, diluted 1 + 3) for 3½ minutes. This was followed by washing in running water for 30 minutes and drying.

Photographs were printed on Ilford paper, developed in Universal developer (Kodak) diluted in 1 + 7 for 1-5 minutes, rinsed in water and fixed in Amfix (Kodak) diluted 1 + 3.

CHAPTER THREE

RESULTS

3.1 Optimising the conditions for radioactive in situ hybridisation

Initial studies were undertaken using a highly repetitive DNA sequence - phr28S-1 - containing the genes for 28S ribosomal RNA, to ensure that a detectable, reproducible signal was obtained with the available protocol. The DNA probe was labelled with ^{3}H-dCTP to a specific activity of 2.9×10^{7} dpm/µg by nick-translation and was hybridised with chromosomal preparations from a normal male at a concentration of 100ng per slide. The slides were exposed for 6 days then were developed and scored for silver grains as described in section 2.6.5. These studies resulted in statistically significant labelling of chromosomes 13, 14, 15, 21, and chromosome 22 ($p<0.001$) where the rDNA genes have been previously mapped (Henderson et al., 1972; Evans et al., 1974). Table 6 shows the statistical analysis of results obtained from such an experiment.

A different highly repetitive sequence, pY3.4, isolated by Lau et al., (1984), specific for the long arm of the Y chromosome, was labelled with ^{3}H-dCTP to a specific activity of 2×10^{7} dpm/µg and was hybridised with a normal male. The preparations were exposed for 6 days and data from 25 cells revealed significant hybridisation with Yq12 (table 7).

Following the apparent success of the above studies attempts were made to map a series of single copy sequences of unknown location. These attempts failed due to extremely high background labelling with silver grains per cell up to 100 [Donlon (1986) considers more than 2 grains per cell excessive]. It should be noted that high background labelling was obtained with the two highly repetitive sequences mentioned above but because of clustering of

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	77	180.69	-103	58.71
2	7.76	83	165.36	-82	40.66
3	6.56	50	139.79	-90	57.94
4	6.13	73	130.63	-58	25.75
5	5.58	75	118.90	-44	16.28
6	5.65	59	120.40	-61	30.9
7	5.00	49	106.60	-58	31.55
8	4.77	54	101.64	-48	22.66
9	4.73	53	100.79	-48	22.85
10	4.35	54	91.69	-38	15.74
11	4.35	47	92.69	-46	22.82
12	4.16	56	88.64	-33	12.28
13	3.59	206	76.50	129	217,52
14	3.28	216	69.89	146	304.99
15	3.11	229	66.23	163	401.62
16	3.11	62	66.27	-4	0.24
17	3.02	55	64.35	-9	1.25
18	2.73	43	58.17	-15	3.86
19	2.58	15	54,97	-40	29.10
20	2.31	37	49,57	-13	3.41
21	1.90	217	40.48	176	765.21
22	1.69	305	36.01	260	1877.25
X	5.14	18	54.26	-72	95.53
Y	2.09	13	22.26	-8	2.87

Table 6. ^{3}H-phr28S-1 in normal male. Statistical analysis of silver grain distribution. Data from 24 cells, total number of silver grains 2146. Chi-square for chromosomes 13, 14, 15, 21 and 22 is highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	19	65	-46	32.5
2	7.76	17	60	-43	31.00
3	6.56	23	50	-27	14.58
4	6.13	16	47	-31	20.44
5	5.58	20	43	-23	12.30
6	5.65	20	43	-23	12.30
7	5.00	30	38.5	-8.5	1.87
8	4.77	19	37	-18	8.75
9	4.73	10	36	-26	18.77
10	4.35	21	33	-12	4.36
11	4.35	6	33	-27	22.09
12	4.16	27	32	-5	0.78
13	3.59	19	28	-9	2.89
14	3.28	9	25	-16	10.24
15	3.11	18	24	-6	1.5
16	3.11	13	24	-11	5.04
17	3.02	13	23	-10	4.34
18	2.73	8	21	-13	8.04
19	2.58	8	20	-12	7.2
20	2.31	7	18	-11	6.72
21	1.90	6	15	-9	5.4
22	1.69	16	13	3	0.69
X	5.14	5	20	-15	11.5
Y	2.09	420	8	412	21218.0

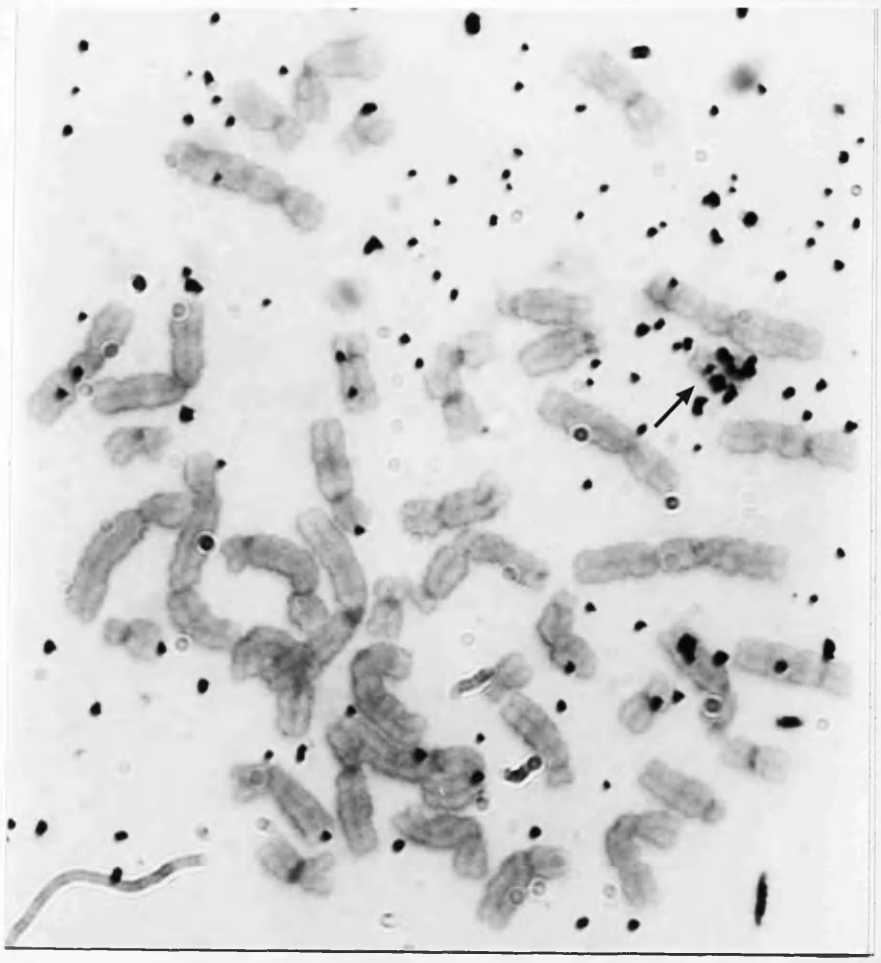
Table 7: ^{3}H-pY3.4 in normal male. Statistical analysis of grain distribution. Data from 25 cells, total number of grains 770. The only chromosome significantly labelled is the Y ($p<0.001$)

silver grains at the chromosome sites of interest due to the repetitive nature of these DNA sequences, following statistical analysis specific chromosome sites were found to be significantly labelled. The high background is demonstrated in figure 5 (probe pY3.4) and figure 7B (probe phr28S-1). An additional problem was that the chromosomal preparations often did not survive the conditions of the procedure and appeared "denatured" after the hybridisation thus making the final scoring for silver grains difficult. Figure 6 shows a metaphase spread with "denatured" chromosomes following hybridisation. The loss in chromosome morphology is not extensive in the metaphase shown but extreme cases are difficult to document by photography as chromosomes are barely seen. Changes were introduced to the available protocol in order to eliminate the high non-specific labelling and improve the morphology of the metaphase chromosomes.

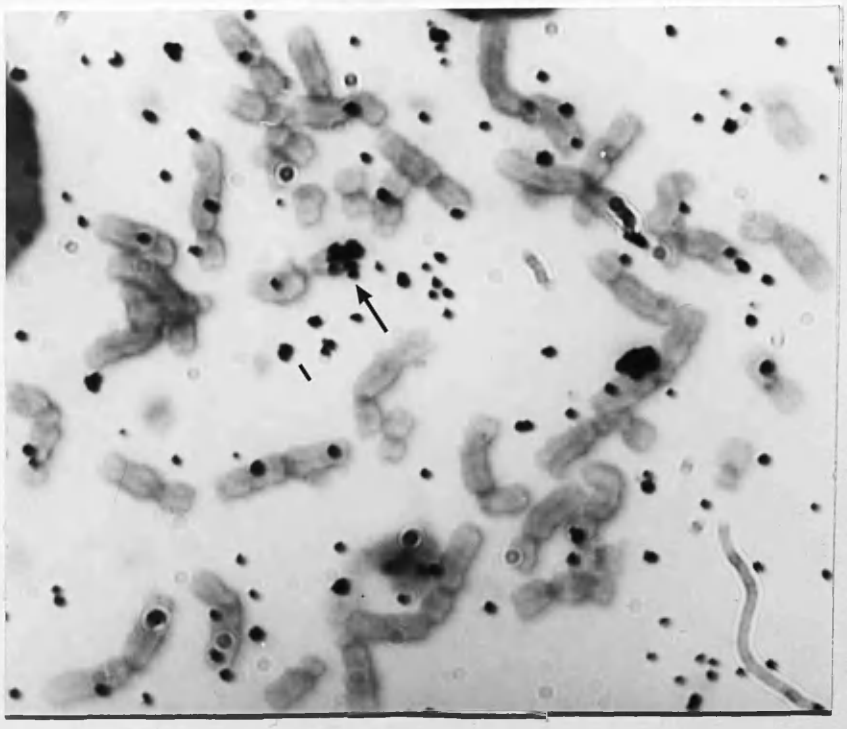
i) High non-specific labelling

Non-specific binding of labelled probe to any part of the preparation must be reduced to an absolute minimum since the autoradiographic signal from the site of specific annealing will always be weak (for single copy probes) and evaluation of an experiment will usually rely heavily on statistics of grain counting and distribution. In order to eliminate the high background the following action was taken:

Post-hybridisation washes: Formamide was introduced to the post-hybridisation washes; thus the post hybridisation washes of several washes in 2xSSC and 0.1xSSC at room temperature, 1 hour 0.1xSSC at 65°C, followed by several changes in 0.1xSSC at 4°C



A



B

Figure 5. High non-specific labelling. Metaphase spreads (A & B) from a normal male following hybridisation with pY3.4. A clustering on the Y long arm did occur, thus despite the high background the Y chromosome was significantly labelled (see table 7).

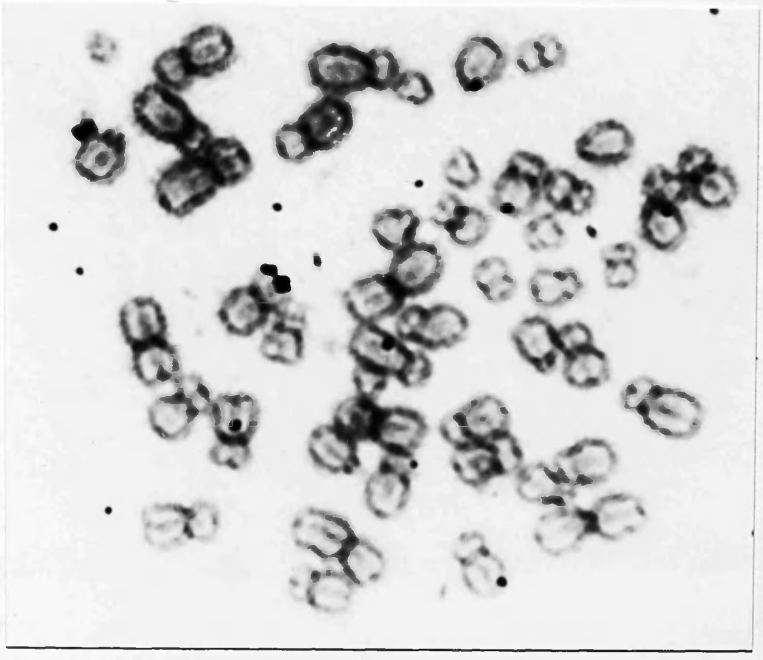


Figure 6 "Denatured" chromosomes following in situ hybridisation.

overnight were replaced by 3 rinses in 50% formamide/2xSSC followed by 3 washes in 2xSSC, all performed at a temperature two ^{o}C higher than that of the hybridisation, according to the method of Harper and Saunders (1981). In this way the duration of each in situ hybridisation experiment was conveniently reduced from 3 to 2 days.

Concentration of probe DNA: 100ng of labelled probe were hybridised with each slide according to the available protocol. Although the effect of the DNA probe concentration on the level of graining was not systematically studied it should be noted that ten (10) ng of labelled ribosomal probe per slide resulted in more clustering of grains over chromosomes 13, 14, 15, 21 and 22 than did 100ng but less than 10ng resulted in very few grains (these data were not quantitated).

Handling of emulsion and autoradiography: Control experiments demonstrated that part of the background labelling was not probe related (silver grains were present in slide where no radioactive probe was added). Autoradiography and slide developing were performed initially in the dark room under an Ilford safelight (see section 2.5.2). According to the manufacturers even exposure to this safelight is not always harmless to the emulsion, therefore it was decided to perform autoradiography and slide developing in complete darkness and every effort was made to ensure that the dark room used for autoradiography was light-tight.

ii) Improving the quality of the metaphase chromosomes

For the improvement of the spreading of the metaphase chromosomes the time of colchicine treatment was decreased from 75 min to 55 min, and the time in hypotonic solution was increased from

10 to 15 min. Following the observation that metaphase spreads obtained when using Iscove's medium were inferior to these prepared in FBS or CSg, only the latter were used for preparation of chromosomes. The quality of the chromosomes was carefully evaluated under phase microscopy and only chromosomes of category II and III as described by Donlon (1986) were used for in situ hybridisation (see section 2.2.3).

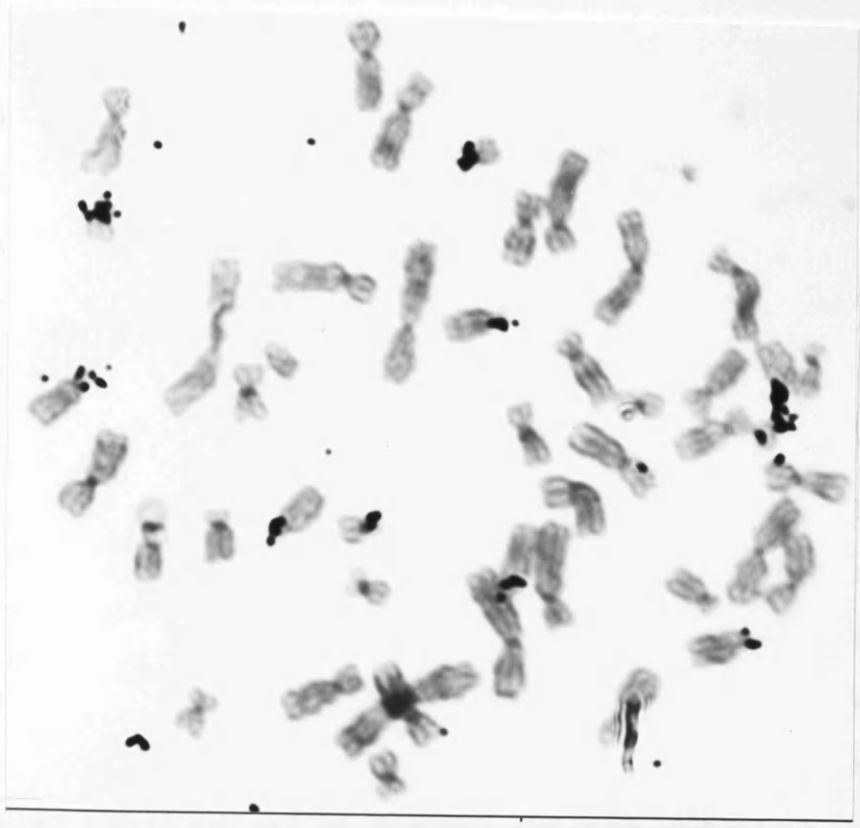
The elimination of background labelling is demonstrated in figure 7A in a metaphase hybridised with the ribosomal probe and after 21 days autoradiographic exposure, as compared to 7B which is a metaphase hybridised with the same probe in the initial studies, exposed for 6 days. It can also be seen that chromosome morphology is best preserved in A.

3.2 Chromosomal localisation of DNA sequences with radioactive ISH

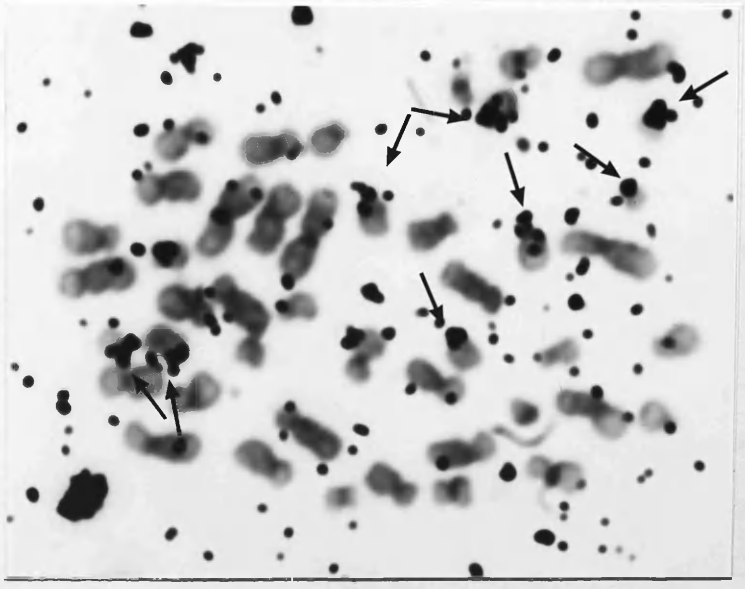
All DNA sequences used were subcloned in a plasmid vector (for description of DNA probes see section 2.1.4 and table 3) and unless otherwise stated were labelled with ^{3}H-dCTP by nick-translation. In every experiment two control slides were included, a positive control with ribosomal DNA probe to ensure that hybridisation had taken place under the given experimental conditions and one without DNA probe to check the probe-not-related background labelling. Grain counts were statistically evaluated using the chi-square test as described in section 2.5.5.

3.2.1 GMGY10 and GMGY7

GMGY10, a 4.5kb insert isolated from a Y-specific library was labelled with ^{3}H-dCTP to a specific activity of $1x10^{7}$ dpm/μ g and



A



B

Figure 7 Elimination of non-specific labelling. Metaphase spreads following hybridisation with the ribosomal probe phr28S-1, washes in 0.1xSSC at 65°C. (A) autoradiography in complete darkness, 12ng of labelled probe. (B) 100ng of labelled probe. Clustering of grains over the D and G chromosomes occurred in both metaphases but there is a dramatic difference in the background labelling which in (A) is reduced to a minimum despite long exposure of 21 days as compared to (B) exposed for 6 days.

hybridised to metaphase spreads from a normal male at concentrations of 60 and 20ng per slide. Autoradiography was performed and the preparations were exposed for 21 days. The slides were then developed and stained for 70 min in 6% Giemsa. A clustering of silver grains was observed over the short arm of the Y chromosome and there was hardly any background labelling (see figure 9) in spite of such a long autoradiographic exposure (in subsequent studies with this probe the chromosomal preparations were exposed for 6 days). Both concentrations, 60 and 20ng, worked equally well; in subsequent studies using this probe 20ng were used per slide for reasons of economy. Thirty cells were relocated and out of a total number of 132 silver grains recorded, 62 were located on the short arm of the Y chromosome. This hybridisation was statistically significant (table 8). The distribution of silver grains over the Y chromosome revealed a peak of 33 silver grains at Yp11.2 and 19 at Yp11.3 (figure 8A). Eleven grains were also recorded at the pericentromeric region of the Y chromosome and 5 at Yq11.2.

GMGY7, a 3.5kb insert, was labelled to a specific activity of 1.4×10^7 dpm/μ g and hybridised to metaphase spreads from a normal male at a concentration of 20ng per slide. Following autoradiography, the slides were exposed for 8 days. For no apparent reason two slides did not have any silver grains at all; from the third slide 11 cells were relocated and out of 26 silver grains recorded, 12 were located at the short arm of the Y chromosome. This hybridisation was statistically significant ($p<0.001$). Six grains were located at Yp11.2 and 5 at Yp11.3 (figure 8B). Metaphase spreads following hybridisation with GMGY7 can be seen in figure 10.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	8	11.18	-3.18	0.89
2	7.76	9	10.24	-1.24	1.36
3	6.56	0	8.65	-8.65	8.65
4	6.13	2	8.09	-6.09	4.58
5	5.58	4	7.36	-3.36	1.53
6	5.65	3	7.45	-4.45	2.65
7	5.00	5	6.60	-1.10	0.18
8	4.77	0	6.29	-6.29	6.29
9	4.73	2	6.24	-4.24	2.88
10	4.35	2	5.74	-3.74	2.43
11	4.35	4	5.74	-1.74	0.52
12	4.16	1	5.49	-4.49	3.67
13	3.59	1	4.73	-3.73	2.94
14	3.28	4	4.32	-0.32	0.02
15	3.11	3	4.10	-1.10	0.29
16	3.11	3	4.10	-1.10	0.29
17	3.02	4	3.98	0.02	0.00
18	2.73	4	3.60	0.40	0.04
19	2.58	3	3.40	-0.40	0.04
20	2.31	1	3.04	-2.04	1.36
21	1.90	0	2.50	-2.50	2.50
22	1.69	1	2.23	-1.23	0.67
X	5.14	0	3.39	-3.39	3.39
Y	2.09	68	1.37	66.63	3240.55

Table 8. ^{3}H-GMGY10 in normal male. Statistical analysis of grain distribution. Data from 30 cells, total number of grains 132. Chi-square for the Y chromosome (one degree of freedom) is highly significant at $p<0.001$.

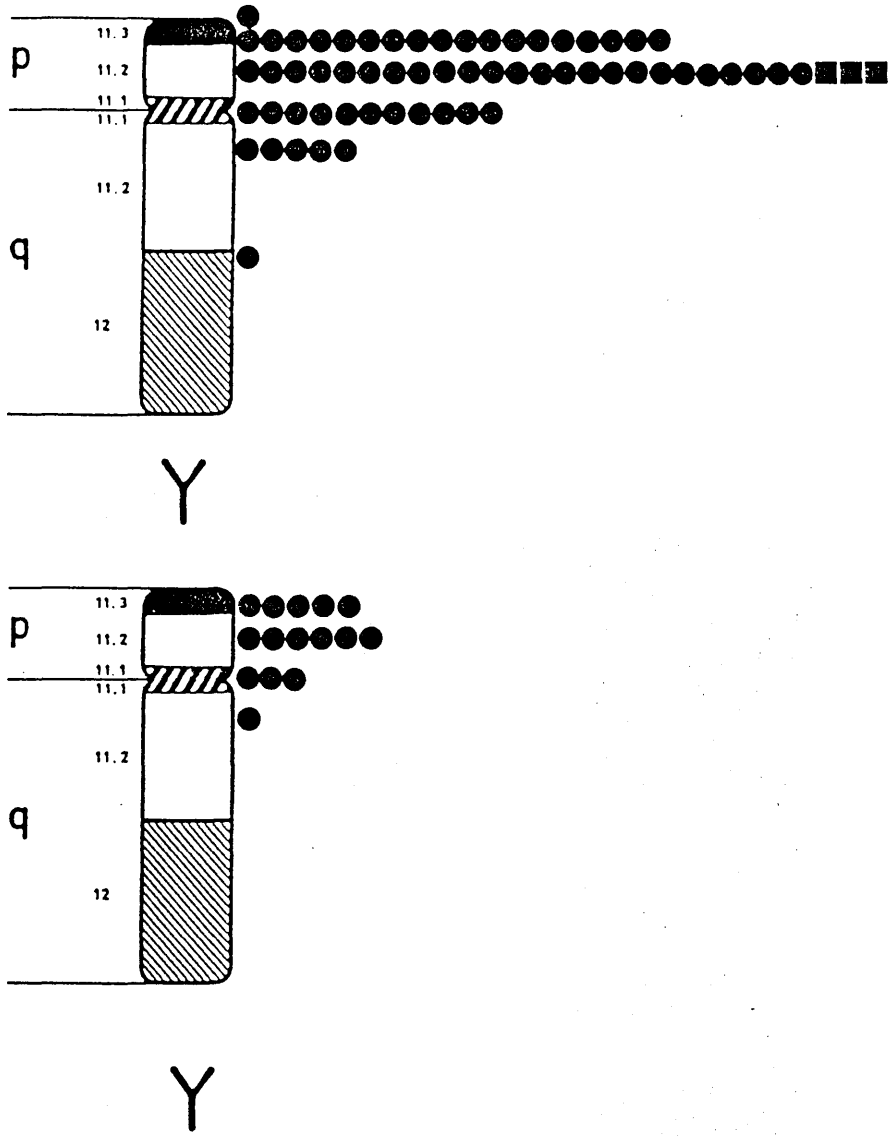


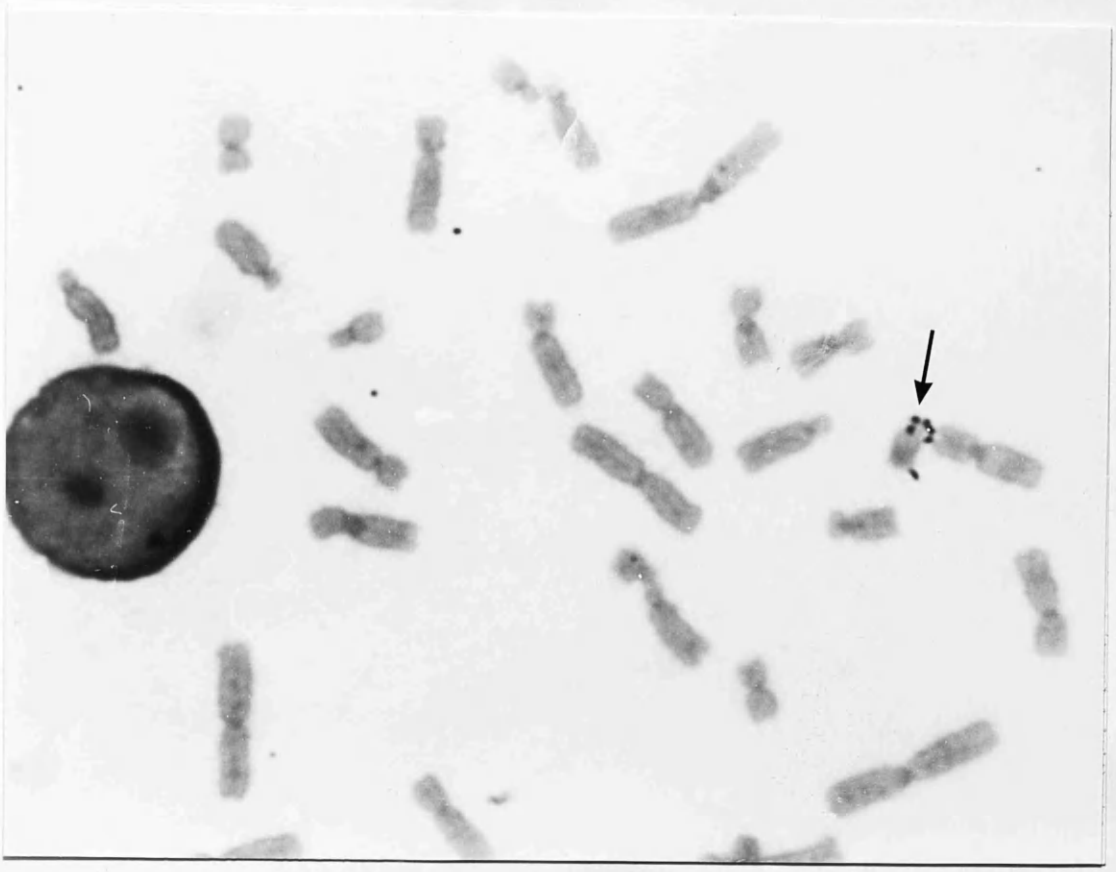
Figure 8 Distribution of silver grains over the Y chromosome.
(A) GMGY10
(B) GMGY7

■: 3 silver grains

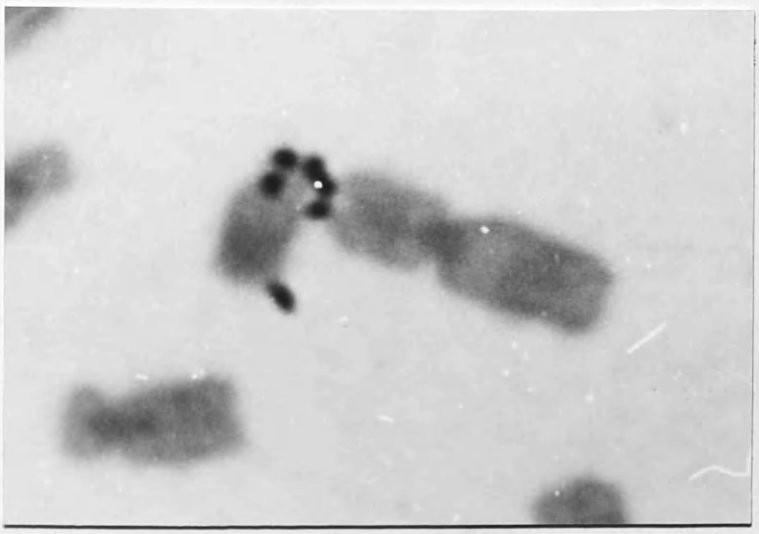
A

B

Figure 9 Metaphase spreads from a normal male following hybridisation with GMGY10. The chromosomes show a C-band pattern following in situ hybridisation; the Y chromosome is easily identified from the large C-band on its long arm.
(A) complete metaphase; note that the only chromosome labelled is the Y. The preparation is incredibly clean from background labelling considering the length of the autoradiographic exposure (21 days).
(B) enlarged Y chromosome from above cell. The whole short arm of the chromosome is covered with silver grains



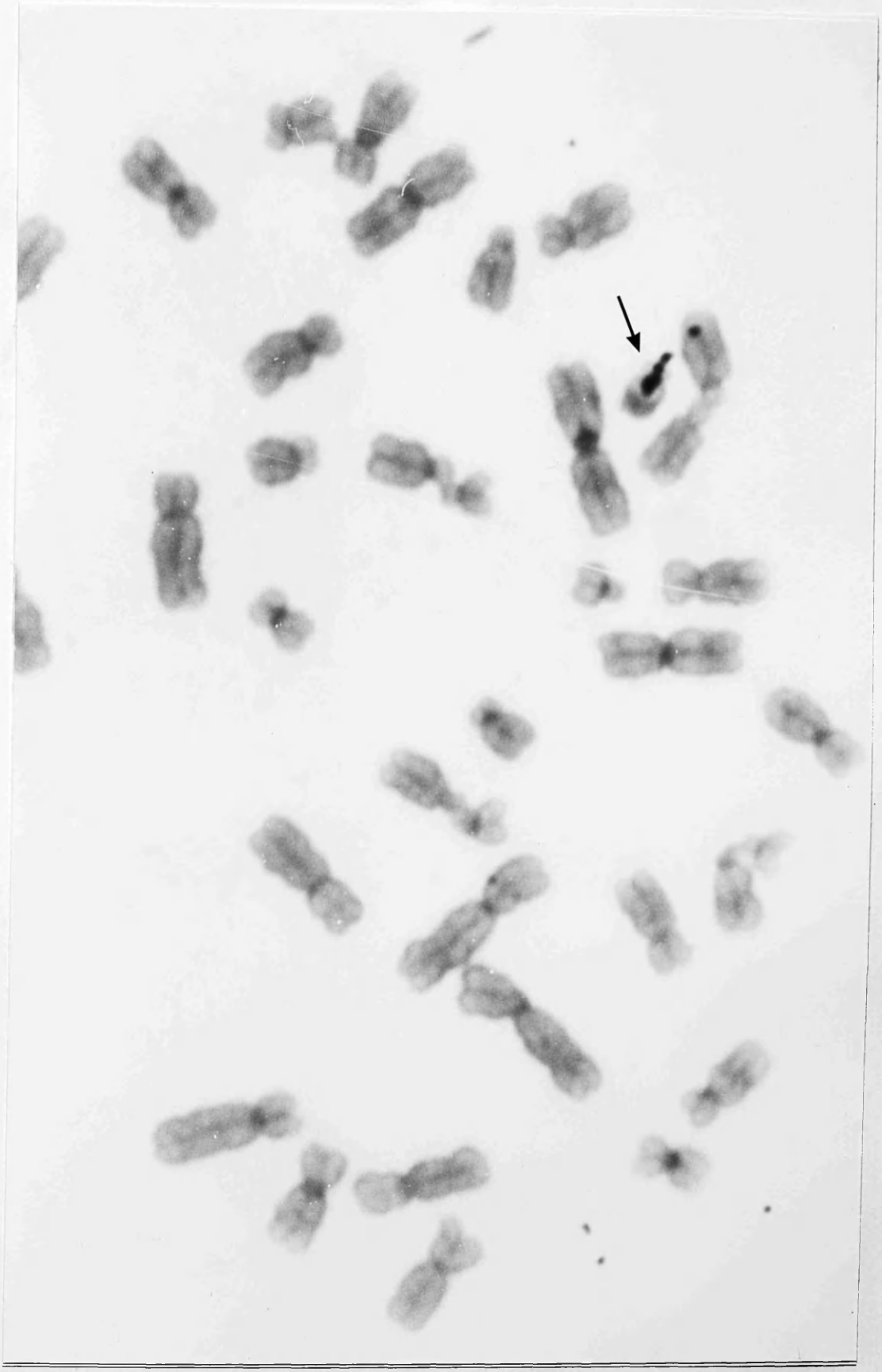
C



D

Figure 9 continued
(C) partial metaphase
(D) enlarged Y chromosome.

Note the distribution of
silver grains on Yp.



E

Figure 9 continued. (E) partial metaphase. Note distribution of silver grains on Yp.

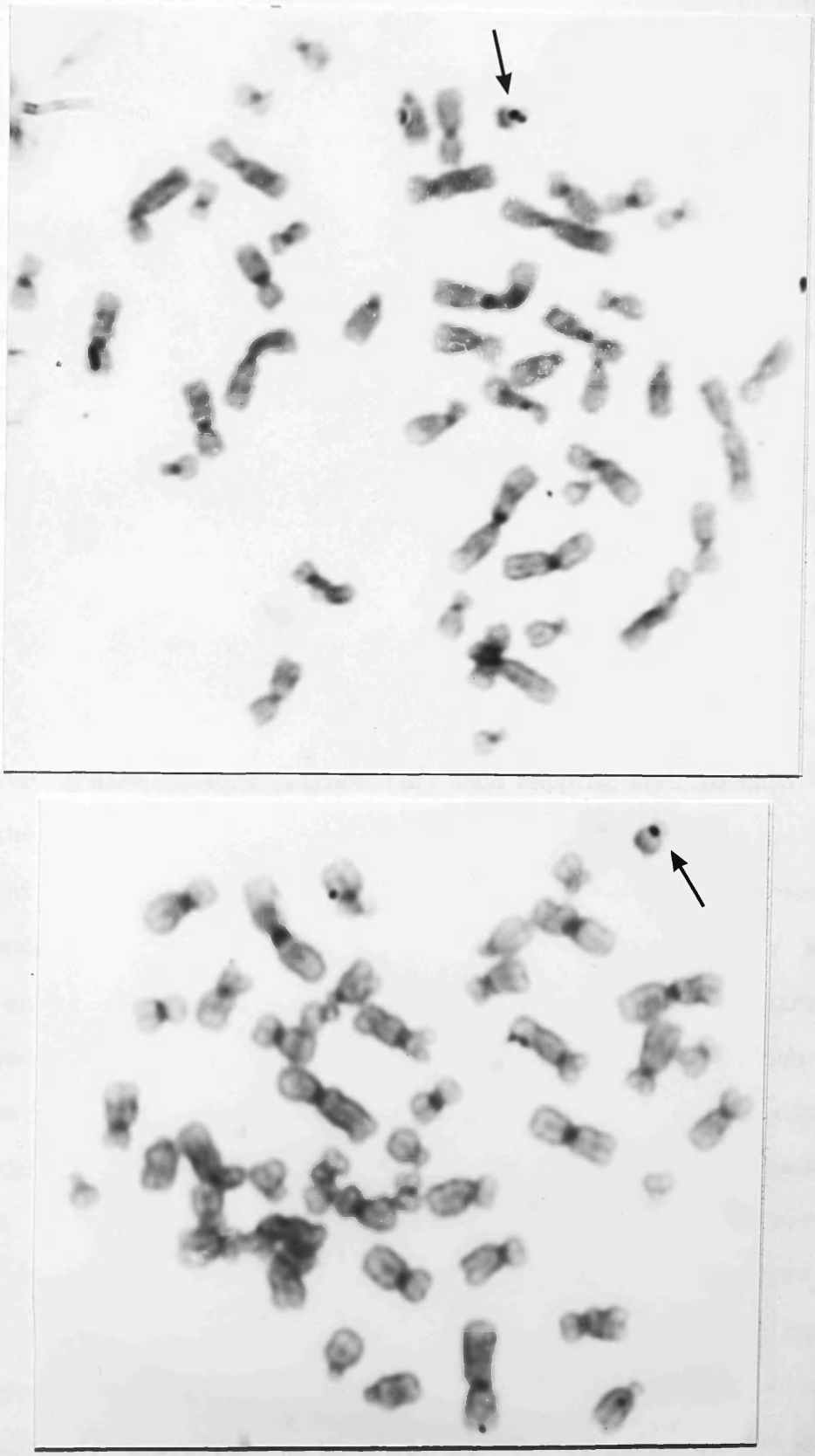


Figure 10 Metaphase spreads following hybridisation with GMGY7 (6 days exposure). As in figure 9 the Y chromosome can be identified from the C-band on the long arm.

3.2.2 P2F2

P2F2, a 1.7 insert, was labelled to a specific activity of $1x10^7$ dpm/μ g. When it was hybridised with metaphase spreads from a normal female and after 28 days exposure time, data from 17 cells showed hybridisation with Xq21 (figures 11A and 12A) but the overall experiment was not very highly significant (table 9). The localisation to Xq21 was confirmed in metaphase spreads from a 46,XX male individual, designated KS. Sixty (60) ng of probe were used per slide and the preparations were exposed for 28 days. Thirty two cells were scored and a total of 702 grains was recorded. Eighty (80) of these were located on the X and this hybridisation was statistically significant ($p<0.001$, table 10). There was a peak of silver grains at Xq21 (figure 11B) thus mapping P2F2 to this region of the X chromosome. Silver grains were also recorded on the short arm at Xp21-pter but is not clear if this hybridisation represents a secondary site for this probe or the presence of Y sequences as KS is an ·XX male (see section 3.4.1). Figure 12 shows pairs of X chromosomes from KS before and after the hybridisation with P2F2 where signal can be seen at Xq21 and at Xp. The same probe was hybridised with a 45X/46X,+mar male individual (RW) and following 35 days autoradiographic exposure, data from 24 cells revealed significant hybridisation with Xq21 ($p<0.001$, table 11). P2F2 was also hybridised with chromosomal preparations from a normal male at a concentration of 10ng per slide. Data from 16 cells revealed significant hybridisation with Xq21 ($p<0.001$); two cells had grains on the short arm of the Y chromosome. Labelling did occur on the short arm of the Y chromosome in other cells not banded before hybridisation as shown in figure 13. The Y chromosome was easily

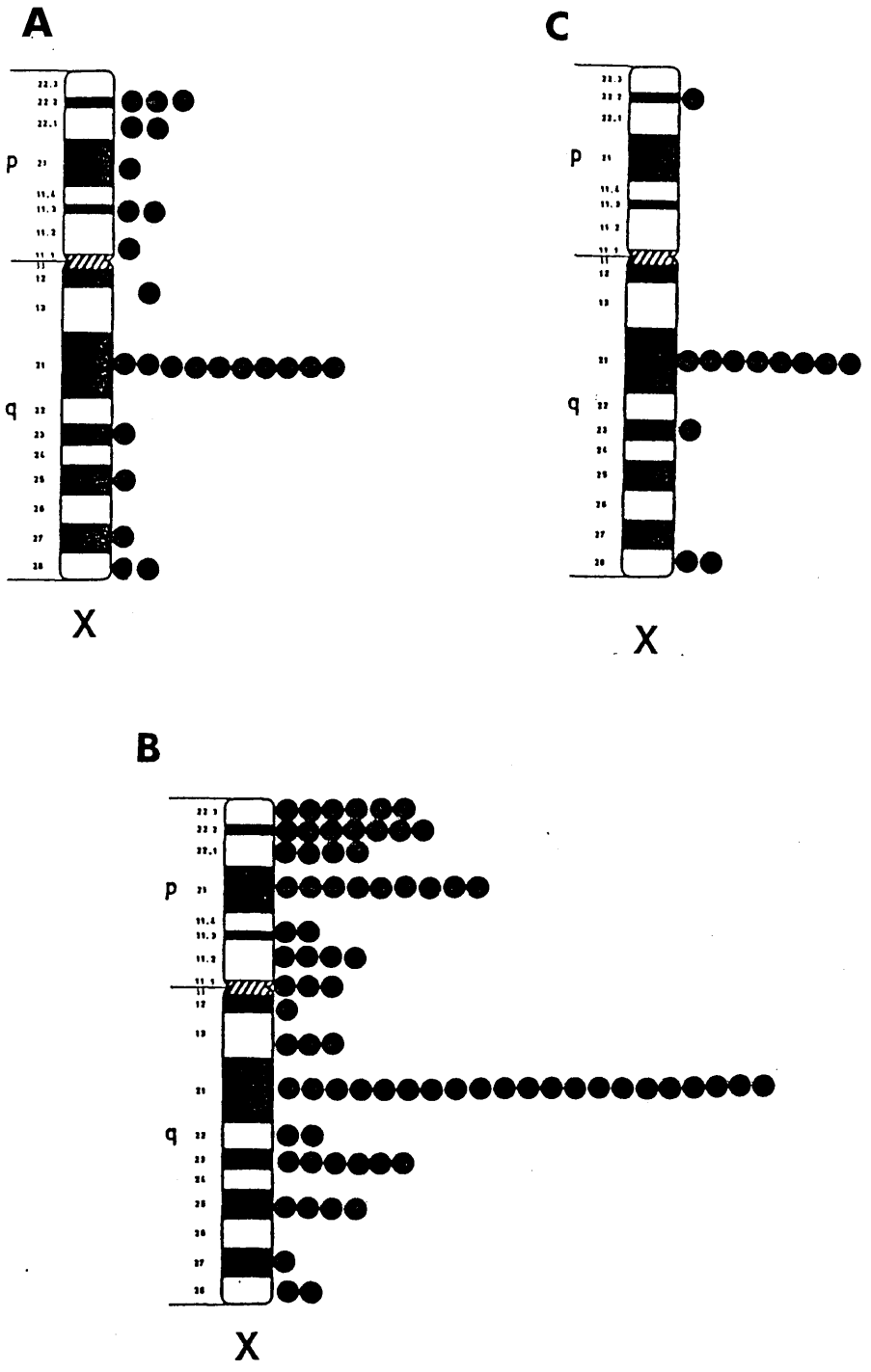
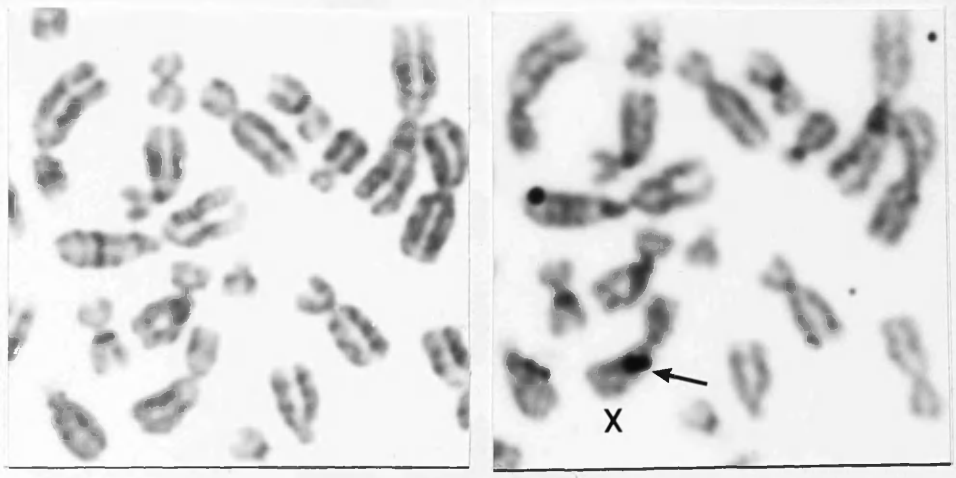
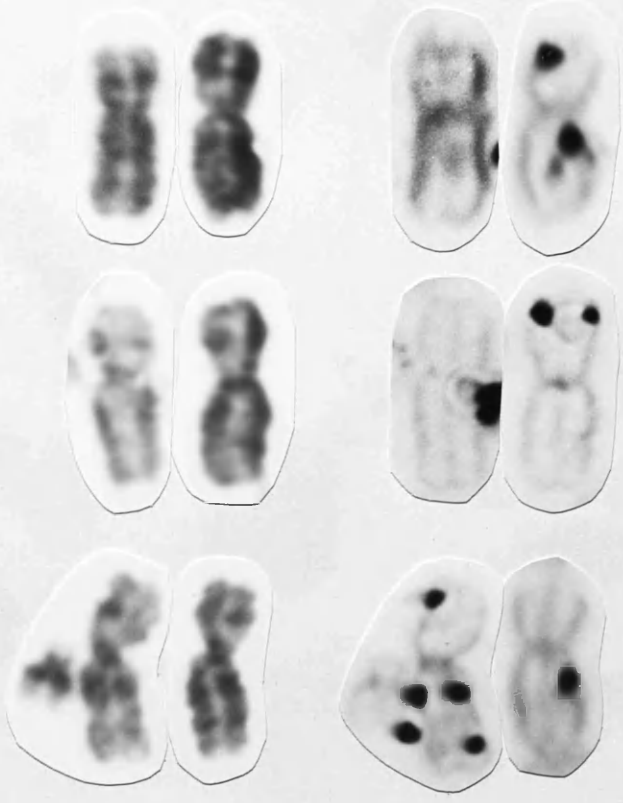


Figure 11 ^{3}H-P2F2: Distribution of silver grains over the X chromosome

(A) normal female, data from 17 cells
(B) XX male KS, data from 32 cells
(C) XO male RW, data from 24 cells

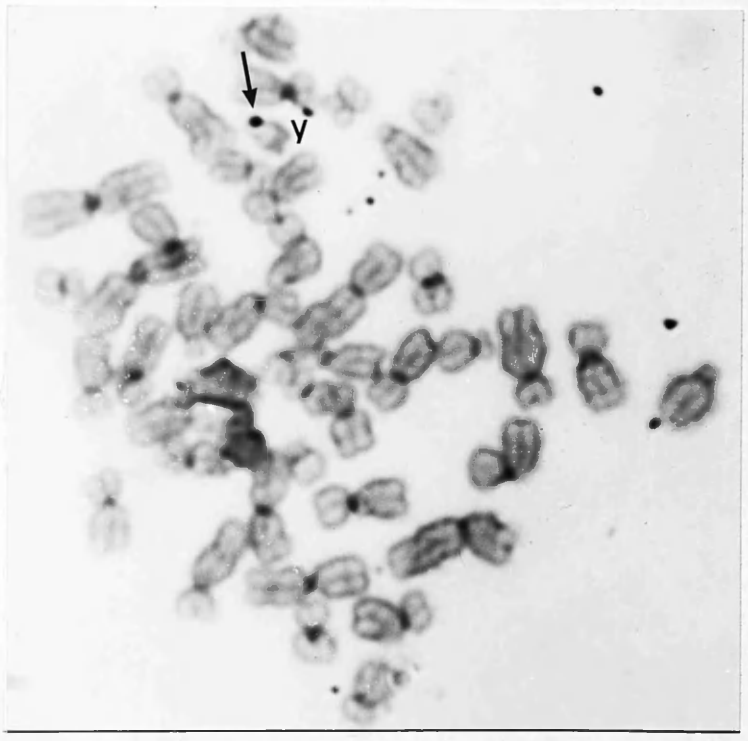


A B

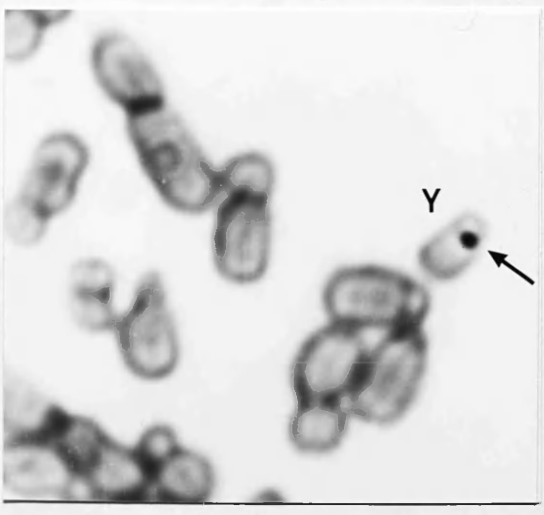


C D

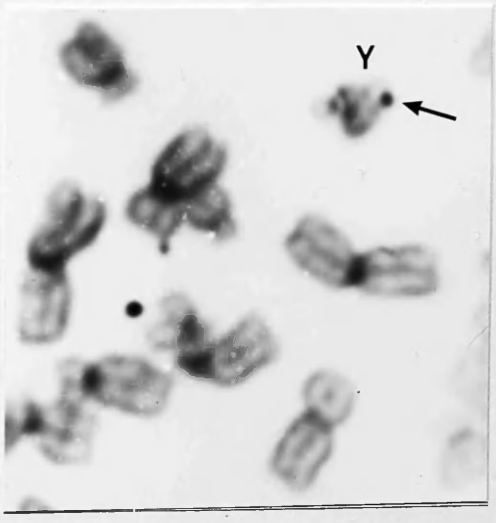
Figure 12 ^{3}H-P2F2 in female and XX male KS.
-Partial metaphase spread from a normal female before (A) and after (B) the hybridisation.
-Pairs of chromosome X from XX male KS before (C) and after (D) the hybridisation.



a



b



c

Figure 13 ^{3}H-P2F2 in normal male.
Complete (a) and partial (b, c) metaphase spreads following hybridisation.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	9	7.69	1.31	0.22
2	4.16	9	7.49	1.51	0.30
3	3.49	8	6.42	1.58	0.39
4	3.32	9	5.98	3.02	1.52
5	3.17	3	5.71	-2.71	1.29
6	2.99	3	5.38	-2.38	1.05
7	2.81	2	5.06	-3.06	1.85
8	2.54	2	4.57	-2.57	1.44
9	2.40	6	4.32	1.68	0.65
10	2.36	3	4.25	-1.25	0.38
11	2.36	1	4.25	-3.25	2.45
12	2.36	2	4.25	-2.25	1.19
13	1.89	4	3.40	0.60	0.10
14	1.81	1	3.29	-2.29	1.59
15	1.72	2	3.10	-1.10	0.39
16	1.61	1	2.90	-1.90	1.24
17	1.47	4	2.65	1.35	0.69
18	1.40	1	2.52	-1.52	0.90
19	1.16	3	2.09	0.91	0.39
20	1.08	4	1.94	2.06	2.19
21	0.77	0	1.39	-1.39	1.39
22	0.89	0	1.60	-1.60	1.60
X	2.69	13	4.84	8.16	13.76

Table 9 ^{3}H-P2F2 in normal female. Statistical analysis of silver grain distribution. Data from 24 cells, total number of grains 90. Chi-square for the X chromosome is highly significant at $p<0.001$. Chi-square for the whole experiment (36.94) and 22 degrees of freedom is significant at $0.025<p<0.01$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	59	59	0	0.00
2	7.76	55	54	1	0.01
3	6.56	38	46	-8	1.39
4	6.13	27	43	-14	0.37
5	5.58	33	39	-6	0.9
6	5.65	27	37	-10	2.7
7	5.00	30	35	-5	0.7
8	4.77	37	33	4	0.48
9	4.73	35	33	2	0.12
10	4.35	35	31	4	0.51
11	4.35	32	31	1	0.03
12	4.16	26	30	-4	0.53
13	3.59	22	25	-3	0.36
14	3.28	24	23	1	0.04
15	3.11	18	22	-4	0.7
16	3.11	25	22	3	0.40
17	3.02	17	21	-4	0.76
18	2.73	22	19	3	0.47
19	2.58	15	18	-3	0.5
20	2.31	22	16	6	2.25
21	1.90	9	13	-4	1.23
22	1.69	14	12	2	0.33
X	5.14	80	36	44	53.77

Table 10 ^{3}H-P2F2 in XX male KS. Statistical analysis of grain distribution. Data from 32 cells, total number of grains 702.

Chi-square for the X chromosome (for one degree of freedom) is highly significant at $p<0.001$.
Chi-square for the whole experiment (67.79) is very highly significant at $p<0.001$, for 22 degrees of freedom.
Omitting the X chromosome, chi-square=14.02 which is not significant (for 21 degrees of freedom, $0.975<p<0.9$). Thus it is the contribution of the X chromosome that makes this experiment statistically significant.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	11	12.12	-1.12	0.10
2	4.16	4	11.81	-7.81	5.16
3	3.49	7	9.91	-2.91	0.85
4	3.32	10	9.43	0.57	0.03
5	3.17	12	9.00	3.00	1.00
6	2.99	7	8.49	-1.49	0.26
7	2.81	7	7.98	-0.98	0.12
8	2.54	4	7.21	-3.21	1.43
9	2.40	6	7.04	-1.04	0.15
10	2.36	5	6.70	-1.70	0.43
11	2.36	9	6.70	2.3	0.79
12	2.36	7	6.70	0.30	0.01
13	1.89	4	5.37	-1.37	0.35
14	1.81	3	5.14	-2.14	0.90
15	1.72	2	4.88	-2.88	1.70
16	1.61	5	4.57	0.73	0.12
17	1.47	4	4.17	-0.17	0.01
18	1.40	6	3.98	2.02	1.02
19	1.16	5	3.29	1.71	0.89
20	1.08	4	3.07	0.93	0.28
21	0.77	2	2.19	-0.19	0.02
22	0.89	1	2.53	-1.53	0.92
X	2.69	12	3.82	8.18	17.52
mar		3			

Table 11 ^{3}H-P2F2 in 45,X/46,X + mar (RW). Statistical analysis of grain distribution. Data from 24 cells, total number of grains 142.
Chi-square for the X chromosome (one degree of freedom) highly significant at $p<0.001$.
Chi-square for the whole experiment 32.89 significant at $0.1<p<0.05$ for 23 degrees of freedom.

recognisable in these cells due to the large C-band on its long arm, as following hybridisation a C-banding pattern could be seen on the chromosomes. According to the above results P2F2 was localised to the X chromosome band Xq21 and to the short arm of the Y chromosome.

3.2.3 GMGY3

This recombinant, containing a 1.3kb insert isolated from a Y-specific library, was labelled with ^{3}H-dCTP using the random priming method, as described in section 2.4.3, to a specific activity of 1.1×10^{8} dpm/μg and was hybridised to metaphase spreads from a normal male at concentrations 5 and 20ng per slide. The preparations were exposed for 19 days then developed and scored for silver grains. Forty five cells were relocated and a total of 366 grains was recorded. The statistical analysis of the grain counts is presented in table 12. Sixteen grains were located on the Y chromosome, 14 of these on the short arm and 2 on the long arm. This hybridisation to Yp was statistically significant ($p<0.001$). Minor hybridisation was observed with the X chromosome, band Xp2.1 though not statistically significant. From the autosomes the only chromosome significantly labelled was chromosome 9 ($0.005<p<0.001$) with 30 grains ($0.005<p<0.0001$). The distribution of silver grains over this chromosome reveals a peak of hybridisation in the short arm, region 9p22-pter. The banding stage of the chromosomal preparations used did not allow subdivision of this region in individual bands. The distridution of silver grains over the Y, X chromosome and chromosome 9 is shown in figure 14. Thus the above recombinant was localised to chromosome Yp and chromosome 9p22-pter.

Chromosome	Relative Length *	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	33	31.26	1.74	0.0969
2	4.16	21	30.45	-9.45	2.9328
3	3.49	21	25.55	-4.55	0.8103
4	3.32	16	24.30	-8.30	2.8350
5	3.17	11	23.20	-12.20	6.4155
6	2.99	16	21.89	-5.89	1.5848
7	2.81	13	20.57	-7.57	2.7858
8	2.54	19	18.59	0.41	0.0090
9	2.40	30	17.57	12.43	8.7937
10	2.36	19	17.28	1.72	0.1712
11	2.36	15	17.28	-2.28	0.3008
12	2.36	22	17.28	4.72	1.2893
13	1.89	13	13.83	-0.83	0.0498
14	1.81	8	13.25	-5.25	2.0802
15	1.72	14	12.59	1.41	0.1579
16	1.61	13	11.79	1.21	0.1242
17	1.47	14	10.76	3.24	0.9756
18	1.40	12	10.25	1.75	0.2988
19	1.16	9	8.49	0.51	0.0306
20	1.08	7	7.91	-0.91	0.1047
21	0.77	4	5.64	-1.64	0.4769
22	0.89	6	6.51	-0.51	0.0400
X	2.69	14	9.85	4.15	1.7485
Y	0.96	16	3.51	12.49	44.4444

Table 12. ^{3}H-GMGY3. Statistical analysis of silver grain distribution. Results from 45 cells total number of grains 366.
Chi-square for the Y chromosome (for one degree of freedom) very highly significant at $p<0.001$.
Chi-square for the 9 chromosome significant at $0.005<p<0.001$.
Chi-square for the whole experiment= 75.67, very highly significant at $p<0.001$ for 22 degrees of freedom.
Omitting chromosomes Y and 9, total chi-square=22.43 not significant for 21 degrees of freedom.

* Expressed as a percentage of the Y-containing haploid genome, taken from Harris et al., (1986)

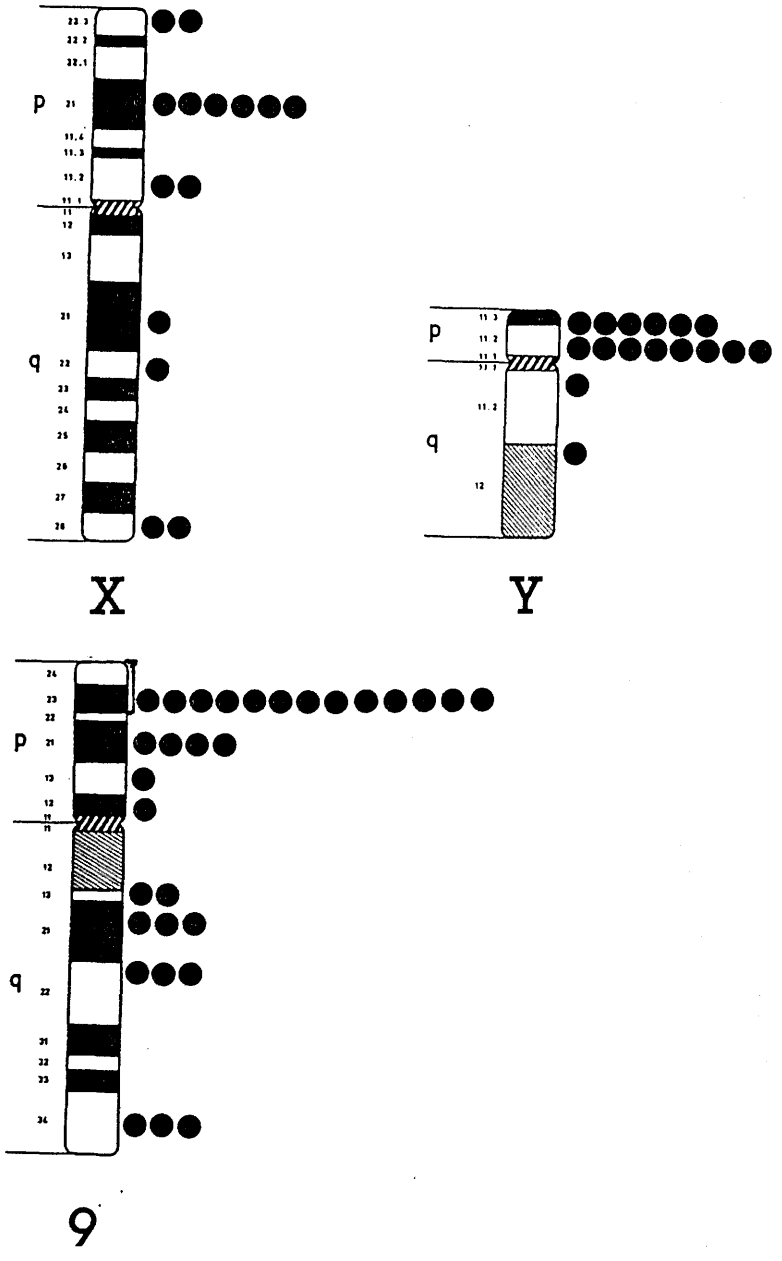


Figure 14 ^{3}H-GMGY3 in a normal male. Distribution of silver grains over chromosomes Y, 9 and X. Silver grains at distal 9p are assigned to region 9p23-9pter

(Data from 45 cells)

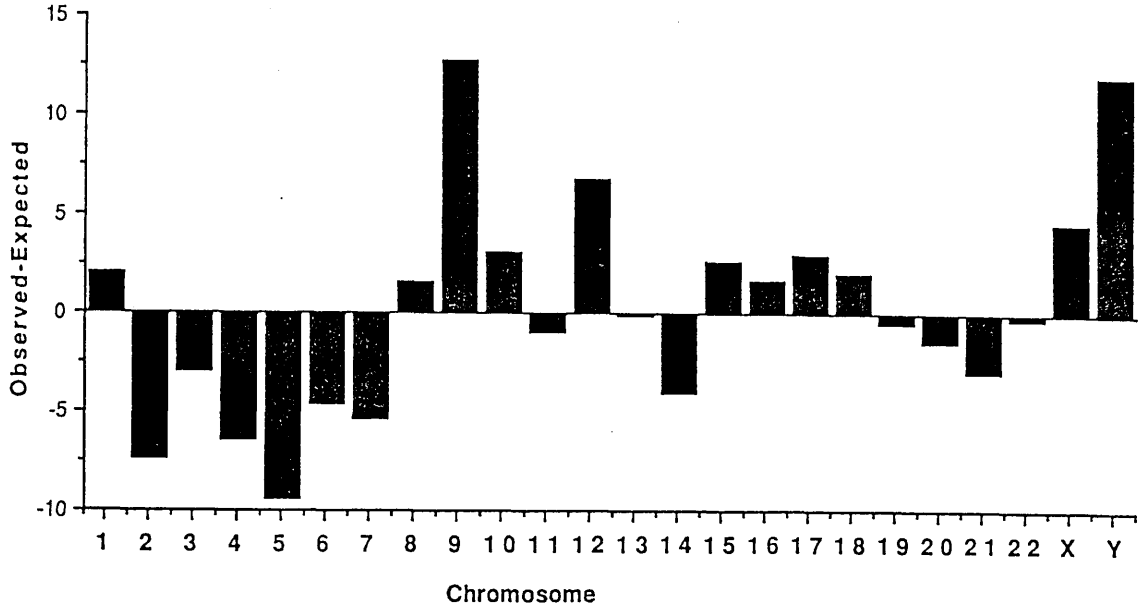


Figure 15 ^{3}H-GMGY3 in a normal male. Histogram showing the difference between observed and expected grain counts for each chromosome (data from 45 cells). The expected grain count for each chromosome was determined by the relative proportion of haploid genome length occupied by the chromosome as measured by flow cytometry.

3.2.4 GMGY4b

This recombinant containing a 1.8kb insert isolated from a Y-specific library, was labelled to a specific activity of 1x107 dpm /µg and was hybridised to metaphase spreads from two different normal males at concentrations of 100 and 60ng per slide. In this particular experiment the post hybridisation washes consisted of washes in 2xSSC at room temperature and 0.1xSSC at 65°C; the preparations were exposed for 22 days. In the slide hybridised with 60ng of labelled probe grains were located at the centromeric region of the Y chromosome. This labelling was heavier in the slide hybridised with 100ng of probe and additional sites of hybridisation were present at the centromeric region of chromosomes 5, 13, 14, 15, 20 and 22. Figure 16 shows the distribution of silver grains obtained with the two different probe concentrations. Statistical analysis on pooled data from both slides revealed that in forty three cells scored and out of a total number of 331 silver grains recorded, 114 were located on the Y chromosome which represents 33.92% of the total hybridisation and is statistically significant ($p<0.001$, table 13). Chromosomes 15 and 22 were also significantly labelled at $p<0.001$ and $0.025<p<0.01$ respectively.

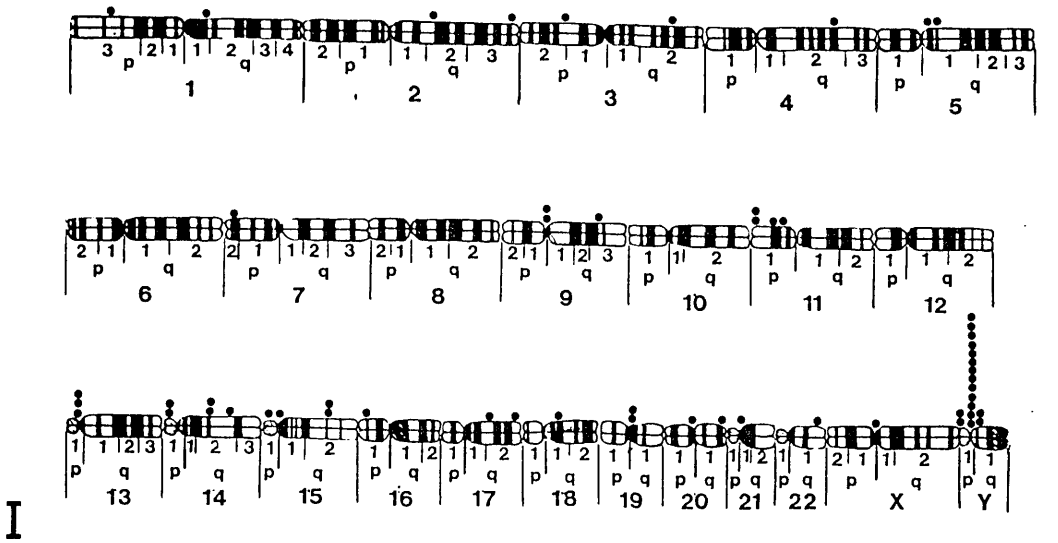
Another study was carried out with post-hybridisation washes according to the method of Cremer et al., (1986) with 3 rinses, 30 minutes each at 38°C. This time the slides were hybridised with 60ng of labelled probe and were exposed for 25 days. Forty two cells were relocated and a total number of 531 silver grains was scored. Chromosomes Y, 22, 21, 14 and 15 showed significant labelling with 62, 33, 26, 33 and 33 silver grains respectively ($p<0.001$). The statistical analysis of the results is shown in table 14. The

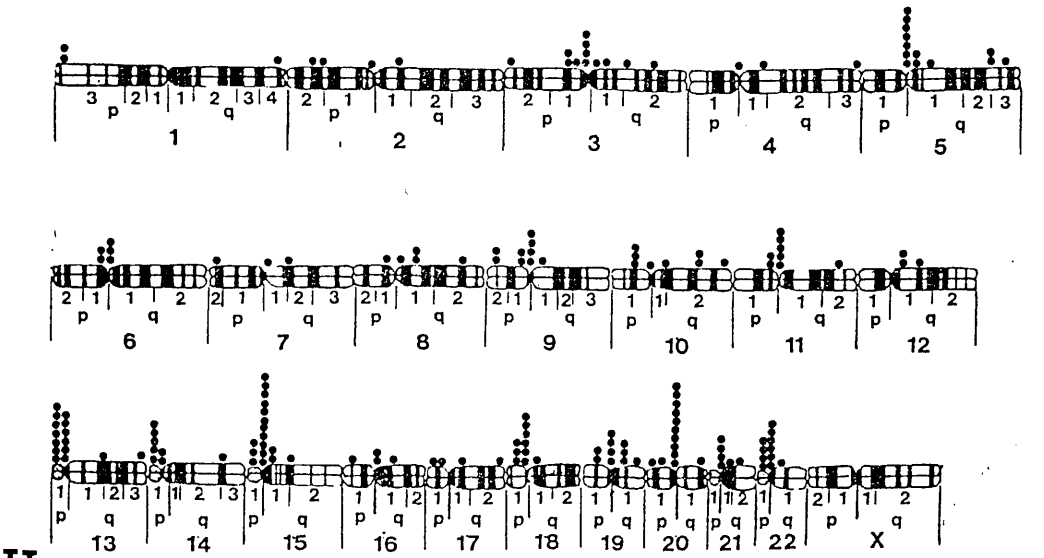
Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	4	28.65	-14.26	7.19
2	4.16	10	27.54	-17.54	11.17
3	3.49	14	23.10	-9.10	3.58
4	3.32	6	21.98	-15.98	11.62
5	3.17	15	20.98	-5.98	1.71
6	2.99	3	19.79	-16.79	14.24
7	2.81	5	18.60	-13.60	9.94
8	2.54	6	16.81	-10.81	6.95
9	2.40	12	15.89	-3.89	0.95
10	2.36	7	15.62	-8.62	4.76
11	2.36	11	15.62	-4.62	1.37
12	2.36	3	15.62	-12.62	10.19
13	1.89	16	12.51	3.44	0.28
14	1.81	13	11.98	1.02	0.09
15	1.72	23	11.39	11.61	11.83
16	1.61	6	10.66	-4.66	2.04
17	1.47	5	9.73	-4.73	2.30
18	1.40	13	9.27	3.73	1.50
19	1.16	11	7.88	3.12	1.23
20	1.08	13	7.15	5.85	4.78
21	0.77	8	5.10	2.9	1.65
22	0.89	12	5.89	6.11	6.34
X	2.69	1	8.90	-7.90	7.01
Y	0.96	116	3.18	112.82	4002.62

Table 13 GMGY4b in normal male. Data from 43 cells, total number of grains 331 (washes: 0.1xSSC at 65°C).
Chi-square for chromosomes Y, 15 very highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	37	45.34	-8.34	1.53
2	4.16	28	44.18	-16.18	5.92
3	3.49	26	37.06	-11.07	3.31
4	3.32	21	36.00	-15.00	6.25
5	3.17	20	33.66	-13.66	5.54
6	2.99	12	31.75	-19.75	12.28
7	2.81	13	29.84	-14.97	9.50
8	2.54	12	26.97	-14.97	8.31
9	2.40	20	25.49	-5.49	1.18
10	2.36	19	25.06	-6.06	1.46
11	2.36	27	25.06	1.94	0.15
12	2.36	18	25.06	-7.06	1.99
13	1.89	15	20.07	-5.07	1.28
14	1.81	33	19.22	13.78	9.88
15	1.72	33	18.27	14.73	11.87
16	1.61	14	17.10	-3.10	0.56
17	1.47	18	15.61	2.31	0.34
18	1.40	11	14.87	-3.87	1.01
19	1.16	10	12.32	-2.32	0.44
20	1.08	14	11.47	2.53	0.56
21	0.77	26	8.18	17.82	38.82
22	0.89	33	9.45	23.55	58.69
X	2.69	9	14.28	-5.28	1.92
Y	0.96	62	5.10	56.90	634.82

Table 14 GMGY4b in normal male (washes: 50% formamide at 38°C)
Data from 42 cells, total number of grains 531.
Chi-square for each of chromosomes Y, 21, 22, 15 very highly significant at $p<0.001$.
Chi-square for chromosome 14 highly significant at $0.005<p<0.001$.





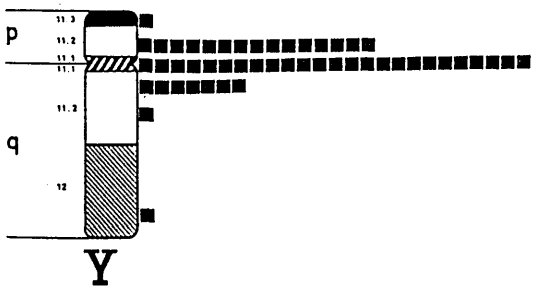


Figure 16 ^{3}H-GMGY4b in a normal male. Distribution of silver grains over the human karyotype (I) 60ng of probe DNA (II) 100ng.
In II the Y chromosome is shown separately.
■: 2 silver grains

centromeric location of the silver grains can be seen in figure 17.

3.2.5 JG51 and JG73

These two recombinants were isolated independently from a flow-sorted chromosome 21 library and have inserts of 4.7kb and 3kb size respectively. Attempts to nick translate these sequences with ^{3}H-dCTP failed repeatedly and eventually 250ng of each probe were labelled with ^{3}H-dCTP and ^{3}H-dATP using the random priming method of Feinberg & Vogelstein (1983) as adopted by Donlon (1986) (see 2.4.4). This resulted in 70 and 36% incorporated radioactivity respectively as was shown by the TCA test.

i) JG73: Radioactive JG73 was hybridised with metaphase spreads from a normal male at a concentration of 14ng per slide and the slides were exposed for 25 days (12 days exposure did not result in enough labelling). Statistical analysis on data from 23 cells showed that chromosomes 19, 21, 22 and Y were significantly labelled ($p<0.001$). The distribution of silver grains over the human chromosome complement is shown in figure 18A. In this figure it can be seen that silver grains were present on many other chromosomes. Cremer et al. (1986) used a probe which under stringent hybridisation conditions maps strictly to the centromeric region of chromosome 18 whereas under conditions of low stringency cross-hybridised with the centromeric regions of the whole chromosome complement. Following their conditions for high stringency which consisted of 3 washes for 30 minutes in 50% formamide/2xSSC at 33^{o}C followed by 3 rinses in 2xSSC at the same temperature, radioactive JG73 was hybridised with metaphases from a

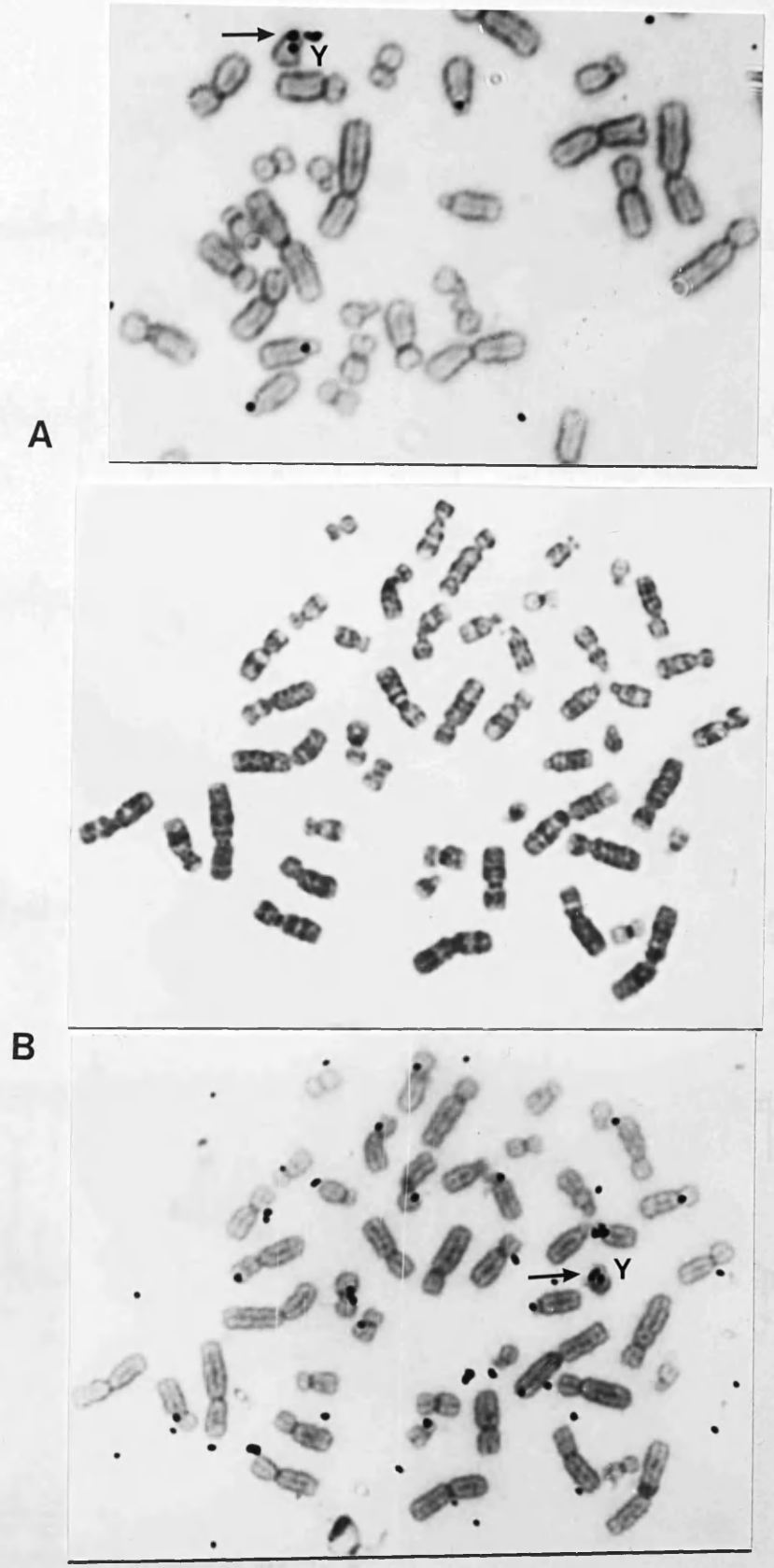
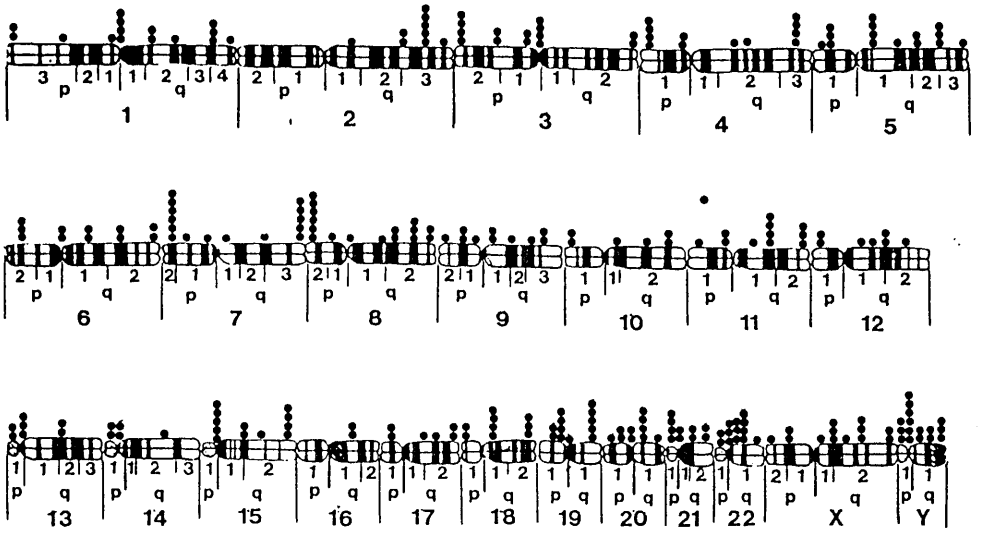
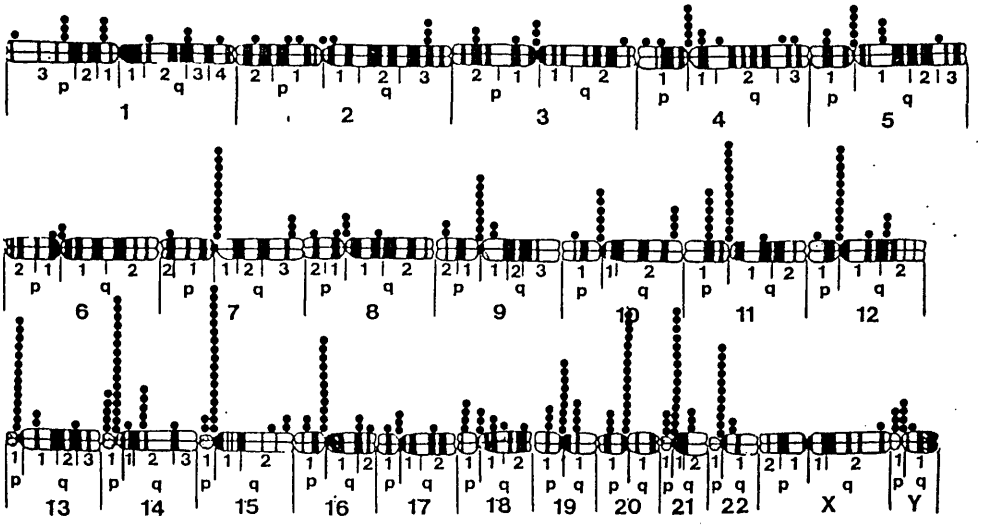


Figure 17 ^{3}H-GMGY4b in normal male.
(A) partial metaphase following hybridisation
(washes: 0.1xSSC at 65°C)
(B) metaphase spread before and after the hybridisation,
(washes: formamide at 38°C)



A



B

Figure 18 ^{3}H-JG73 in normal male. Distribution of silver grains over the human chromosome complement.
(A) washes: formamide at 45°C
(B) washes: formamide at 33°C

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	16	22.55	-6.22	1.9025
2	7.76	12	21.96	-9.96	4.4173
3	6.56	17	18.43	-1.43	0.1109
4	6.13	15	17.53	-2.53	0.3651
5	5.58	14	16.74	-2.74	0.4484
6	5.65	11	15.75	-4.74	1.4530
7	5.00	14	14.84	-0.84	0.0475
8	4.77	18	13.41	4.59	1.5710
9	4.73	10	12.67	-2.67	0.5626
10	4.35	4	12.46	-8.46	6.4431
11	4.35	8	12.46	-44.46	1.5964
12	4.16	7	12.46	-5.46	2.3925
13	3.59	6	9.98	-3.98	1.5873
14	3.28	7	9.56	-2.56	0.6556
15	3.11	11	9.08	1.92	0.4059
16	3.11	2	8.50	-6.50	4.9705
17	3.02	7	7.76	-0.76	0.0744
18	2.73	9	7.39	1.61	0.3507
19	2.58	15	6.12	8.88	12.7184
20	2.31	10	5.70	4.30	3.2438
21	1.90	11	4.07	6.93	11.7997
22	1.69	14	4.70	9.30	18.4021
X	5.14	11	7.10	3.90	2.5723
Y	2.09	15	2.53	12.70	63.7509

Table 15 JG73 in normal male (washes: 50% formamide at 45°C). Statistical analysis of grain distribution. Data from 23 cells, total number of grains 264, 25 days exposure.
Chi-square for chromosomes 19, 21, 22, Y highly significant at $p<0.001$.

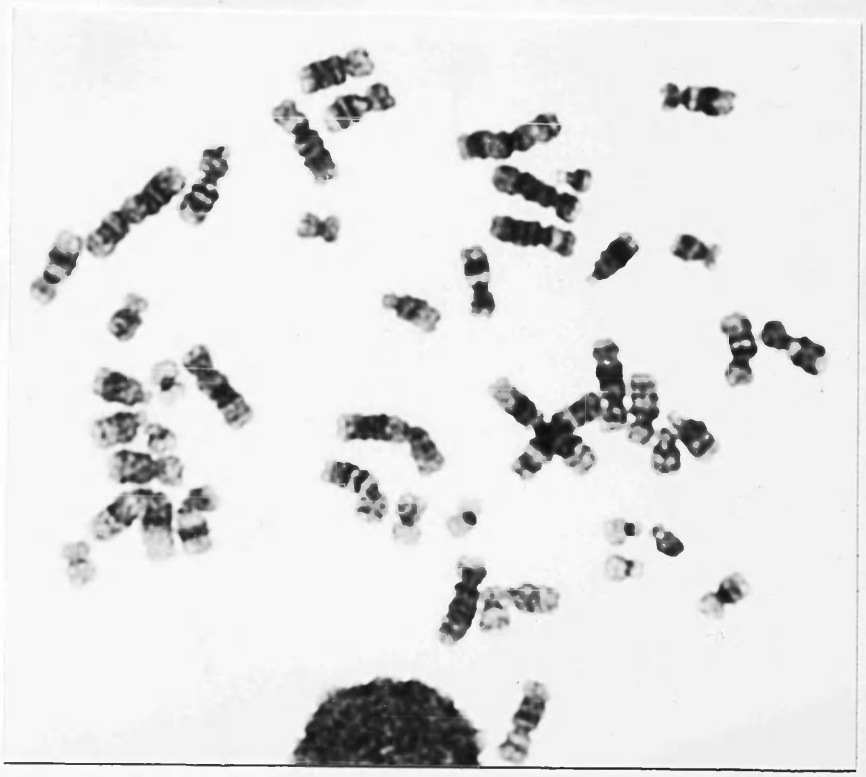
Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	25	42.27	-17.27	7.05
2	4.16	25	41.18	-16.18	6.36
3	3.49	13	34.55	21.55	13.44
4	3.32	20	32.87	-12.87	5.04
5	3.17	20	31.38	-11.38	4.13
6	2.99	12	29.60	-17.60	10.46
7	2.81	26	27.82	-1.82	0.12
8	2.54	10	25.15	-15.15	9.13
9	2.40	18	23.76	-5.76	1.40
10	2.36	19	23.36	-4.36	0.81
11	2.36	20	23.36	-3.36	0.48
12	2.36	20	23.36	-3.36	0.48
13	1.89	24	18.71	5.29	1.49
14	1.81	37	17.92	19.08	20.31
15	1.72	45	17.03	27.97	45.94
16	1.61	22	15.94	6.06	2.30
17	1.47	11	14.55	-3.55	0.87
18	1.40	13	13.86	-0.86	0.05
19	1.16	22	11.84	10.16	8.72
20	1.08	34	10.69	23.31	50.82
21	0.77	30	7.62	22.38	65.73
22	0.89	20	8.81	11.19	14.21
X	2.69	9	13.31	-4.31	1.39
Y	0.96	11	4.75	6.25	8.22

Table 16 JG73 in normal male (washes: 50% formamide at 33°C). Data from 21 cells, total number of silver grains 495.
Chi-square for chromosomes 22, 21, 20, 14, 15 very highly significant at $p<0.001$.
Chi-square for the Y chromosome, chromosome 19 significant at $0.005<p<0.001$.

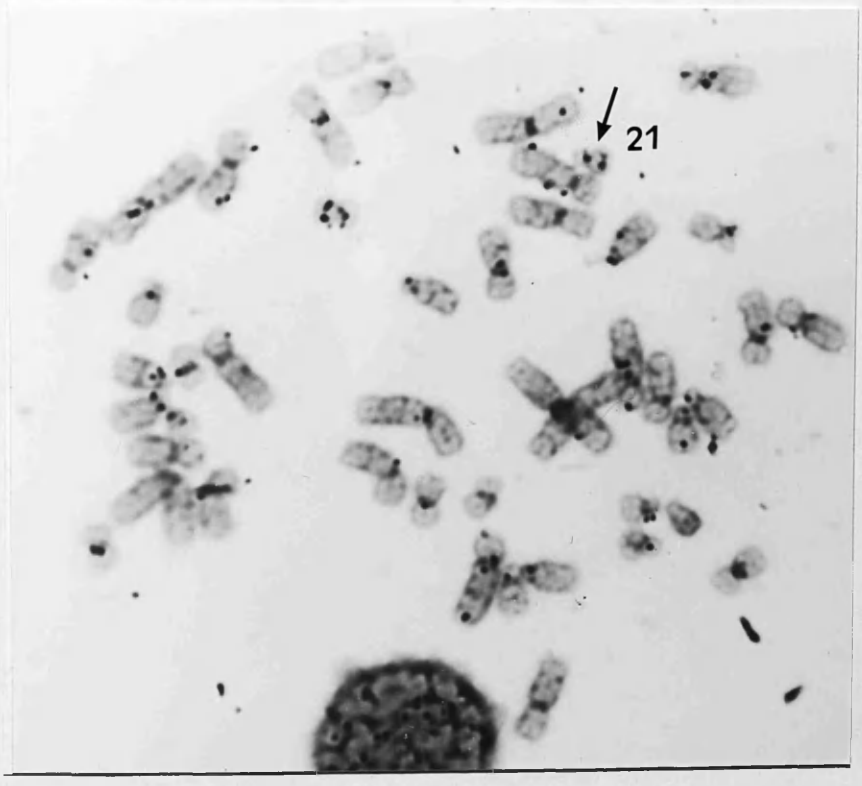
normal male. Data from 21 cells revealed significant labelling of chromosomes 21, 20, 15, 14, 22 ($p<0.001$). These results compared to the ones obtained under the standard washing procedure mentioned above reveal additional statistically significant sites of hybridisation on chromosomes 14, 15 and less labelling on the Y chromosome. In figures 18 however it can be seen that silver grains were present on the centromeres of other chromosomes. this is also shown in figure 19, in metaphases following hybridisation with JG73. It should be noted that in this photograph silver grains appear smaller than they usually do, due to less time in developer during the processing of the slides following autoradiography. These results indicate that the conditions of high stringency used by Cremer et al., (1986) were more "relaxed" than the standard post hybridisation washes used in this project (50% formamide at 45°C).

As the stringency of DNA hybridisation can be increased by raising the temperature of the hybridisation a third study was carried out with the hybridisation performed at 52°C instead of the standard 43°C. Data from 25 cells revealed that the only chromosome significantly labelled was chromosome 21 (table 17). The distribution of silver grains over the chromosome complement can be seen in figure 20.

ii) JG51: Radioactive JG51 was hybridised to chromosomal preparations from a normal male at a concentration of 20ng per slide and the preparations were exposed for 21 days. The post-washes included three washes 30 min each in 50%formamide/2xSSC at 33°C, followed by 3 rinses in 2xSSC at the same temperature, according to the method for higher stringency of Cremer et al. (1986). Data from



a



b

Figure 19 ^{3}H-JG73. Metaphases before (a, c) and after (b, d) the hybridisation. (washes: formamide at 33°C)

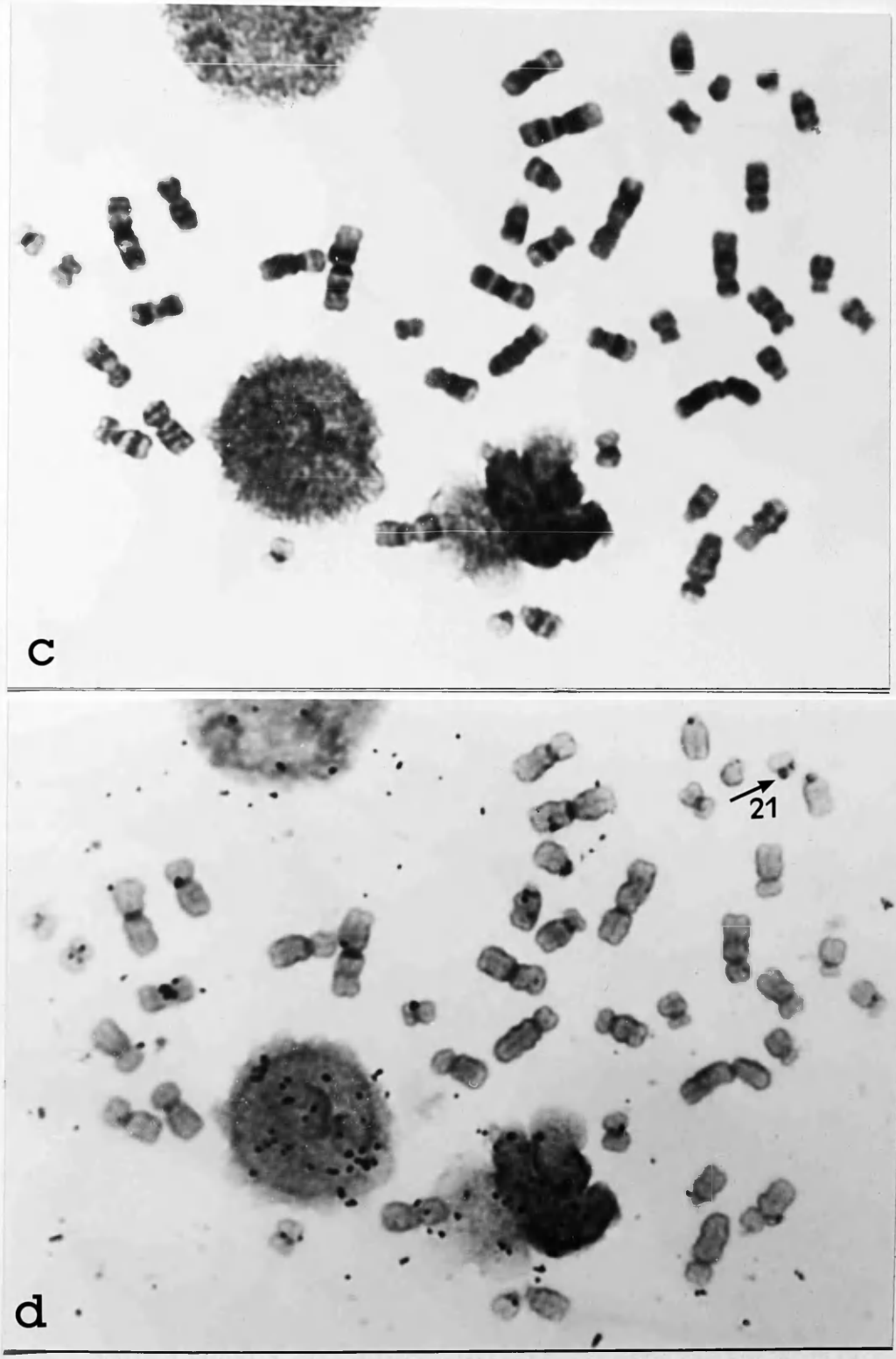


Figure 19 continued

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	16	15.88	0.12	0.00
2	4.16	14	15.47	-1.47	0.14
3	3.49	14	12.98	1.02	0.08
4	3.32	14	12.35	1.65	0.22
5	3.17	5	11.79	-6.79	3.91
6	2.99	14	11.12	2.88	0.74
7	2.81	13	10.45	2.55	0.62
8	2.54	2	9.45	-7.45	5.87
9	2.40	7	8.93	-1.93	0.41
10	2.36	4	8.78	-4.78	2.60
11	2.36	11	8.78	2.22	0.56
12	2.36	7	8.78	-1.78	0.36
13	1.89	5	7.03	-2.03	0.59
14	1.81	3	6.73	-3.73	2.08
15	1.72	6	6.40	-0.40	0.02
16	1.61	5	5.99	-0.99	0.16
17	1.47	6	5.47	0.53	0.05
18	1.40	6	5.21	0.79	0.12
19	1.16	7	4.31	2.69	1.68
20	1.08	8	4.02	3.98	3.94
21	0.77	10	2.86	7.14	17.89
22	0.89	5	3.31	1.69	0.86
X	2.69	2	5.00	-3.00	1.8
Y	0.96	2	1.78	0.22	0.03

Table 17 JG73 in normal male, high stringency (hybridisation at 52°C)
Data from 25 cells, total number of grains 186.
Chi-square for chromosome 21 is highly significant at $p<0.001$.
Chi-square for the whole experiment (44.73) significant at $0.005<p<0.001$ for 22 degrees of freedom.
Omitting chromosome 21, chi-square (26.84) not significant.

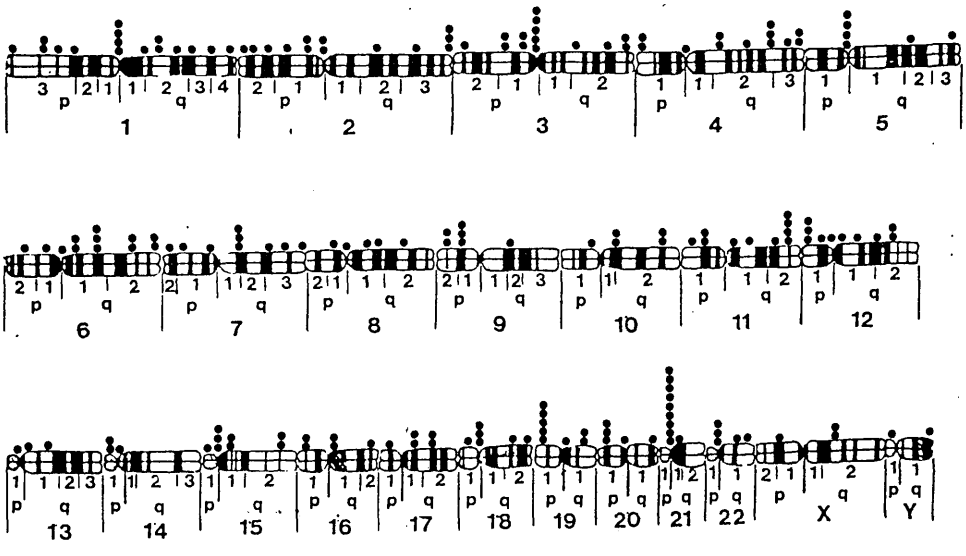


Figure 20 JG73. Distribution of silver grains over the human chromosome complement. Hybridisation at 52°C

28 cells showed that chromosomes 22, 14, 15, 21, 20 and 13 were significantly labelled (table 18) but cross-hybridisation occurred with the centromeres of many other chromosomes (see figure 21A and figure 22). It should be noted that this figure was based only on grain counts from 14 cells. The experiment was repeated with the standard washing procedure (50% formamide/2xSSC at 45°C). Twenty two cells were scored and 510 silver grains were recorded. Chromosomes 13, 14, 15, 20, 21 and 22 were significantly labelled (table 19) but less cross-hybridisation occurred with other chromosomes (figure 21B).

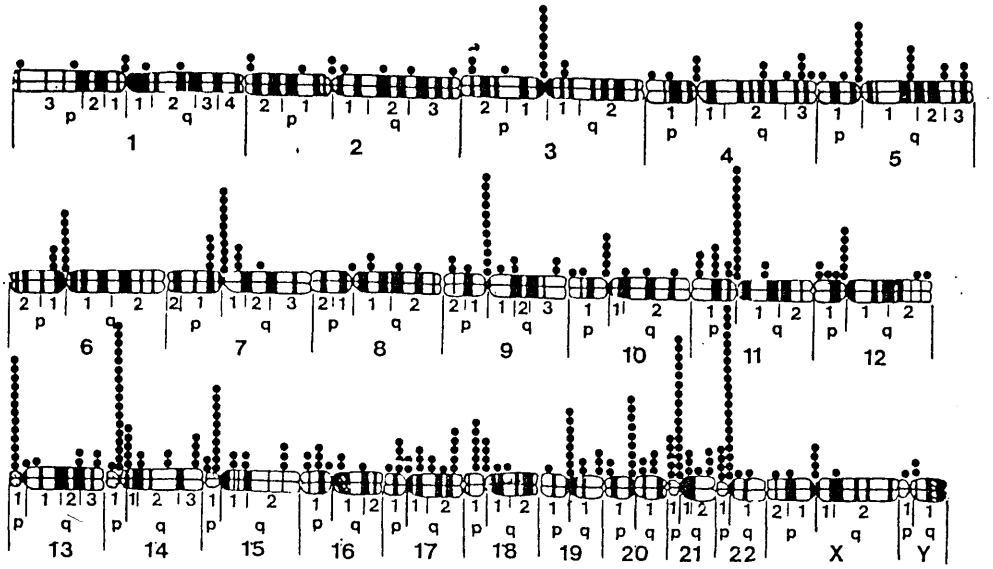
A final hybridisation was performed at 52°C instead of the usual 43°C and the slides were exposed for 17 days. The results showed that JG51 hybridised to chromosomes 22, 13, and 14 (see table 20). The distribution of silver grains in the chromosome complement is showing in figure 23.

3.3 Biotinylated in situ hybridisation

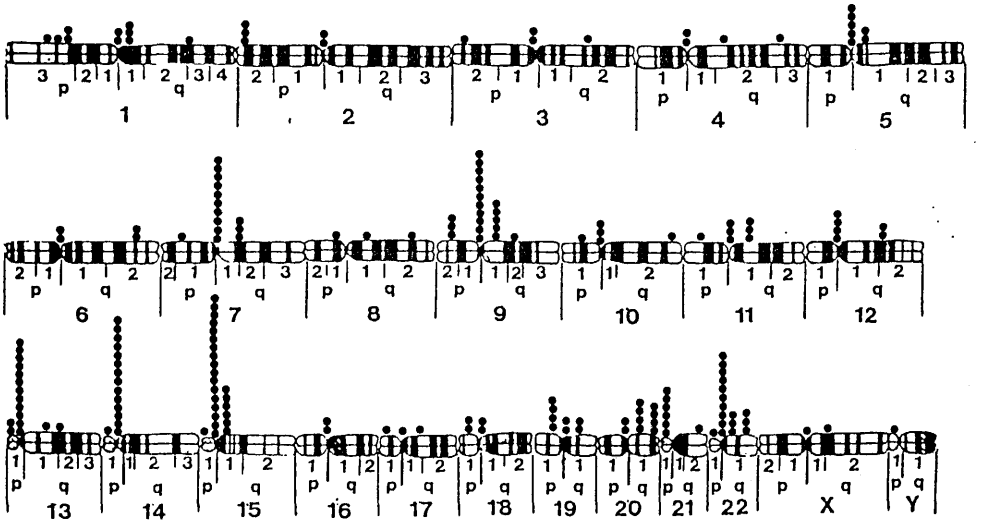
Several protocols have been described for biotinylated in situ hybridisation (see section 1.4.3). For the purposes of this study the most suitable was found to be the one described by Garson et al., (1987). The detection of the formed hybrids by this method takes place in one enzymatic step in contrast to the more complicated several steps of other protocols (Burns et al., 1985). Secondly, in this technique visualization of the hybridisation signal is performed under phase-contrast microscopy (which is available in this department) rather than the more sophisticated approaches such as reflection-contrast or fluorescence. Additionally, this method has been shown to be sensitive enough to

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	21	61.49	-41.49	17.21
2	4.16	29	59.90	-30.90	15.94
3	3.49	29	50.26	-21.26	8.99
4	3.32	8	47.81	-39.81	33.15
5	3.17	42	45.65	- 3.65	0.29
6	2.99	20	43.06	-23.06	12.35
7	2.81	38	40.46	- 2.46	0.15
8	2.54	14	36.58	-12.58	4.33
9	2.40	31	34.56	- 3.56	0.37
10	2.36	15	33.98	-18.98	10.60
11	2.36	39	33.98	5.02	0.74
12	2.36	27	33.98	- 6.98	1.43
13	1.89	46	27.22	18.78	12.96
14	1.81	68	26.06	41.94	67.49
15	1.72	59	24.77	34.23	47.30
16	1.61	22	23.18	- 1.18	0.06
17	1.47	22	21.17	0.83	0.03
18	1.40	15	20.16	-5.16	1.32
19	1.16	26	16.70	9.30	5.18
20	1.08	41	15.55	25.45	41.65
21	0.77	36	11.09	24.91	55.95
22	0.89	52	12.82	39.18	119.74
X	2.69	11	19.39	-8.39	3.63
Y	0.96	9	6.91	2.09	0.63

Table 18 JG51 in normal male (washes: 50% formamide at 33°C)
Data from 28 cells, total number of grains 720.
Chi-square for chromosomes 22, 21, 20, 14, 15, 13 highly significant at $p<0.001$.



A



B

Figure 21 ^{3}H-JG51 in normal male. Distribution of silver grains over the human chromosome complement.

(A) washes: formamide at 33°C
(B) washes: formamide at 45°C

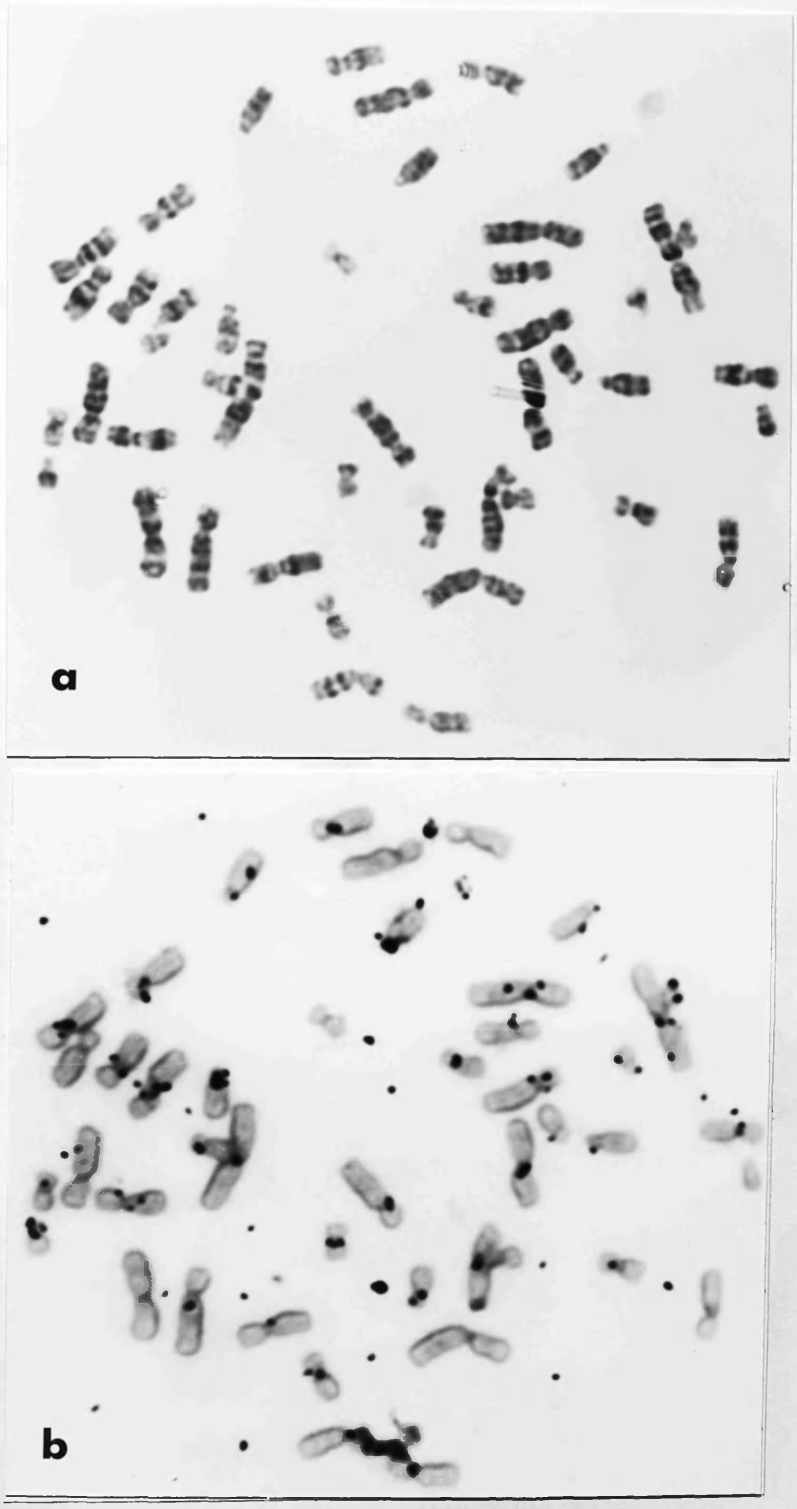


Figure 22 ^{3}H-JG51. Metaphases before (a, c) and after (b, d) the hybridisation. (washes: formamide at 33°C)

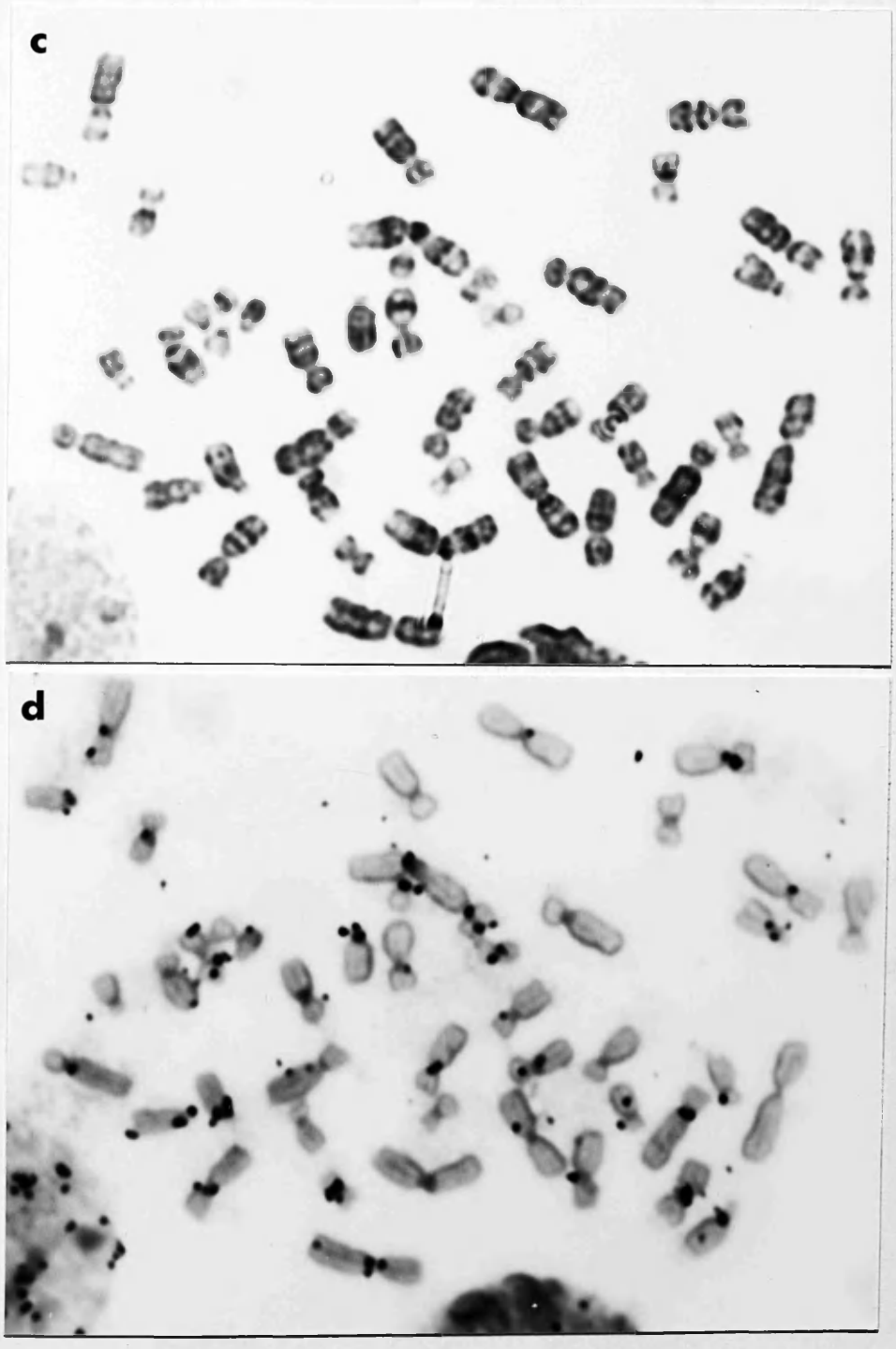


Figure 22 continued

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	15	43.55	-28.55	18.72
2	4.16	15	42.43	-27.23	17.56
3	3.49	14	35.60	-11.60	0.32
4	3.32	11	33.86	-22.86	15.43
5	3.17	21	32.33	-11.33	3.97
6	2.99	5	30.50	-25.50	21.32
7	2.81	26	28.66	-2.66	0.25
8	2.54	8	25.91	-17.91	12.38
9	2.40	37	24.48	12.52	6.40
10	2.36	10	24.07	-14.07	8.22
11	2.36	25	24.07	0.93	0.03
12	2.36	21	24.07	-3.07	0.39
13	1.89	42	19.28	22.72	26.77
14	1.81	54	18.46	35.54	68.42
15	1.72	51	17.54	33.46	63.82
16	1.61	8	16.42	-8.42	4.32
17	1.47	7	14.99	-7.99	4.26
18	1.40	10	14.28	-4.28	1.28
19	1.16	19	11.83	7.17	4.34
20	1.08	25	11.02	13.98	17.73
21	0.77	34	7.85	26.15	87.11
22	0.89	41	9.08	31.92	112.21
X	2.69	6	13.72	-7.72	4.34
Y	0.96	5	4.90	0.1	0.00

Table 19 JG51 in normal male (washes in formamide, 45°C). Statistical analysis of silver grain distribution.
Data from 22 cells, total number of grains 510.
Chi-square for chromosomes 22, 21, 20, 15, 14, 13 highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	15	19.98	-4.98	1.24
2	4.16	10	19.46	-9.46	4.60
3	3.49	13	16.33	-3.33	0.68
4	3.32	8	15.54	-7.54	3.66
5	3.17	15	14.84	0.31	0.23
6	2.99	11	13.99	-2.99	1.56
7	2.81	6	13.15	-7.15	3.89
8	2.54	5	11.89	-6.89	3.99
9	2.40	6	11.23	-5.23	2.43
10	2.36	10	11.04	1.04	0.10
11	2.36	14	11.04	2.96	0.79
12	2.36	11	11.04	0.04	0.13
13	1.89	18	8.44	9.16	9.49
14	1.81	16	8.47	7.53	6.69
15	1.72	13	8.05	4.95	3.04
16	1.61	9	7.53	1.47	0.23
17	1.47	8	6.88	1.12	0.18
18	1.40	9	6.55	2.45	0.92
19	1.16	5	5.43	0.43	0.03
20	1.08	7	5.05	1.95	0.75
21	0.77	6	3.60	2.40	1.60
22	0.89	13	4.16	8.84	18.78
X	2.69	5	6.29	-1.29	0.10
Y	0.96	1	2.25	-1.25	0.14

Table 20 JG51 in normal male (hybridisation at 52°C).
Data from 31 cells; total number of grains 234.
Chi-square for chromosome 22 (one degree of freedom) very highly significant at $p<0.001$.
Chi-suare for chromosome 13 (one degree of freedom) significant at $0.005<p<0.001$
Chi-square for chromosome 14 (one degree of freedom) significant at $0.01<p<0.005$.

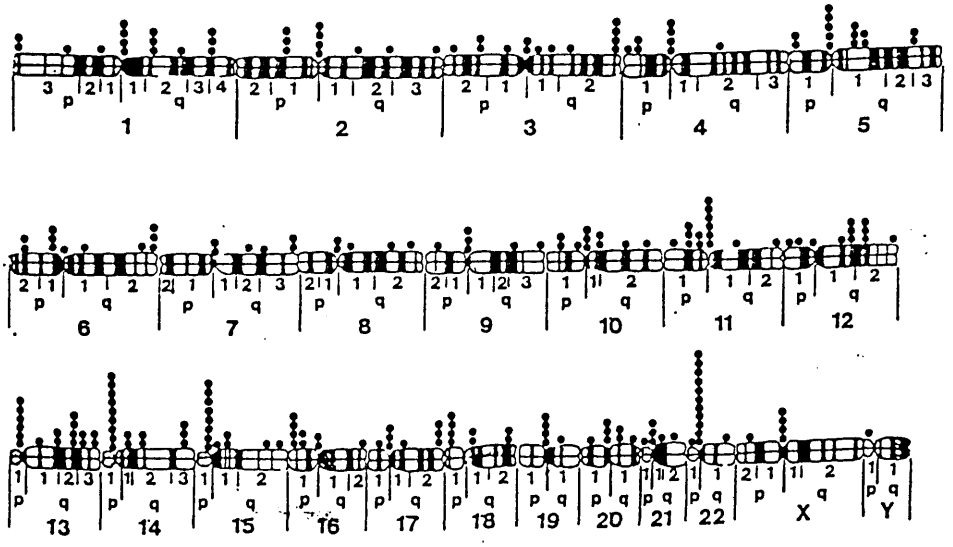
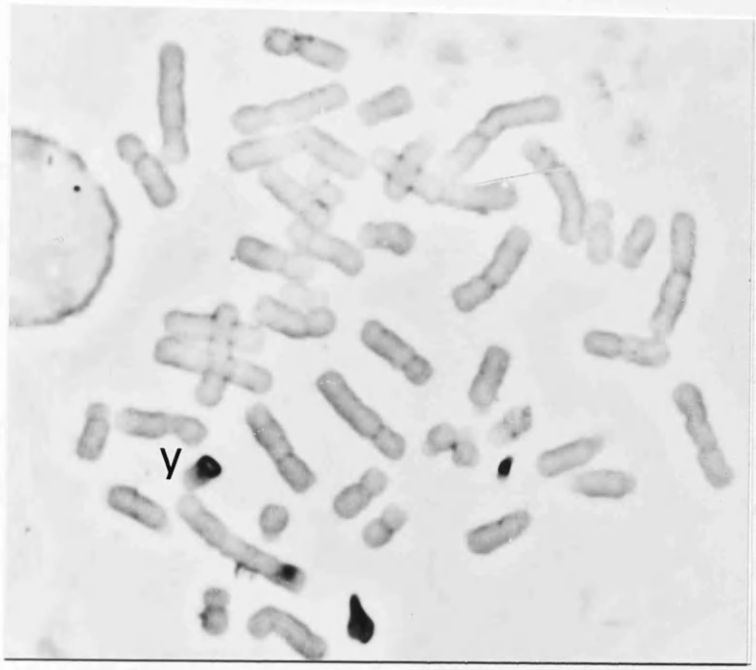


Figure 23 ^{3}H-JG51. Distribution of silver grains over the human chromosome complement (hybridisation at 52°C)

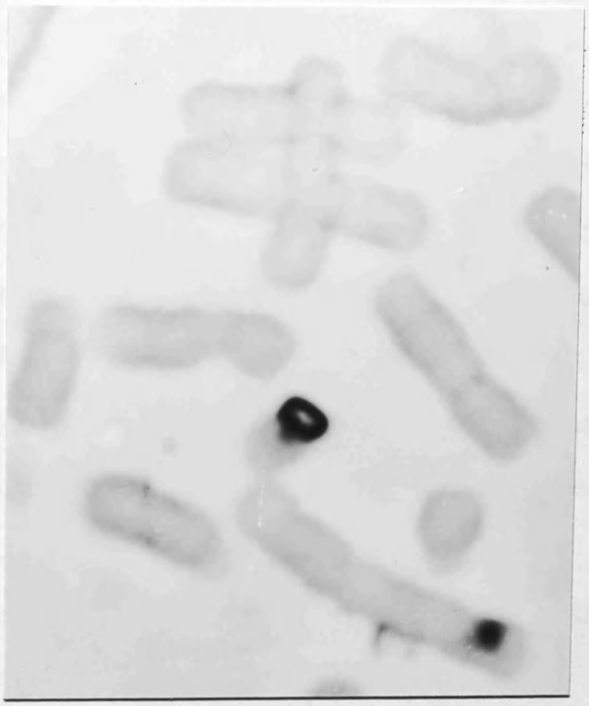
localise as small a DNA sequence as 1kb (Garson et al., 1987).

The reliability and reproducibility of the technique was firstly assessed using the moderate repetitive DNA sequence, GMGY10. DNA probes were labelled with biotin-11-dUTP according to the BRL protocol (see 2.6.1) and was hybridised with chromosomal preparations from a normal male as described in section 2.6.2 at concentrations of 100, 20 and 10 and 2ng per slide. The signal appeared as a yellow-brown "bubble" or as a black spot in chromosomes and interphase nuclei. The word "signal" will be used to refer to both. The higher concentrations of 100ng per slide resulted in an extremely high level of labelling. Strong signal on the Y chromosome was obtained with as little DNA as 1ng per slide with 1 and a half hours incubation in chromogenic substrate (see figure 24). The protocol was straight-forward, easy, and the speed at which results were obtained was amazing when compared with the 6 day autoradiographic exposures required with the same probe and the radioactive approach (section 3.2.1). An advantage of the biotinylated approach is that the reaction can be monitored by examination under phase. If a hybridisation signal is present, the reaction is terminated thus eliminating any non-specific labelling. If inadequate signal was present the slides were incubated longer in chromogenic substrate. Scoring of cells was performed as with the radioactive method. Signals present on any chromosome were recorded and chi-square analysis was performed to assess the level of significance.

A problem associated with this approach was the occasional "dirty" appearance of the chromosomal preparations following hybridisation as under phase contrast dust and stain debris would



A



B

Figure 24 Biotinylated GMGY10 in normal male
(A) complete metaphase following hybridisation
(B) enlarged Y chromosome from above cell.
(phase-contrast microscopy)

Note the great intensity of signal obtained with as little as 1ng of probe DNA.

appear as dark object thus reducing the quality of post-hybridisation photographs. For this reason, the condition of the chromosomal preparations was carefully evaluated under phase-contrast before they were used for in situ hybridisation. In an effort to eliminate phase dark objects from the preparations Garson et al (1987) recommend milliporing of all solutions used for the detection procedure. In initial experiments all solutions were millipored namely PBS, 2xSSC, 0.1xSSS, Buffer 1, Buffer 2, Buffer 3, and stop buffer. Eventually only 1ml of buffer 2 (BSA) that was used to dissolve the streptavidin-alkaline phosphatase conjugate, 1ml of buffer 3 used for the chromogenic substrate solution, and the stop buffer were millipored and the results were as clean.

If traces of stain were left on the slides the preparations were discarded because the documentation of results was difficult. One of the reagents that was found to contribute to the "dirty" appearance of the metaphases after the hybridisation was the BRL reagent BCIP, a component of the BRL detection kit (see section 2.6.2). When stored at $4^{o}C$ following the instructions of the manufacturers this reagent crystallizes and possibly contributes to the above problem. Storage at $-20^{o}C$ eliminated the problem.

Quantitative data from a typical experiment using GMGY10 at a concentration of 1ng per slide and 90 minutes incubation in chromogenic substrate can be seen in table 21.

Biotinylated GMGY7 was also hybridised with chromosomal preparations from two normal males at a concentration of 2ng per slide. The slides were incubated in chromogenic substrate for 2 hours and a positive signal was observed on the short arm of the Y chromosome (figure 25.I). Thirteen (13) cells were scored and from a

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	8	9.30	-1.30	0.18
2	4.16	4	9.07	-5.07	2.83
3	3.49	2	7.61	-5.61	4.13
4	3.32	3	7.24	-4.24	2.48
5	3.17	4	6.95	-2.95	8.70
6	2.99	5	6.52	-1.52	0.35
7	2.81	2	6.12	-4.12	2.77
8	2.54	1	5.54	-4.54	3.72
9	2.40	0	5.23	-5.23	5.23
10	2.36	4	5.01	-1.01	0.20
11	2.36	2	5.01	-3.01	1.81
12	2.36	2	5.01	-3.01	1.81
13	1.89	3	4.12	-1.12	0.30
14	1.81	0	3.94	-3.94	3.94
15	1.72	4	3.75	0.25	0.02
16	1.61	1	3.51	-2.51	1.79
17	1.47	3	3.20	-0.20	0.01
18	1.40	3	3.05	-0.05	0.00
19	1.16	0	2.53	-2.53	2.53
20	1.08	0	2.35	2.35	2.35
21	0.77	2	1.68	0.32	0.06
22	0.89	0	1.94	-1.94	1.94
X	2.69	0	2.93	-2.93	2.93
Y	0.96	26	1.05	24.95	592.86

Table 21 Biotinylated-GMGY10 in normal male. Statistical analysis of signal distribution. Data from 25 cells, total number of signals 109. Chi-square for the Y chromosome is very highly significant at $p<0.001$ for one degree of freedom.

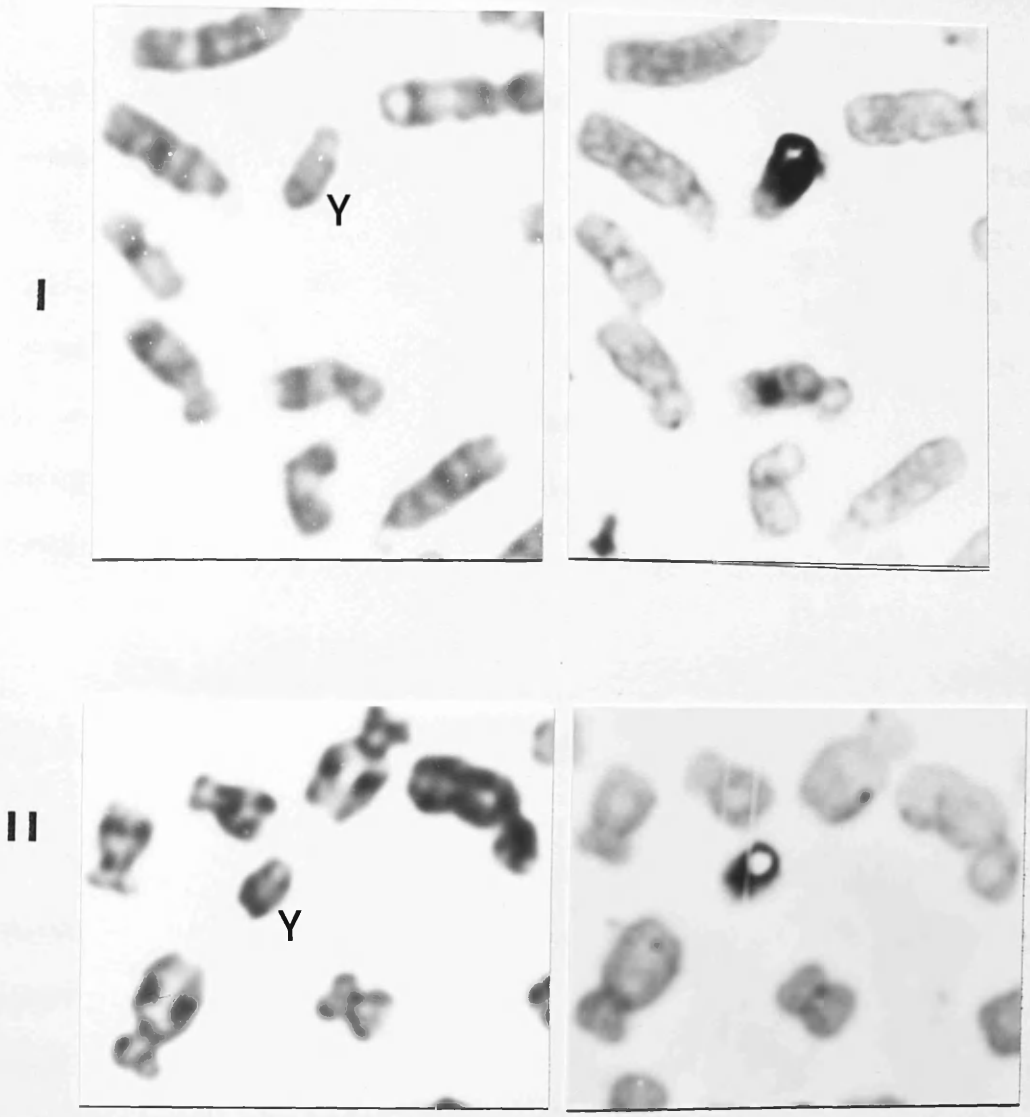


Figure 25 I) Biotinylated GMGY7 in normal male. Partial metaphase before (a) and after (b) the hybridisation (2ng). Note that the signal is extended on the long arm of the Y.
II) With both probes GMGY10 and GMGY7 occasionally the Y chromosome was completely covered by signal; Partial metaphase before (d) and after (c) hybridisation with GMGY10 (2ng).

total number of 50 signals recorded, 12 (24%) were located on the Y chromosome. This hybridisation was statistical significant ($p<0.001$). The quantitative data are presented in table 22. The hybridisation signal observed with both GMGY10 and GMGY7 was too intense, covering the whole short arm of the Y chromosome. With 2ng of labelled probe this signal was extended on the long arm of the chromosome (figure 25.I); occassionally the whole Y chromosome was covered by signal (figure 25.II).

3.4 Localisations using biotinylated in situ hybridisation

3.4.1 p72

The sensitivity of the method to localise single copy DNA sequences was investigated using probe p72, a 3.2kb single copy sequence (Galt, 1988). The probe was biotinylated by nick-translation and was hybridised with chromosomal preparations from a normal male at a concentration of 25, 30 and 50ng per slide. Thirty-two (32) cells were scored and 189 signals were recorded. Twenty-four signals (13% of total) were located at chromosome 21, distal band 21q21. The statistical analysis of the results obtained is presented in table 23 and the distribution of signal over the human chromosome complement and chromosome 21 are shown in figure 26. The more detailed schematic representation of chromosome 21 shown in figure 26ii permits assignment of signal in band 11.2 which is not shown in figure 26i. The above results were obtained with 5 hours incubation in the chromogenic substrate.

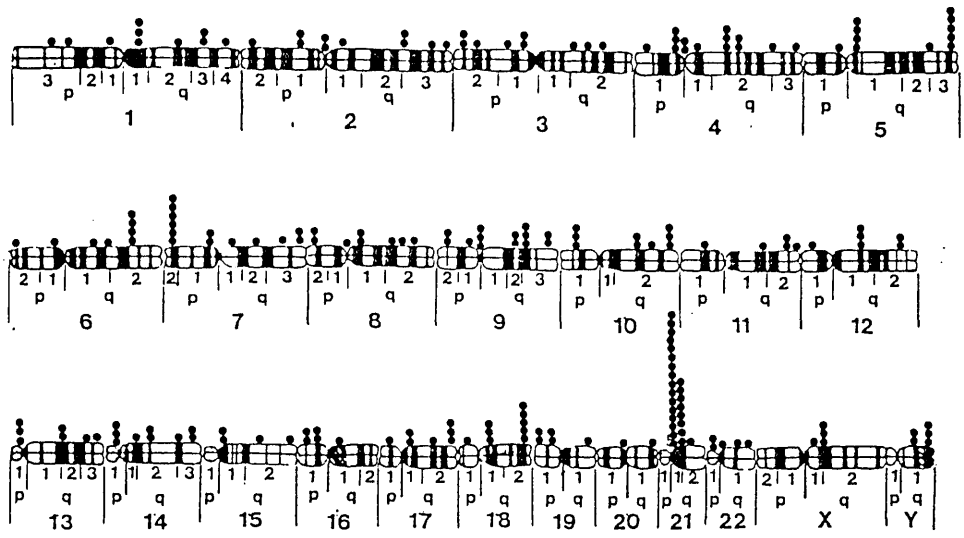
The high topographical resolution of the biotinylated approach is reflected by the presence of distinct hybridisation signal on

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	2	4.27	-2.27	1.21
2	4.16	6	4.16	1.84	0.81
3	3.49	2	3.49	-1.49	0.64
4	3.32	2	3.32	-1.32	0.52
5	3.17	1	3.17	-2.17	1.48
6	2.99	3	2.99	0.01	0.00
7	2.81	4	2.81	1.19	0.50
8	2.54	2	2.54	-0.54	0.11
9	2.40	1	2.40	-1.40	0.82
10	2.36	1	2.36	-1.36	0.78
11	2.36	1	2.36	-1.36	0.78
12	2.36	1	2.36	-1.36	0.78
13	1.89	1	1.89	-0.89	0.42
14	1.81	2	1.81	0.19	0.02
15	1.72	1	1.72	-0.72	0.30
16	1.61	0	1.61	-1.61	1.61
17	1.47	2	1.47	0.53	0.19
18	1.40	1	1.40	-0.40	0.11
19	1.16	0	1.16	-1.16	1.16
20	1.08	1	1.08	-0.08	0.00
21	0.77	0	0.77	-0.77	0.77
22	0.89	2	0.89	1.11	1.38
X	2.69	2	1.34	0.66	0.32
Y	0.96	12	0.48	11.52	276.48

Table 22 Biotinylated GMGY7 in normal male. Statistical analysis of signal distribution. Data from 13 cells, total number of signals 50. Chi-square for the Y chromosome very highly significant, $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	12	17.16	-5.16	1.55
2	4.16	14	16.72	-2.72	0.44
3	3.49	10	14.03	-4.03	1.16
4	3.32	15	13.35	1.65	0.20
5	3.17	9	12.74	-3.74	1.09
6	2.99	9	12.01	-3.01	0.75
7	2.81	12	11.29	0.71	0.04
8	2.54	8	10.21	-2.21	0.48
9	2.40	12	9.65	2.35	0.57
10	2.36	8	9.49	-1.49	0.23
11	2.36	6	9.49	-3.49	1.28
12	2.36	4	9.49	-5.49	3.17
13	1.89	5	7.60	-2.60	0.89
14	1.81	8	7.28	0.72	0.07
15	1.72	8	6.99	1.01	0.14
16	1.61	5	6.47	-1.47	0.33
17	1.47	6	5.91	0.09	0.01
18	1.40	6	5.63	0.37	0.02
19	1.16	5	4.66	0.34	0.02
20	1.08	3	4.34	-1.34	0.41
21	0.77	24	3.09	20.91	141.49
22	0.89	6	3.58	2.42	1.63
X	2.69	2	5.41	-3.41	2.15
Y	0.96	3	1.93	1.07	0.59

Table 23 Biotinylated p72 in normal male. Statistical analysis of signal distribution. Data from 32 cells, total number of signals 201. Chi-square for chromosome 21 is highly significant at $p<0.001$.



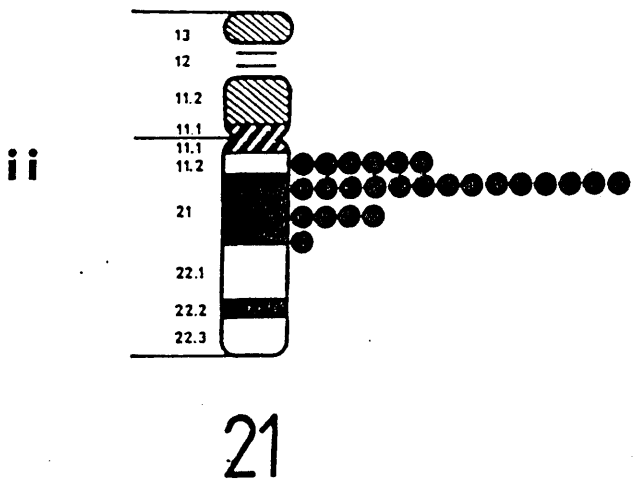


Figure 26 Biotinylated p72 in normal male
i) Distribution of signal over the human chromosome complement
ii) Distribution of signal over chromosome 21
Data from 32 cells

both chromatids (see figure 27). A similar observation has been reported by other investigators (Ambros et al., 1986).

3.4.2 GMGXY8

This recombinant contains a 3kb insert. It was biotinylated and was hybridised with chromosomal preparations from a normal male at a concentration of 40ng per slide and 4 hours in chromogenic substrate. Twenty-three cells were scored and a total of 96 signals was recorded. No chromosome was significantly labelled. A similar result was obtained with 50 and 100ng of labeled probe per slide. Figure 28A shows the distribution of signal over the human complement from these first studies. The probe was labelled again and the experiment was repeated at a concentration of 100 and 150ng per slide with 4 hours in chromogenic substrate. Twenty-five (25%) of the total hybridisation signal was observed on the short arm of the Y chromosome. In the slide hybridised with 150ng the intensity of the signal on the Y chromosome was as high as that obtained with the moderately repetitive probes GMGY10 and GMGY7 (figure 29) although GMGXY8 is thought to be a single copy sequence (Affara et al., 1987). The experiment was repeated once more with 150ng per slide and 4 hours in chromogenic substrate. Strong signal was present on the Y chromosome. Seventeen cells were scored, a total number of 81 signals was recorded and 15% of the total signal was present on the Y chromosome. Table 24 shows the quantitative data obtained at concentrations 100 and 150ng. GMGXY8 has been previously mapped to both short arm of the Y chromosome and chromosome Xq21 by deletion mapping analysis (Affara et al., 1987). It is unclear why in situ hybridisation studies failed to reveal hybridisation to the X

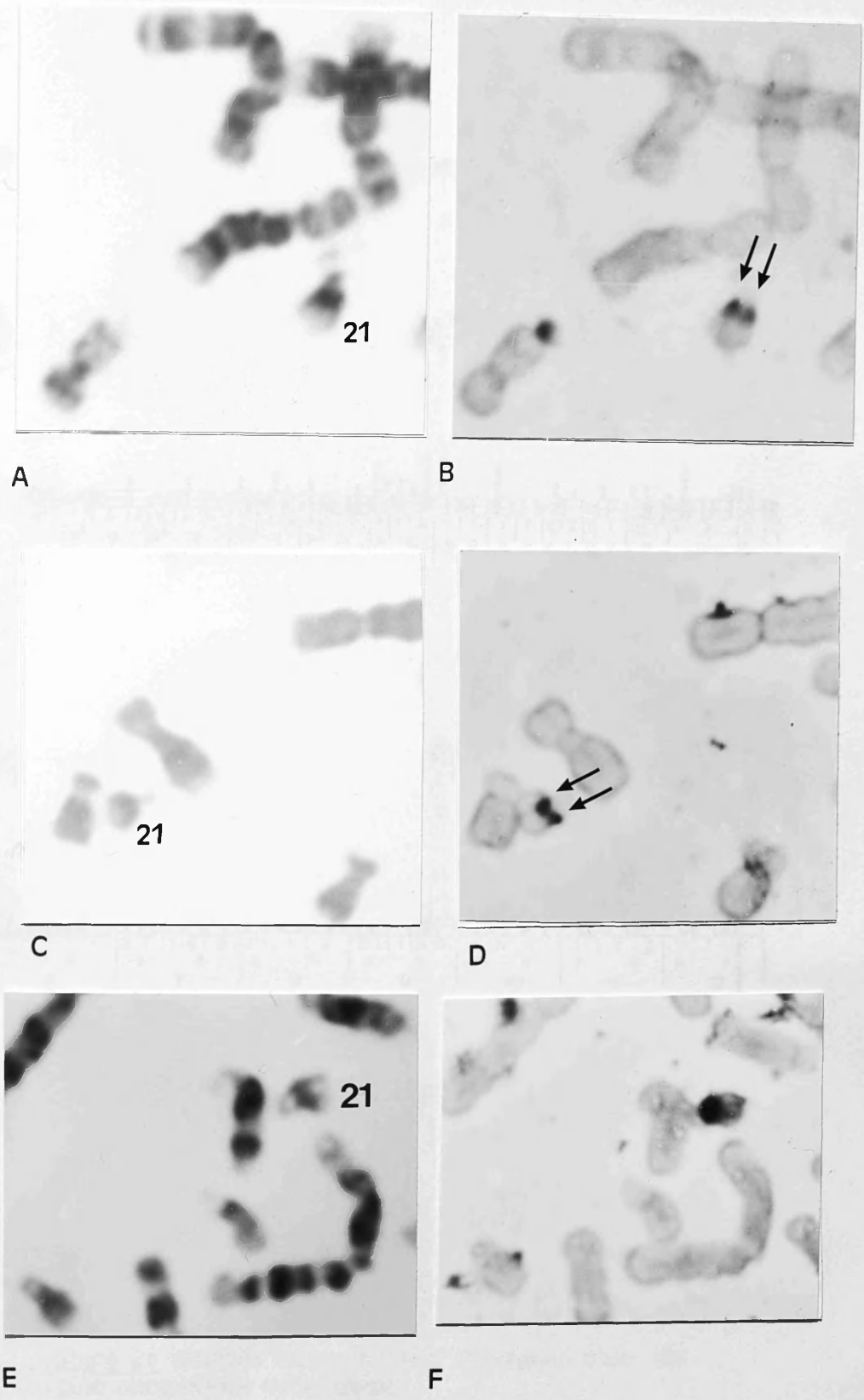
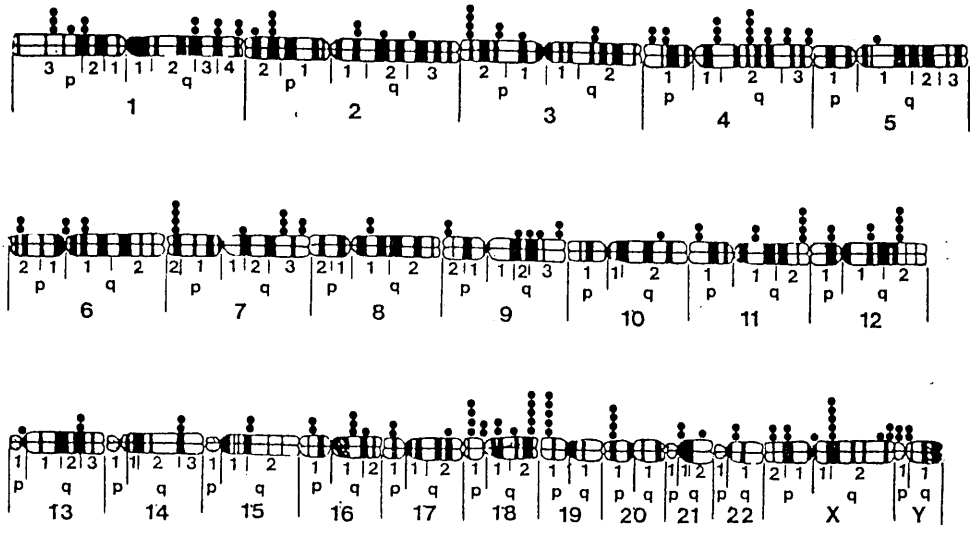
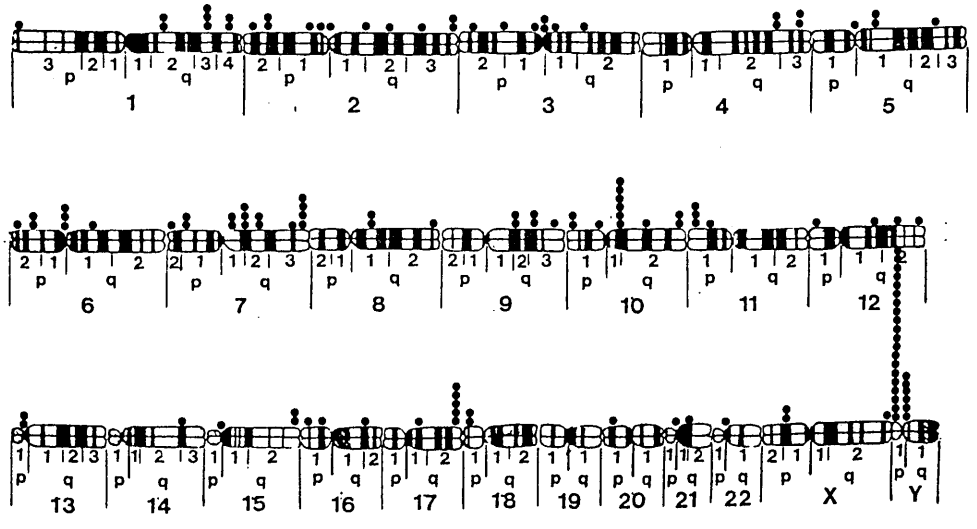


Figure 27 Biotinylated p72: partial metaphase spreads before (A, C, E) and after (B, D, F) the hybridisation; probe concentration 25ng. Note distinct signal on each chromatid (B, D).



A



B

Figure 28 GMGXY8: distribution of signal over the human chromosome complement
(A) 50ng; first nick-translation
(B) second nick-translation, 100ng; pooled data from two experiments

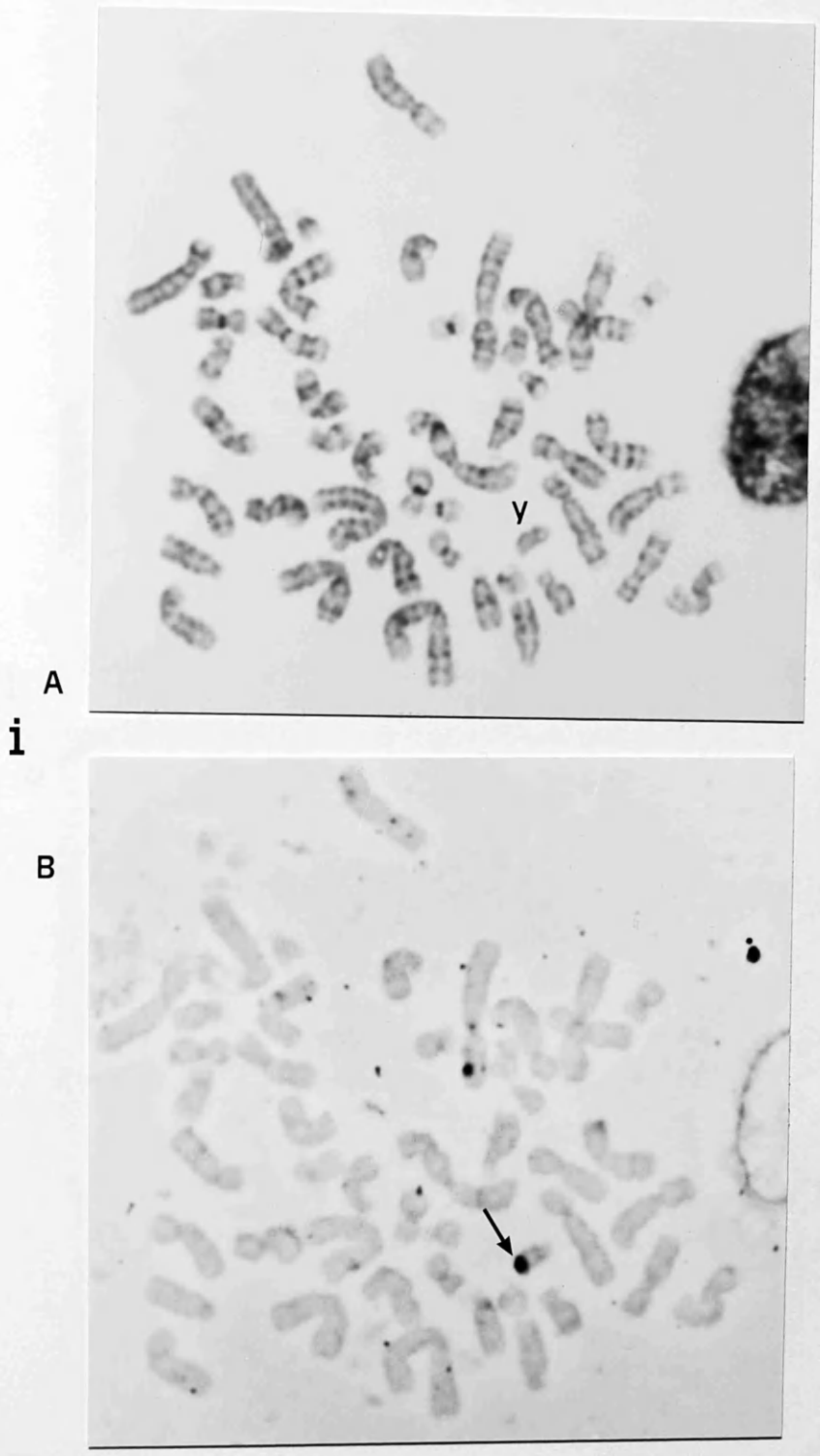
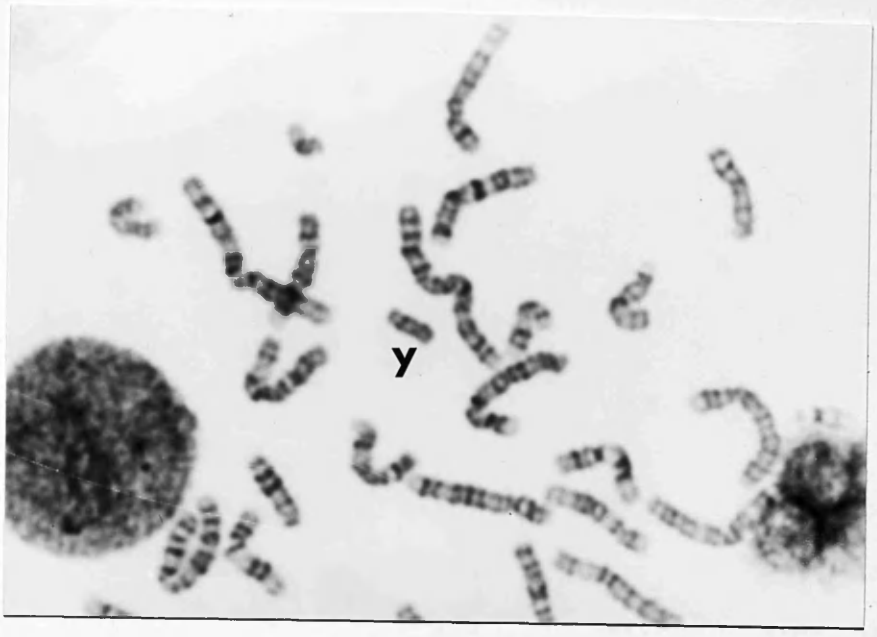


Figure 29 Biotinylated GMGXY8 in normal male.
i) complete cell before (A) and after (B) the hybridisation (100ng of probe)



C

ii

D

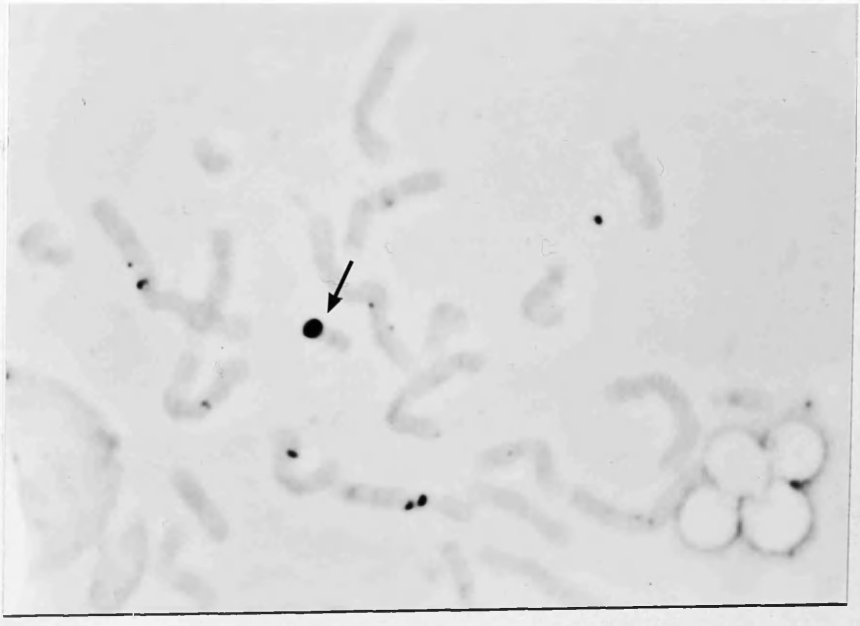


Figure 29 contin ued
ii) partial metaphase spread before (C) and after (D) the hybridisation

Chromosome	Relative Length	Observed O	Expected E	O-E	$(O-E)^2$ E
1	4.27	9	11.78	-2.78	0.66
2	4.16	8	11.48	-3.48	1.05
3	3.49	7	9.63	-2.63	0.72
4	3.32	2	9.16	-7.16	5.60
5	3.17	8	8.75	-0.75	0.06
6	2.99	7	8.25	-1.25	0.19
7	2.81	10	7.75	2.25	0.65
8	2.54	3	7.01	-4.01	2.29
9	2.40	0	6.62	-6.62	6.62
10	2.36	11	6.51	4.49	3.09
11	2.36	3	6.51	-3.51	1.89
12	2.36	5	6.51	-1.51	0.35
13	1.89	0	5.22	-5.22	5.22
14	1.81	4	4.99	-0.99	0.20
15	1.72	2	4.75	-2.75	1.59
16	1.61	6	4.44	1.56	2.43
17	1.47	3	4.06	-1.06	0.28
18	1.40	1	3.86	-2.86	2.12
19	1.16	1	3.20	-2.20	1.51
20	1.08	0	2.98	-2.98	2.98
21	0.77	3	2.12	0.88	0.36
22	0.89	1	2.46	-1.46	0.87
X	2.69	0	3.71	-3.71	3.71
Y	0.96	26	1.32	24.68	461.44

Table 24 Biotinylated GMGXY8 in normal male. Statistical analysis of signal distribution.
Data from 32 cells, total number of signals 138.
Chi-square for the Y chromosome highly significant at $p<0.001$.

chromosome.

3.5 Study of patients with paradoxical sex chromosome complements

3.5.1 XX males

Nine (9) XX males designated RH, JM, TA, AG, JT, GA, WB, DR, KS who were previously shown to be positive for Y-specific DNA sequences (Affara et al, 1986b) were investigated by in situ hybridisation using ^{3}H-labelled GMGY10 or/and GMGY7 in order to determine the chromosomal location of these sequences.

Chromosomal preparations from all cases were obtained from lymphoblastoid lines, hybridisation was performed with 20ng of labelled probe per slide and the preparations were exposed for 6 days. In eight XX males, the Y-specific sequences could be clearly localised to Xp22.3-Xpter (figure 30) and this was the only site of specific hybridisation except one case (DR, see below). The repetitive nature of probes GMGY10 and GMGY7 resulted in the presence of clusters of grains at the tip of Xp as can be seen in figures 31 and 32. In detail, the findings were as follows:

In RH with GMGY10 37 cells were scored and 146 silver grains were recorded; 63 of these (43%) were located on the X chromosome, band Xp22.3. This hybridisation was statistically significant ($p<0.001$) as can be seen in table 25.

In JT with GMGY10, 44 cells were scored and 246 silver grains were recorded. Of these 34 (19%) were located on the X chromosome, band Xp22.3. This hybridisation was significant ($p<0.001$, table 26). Using GMGY7 as a probe, data from 40 cells showed that out of 184 silver grains counted 32 (17%) were located on the X chromosome,

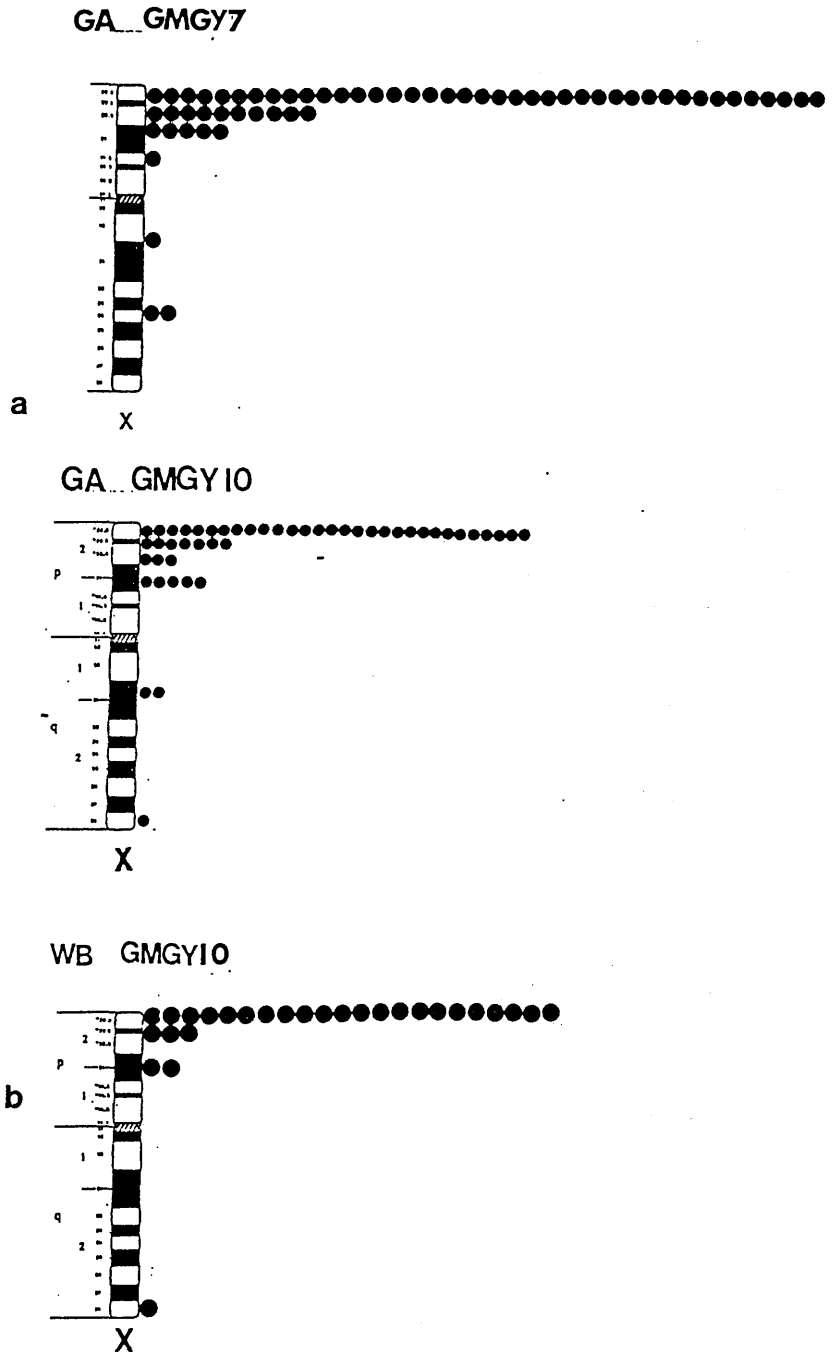


Figure 30 Distribution of silver grains over the X chromosome in XX male GA (a), WB (b), AG (c), TA (d), JT (e), RH (f), DR (g), JM (h).

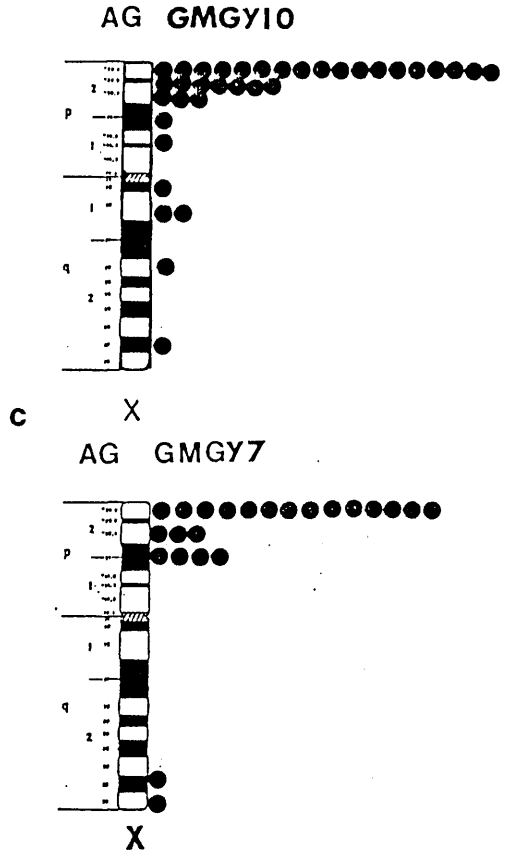
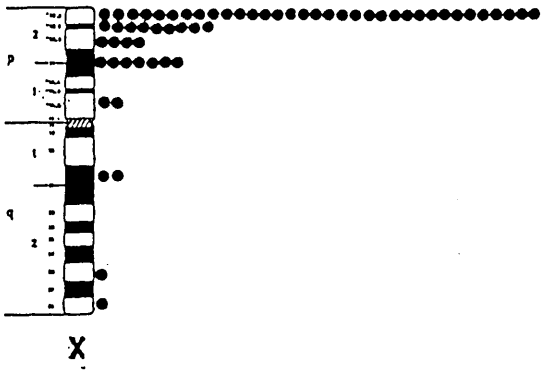


Figure 30 continued

JT GMGY10



e

JT GMGY7

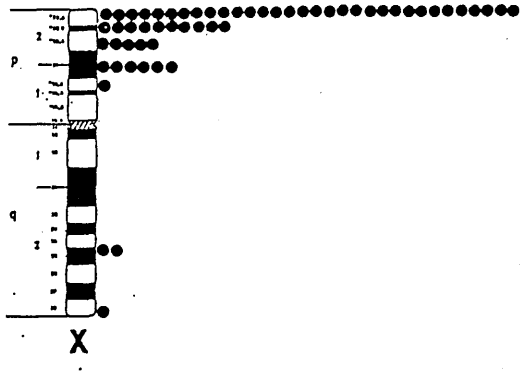


Figure 30 continued

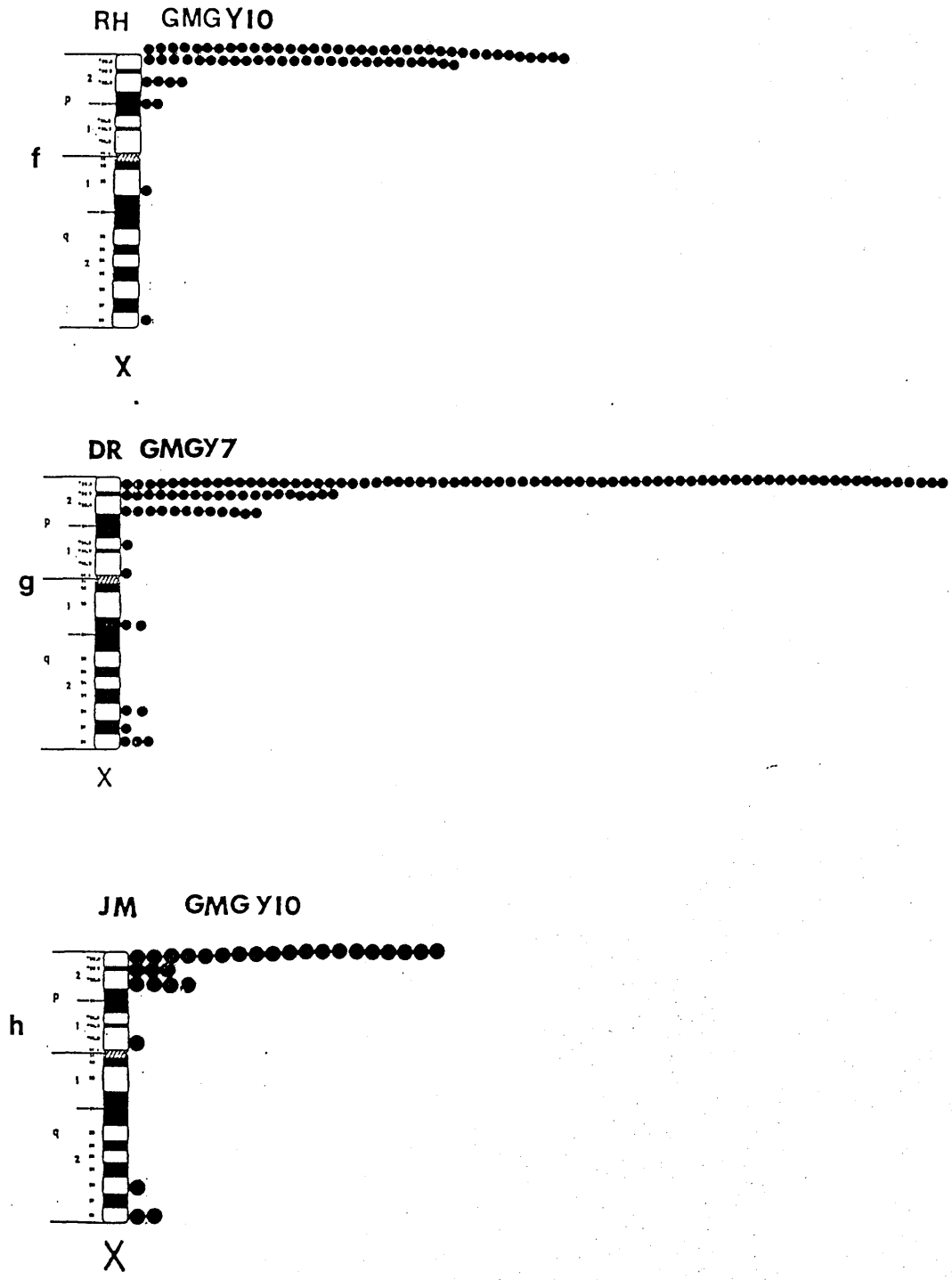


Figure 30 continued

Figure 31 Metaphase spread from XX maleRH before (A) and after (B) the hybridisation with GMGY10.

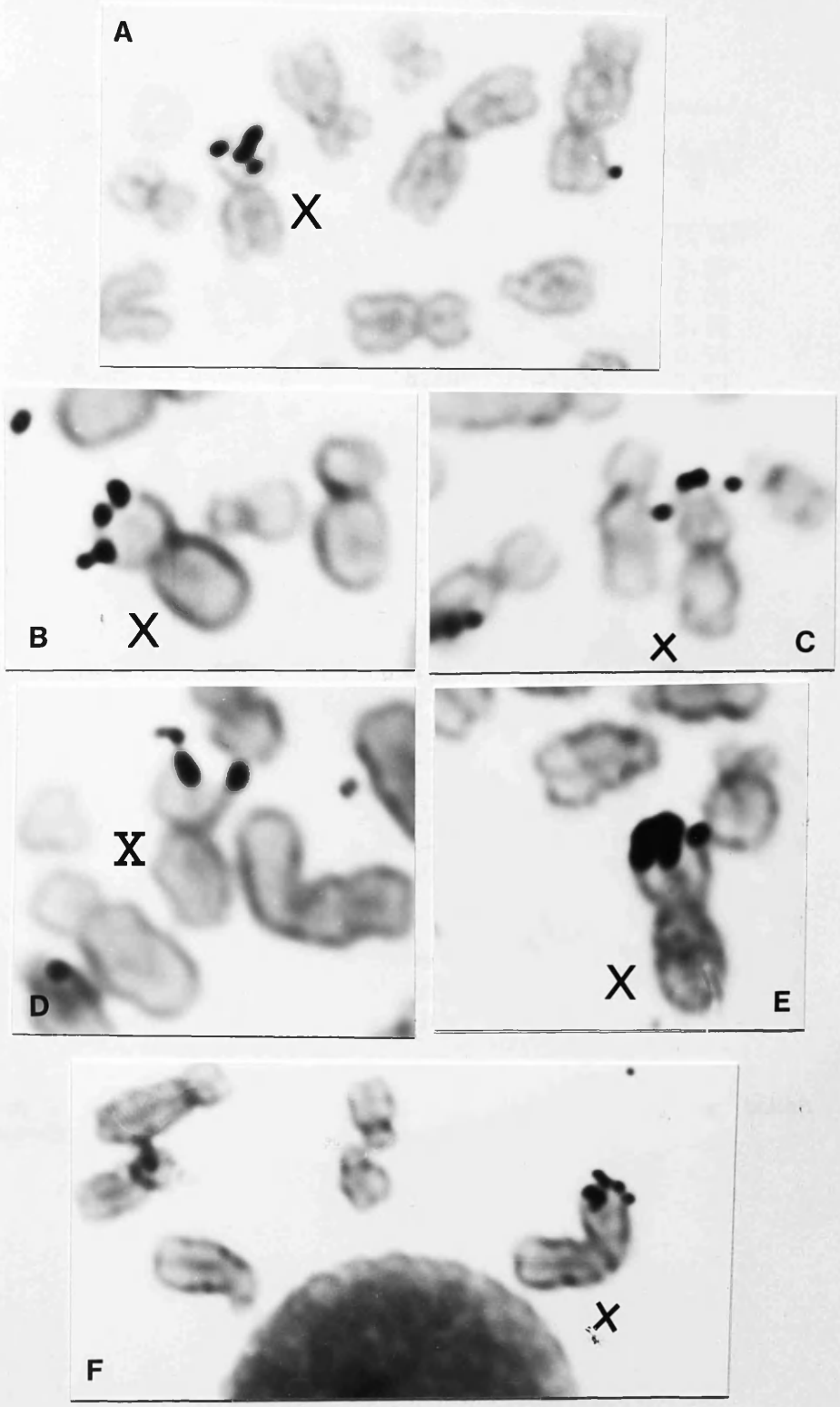


Figure 32 Partial metaphases from XX males showing silver grains on the X chromosome.
A, D, E from DR hybridised with GMGY7
B from GA hybridised with GMGY10
C from JT hybridised with GMGY7
F from RH hybridised with GMGY10

Chromosome	Relative length*	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	11	12.36	-1.36	0.14
2	7.76	5	11.32	-6.32	3.52
3	6.56	7	9.57	-2.57	0.69
4	6.13	2	8.94	-6.94	5.38
5	5.58	6	8.14	-2.14	0.56
6	5.65	7	8.24	-1.24	2.53
7	5.00	3	7.30	-4.30	2.53
8	4.77	3	6.96	-3.96	2.25
9	4.73	0	6.90	-6.90	6.90
10	4.35	5	6.35	-1.35	0.28
11	4.35	2	6.35	-4.35	2.97
12	4.16	8	6.07	1.93	0.61
13	3.59	3	5.24	-2.24	0.84
14	3.28	3	4.78	-1.78	0.66
15	3.11	4	4.54	-0.54	0.06
16	3.11	1	4.54	-3.54	2.76
17	3.02	0	4.40	-4.40	4.40
18	2.73	0	3.98	-3.98	3.98
19	2.58	1	3.76	-2.76	2.02
20	2.31	2	3.37	-1.37	0.55
21	1.90	2	2.77	-0.77	0.21
22	1.69	0	2.46	-2.46	2.46
X	5.14	71	7.50	63.50	537.63

Table 25 GMGY10 in RH. Statistical analysis of grain distribution. Data from 37 cells. Total number of grains 146.
Chi-square for the X chromosome is very highly significant at $p<0.001$.

*Expressed as a percentage of the X-containing haploid genome, taken from Ferguson-Smith (1974)

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	15	20.83	-5.83	1.63
2	7.76	15	19.08	-4.08	0.87
3	6.56	14	16.13	-2.13	0.28
4	6.13	7	15.07	-8.07	4.32
5	5.58	12	13.72	-1.72	0.21
6	5.65	7	13.89	-6.89	3.41
7	5.00	12	12.30	-0.30	0.00
8	4.77	13	11.73	1.27	0.13
9	4.73	6	11.63	-5.63	2.72
10	4.35	9	10.70	-1.70	0.27
11	4.35	7	10.70	-3.70	1.27
12	4.16	11	10.23	0.77	0.05
13	3.59	4	8.83	-4.83	2.64
14	3.28	4	8.06	-4.06	2.04
15	3.11	2	7.65	-5.65	4.17
16	3.11	13	7.65	5.35	3.74
17	3.02	3	7.42	-4.42	2.63
18	2.73	6	6.71	-0.71	0.07
19	2.58	7	6.34	0.60	0.06
20	2.31	5	5.68	-0.68	0.08
21	1.90	5	4.67	0.33	0.02
22	1.69	4	4.15	-0.15	0.00
X	5.14	65	12.64	52.36	216.89

Table 26 GMGY10 in JT. Statistical analysis of grain distribution. Data from 44 cells, total number of grains 246.
Chi-square for the X chromosome is very highly significant at $p<0.001$.

band Xp22.3. This hybridisation is significant ($p<0.001$, table 27).

In GA with GMGY10 38 cells were counted and 134 silver grains were recorded; 30 of these (22%) were located on the X chromosome, band Xp22.3 ($p<0.001$, table 28). A similar result was obtained with GMGY7. Data from 42 cells revealed that out of 156 silver grains 40 (26%) were located at Xp22.3 (table 29).

In DR with GMGY7 43 cells were scored and 503 silver grains were counted ; 74 of these (15%) were located on the X chromosome, band Xp22.3 ($p<0.001$). In table 30 it can be seen that significant hybridisation was also observed with chromosome 12 and chromosome 13. The distribution of grains over chromosome 12 and chromosome 13 is shown in figure 33. The significance of this finding is unclear. Case DR was also studied with biotinylated GMGY10. In twelve cells scored the only chromosome with hybridisation signal was the X. (see figure 34).

In AG with GMGY10 30 cells were scored and 155 grains were counted; 18 of these (12%) were located on the X chromosome, band Xp22.3. The statistical analysis of the silver grain distribution is shown in table 31. When GMGY7 was used as a probe data from 37 cells showed that from a total number of 138 grains, 14 (10%) were located at Xp22.3.

In TA with GMGY10 32 cells were scored and 84 grains were recorded; 9 of these (11%) were observed on the X chromosome, band Xp22.3 (see also table 32).

In WB with GMGY10 43 cells were scored and 175 silver grains were recorded; 30 of these (17%) were located on the X chromosome, band Xp22.3. The statistical analysis of the grain count is presented in table 33.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	9	15.58	-6.58	2.77
2	7.76	11	14.27	-3.27	0.74
3	6.56	14	12.07	1.93	0.30
4	6.13	11	11.27	-0.27	0.00
5	5.58	6	10.26	-4.26	1.76
6	5.65	8	10.39	-2.39	0.54
7	5.00	10	9.20	0.80	0.06
8	4.77	4	8.77	-4.77	2.59
9	4.73	4	8.70	-4.70	2.53
10	4.35	10	8.00	2.00	0.50
11	4.35	6	8.00	-2.00	0.50
12	4.16	6	7.65	-1.65	0.35
13	3.59	4	6.60	-2.60	1.02
14	3.28	2	6.03	-4.03	2.69
15	3.11	4	5.72	-1.72	0.51
16	3.11	9	5.72	3.28	0.19
17	3.02	7	5.55	1.45	0.37
18	2.73	4	5.02	-1.02	0.20
19	2.58	4	4.74	-0.74	0.11
20	2.31	4	4.25	-0.25	0.01
21	1.90	1	3.49	-2.49	1.77
22	1.69	6	3.10	2.90	2.71
X	5.14	40	9.45	30.55	98.76

Table 27 GMGY7 in JT. Statistical analysis of grain distribution. Data from 40 cells, total number of grains 184.
Chi-square for the X chromosome is very highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	7	11.34	-4.34	1.66
2	7.76	13	10.39	2.61	0.65
3	6.56	10	8.79	1.21	0.16
4	6.13	5	8.21	-3.21	1.25
5	5.58	2	7.47	-5.47	4.00
6	5.65	7	7.57	-0.57	0.04
7	5.00	2	6.70	-4.70	3.29
8	4.77	3	6.39	-3.39	1.79
9	4.73	5	6.33	-1.33	0.27
10	4.35	3	5.82	-2.82	1.36
11	4.35	4	5.82	-1.82	0.56
12	4.16	7	5.57	1.43	0.36
13	3.59	4	4.81	-0.81	0.13
14	3.28	1	4.39	-3.39	2.61
15	3.11	4	4.16	-0.16	0.00
16	3.11	2	4.16	-2.16	1.12
17	3.02	1	4.04	-3.04	2.28
18	2.73	0	3.65	-3.65	3.65
19	2.58	2	3.45	-1.45	0.60
20	2.31	0	3.09	-3.09	3.09
21	1.90	3	2.54	0.46	0.08
22	1.69	1	2.26	-1.26	0.70
X	5.14	48	6.88	41.12	245.76

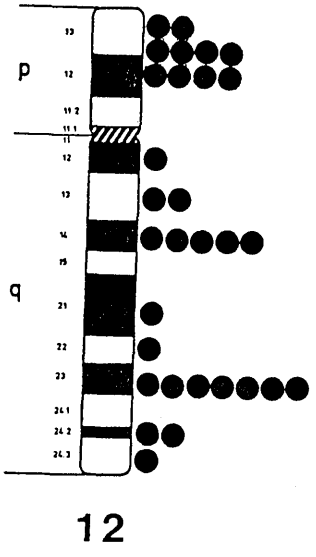
Table 28 GMGY10 in GA. Statistical analysis of grain distribution. Data from 38 cells, total number of grains 134. Chi-square for the X chromosome very highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	5	13.21	-8.21	5.10
2	7.74	4	12.10	-8.10	5.42
3	6.56	4	10.23	-6.23	3.79
4	6.13	6	9.56	-3.56	1.32
5	5.58	3	8.70	-5.70	3.73
6	5.65	4	8.81	-4.81	2.62
7	5.00	5	7.80	-2.80	1.00
8	4.77	8	7.44	0.56	0.04
9	4.73	1	7.37	-6.37	5.50
10	4.35	1	6.78	-5.78	4.92
11	4.35	6	6.78	-0.78	0.08
12	4.16	8	6.48	1.52	0.35
13	3.59	6	5.60	0.40	0.02
14	3.28	3	5.11	-2.11	0.87
15	3.11	3	4.85	-1.85	0.70
16	3.11	4	4.85	-0.85	0.14
17	3.02	3	4.71	-1.71	0.62
18	2.73	4	4.25	-0.25	0.01
19	2.58	1	4.02	-3.02	2.26
20	2.31	4	3.60	0.40	0.04
21	1.90	2	2.96	-0.96	0.01
22	1.69	1	2.63	-1.63	1.01
X	5.14	70	8.01	61.99	479.74

Table 29 GMGY7 in GA. Statistical analysis of grain distribution. Data from 42 cells, total number of grains 156.
Chi-square for the X chromosome is very highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	20	42.60	-22.60	11.98
2	7.76	36	39.03	-3.03	0.23
3	6.56	22	32.99	-10.99	3.63
4	6.13	27	30.83	-4.06	0.47
5	5.58	24	28.06	-4.06	0.58
6	5.65	10	28.41	-10.41	3.81
7	5.00	29	25.15	3.85	0.58
8	4.77	20	23.99	-3.99	0.66
9	4.73	11	23.79	-12.79	6.87
10	4.35	16	21.88	-5.88	1.58
11	4.35	26	21.88	4.12	0.77
12	4.16	34	20.92	13.08	8.17
13	3.59	31	18.05	12.95	9.29
14	3.28	10	16.49	-6.49	2.55
15	3.11	15	15.64	-0.64	0.02
16	3.11	10	15.64	-5.64	2.03
17	3.02	3	15.19	-12.19	9.78
18	2.73	7	13.73	-6.73	3.29
19	2.58	8	12.97	-4.97	1.79
20	2.31	8	11.61	-3.61	1.12
21	1.90	6	9.55	-3.55	1.31
22	1.69	7	8.50	-1.50	0.26
X	5.14	123	28.85	94.15	307.25

Table 30 GMGY7 in DR. Statistical analysis of grain distribution. Data from 43 cells, total number of grains 503. Chi-square for the X chromosome is very highly significant at $p<0.001$. Chi-square for chromosomes 12, 13 highly significant at $0.005<p<0.001$.



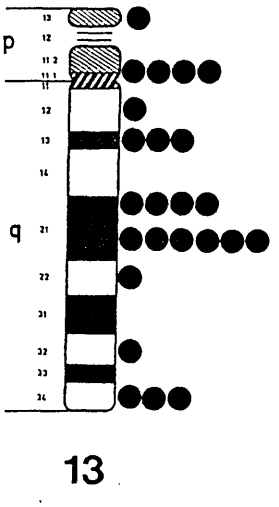


Figure 33 Distribution of silver grains over chromosomes 12 and 13 from DR hybridised with GMGY7

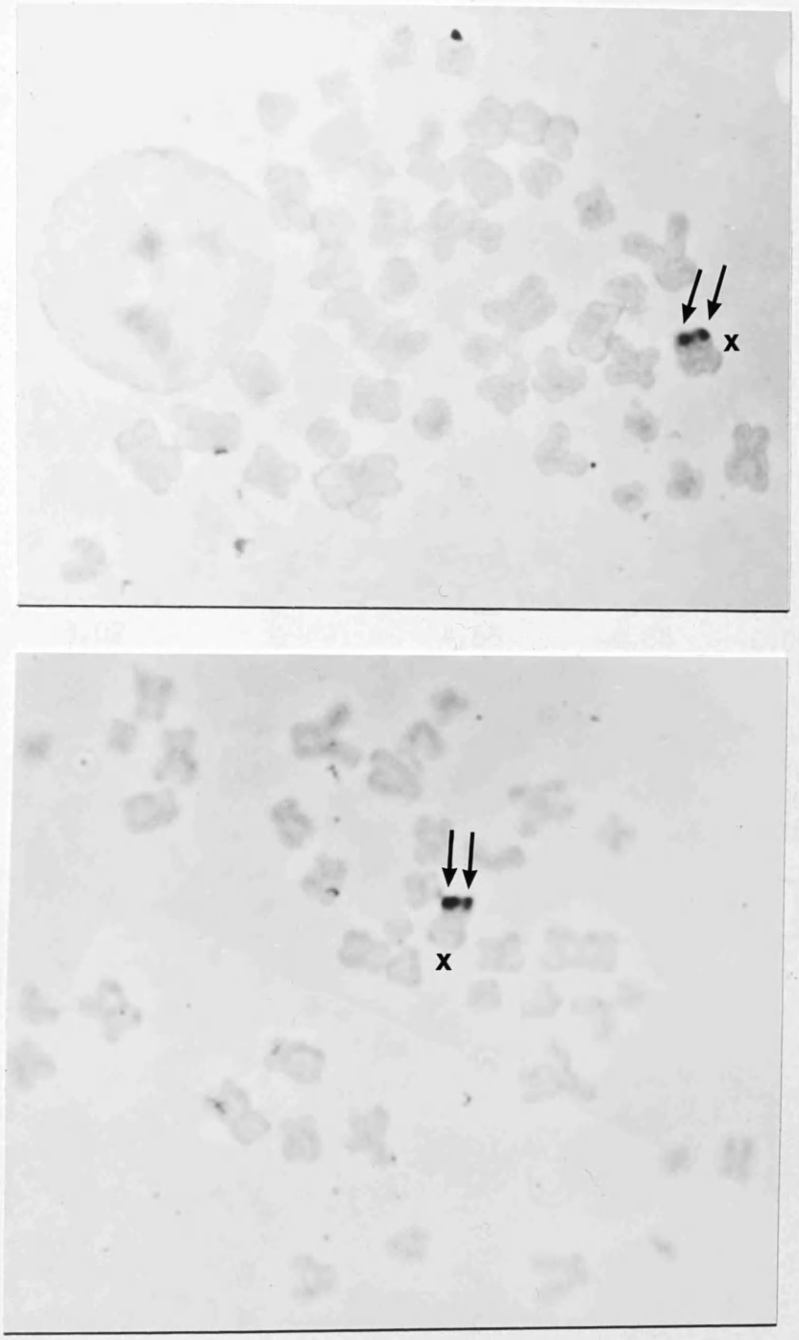


Figure 34 Metaphase spreads from DR following hybridisation with biotinylated GMGY10 (phase-contrast microscopy).

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	11	13.12	-2.12	0.34
2	7.76	9	12.02	-3.02	0.75
3	6.56	8	10.16	-2.16	0.45
4	6.13	8	9.50	-1.50	0.23
5	5.58	6	8.64	-2.64	0.80
6	5.65	11	8.75	2.25	0.57
7	5.00	6	7.75	-1.75	0.39
8	4.77	7	7.39	-0.39	0.02
9	4.73	4	7.33	-3.33	1.51
10	4.35	3	6.74	-3.74	2.07
11	4.35	4	6.74	-2.74	1.11
12	4.16	6	6.44	-0.44	0.03
13	3.59	2	5.56	-3.56	2.27
14	3.28	4	5.08	-1.08	0.22
15	3.11	4	4.82	-0.82	0.13
16	3.11	8	4.82	3.18	2.09
17	3.02	4	4.68	-0.68	0.09
18	2.73	5	4.23	0.77	0.14
19	2.58	3	3.99	-0.99	0.24
20	2.31	4	3.58	0.42	0.04
21	1.90	2	2.94	-0.94	0.30
22	1.69	2	2.61	-0.61	0.30
X	5.14	34	7.96	26.04	85.18

Table 31 GMGY10 in AG. Statistical analysis of grain distribution. Data from 30 cells, total number of grains 155. Chi-square for the X chromosome is highly significant at $p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	2	7	-5	3.5
2	7.76	2	6	-4	2.6
3	6.56	2	5	-3	1.8
4	6.13	4	5	-1	0.2
5	5.58	2	4	-2	1.0
6	5.65	3	4	-1	0.25
7	5.00	7	4	3	2.25
8	4.77	1	4	3	2.25
9	4.73	4	4	0	0.00
10	4.35	3	3	0	0.00
11	4.35	2	3	-1	0.30
12	4.16	3	3	0	0.00
13	3.59	7	3	4	5.3
14	3.28	9	3	6	9.00
15	3.11	3	2	1	0.5
16	3.11	5	2	3	4.5
17	3.02	1	2	-1	0.5
18	2.73	2	2	0	0.00
19	2.58	0	2	-2	2.00
20	2.31	2	2	0	0.00
21	1.90	4	1	3	9.00
22	1.69	0	1	-1	1.00
X	5.14	16	4	12	36.00

Table 32 GMGY10 in TA. Statistical analysis of grain distribution. Data from 32 cells, total number of grains 84.
Chi-square for the X chromosome is very highly significant at $p<0.001$. Chi-square for chromosomes 21, 14 highly significant at $0.005<p<0.001$.

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	11	14	-3	0.6
2	7.76	13	13	0	0.00
3	6.56	8	11	-3	0.8
4	6.13	7	10	-3	0.9
5	5.58	7	9	-2	0.4
6	5.65	5	10	-5	2.5
7	5.00	6	8	-2	0.5
8	4.77	10	8	2	0.5
9	4.73	7	8	-1	0.1
10	4.35	11	7	4	2.2
11	4.35	14	7	7	7
12	4.16	3	7	-4	2.2
13	3.59	3	6	-3	1.5
14	3.28	0	5	-5	-5
15	3.11	4	5	-1	0.2
16	3.11	3	5	-2	0.8
17	3.02	8	5	3	1.8
18	2.73	4	4	0	0.00
19	2.58	5	4	1	0.25
20	2.31	4	4	0	0.00
21	1.90	1	3	-2	1.3
22	1.69	2	3	-1	1.3
X	5.14	30	8	22	60.5

Table 33 GMGY10 in WB. Statistical analysis of grain distribution. Data from 43 cells, total number of grains 175. Chi-square for the X chromosome is highly significant at $p<0.001$.

In JM with GMGY10 28 cells were scored and 127 silver grains were counted; 28 of these (22%) were located on the X chromosome band Xp22.3. With GMGY7, 23 cells were scored (table 34) and out of 130 silver grains recorded, 7 (5%) were located at Xp22.3

The ninth XX male (KS) was investigated with tritium labelled P2F2. The results obtained are presented under the localisation of this DNA sequence, section 3.2.3. Significant hybridisation occurred at Xq21 where this DNA sequence was localised in normal female, an XO male and normal male. Silver grains were also present on the short arm of the X chromosome but in contrast to the findings in the previous eight XX males where the silver grains were located in one chromosome band (Xp22.3) in KS silver grains were located over a wide area of the short arm of the X chromosome including bands Xp22.3, Xp22.2, Xp22.1 and Xp21 (see figure 11B). This case was also studied with tritium-labelled GMGY10. The preparations were exposed for 6 days and from 21 cells analysed, 10% of the total hybridisation signal (17 grains) was present on the X chromosome but this hybridisation was not statistically significant. It should be noted that signal was present at distal short arm (4 grains) and band Xp21. Figure 35 shows a partial metaphase from KS showing silver grains at distal short arm of one X chromosome.

A fold associated with the inactive X chromosome has been proposed by van Dyke et al., (1986) to be a marker of this chromosome in metaphase spreads. In figure 36 partial metaphases from XX male DR are shown with silver grains on the folded X chromosome. Silver grains were observed on not-folded X chromosomes from the same individual (figure 32A,D,E) suggesting that the presence of Y-specific sequences does not affect the random

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	2	11	-9	7.3
2	7.76	13	10	3	0.9
3	6.56	12	8	4	2
4	6.13	5	8	-3	1.1
5	5.58	14	7	7	7
6	5.65	4	7	-3	1.2
7	5.00	8	6	2	0.6
8	4.77	7	6	1	0.1
9	4.73	9	5	4	3.2
10	4.35	2	5	-3	1.8
11	4.35	0	5	-5	-5
12	4.16	3	5	-2	0.8
13	3.59	3	4	-1	0.2
14	3.28	1	4	-3	2.2
15	3.11	1	4	-3	2.2
16	3.11	4	4	0	0.0
17	3.02	7	4	3	2.2
18	2.73	5	3	2	1.30
19	2.58	4	3	1	0.3
20	2.31	1	3	-2	1.3
21	1.90	4	2	2	2
22	1.69	3	2	1	0.5
X	5.14	19	6	13	28.1

Table 34 GMGY7 in JM. Statistical analysis of grain distribution. Data from 23 cells, total number of grains 130. Chi-square for the X chromosome is highly significant at $p<0.001$.

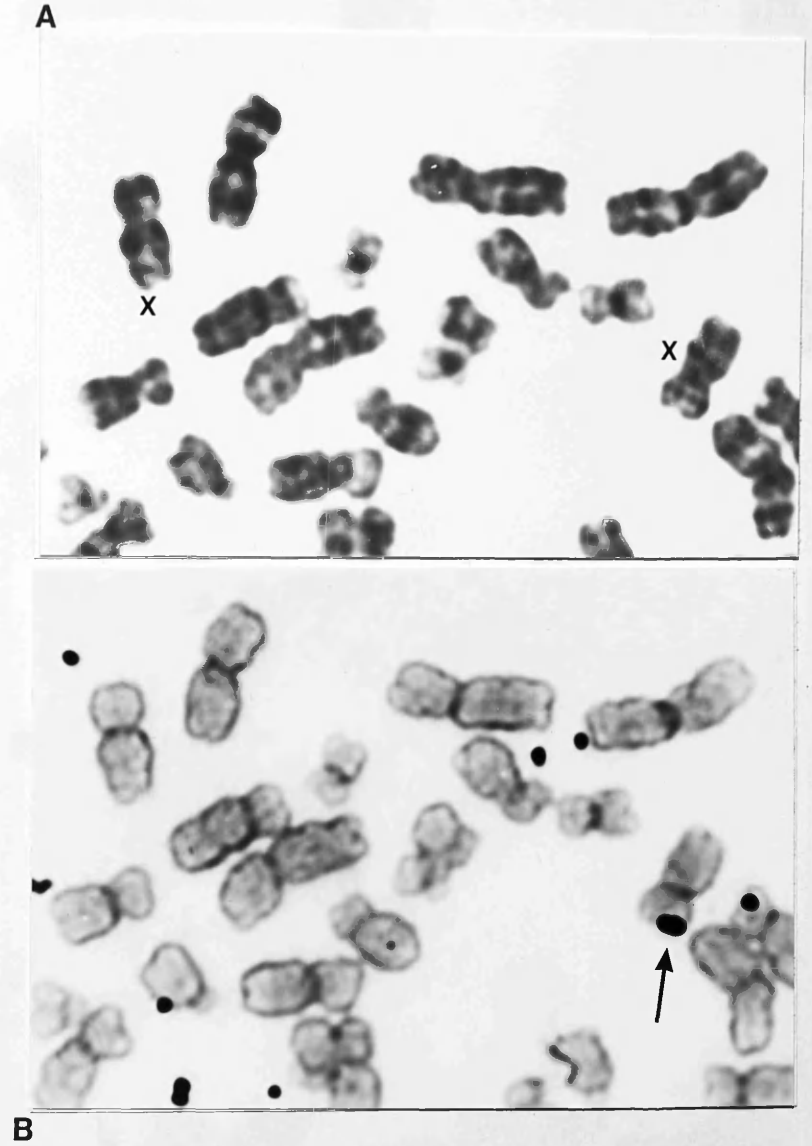


Figure 35 Partial metaphase from KS following hybridisation with GMGY10. Silver grains are present at distal short arm of one X chromosome.

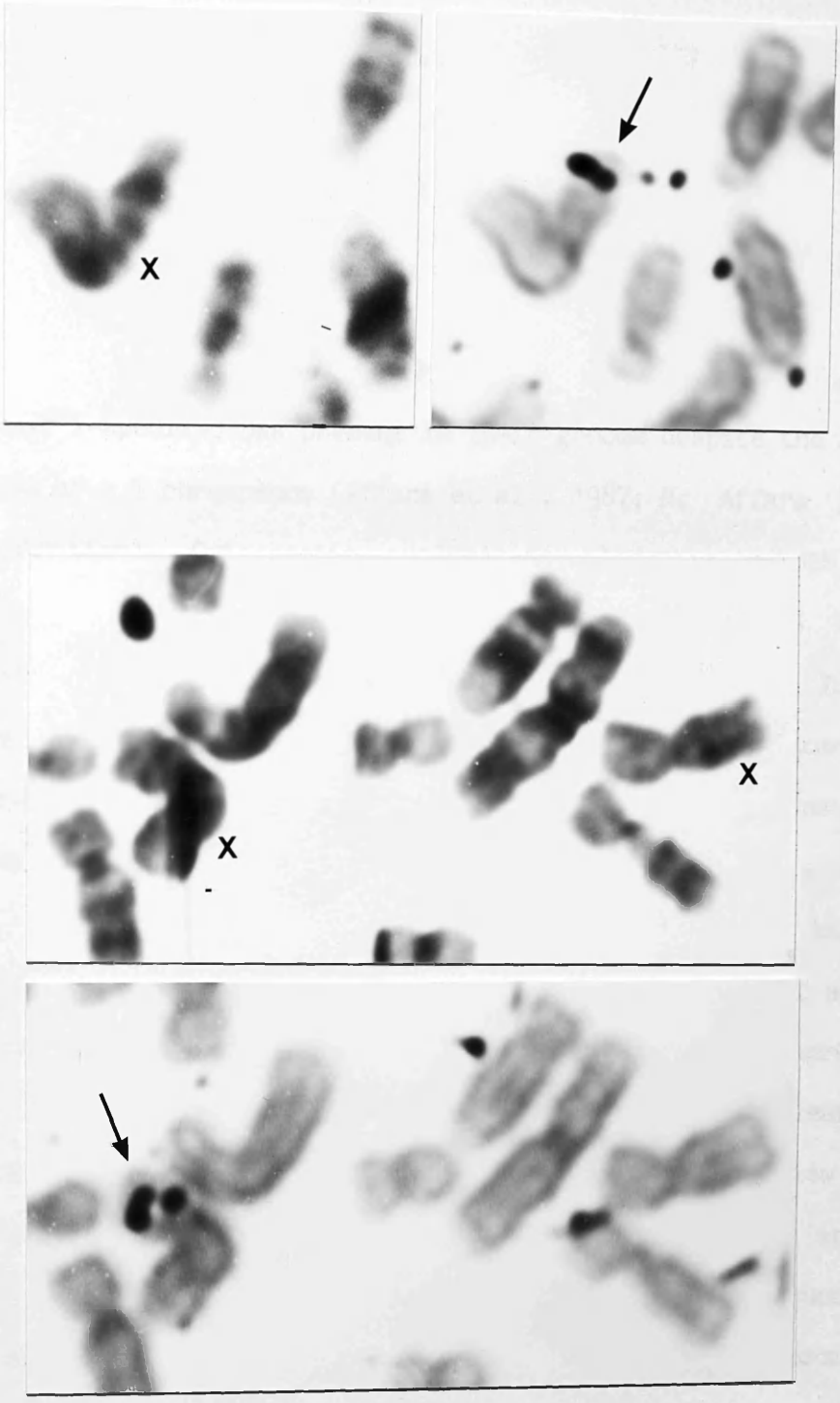


Figure 36 Partial metaphases from DR showing the folded X chromosome with silver grains.

inactivation of the X chromosome in this XX male.

Table 35 summarises the quantitative data obtained in the eight XX males studied with probes GMGY10 or GMGY7.

3.4.2 Two apparent XO males

Two 45,X males, DG and RW, were shown by Southern DNA analysis to have Y-specific DNA present in their genome despite the apparent absence of a Y chromosome (Affara et al., 1987; N. Affara personal communication). Cytogenetic analysis revealed that in both cases a small marker chromosome was present in approximately 80% and 25% respectively of the patients peripheral blood lymphocytes. In DG the marker chromosome had the size of the short arm of chromosome 18 and banding studies suggested that the marker might have originated from a Y chromosome; in RW, the marker was so small, the size of the short arm of a Y chromosome that, in the first karyotype investigation was missed and the patient was then described as 45,X (Ferguson-Smith et al, 1987). The presence of the marker was revealed in a second investigation, but its origin was unclear. The correct description of both cases is 45,X/46,X + mar. In view of the apparent male phenotype of these two individuals in the apparent absence of a Y chromosome and given the presence of Y sequences in their genome, it could be postulated that the marker chromosome had originated from a Y chromosome (Affara et al., 1987). However, the possibility that the Y-specific sequences were located on a different chromosome could not be excluded. Thus, in situ hybridisation was utilized to determine whether the Y-specific DNA was present in the marker chromosome or elsewhere in the chromosome complement.

I. Quantitative evaluation of in situ hybridisation of 46,XX males with the DNA probe GMGY10.

Case	Number of mitoses analysed	Total number of grains	Grains on Xp22.3 No. (%)
RH	37	146	63 (43%)
JT	44	246	34 (19%)
GA	38	134	30 (22%)
AG	30	155	18 (12%)
WB	43	175	22 (13%)
JM	28	127	19 (15%)
TA	32	84	9 (11%)

II. Quantitative evaluation of in situ hybridisation of 46,XX males with the DNA probe GMGY7.

Case	Number of mitoses analysed	Total number of grains	Grains on Xp22.3 No. (%)
GA	42	156	40 (26%)
JT	40	184	32 (17%)
DR	43	503	74 (15%)
AG	37	138	14 (10%)
JM	23	130	7 (5%)

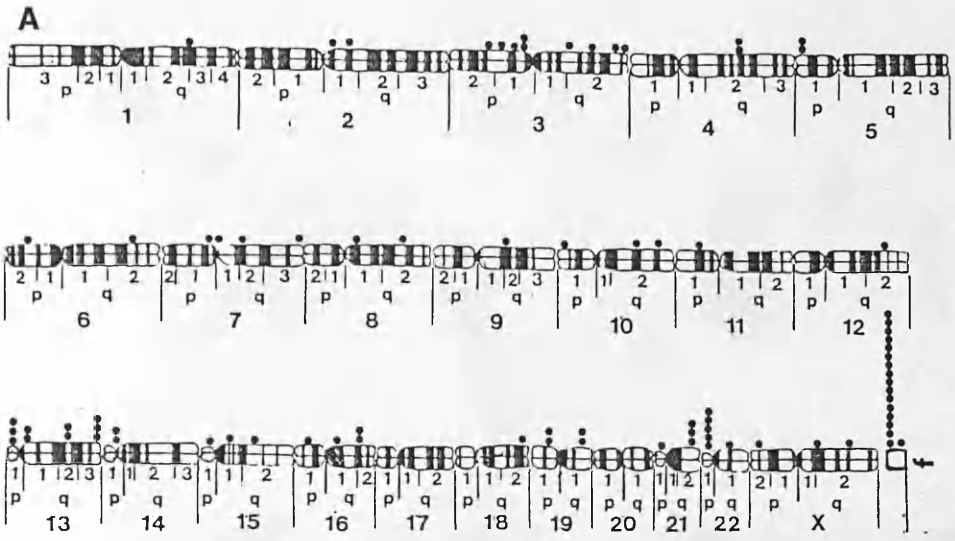
Table 35 Summary of quantitative in situ hybridisation data obtained in XX males with probes GMGY10 and GMGY7.

DG was studied with ^{3}H-GMGY10 previously shown to be present in the genome of this individual (N. Affara, personal communication); chromosomal preparations were obtained from blood lymphocytes and were exposed for 6 days. Twenty four metaphases were analysed and a total number of 85 silver grains were recorded; Seventeen of these (20%) were located on the marker (table 36). Additionally, chromosomes 13 and 22 were also significantly labelled with 10 (12%) and 6 (7%) silver grains respectively (figure 37A). In an independent experiment, data from 23 cells and 7 days exposure showed that from a total of 203 silver grains, 22 grains (11% of total signal) were located on the marker chromosome. No significant hybridisation was observed with chromosomes 13 or 22. A similar result was obtained in a study with biotinylated GMGY10, where data from 10 cells showed that out of 32 signals recorded, 20 (62%) were located on the marker chromosome. No signal was recorded on chromosome 13 or 22. Therefore, the excess of grains over chromosomes 13 and 22 in the first experiment is unlikely to be significant. It is of interest to note that the hybridisation efficiency achieved with the biotinylated approach is markedly higher than the one observed in both radioactive experiments (62% of total signal on the marker as opposed to 20% and 11%). Figure 38 shows partial metaphase spreads from DG with silver grains on the marker chromosome

The second case, RW, was investigated with ^{3}H-GMGY7; metaphase spreads were obtained from lymphoblastoid lines, hybridised with 20ng of labelled probe per slide and the slides were exposed for 32 days; this long exposure was necessary in view of the previous

Chromosome	Relative Length *	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	4.27	1	7.26	-6.26	5.3977
2	4.16	2	7.07	-5.07	3.6265
3	3.49	9	5.93	3.07	1.5893
4	3.32	2	5.64	-3.64	2.3492
5	3.17	2	5.38	-3.38	2.1234
6	2.99	2	5.08	-3.08	1.8674
7	2.81	4	4.78	0.78	0.1272
8	2.54	2	4.31	-2.31	1.2302
9	2.40	1	4.08	-3.08	2.3250
10	2.36	3	4.00	-1.00	0.2500
11	2.36	1	4.00	-3.00	2.2500
12	2.36	1	4.00	-3.00	2.2500
13	1.89	10	3.21	6.79	14.3626
14	1.81	2	3.08	-1.08	0.3787
15	1.72	3	2.92	0.08	0.0000
16	1.61	4	2.74	1.26	0.5794
17	1.47	0	2.50	-2.50	2.5000
18	1.40	1	2.38	-1.38	0.8000
19	1.16	4	1.97	2.03	2.0918
20	1.08	0	1.84	-1.84	1.8400
21	0.77	4	1.31	2.69	5.5237
22	0.89	6	1.51	4.49	13.3510
X	2.69	3	2.29	0.71	0.2201
f	0.76	17	0.64	16.36	418.20

Table 36 ^{3}H-GMGY10 in DG. Statistical analysis of grain distribution. Results from 24 cells, total number of grains 85. For the purposes of this analysis the relative length of the short arm of chromosome 18 (Ferguson-Smith 1974) was taken as the relative length of the fragment.
Chi-square for the fragment is very highly significant at $p<0.001$. Chi-square for chromosome 22, chromosome 13 is very highly significant.



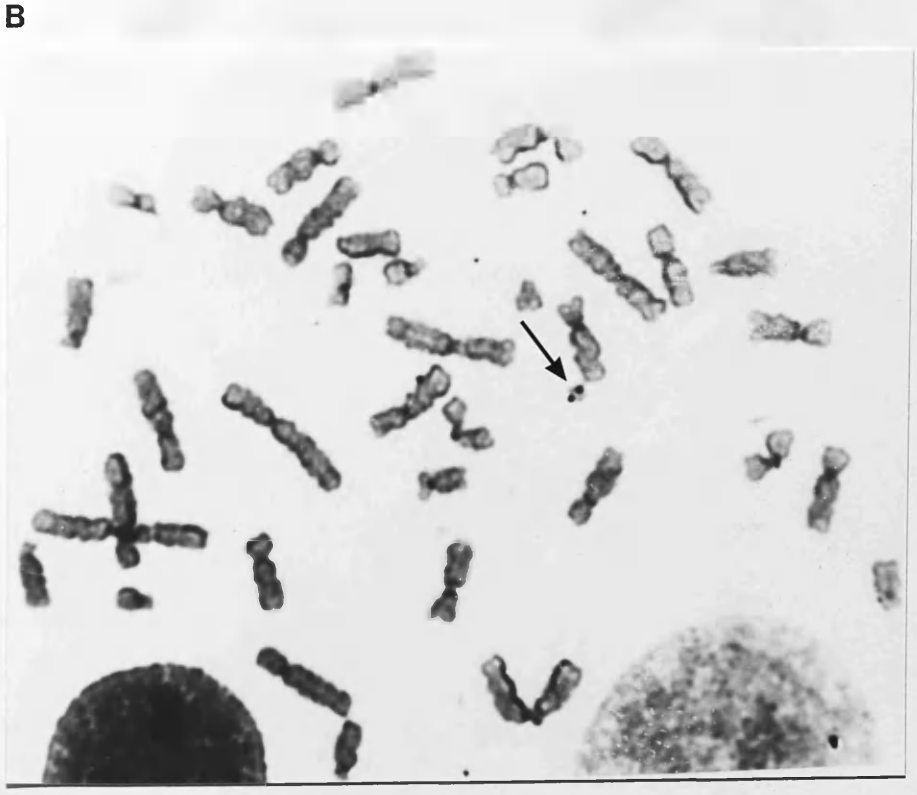


Figure 37 ^{3}H-GMGY10 in DG.
A)Distribution of silver grains over the chromosome complement (Data from 24 cells)
B) Metaphase spread following hybridisation. Note the silver grains on the fragment

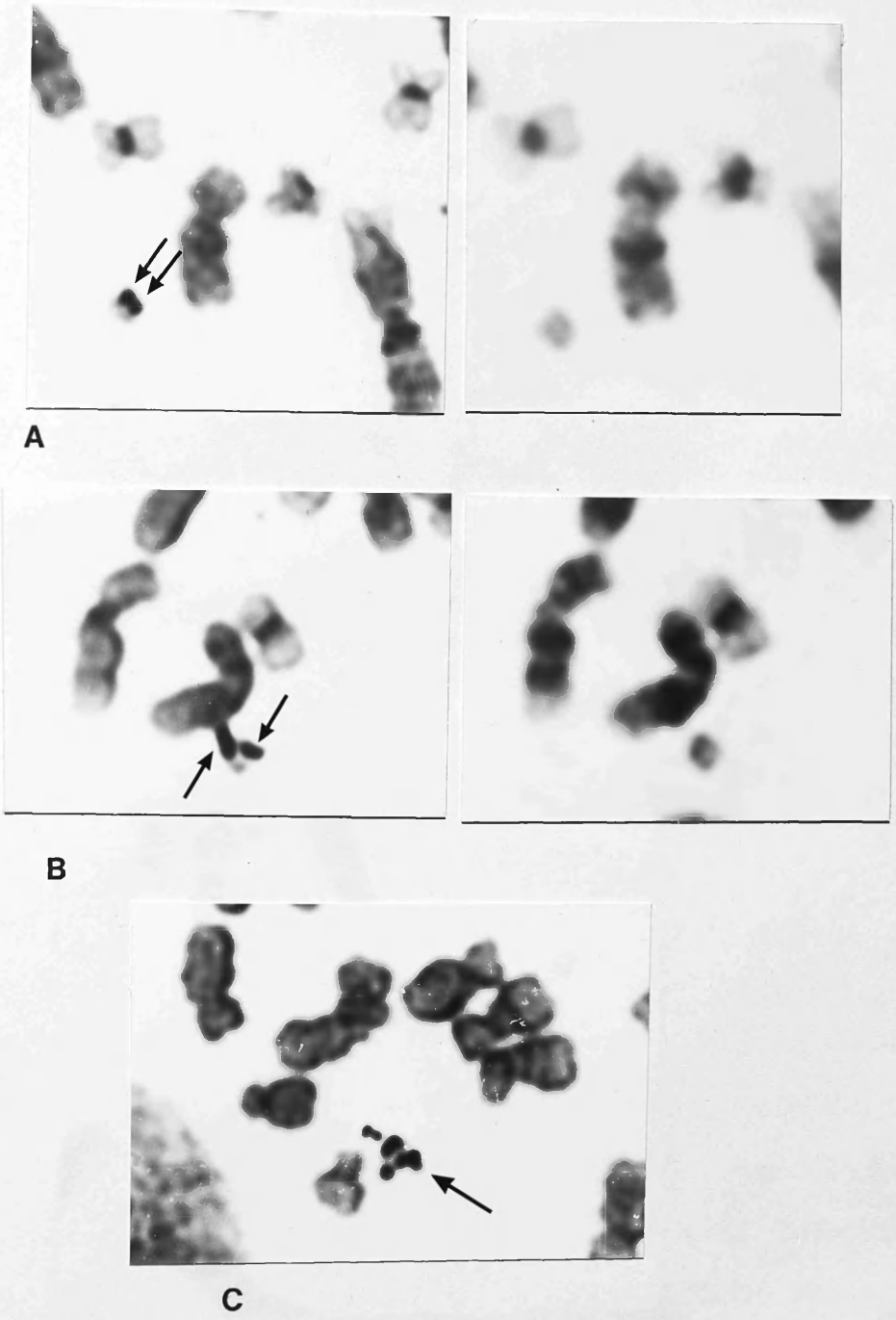
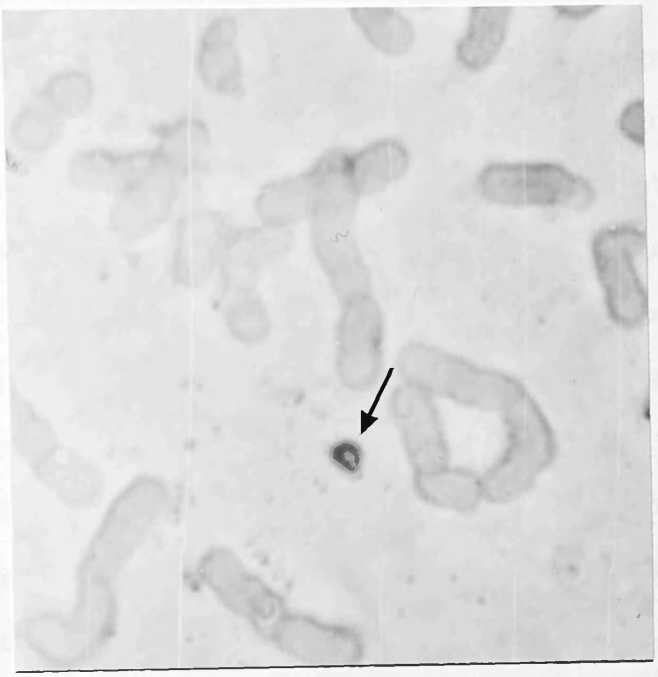
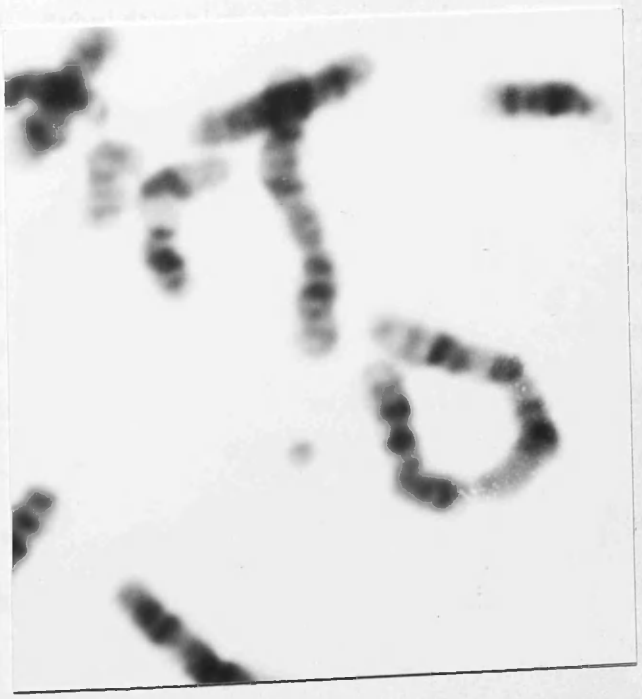


Figure 38 Partial metaphases from DG 3
A, B, C following hybridisation with ^{3}H-GMGY10
D following hybridisation with biotinylated GMGY10



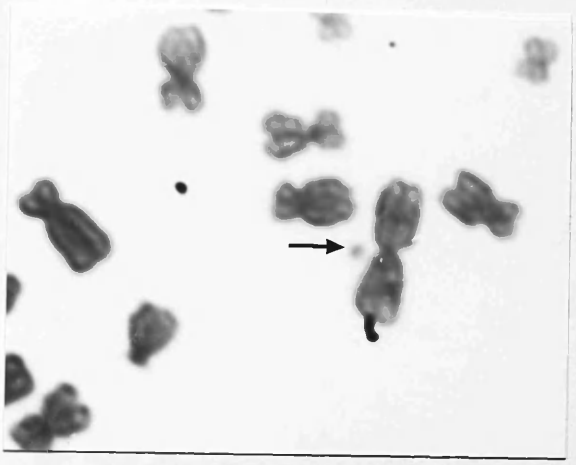
D



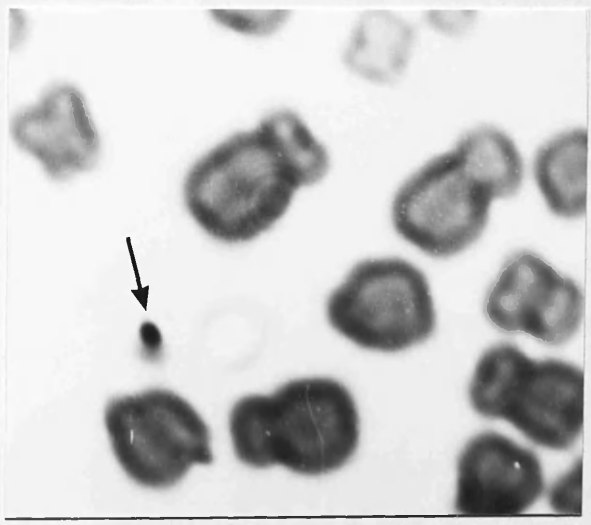
d

Figure 38 continued
D) Partial metaphase from DG following hybridisation with biotinylated GMGY10.
d) same metaphase before the hybridisation

finding that only one band from the several recognised by GMGY7 on Southern blots was present in RW. 42 cells containing the fragment were scored and 7% of the total signal was located on the minute chromosome (figure 39). For statistical analysis, the relative length of the fragment was taken as the relative length of the short arm of the Y chromosome. The hybridisation was statistically significant. RW was also investigated with ^{3}H-P2F2 (10ng per slide), another DNA sequence shown to be present; 24 cells were scored and a total number of silver grains 137 was recorded; only 3 grains were observed on the tiny fragment after an exposure of 35 days although significant hybridisation was recorded at chromosome Xp21 (see also Section 3.2.4). RW was re-investigated with biotinylated GMGY7. The chromosomal preparations used have been kept at room temperature for two years and they would not band after lipsol treatment; this is not surprising as lipsol is reported to be effective on fresh slides up to three months old (Elliot, 1980 and our observations). However, metaphases with the fragment were photographed, their positions were recorded and were hybridised with biotinylated GMGY7. Although the incubation time in chromogenic substrate was the longest possible (5 hours) no signal could be observed over the metaphase chromosomes which it should be noted were severely distorted (figure 40A). Interestingly, hybridisation signal was present in the interphase nuclei (figure 40B). Degradation of chromosomal DNA has been reported as a result of ageing and might be responsible for the absence of hybridisation over metaphase chromosomes. Contrary to this are reports that slides up to 1 year old (Burns et al., 1985), or even 5 year old (Kozma & Adinolfi, 1988) have been used successfully for biotinylated in situ



A

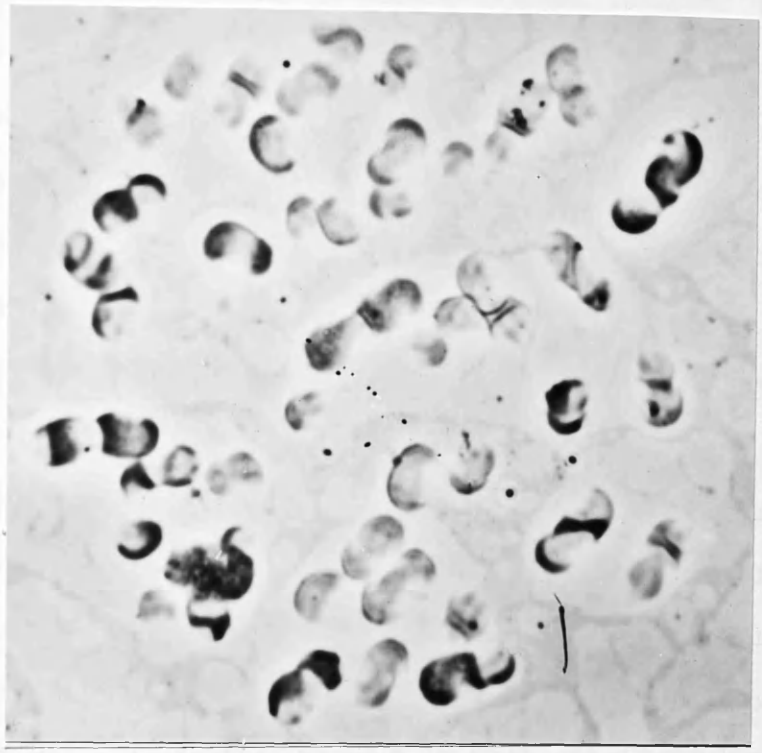


B

Figure 39

(A) Partial metaphase from RW showing the very small size of the marker chromosome
(B) Partial metaphase following hybridisation with tritium-labelled GMGY7. The marker chromosome is barely seen under the silver grain.

A



B

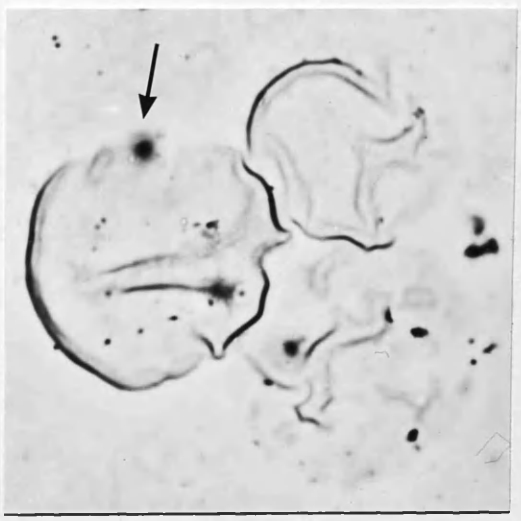


Figure 40 Biotinylated GMGY7 hybridised with 2 year old chromosomal preparations from RW
A) Complete distortion of chromosome morphology
B) Signal is however present in interphase nuclei.

hybridisation.

An occasional association of the chromosomal fragment with the centromere of other chromosomes was observed. A similar observation has been reported by Schmid et al., (1989) for a supernumerary microchromosome found in over half of the metaphases of a CREST scleroderma patient which found this association statistically significant. Seventy-four metaphases were examined for the position of the fragment and 26 associations were recorded. The fragment was considered to be associated to another chromosome when its distance from this chromosome was no greater than three times the size of the fragment itself (the same criterion was used by Schmid et al., (1989). An example of such association is shown in figure 39A. Whenever the fragment appeared to be associated with two or more chromosomes, the associations to each of these were recorded. If the association between the microchromosome and the other chromosomes occurred randomly it should be expected that the total number of associations found would be distributed over the chromosomes in proportion to their relative lengths. The associations however were not found to be statistically significant (table 37).

3.6 The investigation of a 15p+ polymorphism

Following amniocentesis, a chromosome 15 polymorphism was found in a 46,XX amniotic fluid sample. The polymorphic chromosome had an unusually large short arm and is designated here 15p+. An identical polymorphism was present in the father. Cytogenetic analysis revealed that the enlarged short arm of 15p+ had the staining properties of Y chromosome long arm heterochromatic region (Yq12).

Chromosome		Association frequencies			
No.	Relative Length	Observed	Expected	O-E	x^2
1	8.47	3	2.20	0.8	0.29
2	7.76	2	2.01	-0.01	0.00
3	6.56	0	1.70	-1.70	1.70
4	6.13	4	1.59	2.41	3.65
5	5.58	0	1.45	-1.45	1.45
6	5.65	2	1.72	0.28	0.04
7	5.00	1	1.33	-0.33	0.08
8	4.77	0	1.24	-1.24	1.24
9	4.73	0	1.22	-1.22	1.22
10	4.35	0	1.13	-1.13	1.13
11	4.35	0	1.13	-1.13	1.13
12	4.16	0	1.08	-1.08	1.08
13	3.59	1	0.93	0.07	0.00
14	3.28	2	0.85	1.55	2.82
15	3.11	2	0.80	1.2	1.8
16	3.11	1	0.80	0.2	0.05
17	3.02	0	0.78	-0.78	0.78
18	2.73	1	0.70	0.3	0.12
19	2.58	1	0.67	0.33	0.16
20	2.31	2	0.60	1.4	3.26
21	1.90	2	0.49	1.51	4.65
22	1.69	1	0.43	0.57	0.75
X	5.14	1	1.34	-0.34	0.08

Table 37 Association frequencies between marker chromosome and normal chromosomes in RW. No association is staistically significant.

In order to determine conclusively that Y material was present, in situ hybridisation studies were carried out both in the father's chromosomes and the amniotic cells, using the Y-specific DNA probe pY3.4 (Lau et al., 1984).

^{3}H-pY3.4 was hybridised with metaphases obtained from peripheral blood lymphocytes and cultured amniotic fluid cells at a concentration of 30ng per slide. The chromosomal preparations were exposed for 6 days. It should be noted that the probe had been nick-translated a year before and had been kept at -20^{o}C in hybridisation buffer.

In the father, two heavily labelled sites of hybridisation were observed in every cell, one at the short arm of the 15p+ chromosome and the other at the long arm of the Y chromosome (figure 41). Forty seven (47) metaphases were analysed and 391 silver grains were recorded. Ninety-eighty grains ,25% of the total signal, were located on the Y chromosome and 67 (17%) were located on the short arm of chromosome 15p+. The remaining grains were distributed throughout the chromosome complement without any accumulation of grains over any other chromosome or region as shown in figure 42.
A similar study using ^{3}H-labeled pY3.4 on a 46,XY, 15p+ individual was performed by Alitalo et al., (1988). Their quantitative data are in good agreement with the data presented here with one difference: in their study but not this one, significant hybridisation was recorded on chromosome 9. Absence of hybridisation with chromosome 9 was also observed when pY3.4 was hybridised with a normal male (see table 7) and a normal female (table 39).

Quantitative data from the amniotic fluid cells cannot be presented as only five pre-photographed metaphase spreads could be

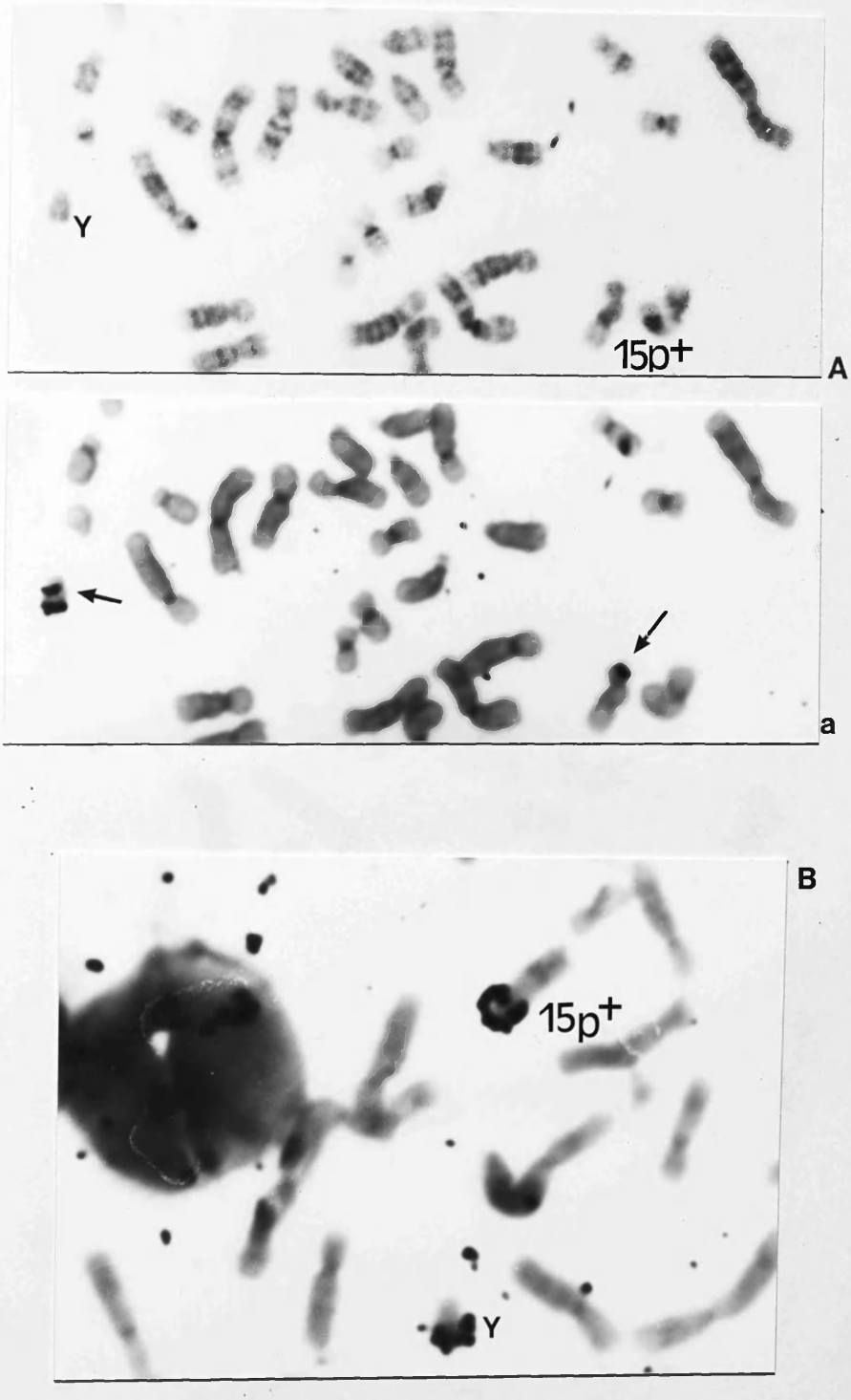


Figure 41 Partial metaphase spreads from the father with the 15p+ polymorphism showing silver grains on Yq and 15p+ following hybridisation with pY3.4
(A) cell before and after the hybridisation (a)
(B) cell after the hybidisation

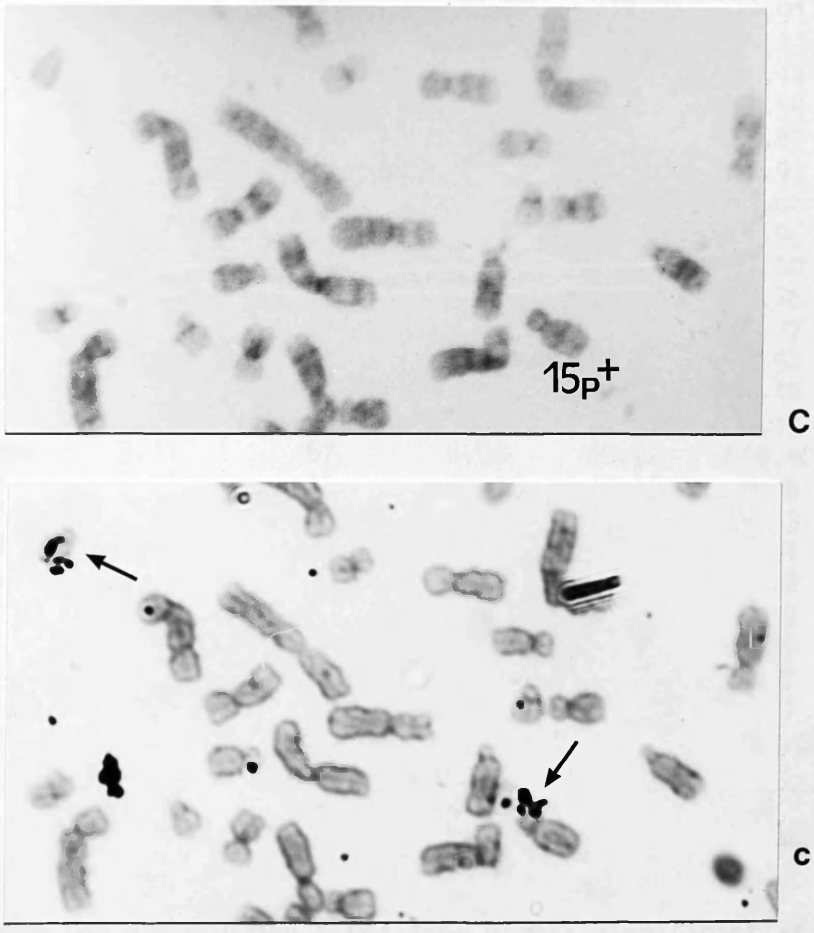
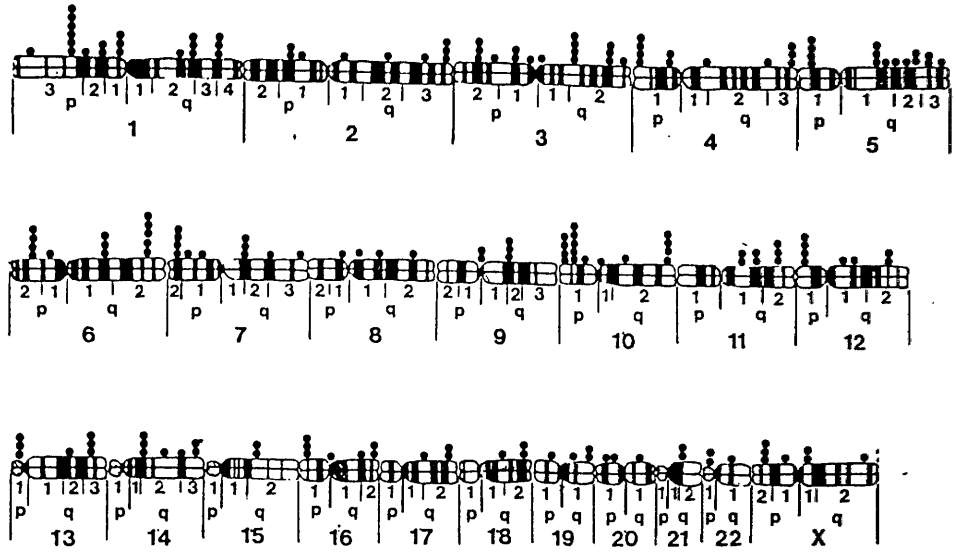


Figure 41 continued
cell before (C) and after (c) the hybridisation

Chromosome	Relative Length	Observed O	Expected E	O-E	$\frac{(O-E)^2}{E}$
1	8.47	25	33.11	-8.11	1.98
2	7.76	13	30.34	-17.34	9.91
3	6.56	15	25.64	-10.64	4.41
4	6.13	14	23.96	-9.96	4.14
5	5.58	16	21.81	-5.81	1.54
6	5.65	12	22.09	-6.09	1.67
7	5.00	12	19.55	-7.55	2.91
8	4.77	8	18.66	-10.66	6.08
9	4.73	8	18.49	-10.49	5.95
10	4.35	17	17.00	0.00	0.00
11	4.35	8	17.00	-9.00	4.76
12	4.16	7	16.26	-9.26	5.27
13	3.59	7	14.03	-7.03	3.52
14	3.28	9	12.84	-3.84	1.14
15	3.11	5	6.08	-1.08	0.19
15p+	3.11	67	6.08	60.92	610.40
16	3.11	10	12.16	-2.16	0.38
17	3.02	2	11.80	-9.80	8.13
18	2.73	6	10.67	-4.67	2.04
19	2.58	7	10.08	-3.08	0.94
20	2.31	5	9.03	-4.03	1.79
21	1.90	3	7.49	-4.49	2.69
22	1.69	9	6.60	2.4	0.87
X	5.14	8	10.04	-2.04	0.41
Y	2.09	98	4.08	93.92	2162.00

Table 38 ^{3}H-pY3.4 in the father with the 15p+ polymorphism.
Statistical analysis of silver grain distribution.
Data from 47 cells, total number of grains 391.
Chi-square for chromosomes Y and 15p+ highly significant at $p<0.001$.



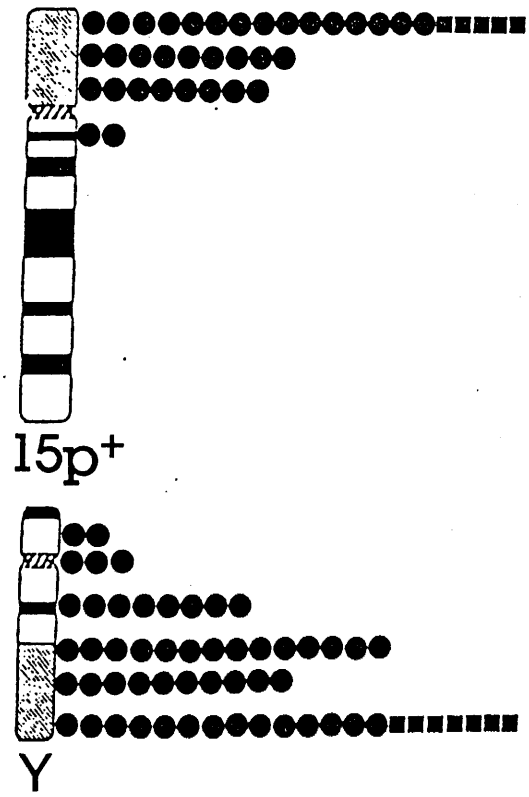


Figure 42 ^{3}H-pY3.4 in the father with the 15p+ polymorphism. Distribution of silver grains over the chromosome complement. Chromosomes Y and 15p+ are shown separately.
■: four silver grains

Chromosome	Relative Length	Observed O	Expected E	O-E	$(O-E)^2$ E
1	8.47	39	41.24	-2.24	0.12
2	7.76	34	37.79	-3.79	0.38
3	6.56	32	31.94	0.06	0.00
4	6.13	29	29.85	-0.85	0.02
5	5.58	27	27.17	-0.17	0.00
6	5.65	28	27.51	0.49	0.00
7	5.00	18	24.35	-6.35	1.65
8	4.77	25	23.22	1.78	0.13
9	4.73	23	23.03	-0.03	0.00
10	4.35	18	21.16	-5.16	1.25
11	4.35	30	21.16	8.84	3.69
12	4.16	19	20.25	-1.25	0.07
13	3.59	16	17.48	-1.48	0.12
14	3.28	11	15.97	-4.97	1.54
15	3.11	26	15.14	10.86	7.79
16	3.11	13	15.14	-2.14	0.30
17	3.02	18	14.70	3.30	0.74
18	2.73	14	13.29	0.71	0.03
19	2.58	13	12.56	0.44	0.01
20	2.31	11	11.24	-0.24	0.00
21	1.90	13	9.25	3.75	1.52
22	1.69	6	8.23	-2.23	0.60
X	5.14	24	25.03	-1.03	0.04

Table 39. ^{3}H-pY3.4 in normal female. Statistical analysis of silver grain distribution. Data from 114 cells total number of grains 487. Chi-square for chromosome 15 is significant at $0.001<p<0.005$. Chi-square for the whole experiment (20) not significant for 21 degrees of freedom.

relocated after hybridisation. This demonstrates the one disadvantage associated with pre-hybridisation banding. Having photographed the metaphase spreads before hybridisation if something goes wrong, the documentation of the results is prevented. Silver grains were present in the short arm of the 15p+ in all five metaphases as were in cells not photographed before the hybridisation (figure 43). In the latter the polymorphic chromosome was easily recognisable because of its enlarged short arm.

The presented in situ hybridisation findings confirmed the suggestion from banding studies that the 15p+ polymorphism is the result of a Yq:15p translocation. Such translocations have no phenotypic effect to the carrier.

3.7 Interphase nuclei topological investigations using in situ hybridisation

This project was not concerned with topological investigations in interphase nuclei. However, hybridisation signal was recorded in interphase nuclei during the in situ hybridisation studies presented in the previous six sections. Examples of such signals are shown in figure 44 as they clearly demonstrate how in situ hybridisation can reveal the position of chromosomes or chromosome regions in interphase. With radioactive pY3.4 the position of the Y long arm is revealed in interphase by the presence of clusters of silver grains, one cluster per nucleus in a normal male individual (figure 44A) and the amniotic fluid sample with the 15p+ polymorphism (fig. 44C) while two clusters are evident in a nucleus from the father with the same polymorphism (see section 3.5).

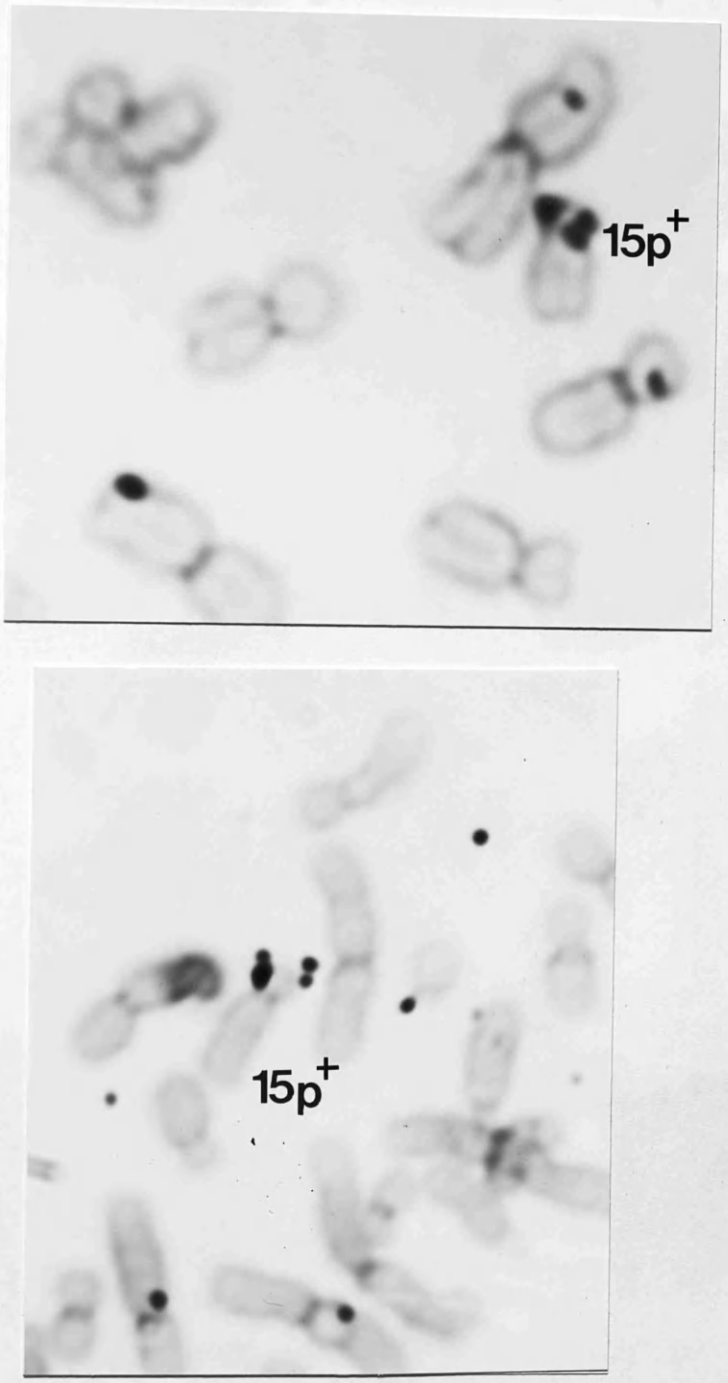


Figure 43 Partial metaphase spreads from the amniotic fluid cells with the 15p+ polymorphism showing silver grains on this chromosome following hybridisation with tritium-labelled pY3.4.

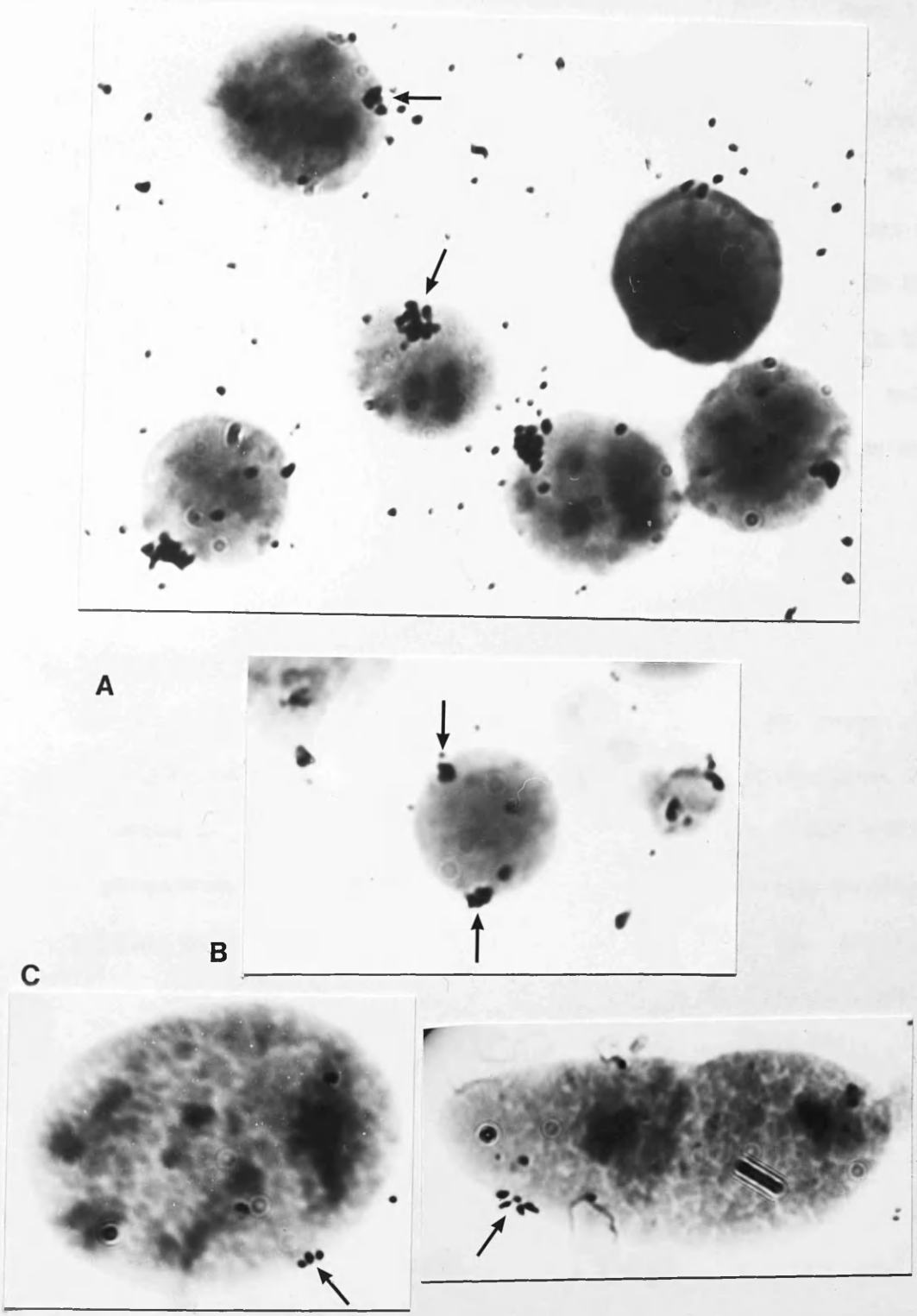


Figure 44 Interphase nuclei topological investigations using in situ hybridisation.

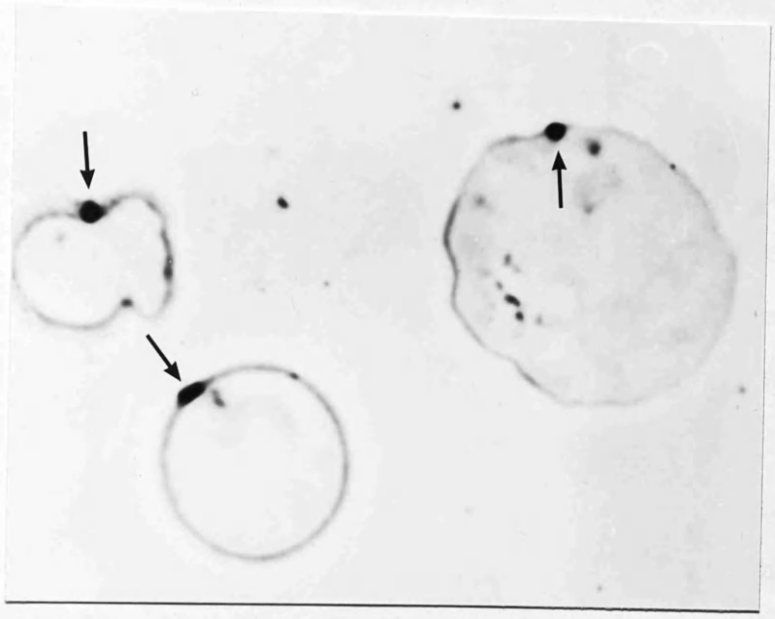
(A) Following hybridisation with tritium-labelled pY3.4 the position of the Y chromosome long arm (Y body) is revealed in interphase nuclei by the presence of clustering of silver grains
(B) The same probe in the father with the 15p+ polymorphism. Two clusters of grains are present in the interphase nucleus shown. In the amniotic fluid cells with the same polymorphism one cluster can be seen (C)

The position of the Y chromosome short arm is revealed in interphase following hybridisation of biotinylated GMGY10 in a normal male (fig. 44D) and XX male DR (fig. 44E) who has Y-specific sequences at the distal part of the X chromosome (see also section 3.5.1). In the normal male the Y chromosome appears to be associated with the nuclear membrane in the three nuclei shown, whereas in the XX male the signal is associated with the nuclear membrane only in one out of the three nuclei shown.

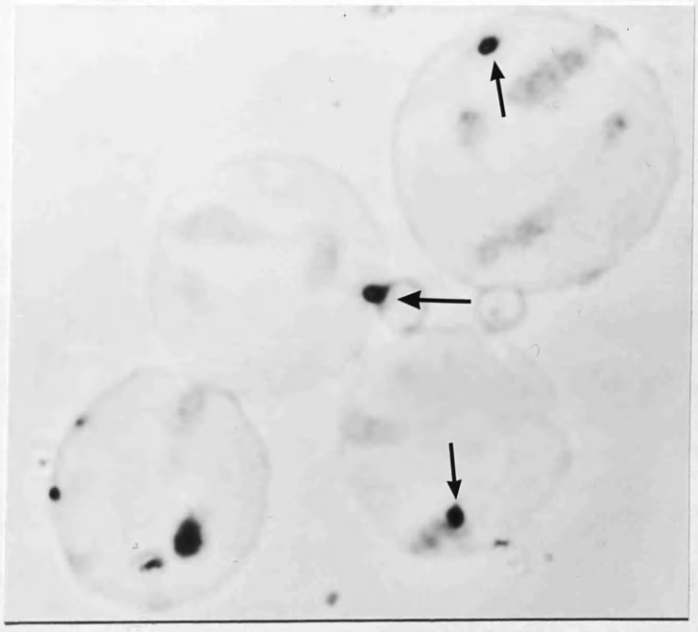
3.8 Comparative mapping in the great apes

For convenience the term gorilla will be used to refer to Gorilla gorilla, chimpanzee to Pan troglodytes and orangutan to Pongo pygmaeus. According to standardised nomenclature [ISCN(1985)] each chromosome of the species is designated by an acronym denoting the species being HSA for man, PTR for chimpanzee, GGO for gorilla and PPY for orangutan followed by its number. For example, HSA1 refers to human chromosome 1 and PTRY refers to the Y chromosome of the chimpanzee.

Comparative mapping studies were carried out on skin fibroblasts from a female and a male gorilla, a male chimpanzee and two male orangutans (designated A and B) using three probes previously mapped in humans, PLB4 and GMGY10 and GMGY7. The latter were previously shown by Southern analysis to be Y-specific in the great apes (K. Kwok personal communication), thus the purpose of the investigation was to determine whether their location within the Y chromosome had been conserved or not in the great apes during evolution. For these studies only the biotinylated approach was used



D



E

Figure 44 continued

(D) With biotinylated GMGY10 the Y chromosome short arm is revealed associated with the nuclear membrane.
(E) When biotinylated GMGY10 was used in an XX male the signal in the nuclei has a different location

because of speed and convenience.

3.8.1 The karyotypes of the great apes

Firstly, the karyotypes of the three species were prepared from lipsol banded preparations following the recommendations of ISCN (1985). Confident identification of the acrocentric chromosomes in each species proved difficult due to lack of experience and the relatively poor quality of available chromosomal preparations. This applied to chromosomes 11, 12, 13, 14, 15 and 16 in the gorilla, chromosomes 12, 13, 14, 15, 16 and 17 in the chimpanzee and chromosomes 10, 11, 12, 13, 14, 15, 16, and 17 in the orangutan. Lipsol banded karyotypes of the gorilla, chimpanzee are shown in the Appendix. It was also difficult to see clearly the short and the long arm of the chimpanzee Y chromosome due to the extremely small size of this chromosome (see below).

Although cytogenetic investigations were not an objective of this project it is worth mentioning that one orangutan, (individual B), was found to be heterozygous carrier of a rearranged chromosome 2 and a rearranged chromosome 9 (figure 45). Both rearrangements have been previously described (Seuanez 1979).

The comparative mapping studies performed during this project were concerned with chromosomes 1 and Y of man and the great apes. Lipsol-banded chromosome 1 of man and the great apes are presented in figure 46. The basic similarity in the banding pattern of this chromosome in the four species mentioned in section 1.9.3 is obvious in this figure. It can also be seen why the arm ratio in the great ape chromosome 1 is the reverse of that in man as mentioned in section 1.9.3; the block of heterochromatin which in the human

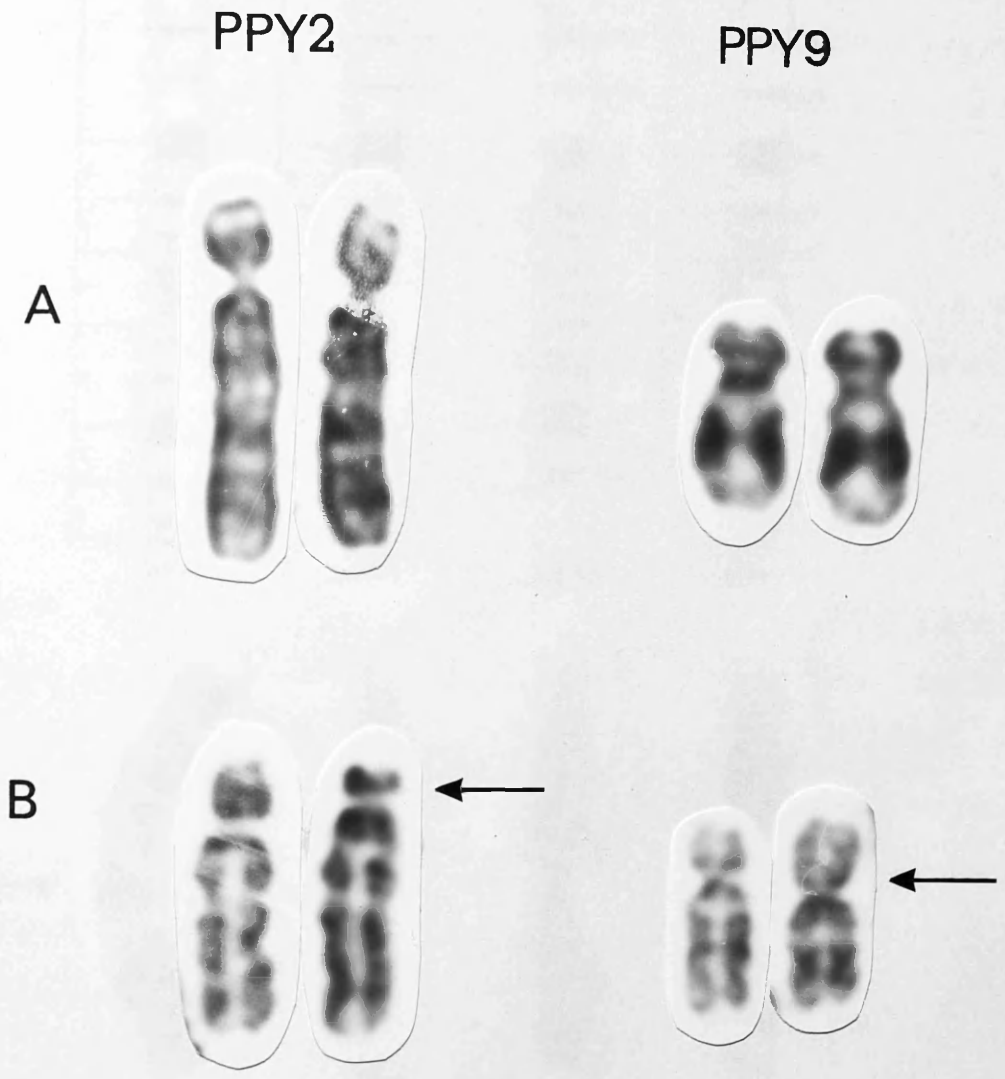


Figure 45 Pairs of chromosome 2 and chromosome 9 from orangutan A and orangutan B. Arrows show the heteromorphic chromosomes.

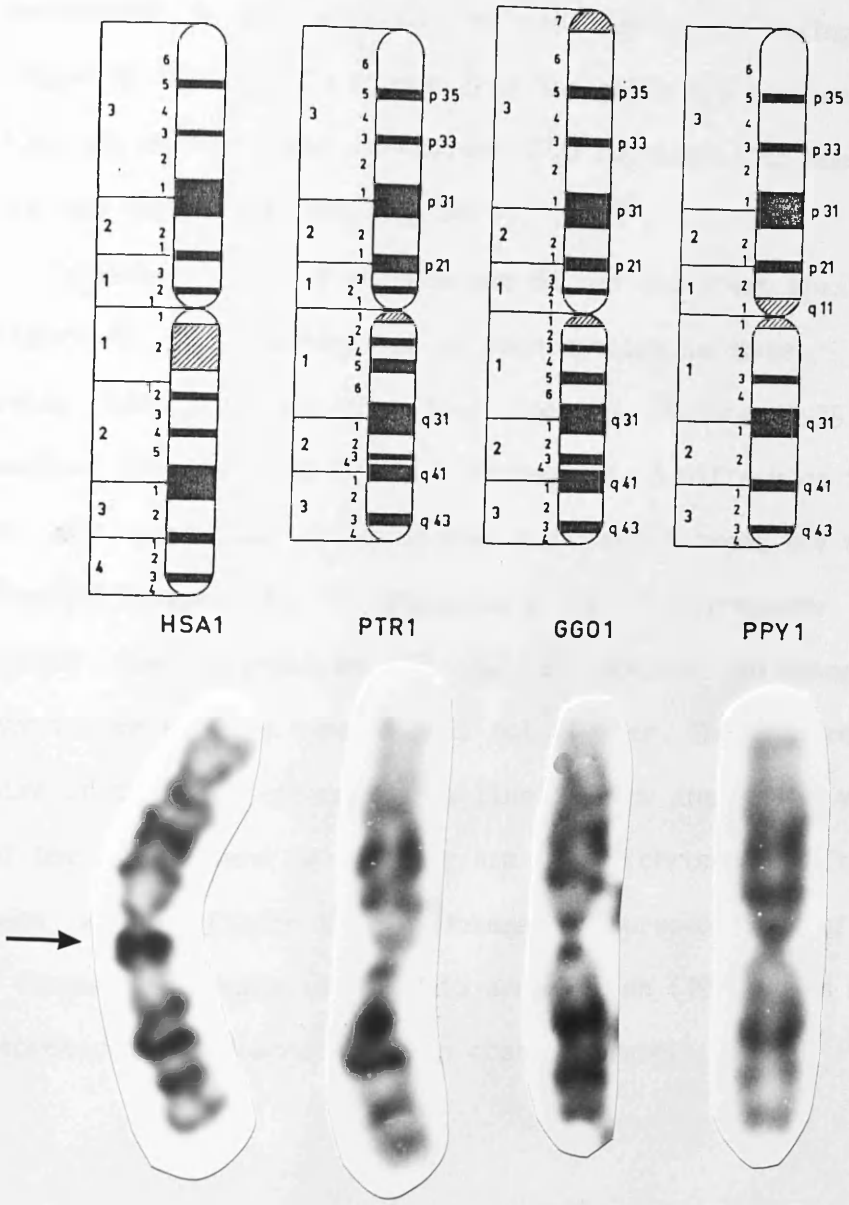


Figure 46 Lipsol banded chromosome 1 of man, chimpanzee, gorilla and orangutan arranged from left to right. In the diagrammatic representation [taken from ISCN (1985)] the great ape chromosome has been inverted to maintain comparisons based on similarity of bands. The arrow indicates the block of heterochromatin present in the human but absent in the great ape chromosome 1.

chromosome 1 is adjacent to the centromere making this arm the longer of the two, is absent from the great ape chromosome. Thus the long arm of the human chromosome 1 is homologous to the short arm of the ape chromosome and vice versa.

Lipsol banded Y chromosomes of man the great apes are shown in figure 47. The X chromosome of each species is shown as by being identical in the four species (Seuanez 1979) serves as a control for the size of the Y chromosome. A difference in the size of the orangutan Y chromosome was noted between the two specimens studied (figure 47). In orangutan A the Y chromosome was clearly larger than chromosome 22 and 23 whereas in orangutan B the Y chromosome was the same size if not smaller. The difference in the size of the Y chromosome seems likely to be due to the variable size of the dark G-band on the long arm of the chromosome. It can also be seen in this figure that the schematic representation of the gorilla Y chromosome suggested by Yunis and Prakash (1982) does not represent the G-banded pattern observed here.

3.8.2 Comparative mapping of a human transfer RNA gene for glutamic acid

PLB4, a 2.4kb human DNA fragment containing a $tRNA^{Glu}$ gene, has been assigned to human chromosome 1p36 with a secondary site of hybridisation at 1q21-q22 (Boyd et al., 1989). It was biotinylated and hybridised with metaphase spreads from the great apes at conditions identical to these used in the human by Boyd et al., (1989) with 25ng of probe per slide and two and a half hours incubation in the chromogenic substrate. Both male and female

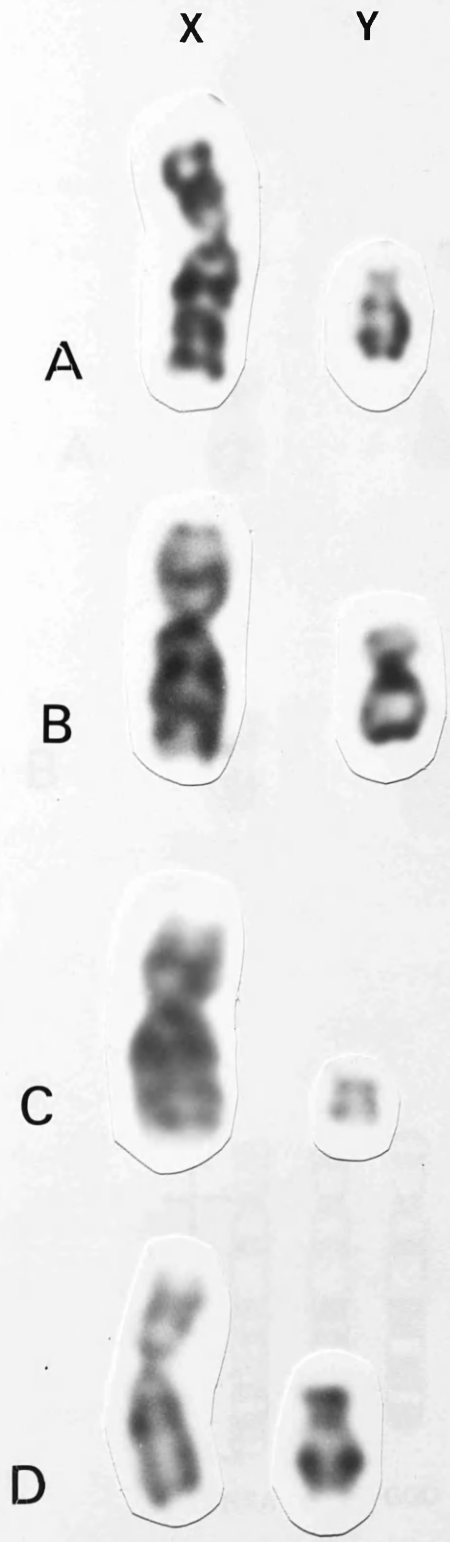


Figure 47 Lipsol banded Y chromosome of man and the great apes. (A) Man (B) Gorilla (C) Chimpanzee and (D) Orangutan. The X chromosome is shown on the left for comparison.

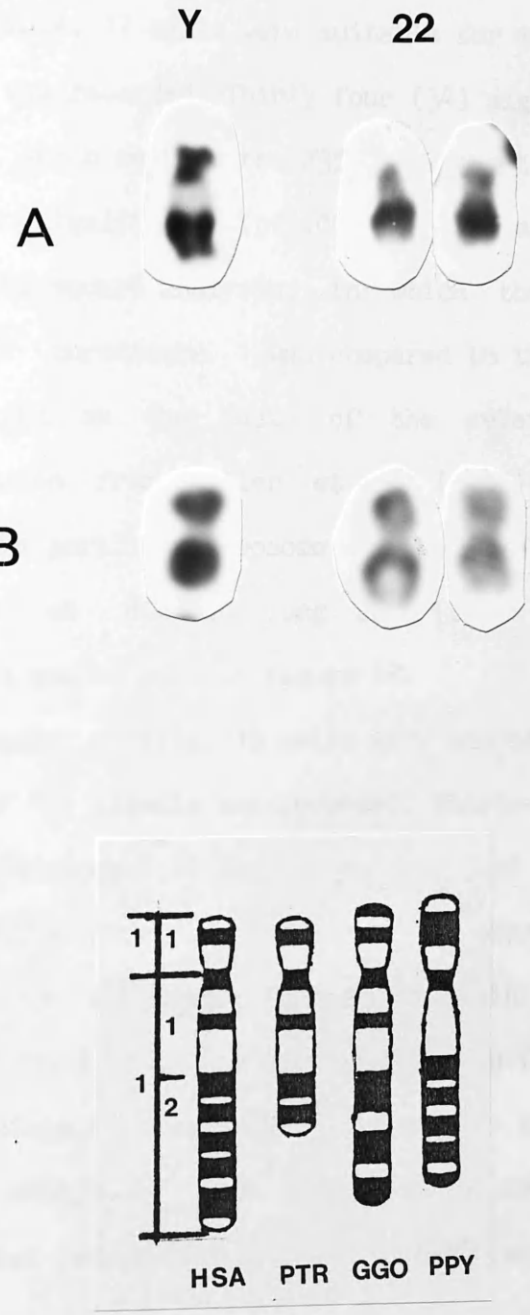


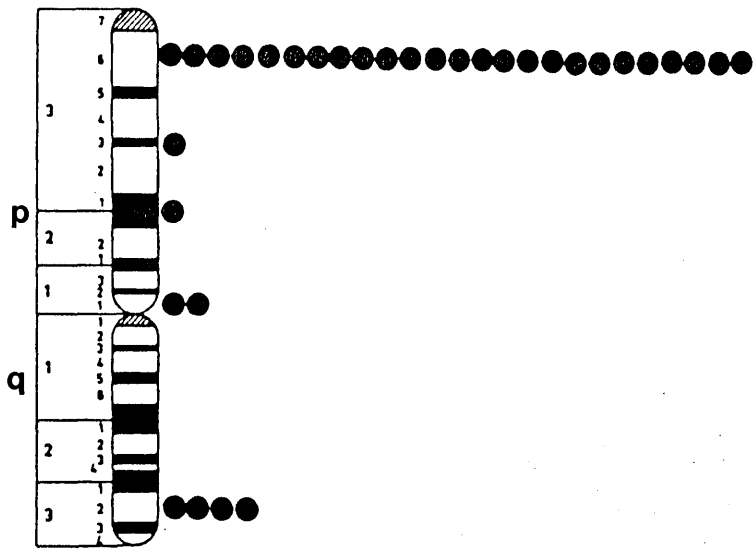
Figure 47 continued
Y chromosomes from orangutan A and B. Chromosomes 22 are shown for comparison in size

gorilla metaphases were used.

In the female, 17 cells were suitable for scoring and a total of 148 signals was recorded. Thirty four (34) signals were observed on chromosome 1, which represents 23% of the total signal and is statistically significant ($p<0.001$). The level of significance was assessed by chi-square analysis, in which the number of grains observed over chromosome 1 was compared to the number expected, a value calculated on the basis of the relative length of the chromosome taken from Miller et al., (1974). The distribution of signal over the gorilla chromosome 1 revealed a single site of hybridisation at distal long arm [band 1p3 6 according to ISCN(1985)] as can be seen in figure 48.

In the male gorilla, 15 cells were scored after hybridisation and a total of 151 signals was recorded. Thirty-three of these were located on chromosome 1 which represents 19% of the total signal and is statistically significant ($p<0.001$). The distribution of hybridisation over chromosome 1 revealed, as in the female, a single peak at 1qter (band 1p36) and this is shown in figure 48. According to these findings the human $tRNA^{Glu}$ gene maps to gorilla chromosome 1p36. Partial metaphases from the gorilla showing hybridisation signal at distal long arm of chromosome 1 are shown in figure 49.

The chimpanzee line was extremely poor; only a limited number of chromosomal preparations were available with few suitable metaphases for the purposes of this study (complete, well-spread, well-banded), on any slide. The number of cells scored was obtained from three different slides. Fifteen (15) cells were scored and a total of 151 signals was recorded. Fifty-nine of these were observed on



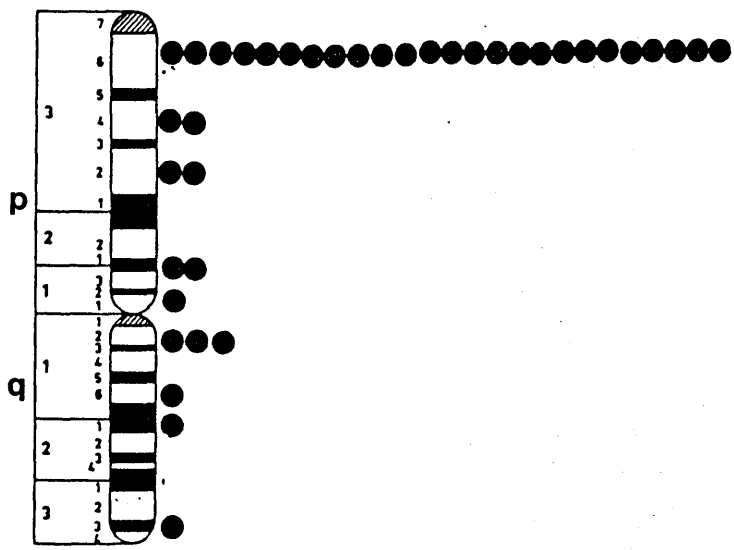


Figure 48 Localisation of a human $tRNA^{Glu}$ gene in the gorilla. Signal distribution over chromosome 1 in the male and female individual. Data from 17 and 15 cells respectively.

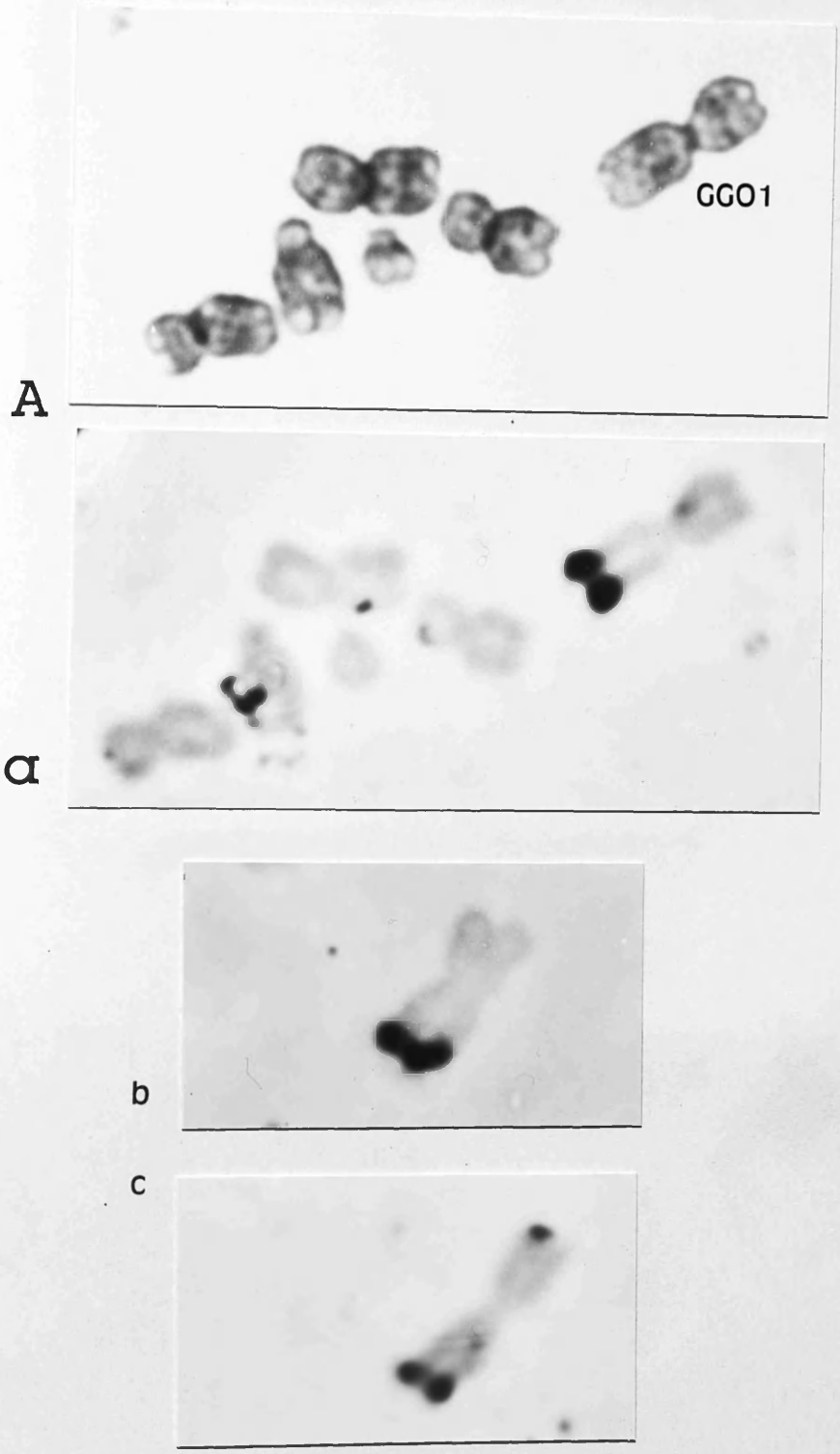
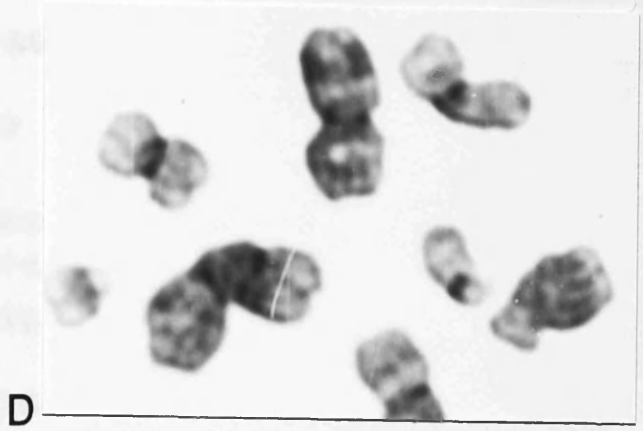
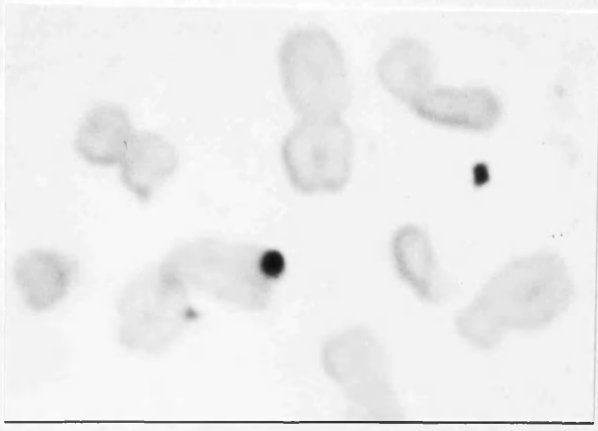
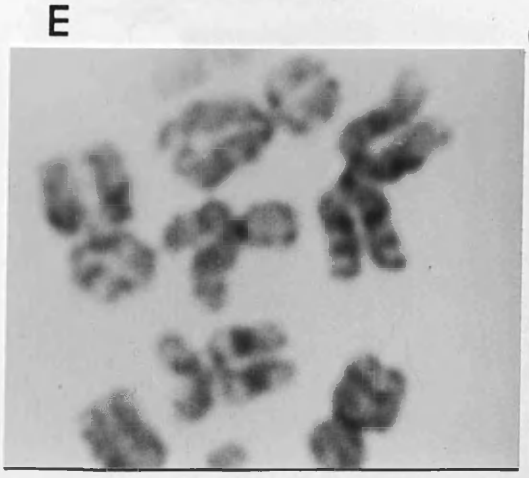


Figure 49 Partial metaphases showing hybridisation signal on gorilla chromosome 1 distal long arm (band 1p36 according to ISCN nomenclature)
Note distinct signal on both chromatids
A, a is the same cell before and after the hybridisation respectively.
b, c chromosome 1 after the hybridisation.







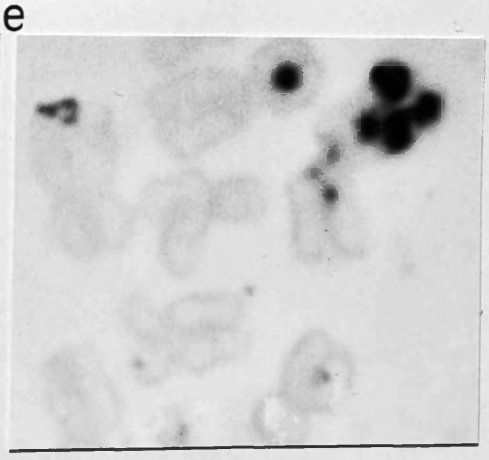
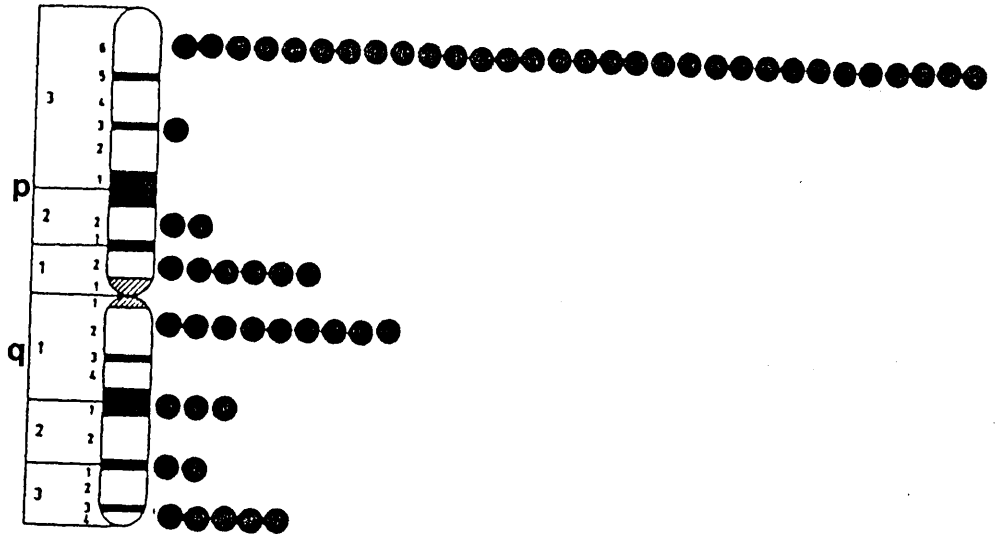


Figure 49 continued
Partial metaphases before (D,E) and after (d,e) the hybridisation with biotinylated PLB4.



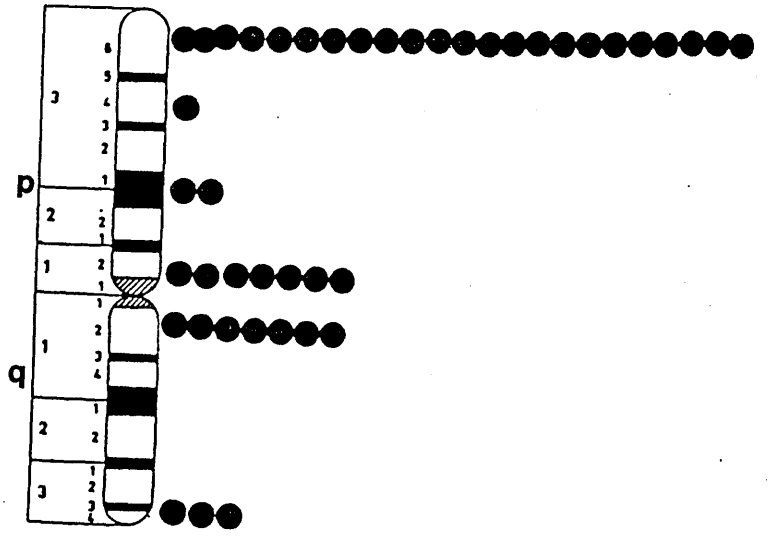


Figure 52 Localisation of a human $tRNA^{Glu}$ in the orangutan. Distribution of signal over chromosome 1 in individual A (data from 24 cells) and B (data from 16 cells).

chromosome 1 which represents 39% of the total signal and is statistically significant ($p<0.001$). The relative length of PTR 1, for chi-square was taken from Warburton et al., (1973). The distribution of hybridisation over the chimpanzee chromosome 1 as shown in figure 50 revealed three sites of hybridisation; one at 1p36 with 31 signals, and two secondary ones, above and below the centromere, with 13 and 8 signals respectively. The great intensity of the hybridisation signal did not permit accurate localisation to specific bands but careful study of all metaphases suggests that it is more likely the hybridisation occurred at region 1p11-p12 and region 1q12-q13. The statistical significance of the hybridisation to the secondary site on the short arm (1q12-q13) was estimated on the basis of the relative length of this arm ($p<0.001$). Since lengths of individual bands have not been measured in the great apes it was not possible to assess the level of significance for the secondary site present on the long arm (1p11-p12). Partial metaphases from the chimpanzee with hybridisation signal on chromosome 1 are shown in figure 51.

In the orangutan, 24 cells from individual A were suitable for scoring after hybridisation and a total of 174 signals was observed. Fifty-six (56) of these were located on chromosome 1 which represents 32% of the total hybridisation signal. Statistical evaluation of this finding was not possible as relative lengths for the orangutan chromosomes have not been measured. Figure 52A shows the distribution of hybridisation over PPY 1 with three hybridisation sites, the main one at 1p36 with 30 signals and two secondary sites above (band 1p12) and below (1q12) the centromere with 8 and 6 signals respectively. This distribution of

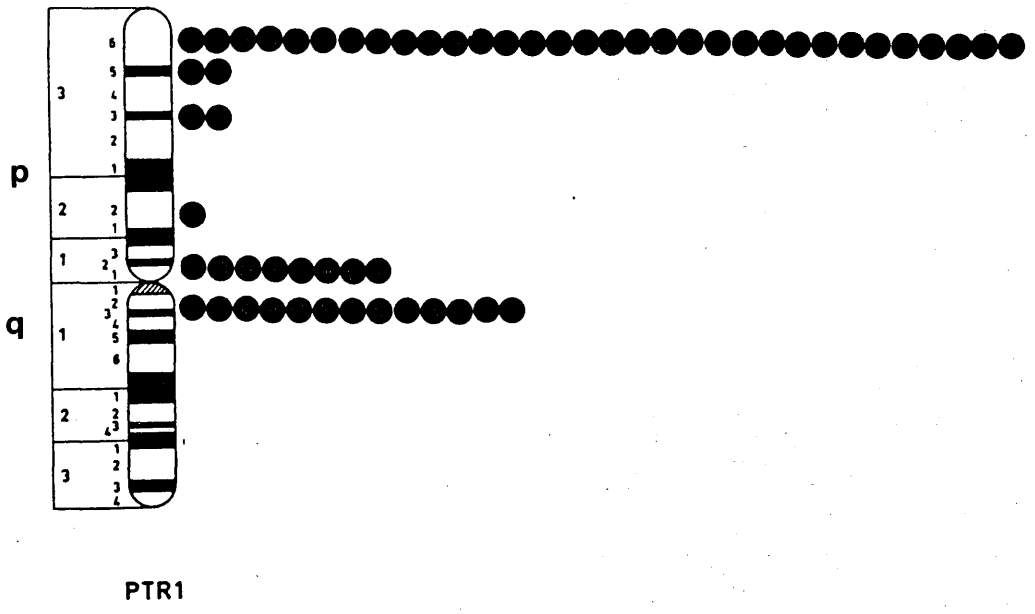


Figure 50 Localisation of a human $tRNA^{Glu}$ gene in the chimpanzee. Distribution of hybridisation signal over chromosome 1 (data from 15 cells).

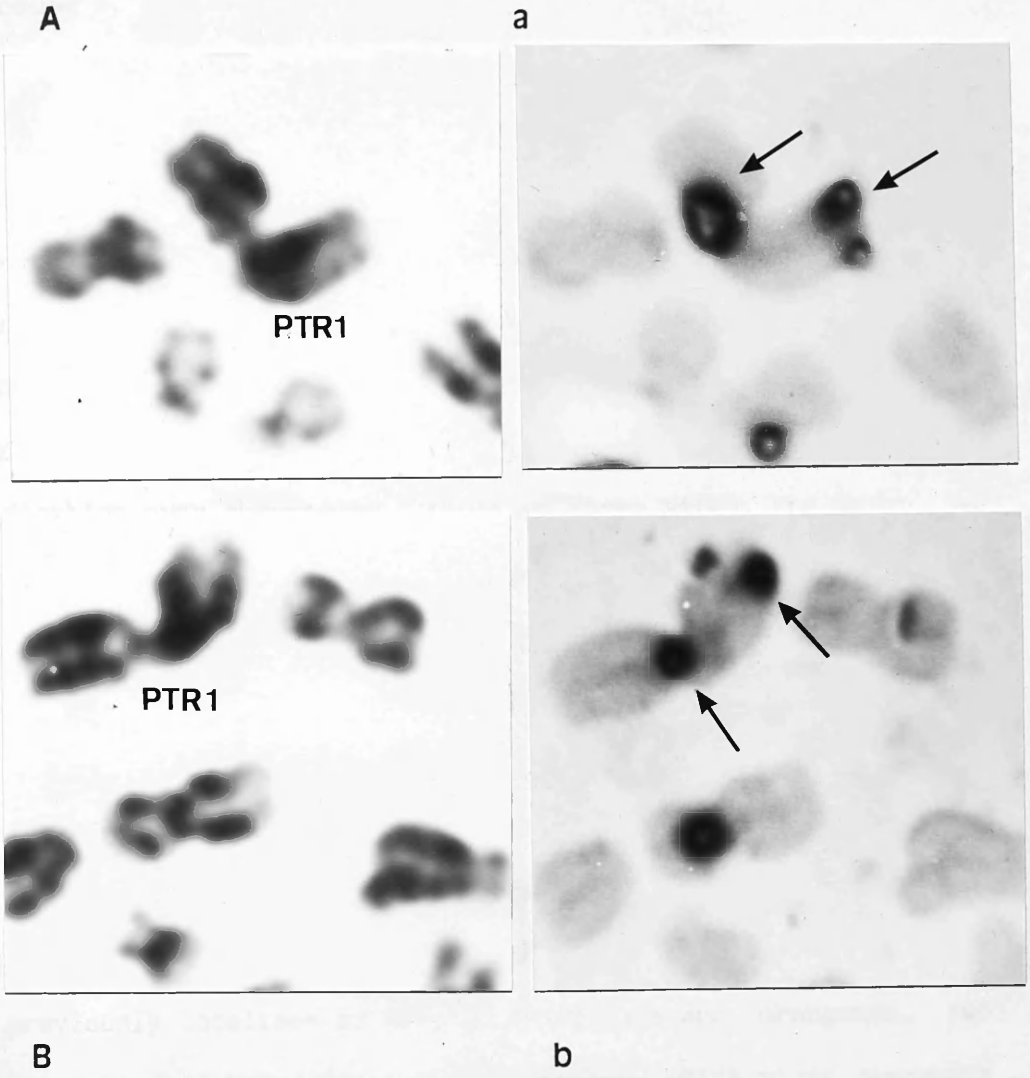


Figure 51 Partial metaphase spreads from the chimpanzee before (A, B) and after (a, b) hybridisation with biotinylated PLB4. Note the two sites of hybridisation on chromosome 1.

hybridisation is markedly similar to that observed in the chimpanzee chromosome 1.

A limitation of comparative studies in the great apes is the small number of animals that are available. To ensure that the results obtained here do not reflect intra-individual variations, an independent experiment was performed on metaphases obtained from the second orangutan (B). Sixteen (16) cells were scored and a total of 149 signals was recorded. Forty-five (45) of these were located on chromosome 1 which is 30% of the total signal. The distribution of hybridisation over chromosome 1 revealed three sites, the major one at 1p36 with 21 signals and two secondary sites (1q12 and 1p12) with 6 signals each, as shown in figure 52B. Figure 53 shows partial metaphases from the orangutan with signal on chromosome 1.

The in situ hybridisation findings taken together indicate that the human gene for glutamic acid transfer RNA is located at distal long arm of chromosome 1 (band 1p36) of the gorilla, chimpanzee and orangutan, a region homoloqous to human 1p3.6 where this gene has been previously localised in man. In chimpanzee and orangutan, two secondary sites of hybridisation were observed which might represent pseudogenes or related DNA sequences in analogy with the secondary site observed in man at 1q21-22. Table 40 summarizes the quantitative data obtained from hybridisation of PLB4 in the great apes. According to these findings the main site of hybridisation of the human $tRNA^{Glu}$ gene in man and the great apes has been conserved during evolution (chromosome 1, band 1p36) whereas there is a variable distribution of the secondary sites in the four species.

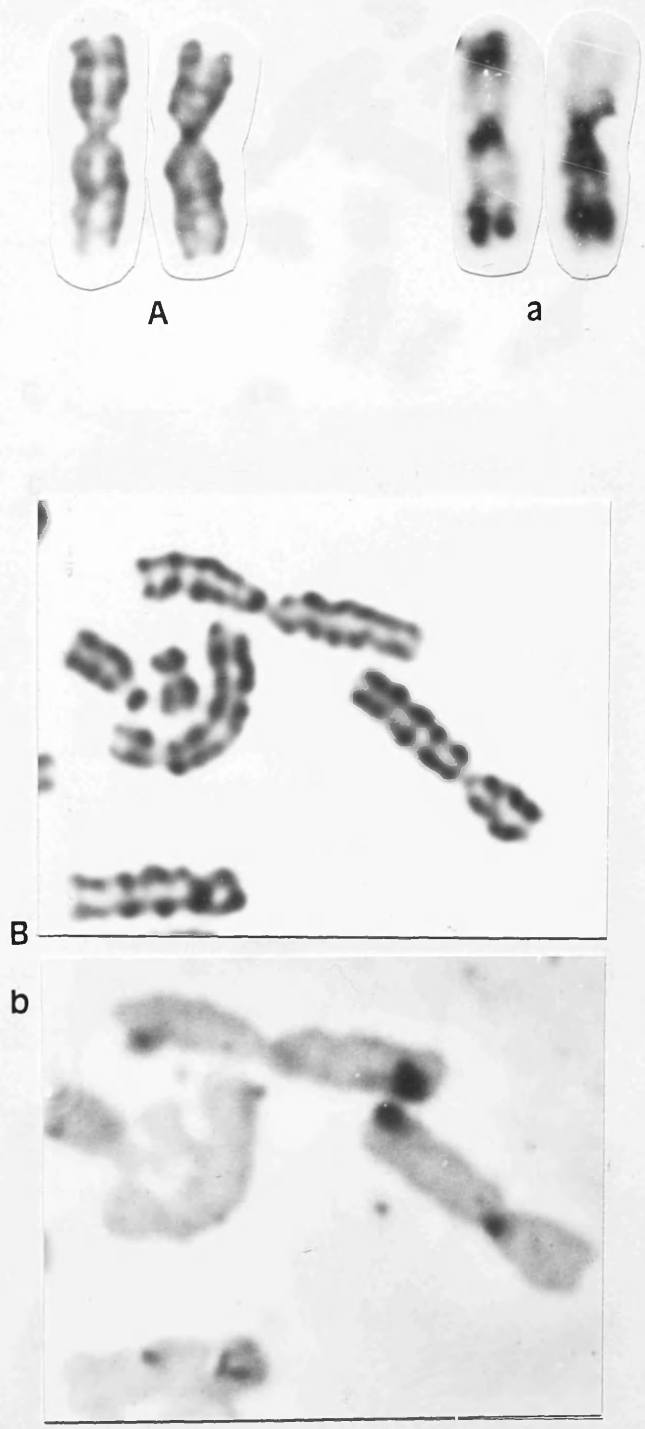
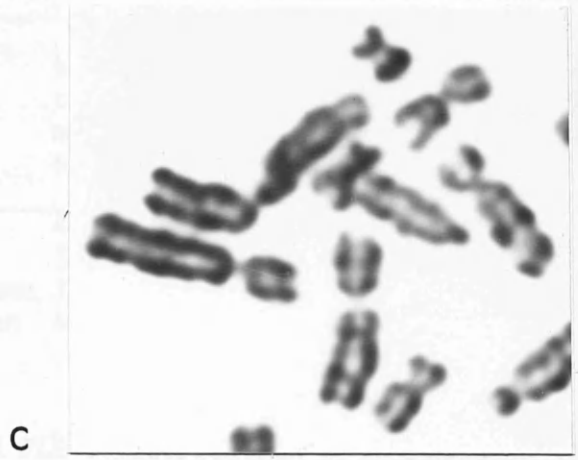
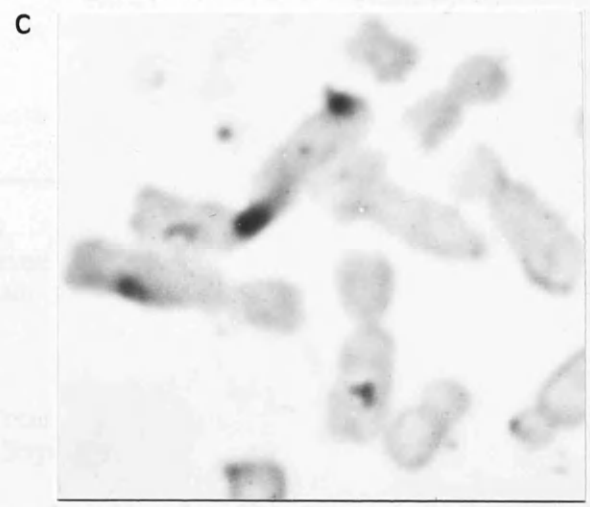


Figure 53 Biotinylated PLB4 in the orangutan.
Pair of chromosomes 1 from individual A before (A) and after (a) the hybridisation
Partial metaphase from orangutan B before (B) and after the hybridisation (b)





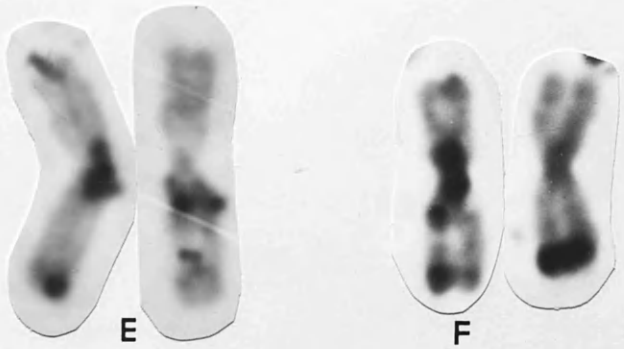


Figure 53 continued
Partial metaphase from orangutan B before and after the hybridisation
Pairs of chromosomes 1 following hybridisation from orangutan A (D) and B (E)

Table 40. Biotinylated PLB4 in man and the great apes

Species	No. of mitoses	Total no. of signals	Signals on chromosome 1 No (%)		Expected signals on chr.1	p
Man1	45	192	77	(40%)	12.26	<0.001
Gorilla	32	322	67	(21%)	25.63	<0.001
Chimpanzee	15	151	59	(39%)	12.91	<0.001
Orangutan	40	323	101	(31%)	*	*

Table 41 PLB4. Distribution of signal over chromosome 1

Species	Total	Signals on					
		1p36		1q12		1p12	
		No	(%)	No	(%)	No	(%)
Man[1]	77	45	(58%)	19	(25%)	3	(4%)
Gorilla	67	50	(75%)	3	(4%)	3	(5%)
Chimpanzee	59	31	(53%)	13	(22%)	8	(13%)
Orangutan	101	51	(50%)	15	(15%)	13	(13%)

[1] Data from hybridisation of PLB4 in man were taken from Boyd et al., 1989.

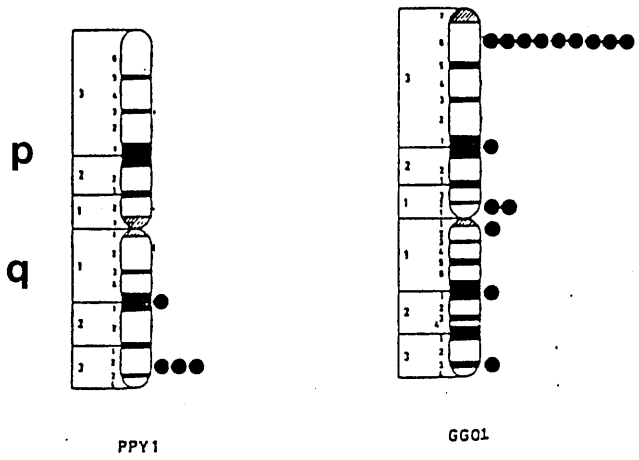
* Relative lengths for the orangutan chromosomes are not available therefore an Expected value cannot be calculated.

3.8.3 Comparative mapping of U1 small nuclear RNA

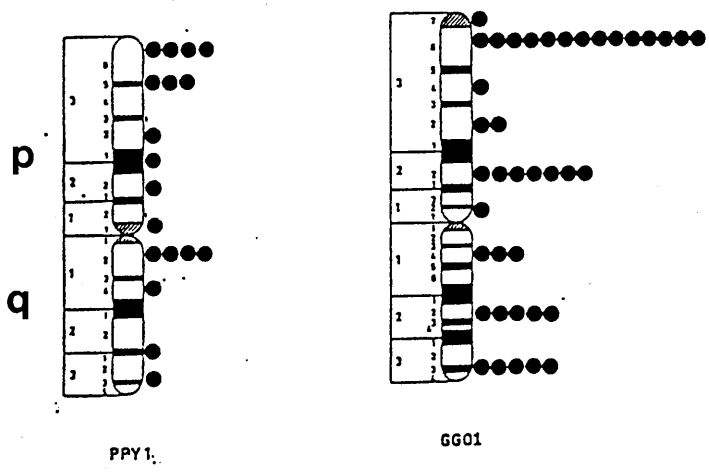
This probe contains a gene for human U1 small nuclear RNA (snRNA) and has been previously mapped to human chromosome 1p36 (Lund et al., 1983). It was biotinylated and hybridised with metaphase spreads from gorilla and orangutan at a concentration of 50ng per slide and 4 and a half hours incubation in chromogenic substrate. The preparations used were banded using the mild trypsin method as described in section 2.2.5, with 3 minutes in trypsin diluted 10^{-5}. When the prebanded metaphases were scored it became apparent that the signal present was weak and rare. Ten cells were scored in the orangutan and from 38 signal recorded, 4 (10%) were located on chromosome 1 which is insufficient for confident localisation. In the gorilla 15 cells were scored and from 119 signals recorded 16 (13%) were located on chromosome 1, with a peak of hybridisation at 1q36. The distribution of signal observed over PPY1 and GGO1 is presented in figure 54A.

The experiment was repeated on lipsol banded preparations of (female) gorilla and orangutan at an increased concentration of labelled DNA probe (75ng per slide) and two and a half hours incubation in chromogenic substrate. The metaphases were covered by hybridisation signal, possibly due to the high concentration of probe DNA used. In the orangutan 12 cells were scored and from a total of 235 signals was recorded, 21 (9%) signals were located on chromosome 1. The observation that the majority of cells had over 20 signals reduces the significance of the previous finding. The distribution of signal observed on PPY1 and GGO1 is illustrated in figure 54B.

A third study was carried out on lipsol banded preparations at a



A



B

Figure 54 Biotinylated U1 in the gorilla and orangutan. Distribution of hybridisation signal over chromosome 1. (A) 50 ng and (B) 75ng of labelled probe per slide.

concentration of 50ng per slide and 4 hours incubation in chromogenic substrate. Too many hybridisation signals were present on any metaphase spread or nuclei. This can not be due to the experimental conditions used, as in the positive control (hybridised with biotinylated GMGY10) specific hybridisation signal was obtained. Fifteen cells were scored in the orangutan and from 261 signal observed, 31 (12%) were located on chromosome 1. Thus no conclusion can be drawn from comparative studies using probe U1.

3.8.3 Comparative mapping of GMGY10

This sequence was shown by Southern analysis to be Y-specific in the great apes (K. Kwok, personal communication). As expected, no signal was detected in chromosomes or nuclei of the female gorilla, following in situ hybridisation with biotinylated GMGY10 (data not shown).

In the male gorilla and male orangutan, initial in situ hybridisation studies using biotinylated GMGY10 at a concentration of 1ng per slide and 1 and a half hours incubation in chromogenic substrate routinely used for this probe in humans, failed to reveal any hybridisation to chromosomes although signal was present in the interphase nuclei. The preparations used were banded using the mild trypsin method of Garson et al., (1987), described in section 2.2.5. Subsequent studies performed on lipsol banded preparations with the same amount of biotinylated DNA probe, revealed weak hybridisation signal on the Y chromosome of both species following 2-3 and a half hours in chromogenic substrate. Later experiments were performed at an increased concentration, 40ng biotinylated GMGY10 per slide and 4-5 hours in chromogenic substrate. The results are as follows:

In the gorilla, 42 cells were relocated after hybridisation and in 21 cells signal occurred on the Y chromosome. Figure 55 shows the distribution of hybridisation over the Y chromosome with 20 signals on the long arm and 3 signals on the short arm of the chromosome. The majority of the signal occurred at proximal long arm. Partial metaphases showing signal on the gorilla Y chromosome are shown in figure 56.

In the orangutan, specimen A, 39 cells were suitable for scoring and in 23 cells hybridisation signal was present on the Y chromosome. Figure 55B shows the distribution of hybridisation over the PPY Y with 21 signals on the short arm and 2 on the long arm. It is interesting to note that 14 signals (10% of the total) were observed on chromosome 1. The significance of this finding cannot be assessed statistically as relative lengths for the orangutan chromosomes are not available.

Metaphase spreads from orangutan B were hybridised with GMGY10, biotinylated in this particular study with Bio-11-dATP instead of Bio-11-dUTP which was used in all other biotinylated studies, as the latter was temporarily unobtainable. At a concentration of 15ng per slide and 2 and a half hours incubation in chromogenic substrate hybridisation signal was observed on the Y chromosome in every cell scored. Eleven cells were scored and from a total number of 28 signals recorded, 12 (43%) were located on the Y chromosome, 11 on the short and 1 on the long arm. In figure 57 partial metaphases from the orangutan are shown with hybridisation signal on the Y chromosome. In metaphase C signal is present on chromosome 1.

In the chimpanzee only a small number of cells was scored due to the poor quality of the available material as mentioned previously.

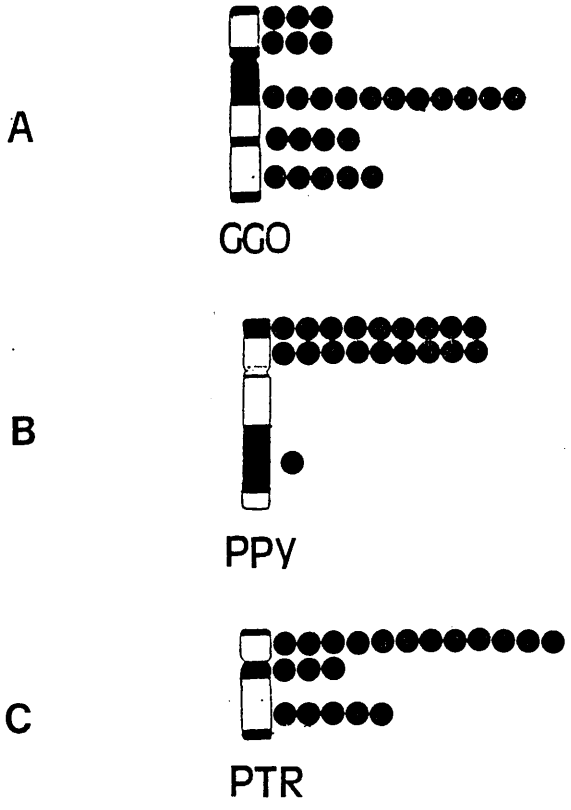


Figure 55 Distribution of hybridisation signal over the Y chromosome in the gorilla (A), orangutan (B) and chimpanzee (C) following hybridisation with biotinylated GMGY10.
For the diagrammatic representation of the great ape Y chromosome see section 4.4.2.I

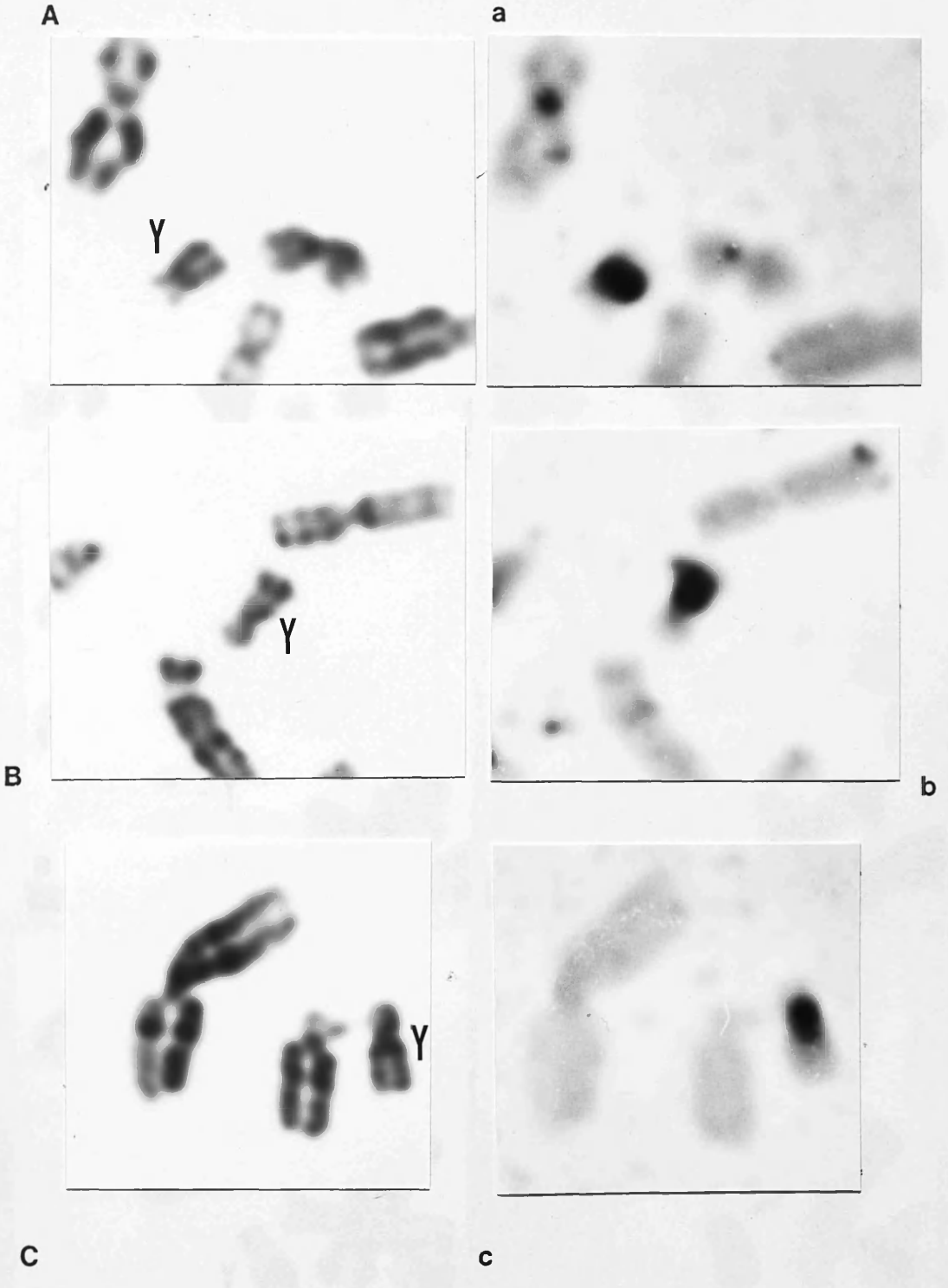


Figure 56 Partial metaphases from the male gorilla showing signal on the Y chromosome following hybridisation with biotinylated GMGY10.

A, B, C before and a, b, c after the hybridisation

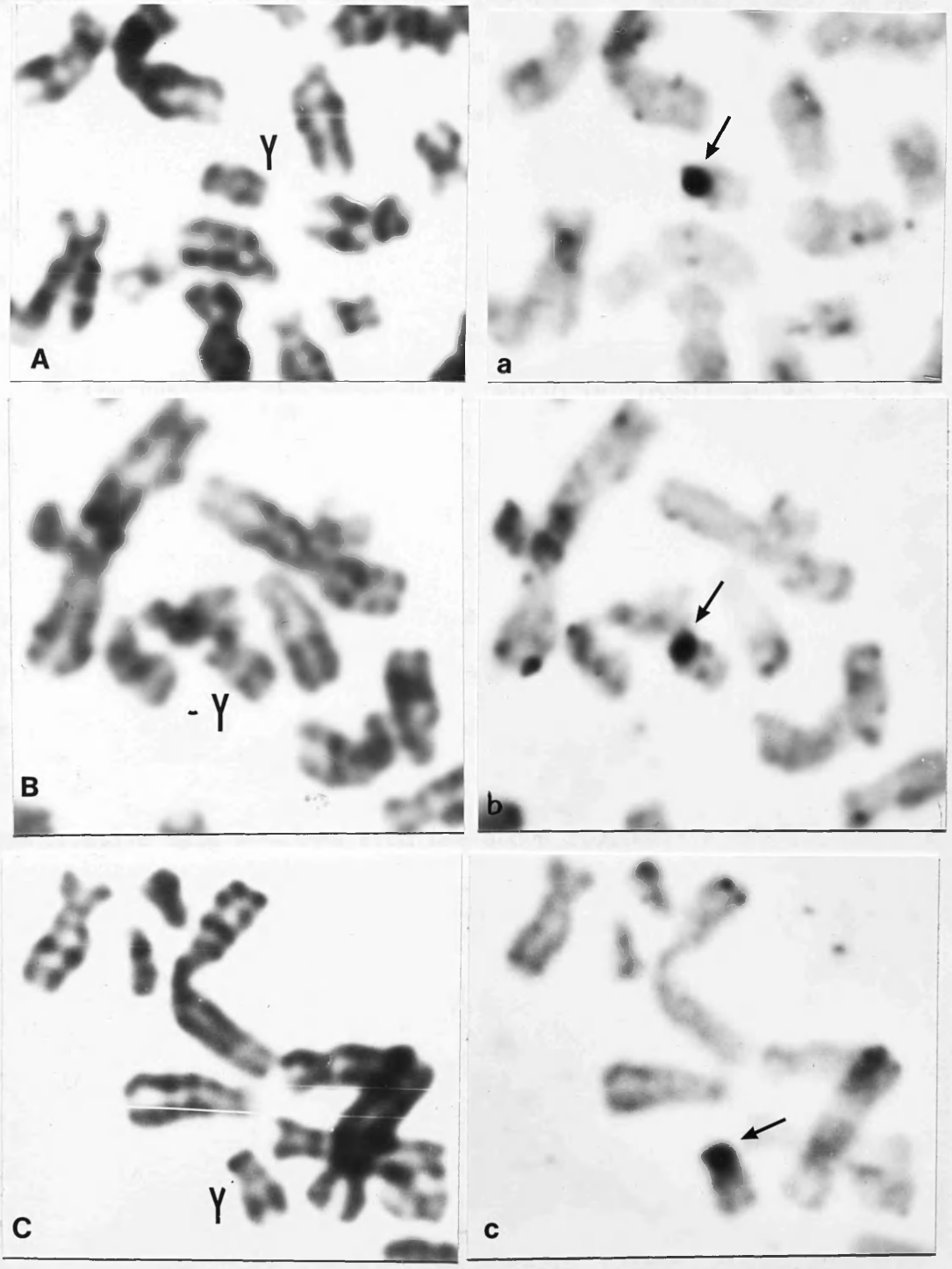


Figure 57 Partial metaphases from the orangutan showing signal on the Y chromosome following hybridisation with GMGY10.
A, B, C before and a, b, c after the hybridisation.

However, from 19 cells which were analysed, 17 showed signal on the Y chromosome. The small size of this chromosome together with the great intensity of the hybridisation signal did not permit a clear-cut localisation to either of the arms of the chromosome. As shown in figure 58 signal did occurred on both arms, and the suggestive distribution of grains given in figure 55C needs to be confirmed after scoring a larger number of cells.

On the basis of the above data, GMGY10 is localised to the Y chromosome of all three species studied, gorilla, chimpanzee and orangutan; localisation to a specific region was possible only in the gorilla and the orangutan. In the orangutan, GMGY10 is located at the the same region as in the human, the short arm of the Y chromosome, but not in the gorilla where it is located at proximal long arm, an interesting finding as orangutan is thought to be more distantly related to man than the gorilla. Table 42 summarises the quantitative data obtained from the above studies.

3.8.5 Comparative mapping of GMGY7

This sequence was shown to be Y-specific in the chimpanzee and orangutan but not in the gorilla (K. Kwok, personal communication). In situ hybridisation studies were performed only in the orangutan, as the chimpanzee line had by then completely deteriorated. Biotinylated GMGY7 was hybridised with metaphase spreads from orangutan B at a concentration of 15 and 25 ng per slide with two and a half hours incubation in chromogenic substrate. Twenty-three (23) cells were scored and in every cell there was signal on the short arm of the Y chromosome (figure 59). In some cases the signal was covering the whole short arm and part of the long arm. From a

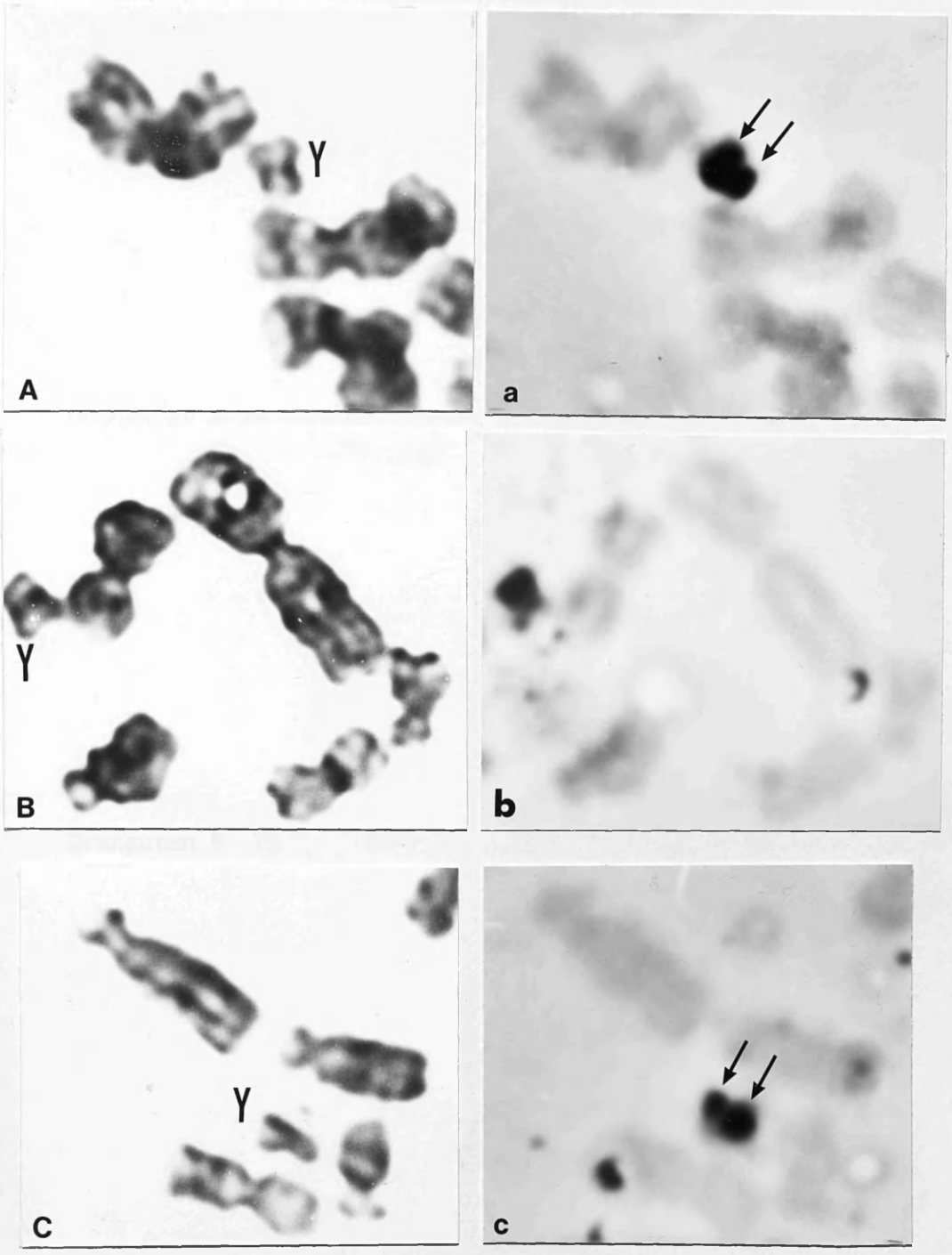


Figure 58 Partial metaphases from the chimpanzee showing hybridisation signal on the Y chromosome following hybridisation with biotinylated GMGY10. A, B, C, before and a, b, c after the hybridisation.

Table 42i: GMGY10 in the great apes.

Species	No. of mitoses scored	Mitoses with signal on Y	Total no. of signals	Signals on Y No. (%)
Gorilla	44	20	324	24 (7%)
Chimpanzee	19	17	94	24 (25%)
Orangutan A	39	23	134	20 (15%)
Orangutan B	11	11	28	12 (43%)

Table 42ii: GMGY7 in the orangutan

	No. of mitoses scored	Mitoses with signal on Y	Total no. of signals	Signals on Y No. (%)
Orangutan B	23	22	380	48 (13%)

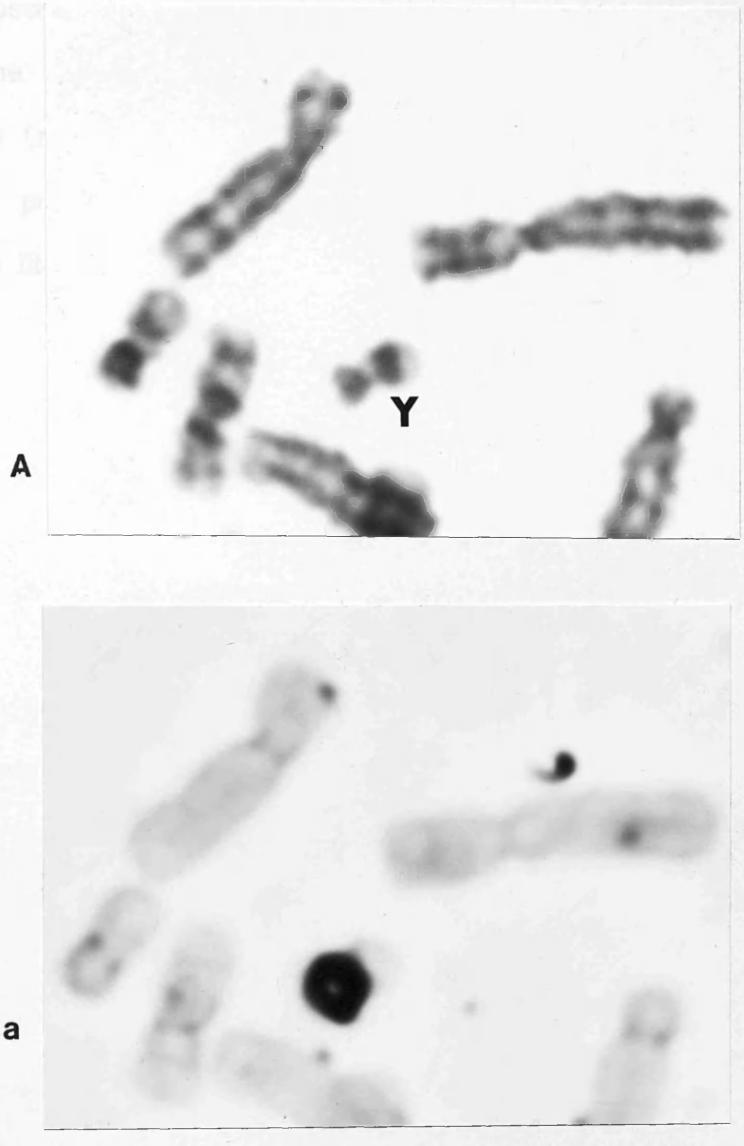


Figure 59 Partial metaphase from orangutan B showing signal on the Y chromosome following hybridisation with biotinylated GMGY7.
A before and a after the hybridisation.

total number of 380 signals recorded, 48 (13%) were located on the Y chromosome, 37 on the short and 11 on the long arm.

The finding that both GMGY10 and GMGY7 are located in the same region (short arm) of the Y chromosome in both the orangutan and the human provides further evidence for homology of human-orangutan Yp, at the DNA level.

CHAPTER FOUR

DISCUSSION

4.1 METHODOLOGY

The protocols for radioactive and biotinylated labelling used in this project are evaluated. Problems encountered with both approaches are presented and a comparison of the radioactive to the biotinylated approach is made. Developments in methodology that have been reported while this project was on are also presented.

4.1.1 The radioactive technique

Since first described and for nearly twenty years in situ hybridisation involved the use of radioactive isotopes for the labelling of DNA probes and autoradiography for the detection of the sites of hybridisation. This conventional in situ hybridisation using tritium labelling was the start-point of this project.

The main problems encountered here with this approach were high non-specific labelling and distortion of the chromosome morphology following hybridisation. Non-specific labelling, as mentioned in section 1.2.6 can be due to hybridisation between the exogenous DNA probe and chromosomal sequences of partial homology; this can be eliminated by increasing the stringency of the hybridisation reaction and/or the stringency of post hybridisation washes. Test experiments however demonstrated that most of the non-specific labelling observed here was of a different nature. Nuclear track emulsions consist of silver halide crystals suspended in a gelatin matrix. When these crystals are exposed to radiation either from a light source or from a radioactive source, or when they are subjected to mechanical friction, they become sensitised in such a way that they are reduced to metallic silver upon treatment with a

photographic developer (ILFORD, 1987). Thus if the emulsion has not been handled properly silver grains will be observed which do not represent hybridisation signal. This type of non-specific labelling can be monitored by taking slides along with the experimental ones that have no radioactive probe added to them. This can also be more readily demonstrated by using blank slides during autoradiography which are developed straight afterwards. Such control slides demonstrated that the major part of non-specific labelling obtained in this study was due to the handling of the emulsion during autoradiography. The dark-room used, designed for the purposes of printing photographs and developing films was not light-tight. Autoradiography and slide developing were later performed in complete darkness and the condition of the emulsion was monitored during each experiment. Following this, the background labelling was eliminated.

Another problem encountered with the radioactive procedure was distortion of chromosome morphology. Thus, although silver grains were present their location on the chromosomes could not be scored. The guidelines set by Donlon (1986) on which type of chromosomes are likely to survive the in situ hybridisation procedure described in section 2.2.3 were found to be extremely helpful. Additionally, the post-hybridisation washes of three rinses in 50% formamide/2xSSC at 45°C would have a milder effect on chromosome morphology than the initially used washes in 0.1xSSC at the high temperature of 65°C.

4.1.2 The biotinylated technique

As outlined in section 1.4.3 several protocols have been described for biotinylated in situ hybridisation in order to overcome the disadvantages associated with the use of isotopes and autoradiography. The possibility of using biotinylated probes was explored during the course of this project. The protocol used has been described by Garson et al., (1987) ; it was chosen because the visualisation of the hybridisation signal takes place under phase microscope which was available in this department and because of the simplicity of the detection procedure which involves one enzymatic step unlike other described methods (Burns et al., 1985) which rely on complex silver enhancement techniques. Moreover, the sensitivity of this protocol to localise a 1kb single-copy sequence was the highest amongst those reported.

The reproducibility of the method was assessed using the repetitive DNA probe GMGY10. Despite the fact that all previous reports had emphasized the convenience and speed with which results are obtained using biotin labelling (Garson et al., 1987; Manuelidis et al., 1982; Brigati et al., 1983) it was still difficult to believe that 24 hours after starting the hybridisation strong signal was present on the Y chromosome, bearing in mind that the equivalent result with the radioactive approach was obtained after 6 days exposure. Positive signal on the short arm of the Y chromosome was obtained consistently with as little DNA as 1 ng per slide. Positive results were obtained for another repetitive sequence, GMGY7, and a single-copy sequence, p72, isolated from a chromosome 21 library (see section 3.3 and below). Positive signal for this single-copy chromosome 21-specific probe was obtained with only 25ng of labelled

probe although Garson et al.,(1987) have used 100ng of labelled probe per slide for single-copy sequences. The high topological resolution of biotinylated in situ hybridisation was demonstrated by the presence of distinct signal on each chromatid on chromosome 21 with probe p72 (section 3.4.1) and chromosome 1 of the great apes with probe PLB4 (section 3.8.2). Other investigators that use biotinylated in situ hybridisation have made a similar observation (Ambros et al., 1986; Pinkel et al., 1986).

The only problem noted with this approach was that as the hybridisation signal is visualised under phase microscopy, it can be obscured by any phase-dark object. It is therefore critical that the chromosomal preparations are checked under phase contrast microscopy before each experiment to ensure that they are free from dust or stain debris. As mentioned in section 3.3.1, one of the components of the detection kit was found to contribute to the "dirty" appearance of the metaphases after hybridisation. The problem was eliminated by storing this reagent at -20^{o}C.

The studies using biotin-labelling performed in this project confirmed that the method used described by Garson et al., (1987) is simple, rapid, of high resolution and sensitive enough to localise single-copy sequences.

4.1.3 Tritiated versus biotinylated in situ hybridisation

The two approaches cannot be directly compared on the basis of the results obtained in this project as both approaches were used only for probes GMGY10 and GMGY7. However, a few observations are noteworthy.

The resolution of signal and overall efficiency of detection

obtained with the biotinylated technique should theoretically be an improvement on that obtained with radioactively labelled probes, because detection occurs directly at the site of hybridisation without the complications of an emulsion layer and electron track lengths. This expected higher efficiency of the biotinylated approach was demonstrated in the present study from the hybridisation of GMGY10 in the 45,X/46,X+mar male, DG (see section 3.5.2). The observed efficiency was 60% with the biotinylated method as compared to 10% and 20% of two studies using radioactive in situ hybridisation.

The high topographical resolution of the biotinylated approach was evident in the XX male DR studied with biotinylated and radioactively labelled GMGY10 (section 3.5.1); although signal was present at distal short arm of the X chromosome with both approaches, the signal obtained with the biotin labelling was confined to band Xp22.3 whereas with tritium-labelling silver grains were located not only within this band but were also scattered around this area of the X chromosome. It should be mentioned however that the resolution obtained with the biotinylated approach for the repetitive probes GMGY10 and GMGY7 was poorer than that of the radioactive, as with the former the signal was intense, covering the whole short arm of the Y chromosome. This was observed in a normal male (section 3.3.1), in the chimpanzee and orangutan (section 3.8.4), and in DG, the 45,X male with the tiny marker chromosome (3.5.2). As this intense signal was obtained following 90 minutes incubation in chromogenic substrate it is possible that shorter incubation might allow finer localisation of the above probes within the short arm of the Y chromosome.

The biotinylated approach used in this project is definitely preferable to the radioactive in terms of speed and convenience of performance. This method in now being used routinely in this department for the localisation of several DNA sequences. Using this approach a 2.4kb fragment containing a glutamic acid transfer RNA has been assigned to chromosome 1p36 (Boyd et al., 1989); the gene for the human glucocorticoid receptor previously mapped to chromosome 5 was further localised to 5q31 using a 4.3kb cDNA probe (Theriault et al., 1989); the human mineralocorticoid receptor gene was assigned to 4q31.1 using a 3.7kb probe (Morrison et al., 1989a); and finally a tRNA gene cluster was assigned to 17p12-17pter using a 4.2kb DNA fragment (Morrison et al., 1989b).

The reported sensitivity of this method is at the range of 1kb (Garson et al., 1987). Although the authors have suggested that unique sequences even smaller than 1kb may be detectable, this remains to be established. With biotin labelling but a peroxidase detection system a 800bp unique sequence has been localised successfully (S. Habeebu, personal communication). Thus it seems that the detection limits of non-isotopic in situ hybridisation is nearing that of high specific activity tritiated probes which can detect as little as 0.5kb of target sequence (Jhanwar et al., 1983).

There has been little attempt to compare systematically the efficiency and the specificity of isotopic and non-isotopic procedures. Mitchell et al., (1986) compared ^{3}H- and N-ACO-AAF labelling for their ability to detect and resolve human DNA transgenomes in transformed cells and also for mapping the site of SV40 integration on the human chromosome 7 of the human-mouse hybrid line C121. This hybrid contains human chromosome 7 as the only human

chromosome but in variable numbers in any one cell. Both techniques detected every chromosome 7 in each metaphase spread but N-AcO-AAF gave finer resolution. On the other hand, tritium labelling was more sensitive as it allowed sublocalisation of the SV40 integration site on the human chromosome 7 (band q31-35) whereas the N-AcO-AAF modified DNA probe did not give convincing site-specific signal. Ambros et al., (1986) used both tritium and biotin labelling to localise a 17kb unique sequence in plant chromosomes. Although the same results were obtained with either tritiated or biotinylated probes, the authors noted that the biotin-labelled probes were clearly superior to tritium-labelled probes; results were obtained within 14h after the start of the experiment, as opposed to 4-8 weeks with tritiated probes and allow more detailed information about chromosome organisation due to the increased resolving power. It should be noted that these reports provide qualitative information on the two systems, radioactive and non-radioactive. Differences between the two systems have not been quantitated. In a recent paper (Boyd et al., 1989) where both tritium and biotin-labelling have been used for the localisation of a human $tRNA^{Glu}$ gene it can be seen that although significant hybridisation was documented at chromosome 1p36 with both approaches, a secondary site of hybridisation (1q21-22) was found to be statistically significant only with the biotinylated method. Although this point was not discussed by the authors it does indicate a higher specificity for the biotinylated approach.

When the cost of each approach is compared it can be clearly seen that the most expensive material in both approaches is the tritiated-nucleotide (prices 1987, in sterling pounds):

	Biotin	Radioactive
Labelled nucleotide	57.80 (125µl)	125.00 (250µl)
Nick translation	65	75
Detection	65	56.95

According to the protocols used in this project, 250µ l of radiosotope, will be sufficient to label 5µg of DNA probe (five nick-translation reactions, 1µg of DNA each). For a repetitive probe such as GMGY10, this amount of radioactively labelled DNA can be hybridised with 125 slides (at a concentration of 20ng per slide). In contrast, 125µ l biotinylated nucleotide will be sufficient to label 50µg of DNA probe (50 nick-translation reactions, 1µg of DNA each) which can be used for hybridisation with 50,000 slides (at a concentration of 1ng per slide). The difference is so great that on the basis of the cost alone, biotin labelling should be the method of choice.

Another advantage of non-isotopic methods of labelling is that the labelled probes are not subject to decay of label as do the radioactively labelled probes. However this was not found to be a problem during this project as radioactively labelled probe was used 1 year later and still gave reproducible results (section 3.6).

Non-isotopic procedures were until recently regarded as a welcome development that did however require further improvement before it could be applied routinely to the localisation of unique sequences as it was not as yet as sensitive as the autoradiographic technique (Buckle & Craig, 1986). As noted in the Introduction, section 1.7, the sensitivity of the described non-isotopic procedures was in the vicinity of 20kb (Pinkel et al., 1986;

Manuelidis & Ward 1984). Improvement of sensitivity could be envisaged at the level of the hybridisation reaction, the immunological detection system and the microscope. Reflection contrast microscopy, which allows the visualisation of reflection rather than absortion of incident light by the dye precipitate, was shown to be more sensitive than absorption or fluorescence microscopy (Landegent et al., 1985a). This type of microscope however is very expensive and not readily available. Other investigators resorted to complex signal amplification techniques i.e. gold/silver amplification of DAB products (Burns et al.,1985a). In contrast, the technique of Garson et al., (1987) for biotin labelling which used in this project, despite its simplicity is efficiently sensitive for the localisation of single-copy sequences and it is predicted that with this increased sensitivity, biotinylated and in general non-isotopic in situ hybridisation will eventually completely replace conventional radioactive in situ hybridisation both for the purposes of gene mapping and molecular diagnosis.

4.1.4 The banding method and the age of the chromosomal preparations

The method for identification of chromosomes used throughout was lipsol banding before hybridisation. As argued by other investigators (Lawrie & Gosden, 1980; Buckle & Craig 1986) such a method is indeed time-consuming but it was the method with which the author had become proficient in analysis.

Lipsol banding works only on freshly made slides (Malcolm 1986; Elliot, 1980; own observations) but this generally did not pose a

problem for the purposes of human gene mapping studies performed here, as freshly prepared slides were available. For the purposes of comparative mapping, the mild trypsinisation method of Garson et al (1987) was attempted. As reported in section 3.6.4 no hybridisation signal was observed in such trypsinised preparations although signal was present in subsequent studies on lipsol banded preparations. On the basis of this observation, it appears that the former method interfered with the hybridisation.

Most of the results obtained with the biotinylated approach, were on freshly prepared chromosomal preparations. In one instance (hybridisation of biotinylated GMGY7 in XO male RW, section 3.5.2) where two year old slides had to be used, no signal was observed on the metaphase chromosomes although signal was present in the interphase nuclei. Thus it can be argued that the chromosomal DNA was degraded during storage because of which hybridisation did not occur between the chromosomes and the probe, whereas in the interphase nuclei where the chromatin is protected by the nuclear matrix, the DNA was intact and hybridisation took place. A recent report of degradation of chromosomal DNA as a result of ageing by Mezzanotte et al., (1988) supports this notion. Contrary to this are reports of efficient detection on slides stored for over five years (Kozma & Adinolfi, 1988) .

Using GMGY10 in a slide stored for three year (data not shown) signal was observed only in a few metaphases (3 out of 25 scored). Thus it seems that the efficiency of detection depends largely on the quality of the metaphase spreads which may differ within the same slide and further work is needed to clarify this point. For purposes of molecular pathology and diagnosis the only available

preparations are often archive material. Therefore it would be essential to establish a technique that permits efficient detection in these cases.

4.2 Recent developments of in situ hybridisation

4.2.1 Choice of probes

The most commonly used probes for in situ hybridisation are recombinant DNA fragments, either complementary DNA or genomic DNA. All probes used in this project were DNA sequences isolated randomly from chromosome specific libraries. Recently the use of cosmid clones and chromosome libraries as probes for in situ hybridisation has been reported.

One of the approaches used to bypass the problem of low sensitivity of the non-radioactive methods in detecting small single copy sequence was to use large clones (cosmid clones 40-50kb long) containing the single copy sequence of interest. As 20-30% of the human genome consists of repeats, large genomic probes cannot be used for in situ hybridisation without precautions as the signal attributed to repetitive elements will mask the signal from the single copy sequence. Landegent et al., (1987) described a method of using several AAF-modified cosmids of the human thyroglobulin gene, Tg, previously mapped to chromosome 8q24 (Landegent et al., 1985b). The contribution of repetitive sequences to the hybridisation signal was eliminated through the addition of competitor DNA in analogy to the procedure used in Southern blot hybridisation (Brison et al., 1982; Fisher et al., 1984). As competitor DNA, an enriched fraction for highly repeated sequences was used -Cot-1 DNA-, added simultaneously with the probe. By using two cosmid clones (total

70-80kb of DNA) specific labelling could be seen at the q telomeres of both chromosomes 8. The signal was less intense when only one cosmid was used, became stronger when three or four cosmids were used but the overall background also increased.

When compared with single-copy probes, this strategy did have the disadvantage of slightly more background and reduced sensitivity since it necessitated 70-80kb of probe DNA as opposed to 20kb when unique probes were used (Landegent et al., 1985b).

The use of entire chromosome-specific libraries as probes has been recently reported independently by two groups (Lichter et al., 1988; Pinkel et al., 1988) for visualizing entire individual human chromosomes both in metaphase spreads and interphase nuclei. Lichter et al., (1988) called their method "chromosomal in situ suppression hybridisation (CISS)" because prior to hybridisation with chromosomal preparations, a preannealing step with total human DNA was included, in order to suppress hybridisation signals from ubiquitous repeated sequences present in the chromosome-libraries such as the Alu and the KpnI elements. Commercially available libraries for chromosomes 1, 4, 7, 8, 13, 14, 18, 20, 21, 22 and X labelled with biotin were assessed for their specificity by means of a computer-assisted method of quantitative densitometry. It was demonstrated that the majority of highly repetitive DNA sequences can be sufficiently suppressed to achieve chromosome-specific labelling both in metaphase spreads and interphase nuclei and that under optimal reannealing conditions (100-200μ g/ml of human competitor DNA, 10-20 minutes renaturation time) a 8:1 signal-to-noise ratio was observed. In the absence of human competitor DNA the signal showed little chromosomal specificity. The

best specificity, in other words no significant cross-hybridisation to other chromosomes, was achieved with libraries for chromosomes 4, 7, 8, 18, 20 and X. The library exhibiting the lowest specificity was the chromosome 13 library with multiple minor binding sites on other chromosomes as well as an exceptionally bright staining of Yq12 that could not be eliminated despite testing of all experimental parameters. In an accompanying paper (Cremer et al., 1988) the utility of this technique to detect numerical changes, deletions and rearrangements of chromosomes 1, 4, 7, 18 and 22 in highly aneuploid tumour cells was demonstrated.

A similar strategy, referred to as "chromosome painting" because at the end of the procedure the target chromosome is specifically stained with hybridisation signal, was reported by Pinkel et al., (1988). They used libraries specific for chromosomes 4 and 21 and human genomic DNA as the competitor. The target chromosome could be made 20 times brighter per unit length than the others. Data were also presented on hybridisation with collections of unique chromosomes from chromosome 4. Using three unique probes the observed hybridisation efficiency was 50% and the hybridisation specificity 50%. When 120 unique sequences were used as a probe, the hybridisation efficiency was 15% and the specificity 27%. Very exciting was the report of detection of trisomy 21 and translocations involving chromosome 4 in metaphase spreads and interphase nuclei. When a leukaemia cell line carrying the translocation t(4;11) was screened with a chromosome 4 specific library approximately half of the nuclei showed three brightly fluorescent domains, presumably produced by the two portions of the involved chromosome 4 and the intact normal chromosome (Pinkel et al., 1988).

Apart from its obvious applications in the detection of chromosome aberrations and the investigation of the organisation of chromosomal DNA in the interphase nuclei, in situ hybridisation with chromosome-specific libraries will also provide another approach to the investigation of genome organisation and evolution in phylogenetically related species.

4.2.2 New methods for non-radioactive labelling

In addition to all the labelling systems outlined in the Introduction (section 1.4) new methods have been recently described. Bromodeoxyuridine (BrdUrd) is a thymidine analogue often used as a substitute for ^{3}H-thymidine in in vitro or in vivo proliferation assays. BrdUrd-labelled DNA probes were used successfully to detect viral DNA in tissue sections (Niedobitek et al., 1988) and satellite DNA sequences on chromosomes (Frommer et al., 1988). In these studies probes were labelled with BrdUTP by nick translation and an in vitro DNA polymerase I reaction. Kitazawa et al., (1989) described in vivo BrdUrd labelling and restriction endonuclease digestion to develop BrdUrd-incorporated DNA probes of defined length. BrdUrd was incorporated into plasmid DNA by inoculating E. coli with culture medium containing 500mg/L of BrdUrd. Probes were then used to detect c-myc mRNA in leukemic cell lines. Hybridised probes were detected with fluorescent microscopy using a FITC-conjugated monoclonal anti-BrdUrd antibody. The sensitivity of this method was however low.

Heiles et al., (1988) reported the use of DNA labelled by means of the random priming method with digoxigenin-UTP, a deoxyuridine triphosphate linked with the steroid

hapten digoxigenin via a chain several C-atoms long for demonstrating human papillomaviruses (HPV) DNA in routinely treated smears of uterine cervix. Hybrids were detected with polyclonal sheep anti-digoxigenin-Fab fragments conjugated to alkaline phosphatase through the formation of a coloured precipitate (NBT/BCIP). The technique sensitive enough to detect even single HPV genome copies, enabled a direct comparison between the hybridisation result and the morphological alterations in the cells thus provided the conditions for a wide use in routine diagnosis.

Additionally, DNA modification procedures based on the bisulphite catalized transamination of cytosine residues have been described. The labelling is accomplished by inserting an antigenic sulphone group into cytosine moieties of single stranded DNA (Sverdlov et al., 1974). The cytosines are sulfonated at carbon 6 by sodium bisulfite at high molarity. Detection of the chemically modified DNA is carried out by a sandwich immunoenzymatic reaction. A monoclonal antibody specifically recognises the sulphone groups on the modified DNA. An enzyme-anti-immunoglobulin antibody conjugate then binds to the monoclonal antibody. The enzyme converts a soluble chromogenic substrate system (NBT-BCIP) into an insoluble dye which precipitates at the exact location of the immune reaction. The coloured product indicates the presence of sulfonated DNA. Sulfonated probes were used in the detection of amylase mRNA using mRNA-DNA in situ hybridisation on sections of human pancreas (Morimoto et al., 1987).

4.2.3 Multiple-label in situ hybridisation

Double labelling and colour microradioautography were used by Haase et al (1985) to identify simultaneously different genes in individual cells. The method was based on the unequal penetration of ^{3}H and ^{35}S into two layers of nuclear track emulsion separated by a thin barrier film (see figure 60). Hybridisation of a ^{35}S-labelled probe specific for one kind of gene results in silver grains in both layers of emulsion whereas the ^{3}H-labelled probe produces silver grains only in the first layer. Silver grains are converted to magenta-coloured grains in the first layer and to cyan-coloured grains in the second with a method based on the principles of developing colour films. Although the technique offers new opportunities to examine gene expression at the single cell level it is a complicated approach if quantitative data are required.

Hopman et al., (1986) described a similar approach but with non-radioactive in situ hybridisation. They simultaneously hybridised mercurated total human DNA and a biotinylated mouse satellite DNA probe to metaphases and interphase nuclei of a human-mouse somatic cell hybrid. The probes were detected immunocytochemically using two different affinity systems, FITC and TRITC fluorescence, or blue (alkaline phosphatase) and brown (peroxidase) precipitated enzyme products. In some metaphase human chromosomes which had integrated mouse DNA were observed. In interphase nuclei the mouse satellite was clearly separated from the human chromosomes. Even a rearrangement between mouse and human chromosomes could be seen which probably would have not been detected with traditional G-11 staining.

Double label hybridisation could be useful in the study of the

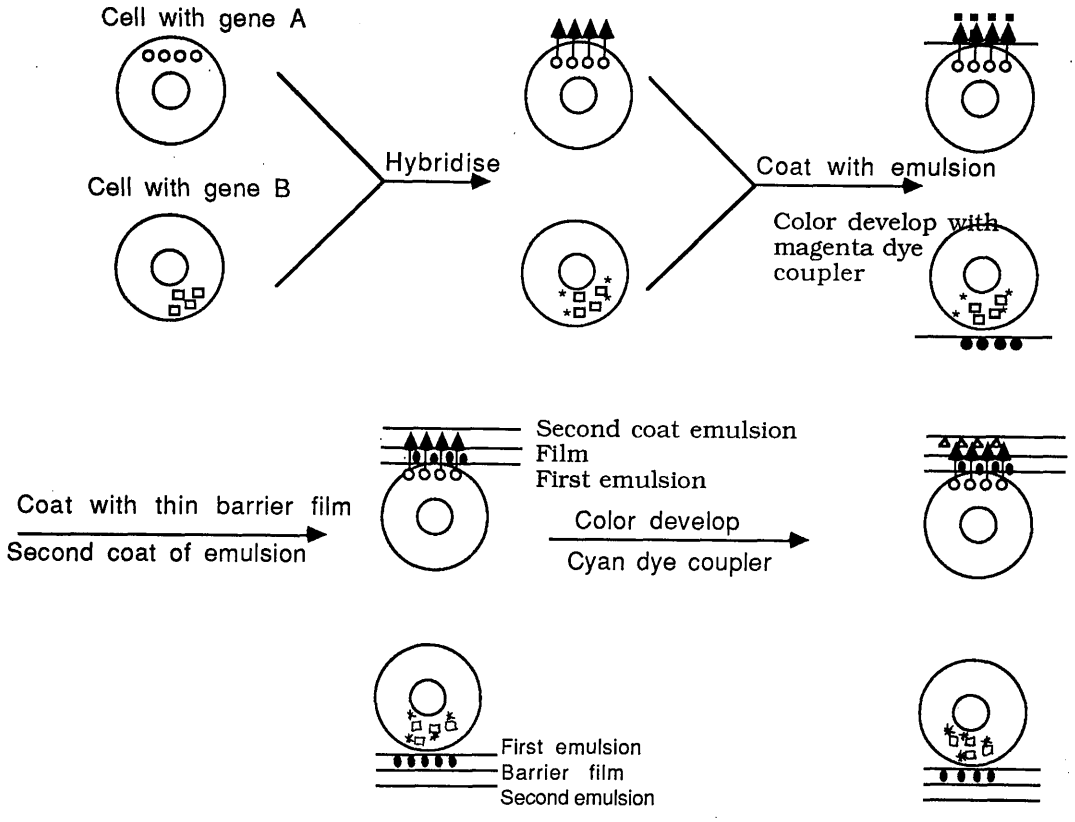


Figure 60

Principles and major steps of double-label hybridisation in situ and color radioautography. Cells with gene A (o) are hybridised to a probe labeled with ^{35}S (▲) cells with gene B (□) are hybridised to a probe labeled with ^{3}H (*). After slides are coated with nuclear track emulsion, the grains in the first layer are color-developed with a magenta dye coupler (●). A thin barrier film and a second coat of emulsion are applied and color developed with a cyan dye coupler (△).
Taken from Haase et. al., 1985.

three dimensional topography of two genes in interphase nuclei, the screening of viral co-infections and investigation of the relative transcription of separate genes in the same cell by detection of multiple mRNAs. Cremer et al., (1988) used a biotinylated chromosome 7 library detected with avidin-FITC and an AAF-modified chromosome 7 alphoid probe detected with TRITC in double CISS hybridisations to define aberrant chromosomes in two glioma cells. In TC 593 cells, five chromosomes entirely decorated with chromosome 7 inserts were regularly observed. Four of these that appeared to represent complete number 7 chromosomes were labelled with the alphoid 7 probe whereas the one that was smaller and metacentric, possibly an iso(7p) was negative for the alphoid probe, suggesting that it did not have a chromosome 7 centromere.

Recently the simultaneous detection of three nucleic acid sequences has been reported using a three-colour fluorescence in situ hybridisation (Nederlof et al., 1989). Three chromosome-specific repetitive probes labelled with either aminoacetylfluorene (AAF), mercury or biotin, were hybridised simultaneously to metaphase chromosomes or interphase nuclei. They were detected using a new blue immunofluorescent label, amino methyl coumarin acetic acid (AMCA) in combination with green and red fluorescing FITC and TRITC. A problem with this technique is that neither propidium iodine nor DAPI can be used for identification of metaphase chromosomes as they both interfere with either the specific TRITC signal or the AMCA signal. However, metaphase chromosomes were faintly visible due to background and autofluorescence of the preparations. An additional problem is that the vector sequences of the different probes will cross-hybridise

and because of the network formation at the hybridisation site especially in the presence of dextran sulphate, mixing of immunofluorescence colours can occur. This problem can be avoided by using inserts or single stranded DNA and RNA probes; for repetitive sequences the use of dextran sulphate is not mandatory. Three-colour in situ hybridisation was applied to the study of numerical chromosome abnormalities in an ovarian solid tumour and showed a high degree of heterogeneity in DNA content. Since chromosomes 13, 18 and 21 are the main autosomes involved in viable postnatal trisomy syndromes, screening for those chromosomes in one triple in situ hybridisation will deal with the prenatal diagnosis of most of the numerical chromosome aberrations. However, before this is extended to routine prenatal diagnosis further studies are required for a reliable quantification of the in situ hybridisation signals as a considerable internuclear variation has been observed (Nederlof et al, 1989).

That the above developments concern non-radioactive procedures is indicative of the amount of research and effort that is being put in this field the last few years due to their increased popularity. In particular, the application of nonradioactive in situ hybridisation with chromosome-specific probes for cytogenetic analysis has increased significantly in recent years. This has been due to the technical convenience of these procedures and to the increased availability of single copy and repetitive probes that hybridise intensely and specifically to selected chromosomes. Such probes are now available for over half of the human chromosomes (Pinkel et al., 1988) and allow identification of all chromosome aberrations.

Beside the well known advantages of nonradioactive in situ hybridisation procedures, such as excellent topological resolution, short processing times and stability of probes, the possibility to detect multiple target sequences simultaneously is very attractive. Simultaneous multiple target detection may give more information than would be obtained by separate single hybridisations in the fields of cytogenetics, oncology and cell biology.

4.3 HUMAN GENE MAPPING BY IN SITU HYBRIDISATION

This section discusses the results from human mapping studies carried out during this project. Emphasis is placed on the homology between the short arms of the Y chromosome and chromosome 9 revealed by probe GMGY3, and on the homology to extensive chromosome regions demonstrated by probes GMGY4b, JG73, JG51. Results obtained from the investigation of cases with sex chromosome aberrations (XX males and XO males) are discussed. The applicability of in situ hybridisation in clinical diagnosis is assessed taking the identification of a Y:15 translocation as an example.

4.3.1 DNA sequences that were mapped to one or two chromosome regions

Probes studied in this project were derived either from the Y chromosome (GMGY10, GMGY7, pY3.4, GMGY3, GMGY4b, GMGXY8) or chromosome 21 (p72, JG73, JG51). On the basis of the in situ hybridisation data these probes fall into two categories; those that showed homology to one or two chromosome sites (GMGY10, GMGY7, pY3.4, P2F2, GMGY3, GMGXY8 and p72) and those that showed homology

with several chromosomes (GMGY4b, JG73 and JG51).

Both Y specific sequences GMGY10 and GMGY7 map to the short arm of the Y chromosome, as shown with both radioactive and biotinylated in situ hybridisation (sections 3.2.1 and 3.3.1). Sub-localisation within the short arm of the above sequences was not possible with the biotinylated approach, because, as already mentioned, the whole short arm of the Y chromosome was covered by hybridisation signal. The radioactive approach allowed localisation of these sequences to region Yp11.2-pter. However, their precise localisation within this region may differ but this could not be detected by in situ hybridisation as the short arm of the Y chromosome is small (1 to $2x10^4$ kb of DNA) and difficult to subdivide accurately by cytogenetic analysis. The distribution of silver grains for GMGY10 suggested a proximal localisation as the heaviest signal was observed in the proximal band Yp11.2; for GMGY7 equal number of grains occurred on proximal and distal short arm (bands Yp11.2 and Yp11.3). Using a Y deletion panel constructed from individuals carrying structural abnormalities of the Y chromosome Affara et al., (1986a) assigned the above sequences to the region between Ycen-Yp11.2. Both probes recognised several Y-specific fragments on Southern blots but it is unclear if all fragments are clustered in the same area of the short arm or they are interspersed throughout the short arm.

The in situ hybridisation quantitative data reported in section 3.6 with probe pY3.4 in a male with a 15p+ polymorphism are in good agreement with those reported by Alitalo et al., (1988) following in situ hybridisation of this probe with a 46,XY,15p+ individual (see table 43). There is however one difference: Alitalo et al., (1988)

	No. of mito-ses	Total no. of grains on all chromo-somes	Grains on		
			Y	15p+	9
			No. (%)	No. (%)	No. (%)
Andersson et al. (1988)	51	295	80 (27%)	58 (20%)	49 (17%)
This study	47	391	98 (25%)	67 (17%)	5 (1%)

Table 43 Grain counts obtained by in situ hybridisation on 46,XY,15p+ individuals investigated with probe pY3.4

reported that 17% of the total signal was located on the pericentromeric region of chromosome 9 whereas in this study no hybridisation was observed with this chromosome in three independent experiments. While pY3.4 is itself from the Y chromosome, it contains sequences that cross-react with DNA from autosomes. Burk et al., (1985) have localised the major autosomal regions of the 3.4 HaeIII Y-repeat to chromosomes 1, 9, 15, 16, 21 and 22. It should be noted that in the case of highly repetitive DNA sequences like pY3.4, different conditions of stringency will produce quite different hybridisation results. The stringency of the in situ hybridisation washes performed by Alitalo et al., (1988) although not described in detail must be similar to the ones performed in this project as both were performed according to the protocol of Harper & Saunders (1981). On the other hand great inter-individual variability has been documented cytogenetically for the heterochromatic region of chromosome 9 (ISCN 1985) which might account for presence of absence of pY3.4 homologous sequences on this chromosome in different individuals.

The findings reported here and these by Alitalo et al., (1988) and Burk et al., (1985) demonstrate that pY3.4 is distributed throughout the length of the long arm of the Y chromosome. However, as shown in figure 61, in this project and the study of Alitalo et al., (1988), a significant concentration of silver grains was recorded at the end of the long arm of the Y chromosome whereas Burk et al., (1985) reported most silver grains in the proximal part of the fluorescent region of the Y chromosome .

In situ hybridisation data from the hybridisation of P2F2 in a

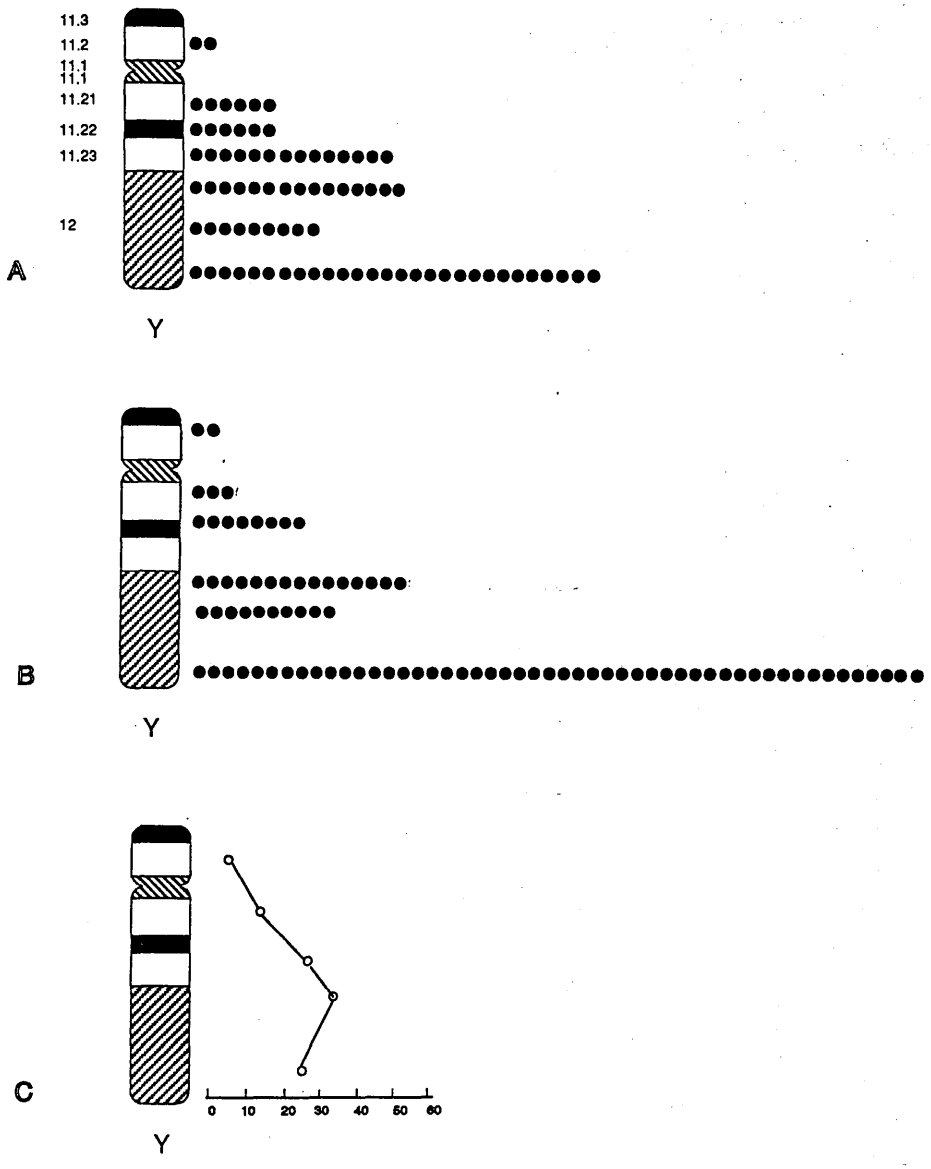


Figure 61

Distribution of 3.4kb repeat on the human Y chromosome

A. Alitalo et al. (1988)
B. This study
C. Burk et al. (1985)

normal female, in the XX male KS, and in the XO male RW demonstrated that this sequence maps to the X chromosome, band Xq21. This sequence recognises on Southern blots one X-specific and a Y-specific fragment which have been assigned to Xq13-Xq24 and Yp11.2-Ypter respectively, using an X panel of somatic cell hybrids (Affara et al., 1986a). Thus, in situ hybridisation findings confirmed the assignments to the X chromosome and allowed a more precise localisation to a single chromosome band.

One peculiarity was observed when the above probe was hybridised with normal male chromosomes. In all cells scored hybridisation occurred either on the X chromosome or on the Y and in no metaphase spread it occurred on both chromosome sites. The distribution of signal over the Y short arm reveals a distal location band in p11.3. although according to molecular analysis data presented by Affara et al., (1986a, 1986b, 1987) P2F2 appears to be one of the proximal sequences As mentioned previously, the small size of the Y short arm makes accurate localisation by in situ hybridisation within this region difficult.

Another X and Y specific sequence, GMGXY8 was shown by biotinylated in situ hybridisation to be located on the Y short arm. Molecular analysis has shown that this probe recognises X and Y-linked fragments which were assigned to Xq13-q24 and Yp respectively. The absence of hybridisation with the X chromosome in the in situ hybridisation studies is difficult to explain; the same result was observed consistently in three independent experiments. In all studies metaphase spreads were obtained from the same male individual, thus a possible deletion on the respective region of the X chromosome of this individual could account for this discrepancy

between in situ hybridisation and molecular findings. No deletion was however visible cytogenetically in lipsol banded preparations. Another explanation is that the respective sequence on the X chromosome shows less homology to the probe and does not hybridise at the stringencies of in situ hybridisation. Magenis et al., (1987b) explained in the same way why their in situ hybridisation studies failed to demonstrate hybridisation to any autosome for a probe (p50f2) known from molecular analysis to show such homology.

Finally, using biotin labelling DNA probe p72 was assigned to chromosome 21 distal band 21q21. This is in agreement with previous findings from Southern analysis using a panel of somatic cell hybrids containing different parts of chromosome 21 which localised this probe to chromosome 21 band q21 (Galt, 1988). In situ hybridisation results were more informative than Southern analysis in that they revealed a distal location of the probe within this band. The chromosome localisation of p72 as already mentioned clearly demonstrates that the biotinylated method of Garson et al., (1987) used in this project is sensitive enough to detect single copy DNA sequences.

4,3.2 Homology between the distal short arm of the Y chromosome and the distal short arm of chromosome 9.

GMGY3 was localised during this project to the distal short arm of the Y chromosome (band Y11.2-ter) and the distal short arm of chromosome 9 (9p22.3-pter) by radioactive in situ hybridisation. The

localisation to distal Yp is in agreement with the one reported by Affara et al., (1986a) who mapped the above sequence by means of deletion mapping using DNA from individuals with structural abnormalities of the Y chromosome. In Southern blots, GMGY3 recognised three Y-specific and four autosomal fragments (Affara et al., 1986a). In situ hybridisation extended the reported molecular findings by indicating the location of the autosomal sequences.

Deletion mapping in XX males has suggested that this sequence is the nearest to the TDF locus in a series of 39 Y restriction fragments (Ferguson-Smith et al., 1987). Additional evidence that GMGY3 resides in the sex-determining region of the short arm of the Y chromosome comes from Page et al., (1987) who reported that GMGY3 is about 100kb distal from ZFY, the Y encoded zinc-finger protein cloned by these investigators which is a strong candidate for the testis-determining factor. A closely related gene, ZFX, exists on the human X chromosome (Page et al., 1987) band Xq21.3 (Muller & Schempp 1989). It is interesting to note that signal -though not statistically significant- was observed in this region of the X chromosome following hybridisation with GMGY3, although Southern analysis did not revealed any homology of GMGY3 to the X chromosome.

Recently Affara et al., (1989) using oligonucleotide sequences based on the amino acid sequence of ZFY isolated a 3.2 kb human testis cDNA sequence, CMPXY4, which was shown by in situ hybridisation to be located at the short arm of the X chromosome (Xp21.2-Xp22.1), the distal short arm of the Y chromosome (Yp11.3) and distal short arm of chromosome 9 (9p22.3-pter). This finding adds further weight to the suggested homology between the short arms of chromosomes 9 and Y. It should be noted however that ZFY does not

recognise autosomal homologies in man (Page et al., 1987) although it does in the mouse (Mitchell et al., 1989) and it was found to be autosomal in marsupials (Sinclair et al., 1988).

The question now arises whether the homology between the sex-determining region of the Y chromosome, where both GMGY3 and CMPXY4 reside, and distal short arm of chromosome 9 reflects functional homology as well. Possible clues might arise from phenotype-genotype correlations .

Frequent features in the 9p deletion syndrome, del(9) (pter-p22 or 21), include hypoplastic labia majora, prominent labia minora and clitoris, cryptorchidism and hypospadias. More than 3/4 of the reported patients were females. In the ring 9 chromosome syndrome, common malformations are hypospadias or ambiguous genitalia and a 3:1 male to female sex ratio was observed in eight cases. These observations however do not consist evidence for involvement of chromosome 9p in sex determination as ambiguous genitalia are frequent in many other chromosomal rearrangements (Schinzel, 1984).

Suggestive evidence for a such a role of chromosome 9p comes from reports of association of 9p22-9pter monosomy with sex reversal in man. Crocker et al., (1988) reported an unbalanced autosomal translocation (7;9) associated with feminization. The karyotype of the patient was 46,XY -9, +der(9), t(7;9)(q31.1;p23). External genitalia were of a normal female; there was a uterus, oviducts but the gonads were testicles with Sertoli cells but no germ cells. As in previous cases with trisomy for 7q31-qter no abnormalities in the internal sex organs have been observed, whereas in cases of del(9p) ambiguous genitalia have been reported and taking into account a case of 3p trisomy/9p monosomy associated with sex reversal (Fryns

et al., (1986), Crocker et al., (1988) suggested a role for genes on 9p in sex determination.

The idea of an autosomal locus (loci) involved in human sex determination is not new. Autosomal mutation that causes sex reversal has been proposed in order to explain the occurrence of human XX males that are negative for all Y DNA sequences tested (de la Chapelle, 1987; Ostrer et al., 1989). GMGY3 has been tested against such XX males (Affara et al., 1986b) but as the main interest was on the Y-specific fragments no information was given on the autosomal fragments. Sex reversal in such cases may be due to mutations in autosomal or X-linked genes whose products function together with or downstream of TDF in the sex determination pathway (Page 1988). The loci on short arm of chromosome 9 identified by GMGY3 may be such autosomal genes. On the other hand, evidence in the mouse suggests that genetic effects which retard development can cause XY individuals to develop as females and the gene loci involved are not specifically concerned with sex determination (Cattanach et al., 1988).

Human sex determination is a complicated issue (de la Chapelle, 1988). As Eisher (1988) noted, it has taken almost 30 years from the observation that the mammalian Y chromosome is involved in testis determination to the accomplishment of obtaining a cloned DNA sequence that is most likely involved in testis determination; in some respects we have come a long way towards understanding sex determination and in other respects we are just beginning. Whether 9p22-9pter plays a role in sex determination either by bearing gene(s) that are sex determining or have effects in retarding development as in the mouse, awaits confirmation. Nevertheless, the

homology between the short arms of the Y and chromosome 9 presented in this project is important as it adds to our understanding of the human genome organisation. Although several single copy sequences that recognise homology with autosomes have been isolated from the Y chromosome (Davies et al., 1987), little is known about the chromosomal localisation of the autosomal fragments.

Affara et al., (1986a) were the only authors to demonstrate that the autosomal fragments of such a sequence (GMGY1) were present on chromosome 1 by using flow sorted material from an individual with a chromosome 1 centric polymorphism. The easiest way at present to identify such autosomal homologies is to use in situ hybridisation which offers direct visualisation of all possible hybridisation sites on metaphase chromosomes. Interestingly, as mentioned in section 3.8.4 when biotinylated GMGY10 was studied in the orangutan, except for the signal on the short arm of the Y chromosome signal was also present on chromosome 1. Such hybridisation was not observed in man; it should be noted the studies in the orangutan were performed at a higher labelled probe concentration and longer incubation in chromogenic substrate (40ng, 3 hours in substrate as compared to 1ng, 1 hour) which might account for this cross-hybridisation.

It would be interesting to see if other Y-specific probes show homology with the short arm of chromosome 9. Additionally, comparative gene mapping studies of GMGY3 might indicate if the autosomal sequences recognised by this probe are conserved during evolution which would suggest an important biological function for these sequences.

4.3.3 DNA sequences that show homology to extended chromosome regions

Three of the sequences mapped in this project, namely GMGY4b, JG51 and JG73 showed homology to extended chromosome regions.

GMGY4b detects a strong Y-specific band with multiple autosomal bands in Southern analysis of EcoR1 digested male genomic DNA, and it was mapped to the short arm of the Y chromosome, at the centromeric region using a panel of somatic hybrids bearing different regions of the Y chromosome (Affara et al, 1986a). The autosomal homologies were not however identified.

The in situ hybridisation studies presented here (section 3.2.4) confirmed the localisation to the centromere of the Y chromosome and defined the autosomal homologies as the centromeric region of chromosomes 15 and 22 under washing conditions of 0.1xSSC at 65^{o}C. At a different stringency (50% formamide at 38^{o}C) additional sites of hybridisation were observed at the centromeres of chromosomes 14 and 21.

Centromeres of human chromosomes are associated with distinct classes of tandemly repeated DNA sequences termed alphoid satellite DNA (Willard, 1985). Recent studies (Wolfe et al., 1985; Waye et al., 1988) indicate that human alphoid DNA is organised into chromosome-specific alphoid subfamilies, formed by the amplification of segments composed of tandemly arranged related copies of the 170-base pair (monomeric) or 340-base pair (dimeric) repeat units. Wolfe et al., (1985) have isolated and characterised an alphoid repeat family from the human Y chromosome, DZY3, which as shown by in situ hybridisation hybridised with the centromeres of the Y chromosome and chromosomes 13, 14, 15 under high stringency (0.1xSSC

at 65°C). Therefore, it seems likely that GMGY4b is related to the Y alphoid DNA although this needs to be confirmed by studying the sequence organisation of GMGY4b. With DZY3 no significant hybridisation was reported with chromosomes 21 and 22. It should be noted however that Wolfe et al., (1985) based their localisation on only ten metaphase cells.

With GMGY4b, in addition to the hybridisation observed with the centromere of the Y chromosome silver grains (20 of 121 recorded on the Y chromosome) were located at proximal long arm as shown in figure 16. This could be due to the track of length of the high energy particle released by the tritium-labelled nucleotide which can be up to 1.0μm from the source (Rogers 1967). On the other hand, it could represent actual homology of GMGY4b with this region of the Y chromosome.

JG51 and JG73, as mentioned previously (section 3.2.5) were isolated independently from a chromosome 21-specific library by Galt (1988); both sequences showed a similar hybridisation pattern of many bands on Southern blots of genomic DNAs and detected homologies with a number of human chromosomes including chromosome 21. Using a chromosome 21 hybrid panel it was shown that both probes are located at the pericentromeric region of chromosome 21 (Galt 1988). The autosomal homologies detected by these two probes were investigated during the course of this project by in situ hybridisation (section 3.2.5).

JG73 was shown to hybridise with the centromeres of chromosomes Y, 19, 21 and 22 at a stringency of 50% formamide at 45°C. At this stringency JG51 hybridised with the centromeres of chromosomes 13,

14, 15, 20, 21 and 22. Thus, probes JG51 and JG73 appeared to have a different distribution in the human chromosome complement. At a lower stringency - 50% formamide at 38°C-, JG73 hybridised to the centromeres of most chromosomes although statistically significant hybridisation was only that to chromosomes 14, 15, 20, 21 and 22 and Y. Less hybridisation was recorded with the Y chromosome than in the previous experiment but considerable variation in the grain distribution between individuals has been observed with similar DNA probes (Choo et al., 1988; Choo et al., 1989). Hybridisation of JG73 to the Y chromosome was confirmed by Southern analysis on a Y chromosome somatic cell hybrid panel (J. Galt, personal communication). At this stringency probe JG51 hybridised to all acrocentric chromosomes (13, 14, 15, 21 and 22). A major difference between the two probes was that JG73 showed no homology to chromosome 13 while JG51 showed no homology with the Y chromosome. By performing the hybridisation at 52°C, a clear difference was observed between the two probes; JG73 hybridised only with chromosome 21 whereas JG51 hybridised with chromosomes 13, 14 and 22.

The cross-hybridisation of probes JG71 and JG53 to the centromeres of many human chromosomes suggests that these probes are alphoid DNA. However, as mentioned above for probe GMGY4b, this could be more clearly demonstrated by cross-hybridisation of these probes to known alphoid DNA or by the presence in these probes of the basic repeating unit of 170bp length characteristic of alpha-satellite DNA (Singer, 1982).

Several alphoid DNA sequences that hybridise to the acrocentric chromosomes have been described. Jorgensen et al., (1988) described

an alpha-repeat subfamily shared by human chromosomes 14 and 22 and a related but distinct subfamily shared by chromosomes 13 and 21 (Jorgensen et al., 1987). Their results established the existence of a very efficient pair-wise homogenis ation of alpha repeat sequences on the human acrocentic chromosome pairs 13/21 and 14/22. Their data however did not exclude the existence of minor species of alpha repeats on these chromosomes not reflecting this pair wise homogenization. Different alphoid sequences have been found on chromosome 14 (Waye et al., 1988) and chromosome 22 (McDermid et al., 1986). Recently Choo et al., (1989) studied by in situ hybridisation five new and one previously described (Choo et al., 1988) alpha-satellite sequences isolated from chromosome 21 libraries. At high stringency (0.1xSSC at 65^{o}C) two sequences hybridised to chromosomes 13, 14, 15, 21 and 22 (also 19 and 20), one sequence hybridised to chromosome 14, and finally three sequences hybridised to chromosomes 13, 14, and 21 with significant but weaker signals on 15 and 22. These results suggested the sharing of alphoid domains between different acrocentric chromosomes and the coexistence of multiple domains on each chromosome. JG51 shows a very similar hybridisation profile to the sequences pTRA-1 and pTRA-4 hybridised to chromosomes 13, 14, 15, 21 and 22 described by Choo et al., (1989). It is interesting to note that hybrdisation with the Y chromosome as observed for JG73 was not reported for any of the previously described alphoid sequences. It should also be noted that the presence of these repetitive alphoid sequences at the centromeres of the acrocentric chromosomes may play an important role in the aetiology of Robertsonian translocations which generally involve recombination of whole acrocentric chromosome arms

by facilitating initial pairing followed by exchange of long arms (Choo et al., 1988; 1989).

Because of the chromosome-specific organisation of the human pericentromeric alphoid repetitive DNA (alpha-repeat DNA), these sequences can be used as chromosome-specific markers. Using in situ hybridisation with such a clone specific for chromosome 18, under conditions of high stringency Cremer et al., (1986) were able to detect trisomy 18 both in metaphases and interphase nuclei from an amniotic fluid. Although minor signals were present in other chromosomes, large clusters of grains occurred at the pericentromeric region of the three chromosomes 18. JG73 does hybridises solely to chromosome 21 under high stringency but it would not be useful as a marker for this chromosome as the signal manifests itself with the intensity of a single copy probe (one grain per chromosome).

4.3.4 Contradictions arisen from in situ hybridisation results

In situ hybridisation is without doubt a powerful method for gene mapping. There have been few instances however, that in situ hybridisation results have been challenged by molecular biologists. This is exemplified by the localisation of the DNA segment D1S1; D1s1 was the first anonymous single copy DNA sequence to be mapped by in situ hybridisation (Harper and Saunders, 1981). In the original investigation, a 14.9 kb human DNA segment estimated to be present in one or two copies per haploid genome was used as a probe, and a highly significant site of hybridisation was observed at band 1p36 of chromosome 1 with 22% of all chromosomal grains. This localisation to human chromosome 1p36 was later confirmed by Donlon

and Magenis with 32% of the total grains (1984); although 9% of the total signal was observed on chromosome 3 (3p21), nonetheless the localisation of D1S1 was reported at chromosome 1p36.

A different study however, Goode et al., (1986) using a panel of human-hamster somatic cell hybrids showed that there are copies of D1S1 on both chromosomes 1 and 3. A similar finding was reported by an independent group (Carritt et al., 1986). The D1S1 clone itself was shown to be derived from chromosome 3; part of it was duplicated at least twice on chromosome 1. The finding that in the great apes, D1S1 detects fragments similar in number and weight to the human chromosome 3 fragments, was additional evidence to that the chromosome 3 D1S1 copy is the ancestral copy and the duplication is of very recent origin.

Following the localisation to 1p36, human chromosome 1p has been implicated in malignant melanoma on the basis of loss of heterozygosity at the HinIII D1S1 polymorphism (Dracopoli et al., 1985). The HindIII polymorphism detected by D1S1 was shown to be part of the chromosome 3 D1S1 copy.

This example demonstrated the importance of using two mapping methods to verify the localisation of a gene or a DNA segment, particularly a polymorphic one which itself may be used as a marker in linkage mapping studies. It also raised the question why in situ hybridisation detected a duplicated portion of a clone but not the chromosomal origin of the clone itself. Such a discrepancy is serious. However, as out of 444 autosomal loci mapped by in situ hybridisation until February 1989 (McKusick, 1989) conflicting reports exist only on three other occasions (Goode et al., 1986), the position of the beta-globin gene, the T-cell receptor beta-chain

on chromosome 7, and the ferritin heavy-chain loci, these could be regarded as isolated cases. Therefore, the conclusion of Goode et al., (1986) that in situ hybridisation appears to be a less sensitive gene mapping method than formerly supposed, is not valid.

4.3.5 Studies of patients with paradoxical sex chromosome complements

4.3.5.I XX males

An XX male is defined as an individual with a male habitus, male external genitalia, gonads of testicular type (always dysgenetic) with absence of ovarian elements and a 46,XX karyotype (de la Chapelle, 1986). XX males are an apparent exception to the rule that testes develop in the presence of the Y chromosome (Ford, 1959; Jacobs & Strong, 1959) under the dominant control of the testis determining factor (TDF) which is located on the short arm of the Y chromosome, band p11.3 (Magenis et al., 1987b)

XX males were originally described in 1964 (Court Brown 1964; de la Chapelle et al., 1964; Therkelsen, 1964) and since then, well over 60 cases have been referred to in the literature. The condition has an incidence of 1 in 20,000 in male newborns. The 46,XX male presents an intriguing puzzle so that despite of its rarity, the aetiological factors responsible for this condition have been the subject of much speculation.

Ferguson-Smith (1966) postulated that an abnormal interchange between the distal parts of the short arms of the X and the Y chromosomes at paternal meiosis transferred the testes determinants to the X chromosome. Thus, the father would pass on a deleted X chromosome bearing a region of the Y chromosome containing the

testes determinants. As postulated, this interchange was facilitated by the pairing of the X an the Y chromosomes in this region during male meiosis and it was the result of an aberrant recombination event. Evidence supporting the X-Y interchange hypothesis comes from cytogenetic studies (Evans et al., 1979; Madan 1976; Magenis et al., 1982) and from measurement of flow-sorted chromosomes (Ferguson-Smith et al., 1986).

That part of the Y chromosome was present in XX males was conclusively shown when Y-specific DNA probes became available (Guellaen et al., 1984; Koenig et al., 1985; Page et al., 1985; 1986; Vergnaud et al., 1986; Muller et al., 1986a, b; Affara et al., 1986b; 1987; Ferguson-Smith et al., 1987; Waibel et al., 1987 Ferguson-Smith & Affara 1988; ; Broendum Nielsen et al., 1988). These studies showed that about 80% of XX males have inherited Y-derived sequences and that the part of the Y chromosome transferrable onto the X varies. The existence of Y sequences in XX males does not however constitute evidence for their location on the (paternal) X chromosome. Magenis et al., (1984) were the first to demonstrate that Y short arm material was transferred to distal Xp in XX males, using in situ hybridisation with a Y-specific DNA probe.

Part of this project was to use in situ hybridisation to study nine XX males previously shown to be Y DNA positive (Affara et al., 1986b; Affara et al., 1987). In eight XX males studied with Y-specific DNA sequences GMGY10 and/or GMGY7, silver grains were clearly localised at the tip of the short arm of one X chromosome (band Xp22.3); therefore the Y-specific sequences present in the patients genome were the result of an X-Y interchange as predicted

by the X-Y interchange hypothesis mentioned above.

Such a conclusion could not be reached for the ninth XX male, KS. This case was investigated with the X-Y specific DNA probe, P2F2. Silver grains were observed at Xq21 where P2F2 is located on the X chromosome (see section 4.3.1). Silver grains were also present on the short arm of the X chromosome but contrary to the finding in all other eight XX males where a peak of silver grains was observed in band Xp22.3 with a few grains over Xp22.2 and Xp22.1, in KS silver grains were observed in all above chromosome bands and Xp21. A similar scattering of silver grain was seen over the entire distal one half of the short arm in two other XX males studied by Magenis et al.,(1987b) using in situ hybridisation and the X-Y specific probe p50f2. When KS was investigated with GMGY10 signal, though not significant, was recorded on the X chromosome on both bands Xp22.3 and Xp21. Signal on the X chromosome was never observed with the above probe in a normal male (data not shown). The fact that the above study did not result in statistically significant labelling of the X chromosome can be explained if we consider that only one band from the several recognised by this probe in Southern blots with normal male DNA is actually present in this KS (Affara et al., 1986b; Ferguson-Smith et al., 1987). Thus the short autoradiographic exposure of 6 days used routinely for GMGY10 throughout this project did not result in sufficient labelling.

The results obtained in KS could be explained by the transfer of Y sequences to distal Xp and a secondary site at Xp21 for probe P2F2. However, there is no evidence for such homology from Southern blot analysis (Affara et al., 1986a) although this cannot be

excluded on the basis of the in situ hybridisation data in a normal female presented here, as a few grains were observed in this region of the X chromosome. A second explanation would be the track length of the high energy particle released by the tritium-labelled nucleotides which, as mentioned previously, can be up to 1.0μm from the source (or about one-half the length of the X short arm). Although this explanation theoretically is acceptable it should apply to all cases studied not only KS. A third explanation is actual insertion of Y DNA in pieces into the short arm of the X. Interestingly, DNA analysis of the Y-specific sequences present in KS has revealed an anomalous transfer of Y-specific sequences which has been explained by the occurrence of a paracentric inversion in his father's Y chromosome (Affara et al., 1986b). The genesis of an XX male is an unusual event and may itself be accompanied by complex rearrangements and interstitial deletions resulting in anomalous patterns of transfer (Affara et al., 1986b). It is not clear what kind of rearrangement would result in insertion of pieces of the Y chromosome into the short arm of the X. This possibility can be however excluded on the basis of cytogenetic analysis by Giemsa banding which clearly demonstrated transfer of Y material to distal Xp (E. Boyd personal communication).

The lack of hybridisation to any chromosome but the X with probe P2F2 together with the demonstration of transfer of Y material at distal Xp indicate that the hybridisation observed at distal Xp in KS is due to the presence of Y-specific seqences. However, because of the signal present in band Xp2.1 further work with different Y-specific probes is required to clarify if the presence of Y-specific sequences in KS is the result of an X-Y interchange.

A fold on the X chromosome has been suggested to be a practical metaphase marker of the inactive X chromosome (van Dyke et al, 1986). The observation that silver grains were present both on folded and not-folded X chromosomes in the XX male DR would suggest that the presence of the Y sequences does not affect random inactivation of the X chromosome. The position of signal observed in a limited number of interphase nuclei following hybridisation of biotinylated GMGY10 with DR would also suggest random inactivation as signal was observed attached with the nuclear membrane indicating the inactive X chromosome (Barr body) as well as in different positions in the nucleus (the active X chromosome). This has been recently demonstrated by Schempp et al., (1989). The authors studied simultaneously the localisation of Y-specific DNA and X-inactivation by combining in situ hybridisation and late replication banding on a sample of eleven XX males. In all but two cases the presence of the Y DNA did not result in a deviation from random X inactivation. In the two exceptions, the Y DNA was found only on the late replicating X chromosome in metaphases obtained from fibroblasts although random inactivation of the Y DNA carrying X chromosome was observed in metaphases obtained from lymphocytes. It was suggested that X inactivation in XX males depends on the size of the X segment deleted as a result of the X-Y interchange (part of the X-Y interchange hypothesis is that some X sequences will be transferred to the Y chromosome). If the deletion on Xp results in loss of X loci which do not escape inactivation, cells survive only if the X-Y translocation chromosome is inactivated (Schempp et al., 1989).

Other investigators have used in situ hybridisation to test the X-Y intechange hypothesis in XX males (Andersson et al., 1986;

Magenis et al. 1987b ; Buckle et al., 1987) and all data presented supported the X-Y interchange hypothesis. Recently Kozma & Adinolfi (1988) studied one XX male with non-radioactive hybridisation. A bright fluorescent spot was seen on one of the X chromosomes in about 40% of the metaphases examined. Table 44 summarises all XX males studied so far with Southern blot analysis and in situ hybridisation. Of 51 XX males shown to have Y DNA present in their genone, only 27 have been investigated by in situ hybridisation and of these, 26 cases were shown to be the result of an X-Y interchange. Therefore, it is only appropriate that these males are called X-Y interchange males instead of XX males as proposed by Ferguson-Smith et al.,(1987).

4.3.5.II Two 45,X/46,X+mar males

In this study, marker chromosomes in two 45,X/46,+ mar males, RW and DG, were identified as Y fragments utilising in situ hybridisation and Y-specific probes (see section 3.5.2). DNA analysis had shown that Y DNA was present in these individuals (Affara et al., 1987) . but the possibility that this DNA was located on a chromosome other than the fragment could not be ruled out. In situ hybridisation demonstrated that the Y-specific sequences were present on the minute chromosome and had not been translocated to another chromosome. Thus the male phenotype of the above individuals is due to the presence of a small part of the Y chromosome apparently bearing the male determinants (TDF).

Small marker chromosomes may originate either from acrocentric autosomes or from sex chromosomes (Fryns et al., 1982; Chudley

XX males investigated by DNA analysis

	No of XX males studied	No of XX males with Y DNA
Guellaen et al. (1984)	4	3
Page et al. (1985)	3	3
Vergnaud et al. (1986)	19	12
Muller et al. (1985a,b)	11	9
Ferguson-Smith & Affara (1988)	23	19
Waibel et al. (1987)	11	11
Buckle et al. (1987)	2	2
Broendum Nielsen et al. (1988)	3	3
Total	77	62 (80%)

XX males studied by in situ hybridisation

Andersson et al. (1986)	3	
Magenis et al. (1987)	3	
Buckle et al. (1987)	2	
Kozma & Adinolfi (1988b)	1	
Schempp et al. (1989)	11	
this study	9	
Total	29	47% of Y positive XX males

Table 44 Summary of XX males studied wih DNA analysis and in situ hybridisation.

et al., 1983; Benn and Hsu, 1984). Before the advent of molecular biology, cytogenetic studies were the only means for elucidating the origin of small markers. NOR staining detects transcriptionally active stalk regions of acrocentic-derived fragments, and Q-band staining demonstrates bright fluorescence of satellites or of Yq-derived heterochromatin. G-11 staining allows the distinction between the X- and Y-derived chromosomes as long as the centromeric heterochromatin is intact (Magenis and Donlon, 1982) but is a difficult technique to reproduce consistently, particularly over periods of time (Bobrow et al., 1972; Wyandt et al., 1976).
However, in most cases the small marker chromosomes cannot be characterised sufficiently by conventional staining methods. Y fragments in particular are often not recognizable as such because they may contain only pericentromeric euchromatin that stains non-specifically.

Marker chromosomes can be identified as Y fragments when at least some masculinisation is present in the respective individual or when the centromere is consistenly C-negative. Davis (1981) suggested that although difficult to prove, Y fragments are always rings. More than 20 cases have been reported in the literature (Buhler 1985), with a variable loss of Yq and Yp, XO mosaicism and a similar clinical picture with Y rings. The appearance of external genitalia may vary from normal female over any degree of masculinization to normal male. The isolation of DNA clones from the sex chromosomes provided a new tool for the identification of such fragments.

Patient RW was shown by DNA analysis to lack the Y centromeric sequence GMGY4b, thus it has been suggested that he may not have a Y

centromere (Affara et al., 1987). Cytogenetic analysis was not informative in this respect. The presence of the fragment only in 25% of the patient's lymphocytes could be a result of it being acentric. Although acentric fragents are generally lost during mitosis, there is areport in the literature of a microchromosome that was situated near or in close proximity to centromeres (Patil & Waziri, 1982). The authors interpreted this microchromosome as acentric chromatin of unknown origin which in the absence of its own centromere might "hitchhike" along with any other chromosome. Such an association of the fragment appeared to be present in RW studied here, but 26 associations recorded in 74 metaphases were not found to be statistically significant (see section 3.5.2). This could be due to the relatively small number of metaphases scored. Schmid et al (1989) found a significant centromeric association of a supernumerary microchromosome in a CREST scleroderma patient after scoring 5,000 metaphases.

Two other cases of minute chromosomes have been identified as Y-fragments by means of in situ hybridisation (Münke et al. 1985). Patients were males, one with sterility owing to azoospermia and the other with stature at the 10th centile, otherwise phenotypically normal with the karyotype of 46,X,min. In situ hybridisation demonstrated that the sequence pDP31 was present on the minute and had not been translocated to another chromosome. The proportion of silver grains observed on the minute chromosomes was 6% and 7%. The minutes were assumed to contain a centromere since they segregated normally at mitosis.

In situ hybridisation is the only technique presently available for revealing the origin of small marker chromosomes that are not

readily recognisable by standard karyotype analysis. The identification of the chromosomal fragments in this study and that of Munke et al., (1985) illustrates the power of the technique to resolve such problems. This study as well as the previously mentioned study of 8 XX males presented in this project also demonstrated the usefulness of DNA probes GMGY10 and GMGY7 as specific markers of the short arm of the Y chromosome.

4.3.6 In situ hybridisation in clinical diagnosis -Identification of a Y/15 translocation.

During this project a 15p+ polymorphism present in amniotic fluid cells and in paternal lymphocytes with morphology and staining properties compatible with it carrying a piece of Yq12 on its short arm, was identified as a Yq:15p translocation by means of in situ hybridisation with Y-specific probe pY3.4. The presence of this translocation in the father's chromosomes indicates that it does not affect fertility.

According to Nielsen and Rasmussen (1976) the frequency of Y:autosomal translocations in the general population is approximately 1 in 2,000. Familial transmission has been reported both in males and females with the extra Y material translocated most frequently onto the short arm of a chromosome 15 (Fryns et al., 1985). In general, phenotypic expression of Y/A translocations with familial occurrence is minimal, and most of them have been detected accidentally. The most logical explanation for this "harmlessness" is that the additional Y-chromosome material most frequently involves the distal heterochromatic Yq12-Yqter portion (Fryns et al, 1985).

Before the advent of molecular biology Y:acrocentric translocations could only be investigated by conventional cytogenetic analysis. When the short arm of an acrocentric chromosome was large and brilliantly fluorescent by quinacrine staining the question was raised as to whether it represented material translocated from Yq12 or a normal polymorphism as the short arms and satellite regions of the human acrocentrics chromosomes are highly heteromorphic with respect to size and staining properties (ISCN 1985).

The use of Y-specific probes provided diagnostic tools for unambiguous identification of Y:acrocentric translocations both by Southern hybridisation (Cooke & Noel 1979; Schmidtke & Schmid 1980; Burk et al., 1983) and by in situ hybridisation (Lau 1985; Lau et al., 1985; Alitalo et al., 1988). The use of non-radioactive in situ hybridisation has permitted rapid diagnosis of Y:autosomal translocations in less than 35 hours (Kozma & Adinolfi, 1988).

Although it was generally assumed that the breakpoint on the Y chromosome lies within band Yq12, this was demonstrated for the first time by Alitalo et al (1988) who characterised molecularly a Y:15 translocation segregating in a family in an autosomal dominant manner. It was demonstrated that Y-specific fragments recognised by probes located in the euchromatic portion of the Y, including Yp, the pericentromeric region and Yq11 were absent in 15p+ carriers therefore it was concluded that the breakpoint in the ancestral Yq was probably close to the end of the euchromatic region. The breakpoint in chromosome 15 was not determined by molecular methods. Since the 15p+ had a centromere which was not that of the Y by virtue of the absence of Y DNA probes that map to the

pericentromeric region, the breakpoint must be in the short arm. The translocation is unbalanced since it entails the loss of most of 15p an the gain of Yq12. This study also provided quantitative evidence that the heterochromatic band Yq12 present on the 15p+ chromosome was of the same size as that of the Y chromosome segregating in this family. A similar finding has been previously reported by Cooke & Noel (1979) on the basis of DNA measurements by densitometry. The quantitative data obtained in this study from the hybridisation of pY3.4 in the paternal lymphocytes are in good agreement with these presented by Alitalo et al., (1988) in a similar study (table 43). The lack of hybridisation with chromosome 9 observed here has been discussed earlier (section 4.3.1).

Somatic pairing of chromosomes Y and 15 has been demonstrated by Schmid et al., (1983) in lymphocyte cultures treated with 5-azacytidine; direct contact was observed between the short arm of chromosome 15 and the long arm of the Y. This association may be the result of homologous DNA sequences located in the long arm of the Y chromosome and in the short arms of the acrocentric chromosomes. Stahl et al (1984) have shown a non-random association of the nucleolus and short arm of the acrocentric chromosomes with the XY pair in human spermatocytes. The authors observed close relationships between the sex vesicle chromatin and that of the associated acrocentric bivalent, especially in the short arm region, from early zygotene to late pachytene. These relationships might explain the frequent involvement of acrocentrics in Y:autosome translocations.

The application of in situ hybridisation in clinical diagnosis has increased significantly in recent years and it is impossible to

cite all published reports. This has been due to the increased availability of single copy and repetitive probes that hybridise intensely and specifically to selected chromosomes. Such probes are now available for over half of the human chromosomes and allow identification of many chromosome aberrations (Pinkel et al., 1988). The introduction of non-radioactive procedures which decrease dramatically the time that results are obtained (6 days required for the identification of the Y:15 translocation using isotopic in situ hybridisation reported here as compared to 35 hours non-isotopic detection reported by Kozma & Adinolfi, 1988) will greatly augment the value of in situ hybridisation as a diagnostic tool.

4.3.7 New Applications of in situ hybridisation

4.3.7.I Interphase cytogenetics

Observations that the DNA of individual chromosomes occupies focal territories or spatially cohesive domains within mammalian interphase nuclei made investigators to consider the possibility of detecting numerical or structural aberrations of chromosomal domains in non-mitotic cells using chromosome-specific probes, an approach for which the term "interphase cytogenetics" has been coined (Cremer et al., 1986). Such an approach will be valuable in prenatal diagnosis where rapid results are desirable and in the investigation of human solid tumours where cytogenetic investigations are tedious and difficult to perform due to both the complexity of chromosomal changes and the lack of sufficient number of suitable metaphase spreads for reliable analyses. Earlier studies demonstrated the prenatal diagnosis of trisomy 18 in interphase cells using a repetitive chromosome-18 specific probe (Cremer et al., 1986)

Repetitive probes are not useful for detection of structural alterations since the probability that the aberrations will involve the region to which the probe hybridises is low (Pinkel et al., 1988). With the development of methods that allow the specific staining of entire individual chromosomes using chromosome specific libraries as probes (see section 4.2.1) interphase cytogenetics is now feasible. The preliminary results are encouraging (Pinkel et al., 1988; Cremer et al., 1988). Whether the hybridisation patterns observed in interphase nuclei are sufficiently reliable for routine prenatal diagnosis remains to be determined. Complications may result from clustering, overlapping and/or distortion of the fluorescent domains. The use of sophisticated computer assisted microscope systems like the laser-scanning confocal fluorescence microscope assembly used by Lichter et al., (1988) for optical sectioning and 3-D image reconstruction of labelled chromosome domains in nuclei allows accurate documentation of the hybridisation signals but the initial investment is very costly.

In comparison with classical cytogenetic analysis based on the evaluation of banded chromosomes, "interphase cytogenetics" has the disadvantage that it cannot be used, at least presently, for the identification of any possible chromosome aberration but only for specific ones (i.e. trisomies).

4.3.7.II Physical mapping by in situ hybridisation

A very interesting new application of in situ hybridisation has been recently reported by Trask et al., (1989). The authors described the use of fluorescence in situ hybridisation for determining the physical distance between DNA sequences in

interphase nuclei. Eight cosmids whose relative position had been previously determined by restriction enzyme mapping were used containing fragments of the Chinese hamster genome spunning 273 kilobase pairs surrounding the dihydrofolate reductase gene. The distance between DNA sequences in interphase nuclei was correlated to molecular distance over a range of 25 to at least 250 kb. The observed relationship was such that genomic distance could be predicted to within 40 kb from interphase distance. The correct order of seven probes was derived from interphase distances measured for 19 pair-wise combinations of the probes. If the observations made with this set of cosmids can be extended to other probes in other regions interphase chromatin mapping may be used to complement other physical mapping techniques. with the advantages of speed (the seven cosmids encopassing 250kb were mapped within a week) and simplicity (it requires only a good quality fluorescence microscope).

4.4 COMPARATIVE GENE MAPPING IN THE GREAT APES

In this section the findings from the comparative gene mapping studies of a human $tRNA^{Glu}$ gene and Y-specific sequences GMGY10 and GMGY7 are discussed. The cytogenetic and molecular information available on the Y chromosome of the great apes is reviewed and a diagrammatic representation of the Y chromosome of the great apes showing G-bands is proposed. Heteromorphisms of the orangutan chromosomes observed in the two specimens studied are presented. Finally problems associated with interpreting the results from comparative gene mapping studies are addressed.

4.4.1 Comparative gene mapping of a human $tRNA^{Glu}$ in the great apes

Goddard et al., (1983) isolated and characterised a human transfer RNA gene for glutamic acid ($tRNA^{Glu}$) which was assigned to human chromosome 1p36 by in situ hybridisation with a secondary site at 1q21-q22 (Boyd et al., 1989).

Comparative mapping studies presented here showed that the above human $tRNA^{Glu}$ gene is conserved in the gorilla, chimpanzee and orangutan. A main site of hybridisation was observed at distal long arm of chromosome 1 in the gorilla, chimpanzee and orangutan, a region homologous to human 1p36 in view of the reversed arm ratios of chromosome 1 in man and great apes. In order to maintain the homology between the arms of this chromosome despite the difference in length, standard nomenclature (ISCN 1985) suggests that long arm of the great ape chromosome is referred to as 1p and vice versa. Thus the site of hybridisation of the $tRNA^{Glu}$ gene in the great ape chromosome 1 is band 1p36. Interestingly, in the chimpanzee and orangutan two additional sites of hybridisation were observed, above and below the centromere. These sites might represent related sequences or pseusogenes as has been proposed for the secondary site observed in man (Boyd et al., 1989).

The sites of hybridisation of the $tRNA^{Glu}$ gene in the human, coincide with the sites of true genes and pseudogenes for the U1 small nuclear RNA (snRNA). Most or all of the 30 U1 genes are known to be clustered in band p36 of human chromosome 1 (Lund et al., 1985; Naylor et al., 1984). In addition to the active genes the U1 multigene family contains at least 500-1000 unexpressed pseudogenes with imperfect coding sequences that can be divided into at least three classes based on their structure. Lindgren et al., (1985)

mapped four class I pseudogenes both by in situ hybridisation and somatic cell hybrids to the long arm of human chromosome 1, bands q12-q22.

By analogy to the distribution of U1 snRNA true genes and pseudogenes, the secondary site observed in man and the great apes may represent pseudogenes [the same assumption has been made for the secondary site observed on chromosome 1 with the human asparagine transfer RNA genes which were also shown to map to human chromosome 1p36 and 1q21-22 (Buckland et al., 1989)]. This however needs to be confirmed by studying the nucleotide sequence of the $tRNA^{Glu}$ gene.

Knowledge of the chromosomal position of the genes and pseudogenes is of interest as it might allow to distinguish between various theories regarding the homogenization of multigene families. For the tRNA genes it has been suggested that a transposition event could have facilitated the generation of a second locus from what was originally one gene cluster (Bernstein et al., 1985). The split into two distinct loci could have also resulted from a pericentric inversion prior to the evolution of the great apes (Buckland 1989). If such a pericentric inversion had taken place before the divergence of the great apes from the common stock the observed distribution of secondary sites in the four species would require loss of the second locus in the gorilla and independent amplification in the lineage of chimpanzee and orangutan.

The human haploid genome contains approximately 1,000 tRNA genes (Hatlen & Attardi, 1971) representing complex multigene families with on average 10-20 copies of about 60 different genes. When studying the chromosomal distribution of multigene families the question arises whether this has originated from an ancestral distribution or

independent amplification has occurred in each lineage. The genes coding for ribosomal RNA in the great apes have also revealed variable distribution in any species. In the chimpanzee these sequences are distributed in five chromosome pairs as in man, but not in the exactly corresponding homologous chromosomes (Henderson et al., 1974). In the orangutan all satellited acrocentric chromosomes carry rDNA genes (chromosomes 12,11, 13, 14, 15, 16, 17, 22 and 23) (Gosden et al., 1978) whereas in the gorilla these genes are found on only two chromosomes, 22 and 23. A comparison among species within the Hominidae did not allow to suggest whether this distribution have been derived either from an ancestral "distribution" of these sequences prior to speciation or from independent amplification in each species. Comparison with other primate species has however indicated that the amplification of these repetitive sequences in the chromosomes must have been independent in each primate lineage (Seuanez 1979). Further comparative mapping studies in lower primates are therefore required to clarify the evolution of the $tRNA^{Glu}$ multigene family.

Whether or not the secondary sites of hybridisation on chromosome 1 observed for $tRNA^{Glu}$ represent pseudogenes, their variable distribution (one in man, none in the gorilla and two in the orangutan and chimpanzee) suggests that differences do exist at the DNA level between chromosome 1 of man and the great apes, despite the remarkable similarity in the banding pattern. A similar observation was reported on the basis of the AluI-induced bands in great apes and man (de Steffano et al 1986; Ferrucci et al., 1987). These are specific banding patterns induced in metaphase chromosomes after treatment with the restriction enzyme AluI. This enzyme digests

chromosomal DNA directly in relation to the presence or absence of its recognition sequence along the chromosomal axes and the induced bands seem in all species tested to mirror the distribution patterns of their of highly repetitive DNA sequences (satellite DNAs) (Bianchi et al., 1985; Mezzanotte, 1986). In the human chromosome 1 there was a dark band on the long arm, adjacent to the centromere which was present in the gorilla although smaller but was absent in the chimpanzee and orangutan; these patterns suggested qualitative differences (the presence or absence of specific DNA sequences resistant to AluI digestion) for chromosome 1 of man and the great apes contrary to classical banding methods which had revealed only quantitative variations in the heterochromatic component of this chromosome (Ferrucci, 1987).

4.4.2 The Y chromosome of the great apes

4.4.2.I Cytogenetic findings

Despite numerous comparative cytogenetic studies in man and the great apes, the Y chromosome of the great apes was until recently only poorly characterised. This is exemplified in the report of the standing committee of chromosome nomenclature [ISCN (1985)] shown in figure 3, which fails to illustrate the appearance of the Y chromosome of gorilla, chimpanzee and orangutan stained to show Q- and G-banding. This schematic presentation gives the false impression that the gorilla Y chromosome is basically similar to the human Y. In fact, the gorilla Y has a more complicated banding pattern than the human which in turn is more complicated than that of the chimpanzee or orangutan (Miller 1977).

Two other diagrams of the great ape Y chromosome are available

from subsequent studies; one showing G-bands (Yunis and Prakash 1982; see figure 62A), and one showing early replicating bands (Weber et al., 1986; see figure 62B). Both diagrams were based on prometaphase chromosomes and although not directly comparable - they provide different information in terms of banding - they should be consistent as regards the relative sizes of the ape Y chromosomes. However, there is a remarkable difference regarding the orangutan Y chromosome: in the Yunis & Prakash (1982) study this chromosome is clearly submetacentric with a centromeric index (ratio of the length of the short arm over the total length of the chromosome) of 0.29 whereas it looks more metacentric with a centromeric index of 0.38 according to Weber et al., (1986). The centromeric indices were determined by ruler measurements of the arms of the chromosome in question as they appear in the published diagrams under the assumption that in both diagrams chromosomes were drawn to scale.

As reported in section 3.8.1 a difference was noted in the size of the Y chromosome in the two orangutans studied here which was apparently due to a difference in the size of the light G-band in proximal long arm. Although the possibility that the smaller Y chromosome in orangutan B is the result of a chromosomal aberration (i.e. deletion) cannot be ruled out, an alternative explanation would be that proximal long arm of the orangutan Y chromosome is polymorphic in size; such a suggestion is plausible if we consider that proximal long arm of the orangutan Y is heterochromatic as it stains darkly with C-band (Seuanez 1979), and that polymorphic variations of heterochromatin are commonly found in man and the great apes (Yunis & Prakash 1982). Such a polymorphism could also account for the difference noted in the diagrams mentioned above regarding

this chromosome. On the other hand, as aberrant Y chromosomes have been reported in the chimpanzee and pygmy chimpanzee (Weber et al., 1988) unless a large number of animals are studied this polymorphism can only be speculative.

The diagram of the great ape Y chromosome proposed by Weber et al., (1986) although based on the most detailed cytogenetic investigation of the great ape Y chromosome reported todate, cannot be of wide use as it mainly shows early replicating bands, a banding method not commonly used. The G-banding pattern of the gorilla Y proposed by Yunis & Prakash does not agree with the pattern observed in this study and that in previously reported gorilla karyotypes [ISCN (1985); Miller et al., 1974; Seuanez 1979]. Additionally, there is a difference regarding the G-banded pattern of the oranutang Y between the Yunis & Prakash and own observations but this could due to different degree of chromosome condensation as the former study was based on prometaphase and the latter on metaphase chromosomes.

Thus, a diagram of the ape Y chromosomes showing G-bands in metaphase is proposed (figure 62C) which is consistent with the banding patterns seen here and in previously published ape karyotypes. As this diagram was not based on measurements the sizes of gorilla and chimpanzee Y chromosome are drawn according to Weber et al (1986) while the size of the orangutan Y is drawn according to Yunis & Prakash (1982). It should be also noted that the proposed G-banded pattern for the chimpanzee Y could not be compared with that in previously published karyotypes of the chimpanzee as G-bands were not clearly seen in these studies.

According to this diagram, there is a large dark band below the

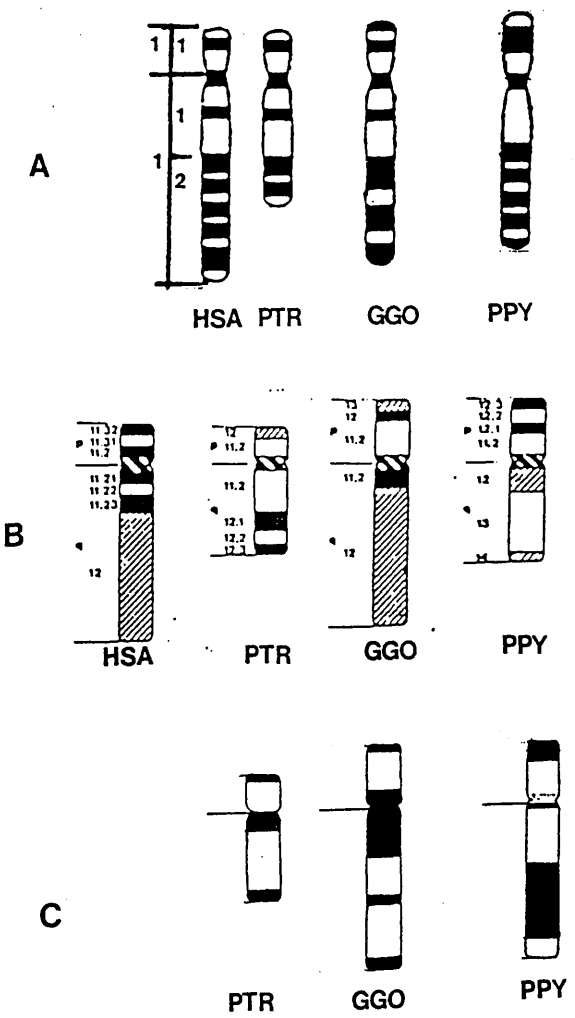


Figure 62 Diagrammatic representation of the great ape Y chromosome
(A) after Yunis & Prakash, 1982
(B) after Weber et al., 1986
(C) this study

centromere at proximal long arm of the gorilla Y, a dark band in the middle of the long arm -not present in all metaphases studied possibly due to different degree of condensation of the chromosomes- and finally a dark band at distal long arm. In the orangutan Y, the light band in proximal long arm is proposed to be polymorphic thus accounting for the difference in the size of this chromosome noted in orangutan B in the present study and most probably in the diagram proposed by Weber et al., (1986).

4.4.2.II Molecular findings

The in situ hybridisation findings presented in this study from comparative mapping studies of GMGY10 and GMGY7 in the great apes confirmed that these probes are located on the Y chromosome of the gorilla, chimpanzee, and orangutan (GMGY10) and orangutan (GMGY7) as was previously shown (K Kwok personal communication) by Southern analysis. The localisation of GMGY10, which in the human is located at Yp, to proximal long arm of the gorilla Y chromosome provides further evidence to the recent view that rearrangements have occurred in the euchromatic part of the Y chromosome of the four species which suggested to share a basic homology. Earlier banding studies had documented a difference in the heterochromatic region of the Y chromosome (for review see Seuanez 1979) but as regards the euchromatic parts insuffient information was available although it has been suggested that these parts of the Y chromosome man and the great apes share a basic homology, (Yunis & Prakash 1982), However, Weber et al., (1986) using a variety of banding methods demonstrated that structural alterations have caused enormous differences in both the euchromatic and heterochromatic regions of the Y chromosome. This

was more obvious with the early replicating pattern (RBA bands): the fully expressed R-type replication pattern of the Y chromosome consists of four early replicating bands in man, two on Yp and two on the proximal portion of Yq, two in the chimpanzee both on the distal long arm, two in the gorilla Y, one in the short arm and the other in the proximal region of the long arm, and finally two bands in the orangutan located in the short arm. Differences were also observed in the Q-, DA/DAPI and C-banding patterns of the short arm and proximal long arm of the ape Y chromosome. The differences between the Y chromosome in the four species were documented further by the localisation in the great apes of a repeated element, DXYZ2, specific for the human pseudoautosomal region that is the pairing region between the X and Y chromosomes. Using in situ hybridisation, DXYZ2 was localised to distal short arm of the orangutan Y (same region as in the human), distal long arm in the chimpanzee Y, and proximal short arm of the gorilla Y (Weber et al, 1987). The localisation in GMGY10 in different parts of the Y chromosome in man and gorilla demonstrates further differences in the Y chromosome of the two species.

That rearrangements have taken place on the Y chromosome during evolution has also been suggested on the basis of molecular findings that human X and Y homologues sequences are present only on the X chromosome of the great apes (Page et al., 1984; Mitchell 1989). which suggested that a transposition of DNA sequences from the X to the Y must have occurred after the divergence of orangutan, gorilla and chimpanzee from the common stock.

It is interesting to note that GMGY10, GMGY7 and the previously reported sequence DXYZ2 (Weber et al., 1987) are the only repeated

human Y-specific sequences thus far shown to be conserved in the great ape Y chromosome. The human male specific 3.4 kb Hae III sequences (DYZ1) which accounts for 40% of the DNA of the human Y chromosome were not found within the Y chromosome of apes but they were present in autosomes (Kunkel and Smith, 1982). Related sequences to the 2.47 kb repeated sequence which constitutes 20% of the DNA of the human Y chromosome were found in the gorilla and chimpanzee (Cooke et al., 1982) but not on the Y chromosome. Furthermore, the human Y alphoid repeat was not seen in gorilla, orangutan or chimpanzee male DNA (Wolfe et al., 1985). This conservation could argue in favour of a functional importance of GMGY10 and GMGY7 as has been suggested for DXYZ2 (Weber et al., 1987) or the satellite DNAs (Jones 1977). Evolution proceeds by the occurrence and fixation of quantitative or qualitative changes in DNA. As the great majority of genes are found on homologous chromosomes in the different species (Lalley & McKusisck, 1985), it may as well be that the DNA changes expected to produce evolutionary modifications may not be found at the level of the structural genes (Wilson et al., 1974) but that of the repetitive DNA.

The short arm of the orangutan Y chromosome is clearly larger than that of the human. In order to explain this difference Yunis & Prakash (1982) suggested that the orangutan Y chromosome differs from the human by a pericentric inversion (p11.32q11.23). However, the finding presented here that both DNA probes, GMGY10 and GMGY7, are located in the short arm of the Y chromosome both in man and orangutan is against the above suggestion.

4.4.3 General comments on comparative mapping

If we look at the chromosomal distribution of the human $tRNA^{Glu}$ gene and GMGY10 in the great apes as presented in this study in relation to accepted phylogeny of the Hominidae we are confronted with a problem in interpretation. With both DNA sequences it was the gorilla which showed a different hybridisation pattern to that observed on one hand in man and on the other hand in the chimpanzee and the orangutan. These findings are not isolated cases. GMGY7 was found to be Y-specific in chimpanzee and orangutan as in man, but not in the gorilla (Kenny Kwok, personal communication). Furthermore, DNA sequences that recognise a Yq/Xp22.3 homology were found on the X and Y chromosomes of the orangutan and chimpanzee but only on the X chromosome in the gorilla (Mitchell 1989).

The above data taken together would suggest that gorilla diverged from the ancestral common stock prior to orangutan. This however conflicts with morphological, paleontological, cytogenetic and molecular evidence that gorilla is more related to man than orangutan (Yunis and Prakash, 1982). Evolutionary theory of primates argues that the orangutan diverged first from the common ancestor of man, then gorilla and finally chimpanzee (Sibley & Alquist, 1987). Therefore an acceptable explanation would be the loss of DNA sequences GMGXXY3 and GMGXY19 as was postulated by Mitchell (1989), GMGY7 and the second locus for the human $tRNA^{Glu}$ gene in the gorilla lineage. Additionally, a chromosomal rearrangement (pericentric inversion) must have transferred GMGY10 to the long arm of the gorilla Y chromosome.

The above suggestions are made within the limitation of comparative mapping in the great apes, the few animals available for

study. Further comparative mapping studies using different DNA probes and different specimens should clarify if the different hybridisation patterns noted above in the gorilla as compared to man and orangutan apply to all the chromosomes of the gorilla, or are restricted to the specific regions of chromosome 1 and the Y chromosome homologous to the probes used here.

4.4.4 Chromosome heteromorphisms in the great apes

A number of chromosome abnormalities have been reported in higher primates, including sex chromosome abnormalities, autosomal trisomies and pericentric inversions (for review see Miller 1977).

One type of rearranged chromosome corresponding to human No.12 has been described in the orangutan (Dutrillaux et al., 1975; Turleau et al. 1975; Seuanez et al., 1976a and b; Seuanez 1979). In the latter study, the variant chromosome 9 was found in 13 out of 29 animals studied, 10 heterozygous and 3 homozygous carriers. A similar rearrangement was found in orangutan B studied here in the heterozygous condition. This must therefore be a common type of variant chromosome with a high incidence in the population of the orangutan. One suggestion is that it was produced by an inversion within an inversion (Seuanez et al., 1976b). Since the rearranged chromosome was found in orangutans from both Borneo and Sumatra, both forms of chromosome 9 appear to have existed in the orangutan population before the two groups were isolated geographically, perhaps 8000 years ago (Seuanez 1979).

Variant chromosomes 2 have also been described in the orangutan. In the previously mentioned study of 29 specimens two different kinds

of chromosome 2 were observed: one carried by all Bornean animals studied (9 specimens) in the homozygous condition consisted of a subtelocentric chromosome 2 with a centromeric index=10.2 with one G-band region in its short arm and two G-band regions in the subcentromeric region. In all animals of Sumatran origin studied (16 specimens), the homologue to this chromosome corresponded to a more metacentric chromosome 2 with two G-band regions in its short arm and one in the subcentromeric region. Either type of chromosome 2 could be derived from the other by a pericentric inversion. Seuanez (1979) suggested that each chromosome type has become fixed in each subpopulation and thus each chromosome should be designated as the "Bornean" and "Sumatran" chromosome 2 in the orangutan and that it might be prudent to avoid matings between Bornean and Sumatran orangutans in captivity and to maintain separate breeding for the two types.

The finding of inversions but not translocations in the orangutan is especially interesting as it shows that pericentric inversion involving euchromatic regions is a mechanism by which new chromosome types have been formed in this species and supports the view that inversion, but not translocation, was of importance as a mechanism for evolutionary divergence of man and the great apes from the common stock (Seuanez 1979). In contrast, common pericentric inversions in man are small and confined to heterochromatic regions. Pericentric inversions that involve euchromatic regions have been reported but often are associated with pathological conditions.

It is of interest to note that chromosome 3 in man is involved relatively often in pericentric inversions with breakpoints at 3p25 and 3q21 or 3q25 (Schinzel 1984). Human chromosome 3 is homologous to

orangutan chromosome 2 and according to Seuanez (1979) it can be derived by the Bornean type of orangutan chromosome 2 by a single pericentric inversion p11-q21 (see figure 63). The proposed breakpoint of this ancestral inversion in the short arm of PPY2 (Bornean) coincides with the breakpoint in the pericentric inversions in HSA3 observed today. In a pericentric inversion]inv(3) (p25q21)Ω presented by Allderdice et al., (1975) the inverted chromosome 3 appears remarkably similar to the Sumatran orangutan chromosome 2. Should Seuanez (1979) have been wrong and the human chromosome 3 has been derived from the Sumatran type orangutan chromosome 2 then the inversion observed in man today could represent a reverse mutation.

4.5 CONCLUSIONS

In situ hybridisation for Human Gene Mapping

In situ hybridisation is increasingly being used to determine the chromosomal location of newly cloned DNA sequences. In table 45 it can be seen that in the last two years the number of human autosomal loci mapped by in situ hybridisation has doubled and in situ hybridisation has now the second position among other methods of gene mapping. This is a remarkable progress considering that the first localisation of single copy gene using this method was reported in 1981. The value of in situ hybridisation in human gene mapping was demonstrated in this project by the mapping of nine anonymous DNA sequences to specific chromosome regions. These sequences were previously mapped in this department using somatic cell hybrdis. In situ hybridisation was more informative as it defined autosomal homologies or homologies between extended chromosome regions that were not clarified by somatic cell hybrids analysis. Thus, the short

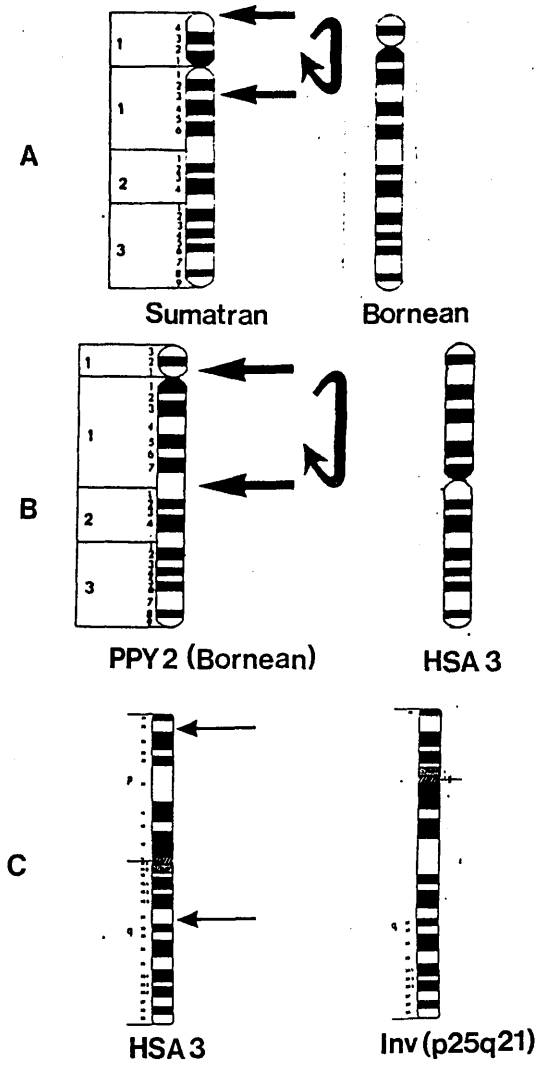


Figure 63 Pericentric inversions in orangutan and man

A) Chromosome 2 in orangutan Sumatran and Bornean. The Bornean can be derived from the Sumatran by a pericentric inversion (curved arrow) (after Seuanez et al., 1979)
B) Derivation of chromosome 3 in man (HSA 3) from the Bornean chromosome 2 in the orangutan by a single pericentric inversion, proposed by Seuanez et al., (1976)
C) Pericentric inversion in human chromosome 3 (p25q21) reported by Allderdice et al., (1975). A reverse mutation?

Table 45 Number of Autosomal Loci Mapped by Several Methods

August 15, 1987

Method	No. of loci mapped
Somatic cell hybridisation	- 628
Family linkage study	- 285
In situ hybridisation	- 274
Dosage effect	- 102
Chromosome aberrations	- 73
Restriction enzyme fine analysis	- 43
Homology of synteny	- 62
Radiation induced gene segregation	- 18
Others	- 89
Total (many mapped by 2 or more methods)	1574

February 13, 1989

Method	No. of loci mapped
Somatic cell hybridisation	- 889
In situ hybridisation	- 444
Family linkage study	- 382
Dosage effect	- 132
Chromosome aberrations	- 99
Restriction enzyme fine analysis	- 109
Homology of synteny	- 76
Radiation induced gene segregation	- 18
Others	- 128
Total (many mapped by 2 or more methods)	2277

Taken from McKusick, The Human gene Map, August 15, 1987; February 13, 1989

arm of the human Y chromosome appears to share homologous DNA sequences with the short arm of 9p (9p22.3-pter) with possible implications of 9p in sex detrmination. The Y centromeric region shares homologous sequences with the centromeric region of chromosomes 15 and 22. Homologies were also revealed for the centromeric region of the acrocentric chromosomes (13, 14, 15, 21, 22). These homologies may be relevant to the frequent involvement of chromosomes 15 and 22 in Y: autosomal translocations on one hand and on the other hand the frequent involvement of the acrocentric chromosomes in Robertsonian translocations.

In situ hybridisation can extend current methods of cytogenetic analysis as was demonstrated by the identification of the origin of a tiny marker chromosome in two cases of 45X/46,X +mar males and as demonstrated by the identification of a Yq:15p translocation can provide a powerful and reliable tool in clinical diagnosis.

XX males

The localisation of Y-specific sequences to distal short arm of the X chromosome in 8 XX males provided convincing evidence that an X-Y interchange at paternal meiosis has transferred part of the short arm of the Y chromosome to distal short arm of the X. This could not be demonstrated in one case (XX male KS) and further work is required to clarify wheter or not this XX male is the result of an XY interchange.

In situ hybridisation for Comparative Mapping

Comparative mapping studies in the great apes of a human $tRNA^{Glu}$ gene confirmed the homology between distal short arm of the great ape

chromosome 1 and distal short arm of this chromosome in man, proposed by banding studies, at the DNA level. However, findings presented here and those reported by other investigators suggest that differences also exist between chromosome 1 of man and the great apes that earlier comparative banding studies were not able to detect. According to the data presented here and these of other investigators changes must have occurred in the euchromatic parts of the Y chromosome in the four species contrary to earlier suggestion based on banding studies that these parts of the Y chromosome share a basic homology. Thus, although comparative banding studies have generated a wealth of information regarding chromosome phylogeny and have established the homologies between the karyotypes of man and great apes the availability of molecular probes allows a rigorous investigation of chromosome evolution which can corfirm or infirm the proposed homologies at the DNA level.

Biotin-labelling

The method for biotinylated in situ hybridisation used in this project was found to be superior to isotopic in situ hybridisation in terms of speed, convenience and topographical resolution. Moreover, it was sufficiently sensitive to map a 3.2kb single copy sequence. The sensitivity of this technique has been reported to be in the range of 1kb, which is nearing the current sensitivity of the radioactive techniques (0.5kb). Future work should therefore be directed in establishing the sensitivity limits of this method. In view of the advantages and increased sensitivity of non-isotopic methods it is predicted that isotopic labelling will become redundant both for the purposes of gene mapping and clinical diagnosis.

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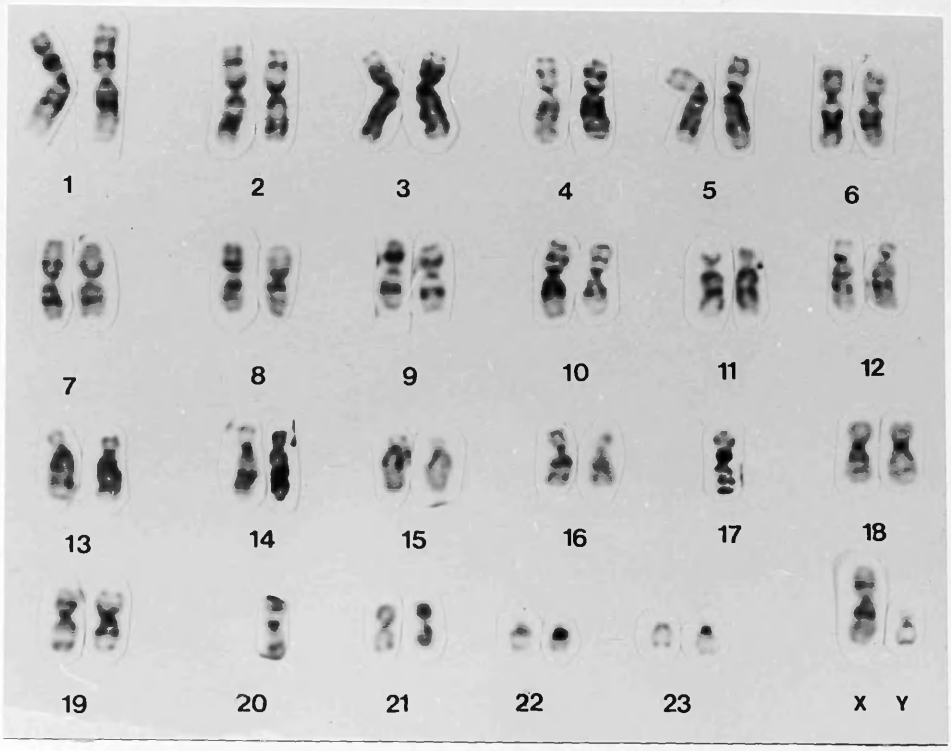
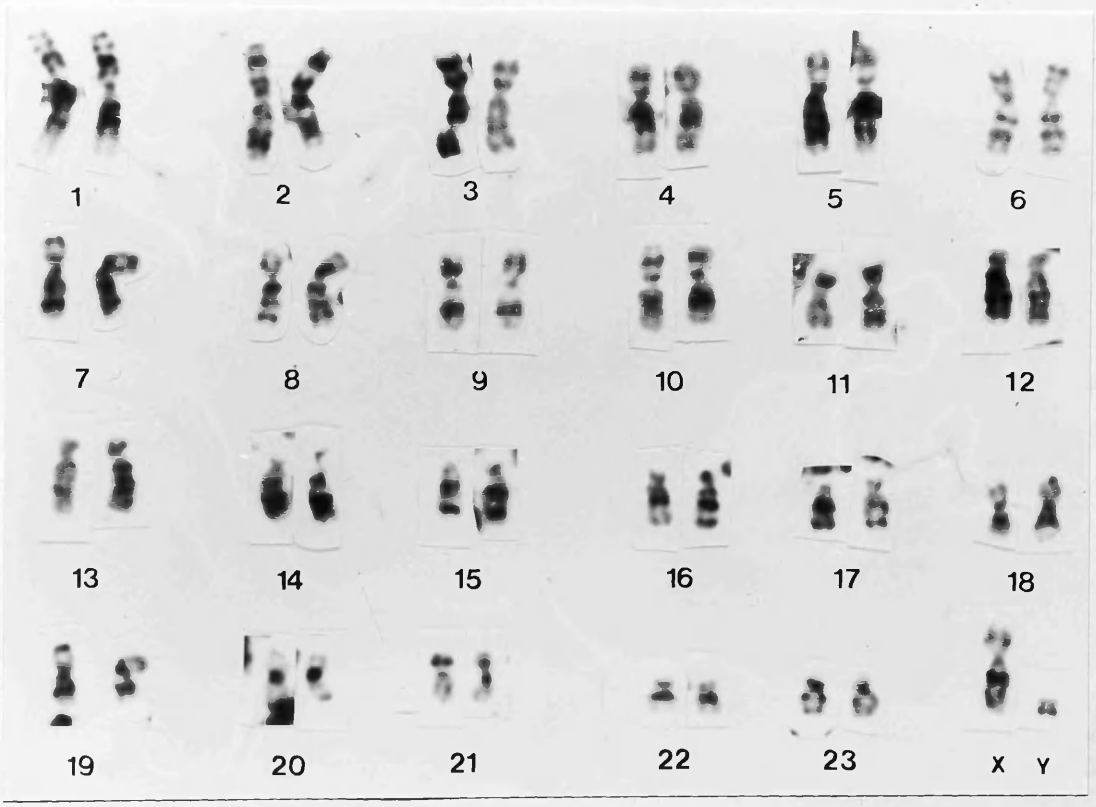
APPENDIX





Lipsol banded karyotypes of the great apes
(A) Gorilla
(B) Chimpanzee