THE ISOLATION AND LOCALIZATION OF HUMAN GENE SEQUENCES AND THEIR USE IN THE DIAGNOSIS OF CHROMOSOMAL ABNORMALITIES

By

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ABSTRACT

Normal human Y chromosome-specific gene sequences of repetition frequency upto 100 copies per genome were isolated by extensive hybridization of radio-labelled total male DNA to an excess of unlabelled female DNA. These sequences represent an average of 0.84% of the total male haploid genome. Similarly, human X chromosome-specific gene sequences repeated upto 300-fold were isolated by extensive hybridization of radio-labelled mouse-human hybrid cell DNA to a vast excess of unlabelled parent mouse DNA. Both Y and X chromosome-specific gene sequences were contaminated with degraded labelled and unlabelled DNA sequences. The Y chromosome-specific gene sequences were found to be derived mainly from the distal fluorescent segment (Yq12 region) of the Y chromosome, by *in situ* hybridization to normal 46,XY male chromosome preparations. These sequences were also found to be distributed mainly on the Yq12 region of a normal male with 46,XYq+ karyotype.

The nature of the Y chromosomes from a 48,XYYY male with mental retardation was studied by *in situ* hybridization to the Y chromosome-specific gene sequences. This revealed similar localization to that observed on a "normal" Y and a Yq+ without any significant difference between sequence distribution on any of the Y chromosomes. It is concluded that the Y chromosomes in this individual are equivalent to each other and to a "normal" Y chromosome in their hybridization behaviour.

Radio-labelled Xlr101 plasmid DNA containing *Xenopus laevis* 28S and 18S ribosomal genes was used to study the nature of an extra marker chromosome in two phenotypically normal women with 47,XX + marker, by hybridizing the plasmid *in situ* to chromosome preparations. The results were compared to observations of silver staining, banding techniques, and satellite association of the chromosomes. It was concluded that the marker chromosome in each case, introduced extra dosage of functioning ribosomal genes without any phenotypic abnormality. Cloned gene sequences can, therefore, be used as tools for assisting in the diagnosis of gene-specific chromosome abnormalities.

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CONTENTS

List of Figures

Continued

LIST OF TABLES

SECTION I

1.1. GENE AND GENE SEQUENCES

1.1.1. The Gene Concept:

a) General view:

The traditional concept of genes is that they are the unit determinants of biological inheritance which are arranged linearly on chromosomes and occupy specific positions or 'loci' (Stevenson & Clare Davison, 1976). They were first called 'genes' in 1909 by Johannsen, referring to what Gregor Mendel termed 'factors of inheritance' in the 1860's. Alternative forms of genes which can occupy the same gene locus (or pair of loci) are termed 'alleles'. The gene was conceived of as the ultimate unit of heredity in that it was the smallest unit which could exert any individual function; that it was the smallest unit at which mutation could occur; and that crossing-over in meiosis (exchange in genetic material between chromatin from the opposite chromosomes of a pair) could occur between genes, but not within genes.

This concept could readily be justified by the great majority of the experimental evidence and it still remains adequate for a high proportion of experimental work with multicellular organisms and in human genetics. However, following the new developments in bacterial genetics and an understanding of the chemical nature of the hereditary material, it is clear that this concept of an all-

purpose unit of heredity is no longer tenable. The genes, chemically, are formed of double-stranded, spirally arranged deoxyribonucleic acid molecules (DNA) in which genetic information is stored by specifying the sequences of amino acids that make up a polypeptide chain in an enzyme or structural protein. However, a finite length of nuclear DNA does not correspond to a gene in the traditional sense. Thus a length of DNA may ultimately be responsible for specification of a polypeptide chain or a protein, but changes or mutations at any point in that length may cause the specification of a different amino acid in the chain. In other words, mutation can take place within what corresponds to a 'functional' gene. Again, crossing over, with exchange of material can occur within such DNA sequences in chromatids, i.e. within a 'functional' gene.

As each individual results from a zygote formed by the union of a paternal and maternal haploid gamete, he (or she) receives one of each chromosome pair from each parent, i.e. two genes which occupy a pair of gene loci on each of the two homologous chromosomes. The haploid human genome consists of some 3×10^9 base pairs which is equivalent to 3 picagrams of DNA, or 10^9 potential amino acid codons (see Lewin, 1974). Only a small proportion of the DNA codes for proteins. The rest may have other functions, possibly in control of gene function, and/or having structural significance in the chromosome which might, to some extent, be independent of precise nucleotide.

b) Gene-Cytoplasm Relationship:

Genetic information is carried in the genes in the chromosomes by a code made up of the four nucleotides of which DNA is composed. Each amino acid is represented in this code by several alternative sequences of three nucleotides. The information in DNA is transcribed to a molecule of RNA, called messenger RNA (mRNA). This subsequently becomes associated with ribosomes in the cytoplasm and acts as a template on which amino acids are assembled in the correct order by adaptor tRNAs to form proteins. In this way the information in the genes is transcribed and translated into the sequences of protein molecules.

Ribosomal RNA and tRNA are also made in the nucleus and transported to the cytoplasm to participate in protein synthesis. Other kinds of RNA are made in the nucleus but are not transported to the cytoplasm; their function is not known. Besides the movement of RNA from nucleus to cytoplasm, there is movement of some proteins, such as histones, from cytoplasm to nucleus.

1.1.2. The Gene Sequences:

In order to define and understand the gene sequences, the re-association profiles of that genomic DNA should be followed, i.e. the Cot curve (Cot is a parameter denoting concentration of the nucleotides in moles times seconds per litre, so that a Cot of 1 mol X second/litre results if DNA is incubated for 1 hour at a concentration

which corresponds to an optical density of about 2 at 260 nm per ml (Britten & Kohne 1968). The kinetics of reassociation of an organism's DNA provide a useful tool in its characterisation and an important first step in the detailed analysis of its component families (Rice, 1970).

1.1.2-1. Renaturation of DNA:

When DNA in solution is heated to 100°C, it separates into its two complementary strands. On reassociation the stable duplexes formed at different Cot values can be detected by analysis by calcium phosphate (hydroxyapatite) column chromatography, utilizing the fact that single and double stranded DNA bind differently to such a column. The use of hydroxyapatite for the fractionation of nucleic acids has been demonstrated by Main and Cole (1957), Main et al. (1959), Bernardi (1961, 1962, 1965) , Walker and McLaren (1965) and Miyazawa and Thomas (1965).

The non-reannealed single stranded material can be separated from the double-stranded by washing the column with low and then high concentration of phosphate buffer successively; the single stranded DNA elutes at lower salt concentrations.

The percentage of the DNA reannealed at each point is plotted against its Cot value. A reannealing curve is drawn from which the transcription of different sequence components of the genome can be estimated from the Cot curve according to the degree of the repitition of the DNA.

The process of reannealing of DNA molecules starts by the formation of one or a few correct base pairs at certain points along the two strands. This process which is called "nucleation" is followed by "Zipping" when the base pairs will form duplex (Wetmur and Davidson 1968). The parameters which affect the rate of reassociation and which must be controlled include the temperature of the reassociation process, the size of the DNA fragments, the concentration of DNA in solution, the ionic strength of the solution and the time allowed for the reannealing process (Britten et al., 1973).

1. The temperature of the reassociation process:

At very low temperatures ($4^{o}C$) reannealing is much reduced in rate, while if DNA in solution is heated to above the Tm (melting temperature*) then cooled quickly, it will remain single-stranded. Once the temperature is below the Tm then the DNA starts to renature. It was found that the optimum temperature for following re-association is $20-25^{o}C$ below the Tm, i.e. at $60-65^{o}C$ for human DNA.

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*Tm (the melting temperature) of a DNA is the temperature at which half of the maximal hyperchromicity is produced during the process of denaturation of the DNA which means the temperature for half-strand separation (Britten et al., 1973). It depends on the G-C content of the DNA, ionic strength of the solution and the nature of the DNA.

a) The G-C content of the DNA:

Since there are three hydrogen bonds between the G (Guanine) and C (Cytosine) bases on the two complementary strands of the DNA, and only two bonds between T (Thymine) and A (Adenine), therefore more energy is required to separate the G-C and therefore a higher Tm is characteristic of DNA of higher G-C content.

b) Ionic strength of the solution:

At high ionic concentration, the Tm is raised, and vice versa.

c) Nature of the DNA:

Homogenous DNA like viral DNA, has a sharp Tm while DNA of heterogenous sequence melts over a wider temperature range.

2. The size of DNA fragment:

The ideal size for reannealing DNA would be about 400 nucleotides in length (Britten et al., 1973),which could be obtained by shearing high molecular weight DNA. DNA of longer sequences contain a mixture of unique and repeated sequences, and therefore they will reanneal too fastly due to the rapid reannealing of their complementary strands. Short strands are unstable and it is more difficult for them to find their complementary partners to reanneal to. Moreover, the rate of reassociation is inversely proportional with the square root of the molecular weight of the reannealing DNA in solution.

3. The concentration of the DNA in solution:

Higher concentration results in higher probability of reannealing for two complementary strands, and thus the rate of reannealing increases.

4. The ionic strength of the solution:

Since the DNA is a highly negatively charged molecule at neutral pH, it is likely that two strands would repel one another and therefore it is necessary to mask such repulsion by a salt solution during the process of hybridization. The reassociation is increased when the monovalent cation concentration increases at a particular temperature (Britten et al., 1973).

5. Time allowed for the reannealing process:

With increasing time, the proportion of DNA reannealing at a given temperature is seen. The term Cot has been defined for the study of reannealing of DNA and for the formation of hybrids, because if renaturation is allowed under ideal conditions, two DNA samples of identical concentration should take different times to reanneal in proportion to their genomic complexity.

In general human, as other eukaryotic, DNA can be divided into three components:-

1) Highly repetitive (reiterated) sequences that reassociated at very low Cot values, and fail to hybridize with cellular RNA. This fraction consists of more than 10^6 repetitions of rather short sequences of the order of 100-nucleotide long.

2) Mid-repetitive sequences that reassociate to a Cot 10-100 and are present in from 10^3 - 10^5 repetitions; the range of frequencies depends upon the organism.

3) Unique sequences that are recovered in duplex from after renaturation at Cot values greater than 1000. They are present in only one copy or a very few copies per haploid genome (i.e. low repetitive sequences).

1.1.2-2. Repetitive Gene Sequences:

It was argued by Britten and Waring (1965) that some nucleotide

sequences were often repeated in the DNA of vertebrates, a hypothesis that was supported by the observation that 10% of the DNA of the mouse reassociated extremely rapidly. This fraction identified as "mouse satellite" was shown by Waring and Britten (1966) to consist of a million or so copies of approximately 300 nucleotide long. It was found later that repeated nucleotide sequences occur widely and probably universally in the DNA of higher organisms (Britten and Kohne, 1966, 1967, 1968). The repetitive sequences include sequences like the ribosomal and transfer RNA genes.

1.1.2-3. Satellite DNA:

Early work by Meselson et al. (1957) demonstrated a pronounced skewed distribution towards the heavier side in a neutral CsCl (caesium chloride) density gradient for calf thymus DNA. Later a minor light component was found in mouse DNA by Kit (1961, 1962), who also noted a heavy component in guinea-pig DNA, as did Sueoka (1961) in the calf. Because of the political and astronautical climate of that period, these minor fractions were termed "satellite DNA". Later, further fractionation of mouse DNA using the hydroxyapatite procedure revealed that 16% of the DNA reassociated more rapidly than small virus DNA, and this fraction was identified as identical to mouse satellite DNA (Waring and Britten, 1966). Mouse satellite DNA has the following characters (Flamm et al., 1969):-

1) It has a defined frequency, about a million copies of a sequence approximately 300 nucleotides long.
2) It gives discrete bands in a caesium chloride equilibrium density

gradient, due to its particular nucleotide composition and its occurrence in clusters.

3) When reassociated after denaturation (as well as native) it exhibits a narrow range of melting temperature.

4) It has a striking base asymmetry in the composition of its strands, which distinguishes it from most of the DNA of the mouse.

5) It is not transcribed in at least some circumstances. Pardue and Gall (1969) and Jones (1970), found that the mouse satellite DNA was localised close to the centromere of mitotic chromosomes by *in situ* reannealing of labelled single-stranded mouse satellite DNA to metaphase figure of mouse cells and observing the centromeric location of autoradiographic grains. It was found, by neutral caesium chloride gradient studies that the human satellite DNA are of at least four types Viz. satellites I, II, III and IV and were classified according to their densities in the gradients (Corneo *et al.*, 1968, 1970, 1971 and 1972) as shown by table 1.1.

1.1.2-4. Function and role of repetitive sequences:

The sequences of RNA transcribed in eukaryotic cells appear to correspond only to the unique and intermediate sequences but not to the highly repetitive regions, for Flamm, *et al.* (1969) found no case where an excess of RNA extracted from the liver, spleen or kidney of the mouse hybridizes to satellite DNA. This situation also applies in humans, adding to the puzzle of their possible function,

TABLE 1.1.

Types of Human Satellite

Identified by Neutral Caesium Chloride Gradients

TABLE 1.1.

HUMAN SATELLITE	CsCl Density g/ml	% of the total genome	Reference
I	1.687	0.5%	Corneo et al., 1968
II	1.693	2%	Corneo et al., 1970
III	1.696	1.5%	Corneo et al., 1971
IV	1.7	2%	Corneo et al., 1972

about which there is much speculation but nothing established.

Britten and Davidson (1971) have suggested that the highly repetitive sequences . (including the satellite DNA) may represent a DNA which is not in genetic use but which may be utilised in further evolution, or may be elements recognised by molecules which control gene expression. Highly repeated sequences might also provide sites at which transcription or replication is initiated (see Lewin, 1974). However, these suggestions would imply that satellite sequences should be found at many chromosomal locations, rather than only at centromeres.

The centric concentration of the satellite DNA (Jones, 1970; Pardue and Gall, 1970) is also potentially significant in relation to the suggestion that it may be a factor helping the chromosomes to pass successfully through the movements demanded of them at cell division in resisting breakage when chromosomes are pulled to the poles by the spindle fibres during mitosis (Walker, 1971) or in meiosis as suggested by Walker et al. (1969) assisting in the recognition of the homoglous centromeres and folding of the chromosome fibre.

1.1.2-5. The intermediate repetitive gene sequences:

They are present in from 1000-100000 copies and located in all the regions of the chromosome as shown by in situ hybridization technique. They hybridize to RNA, but the nature of the sequences

is not entirely clear. They consist of families each comprising many sequences which are related to varying extents in that each consists of a set of nucleotide sequences which have sufficient similarity with each other to renature, but which are not identical, and therefore renaturation is accomplished by the association of sequences comprising different members of a family. The renatured duplexes are not perfectly matched as is shown by the low thermal stability. Duplexes are formed by the association of strands which are derived from related but not identical sequences (see Lewin, 1974).

1.1.2-6. Unique Sequences:

In humans 40% of the total genomic DNA is repetitive to some extent. The remainder is unique sequences, although it is very difficult, using the current techniques, to establish this "uniqueness", because on the one hand differences between closely related sequences may not be readily detected, and on the other the kinetics of reassociation cannot distinguish easily between, say, one and two copies.

The unique gene sequences are present in only one or very few copies per haploid genome. They are composed of perhaps 10^6 different globin gene-sized sequences. They bind to mRNA (Gelderman et al., 1969, 1971) and in DNA-DNA reassociation experiments, sequence homologies can be detected between closely related species (Britten and Kohne 1967, McConaughy and McCarthy, 1970). The hybrids and duplex molecules formed from this kind of DNA have a higher stability than those

made from most intermediate repetitive fractions.

The unique sequences include many genes coding for proteins but it is not known what proportion of the unique sequences codes for protein and whether it has any other functions. There are some 50,000 structural genes postulated for man, which are those determining the basic amino acid structure of:-

1) All the enzymes of intermediary metabolism.
2) All the structural proteins such as the collagens.
3) All the proteins of special function such as the haemoglobins and the immunoglobulins. They also determine the amino acid structure of many enzymes involved in processing of proteins such as procollagen peptidase, glycosyl transferase, lysyl oxidase and proline lysine hydroxylases, necessary for formation of collagen fibres.

The single copy sequences are interspersed with spacer-DNA of unknown function which could be merely structural and may have been amplified to form the satellite sequences during evolution. It is thought that intermediate repetitive sequences partly code for related polypeptides and partly control the expression of genes and that the satellite DNA has an important function in chromosome folding and pairing (Walker, 1971).

SECTION II

1.2. THE HUMAN CHROMOSOMES

1.2.1. The human karyotypes:

It was possible to study chromosomes by the introduction of two modifications of techniques:-

1) The use of colchicine in cell culture to cause an accumulation of cells in metaphase of mitosis, the stage most favourable for counting the chromosomes.

2) The use of hypotonic solutions to produce swelling of the nucleus and separation of the chromosomes. The cells studied may be derived from bone marrow by the usual aspiration technique or from explants of skin or other tissue. Cells grown from the peripheral blood in short-term culture have been particularly useful for family studies and surveys. Use of the mitosis-stimulating properties of phytohaemaglutinin is another technique that has facilitated chromosome study. The chromosomes are studied by light microscopy after appropriate fixing and staining.

Chromosomes are present in 23 pairs in every somatic cell, except for the Y and X in a normal male. One member only of each pair is found in the reproductive cells or gametes (the sperm or the egg) which contain the haploid number (n). After fertilization, one

of each pair of chromosomes in the gamete in each cell of the future individual, is derived from each of the individual parents. Thus each individual inherits two copies of each chromosomes and hence two copies of each gene.

A given gene resides at a specified genetic locus on one particular chromosome. For example, the genetic locus for the Rh blood group is on the short arm of chromosome 1 (Jacobs et al., 1970); at this chromosomal site there are two Rh genes, one on chromosome 1 derived from the mother and the other on the other chromosome 1 derived from the father. When two alleles at the same genetic locus are identical, the individual is a homozygote. When the two genes differ (i.e. two alleles present at the locus) the individual is a heterozygote. Each individual is homozygous at some loci and heterozygous at others.

Identification of individual human chromosomes used to be far from easy by the application of the traditional staining techniques. They were classified into seven groups (A-G), according to centromere position and their size (starting from the largest and finishing with the smallest, plus the sexual chromosomes (X and Y). They are also classified according to number from 1 to 22. Both classifications are used today in the standard nomenclature of chromosomes adopted by the Chicago conference of 1966.

Paris Conference in 1971, recommended a standardized system of designating specific bands and regions on individual chromosomes. Whether stained or not, a chromosome is considered as a continuous series of hands. The arms of the chromosome are designated p (Petit) for short arms and q for long arms.

Both arms are divided into regions by using some of the most consistent and distinct bands together with the centromeres and distal ends of each arm as landmarks. This system is of particular value in describing break points and gene localization.

Modern banding technique in use since 1970, enabled each chromosome to be identified and also enabled various chromosomal derangements such as translocations or deletions to be identified. Those techniques are:-

1. Staining with quinacrine dyes for fluorescent microscopy:

It was noted by Caspersson et al. (1968) that the plant chromosomes, showed specific fluorescent banding pattern after treatment with certain fluorescent derivatives of acridine. Later, Zech (1969) noted that the human Y chromosome showed specially bright fluorescence when examined under an ultra-violet light after staining with quinacrine mustard. Subsequently, it was shown that the number of Y chromosomes could be identified by similar staining in the interphase nuclei of human cell and that the distal ends of the long arm of the Y chromosome, fluoresced intensely throughout all stages from spermatogonia to spermatozoa, besides some fluorescence noted

on large numbers of segments or bands in the autosomes. Study of cells in meiotic prophase confirmed that the short arms of the Y chromosome associated with X-chromosomes at this stage. This phenomenon raises the possibility of crossing over between the X and the Y and the possible transfer of genetic materials between those 2 chromosomes.

Present evidence suggests that quinacrine staining of the Y in cultures of blood lymphocytes, skin fibroblast and amniotic cells is a reliable method for detecting the Y chromosome, and the anomalies associated with its structure (e.g. deletion, translocation... etc.) or anomalies of the number of the Y chromosome.

2. Various modified Giemsa staining methods which yield chromocomes showing a banding pattern not produced by other methods:

Various techniques have been described which produce characteristic bands along the length of chromosomes. These methods are basically performed by either staining with buffered Giemsa stain after a variety of pre-treatments, or by using enzymatic pre-treatment with either RNAse or trypsin before staining with Giemsa. Different methods were described and modified to give various banding patterns.

a) The identification of the G bands:

A whole variety of modifications have been published giving apparently similar results. One of the earliest and simplest of

these methods was described by Sumner et al. (1971) which was referred to as ASG (Acetic/saline/Giemsa) technique. The air-dried chromosome preparations are incubated for 1 hour at 60°C in 2 X SSC followed by washing in deionized distilled water and staining in Giemsa in phosphate buffer (pH 6.8). G-banding patterns with high quality can be made by treating the chromosome preparation with trypsin (as a proteolytic enzyme) followed by Giemsa staining (Seabright, 1972). This method was found to be simple and reliable and consistently gives better resolution than the ASG method in terms of number of bands and sub-bands (Ferguson-Smith, 1974).

b) Identification of the C-bands:

It was shown by Arrighi and Hsu (1971) that the centromeric heterochromatin of the human chromosomes stained more densely with Giemsa than the rest of the chromosomes when the chromosome preparations were pre-treated with sodium hydroxide (to denature the DNA) and incubated in saline or 2 X SSC. They also found that chromosome 1, 9 and 16 have large centromeric heterochromatin segments whereas chromosomes 2,4 and 8 have relatively small amounts. This technique also stains specifically the distal part of the long arm (region q12) of the Y chromosome including the quinacrine fluorescent region. This method, therefore, could be used to study Y-chromosome aberrations and rearrangements.

This C-banding technique was later modified by Bobrow et al., (1972) introducing the Giemsa-11 technique (S bands). This method differentially stains the satellite region of the acrocentric chromosomes (hence "S" bands), the paracentric heterochromatin of the long arm of chromosome 9, much of the Y chromosome and smaller heterochromatic regions in the long arm of chromosome 1 and less consistently in other chromosomes. The largest blocks of chromatin which are stained a brilliant magenta by this method are in chromosome 9 and these are so striking that they can be detected in interphase nuclei, sperm and throughout meiosis. The technique is based on treating the chromosome preparations with Giemsa in 20 parts sodium hydroxide at pH 11.0.

There are various other modifications of those techniques described by Dutrillaux and Lejeune (1971), Bobrow and Madan (1973) and others.

1.2.2. SATELLITE ASSOCIATION OF THE CHROMOSOMES AND THE RIBOSOMAL GENES.

1.2.2-1. The satellite association of the chromosomes:

The satellites of the chromosomes are small circumscribed areas of heterochromatin found on the free ends of the short arms of the acrocentric chromosomes (Group D and G, i.e. chromosomes No. 13, 14, 15, 21 and 22) above short secondary constriction.

It was observed by Ferguson-Smith and Handmaker (1961) that the chromosomal satellites associate with (attach or come close to) each other in metaphase frequently and may associate to specific regions on the other chromosomes. Much evidence has accumulated to correlate the frequency of satellite association (SA) of the chromosomes with the occurrence of non-disjunction and the breakpoints in translocations and therefore the occurrence of trisomies (Ferguson-Smith and Handmaker, 1963; Ford, 1968; Bishum, 1966; Hansson and Mikkelsen, 1974a; Nakagome, 1973). Hansson and Mikkelsen (1974b) found a significantly increased association tendency of chromosome No. 21 in mothers of children with Down's syndrome. Henderson et al. (1972) by in situ hybridization of (^{3}H) - labelled RNA to normal chromosome preparation found that the silver grains were clustered over the short arms of the acro-centric chromosomes, which indicated that those regions are the sites for ribosomal genes. Later in 1974, Evans et al. was able to show, by in situ hybridization

that the satellite stalks, rather than the satellites themselves, were the sites for 28S and 18S ribosomal genes. They also found that the frequency of SA is not well correlated with the amount of rDNA, but other factors had to be considered. It was suggested that the predisposition to breakage and translocation, which may be brought about by the phenomenon of satellite association, is increased when the short arm is enlarged (Ohno et al., 1961; Ferguson-Smith and Handmaker, 1963; Bauchinger and Schmid, 1970; Hamerton, 1971). A significantly higher incidence of enlarged satellites amoung children admitted to a psychiatric hospital was noted by Christensen and Nielsen (1974). On the other hand, it was shown by Lubs and Ruddle (1971) that variations in length of the short arms and satellite regions of the acrocentric chromosomes are simple human polymorphisms without showing any phenotypic effects.

1.2.2-2. The structure of human ribosomal genes:

In the human genome, the 18S and 28S ribosomal genes were found to be 180-220 in number per haploid genome by molecular hybridization technique using labelled rRNA (Scherberg and Rifetoff,1973; Gaubatz and Cutler, 1975; Gaubatz et al., 1976), while in another study (Young et al., 1976) they were found to be around 50 per haploid genome. The differences in the findings were of technical origins. It seems that Young et al.'s estimate (1976) was more accurate as they overcame the technical limitations of the previous authors by using

highly specific radioactive cDNA of human ribosomal RNA. Both the high specific activity and the greater stability of the cDNA means that it can be hybridized to total human DNA in solution for relatively long periods. Further they based their estimates on two separate and independent techniques, a kinetic and a saturation method, which gave similar results for the ribosomal gene number. Ribosomal DNA consists of repeats of 18S and 28S with spacer DNA in between. Arnheim and Southern (1977) found that human ribosomal DNA is heterogenous in structure and that the repeat size for human DNA is 20×10^6 daltons. By Eco R1 digestion and hybridization to radio-iodinated RNA they showed that DNA had three distinct bands, viz. a 14×10^6-dalton band that was shown to contain 18S genes and a spacer, a 4.9×10^6-dalton containing 28S genes. The authors suggested that genetic exchanges may occur between ribosomal repeats on non-homologous chromosomes, which was explained by the phenomenon of satellite association.

1.2.2-3. Silver stainability of the nucleous organizer regions (NORs):

It was shown by Howell et al., (1975) that the nucleolus organizer regions of human D and G group of chromosomes were selectively stained with silver nitrate. The technique was modified by Goodpasture and Bloom (1975) and Bloom and Goodpasture (1976), and combined with trypsin banding (Zankl and Bernhardt, 1977). It was shown by Goodpasture et al. (1976) that the satellite stalks (i.e. the region of the secondary constriction) rather than the satellites

themselves were silver stainable, and therefore the NOR region which was the region previously shown by Evans et al. (1974) to be the sites of nucleolus organization by in situ hybridization. Miller et al. (1976, a and b) showed in studies of somatic cell hybrids, that only the ribosomal genes which are active in RNA synthesis were stained with silver. The frequency of silver-stained nucleolus organizer regions in the acrocentric chromosomes of man was studied by Mikelsaar et al. (1977). They found in lymphocyte cultures that ninety percent of the individuals they studied had a modal number of 8-10 silver-positive NORs per cell, and that a consistent pattern of silver-positive NORs was found in the lymphocytes of each individual. Further study by Markovic et al. (1978) suggested that the degree of silver staining is characteristic for a particular chromosome.

In another study, Miller et al., (1977) showed that the frequency of satellite association of human chromosomes is correlated with amount of silver-staining of the nucleolus organizer region.

1.2.3. GENE MAPPING ON THE CHROMOSOMES

1.2.3-1. Definition and history:

Gene mapping means locating a specific locus to a specific region of a particular chromosome. Besides its academic interest, knowledge of the chromosome map gives insight into evolution, chromosomal organisation in relation to genetic control mechanism, and the pathogenesis of neoplasms and malformation (McKusick and Ruddle 1977).

Sturtevant, in 1911, at Columbia University, constructed the first genetic map of the X chromosome of Drosophila based on recombination study following breakage of homolgous strands of the chromosome during meiosis. At the same time, the first human gene assignment was made when the gene for colour-blindness was assigned by pedigree studies to a specific human chromosome, the X.

More than 100 loci on the X chromosome and more than 1100 on autosomes have been identified, which are only a small proportion of the human genes (perhaps no more than a fiftieth) (Mckusick and Ruddle, 1977). The only gene assigned to the Y chromosome is the histocompatibility (H-Y) antigen-determining gene, found on the Y chromosome of the mouse, rat and guinea pig besides the human Y (Wachtel et al., 1974).

1.2.3-2. Methods for gene mapping:

a) evidence from family linkage analysis and the pattern of inheritance of certain characters or disease.

b) somatic cell genetics.

c) in situ hybridization.

d) other means.

a) Evidence from family linkage analysis and the pattern of inheritance of certain characters or disease:

Gene loci are said to be linked if they are on the same chromosome pair. If two linked genes are on the same chromosome of the pair, then the linkage is in coupling. The linkage will be in repulsion if the two genes are on opposite chromosomes of the pair (Stevenson and Clare-Davison, 1976).

The phenomenon of crossing-over during meiosis will result in the exchange of chromosomal material between homolgous chromosomes, and hence two different genes located originally on different chromosomes of a particular chromosome pair, as a result of crossing-over, may come to be located on the same chromosomes. On the other hand, two genes, originally located on the same chromosome, of a particular chromosome pair may become separated as a result of crossing-over. Therefore by finding the frequency with which crossing-over occurs between these genes, the relative distances between genes on any particular chromosome can be determeined. This is measured in map units, one map unit being equal to 1 percent chance of crossing-over. It is necessary in linkage studies, to depend on the information obtained from studying the segregation of the "marker" genes in families with a particular hereditary disorder.

Family studies have been used to map certain genes where there

informative matings which have occurred by chance. For example, in order to find the distance between loci on the X chromosome of the genes for X-linked haemophilia and Duchenne muscular dystrophy, it would be necessary to study families in which both diseases occurred together, but these diseases are rare and the chances of their occurring in the same family, are small. By the family studies method it was possible to show the linkage between the loci for the Rh and elliptocytosis (Lawler, 1954), and those for the ABO blood groups and nail-patella syndrome (Renwick and Lawler, 1955). Family study was, until as recently as 1967, essentially the only method for gene mapping and linkage studies. Later the Rh gene was assigned to the short arm of chromosome 1 (Jacobs et al., 1970) and the ABO to the distal end of the long arm of chromosome 9 (Ferguson-Smith et al., 1976). The X-linked marker traits include Xg blood group system, glucose-6-phosphate dehydrogenase production, colour-blindness, haemophilia, the Duchenne type of muscular dystrophy, agammaglobulinaemia and Xm serum protein group (See McKusick, 1975). The linkage between the loci for colour blindness and glucose-6-phosphate dehydrogenase (G-6-PD) was studied by Porter et al., (1962).

b) Gene mapping by somatic cell hybridization:

In recent years, somatic cell genetics has provided a highly productive approach to chromosome mapping (Ruddle and Creagan, 1975).

The first step in this method is fusion of human cells with those from rodent species. The nuclei of the hybrid cells will contain the chromosomes of both parent cells, but in subsequent cell divisions, progressive and preferential loss of human chromosomes from the hybrid cells, will take place. An essential requirement for many of these studies is a selective system to enable the hybrid cells to be isolated and recognised (Paul, 1975). Production of useful hybrids depends on promotion of cell fusion, mutation and chromosome elimination (Paul, 1975).

Although cell fusion may occur spontaneously as was first shown by Barski et al. (1960), there are methods used to promote fusion such as the use of Sendai virus which was first described by Okada (1962) or by the use of polyethylene glycol (Pontecorvo 1975; Davidson and Gerald, 1976). There is, for an unknown reason, a variation in the efficiency with which cell lines will fuse. In some 60-70% of cells will form synkaryons or heterokaryons, while some will completely fail to fuse (Paul, 1975).

The use of selective media in somatic cell hybridization:

The fact that for an unknown reason, preferential and extensive loss of human chromosome gradually takes place from the hybrid cells between human and mouse, was utilized in an attempt to preserve certain human chromosomes.

Although it is possible, without using selective agents, to try

to relate the presence of a human gene product to the human chromosomes persisting in a mouse-human hybrid, the cell populations will probably be heterogeneous with respect to human chromosome content (Matsuya et al., 1968). The problem of heterogeneity can be dealt with only by selection. A variety of variant mouse lines must be prepared, each of which lacks a gene with survival value under given cultural conditions. Each line is then hybridized with human diploid cells and, by continuous growth under the respective selective conditions, the unnecessary human chromosomes are eliminated while the one bearing the gene with survival value is retained.

By testing for the presence of human gene products, chromosomal assignments can be made for the corresponding gene. The first chemical selection procedure was invented by Szybaliski et al., (1962) and developed by Littlefield (1964), who used aminopterin as the selective agent and depended on a knowledge of the pathways of purine and pyrimidine biosynthesis. Aminopterin blocks the in vivo synthesis of inosine monophosphate (IMP), a precursor of adenosine monophosphate (AMP), and guanosine monophosphate (GMP) from the glycine and phosphoribosyl pyrophosphate in the presence of glutamine. It also blocks the de novo synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP) by blocking the action of the enzyme thymidylate synthetase (Fig. 1.1.). Adenine and guanine are purine bases while thymine is a pyrimidine base. Hence one would

FIG. 1.1.

The site of action of aminopterin and FUDR in blocking nucleic acid synthesis.

FUDR = fluorodeoxyuridine

IMP = inosine mono-phosphate

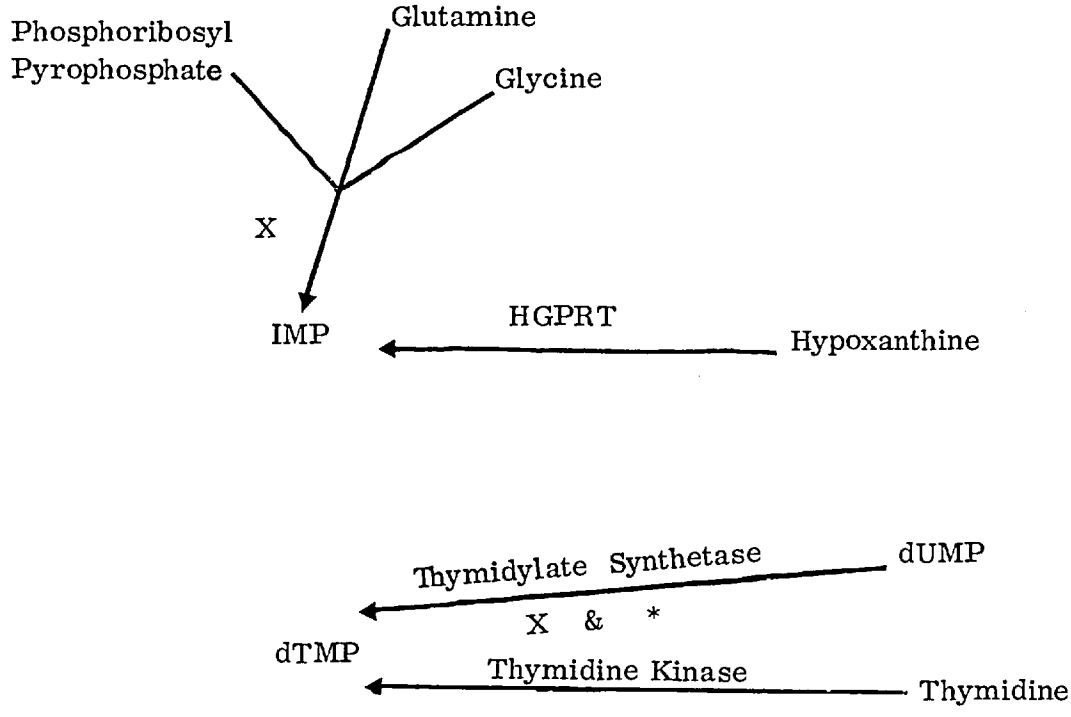
dTMP = deoxythymidine monophosphate

dUMP = deoxyuridine monophosphate

HGPRT = Hypoxanthine Guanosine phosphoribosyl transferase

HGPRT blocks the de novo synthesis of IMP and dTMP. FUDR blocks the de novo synthesis of dTMP.

Fig. 1.1



X : Site of action of Aminopterin
* : Site of action of FUDR

expect a complete blockage of the synthesis of the DNA by cells in media containing aminopterin and therefore death of these cells will result, unless those cells can find other means for the synthesis of purines and pyrimidines.

Cells that have the gene for the enzyme HGPRT (Hypoxanthine guanosine phosphoribosyl transferase) can synthesize inosine monophosphate from hypoxanthine, provided this substance (hypoxanthine) is supplied in the growth medium as the cells are unable to synthesize deoxythymidine monophosphate from exogenous thymidine, if the latter is supplied in the nutrient medium. The gene for HGPRT is carried on the long arm of human X chromosome, and that for thymidine kinase on the long arm of chromosome 17 (Boone et al., 1972). Therefore in blocking the synthesis of purine and pyrimidine bases by aminopterin only cells in culture will survive which can synthesize nitrogenous bases by using the enzymes HGPRT and thymidine kinase (TK) and the exogenous source of hypoxanthine and thymidine. Glycine should be included in the HAT medium since the conversion of serine to glycine is blocked by aminopterin (Littlefield, 1964).

The same principle will hold if the synthesis of dTMP from dUMP is blocked by blocking the enzyme thymidylate synthethase by FUDR (fluorodeoxyuridine), in which case the cells can survive if they have the genes for thymidine kinase besides the presence of thymidine in the growth medium. These principles were used to obtain mouse-human hybrid cells retaining human X in the presence of HAT (hypoxanthine, aminopterin, thymidine) after fusion of HGPRT deficient mouse cells

with human cells.

It is therefore possible to identify the chromosomes which carry certain genes, by correlating the loss or retention of particular chromosomes with the disappearance or maintenance of particular gene functions. Because human chromosomes are preferentially lost from the hybrids, with mouse or Chinese hamster cells, this technique has proved particularly useful for locating genes.

Identification of human X chromosome in the mouse-human hybrid cells:

It is necessary to distinguish between rodent and human chromosomes in somatic cell hybrids. The fluorochrome dye Hoechst 33258 (bisbenzimid) which is a benzimidazol compound binding to DNA, was used by Hilwigg and Gropp (1972) to visualize the site of repetitive DNA in the mouse. It stains the constitutive heterochromatin of the mouse specifically and hence the centromeres of metaphase chromosomes and the chromocenters of interphase nuclei fluoresce brightly in mouse cell lines. This staining property has been used to distinguish between human and mouse interphase nuclei in heterokaryons (Moser et al., 1975) and between human and mouse metaphase chromosomes in somatic cell hybrids (Kucherlapati et al., 1975). Both the Hoechst 33258 and the G-11 technique (the latter described by Bobrow and Cross, 1974) distinguish mouse and human chromosomes on the basis of species-specific staining differences. The G-11 technique differentiates between the human and mouse chromosomes through

a colour difference observed after alkaline Giemsa staining.

c) Gene mapping by *in situ* hybridization:

In situ hybridization is hybridization of labelled nucleic acids to cytological preparations containing previously denatured DNA. The specific complexes formed between the radioactive nucleic acids and the chromosomal DNA *in situ* are then detected by autoradiography. This method was developed by Gall and Pardue (1969) and John *et al.* (1969). The method was mainly used in the early days for the localization of highly repetitive DNA sequences which were found on the heterochromatic regions of the mouse chromosomes, mainly the centromere (Jones, 1970).

The chromosomal DNA on the slides is denatured into single strands followed by hybridization to the labelled single stranded nucleic acid (RNA, cRNA, DNA or cDNA), and washing thoroughly in buffer (usually twice the concentration standard saline citrate, 2 X SSC which is 0.3 m NaCl, 0.03m trisodium citrate) to get rid of the non hybridized nucleic acids. A photographic emulsion is then applied onto the slides so that after developing those slides, it is possible to localize the sites of hybridization associated with the silver grains. The presence of background cannot be avoided which forms one of the difficulties in this technique. The background may be due to random distribution of the labelled probe and tends to be particularly high when iodine is used. This is much less with tritium

(^{3}H) which, besides being cheaper, gives lower specific activity. One should not forget the safety precautions which are required in handling radioactive iodine. A high specific activity is essential in *in situ* hybridization which can be achieved by enzymatic methods including nick-translation (Kelly *et al.*, 1970).

Much interest is focussed on the possibility of the use of *in situ* hybridization to locate low-repetitive or unique gene sequences, but the main obstacle to locating such sequences, is the lack of probes sufficiently labelled to detect such short DNA units, and mRNA probes.

It was possible to identify the location of the ribosomal genes on the satellites of groups D and G chromosomes by the use of (^{3}H) rRNA probes, *in situ* hybridized to normal human chromosomes (Henderson *et al.*, 1972; Evans *et al.*, 1974). *In situ* hybridization, therefore, may be used in identifying the nature of the diseases associated with derangements in those genes.

The problem of using highly labelled probe for *in situ* hybridization has been overcome to some extent by using recombinant plasmids containing specific gene inserts, which increased the efficiency of *in situ* hybridization, and hence shorter time of exposure, without increasing the background. This was explained by the fact that both the plasmid and the gene insert would contribute to the signal (Malcolm *et al.*, 1977). In order to allow for the self-reannealing

of the complementary strands of the plasmid that would compete with the hybridization to chromosomal DNA an excess of radioactive probe is used to allow for this (Malcolm et al., 1977). Recently it was possible to use plasmids that contain a ribosomal gene insert for locating the ribosomal gene loci on normal human chromosomes by in situ hybridization (Malcolm et al., 1977). Previously, cloned Drosophila melanogaster DNA has been used for in situ hybridization to Drosophila polytene chromosomes where there were many copies of each gene (Glover et al., 1975).

d) Gene mapping by other means:

Genes may be mapped by other means such as studying cases with chromosomal anomalies which will lead to changes in the gene dosage, such as deletion (deletion means loss of a part of a chromosome). By this means the gene for the enzyme red cell acid phosphatase has been localized to the distal end of the short arm of chromosome 2, as the first assignment by deletion mapping - (Ferguson-Smith et al., 1973). The first assignment was later confirmed by family studies of chromosomal rearrangements (Mace et al., 1975), by studies of somatic cell hybrids (Povey et al., 1975) and by trisomy mapping (Magenis et al., 1976).

Other methods of utilizing changes in the gene dosage in studying gene mapping are based on trisomy or duplication mapping (Mckusick and Ruddle, 1977) whereby a person trisomic for part or all of a

chromosome has both alleles of one parent or has approximately 50 percent more of a particular gene product. This method like the deletion mapping method, depends on the gene dosage. It was possible to assign the locus for the enzyme nucleoside phosphorylase to chromosome 14 by studying partial trisomies of chromosome 14 (George and Francke, 1976).

By quantitative studies on red cell adenylate kinase (AK-1) in cases of partial duplication or deletion of chromosome 9, it was possible to assign precisely the AK-1 locus to the distal end of the long arm of chromosome 9 and to assign its linkage to ABO blood group system and nail-patella locus (Ferguson-Smith et al., 1976).

1.2.4. THE SEX CHROMOSOMES

1.2.4-1. The X Chromosome:

The X chromosome occurs in a single dose in the normal male (XY) and in a double dose in the normal female (XX). Abnormalities in the number of the X chromosome include Turner's syndrome (45, X) or superfemale (47,XXY) and even tetra X (48, XXXX) and males with Klinefelter syndrome (47, XXY). These conditions show various degrees of clinical abnormalities. Structural anomalies of the X chromosome itself such as translocations to or from other chromosomes were reported. Garcia et al. (1977) reported a case of premature menopause with a 5/X chromosomal translocation. Cases of Y to X translocations in phenotypically females were reported also (Khudr et al., 1973; Borgaonkar et al., 1974 and Berghe et al., 1977). The X chromosome is large, measuring about six percent of the total haploid genome length (Mckusick and Ruddle 1977). More than 100 gene loci have been located on the X chromosome including the genes for glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate kinase (PGK), Hypoxanthine Guanosine phosphoroibosyl transferase (HGPRT), α - galactosidase , Xg blood group, tryosine aminotransferase regulator, etc. (See Mckusick, 1978).

There are diseases which are specifically X-linked and therefore are recessive disorders transferred by the mothers to their sons so that their sons will carry the stigmata. Examples of such diseases are Haemophilia A, Duchenne muscular dystrophy, colour blindness etc.,

and other diseases associated with the deficiency of the enzymes which genes are carried on the X, some of which mentioned already.

1.2.4-2. The X-Chromosome inactivation:

During early embryogenesis there is a random inactivation of one of the two X chromosomes. This was first hypothesized by Lyon (1961). The inactivated X remains visible during the interphase as a highly condensed body (Barr body). Therefore a normal (46,XX) female has one Barr body, which can be detected in Turner's syndrome (45,X) and a (47,XXX) female has two Barr bodies.

The X chromosomes inactivation is supported by the fact that a normal male with XY is a normal individual despite the fact that he has one X only and hence a (46,XX) female is normal with one active X only. This brings into the view, the fact that the Y chromosome, which is only a small chromosome, bears no significant physiological action except for male determination. However the cause, the biochemical and biophysical basis of X inactivation are obscure.

1.2.4-3. The Y Chromosome:

Although normal male individuals possess one Y only, cases of males with more than one Y have been reported. There have been reports of males with (47,XYY) karyotypes (Jacobs et al., 1965; Price et al., 1966; Casey et al., 1966; Court Brown, 1968;

Nielsen et al., 1971; Fründ et al., 1972 and others). There have also been reports of males with (48,XYYY) karyotypes (Townes et al., 1965; Pozsonyi and Sergovich, 1971; Schoepflin and Centerwall, 1972; Ridler et al., 1973). The length of the Y chromosome was found to be subject to variations between different racial groups (Cohen et al., 1966). Unnerus et al (1967) concluded that the size of the Y chromosome has a normal distribution in a random population. The origin and cause of this variation stimulated further research. Bobrow et al., (1971) and Schnedl (1971) indicated that the size differences in the Y chromosomes were due to variation in the fluorescent area which correspond to region q12. There were many reports on cases of long Y chromosome i.e. Yq + (Cohen et al., 1966; El-Alfi, 1970; Bobrow et al., 1971; Schnedl, 1971 and others). Abnormalities of the structure of the Y, in the non-fluorescent portion, besides the translocation of Y to X, do occur such as Yq- or deletion of the long arm, (Muldal and Ockey, 1962; Meisner and Inhorn, 1972; Yunis et al., 1977 and others). Cases of Yp- (or deletion of the short arm of the YO were also reported in female phenotypes either as isochromosome Y* (Böök et al., 1973; Mangelli et al., 1974; Ferguson-Smith et al., 1969) or as Yp- due to an unbalanced translocation (Buhler et al., 1971).

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* Isochromosome: a chromosome with two morphologically identical arms. They are the result of transverse, rather than longitudinal division of the centromere during cell division. Therefore the two arms of an isochromosome are of equal lengths and contain the same genes.

Those findings of Yp- in phenotypically female subjects, supported the view made by Jacobs and Ross (1966) that the short arm of the Y (the p region) carries the genes for testicular differentiation. Those genes must be close to the centromeres as testicular differentiation existed in cases of loss of the distal portion of the Yp (the short arm) either by Y-ring (Chandley and Edmond, 1971; German et al., 1973; Maeda et al., 1976) or by Y-autosome translocation (Krmpotic et al., 1972). The occurrences of satellites on the distal end of the long arm of the Y chromsome has been reported (Genest et al., 1967; Schmid, 1969). The proband of family A reported by Genest et al., (1967) was a male trisomy 21 patient. Howard-Peebles and Stoddard (1976) reported the satellited Yq in a proband with trisomy 21 and inversion of chromosome 9. Later, Howell et al., (1978) by silver staining technique found that the satellite on that particular case of satellited Yq carried functionally active NORs.

Other Y chromosome anomalies occur and as they are not associated with clinical abnormalities, variations concerned with differences in the length of the fluorescent portion of the long arm (i.e. the distal portion) are generally accepted as "normal" (Bobrow et al., 1971; Hamerton et al., 1975). The length of the Y chromosome shows a considerable variation which is probably distributed in populations according to a Gaussian curve (Unnerus et al., 1967). Cohen et al., (1966) found the presence of geographical differences in the length of the Y, depending upon differences in race. There are contradictory

results of the reports as to the associations of the length of the Y chromosome with criminality, character disorder or alcohol abuse. Positive results (i.e. long Y associated with criminality) were found by Nielsen (1968) , Nielsen and Friedrich (1972) and Soudek and Laraya (1974). Those findings were contradicted by the works of Urdal and Brogger (1974), Schwinger and Wild (1974), Benezech et al., (1976), Brogger et al., (1977) and Akesson and Wahlstrom (1977).

The only condition described as being Y-linked is that characterized by the growth of hair on the outer rim of the ear. It is fairly common in parts of India and possibly some other areas as well (Slatis and Apelbaum, 1963; Dronamraju, 1965).

1.2.4-4. The H-Y antigen and sex differentiation:

Usually the presence of a Y chromosome result in a male subject regardless of the number of X chromosomes present, although supernumerary X chromosomes in a male lead to a variety of abnormalities including small, azospermic testes as in case of Klinefelter's syndrome (47,XXY). Testicular differentiation seems to be the only function of the Y-chromosomal male-determining gene, since subsequent male differentiation takes place under the influence of androgen which is secreted by the newly formed testis. The individual becomes a female in the absence of androgen.

It was found that when female mice are sensitized with skin

grafts or lymphoid cells from males of the same inbred strain, they produce antibody which identifies a plasma membrane component called H-Y (histo-compatibility-Y antigen) (Gasser and Silvers, 1972). It was also found that a structural or regulatory gene for H-Y antigen expression was located on the Y chromosome of man and that with white blood cells from human males with two Y chromosomes expressed more H-Y antigen than cells from normal XY males (Wachtel et al., 1975). The exact locus of the human H-Y gene is still obscure, although it was supposed to be on the short arm of the Y chromosome. Koo et al. (1977) confirmed that the position of H-Y gene on the short arm of the Y in most individuals they studied but could not rule out the location of the gene on the long arm in at least one of 17 individuals studied, with structural abnormalities of the Y. Despite the male-determining role of the mammalian Y chromosome, testicular differentiation and subsequent phenotypically male or hermophroditic development have been observed in subjects with a female (46,XX) karyotype (Chapelle, 1972). In a study carried out on a family with three of their female members who had streak gonads (gonadal dysgenesis) and a (46,XY) karyotype, German et al., (1978) concluded that the Y chromosome alone was not sufficient to form the indifferent embryonic gonad into a testis. They suggested the possibility of the presence of an interaction between a locus near the centromere of the Y and another locus on the X, and that either the locus on the Y would carry the gene responsible

for testis differentiation which is induced by a controlling element on the locus on the X or vice versa. They also pointed out that although a single human X is sufficient for differentiation of ovary, it is insufficient for production in adult life of normal oogenesis. However, the prevailing view of sex determination is that a structural gene coding for a cell surface antigen (H-Y), which is responsible for testis formation, is on the Y chromosome (Koo et al., 1977; Silvers and Wachtel, 1977; Ohno, 1976; Wachtel, 1977 and others).

1.2.5. AUTOSOMAL ANOMALIES

Anomalies of the autosomes could be either anomalies of their numbers or of their structures.

a) Numerical abnormalities are due to loss or gain of one or two chromosomes (aneuploidy), or even the gain of the whole chromosome set (polyploidy). Aneuploid and polyploid cells have been found in tissues from some abortuses and stillbirths, in leucocytes from patients with acute leukaemia, and in cancer cells. Monosomy (or the loss of an autosome) appears to be lethal since no case of 45 chromosomes due to total loss of an autosome has been found. Trisomy (or the addition of an extra autosome) has been described in several disorders, such as Down's syndrome or mongolism (trisomy-21), Patau's syndrome (trisomy-13), Edward's syndrome (trisomy-18). Trisomies of chromosomes 8, 9, 22 and partial trisomies of short arms of chromosomes 4 and 9 (i.e. trisomies - 4p and 9p) have also been described besides many other partial trisomies (See Valentine, 1969; Riccardi, 1977). The commonest trisomy case so far described is trisomy-21 (one case for every 650 births), and trisomies 18 and 13 which are 20 times less frequent (Grouchy, 1976). The frequency of all the other autosomal trisomies is comparatively low. In all those conditions, no real cause for the clinical manifestations associating the genotypical abnormality was described.

Creasy and Crolla (1974) found that over half of pregnancies

with trisomy-21 fail to survive to term which suggests that the extra chromosome also interferes with intrauterine development.

b) Structural abnormalities of the autosomes are also associated with clinical manifestations. Examples of translocation of a segment of a chromosome to a non-homologous one. Deletion indicates loss of a segment of a chromosome. Amongst the cases described to have translocations are translocations 14/21 or 15/21, 21/21 translocations of portion of the short arm of chromosome 9 to the short arm of chromosome 7. Examples of cases of deletions associated with identifiable syndrome are 4p-(deletion of the short arm of chromosome 4), 5p-(Cri du chat syndrome), 13q-(or deletion of the long arm of chromosome 13), 18q- or 18p- (De Grouchy's syndrome), 21q- and 22q- (See Grouchy, 1976).

Ring Chromosomes:

A ring chromosome has been found in some infants with multiple congenital abnormalities. A ring chromosome results when there is a break at both ends of a chromosome and the rejoining of the broken ends to each other to form a ring. The acentric fragment is lost. Ring formation with assumed deletions has been described for the X and some of the autosomes.

Extra "marker" Chromosome:

The occurrence of an abnormal extra marker chromosome has often been reported since the first report by Ilbery et al., (1961). Surveys showed that a marker chromosome can occur in phenotypically normal individuals and their families (Walzer et al., 1969; Friedrich and Nielsen, 1974; Nielsen and Ramsmussen, 1975) or may be associated with a wide range of clinical abnormalities (Bain, 1973). The presence of a metacentric bisatellited michrochromosome in amniotic cells was reported by Bernstein et al. (1978).

These extra chromosomes may have various origins which cannot be identified easily (Wahrman et al., 1978). However, the identification of the marker chromosome as a derivative of one of the acrocentric chromosomes is suggested by the presence of satellites on one or both arms. This can be studied further by the method of in situ hybridization using a probe specific for the 18S and 28S ribosomal RNA genes which have been shown to be located on the short arms of the acrocentric chromosomes in man (Henderson et al., 1972; Evans et al., 1974) and by the silver staining technique of Bloom and Good pasture (1976) which specifically stains functionally active NORs (nucleolus organisers) (Miller et al. 1976 a and b).

1.2.6. THE USE OF RECOMBINANT DNA IN THE STUDY OF HUMAN GENETIC DISEASES.

A plasmid is an extrachromosomal genetic material that has the ability of autonomous replication, and therefore maintains itself, for many generations, in the cytoplasm of a bacterium (Spp. Escherichia, Salmonella, Schigella etc). It is usually a piece of circular double stranded DNA, and is much smaller than a bacterial chromosome, and contains 50-100 genes. The genes that it contains are not essential to the cell, but yet it is has many of the properties of a small chromosome and contains genetic information for controlling its replication and ensuring a high enough multiplicity to give segregation of one copy into each daughter cell at cell division. A plasmid often has the ability of integrating its DNA into the host genome.

A recombinant DNA results when a specific foreign gene (or insert) is inserted into the plasmid. Such a recombinant DNA will replicate and therefore will be produced in large amounts if it infects a bacterial host. A foreign gene is inserted into a plasmid by utilizing the properties of restriction enzymes and DNA ligase. A restriction enzyme causes a break at a single specific site in the plasmid DNA, often with overlapping base-paired ends. DNA from another species (such as human DNA) is restricted using the same enzyme and the foreign DNA integrated into the site, making use of the "sticky ends" for binding. Sometimes synthetic A-T or G-C

sequences are added and used instead. Phsophatase treatment is necessary to digest the phosphates at the broken "sticky" ends of the plasmid to prevent their recombination with each other before adding the insert. Various restriction enzymes are available; each will cleave DNA at a specific site, as listed in Fig. 1.2. The recombinants can be used to sequence DNA or for the isolation of specific genes, such as β-globin genes (Tilghman et al., 1977, Van den Berg et al., 1978) and immunoglobulin genes (Tonegawa et al., 1977). This has proved invaluable in revealing important details of the organisation and expression of eukaryotic genomes. However, a single copy structural gene accounts for less than 10^{-6} part of mammalian genome. Most workers, therefore, have partially purified the structural gene plus surrounding DNA sequences before cloning. The recombinant genes have been used for the study of human genetic diseases. Little et al. (1978) used human globin cDNAs (complementary DNAs) inserted into plasmid pCRI, as specific hybridization probes and for the partial sequencing of α-, β- and γ-globin genes. Wilson et al. (1978) described similar human globin cDNAs incorporated into another plasmid pMB9 and sequenced the β-globin gene insert. Gene mapping studies of the molecular genetics of haemoglobin synthesis in normal and abnormal globin gene expression as in thalassaemia are greatly facilitated by the availability of pure chain specific human globin cDNA. Malcolm et al. (1977) used labelled Xenopus laevis ribosomal genes incorporated into plasmid (Xlr 101) as a probe for in situ hybridization to normal human chromosome

FIG. 1.2.

Example of some of the restriction enzymes.

Arrow indicate their sites of action.

Py Pyrimidine

Pu Purine

G Guanine

A Adenine

C Cytosine

T Thymine

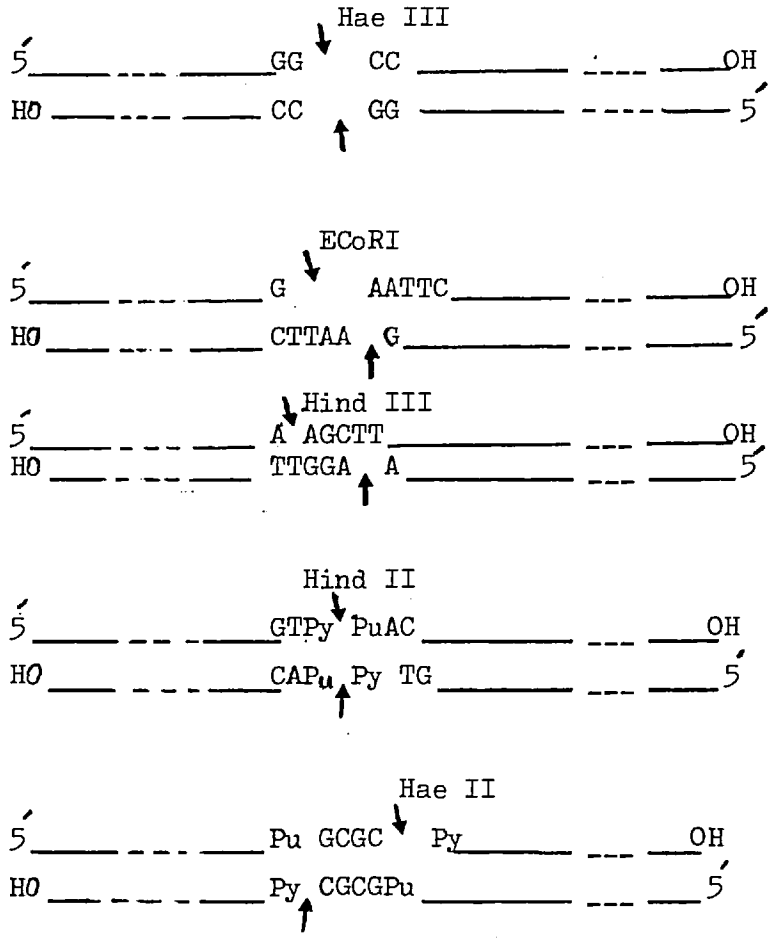
Hae III *Haemophilus aegyptius* III

Hae II *Haemophilus aegyptius* II

Hind III *Haemophilus influenzae* Rd.

ECo RI *Escherchia coli* RI

FIG. 1.2.


Hae III
5'
GG
CC
OH
HO
CC
GG
5'
ECoRI
5'
G
AATTC
OH
HO
CTTAA
G
5'
Hind III
5'
A
AGCTT
OH
HO
TTGGA
A
5'
Hind II
5'
GTPy
PuAC
OH
HO
CAPu
Py TG
5'
Hae II
5'
Pu GCGC
Py
OH
HO
Py
CGCGPu
5'

and found that they hybridized preferentially to the satellites of the D and G groups at high efficiency. The recombinant genes may also be used to produce human proteins or hormones such as somatotropin or insulin.

1.2.7. THE ISOLATION AND STUDY OF HUMAN CHROMOSOME-SPECIFIC GENE SEQUENCES

The revolution in the field of medical genetics on the isolation and studying of human chromosome-specific gene sequences was started by the works of Kunkel et al. (1976) and Cooke (1976). The former (Kunkel et al.) were able to isolate human Y-chromosome-specific reiterated gene sequences by extensive reassociation between radio-labelled male DNA and an excess of non-labelled female DNA. They supported their view that the sequences are Y-specific by the finding that the amount of Y-specific sequence isolated from an XYY individual was almost double that isolated from an XY. Later, Kunkel et al. (1977) located the reiterated Y-specific sequences on the long arm of the Y chromosome and found that the sequences played no evident role in male determination. Cooke (1976) on the other hand, was able to show, by restriction enzyme analysis and nucleic acid hybridization, the existence of a human male specific repetitive sequence which was not present in the female. This sequence constitutes about 50% of the total Y genome and was a simple tandemly repetitive DNA. The male DNA exhibited a major restriction fragment of molecular weight 2.2×10^6 Daltons, which increased in amount in DNA from males with more than one Y-chromosome. A similar study by Kinross et al.(1978) used the restriction enzyme Bsu (Bacillus subtilis) which is an isoschizomer of the enzyme HaeIII used by Cooke (1976) in his study. They studied various cases of translo-

cations involving the Y-chromosome. These confirmed the origin of the translocated fluorescent segment as of Y-chromosome origin by the presence of the Bsu digest band on agarose gel corresponding to the Y-specific band of 2.2×10^6 Daltons. They also confirmed the localization of this sequence predominantly on the Yq region. It was shown by McKay et al. (1978) that individual men are polymorphic for the amounts of two different repeated DNA sequences of 3.41 kb and 2.1 kb, as measured on agarose gel after Hae III digestion of male DNA. They found that the amount of the 3.41 kb sequence is a function of the size of the Y chromosome and hence the length of the fluorescent distal segment while the smaller fragment of 2.1 kb does not vary in amount in the same way.

Bostock et al. (1978) also identified a 3.5 kb DNA segment unique to men after digesting DNA with Hae III and showed that such a segment co-purifies with human satellite III. They found that this DNA sequence is located on the regions q11 and the most proximal part of the fluorescent segment, q12, of the Y chromosome.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Materials:

All chemicals, unless otherwise stated, were purchased from British Drug House Laboratories (AR Grade).

Isotopes:

All isotopes were obtained from the Radiochemical Centre, Amersham, England.

Buffers:

Tris (Trizma Base Reagent Grade) was obtained from Sigma Co.Ltd., St. Louis, Missouri, U.S.A.

Nucleotides:

These were purchased from Sigma Co. Ltd., St Louis, Missouri, U.S.A.

Enzymes:

The enzyme DNA polymerase Grade 1 was obtained from Boehringher Mannheim G mbH - West Germany. All the following enzymes were purchased from Sigma Chemical Ltd., U.S.A : Ribonuclease (Type I-A), Protease (Type VI, from *Streptomyces griseus*), Deoxyribonuclease (Grade DN-EP).

Chromotography:

HAP DNA-Grade for column chromotography, Biogel, HTP was purchased from Biorad Lab., Richmond, California.

G-50 Sephadex was obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden.

Tissue Culture and Cytogenetics:

All culture media, animal sera, trypsin, versine, Hepes buffer, sodium bicarbonate, glutamine, antibiotics and phytohaemagglutinin (M form) were purchased from Gibco-Biocult, Glasgow, Scotland.

Sterile pipettes were from Sterilin, Teddington, Middlesex, England.

Sterile plastic flasks and petridishes were purchased from Falcon Plastics, Division of Becton Dickinson & Co.

Quinacrine mustard and Colcemid (Demcolcin) were purchased from Sigma Co.Ltd., St.Louis, Missouri, U.S.A.

Giemsa Stain (Gurr improved R66) and Gurr phosphate buffer tablets were obtained from Searle, High Wycombe, Bucks., England.

Heparin for injection, mucous, phenol-free (B.P) 1000 units/ml was obtained from Paines & Byrne Ltd., Greenford, Middlesex, England.

Hoechst 33258 (Bisbenzimid fluorchrom) Batch No. 706857, was purchased from Hoechst Company, Hounslow, Middlesex, England.

Photographic Materials:

All the photographic films and developing and fixing materials for in situ hybridization slides were obtained from KODAK.

Qualitol developer and Super Amfix fixer for developing and fixing the films and prints were purchased from May & Baker. Ltd., Dagenham, England.

The printing papers were purchased from ILFORD Ltd., Ilford, Essex.

2.2 METHODS

2.2.1. Determination of Radioactivity:

Radioactivity was measured using a Packard Tricarb Liquid Scintillation Counter. Radioactive samples precipitated onto Millipore or glass fibre filters were dried, and then counted in 10 ml toluene-based scintillator (TBS), containing 0.5% of 2,5-diphenyloxazolyl (PPO, scintillation grade, Nuclear Enterprises,Edinburgh), 0.03% 1,4 bis(2-(5-phenyloxazolyl) benzene) (POPOP, scintillation grade, Nuclear Enterprises, Edinburgh) dissolved in sulphur-free toluene (May and Baker, Dagenham, England).

Radioactive samples in aqueous solution were made up to 1 ml with water and 10 ml TXBS (2 parts TBS to 1 part Triton X-100, Lening Chemicals, Croydon) added. The mixture was shaken and then counted. Formamide gel fractions from the Gilson gel slicer were eluted with Soluene-350 tissue solubilizer (0.5 quaternary ammonium hydroxide in toluene-Packard) into plastic vial inserts and 3.2 ml TBS were added. The solution was shaken and left for 24 hours at room temperature before counting. Aqueous samples of 6 ml with relatively low radioactivity were counted in an equal volume of Instagel (Packard, Zurich, Switzerland).

Corrections were made for counting efficiency of (^{3}H) using $^{3}H_2O$ standard quench curves (4.84×10^{6} dpm = 2.18 µCi/g. December 1st, 1976). The efficiency of counting of (^{3}H) was approximately 32% in TBS on Whatman GF/A filters, and 25% in TXBS.

2.2.2. DNA PREPARATION:

a) From Tissues:

DNA was prepared from different tissues by phenol/chloroform/octanol extractions. The fresh tissue, cleaned from fibrous materials (within one hour after delivery in the case of human placenta, or immediately after killing the animal) was weighed, chopped and homogenized at 4°C in a homogenizing buffer (0.15M sodium acetate, 0.1M EDTA, pH 8.0). The nuclei were then lysed by the addition of SDS solution to a final concentration of 1% and left at room temperature for 15 minutes. An equal volume of freshly distilled phenol saturated with homogenizing buffer, was added, mixed well and centrifuged at 2,000 rpm for 30 minutes at room temperature in MSE centrifuge. The supernatant was re-extracted with equal volume of phenol:chloroform (1:1). This was repeated until no interphase was present. For maximum yields of DNA the interphase was also re-extracted with phenol/chloroform. To the final supernatant, sodium acetate was added to a final concentration of 0.3M followed by two volumes of filtered absolute ethanol and the crude DNA spooled on a glass rod. The spool was washed with 70% ethanol, dried in nitrogen and dissolved in 0.03M sodium acetate, 0.02M EDTA (pH 8.0). Ribonuclease (previously boiled to destroy any trace of DNase) was added to a final concentration of 50 µg/ml and incubated for 2 hours at 37°C, followed by Protease (Pronase) to a final concentration of 100 µg/ml for 2 hours at 37°C. This was followed by phenol/chloroform (1:1) and chloroform/octanol (24:1) extractions and spooling the DNA from two volumes ethanol as before. The DNA was dissolved in 0.001 M Tris,

0.001 M EDTA and the optical density was measured.

b) DNA Extractions from cells:

The cells were collected after centrifuging the cell suspension obtained by trypsinization of cells in tissue culture flasks. The pellet was suspended in not more than 1 ml homogenizing buffer (0.15M NaCl, 0.1M EDTA, pH 8.0). The nuclei were then lysed by adding 10% SDS to a final concentration of 1% for 10 minutes at room temperature. 5M solution of sodium perchlorate was added to a final concentration of 0.25M in order to precipitate protein. This was followed by thorough mixing in an equal volume of phenol which was saturated with the homogenizing buffer and 0.25M sodium perchlorate. After spinning for 5 minutes at 10,000 rpm at room temperature, the supernatant was mixed with an equal volume of chloroform:octanol (24:1) and shaken thoroughly. This was followed as above, by chloroform/octanol extraction to the supernatant and centrifugation. To the supernatant 3M sodium acetate was added to a final concentration of 0.3M and two volumes of absolute ethanol and the crude nucleic acid was spooled on a glass rod. The spool was then dissolved in 1 ml of 1/10 concentration of the homogenizing buffer. Ribonuclease was added to a final concentration of 50 µg/ml for 2 hours at 37°C followed by protease (Pronase) to a final concentration of 100 µg/ml for another 2 hours at 37°C. This was followed by one phenol extraction and one chloroform:octanol (24:1) extraction. The DNA was then spooled from two volumes ethanol as before.

2.2.3. Sonication of DNA:

Native DNA was sheared to an approximate size of about 800

nucleotides (as determined by formamide gel electrophoresis) in 10 ml solution of 0.1M NaCl by sonication using the macroprobe of Dawe Soniprobe (Type 7532A) for six 15-second bursts at a power of 90 watts with one minute rest in ice between each two successive sonications.

2.2.4. Desalting of DNA:

DNA was desalted by passing through a G50-Sephadex medium-grade chromatography column on the top of which was a small layer of Chelex resin, and eluted with distilled water.

Chelex (Dowex chelating resin, dry mesh, 50-100 obtained from Sigma Co. Ltd., USA). Chelex was prepared by mixing with water and the pH taken to 7.0 with concentrated hydrochloric acid. The resin was then stirred and the pH corrected until a steady pH of 7.0 is reached. It was, then thoroughly washed with distilled water to remove any trace of acid. Chelex was stored in distilled water at $4^{o}C$ for future use.

2.2.5. Nick Translation:

Nick translation of native DNA was according to the method of Maniatis et al.(1975). 180 pmoles each of dATP, dGTP, (^{3}H) TTP and (^{3}H) dCTP, were lyophilized in a silicone-treated tube and 1 µg native, desalted DNA was added. The mixture was dissolved in 10 µl buffer 50mM Tris-HCl, pH 7.8, 5mM $MgCl_2$., 1mM beta-mercapto-ethanol

and 50 µg/ml desalted BSA (Sigma), and the volume was made up to 100 µl with sterile distilled water. The mixture was pre-incubated for 10 minutes at 15°C and the reaction was started by adding 1 µl of DNA-polymerase (1 µl = 5 units) and 1 µl of 10^{-7} g/ml of DNase (freshly diluted from 2 mg/ml stock in distilled water) incubated for one hour at 15°C at the end of which 1 µl was taken from the reaction mixture onto GF/A filter to measure the total counts. Another 1 µl aliquot was added to 1 ml chilled 5% TCA in ice together with 50 µg E.coli RNA as a carrier, left for 10 minutes then filtered on a 2.5 cm GF/A filter after washing with 20 ml chilled 5% TCA. The filter was counted in TBS to measure the amount of radioactivity incorporated in the DNA. After one hour of incubation, the reaction was terminated by adding an equal volume of distilled phenol, mixed well and centrifuged at 6,000 rpm at room temperature. The supernatant was re-extracted with phenol and passed through a G-50 Sephadex column, eluting with 50mM Tris buffer. 1 ml fractions were collected to separate the DNA from the nucleotides that eluted later. The fractions containing the DNA were pooled, lyophilized and desalted on G-50 Sephadex column.

The specific activity of the nick translated DNA (counts/minute per µg DNA) was calculated from the activity of the radio-labelled nucleotides in the reaction mixture, the percentage incorporation and the efficiency of counting of the GF/A filters (32%).

2.2.6. Estimation of DNA size:

The size of the sonicated or nick-translated DNA was measured by fractionation on formamide gels, which were prepared according to a modification of the procedure described by Staynov et al. (1972). Before use, formamide was deionized by stirring with a 5% w/v suspension of mixed bed resin (Bio.Rad AG 501 X 8 (D) for 2-3 hours at 4°C. The resin was removed by filtration and 20 ml formamide were mixed with 0.91 g recrystallised acrylamide, 0.09 g recrystallised bisacrylamide, 0.092 g diethylbarbituric acid and 60 µl TEMED (N, N, N', N' - tetramethylethylenediamine) pH 9.0. Then 0.2 ml of fresh 18% ammonium persulphate was added and mixed, and the acrylamide solution immediately pipetted into 0.7 X 12 cm perspex tubes sealed with parafilm and overlayed with 70% buffered deionized formamide during polymerization (about 30 minutes) to ensure a flat surface, after which it was replaced by 100% buffered formamide and the parafilm was replaced by muslin.

Buffered formamide for dissolving samples and overlaying gels was prepared by mixing 20 ml 100% deionized formamide with 0.092 g diethylbarbituric acid, pH adjusted to 9.0, then the volume made up to 25 ml with dionized formamide. 70% buffered formamide was made by dilution with water. Samples of salt-free lyophilised DNA or marker RNA were dissolved in 30 µl 100% buffered formamide containing 10% sucrose and a trace of bromophenol blue as a tracking dye, then samples were heled at 100°C for 2 minutes in order to ensure complete

denaturation and to aid in dissolving the nucleic acids. This was followed by rapid cooling in ice before application onto the gel through its overlay of buffered formamide. After application of the samples, the electrophoresis chambers were filled with 20mM aqueous NaCl to act as supporting electrolyte; thus the samples never came into contact with water, being separated from the electrolyte by the overlay of buffered formamide. Electrophoresis was at the low current of 1.25 mA per gel to improve the quality of separation. The 20mM NaCl was circulated between the electrophoresis compartments throughout the run.

2.2.7. Scanning and fractionation of the gels:

The gels containing only unlabelled samples were washed for 1-2 hours with several changes of water before scanning with a Gilford Spectrophotometer 250 at 260 nm. Gels containing labelled samples were sliced directly into vials using a Gilson automatic gel slicer (Villiers-Le-Bel, France). Radioactive components were eluted from the gel fragments and counted as described above.

2.2.8. Hybridization of DNA:

Desalted sonicated DNA was lyophilized and dissolved in 0.12M PB (the phosphate buffer is composed of an equimolar mixture of Na_2HPO_4 and NaH_2PO_4, pH 6.8) at a concentration of 10 mg/ml (1 O.D. double-stranded DNA = 50 µg). Small amounts of DNA (400 µg or less in 0.12 M PB) were put in siliconized capillary tubes and sealed at both ends, while

larger amounts were put in longer narrow glass (Sorval) siliconized tube and overlayed with liquid paraffin. The tubes containing the DNA were held at 100°C for 10-15 minutes in order to denature the DNA followed by holding at 60°C for the appropriate time or Cot value, when they were cooled immediately in ice. The hybridization samples were then flushed from the capillaries with about 600 µl of 0.12M NaCl and stored frozen at -20° for later analysis. For larger volume samples, at the appropriate Cot value, the liquid paraffin was removed from the top of the DNA solution and the concentration of PB was reduced to 0.03M by adding distilled water.

2.2.9. Preparation of HAP (Hydroxylapatite) for use in analysing hybrids:

100 ml of DNA - Grade HAP was washed with 400 ml of each of 0.5M PB, distilled water and 0.16M PB successively. This was followed by re-suspension in 0.16M PB, boiling for 15 minutes, washing again with distilled water and then with 0.03M PB. The HAP was finally resuspended in 200 ml 0.03M PB and stored at 4°C ready for use. Each batch of HAP was tested before use. This was done by washing native and denatured DNA through the HAP separately with increasing concentrations of PB and finding the cut off point between the concentrations of PB at which single stranded and double stranded material is eluted.

2.2.10. Analysis of hybrids:

Analysis of hybrids on HAP was carried out using a ratio of 200 µg of DNA per ml of HAP as described by Britten et al, (1973). Columns were kept at 60°C and washed before and after loading the sample with 6 ml of 0.03M PB then eluted twice with 3 ml 0.14M PB and

finally twice with 3 ml of 0.4M PB.

Absorbance measurements of fraction of eluate at 260 nm were made and in the case of labelled samples, fractions were either counted in equal amounts of Instagel (when the radioactivity was expected to be low) or 1 ml of each fraction was added to 1 ml chilled 10% TCA, left for 10 minutes in ice, precipitated on GF/A filters and washed with 20 ml of 5% TCA and counted in TBS as described previously.

TISSUE CULTURE

All the tissue culture techniques were performed in a vertical flow cabinet with prefiltered air. The cabinet was tested before use for the first time with bacteriological plates to ensure sterilisation. Before each use the air flow and U.V. lamp were switched on for 30 minutes and the cabinet inner surfaces were swabbed with 70% ethanol. The air flow was left on during use and the tops of the flasks were flamed before and after each use immediately. Cultures were incubated at $37^{\circ}C$ in anhydric CO_2 incubators (Charles Herason & Co. London).

2.2.11. Conditions and media for growing HORL 9.X 4 2.2 cells:

The human-mouse hybrid cells, HORL 9.X 4 2.2 cells were grown in monolayer culture at $37^{\circ}C$ in HAM-F12 medium containing 10% foetal bovine serum, 29.2 mg% glutamine, 200 units penicillin and 200 µg streptomycin per 1 ml medium, 0.025M Hepes buffer (pH 7.3) and 0.01M NaOH. To the above cocktail 1×10^{-4}M hypoxanthine, 4×10^{-7}M aminopterin and 1.6×10^{-5}M thymidine (HAT) and 3×10^{-6}M glycine

were added (Littlefield, 1964).

The medium was changed every 2-5 days depending upon the state of the growth or if there was a change in the colour of the medium towards an acid pH (more yellowish in colour). Each culture was examined daily under a phase contrast microscope for signs of viability and growth of the cells. Cultures were trypsinized whenever they were found to be confluent.

2.2.12. Trypsinization:

After the growth medium was removed, the culture was washed with calcium and magnesium-free BSS then incubated at 37°C for a few minutes with 5-10 ml of 0.05% trypsin in 1/5000 versene solution containing 1% v/v chicken serum pre-warmed to 37°C, sometimes with gentle shaking until the solution became slightly turbid or the cells looked rounded under the phase contrast microscope. Then the trypsin/versene solution was replaced by fresh growth medium and cells suspended by gentle pipetting and split between 2 or 3 larger or identical flasks according to the size of the culture.

2.2.13. Preservation of the cells by freezing:

Cells were preserved for future culture by freezing. Cells from one large flask 250 cm^3 size were trypsinized (to give more than 10 X 10^6 cells). The cell suspension was then centrifuged in a sterile uni-

versal container at 1000 rpm for 10 minutes. The pellet was resuspended in culture medium containing sterile 10% v/v dimethylsulphoxide (DMSO), transferred into sterile screw-capped vials and frozen down immediately at 70°C. The vials were then transferred into a liquid nitrogen freezer to store at -191°C.

CYTOGENETIC TECHNIQUES

2.2.14. Chromosome preparation from peripheral blood lymphocytes:

The technique is based on the method of Moorhead et al (1960). 5-10 ml venous blood was mixed immediately with 0.5 ml Heparin (500 i.u.). 0.3 ml of the whole heparinised blood was added to a sterile universal container which contained HAM F10 cocktail medium containing 10% FBS, 29.2 mg% glutamine, 200 units penicillin and 200 mg streptomycin per 1 ml medium, 0.025M Hepes buffer (pH 7.3). To this cocktail 0.1 ml phytohaemaglutinin was added as a mitogen and incubated at 37°C for 72 hours, during which the culture was shaken gently every 12-24 hours in order to ensure good mixing of the cells and the medium. After the third period of incubation, 0.2 ml of an 80 µg/ml solution colcemid (Demcolcine) was added for a period of 3 hours at 37°C to arrest cells at the metaphase stage of mitosis, after which the culture was transferred into a siliconised conical centrifuge tube and centrifuged for 10 minutes at room temperature. The pellet left in a little supernatant, was mixed with pre-warmed 10 ml 0.075M potassium chloride and incubated for 12 minutes at 37°C and then centrifuged at 600 rpm for 10 minutes. The pellet was suspended in freshly prepared methanol: glacial acetic acid (3:1) fixative, mixed thoroughly, left for 15 minutes and cetrifuged. Many changes of the fixative followed by centrifugation were carried out until a clear colourless supernatant resulted, after which the pellet was suspended in fresh fixative enough to give a milky appearance. Two drops of the suspension were

dropped from a 30 cm distance onto a clean, wet specially-prepared slides and left to dry on a hot plate. Slides were examined with the phase contrast microscope for identification of the mitotic spreads. (For future use of the chromosome preparations for in situ hybridization techniques, the slides were prepared before spreading the chromosomes by washing them thoroughly in detergent then in distilled water followed by immersing them in concentrated chromic acid for 48 hours then washing them in cold running tap water for 2 hours. The slides were then either given 2 washes with distilled water and used immediately or stored in absolute methanol for future use).

2.2.15. Preparation of chromosomes from hybrid cells:

The cells were left to grow confluent for a few days in order to be synchronized. The day before harvesting, the cells were trypsinized and subcultured into three or four flasks, and fresh medium was added. 18-24 hours later a flask with a high number of mitotic cells was chosen, and Demcolcin was added to a final concentration of 4 µg/ml medium for 90 minutes at 37°C. Following this, the cells were trypsinized, collected in 10 ml fresh medium and centrifuged in a siliconized conical centrifuge tube for 10-15 minutes at 600 rpm. To the pellet in a little supernatant, 10 ml of pre-warmed hypotonic (1:3 of nutrient medium without foetal bovine serum:distilled water) was added; a few drops first, then the rest added with whirlimixing and held at 37°C for 40 minutes. After incubation the suspension was centrifuged at 600 rpm for 10

minutes at room temperature. To the pellet 8 ml of fresh fixative of glacial acetic acid:methanol (1:3) was added (the first few drops were added with thorough mixing by a Pasteur pipette). After 10 minutes centrifugation at 600 rpm, the pellet was resuspended in 8 ml fresh fixative for another minute followed by centrifugation as above. The pellet was then resuspended in 8 ml fresh fixative held at $4^{o}C$ for 2 hours, centrifuged and resuspended in a few drops of fresh fixative to give a milky appearance. The suspension was dropped by a Pasteur pipette on wet slides from 30 cm. The slides were then dried on hot plate.

STAINING TECHNIQUES FOR CHROMOSOME PREPARATIONS FROM LYMPHOCYTES

2.2.16. Giemsa staining:

Dried preparations were stained with 2% Giemsa stain in phosphate buffer (pH 6.8) for 10 minutes, then washed with water and air dried.

2.2.17. Trypsin-Giemsa banding techniques:

In order to identify G-banding, the method of Seabright (1972) was used. The slides, at least a week old, were dipped for 2 minutes in 2 X SSC buffer, washed quickly in normal saline and covered by freshly prepared 0.25% trypsin solution in normal saline for 8 seconds (the time varies with different preparations), washed in saline and stained for 5 minutes with 25% Giemsa in phosphate buffer (pH 6.8) and washed in water before glass mounting in DPX.

2.2.18. Identification of Q-bandings on the Y-chromosomes:

The standard slide preparation (at least a week after preparation) was left in 2% KCl solution for a few minutes followed by staining for 20 minutes with freshly prepared Quinacrine mustard solution (0.04g% Quinacrine mustard in 1:1 mixture of absolute methanol:2% KCl).

The slide was then washed in saline and covered immediately by a cover slip sealing the ends with nail polish. The slide was immediately examined for the brilliant fluorescent Q-band in the dis-

tal segment of the Y chromosome under a Leitz fluorescent microscope (Ploemopak 2.1a.2.2) with a vertical illuminator, and supplied with a mercury vapour lamp and 510 nm barrier filter. Photography was done by using Ektachrome slide film ASA/200-400, printed by Kodak.

2.2.19. Identification of C bands:

The slide was incubated in the filtrate of 3% (w/v) barium hydroxide in distilled water at 60°C for one hour. The slide was then washed in tap water and incubated in 1 X SSC at 60°C for another hour. This was followed by washing thoroughly in distilled water then staining in 2% Giemsa in phosphate buffer (pH 6.8) for 75 minutes, then washing briefly in water.

2.2.20. Silver staining of nucleolar organizer regions in human chromosomes:

The method is bases on the method used by Bloom and Goodpasture, (1976). The slides to be stained were set in a moisture tight container (plastic or metal) and flooded with 50% silver nitrate solution in distilled deionized water that was prepared in light-tight tubes. The slides were covered immediately by cover slips. In order to ensure moisture, a piece of filter paper soaked in silver nitrate solution and distilled water was left in the container. Slides in such a chamber were incubated in a water bath at 55°C for 22 hours. After incubation, the slides were washed in deionized distilled water thoroughly to remove excess silver stain. This was followed

by staining either with the 2% Giemsa in phosphate buffer or treated by trypsin and Giemsa as mentioned before.

2.2.21. Identification of the human X chromosome in mouse-human hybrids by Bisbenzimid fluorochrome (Hoechst 33258):

Slides containing the HORL 9 X 4 2.2 chromosomes, at least a week after their preparation, were rinsed in freshly prepared solution of 0.5 µg/ml Bisbenzimid in a solution of 0.09% NaCl, 2% KCl, for one hour at 4°C in the dark. This was followed by washing in distilled water then in the mounting buffer (0.08M sodium phosphate, 0.12M sodium citrate, pH 4.1). The slide was then glass-mounted in the mounting buffer and the edges sealed with nail polish. Immediately after mounting, the slide was scanned for the suitable mitotic figure with fluorescent regions in a Leitz (Ploemopak 2.1a.2.2) fluorescent microscope with vertical illuminator, supplied with a mercury vapour lamp and 510 nm barrier filter. Photography was by using Ektachrome slide film ASA/200-400 and printed by KODAK.

2.2.22. In situ hybridization:

Suitable microscopic slides with many metaphase spreads, were chosen by phase-contrast microscope. Each slide was incubated at 37°C for 30 minutes in 200 µl of 2 X SCC containing 100 µg/ml ribonuclease underneath a 22 X 40 mm coverslip. (The ribonuclease was freshly prepared from 2 mg/ml stock which was previously boiled for 5 minutes to inactivate any contaminating DNase). All the incubations were done in a stainless steel tray partially immersed in a water

bath. The slide after being washed thoroughly in 2 X SSC at room temperature, was dehydrated in 50%, 75%, 95% and absolute alcohol susseccively. 200 µl of 95% deionized formamide in 0.05% SSC was applied onto the slide surface, covered by a 22 X 40 mm coverslip and incubated at 65°C for 2½ hours to denature the DNA. This was followed by washing thoroughly in 2 X SSC and dehydration in alcohols as before. The desalted radio-labelled DNA, to be hybridized to the chromosomal DNA, was dissolved in 4 µl of the hybridization buffer (0.5M NaCl, 0.025M Hepes, 0.01M EDTA adjusted to pH 7.0, 50% formamide), held at 100°C for 2 minutes (to ensure denaturation of the DNA) then applied immediately on the slide (pre-incubated at 43°C), covered by a 22 X 22 mm coverslip and sealed around the edges by a rubber solution (Cow Gum).

The slide was incubated at 43°C for 16 hours. After the period of incubation, the tray that contained the slide was kept on ice for 5 minutes. The slide was then washed thoroughly in 2 X SSC at room temperature, and then held at 65°C in 2 X SSC in a shaking water bath for 6 hours, changing the SSC each 2 hours, in order to remove the non-hybridized labelled DNA as much as possible, and dissociate the non-specific hybrids. This was followed by washing in cold 2 X SSC then stirring at 4°C in 2 litres of 2 X SSC for 16-24 hours. The slide was dehydrated in series of alcohol and then covered by photographic emulsion. The emulsion was prepared by mixing equal amounts of distilled water and photographic gel (K2 emulsion in gel form, Ilford Ltd., Mobberley, Cheshire, England), at 50°C. The slide then was kept in

sealed, light-proof box, in which some crystals of calcium sulphate were left to avoid humidity. After an appropriate time of exposure at 4°C, the slide was developed in D19 developer:distilled water (1:1) for 5 minutes, washed in tap water and fixed in Hypam fixer: distilled water (1:4) for 4 minutes then washed in running tap water for one hour. The slide was stained for 40 minutes with 5% Giemsa in phosphate buffer (pH 6.8) followed by washing in tap water. The slide was then examined under a Leitz microscope and suitable figures were photographed using Recordak 35 mm microfile film (Type 5460) ASA/12 (KODAK).

SECTION 1

3.1 THE ISOLATION OF HUMAN CHROMOSOME SPECIFIC GENE SEQUENCES

3.1.1. Principle:

Cross reannealing of DNA from sources which differ in a single chromosome should make it possible to isolate DNA sequences specific for that chromosome in single stranded form.

The presence of the Y chromosome in normal male subjects and its absence in normal females is the basis for the isolation of Y-specific gene sequences after cross-reannealing to a certain Cot value. If the genomic differences between a normal male and a normal female (other than the presence of the Y in male and its absence in female) are excluded, then hybridizing the labelled male DNA to 10^4-fold excess of unlabelled female DNA to Cot 10^4 will, in principle, let all the complementary strands in the two genomic sets reanneal, leaving behind any DNA that cannot find its complementary strand, i.e. DNA specific to the Y genome and present at low multiplicity. Hybridization is to a vast excess of unlabelled female DNA in order to prevent or reduce to a minimum the self-reannealing of the male DNA. The same principle will hold for the isolation of other chromosome-specific gene sequence utilizing somatic cell hybrids (between human and rodent cells) which have been grown on selective media in order to retain a single chromosome besides the rodent parental chromosomal set. Labelled total DNA from such hybrids is reannealed to an excess of unlabelled mouse DNA. The mouse parental genome from both sources reanneals, leaving the human chromosome-specific low reiteration class of DNA sequences non-hybridized.

3.1.2. THE ISOLATION OF HUMAN Y-CHROMOSOME SPECIFIC GENE SEQUENCES

Labelled normal male DNA was hybridized to 10^4-fold excess of unlabelled normal female DNA. The hybridization was carried out in 0.12M PB at 60°C to Cot 10^4 for the unlabelled female DNA (100 hours, concentration of 10 mg/ml unlabelled DNA). Analysis of the hybrids was by HAP chromatography at 60°C. The single stranded non-hybridized material, eluted with 0.14M PB, was dialysed against 3 changes of 5 litres of distilled water at 5, 16 and 24 hour intervals. After being ethanol-precipitated (or lyophilized), the material was desalted on a G-50 Sephadex column, washed with distilled water, and the DNA eluate collected. The radiolabelled DNA was measured and a further portion of sheared desalted female DNA was added to make the ratio of the female DNA to labelled male DNA 10^4 once again. The DNA was lophilized and dissolved in 0.12M PB (10 mg/ml) and hybridized to Cot=10^4 at 60°C. The material that did not reanneal in the second cycle of hybridization was collected by HAP chromatography and the female DNA was made up to 10^4-fold excess over labelled male DNA and reannealed to Cot=10^4 for the third time.

The non-hybridized DNA after the third cycle was pooled after analysis on HAP. This was tested for its male specificity by reannealing it to 40,000-fold excess of unlabelled male DNA and to 36,000-fold excess of unlabelled female DNA (and about 4,000-fold excess unlabelled female which was still present in the male-enriched probe). The reannealing was carried out in two separate reactions run together in 0.12M PB at 60°C at 10 mg DNA/ml.

The experiment of enriching for male-specific gene sequences was repeated three times.

3.1.2.-1. DNA PREPARATION & LABELLING:

Both male and female DNA were prepared from placentae. About 400 mg DNA was obtained from 600g of tissue. The DNA was labelled by nick-translation. Nick translation depends upon the ability of the enzyme E.coli polymerase I to catalyze a sequential addition of nucleotide residues to the 3-hydroxyl terminus of a nikc and the elimination of nucleotide units from the cleared 5'-phosphoryl terminus (Kelly et al., 1970). The enzyme DNase is used to nick the DNA. The nick translation was performed as described in the Methods section.

The (^{3}H) TTP and (^{3}H) dCTP used were of specific activities 50 and 20 Ci/mmol respectively. The results after one hour nick translation of 5 µg DNA at 15°C were:-

	cpm	dpm
Total counts	105600	330000
DNA (TCA ppt)	62916	196612

(quench factor = 32% on GF/A filters counted in TBS)

percentage incorporation of counts incorporated into DNA was $\frac{62916}{105600} = 59.6\%$.

The radioactivity used in the reaction was 19 + 56 = 75 µCi for 5 µg DNA, or 15 µCi for 1 µg DNA; therefore the specific activity = $2.22 \times 10^6 \times \frac{59.6}{100} \times 25 = 19.8 \times 10^6$ dpm/µg DNA.

After two phenol extractions, the supernatant was lyophilized, dissolved in 50 mM Tris buffer and passed through G-50 Sephadex column to separate the unincorporated nucleotides from the labelled DNA (Fig. 3.1.1). [The purity of the pooled desalted DNA fractions on G-50 Sephadex is shown in Fig. 3.1.2.] The final yield of the DNA was 3 µg out of a starting 5 µg, or 60%.

3.1.2.-2. MEASUREMENT OF THE SIZE OF THE LABELLED DNA:

The size of the DNA was measured by formamide gel electrophoresis compared to 28S, 18S and 5S RNA as markers. This enabled an approximate size of 700 nucleotides to be deduced (Fig. 3.1.3). By the same technique the size of the other nick translated DNA preparations was found to vary from 600-800 nucleotides.

3.1.2.-3. SNAP BACK PHENOMENON IN NICK-TRANSLATED DNA:

During the process of nick-translation, the DNA polymerase I may reverse direction and copy the chain already synthesized and hence synthesize palindromic structures (i.e. inverted repeat sequences, or sequences that "read the same" both backwards and forwards), which can form intrastrand hybrids after denaturation, and therefore reanneal at low Cot values as if satellite DNA.

In order to measure the degree of snap-back, nick-translated DNA was dissolved in a large volume of 0.12M PB, denatured by boiling and HAP chromatographed immediately. Only snap-back sequences will reanneal and appear double-stranded in the conditions used. For this purpose 5,000 cpm of nick translated male DNA was lyophilized and

Fig 3.1.1

Separation of nick translated DNA from un-incorporated nucleotides on Sephadex G-50 column chromatography. The (^{3}H) DNA was eluted in the fractions between 6-9 inclusive.

Fig. 3.1.1

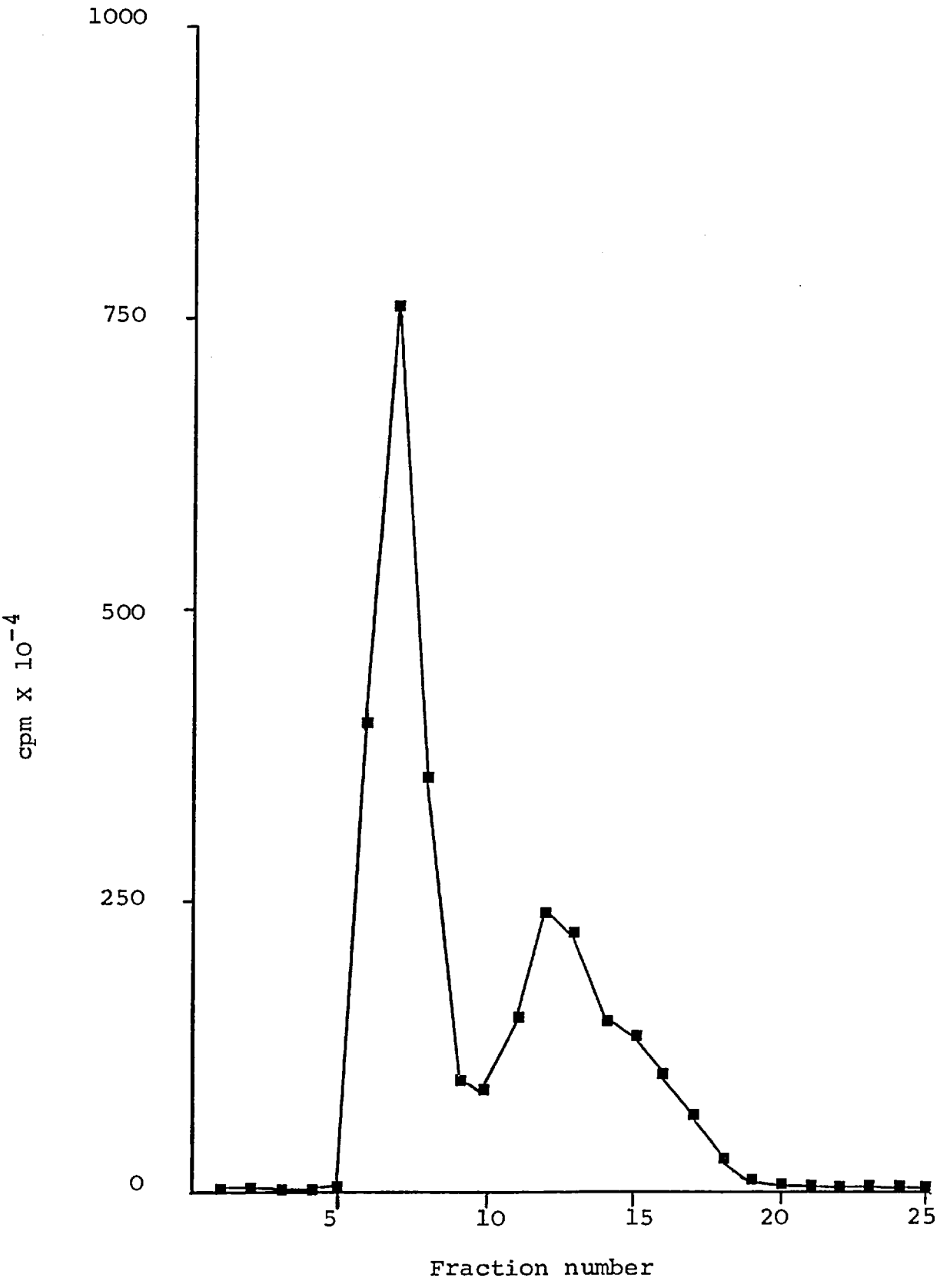


Fig. 3.1.2

Desalting nick translated DNA (specific actvity 1.98×10^7 dpm/µg DNA) on Sephdex G-50 column chromatography.

Fig.3.1.2

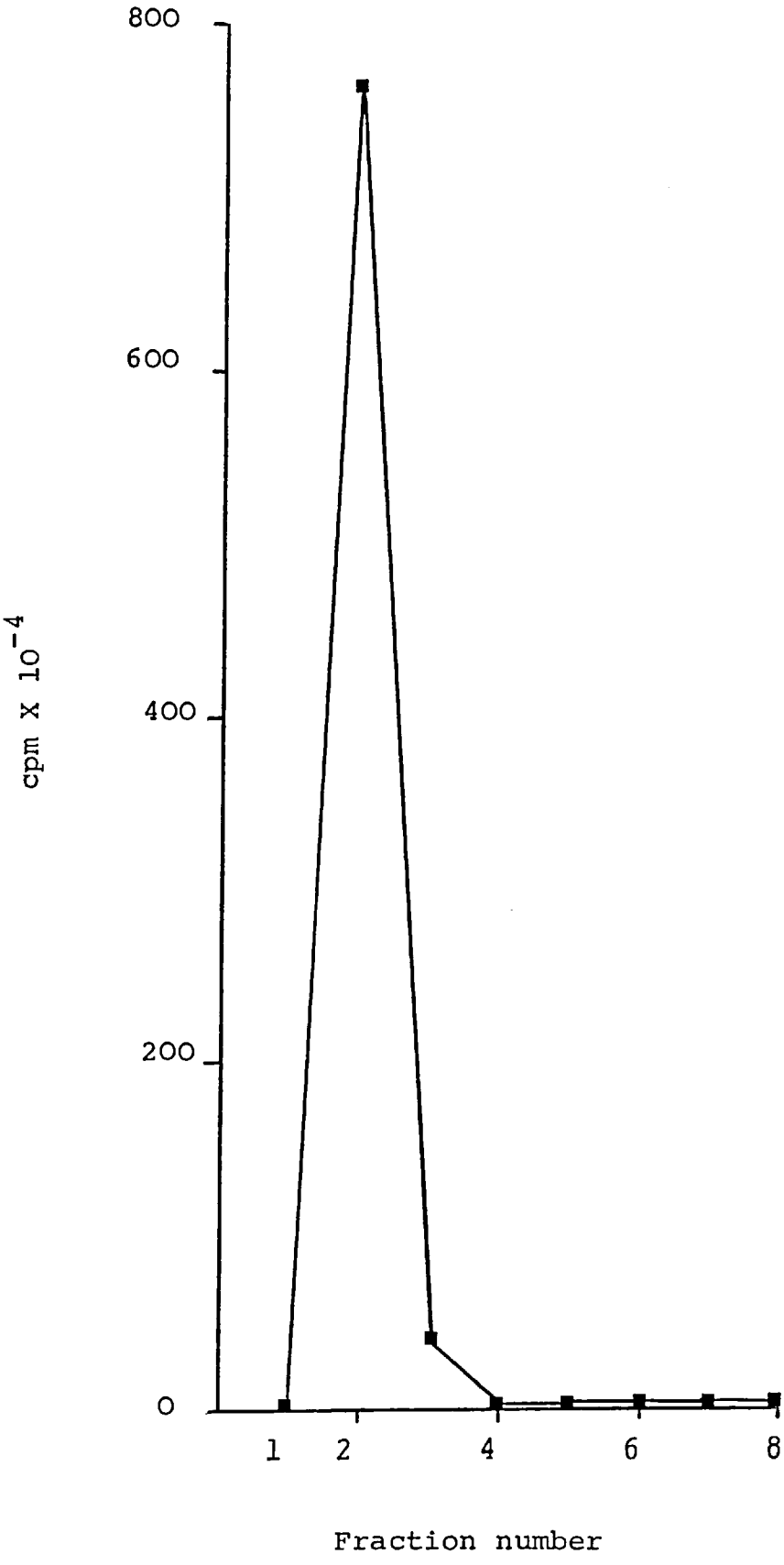
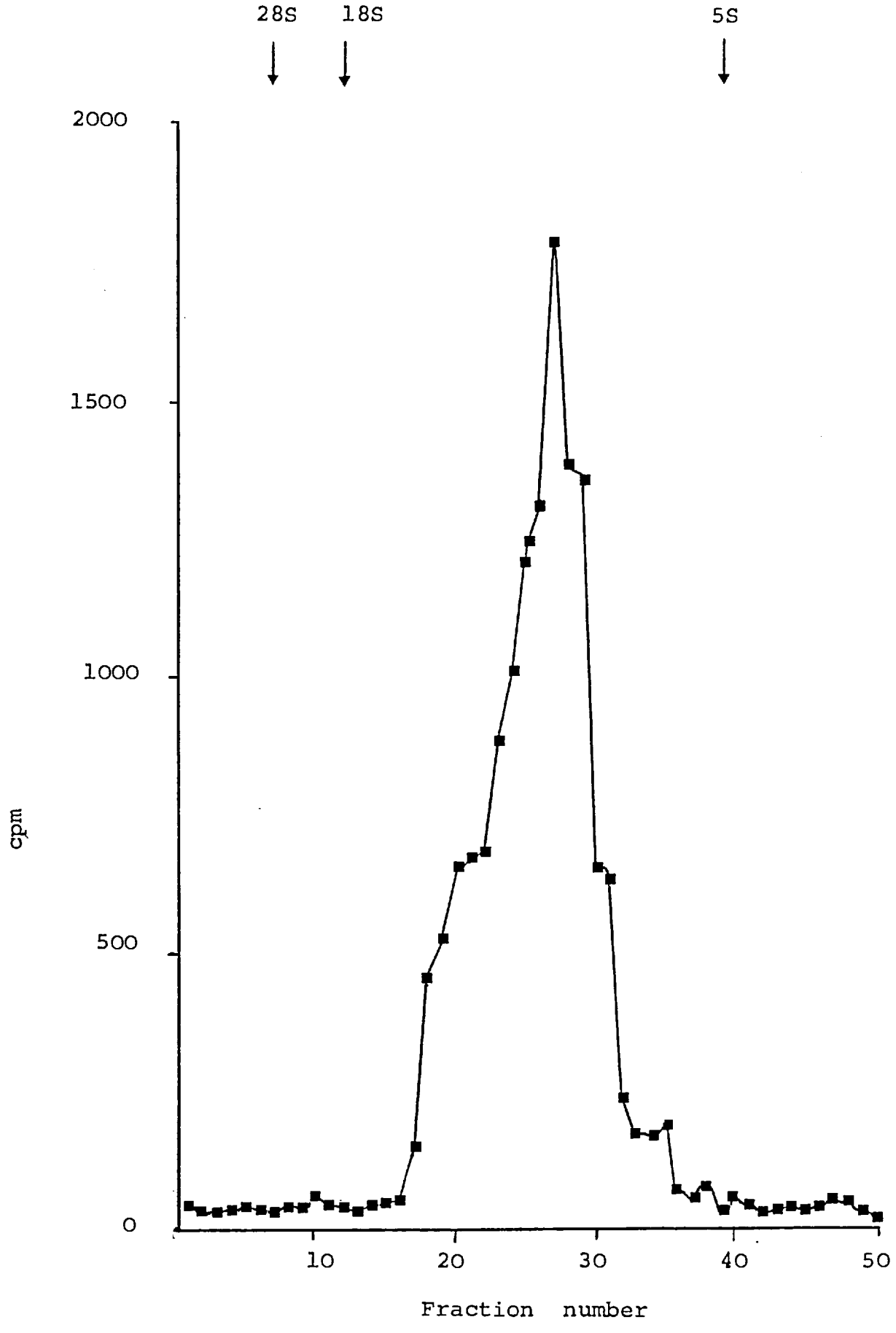


Fig. 3.1.3

Formamide gel electrophoresis of nick translated DNA (specific activity 1.98×10^7 dpm/µg DNA) . 50,000 cpm (^{3}H)DNA was used and 30 µg of 28S,18S and 5S RNA was used as a marker,in order to obtain an approximate size of the DNA. Each sample was dissolved in 30µl buffer.

The positions of the RNA marker on the gel is represented by arrows.

Fig. 3.1.3


28S
18S
5S
2000
1500
1000
500
0
cpm
10
20
30
40
50
Fraction number

dissolved in 0.2 ml of 0.12M PB, boiled for 10 minutes, cooled immediately in ice and diluted to 0.03M PB with 0.6 ml distilled water. (This represents a Cot of approximately 10^{-6}.) After HAP analysis at 60°C, the results were:-

PB	cpm
0.03M	111
0.14M	4442
0.4M	182

The degree of snap back is at most 3.9%.

3.1.2.-4 HYBRIDIZATION OF THE LABELLED MALE DNA WITH UNLABELLED SHEARED MALE DNA:

This is demonstrated by Fig. 3.1.4 which shows that the labelled male DNA reannealed to the unlabelled male DNA with the same kinetics as the unlabelled DNA to itself (Cot 1/2 = 10^3 for non-repeated sequences).

3.1.2.-5 HYBRIDIZATION OF LABELLED MALE DNA TO UNLABELLED FEMALE DNA (First Cycle):

The labelled male DNA reannealed to the unlabelled female DNA with the same kinetics as the unlabelled female DNA to itself (Cot 1/2 = 800). This is shown in Fig. 3.1.5, the reannealing profile of the first cycle of hybridization of the labelled male DNA to the unlabelled female DNA. Cot 1/2 for the self-reannealing of the female DNA is 900 for non-repeated sequences.

Fig. 3.1.4

Reannealing profile of the (^{3}H)total male DNA to sheared unlabelled DNA.(Specific activity of the labeeled DNA was 1.98 X 10^{7} dpm/µg DNA).

▲——▲ Reannealing of (^{3}H)male DNA to unlabelled male DNA.

□——□ Reannealing of unlabelled male DNA to itself.

Fig. 3.1.4

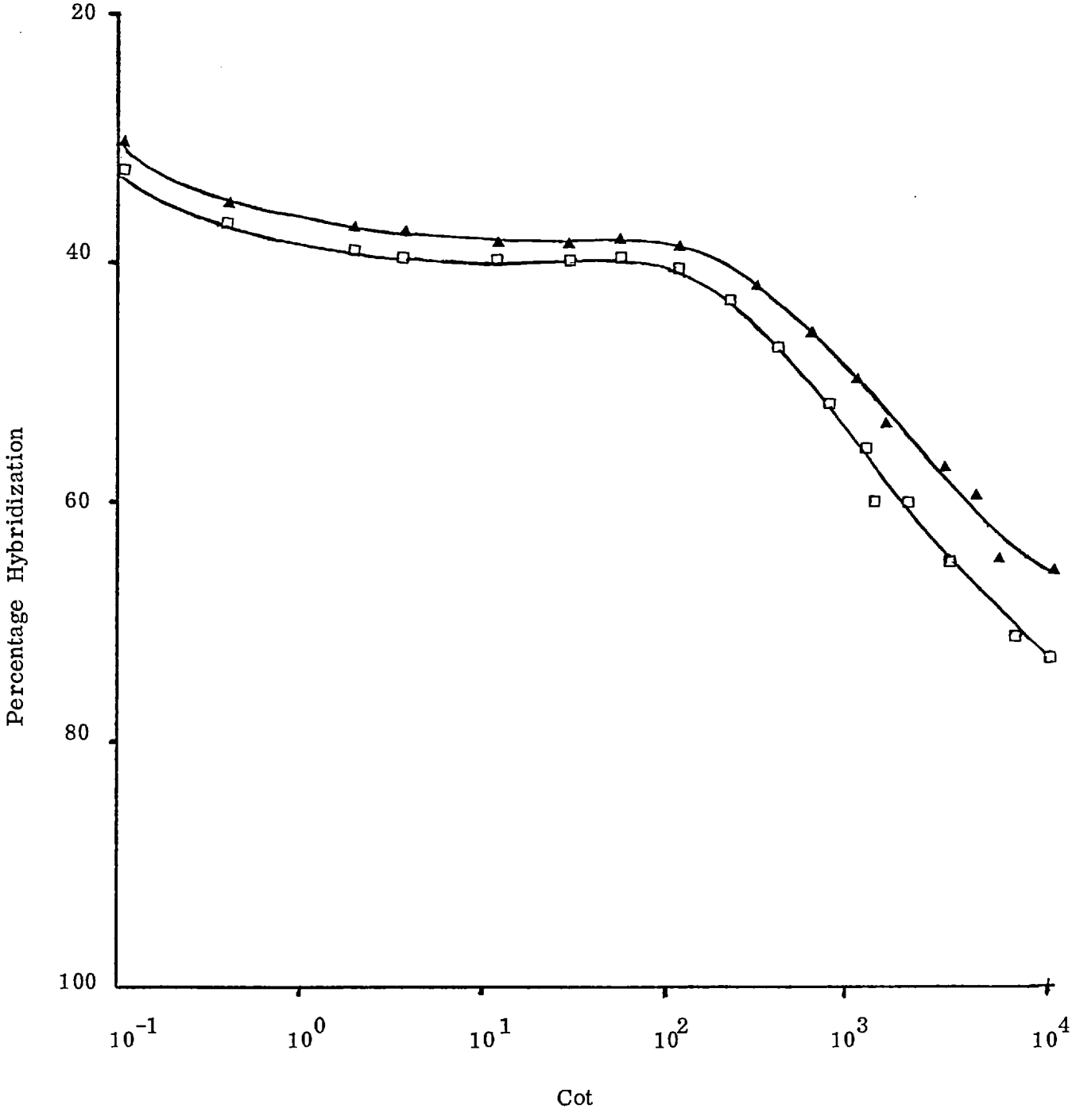

20
40
60
80
100
Percentage Hybridization
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
Cot

Fig. 3.1.5

Reannealing profile of (^{3}H)male DNA to 10^{4}-fold excess of sheared unlabelled female DNA (first cycle hybridization).

●——● Reannealing of labelled male DNA to unlabelled female DNA.

▲——▲ Reannealing of unlabelled female DNA to itself.

Fig. 3.1.5

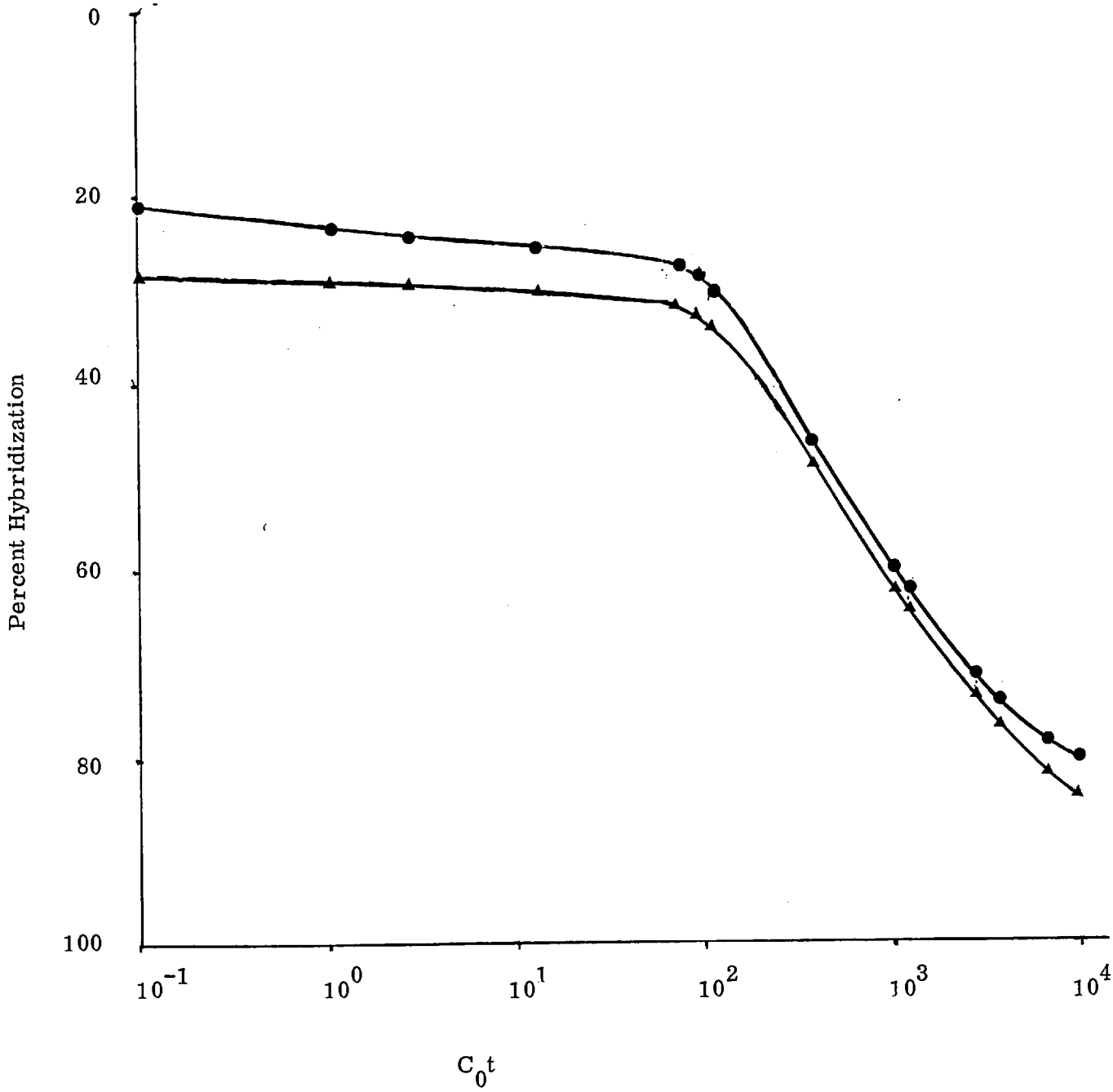

0
20
40
60
80
100
Percent Hybridization
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
C_0t

Table 3.1.1. shows the results of the hybridization to Cot 10^4 of 17 mg sheared female DNA to 1.7 µg (10.8×10^6 cpm) labelled male DNA in 1.7 ml of 0.12M PB.

The hybridization percentage of the unlabelled female DNA to itself was calculated from table 3.1.1 as:-

$$\frac{13.6}{13.6 + 2.4} = 85\%$$

The percentage of reannealing of the labelled male DNA to the unlabelled female DNA is calculated as:-

$$\frac{8283240}{8283240 + 2110752} = 80\%$$

After dialyzing against distilled water, ethanol precipitation then desalting on Sephadex G-50 column the single stranded material (eluted at 0.14M) was found to contain 1.75 mg female DNA and 1.785×10^6 cpm (5.578×10^6 dpm), which is equivalent to 0.2811 µg male DNA. In order to make the ratio between the labelled male DNA/ unlabelled female DNA = $1/10^4$, a further 1.061 mg female DNA was added. The hybridization mixture of the second cycle was:-

0.2811 µg (^{3}H)-labelled

2.811 mg female DNA

which was hybridized to Cot 10^4.

3.1.2.-6. ANALYSIS OF THE HYBRIDS OF THE SECOND CYCLE:

Table 3.1.2. shows that 75% of the unlabelled female DNA had reannealed to itself ($\frac{1.85}{1.85 + 0.62}$) while 50% of the labelled male DNA reannealed to the female DNA ($\frac{753912}{753912 + 755280}$). The DNA, after dialysis against distilled water and desalting as before, was found to contain 405330 cpm (0.06383 µg) male DNA and 318.3 µg female DNA.

Table 3.1.1

Result of the first cycle hybridization of labelled male DNA to unlabelled excess female DNA. The table shows the amount of the DNA eluted on HAP chromatography at 60°C by PB during analysis of the hybrids ($C_o t\ 10^4$).

Table 3.1.1

PB	mg toatl DNA eluted	Total cpm
0.03M	0.529	200088
0.14M	2.4	2110752
0.4M	13.6	8283240

Table 3.1.2

Result of the second cycle hybridization of labelled male DNA to excess unlabelled female DNA. The table shows the amount of the DNA eluted on HAP chromatography at 60°C by PB during analysis of the hybrids ($C_o t\ 10^4$).

Table 3.1.2

PB	mg total DNA eluted	Total cpm
0.03M	0.108	53424
0.14M	0.62	755280
0.4M	1.85	753912

A further 320 μg of female DNA was added in order to keep the female DNA in 10^4-fold excess to the male DNA. The DNA mixture was lyophilized and hybridized at 60°C to Cot 10^4 in 0.12M PB for the third time.

3.1.2.-7 RESULTS OF THE THIRD CYCLE HYBRIDIZATION (TABLE 3.1.3):

72% of the female DNA reannealed to itself, while only 40% of radio-labelled male DNA reannealed to the female DNA. In 0.03M PB no unlabelled DNA was detected by optical density measurements while a total of 7440 cpm were eluted. The radio-labelled DNA eluted by 0.14M PB was regarded as "male enriched" (60% of the input into the third cycle). The final yield of male-enriched DNA after dialysis against distilled water and desalting as before was approximately 120,000 cpm containing 40.7 μg of unlabelled female DNA.

3.1.2.-8 ESTIMATION OF THE SIZE OF THE MALE-ENRICHED DNA PROBE:

The size as measured by formamide gel electrophoresis, was about 180 nucleotides (Fig. 3.1.6) although it had also some smaller material. This is about one-third the size of the original (^{3}H) male DNA before hybridization to female DNA (Fig. 3.1.3).

3.1.2.-9 SPECIFICITY OF THE MALE-ENRICHED PROBE:

The specificity of the male-enriched DNA was tested by comparing the reannealing profiles of the male-enriched probe to vast excess of unlabelled male DNA and of unlabelled female DNA. This was compared, also, to its reannealing to excess E.coli DNA. Fig. 3.1.7 shows the presence of a difference in kinetics of reannealing over the entire length of the two curves (more obvious after Cot 10^2), and the presence of gene sequences which reannealed to the unlabelled male

Table 3.1.3

Result of the third cycle hybridization of labelled male DNA to unlabelled excess female DNA. The table shows the amount of the DNA eluted on HAP chromatography at 60°C by PB during analysis of the hybrids ($C_o t\ 10^4$).

Table 3.1.3

PB	mg total DNA eluted	Total cpm
0.03M	___	7440
0.14M	0.165	216792
0.4M	0.425	144360

Fig.3.1.6

Formamide gel electrophoresis of the Y chromosome-enriched DNA isolated after the third cycle hybridization of labelled male DNA to unlabelled female DNA.The positions of the 28S ,18S and 5S represent the O.D. scanning of the gel in which the marker RNA was used.

Fig.3.1.6

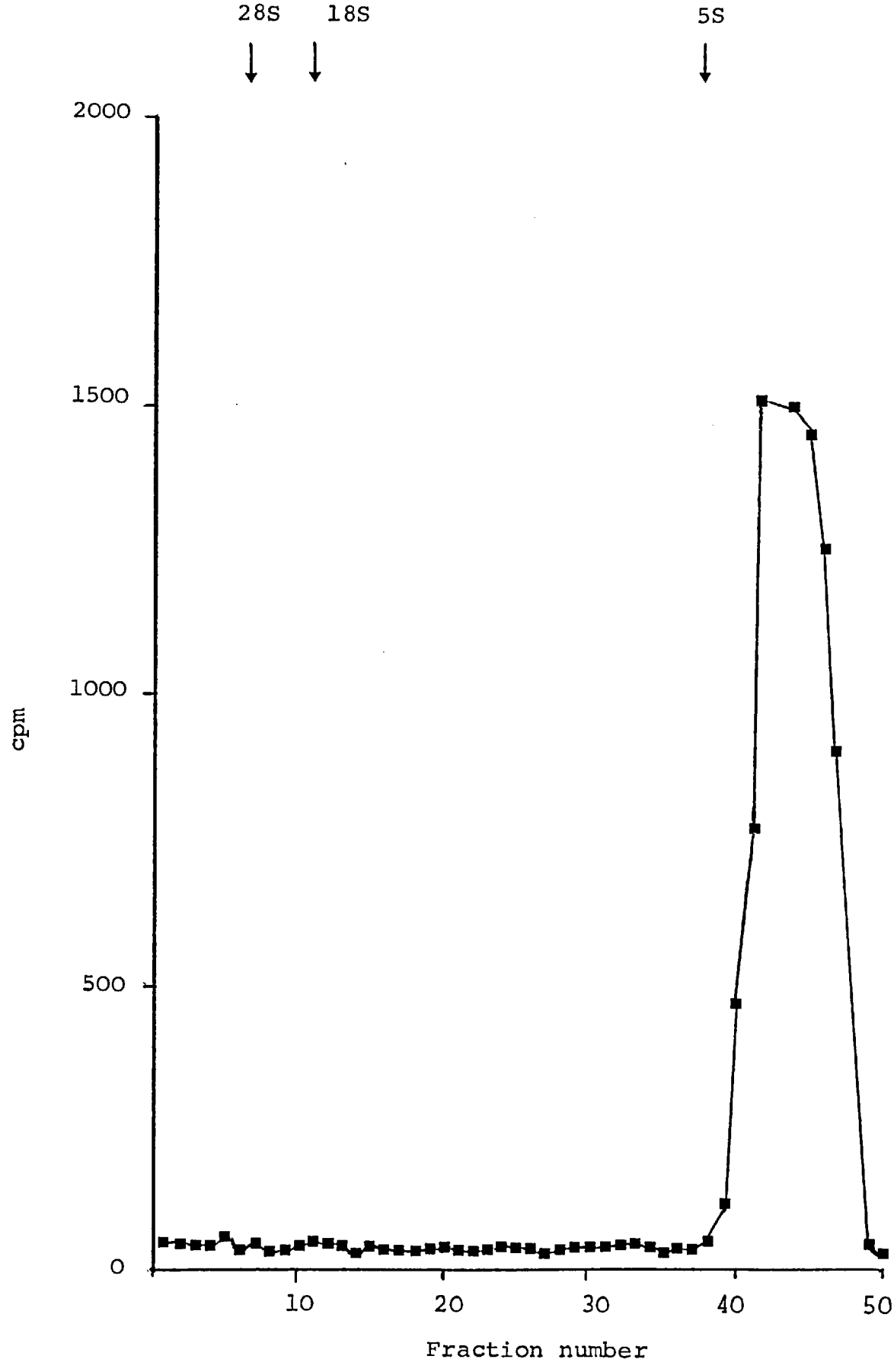
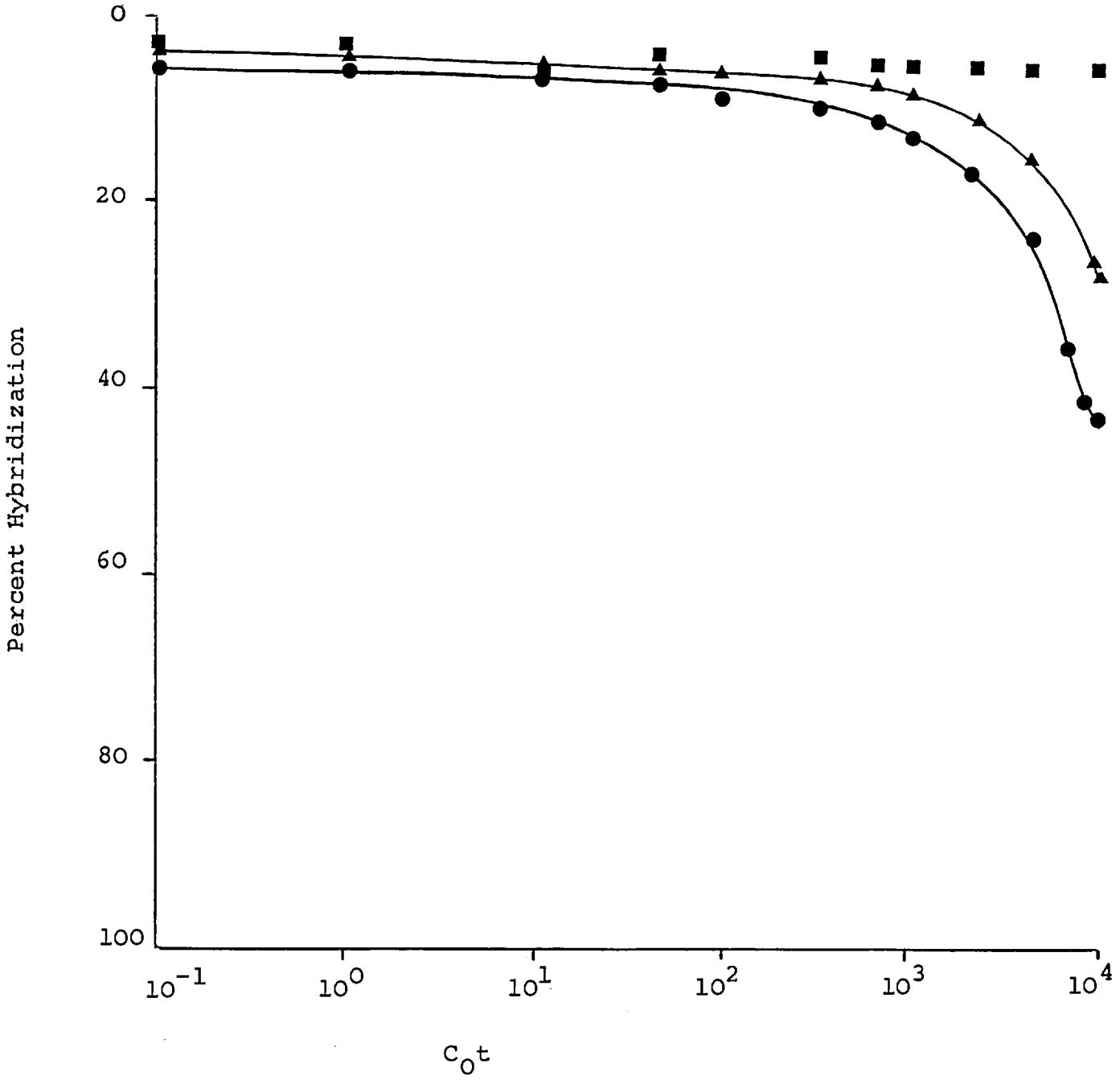

28S
18S
5S
2000
1500
1000
500
0
cpm
10
20
30
40
50
Fraction number

Fig.3.1.7

Reannealing profiles of labelled Y chromosome - enriched gene sequences to excess unlabelled female DNA (▲——▲);excess unlabelled male DNA(●——●) and to E.coli DNA(■ ■).

Each reaction point determines hybridization of 2,000 cpm (315ng) labelled Y-enrched DNA to 12.6µg unlabelled female DNA (4 X10^4-fold excess). In the case of reannealing to unlabelled male DNA , 11.6µg of the latter was added to each point(36X10^3-fold excess). This is due to the unlabelled female DNA already present in the Y-enriched probe(1.44µg at each point,or about 4X10^3-fold excess) which together with the unlabelled male DNA added make up a 4 X10^4-fold excess. At the same time the Y-enriched gene sequences were hybridized to 10^4-fold excess of E.coli DNA. The hybridization was carried out in 0.12M PB (at a concentration of 10 mg DNA/ml) at 60°C in siliconized sealed capillaries,and analysed on HAP.

Fig. 3.1.7


0
20
40
60
80
100
Percent Hybridization
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
C_0t

DNA but not to the female DNA. This demonstrates the existence of gene sequences in the male genome which do not occur in the female. At Cot 10^{-1}, as could be seen, the percent reannealing of the male-enriched sequence to the unlabelled female DNA is only 4% and to the unlabelled male DNA was 5.0% compared to 22% hybridization of the labelled male DNA to the unlabelled female DNA during the first cycle of enriching and 28% reannealing of the unlabelled female DNA to itself. This suggests that almost all the highly repetitive DNA sequences were removed during the purification process.

The $Cot_{\frac{1}{2}}$s of the two reannealing curves cannot be compared directly as they have not reannealed to the same extent at Cot = 10^4. If it is assumed that hybridization of male-enriched sequences to total female DNA will reach 42% (as for their hybridization to total male DNA) at Cot = 3 X 10^4, then the $Cot_{\frac{1}{2}}$ to male DNA is 7,000 and to female DNA is approximately 9,000. All of these values must be regarded as inexact until the nature of the non-hybridizing material in the male-enriched sequences is known.

sequences in the male-enriched probe which do not occur in the female DNA and, therefore are specific to the male genome. It is also shown in Fig. 3.1.7 that the male-enriched sequences do not reanneal significantly to the E.coli DNA, the hybridization percentage at Cot 10^4 to E.coli DNA is only 4%.

3.1.2.-10 FURTHER EXPERIMENTS TO ENRICH THE MALE GENE SEQUENCES:

Another two experiments to enrich male gene sequences were performed following the same principles and procedures as already described. The labelled male DNAs used were of specific activities of 2.855 x 10^6 cpm/µg DNA and 3.528 cpm/µg DNA for the second and third experiments

respectively. In each of the three cycles of hybridization, the ratio of the unlabelled female DNA/labelled male DNA entering the reannealing process was 10^4. Table 3.1.4 shows a comparison of the results of the three cycles of reannealing during the process of isolation of the male-enriched gene sequences for the three experiments. There was not much difference in the percentage reannealing of the labelled male DNA to the unlabelled female DNA at each cycle except for the second experiment where the results were 40%, 50% and 50% for the three cycles of hybridization respectively (average 46.6%). It was possible to test the specificity of the male-enriched material isolated from the third enrichment experiment; the final yield from the second experiment was too low. This was done as described previously and is shown in Fig 3.1.8. At Cot 10^4 only 29% of the male-enriched gene sequences had reannealed to excess of total female DNA while the percent reannealing to excess of total male DNA was 40%.

3.1.2.-11 ESTIMATION OF THE Y CHROMOSOME-SPECIFIC GENE SEQUENCES:

As it is apparent from the kinetic analysis of the male-enriched DNA isolated from the first and third enrichment experiments (Fig 3.1.7 and 3.1.8) that there exist sequences that hybridized to excess unlabelled male DNA but not to unlabelled female DNA; these sequences are male-specific. If other genomic differences between the male and female subjects are excluded (apart from the existence of the Y chromosome in the male and its absence from a female) then these male-specific sequences may be assumed to be Y chromosome-specific. The percentage of the total male genome isolated at Cot = 10^4 as Y chromosome-specific gene sequences was determined by the difference between the reassociation of the (^{3}H) male-enriched sequence with excess unlabelled female DNA (Kunkel *et al.*, 1976). This difference

Table 3.1.4

Comparison between hybridization percentages at $C_o t\ 10^4$ of labelled male DNA to excess unlabelled female DNA and of unlabelled female DNA to itself in each of the three hybridization cycles .

(The results are from three enrichment experiments).

Hybridization cycle	Percent hybridization of							
	unlabelled female DNA to itself in the				labelled male DNA to excess unlabelled female DNA in the			
	first*	second*	third*	mean	first*	second*	third*	mean
first	85%	82%	78%	81.7%	80%	81%	80%	80.3%
second	75%	78%	72%	75%	50%	50%	40%	46.6%
third	72%	72%	70%	71.3%	40%	42%	40%	40.7%

* enrichment experiment.

Table 3.1.4

Fig. 3.1.8

Reannealing profiles of the (^{3}H) Y chromosome-enriched gene sequences ,isolated from the third enrichment experiment,to excess unlabelled female DNA (▲——▲) ; excess unlabelled male DNA (●——●); excess unlabelled E.coli DNA (■ ■).

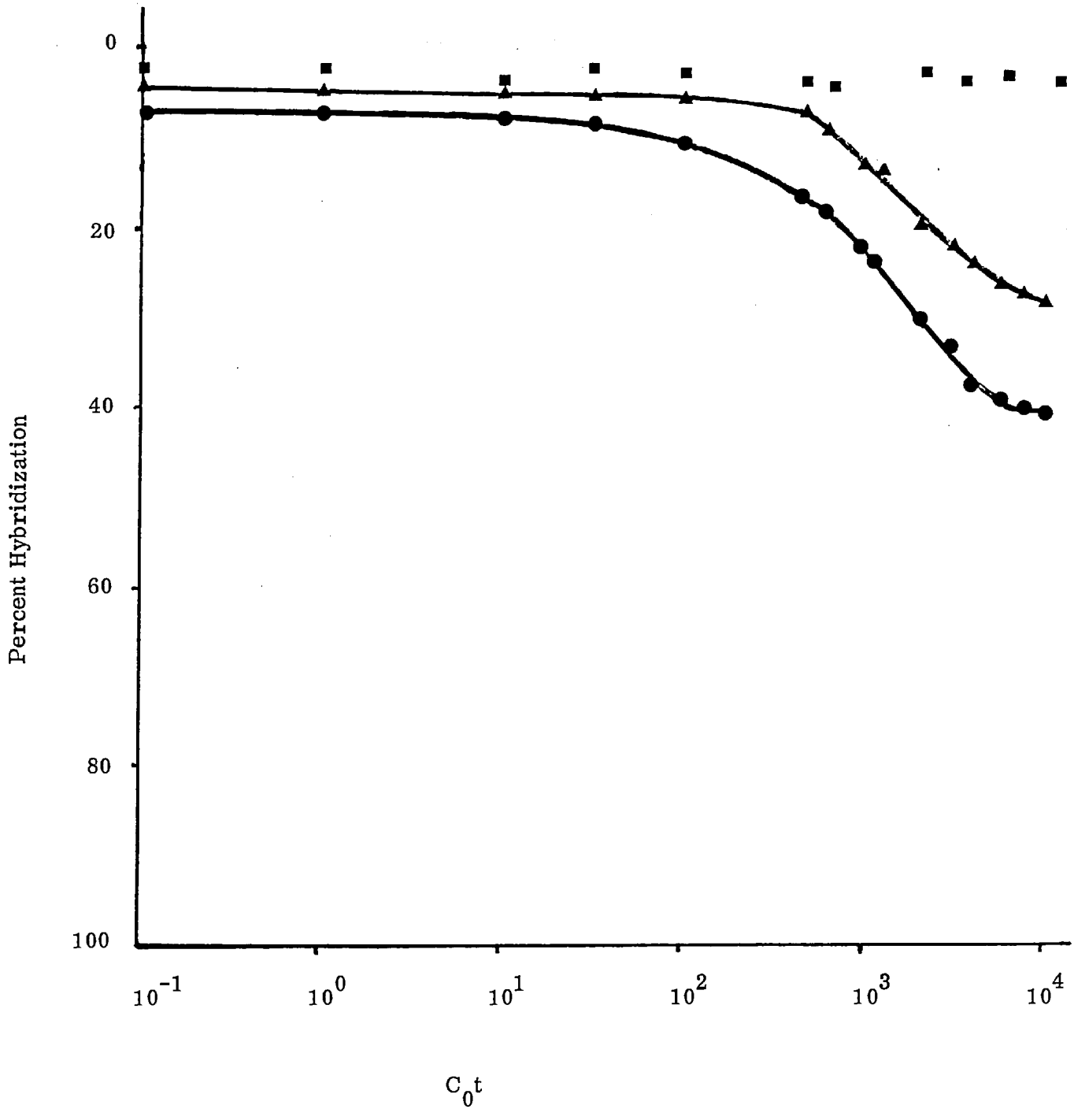

0
20
40
60
80
100
Percent Hybridization
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
C_0t

Fig. 3.1.8

was then calculated as the percentage of the material that did not form stable duplexes in each step of the hybridization reactions and calculated as percentage of the original genomic input. Therefore, in the first experiment of enriching the Y chromosome specific sequence was:-

20% x 50% x 60% x (42% - 28%) = 0.89% of the total genome

The Y chromosome specific sequence isolated from the third enrichment experiment was:-

20% x 60% x 60% x (40% - 29%) = 0.79% of the total genome

(See Table 3.1.5).

Table 3.1.5

Percent hybridization of Y chromosome-enriched gene sequences to excess unlabelled male and female DNAs in two enrichment experiments at $C_o t\ 10^4$. The table also shows the amount of the Y chromosome-specific gene sequences estimated in each experiment.

Table 3.1.5

Enrichment experiment	percent hybridization to		Y chromosome specific gene sequences*
	male DNA	female DNA	
first	42 %	28 %	0.89 %
third	40 %	29 %	0.79 %

* Y chromosome-specific gene sequences are expressed as percentage of the total male genome.

3.1.3. THE ISOLATION OF HUMAN X CHROMOSOME-SPECIFIC GENE SEQUENCES:

3.1.3.-1. PRINCIPLE:

The human X chromosome-enriched gene sequences were obtained by extensive hybridization of labelled DNA isolated from mouse-human hybrid cells HORL9X (which contain the human X chromosome only, besides the mouse chromosomes) to 10^4 - fold excess of unlabelled parent mouse DNA. The principle is similar to that for the isolation of the human Y chromosome-specific gene sequences. The hybridization was carried out to Cot 10^4 and the non-hybridized material was isolated and rehybridized to 10^4-fold escess of unlabelled parent mouse DNA to Cot 10^4. The single strand material that does not reanneal at this step was regarded as X-enriched, as the percentage reannealing in the second cycle was very low. It was tested for its X-specificity by following its reannealing profiles to excess unlabelled DNA from both the human-mouse hybrid cells and parent mouse cells.

The human-mouse hybrid cells were hybrids (HORL 9.X 4 2.2) obtained from Dr V. Van Heyningen, Edinburgh, which were originally grown in the Genetics Laboratory, Biochemistry Department, Oxford University. We thank her for her geneous gift of this cell line, which we then maintained in our laboratory. For convenience those cells will be named HORL 9.X. The mouse from which the hybrids were obtained was a C3H strain of mice.

3.1.3.-2 THE IDENTIFICATION OF THE HUMAN X CHROMOSOME IN THE HORL 9X CELLS:

Fig 3.1.9 and 3.1.10 show the result of Hoechst 33258 staining of two different metaphase spreads from HORL 9.X cells. The centromere of the

Figures 3.1.9 and 3.1.10

Hoechst 33258-fluorescent staining of HORL 9.X chromsome preparations. The arrows indicate the human X chromosome. Note that all the centromeres of the metacentric and telocentric mouse chromosmes are brightly fluorescent, while the human X chromosome does not show the centromeric fluorescence. The human X chromosome shows an intense fluorescent band in the middle of each of its arms; this is well shown in Fig. 3.1.10(page **100**).

N.B.; Metacentric mouse chromosomes are two joined telocentric chromosomes.

Fig.3.1.9

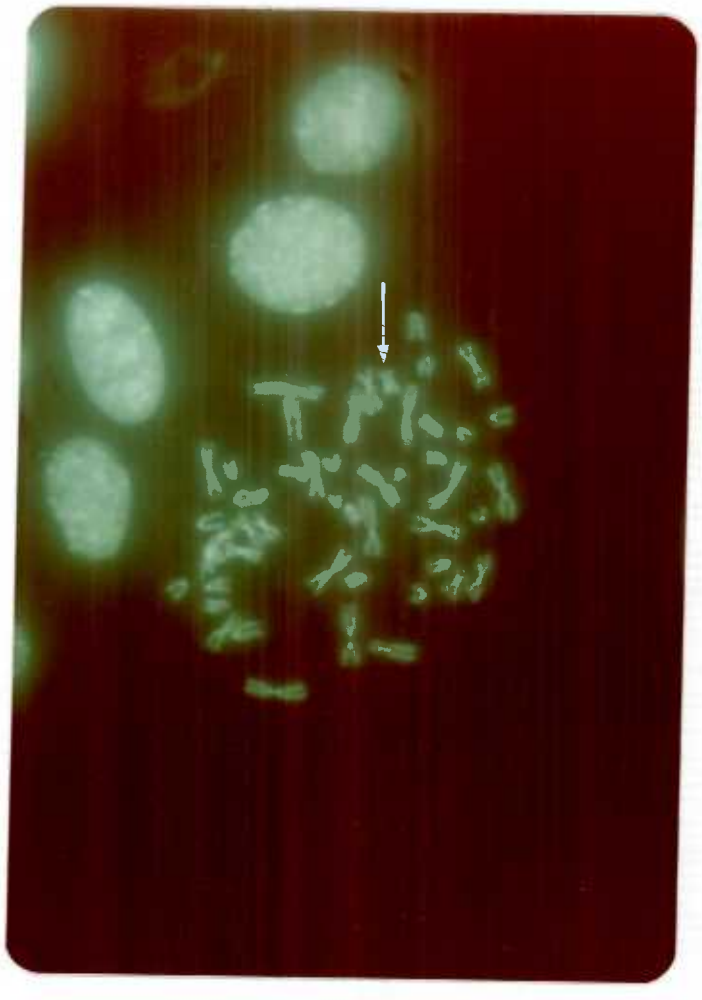
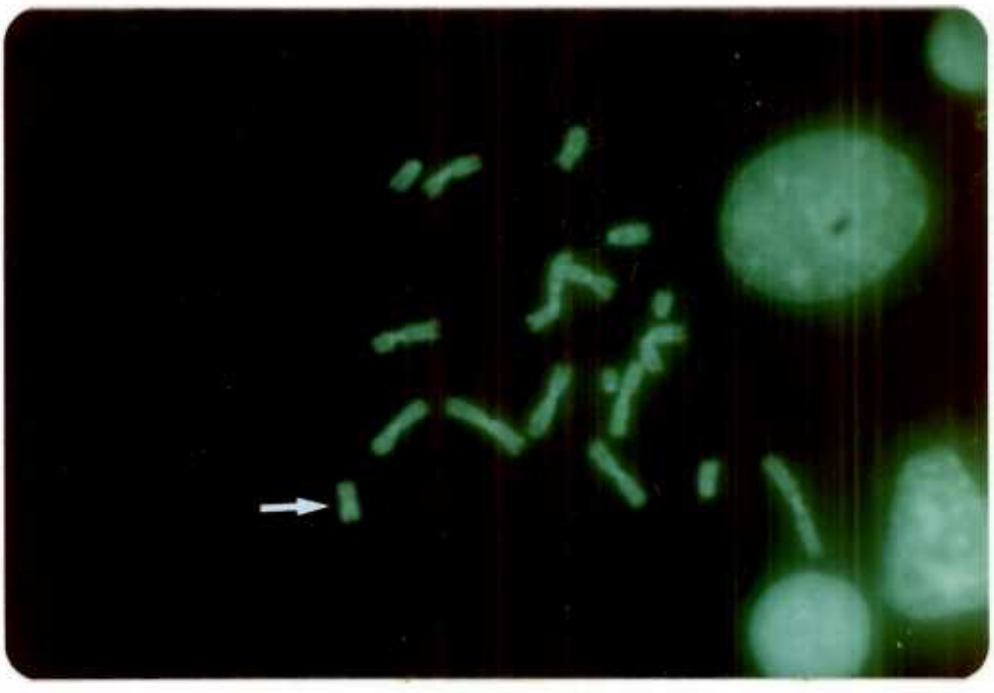


Fig.3.1.10



human X chromosome does not show bright fluorescence, while a fluorescent band maybe shown in the short arm and another in the long arm of the human X.

3.1.3.-3 FIRST CYCLE HYBRIDIZATION:

10 mg unlabelled parent mouse DNA was reannealed to 1 µg labelled HORL 9.X DNA (specific activity 5.9 x 10^6 dpm/µg DNA, size 600 nucleotides) to Cot 10^4 (Fig 3.1.11). The result of HAP analysis of the hybrids at 60°C is shown in table 3.1.6, where the percentage reannealing of the labelled HORL 9.X DNA to the unlabelled mouse DNA was found to be 72%, and hence 28% did not form stable duplexes. The hybridization percentage of the unlabelled parent mouse DNA to itself was 80%. Cot $\frac{1}{2}$ of reannealing of the labelled HORL 9.X DNA to the parent mouse DNA was 10^3 for the non repeated sequences.

After dialysing and desalting the single-stranded DNA, only 304000 cpm of (^{3}H) HORL 9.X DNA (equivalent to 0.16 µg) and 500 µg unlabelled DNA were obtained. The unlabelled DNA was made up to 1.6 mg (i.e. 10^4-fold excess), lyophilized and dissolved in 160 µl of 0.12M PB and hybridized at 60°C to Cot = 10^4 following boiling for 15 min.

3.1.3.-4 ANALYSIS OF THE SECOND CYCLE HYBRIDIZATION PROCESS:

Table 3.1.7 shows the results of the analysis on HAP chromotography. 71.5% of the unlabelled DNA reannealed to itslef at Cot = 10^4. This is less than the hybridization percentage obtained during the first cycle, 80%. The percentage reannealing of the labelled DNA to the unlabelled DNA was 25.5% which means that 74.5% of the labelled material that entered the second cycle did not reanneal to the

Fig. 3.1.11

First cycle hybridization of (^{3}H)HORL 9.X DNA to unlabelled sheared parent mouse(C_3H strain) DNA. 10 mg of unlabelled sheared mouse DNA and 1 µg of labelled HORL 9.X DNA(specific activity 5.94 X 10^6 dpm/µg DNA) were used. The hybridization was carried out in 1 ml of 0.12M PB at 60°C.

▲——▲ Reannealing of (^{3}H)HORL 9.X DNA to unlabelled parent mouse DNA.

●——● Reannealing of parent mouse DNA to itself.

Fig.3.1.11

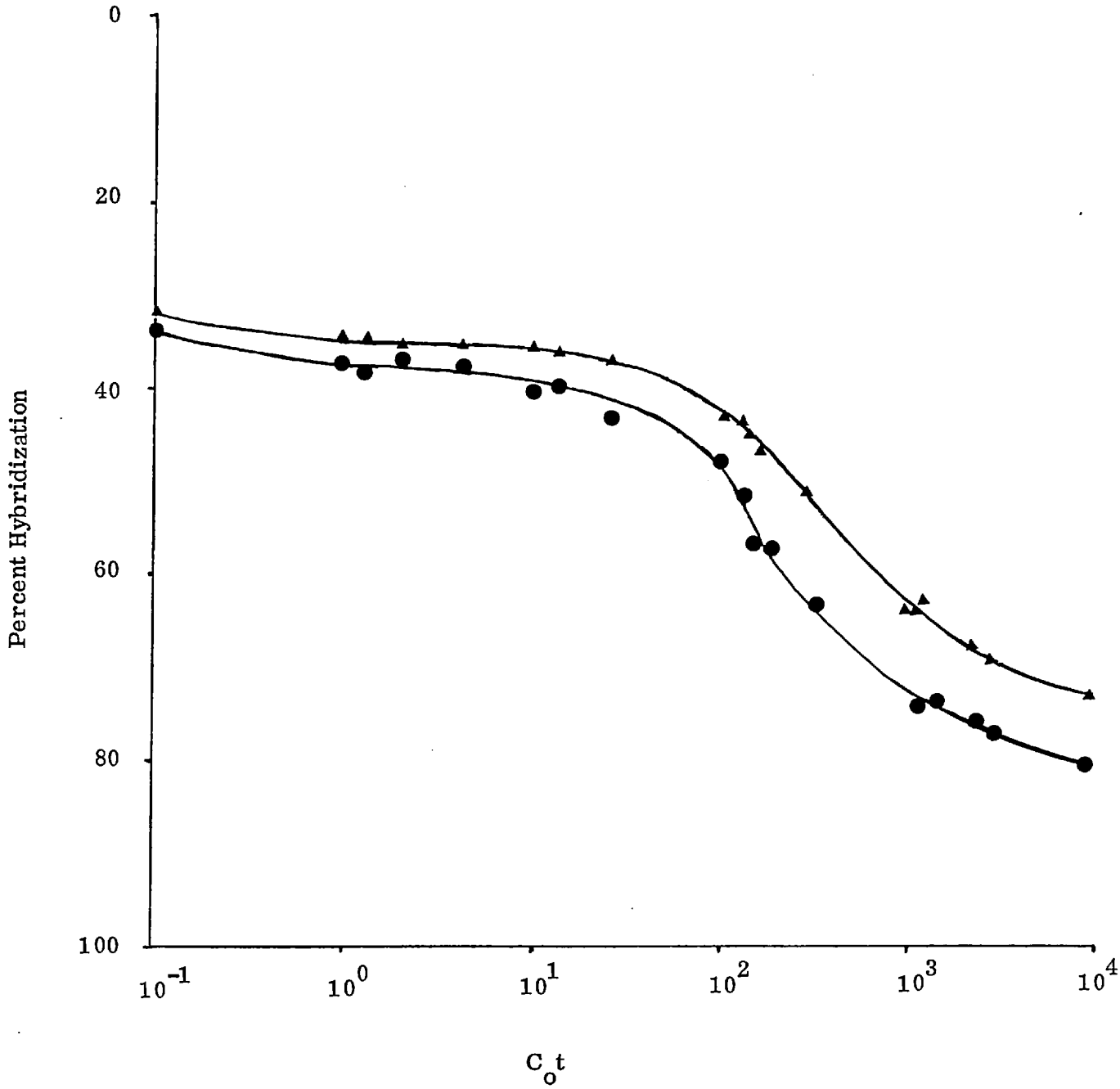

0
20
40
60
80
100
Percent Hybridization
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
$C_o t$

Table 3.1.6

Results of analysis of the first cycle hybridization on HAP column at 60°C , of labelled HORL 9.X DNA and unlabelled parent mouse DNA.

Table 3.1.6

PB	Total DNA eluted mg	Toatl cpm
O.O3M	O.561	26640
O.14M	1.77	475130
O.4M	7.O8	1221363

Table 3.1.7

Analysis of the second cycle hybridization on HAP column at 60°C of labelled HORL 9.X DNA and unlabelled parent mouse DNA.

Table 3.1.7

PB	Total DNA eluted mg	Total cpm
0.03M	0.227	37282
0.14M	0.422	180245
0.4M	1.057	61827

unlabelled mouse parent DNA. Only 119000 cpm and 25.53 µg of the unlabelled DNA remained after dialysis and desalting. This probe was tested for its specificity for the X chromosome. During both cycles of hybridization the material that wereeluted at 0.03M PB were discarded, so they did not enter a further hybridization cycle and be pooled with the X-enriched probe.

3.1.3.-5. SPECIFICITY OF THE X-ENRICHED PROBE:

The reannealing profile of the X-enriched probe to 10^4-fold excess of unlabelled HORL 9.X DNA was compared with its reannealing to 10^4-fold excess of unlabelled parent mouse (C3H strain) DNA. The result of HAP analysis of the hybridization curves (Fig 3.1.12) indicates the existence, in the probe tested, of DNA sequences that do not hybridize to the parent mouse DNA but hybridize to the HORL 9.X DNA. As the latter contains the human X chromosome as well as the mouse chromosomes, it is concluded that the probe contains sequences specific for the human X-chromosome. It is observed that almost all the highly repetitive sequences,of whatever origin, have been removed from the X-enriched probe, as 6% of it has reannealed to the unlabelled HORL 9.X DNA and only 5% has reannealed to the mouse parent DNA at Cot = 10^{-1} (Fig 3.1.1). This can be compared to the reannealing of the (^{3}H) HORL 9.X DNA to the parent mouse DNA in the first cycle of hybridization, which is 32%, and also the self-reassociation of both mouse parent DNA and HORL 9.X DNA at Cot = 10^{-1} (23% and 30% respectively) (Fig 3.1.12).

3.1.3.-6. ESTIMATION OF X CHROMOSOME-SPECIFIC GENE SEQUENCES:

X chromosome-specific gene sequences were estimated in the same way as previously described for the human Y chromosome-specific gene sequences. This was calculated from the difference between

Fig.3.1.12

Specificity of the X chromosome-enriched probe. This was determined by comparing the reannealing profiles of (^{3}H)X chromosome-enriched DNA to excess unlabelled parent mouse DNA(▲——▲) and to unlabelled HORL 9.X DNA (■——■).

●——● represents reannealing of unlabelled mouse parent DNA to itself.

□——□ represents reannealing of unlabelled HORL 9.X DNA to itself.

Each experimental point represents the hybrdization of 7600 cpm(4 ng)of (^{3}H)X chromosome-enriched DNA which already had 1.064 µg of parent mouse DNA (266-fold excess). The amount of the unlabelled DNA was made upto 40 µg for each point by adding excess of either parent mouse DNA or unlabelled HORL 9.X DNA to obtain a final ratio of 10^4. Each mixture was lyophilized and dissolved in 4 ul of 0.12M PB and allowed to hybridize in sealed siliconized capillaries at 60°C after 15-minute incubation at 100°C.

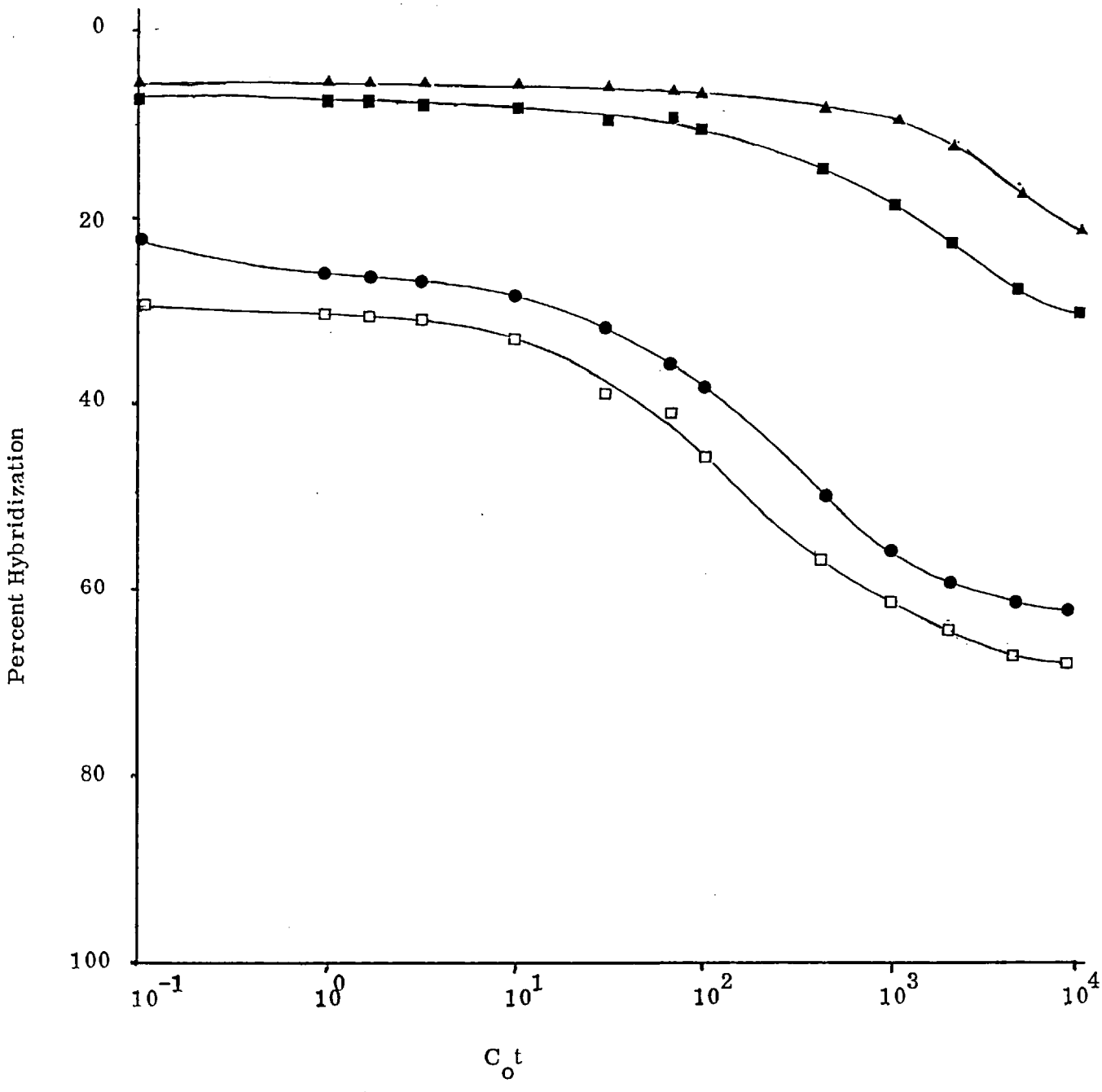

0
20
40
60
80
100
Percent Hybridization
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
$C_o t$

Fig.3.1.12

the percent reannealing at Cot = 10^4 of the X-enriched probe to excess unlabelled HORL 9.X DNA and its reannealing to excess parent mouse DNA. This difference was then calculated as a percentage of the amount of single stranded material that did not form stable duplexes in each of the two enrichment cycles. The result will obviously reflect the amount of human X-specific genome as a percentage of the HORL 9.X genome (and not as a proportion of the human genome) at Cot = 10^4. It is 28% x 74.5% x (31% - 21%) = 2.08%. As at Cot = 10^4 only the low repetitive sequences are assayed, this reflects that the human X-chromosome specific DNA is approximately 2% of the mouse low-repetitive DNA. Such sequences occupy about 70% of the total mouse genome (Britten and Kohne, 1968). Therefore the total proportion of human X-specific sequences (low and mid-repetitive) is approximately 1.5% of the mouse genome in this hybrid cell line.

SECTION II

3.2. THE CHROMOSOMAL LOCALIZATION OF HUMAN Y CHROMOSOME-SPECIFIC GENE SEQUENCES

Y chromosome-specific gene sequences, isolated as described in Section I, were localized to a particular chromosome by the method of molecular *in situ* hybridization. Y chromosome-enriched gene sequences were hybridized to normal male chromosomes isolated from the peripheral blood lymphocyte culture. The distal fluorescent segment of the Y chromosome (Yq12) stained brightly with quinacrine mustard in chromosome preparations (Fig. 3.2.1) was found to measure one-third of the Y chromosomal length. The relative length of the Y chromosome to other chromosomes was measured for 10 quinacrine-banded cells, and was found to average 2.01% $\pm$ 0.145% of the total haploid length. Therefore the relative length of Yq12 region (the fluorescent segment) will be approximately 0.66% of the total haploid length.

After *in situ* hybridization, the distribution of the silver grains over different chromosomes was studied by direct observation of photographic prints and their microscopic figures. Only figures with complete chromosome spreads were photographed and scored for the chromosomal distribution of silver grains. A total of 17 cells were scored. The Y chromosome was covered by grains in 16 cells. Each figure was karyotyped, in general according to chromosomal groups, except for the chromosomes which could be reliably identified, chromosomes 1, 2, 3, 9 and 16. Figure 3.2.2. is an example of the figures printed, while its karyotyping is represented in Fig. 3.2.3. Some of the Y chromosomes from other cells are shown in Fig. 3.2.4. A total of 41 grains on the Y chromosomes (35% of the total of 117 grains) were found. The distal

Fig. 3.2.1

Quinacrine mustard staining of chromosome preparation from the peripheral blood lymphocytes of a normal 46,XY male subject. The arrow indicates the Y chromosome. Note that the distal fluorescent segment is one-third of the total Y length.

Fig.3.2.1

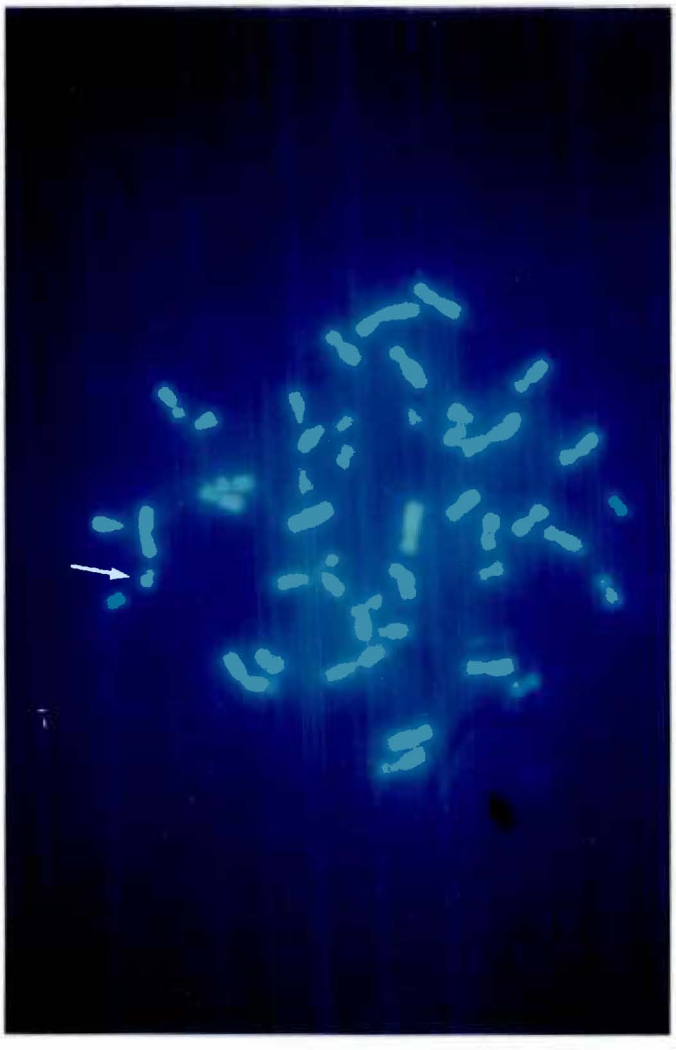


Fig.3.2.2

Normal 46,XY chromosome preparation(from the peripheral blood lymphocytes) after hybridization *in situ* to (^{3}H)Y chromosome-enriched gene sequences(specific activity 1.98 X 10^{7} dpm/μg DNA). 7.1 ng (140,625 dpm) of probe was dissolved in 8 μl of formamide hybridization buffer and hybridized *in situ* to two slides of the normal male chromosome preparations. The exposure time to photographic emulsion was eight weeks.
The arrow indicates the Y chromosome.

Fig.3.2.2

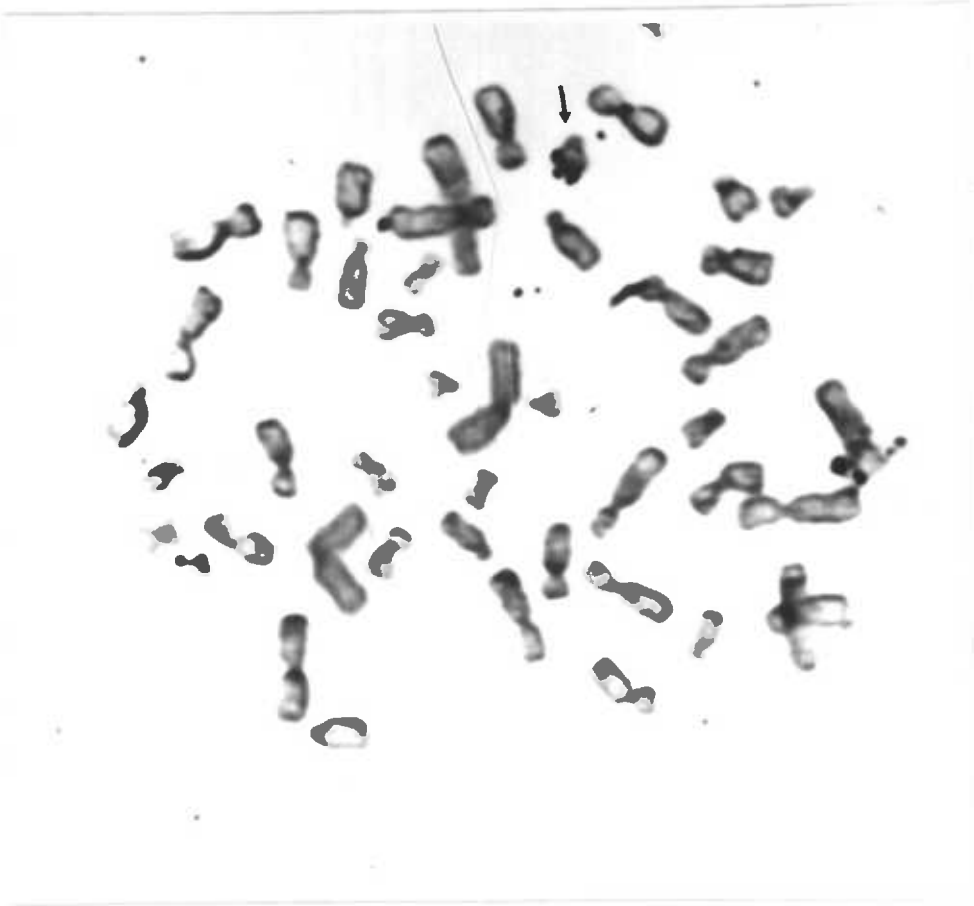


Fig. 3.2.3

Karyotype of Fig.3.2.2 .
Group A includes chromosomes 1-3;group B, chromosomes 4 and 5 ;group C,chromosomes 6-12 ; group D,chromosomes 13-15 ; group E,chromosomes 16-18 ; group F,chromosomes 19 and 20 ;group G,chromosomes 21 and 22. Note that two grains are found on the centromeric region of a chromosome thought to be an X . The X chromosome is usually regarded as a member of the C group.

Fig.3.2.3

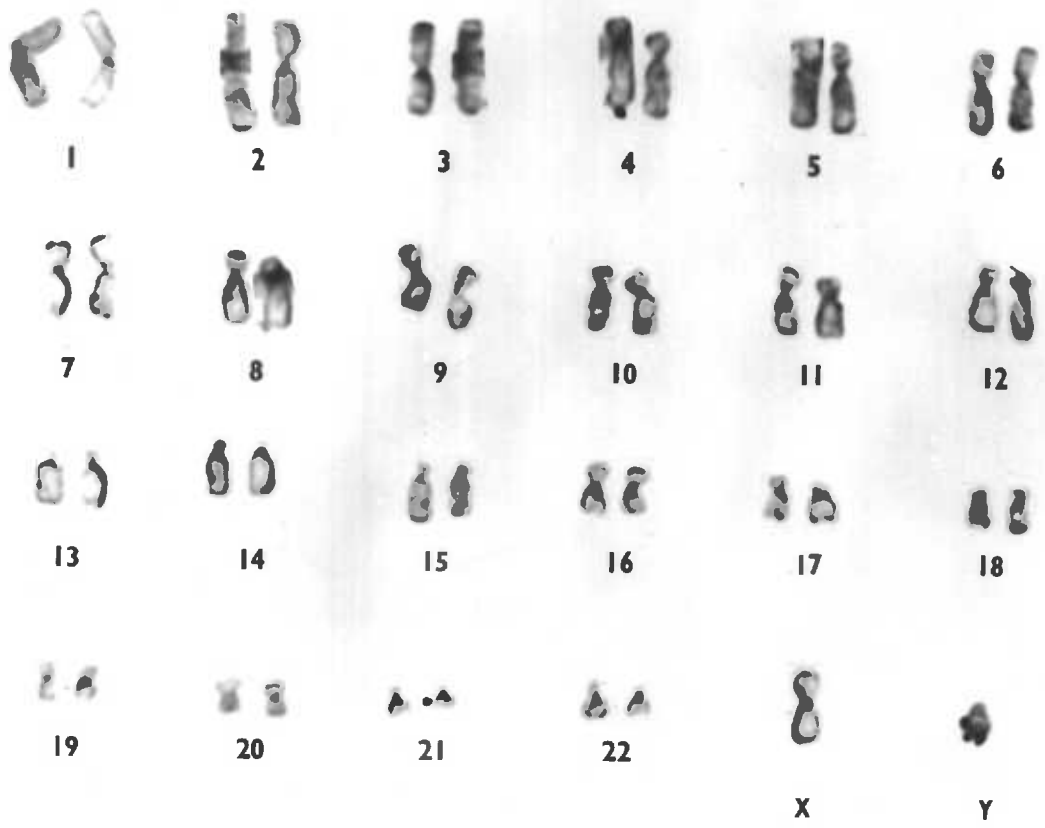
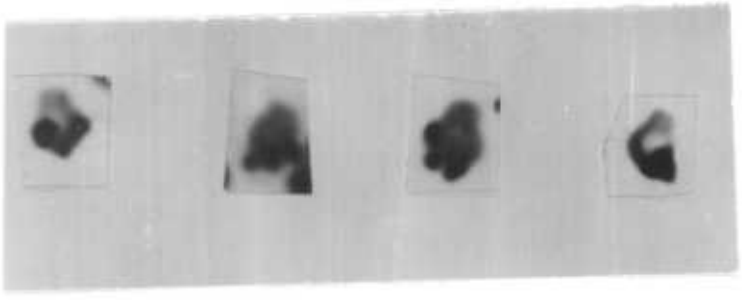


Fig.3.2.4

Some Y chromosomes from chromosome preparations of the peripheral lymphocytes from a normal 46,XY male after *in situ* hybridization to (^{3}H)Y chromosome-enriched gene sequences. The silver grains are specifically found over the distal segment of the long arm of the Y (Yq12).

Fig.3.2.4



segment of the long arm of the Y (Yq12) which corresponds to the fluorescent segment, ranked highest (81% of the grains on the Y) compared to 17% on region Yq11 and 2% on the short arm (Yp), as shown in Fig. 3.2.5. Regions Yq11 and Yq12 were defined by measuring the length of each Y chromosome scored when the distal third was regarded as region Yq12 .

The distribution of the silver grains on different chromosomal groups is shown in Fig. 3.2.6 and that on chromosomes 1, 2, 3, 9, 16 and Y in Fig. 3.2.7. No silver grains were detected on the centromeres of any of the chromosomes except for a chromosome from the C group that resembles an X chromosome and which had two silver grains on the centromere (Fig. 3.2.2 and 3.2.3).

To allow for extra random background being found in larger chromosomes the number of the grains on each chromosomal group has been divided by the sums of the relative lengths of the individual chromosomes in each group, as measured for ten quinacrine-banded cells. This was compared to the number of the grains scored on the Y chromosome adjusted according to its length (Fig. 3.2.8 and 3.2.9). The background in each cell was estimated by dividing the total number of the grains on the chromosomes (except the Y) in each cell by their added relative lengths. The average background, therefore, is equal to 0.023 grains/relative length/cell, while the number of grains on the Y chromosome divided by its relative length per cell is 1.21 (which is 52 times higher than the background). X^2 (Chi square) analysis was applied to compare the grains on the Y chromosome/relative length in each cell with the background in each cell. The null hypothesis was to assume that both densities (the

Fig. 3.2.5

Distribution of the silver grains on different regions of the " normal " Y chromosome for the 46,XY male.

Fig. 3.2.5

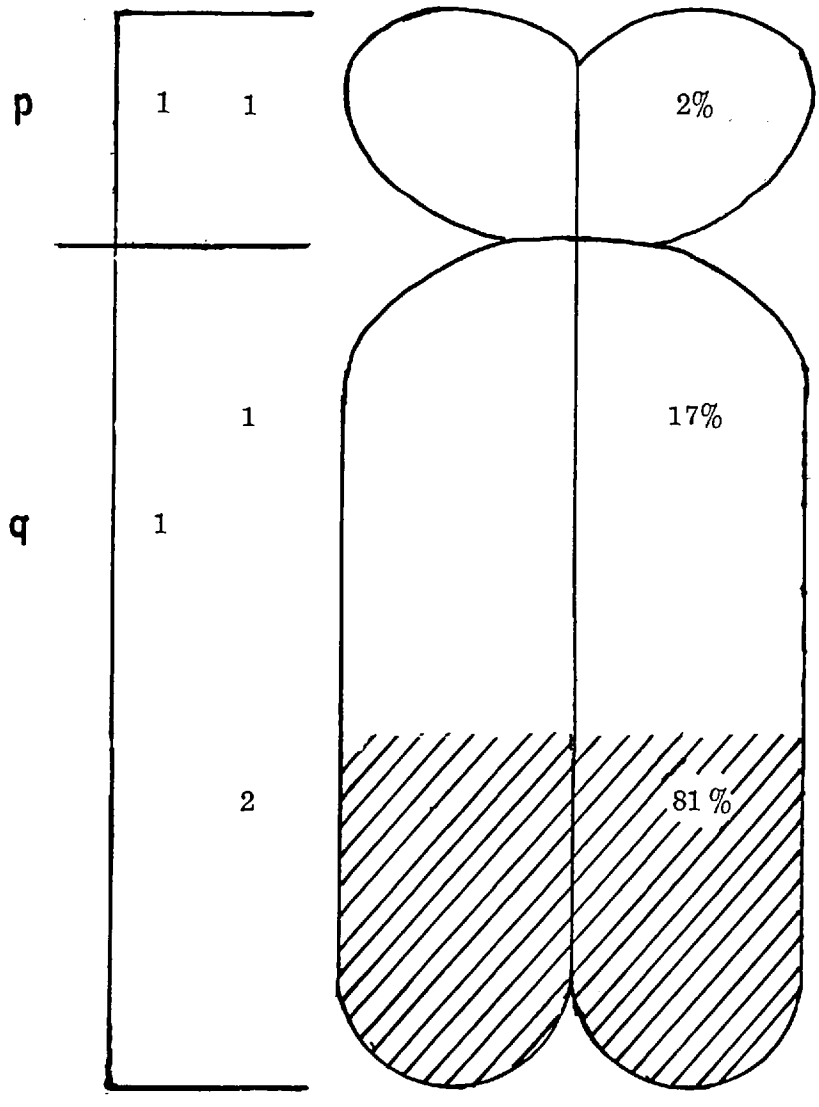


Fig. 3.2.6

The frequency of the distribution of silver grains over the Y chromosome and other chromosome groups after *in situ* hybridization of (^{3}H)Y chromosome-enriched gene sequences to normal 46,XY male chromosome preparations.
The X chromosome is included within the C group.
Figures within the histogram indicate the number of the silver grains actually incorporated into each group.

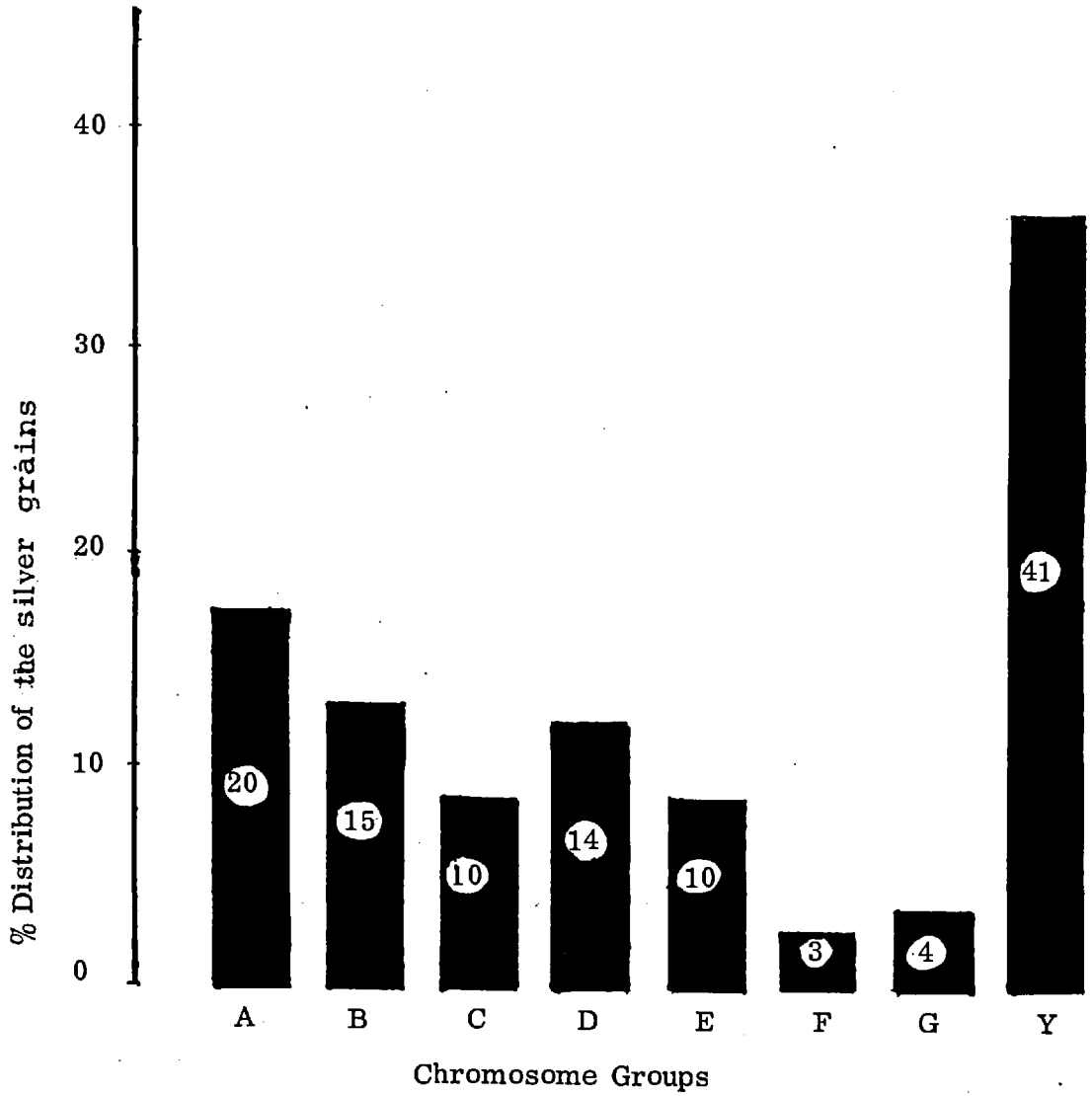

% Distribution of the silver grains
40
30
20
10
0
20
15
10
14
10
3
4
41
A
B
C
D
E
F
G
Y
Chromosome Groups

Fig.3.2.6

Fig. 3.2.7

Distribution of the silver grains on the Y chromosome and on chromosomes 1, 2, 3, 9, and 16 after *in situ* hybridization of (^{3}H)Y chromosome-enriched probe to normal 46,XY male peripheral lymphocyte chromosome preparations.

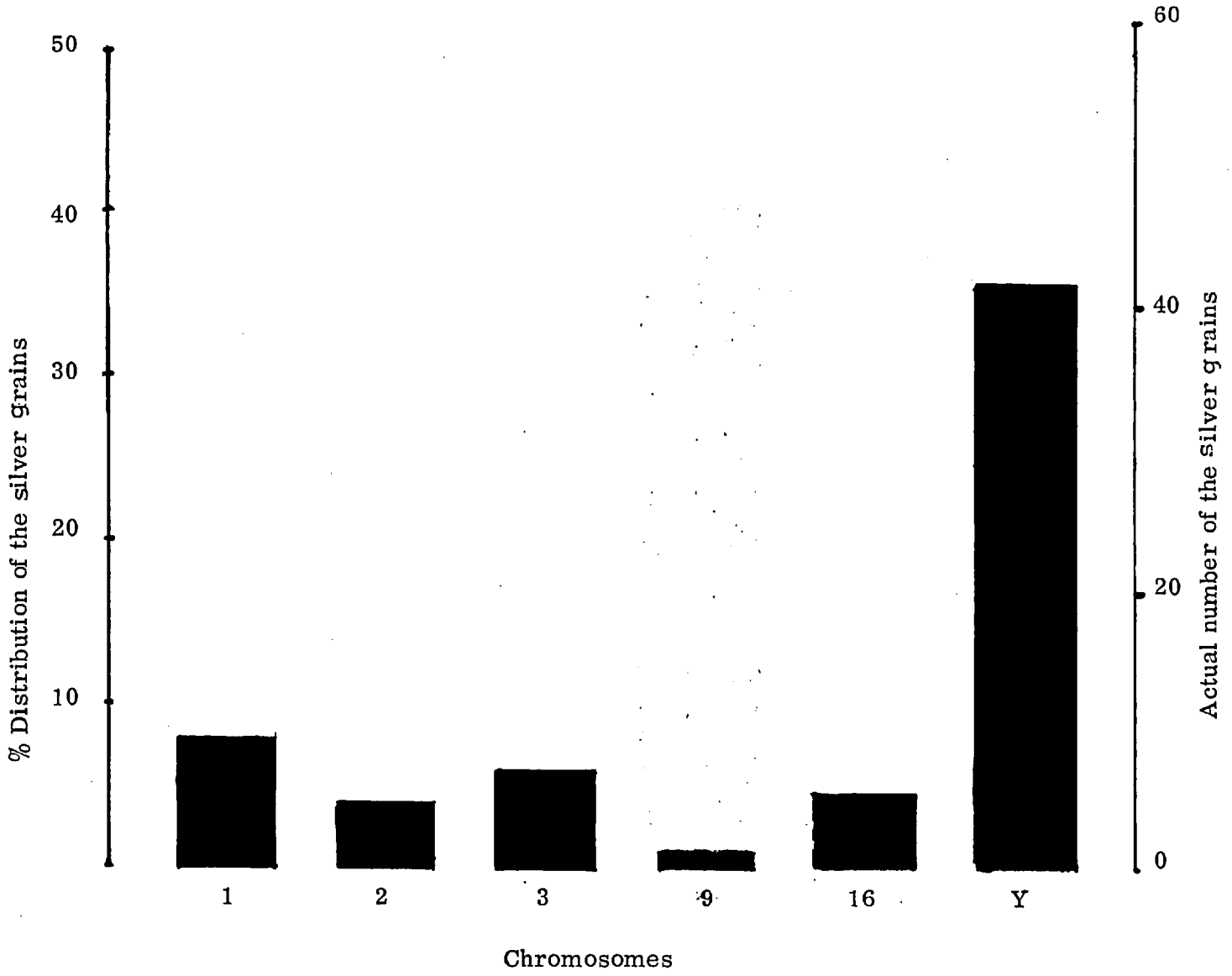

50
40
30
20
10
% Distribution of the silver grains
60
40
20
0
Actual number of the silver grains
1
2
3
9
16
Y
Chromosomes

Fig.3.2.7

Fig. 3.2.8

Distribution of the silver grains on the Y chromosome and the other chromosome groups adjusted according to their relative lengths in a normal 46,XY male after *in situ* hybridization to (^{3}H)Y chromosome-enriched gene sequences.

Fig. 3.2.8

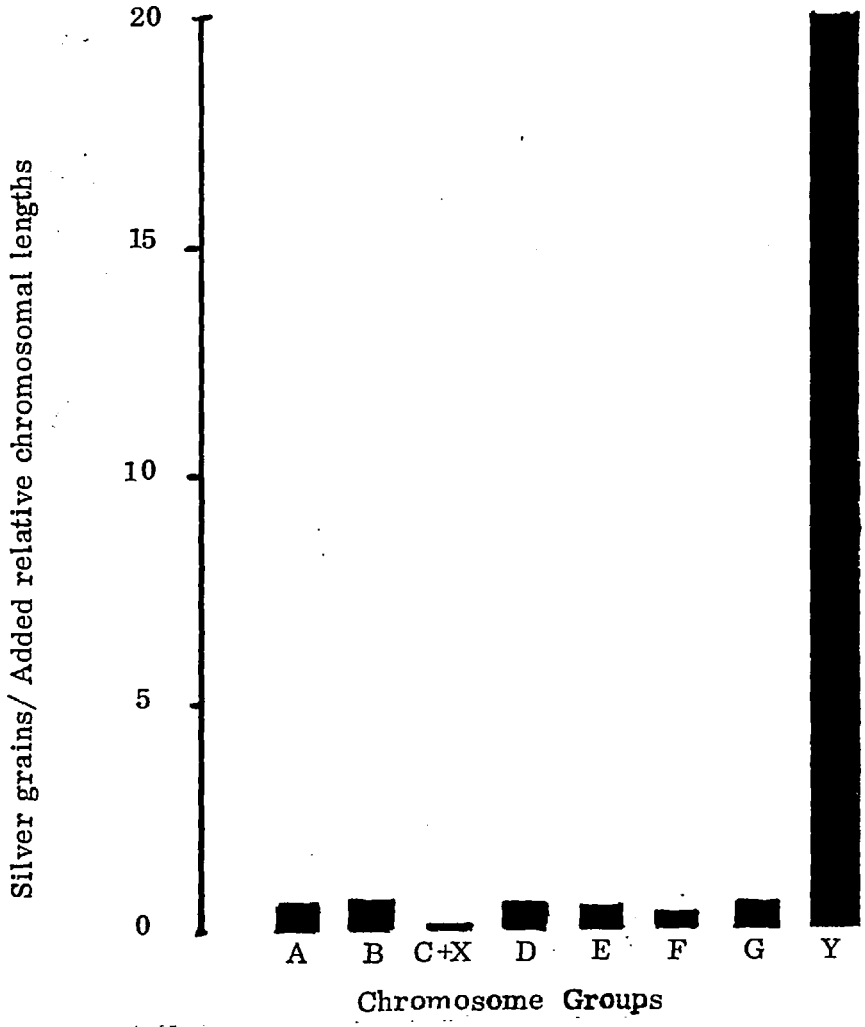
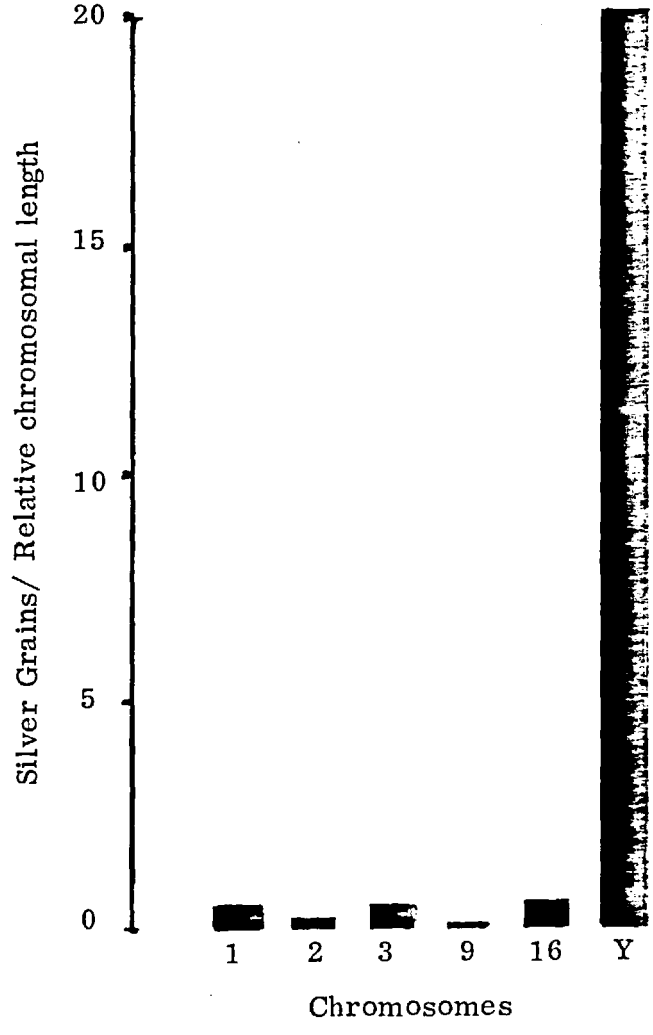

20
15
10
5
0
Silver grains/ Added relative chromosomal lengths
A
B
C+X
D
E
F
G
Y
Chromosome Groups

Fig. 3.2.9

Distribution of the silver grains on the Y chromosome and chromosomes 1, 2, 3, 9 and 16 as adjusted according to their relative lengths in a 46,XY male after *in situ* hybridization to (^{3}H)Y chromosome-enriched gene sequences.

Fig. 3.2.9


20
15
10
5
0
Silver Grains/ Relative chromosomal length
1
2
3
9
16
Y
Chromosomes

grains/relative length on the Y chromosome and the background) are the same in the two variants compared. The X^2 was found to be 13 ($P < 0.01$).

This is highly significant and indicates that the silver grains are significantly and specifically found over the Y chromosome.

SECTION III

3.3. THE USE OF Y CHROMOSOME-SPECIFIC GENE SEQUENCES IN STUDYING CASES OF 46, XYq+ AND 48, XYYY MALES

3.3-1. LOCALIZATION OF THE Y CHROMOSOME SPECIFIC GENE SEQUENCES IN A 46, XYq+ MALE

The length of the Y chromosome was measured from 8 C-banded cells from a (46, XYq+) male as a percentage of the total haploid lengths (Fig. 3.3.1). This was found to be 2.84 ± 0.10. The fluorescent segment (Yq12 region) measured 54% of the total Y length (1.624% of the total haploid chromosomal length). Fig. 3.3.2. demonstrates the quinacrine mustard staining of the chromosome preparation from the peripheral lymphocytes of this phenotypically normal male subject with a 46,XYq+ karyotype. In situ hybridization of Y chromosome enriched sequences (specific activity 6.3×10^6 dpm/µg DNA) to chromosome preparations from the 46,XYq+ case was carried out. Fifteen cells were scored for grain distribution and the Y chromosome was covered by grains in 14 only. Scoring was directly compared to the microscopic figure of each cell. Only cells with complete chromosome numbers were scored for the grain distribution. As in the case of "normal" Y chromosomes (Section II), region Yq12 was defined as the distal half of the Y which corresponds to the fluorescent segment. Fig. 3.3.3 shows an example of one of the cells scored for the silver grain distribution after in situ hybridization. Some of the Y chromosomes showing the mode of the distribution of the silver grains are shown in Fig. 3.3.4. A total of 174 grains were scored on all the chromosomes, of which the Y had 69 grains (40%) (Table 3.3.1). 80% of the grains scored on the Y chromosome were on the Yq12 region corresponding to the fluorescent segment. Regions Yq11 and Yp scored 18.6% and 1.4%

Fig. 3.3.1

C-banded peripheral lymphocyte chromosome preparations of 46,XYq+ male. The arrow indicates the Y chromosome. The deeply-stained Yq+ region occupies 54% of the total length of the Y chromosome.

Fig.3.3.1

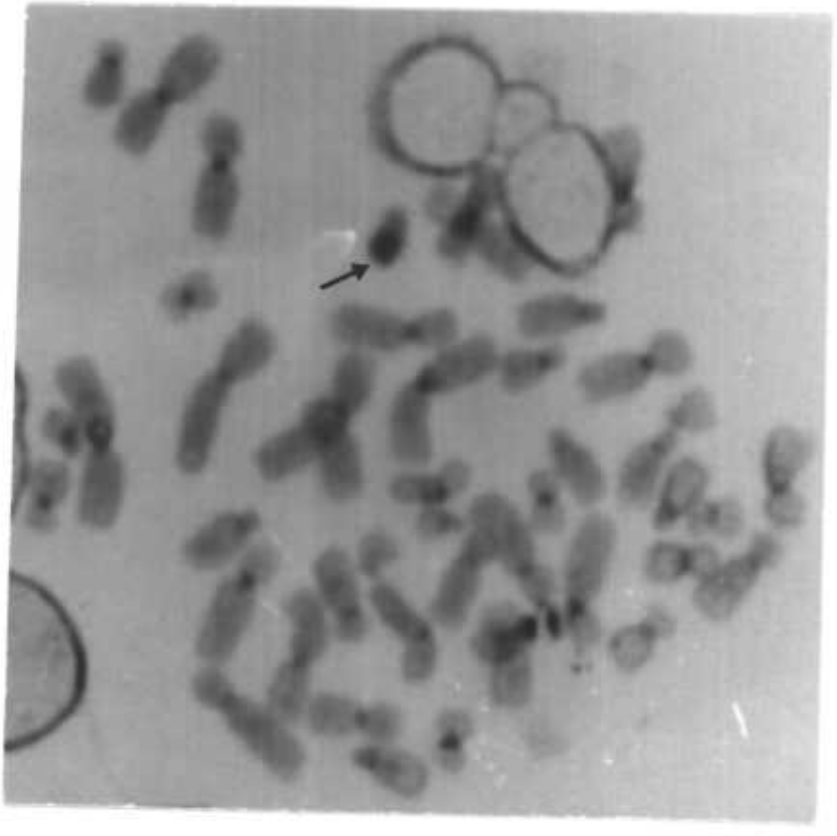


Fig. 3.3.2

Quinacrine mustard -stained peripheral lymphocyte chromosome preparation from the male with 46,XYq+ karyotype. The arrow indicates the Y chromosome.

Fig.3.3.2

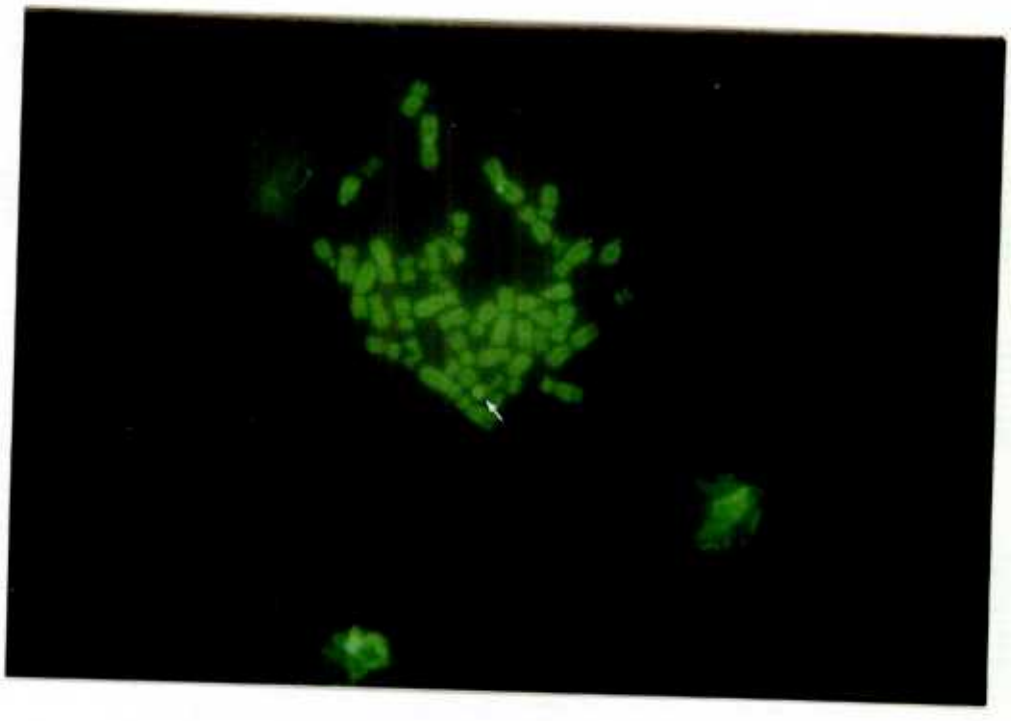


Fig. 3.3.3

46,XYq+ male chromosome preparation after *in situ* hybridization with (^{3}H) Y chromsome-enriched gene sequences(specific activity 6.3 X 10^{6} dpm/µg DNA). The (^{3}H) Y-enriched probe also contained 4×10^{3}-fold excess of unlabelled DNA. 6 ng of the probe was used on each slide .The exposure time was 8 weeks at 4°C. The arrow indicates the Y chromosome.

Fig.3.3.3

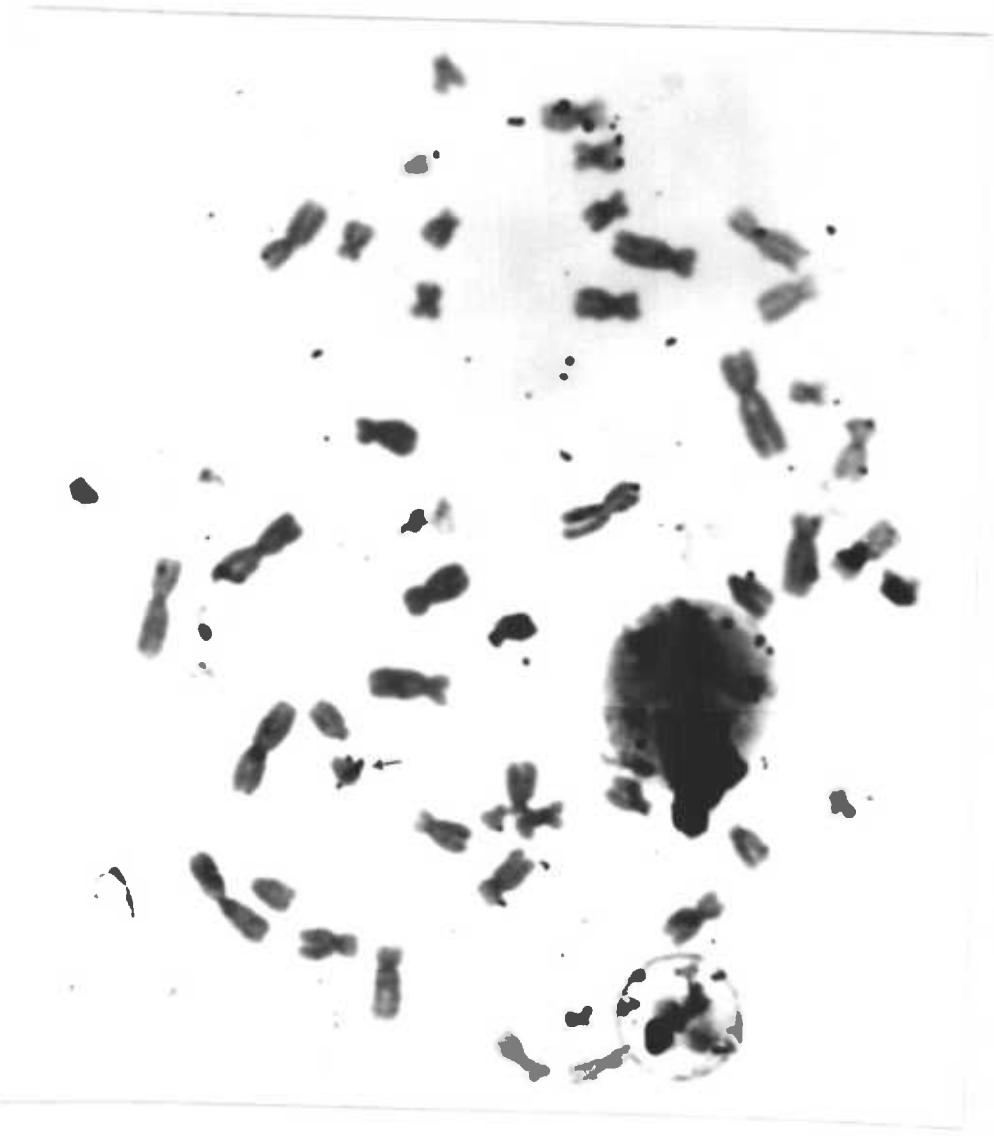


Fig. 3.3.4

Distribution of the silver grains on some of the Y chromosomes in46,XYq+ male case after *in situ* hybridization with (^{3}H) Y chromosome-enriched gene sequences.

Fig.3.3.4

Table 3.3.1

Distribution of the silver grains on the Y chromosme and other chromosomal groups in the 46,XYq+ male after *in situ* hybridization to Y chromosome -enriched gene sequences (results from 15 cells).

Table 3.3.1

Chromosomal group	number of grains	percentage	grain/chromosome
A	28	16%	4.7
B	4	2.3%	1
C + X	41	23.4%	2.7
D	14	8%	2.3
E	6	3.4%	1
F	7	4%	1.75
G	5	2.9%	1.25
Y chromosome	69	40%	69
total	174	100%	83.7

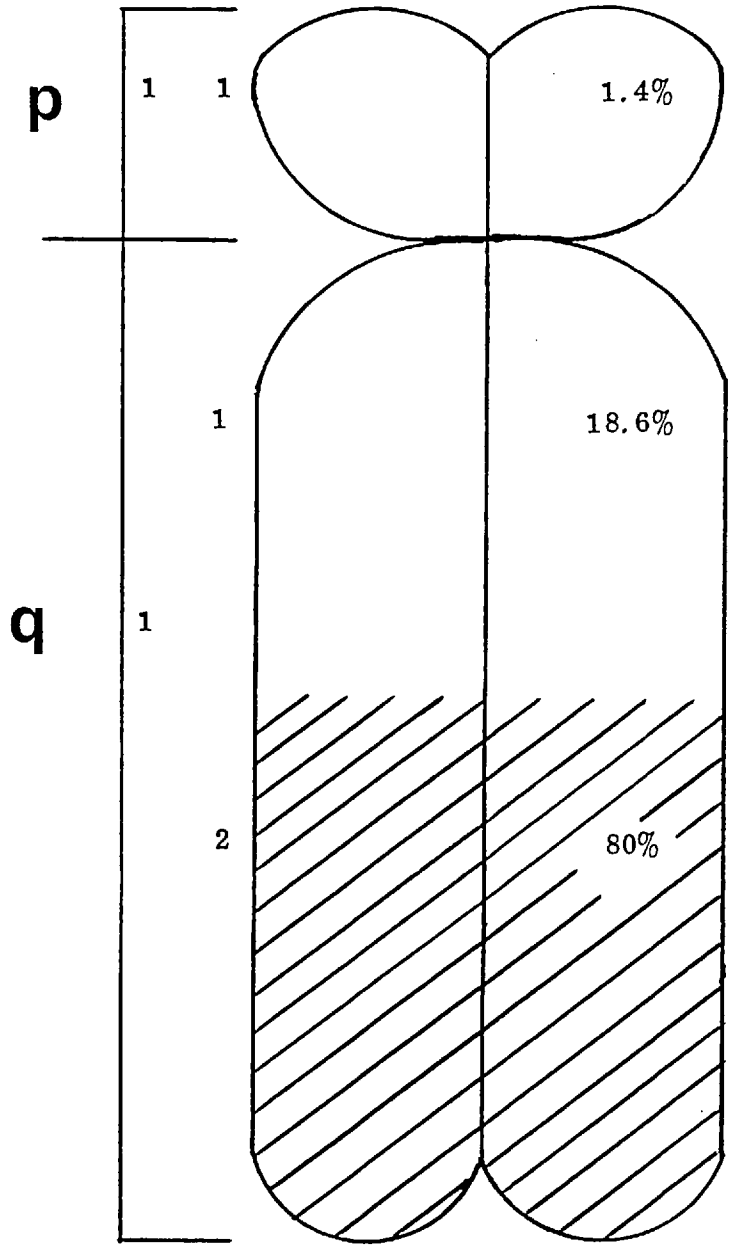
respectively (Fig. 3.3.5). The average distribution of grains on the Y chromosome per relative lengths of the Y per cell = 1.8. This figure was 25 times higher than the background (grains/relative chromosomal length other than the Y chromosome) which was found to be 0.074. This figure was significantly different from density of the grains on the Y (X^2 = 13; $P < 0.01$) when the background and the density of the grain were assumed to have equal distributions.

Those results, besides confirming the DNA as containing Y chromosome specific gene sequences, suggest that the Y chromosome-specific gene sequences contained in the probe isolated as in Section I is distributed over the fluorescent segment of a "big" Y chromosome (Yq+) similarly to that over a "normal" Y.

Fig. 3.3.5

Distribution of the silver grains on different regions of the Y chromosome for the 46,XYq+ male.

Fig. 3.3.5



3.3-2. STUDYING THE NATURE OF THE Y CHROMOSOMES IN A 48,XYYY MALE SUBJECT

The case studied, which was previously reported by Ridler *et al.* (1973), has a 48, XYYY karyotype. Fig. 3.3.6 shows the peripheral lymphocytes chromosomes stained by quinacrine mustard. It was impossible to distinguish each Y chromosome separately by routine cytogenetic techniques including quinacrine staining. This made it difficult to determine the relative length of each when more than one quinacrine-stained cell was scored for this purpose. The total relative lengths of the three Ys were determined from 5 cells stained with quinacrine mustard and found to be $6.1 \pm 0.16\%$ of total haploid length. The fluorescent segment (Yq12 region) measured one third of length of the Y in each Y chromosome. Radio-labelled Y chromosome-enriched gene sequences (specific activity 6.3×10^6 dpm/μg DNA) were hybridized *in situ* to chromosome preparations from a peripheral blood lymphocyte culture. Scoring for the grain distribution on the Y chromosomes and the other chromosomal groups was performed in the same way as previously described for the normal 46,XY male and 46,XYq+ male. Only cells with complete chromosome numbers were photographed, karyotyped and scored. Scoring was done on the karyotypes while comparing directly with a microscopic view. Figure 3.3.7 shows one of the 27 cells scored for the distribution of silver grains. Figure 3.3.8 shows the spread of the grains on the Y chromosomes from some of the cells scored. There was no significant variation in the distribution of the silver grains on any of the three Ys in the cells scored. The number of the Y chromosomes covered by silver grains varied from cell to cell, as can be seen in Table 3.3.2. In 70% of the cells scored, all three Y chromosomes were covered by silver grains in the same cell. The distribution of the grains on different chromosomal groups and the Y chromosomes is shown in Table 3.3.3. The Y chromosome

Fig. 3.3.6

Quinacrine mustard staining of peripheral lymphcyte chromosomes of a male with 48,XYYY karyotype. Arrows indicate the Y chromosomes. The fluorescent segment in each Y chromosome occupies about one-third of the total length of the Y chromosome.

Fig.3.3.6

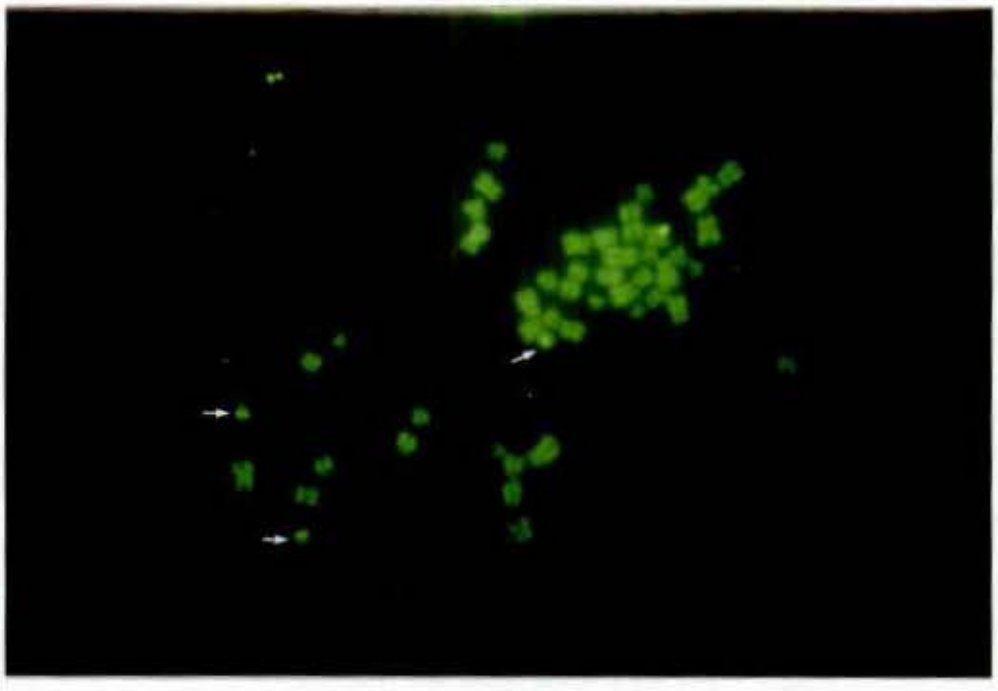


Fig.3.3.7

48,XYYY chromosome preparation after *in situ* hybridization to (^{3}H)Y chromosome-enriched gene sequences(specific activity 6.3 X 10^6 dpm/µg DNA).

6 ng of (^{3}H) DNA was applied to each slide in 4 µl formamide hybridization buffer. The exposure time was 8 weeks at 4°C. Arrows indicate the Y chromosomes.

Fig.3.3.7

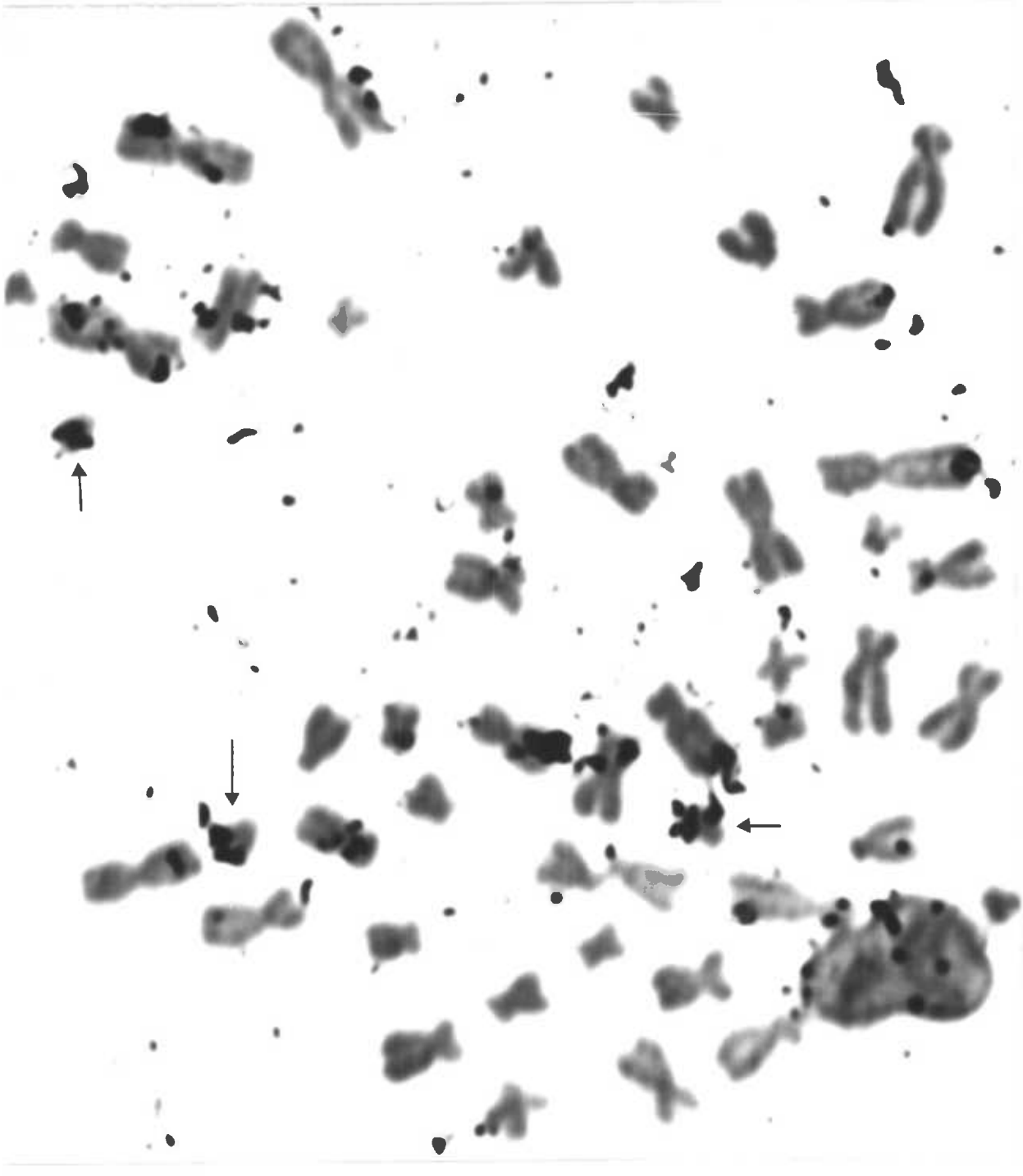


Fig. 3.3.8

Distribution of the silver grains on the Y chromosomes from some of the cells scored from the male with 48,XYYY karyotype, after *in situ* hybridization to (^{3}H) Y chromosome-enriched gene sequences.
The three Y chromosomes in each line are from one cell.

Fig.3.3.8

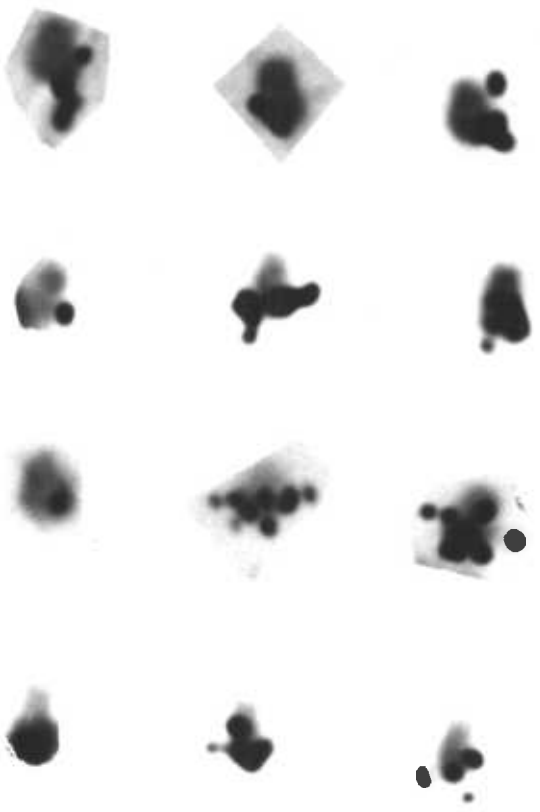


Table 3.3.2

Frequency of cells that show various number of Y chromosomes with silver grains for the 48,XYYY male after *in situ* hybridization to (^{3}H)Y chromosome-enriched gene sequences.

Table 3.3.2

number of Y chromosomes covered by silver grains in a single cell	number of cells observed	percentage of cells observed
3	19	70%
2	5	19%
1	3	11%
Total	27	100%

Table 3.3.3

Distribution of the silver grains on the Y chromosomes and other chromosomal groups in the 48,XYYY male after *in situ* hybridizationto (^{3}H)Y chromosome-enriched gene sequences.

Table 3.3.3

Chromosomal group	number of grains	percentage distribution of grains	grain/chromosome
A	43	9.7%	7.2
B	19	4.3%	4.75
C + X	61	13.7%	4.1
D	21	4.7%	3.5
E	13	2.9%	2.2
F	12	2.7%	3
G	6	1.4%	1.5
Y chromosomes	269	60.6%	89.7
Total	444	100%	115.95

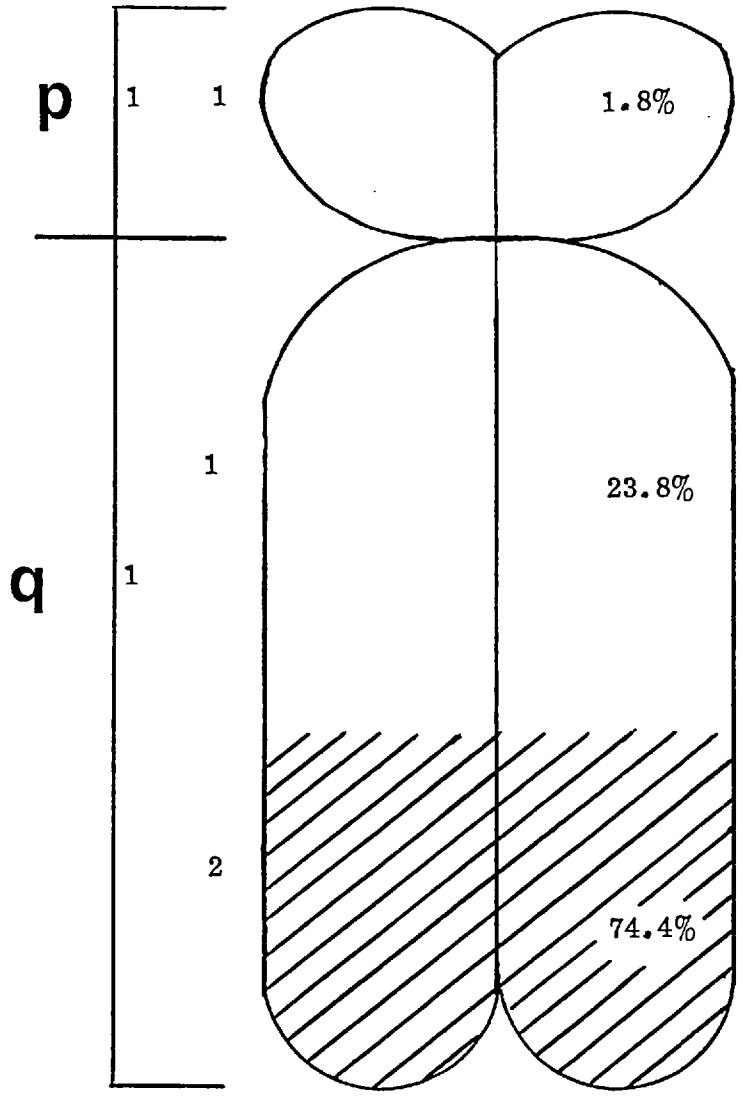
was associted with 60.6% of the total grains (444) scored. As with the 46, XY case studied (Section II), it was noted that the silver grains were specifically spread over region Yq12 which was defined, as previously, by measuring the grains spread over the distal third of each of the Y chromosome corresponding to the fluorescent segment (Fig. 3.3.9). The background was calculated, as previously done, by dividing the number of the grains on the chromosomes (except the Ys) in each cell by their added relative lengths. The average background per cell was found to be 0.036.

The distribution of the grains on the Ys/added relative lengths of the Ys per cell = 1.74. This figure was found after taking into consideration the variation in the frequency of grains over the different Y chromosomes in different cells. However, this figure is 48 times higher than the background. By comparing the density of the grains on the Y (grains/relative length) with the background a significant difference was found ($X^2 = 22$, $P < 0.005$) indicating that the silver grains are virtually confined to the Y chromosomes. From the above results it is concluded that almost all the three Y chromosomes had similar distributions of silver grains, which were mainly concentrated on the fluorescent segment. As the probe used for <u>in situ</u> hybridization contained Y chromosome-specific gene sequences, therefore all these Y chromosomes are carrying such sequences in their fluorescent segments without any significant variation in the density of grains amongst different Y chromosomes in the same cell.

Fig. 3.3.9

Distribution of the silver grains on different regions of the Y chromosome for the 48,XYYY male.

Fig. 3.3.9



SECTION IV

3.4. THE USE OF CLONED GENE SEQUENCES IN STUDYING THE STRUCTURE AND NATURE OF AN EXTRA MARKER CHROMOSOME IN TWO PREGNANT WOMEN

The preceeding sections have outlined the use of chromosome-specific probes in the study of gene arrangement on the human X- and Y-chromosomes. It is also possible to study the arrangement of genes on human chromosomes using gene-specific probes which have been cloned in bacterial plasmids or phage. In this section I discuss the use of Xenopus laevis ribosomal gene sequences in plasmid recombinants to localise human ribosomal genes in cases where an extra marker chromosome is found. These results were compared to results obtained from studying the phenomena of satellite association and other staining techniques including silver staining of the nucleolus organizer regions (NORs).

3.4.1. CASE REPORTS:

CASE I (Mrs.A.)

Mrs.A., a 26-year old woman, visited the genetic counselling clinic at the Royal Hospital for Sick Children, Glasgow, Scotland in 1975, during the first trimester of her second pregnancy. Amniocentesis was performed to exclude Niemann-Pick disease in the foetus, as her first baby (girl) had died during infancy of this disease. Chromosome analysis of the amniotic fluid cells was also carried out as a routine procedure. Parental blood was taken for chromosome analyses. A normal female baby was born later and is developing normally.

CASE II (Mrs.D.)

A sample of amniotic fluid from a 35-year old woman, Mrs. D., was sent to the Medical Genetics Department, the Royal Hospital for Sick Children, Glasgow, Scotland, for analysis. The indication for amniocentesis was

that she had given birth to a child with spina bifida in her third and previous pregnancy. Routine chromosome analyses were carried out on the amniotic fluid cells and the parental lymphocytes. The pregnancy went to term with the delivery of a normal baby.

3.4.2. RESULTS FROM CYTOGENIC STUDIES:

Giemsa staining, trypsin-Giemsa banding and C-banding revealed a karyotype of 47, XX + a marker chromosome in the chromosome preparations from the amniotic fluid cells of both Cases I and II and from the peripheral blood lymphocyte cultures of both Mrs. A. and Mrs. D. There were no structural abnormalities in any of the chromosomes except for the presence of a supernumerary chromosome (Figs. 3.4.1a & b, 3.4.2a & b). Chromosome preparations from peripheral blood lymphocyte culture of both husbands Mr. A. and Mr. D. showed normal 46, XY karyotypes. The marker chromosome in each case was a small bisatellited metacentric chromosome with two distinct heterochromatic bands in each subsatellite region (Fig. 3.4.3).

Further studies were only carried out on the chromosome preparations of Mrs. A. and Mrs. D.

3.4.3. RESULTS FROM SILVER STAINING TECHNIQUE:

The presence of NOR regions on both ends of the marker chromosome was confirmed by the silver staining technique. Twenty cells from Mrs. A. and 22 cells from Mrs. D. were scored after silver staining followed by trypsin-Giemsa or Giemsa staining (Figs. 3.4.1a , b; 3.4.2a , b). This showed that both satellite regions of the marker chromosome in Mrs. A. stained heavily and equally in all the cells scored, while for Mrs. D. one satellite region of the marker stained more heavily than the other, which stained only faintly (Fig. 3.4.4).

Fig. 3.4.1a

Silver staining followed by trypsin-Giemsa banding of the peripheral blood lymphocyte chromosome preparation (Mrs.A.). The arrow indicates the marker chromosome. Note that both satellite regions of the marker chromosome are stained with silver, and that one of its satellites is in association with chromosome 22.

Fig.3.4.1a

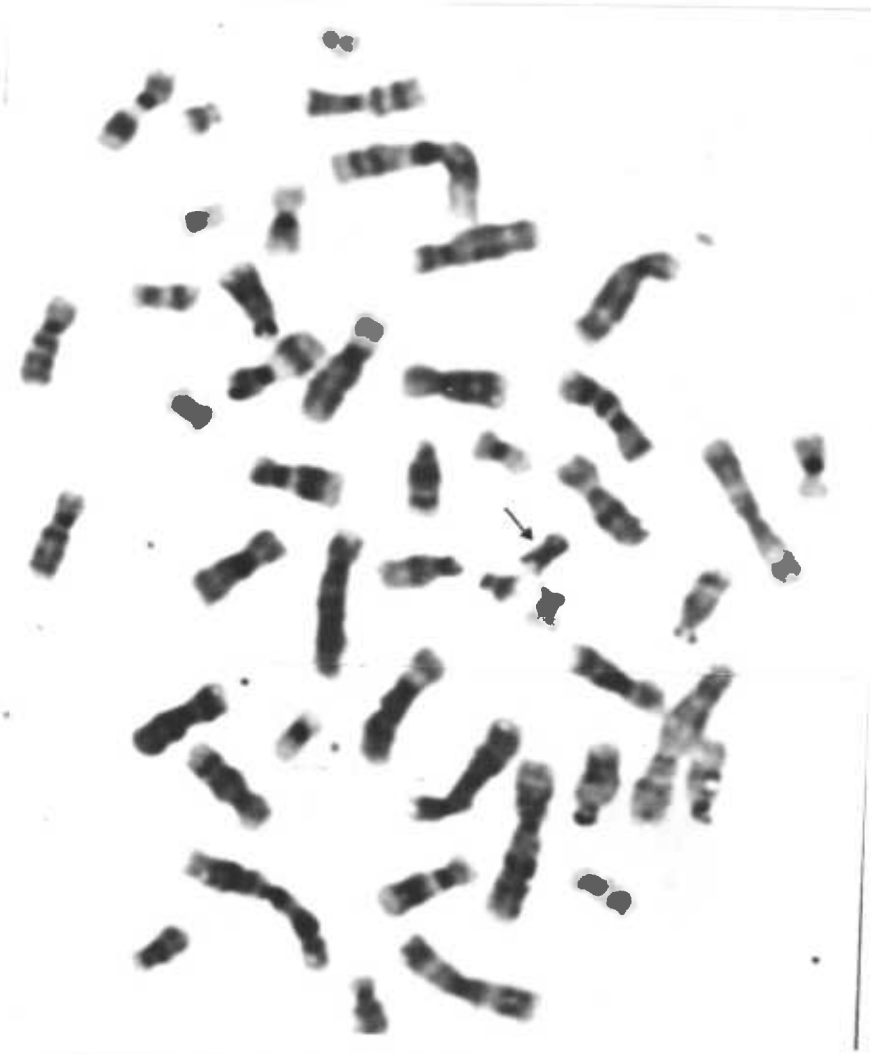


Fig 3.4.1b

Karyotype of Fig.3.2.1a.
Note the silver staining of the satellites of both chromosomes 13,one of the 14s,both 21s and one of the 22s in addition to the marker. Arrow indicates the marker chromosome.

Fig.3.4.1b

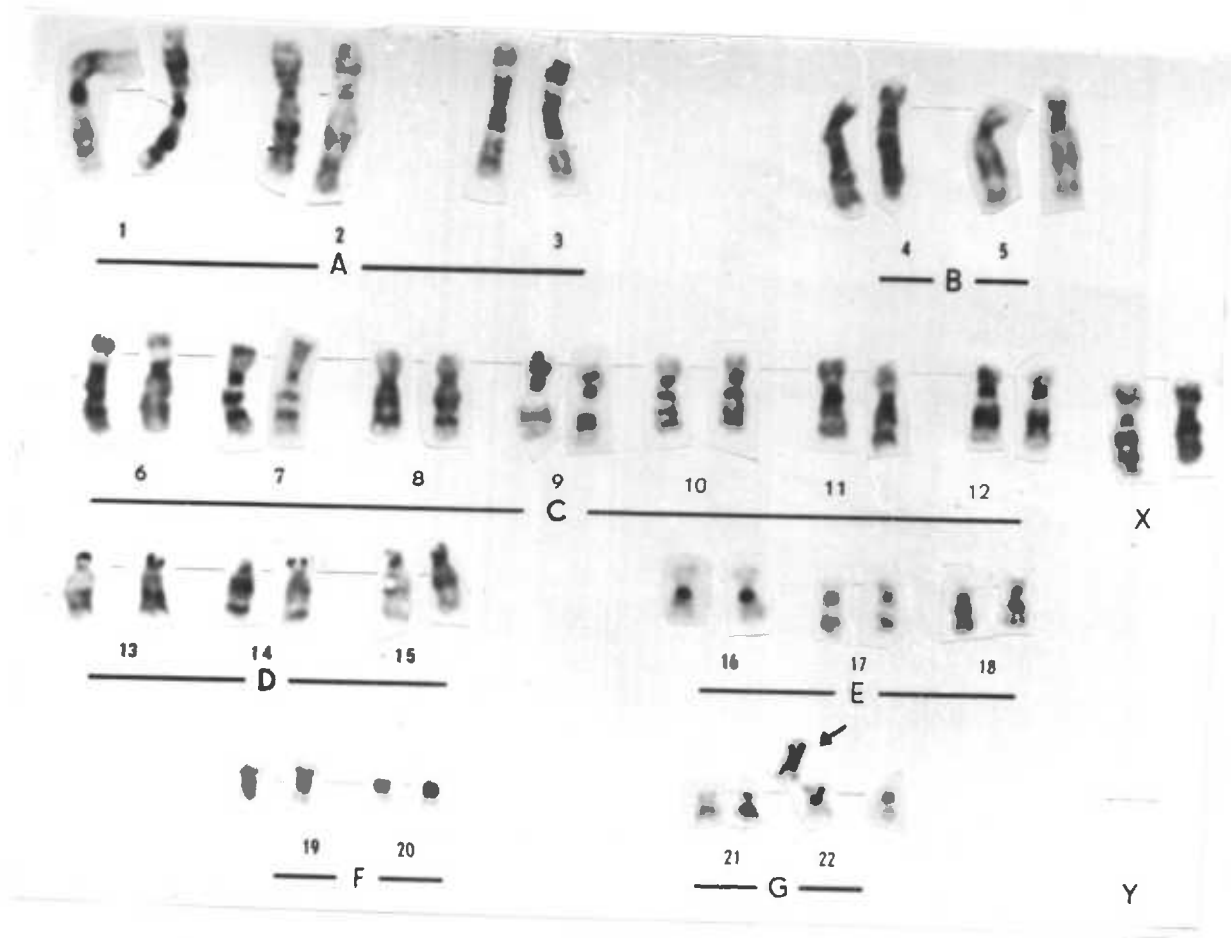


Fig. 3.4.2a

Peripheral lymphocyte chromosome preparation from Mrs.D. after silver staining and trypsin-Giemsa banding. The arrow indicates the marker chromosome. Note that one satellite region of the marker is more deeply stained with silver than the other.

Fig.3.4.2 a

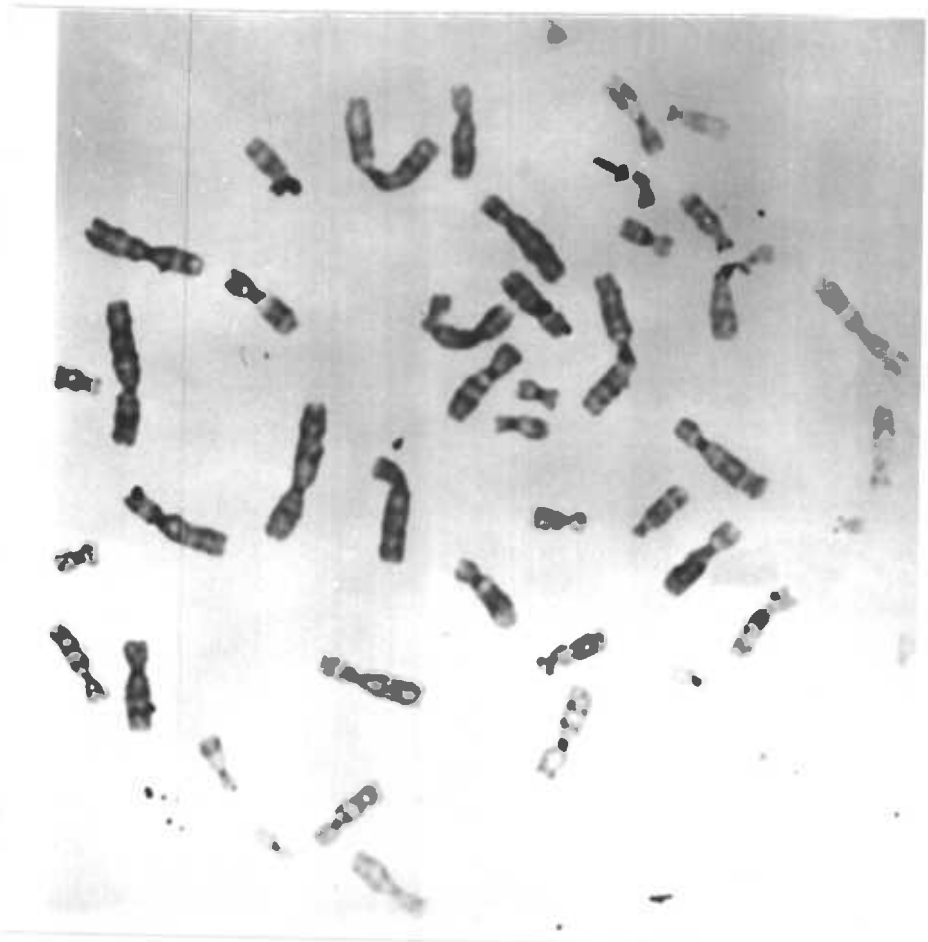


Fig.3.4.2b

Karyotype of Fig.3.4.2a .
Note the silver staining of the satellites of both chromosomes 13 , one 14 ,both 15s and one of each of 21 and 22.
One of the chromosomes 13 is in satellite association with a chromosome 14 and a 22.
Arrow indicates the marker chromosome.

Fig.3.4.2b

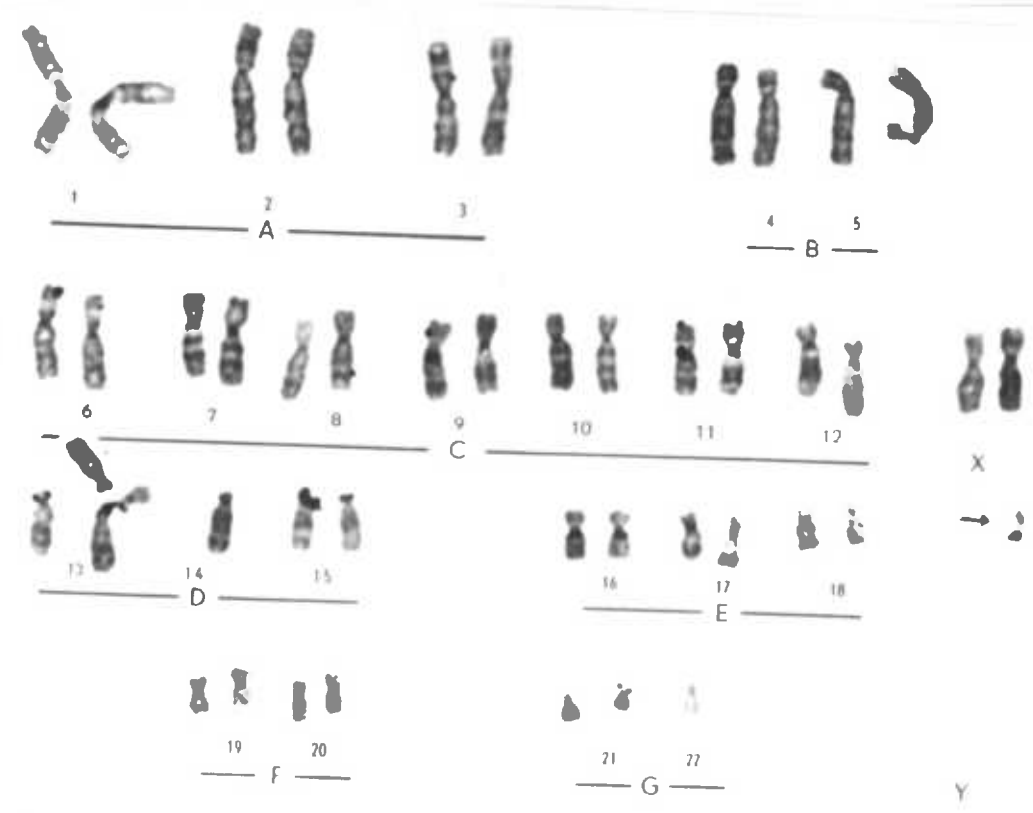


Fig. 3.4.3

Comparison of some of the cytogenetic staining techniques of the marker chromosome in Mrs.A. and Mrs.D. . The marker in each case is a bisatellited metacentric chromosome with a heterochromatic band in each subsatellite region, which is best demonstrated by C-banding. Note the silver staining of both satellite regions.

Fig.3.4.3

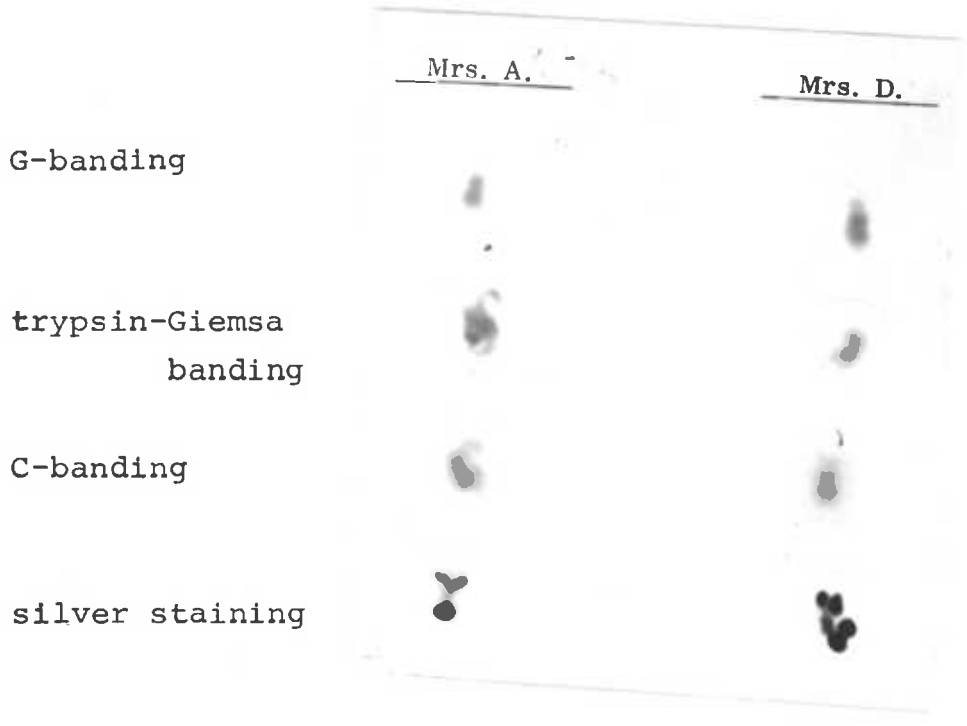
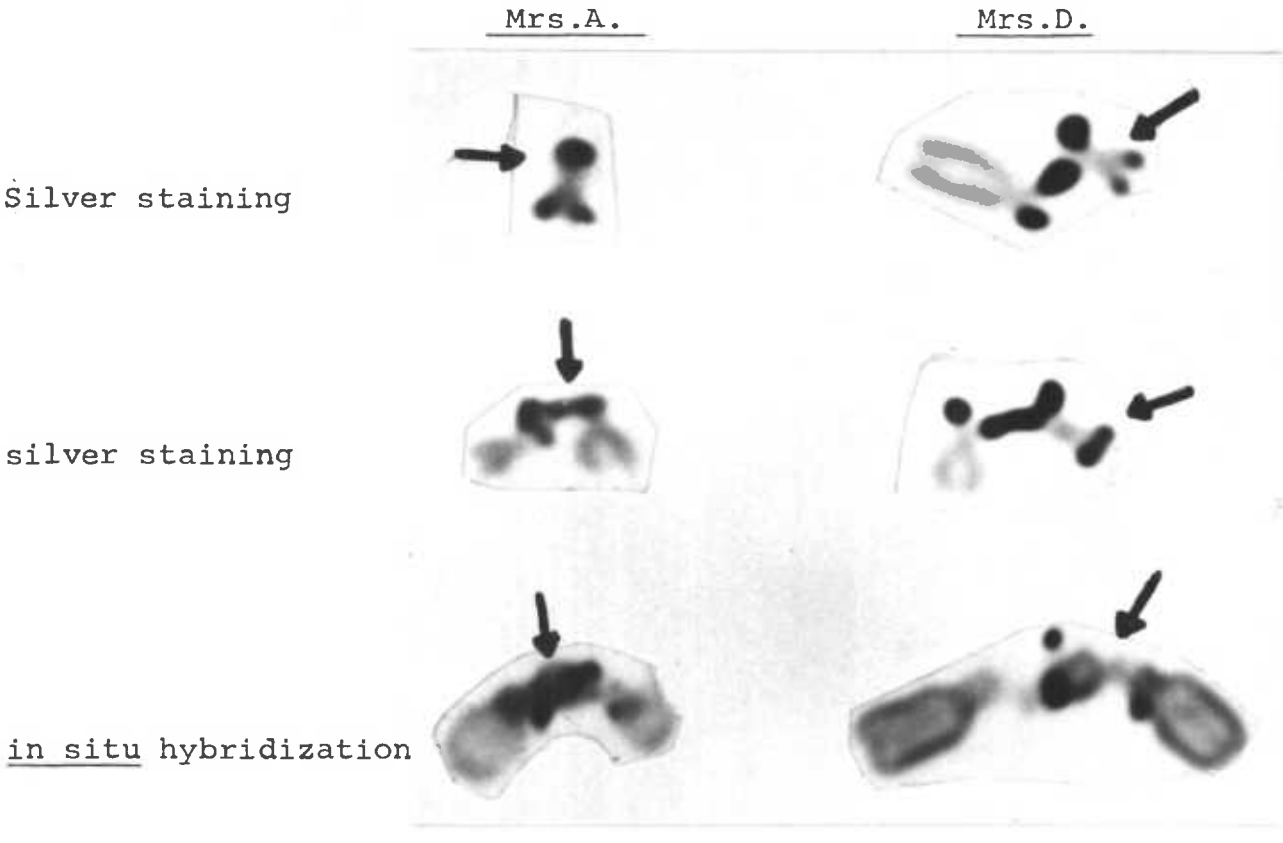

Mrs. A.
Mrs. D.
G-banding
trypsin-Giemsa banding
C-banding
silver staining

Fig. 3.4.4

Examples of silver staining and *in situ* hybridization of Mrs.A.'s and Mrs.D.'s peripheral lymphocyte chromosome preparations. The marker chromosomes are indicated by arrows. Note the difference in the intensity of silver staining between the satellite regions of the marker chromosome in Mrs.D. . The markers are in satellite association with a D and a G group chromosome in Mrs.D.,and with 2 G group chromosomes in one case for Mrs.A. .The satellite association sites in both cases are heavily silver-stained.
In situ hybridization was to (^{3}H)Xlrl01 plasmid containing *Xenopus laevis* 28S and 18S ribosomal genes (specific activity 10^{7} dpm/μg DNA), is demostrated. Note the incorporation of the silver grains into the satellite regions of the marker chromosomes and the other acrocentric chromosomes. The marker is in satellite association through its each satellite regions to 2 D chromosomes in Mrs.D.,and to a D and a G chromosome in Mrs.A.

Fig. 3.4.4


Mrs.A.
Mrs.D.
Silver staining
silver staining
in situ hybridization

In Mrs. A. three D and two G chromosomes were found to stain with silver in 4 out of 5 cells scored, and four D and two G in the remaining one. These were identified by trypsin-Giemsa banding as both chromosomes 13 and one 14, one 21 and one 22. The other chromosome 14 was stained by silver only in one cell scored.

In Mrs. D. four Ds and three Gs were stained by silver in eight out of 12 cells scored, which were shown by trypsin-Giemsa banding to be both 13s one each of 14 and 15, both 21s and one 22. In the remaining 4 cells scored an additional chromosome 14 was found to be stained by silver (Figs. 3.4.1a, 3.4.1b, 3.4.2a and 3.4.2b).

3.4.4. RESULTS FROM IN SITU HYBRIDIZATION

(^{3}H) X1r101 plasmid, containing Xenopus leavis 28S and 18S ribosomal genes as an insert, was used as a probe for in situ hybridization to chromosome preparations from Mrs. A. and Mrs. D. A total of 8 cells from Mrs. A. and 18 from Mrs. D. were scored for the distribution of silver grains. There was specific hybridization of the probe to the marker chromosome of both Mrs. A. and Mrs. D. as well as to the NOR regions of the D and G chromosomes (Figs. 3.4.5 and 3.4.6; 3.4.7a, b; 3.4.8a, b). The exposure of slides to photographic emulsion before development was 1-3 weeks although one slide from Mrs. A. was developed after 8 weeks' exposure (Fig. 3.4.7a, b). However, silver grains were detected as early as after one week's exposure only.

The relative lengths of the marker chromosome were found to be 1.35% and 1.22% of the total haploid length in Mrs. A. and Mrs. D. respectively. The distribution of the grains per relative length in the chromosomal

Fig. 3.4.5

Distribution of silver grains on different chromosomal groups and that adjusted according to added relative chromosomal lengths in each group, compared to those on the marker chromsome(m) in Mrs. A.

(Total number of grains = 174).

■ Actual number of silver grains

□ Grain/relative chromosomal length

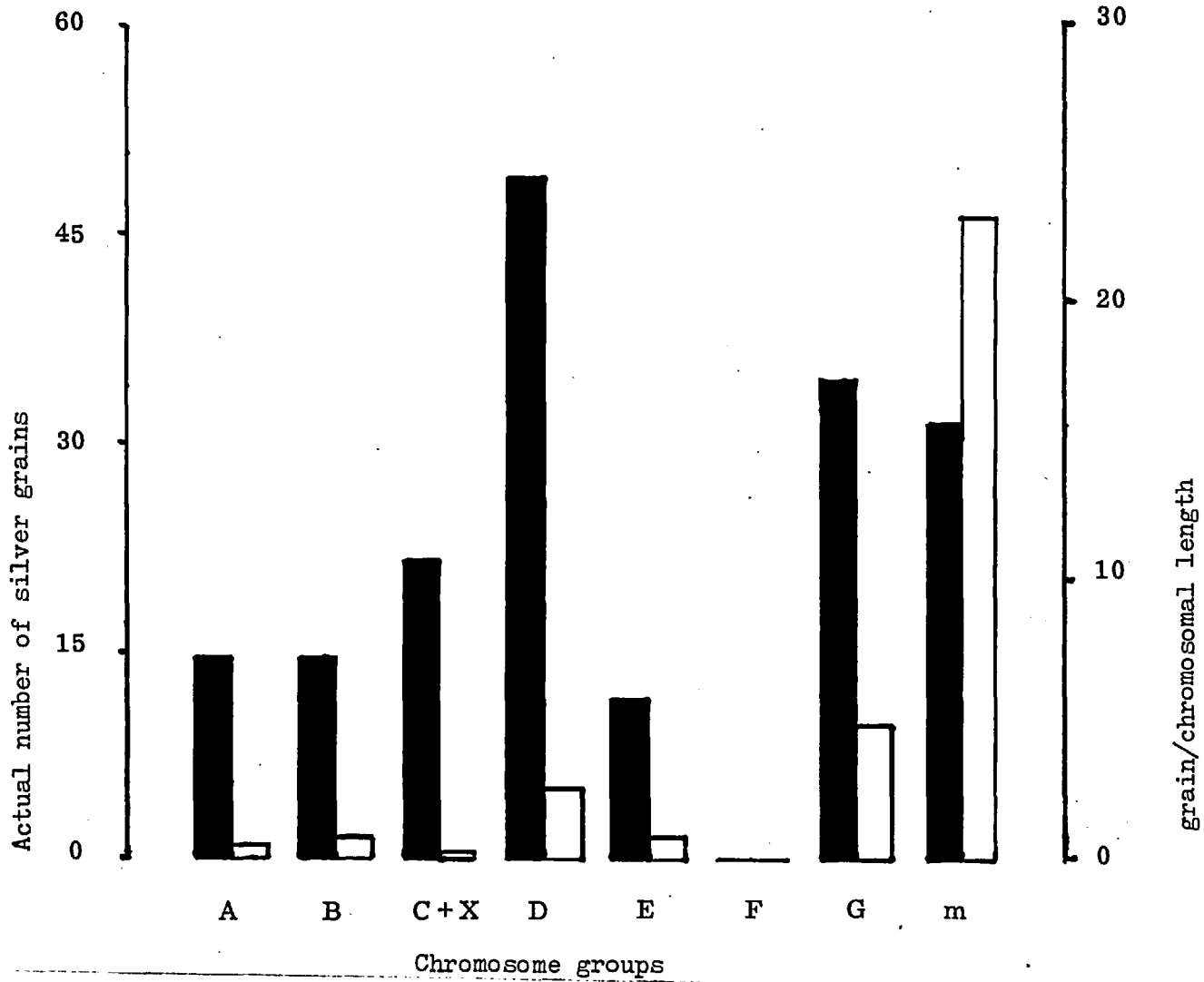

60
45
30
15
0
Actual number of silver grains
30
20
10
0
grain/chromosomal length
A
B
C+X
D
E
F
G
m
Chromosome groups

Fig. 3.4.5

Fig. 3.4.6

Distribution of silver grains on different chromosomal groups and that adjusted according to added relative chromosomal lengths in each group, compared to those on the marker chromosome(m) in Mrs. D.

(Total number of grains = 396).

Actual nuber of silver grains

Grain/relative chromosomal length

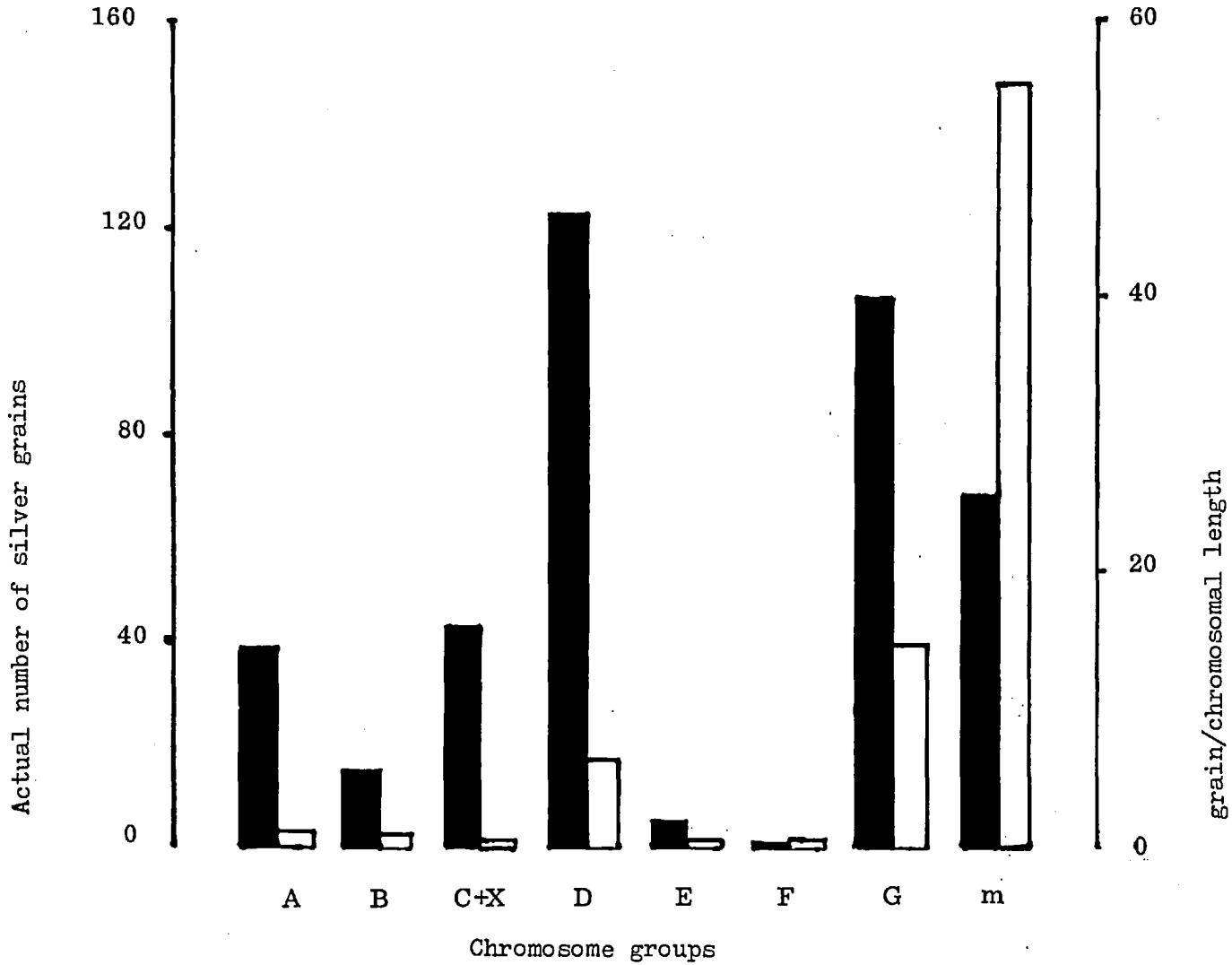

160
120
80
40
0
Actual number of silver grains
60
40
20
0
grain/chromosomal length
A
B
C+X
D
E
F
G
m
Chromosome groups

Fig. 3.4.6

Fig.3.4.7a

Peripheral blood lymphocyte chromosome preparation from Mrs.A.(47,XX + a marker) after *in situ* hybridization to (^{3}H)X1r1O1 plasmid containing *Xenopus laevis* 28S and 18S ribosomal genes (specific activity 10^7 dpm/μg DNA). 150 ng of the (^{3}H) plasmid, labelled by nick translation,was dissolved in 4 μl of formamide hybridization buffer and applied to each slide.(150 ng plasmid contains 19.2 ng of each strand of the ribosomal gene,as the plasmid contained 16.7 kb of DNA; 4.3 kb of it represents the *Xenopus* 28S and 18S ribosomal genes). The exposure time in this particular example was 8 weeks. The other cells scored from Mrs.A. were obtained from slides exposed for 1-3 weeks.
The arrow indicates the marker chromosome in which both satellite regions are covered by silver garins.

Fig.3.4.7a

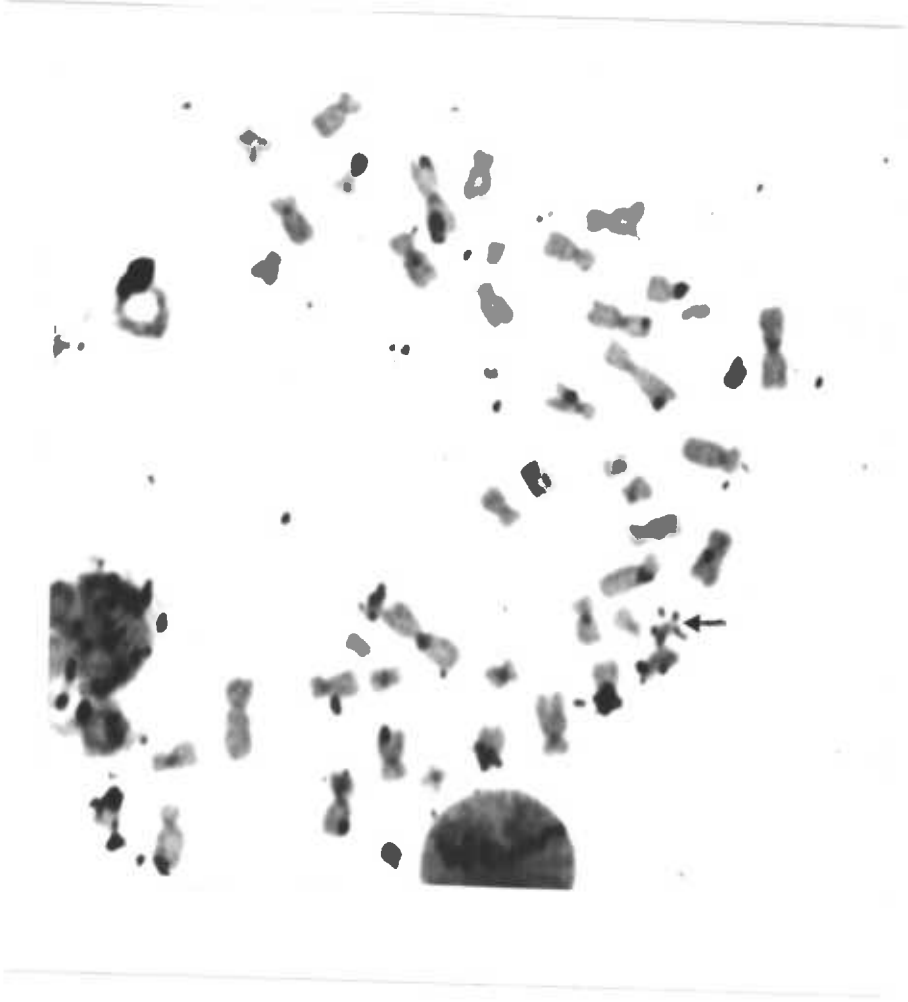


Fig. 3.4.7b

Karyotype of Fig.3.4.7a .
The chromosomes are divided to groups as it is difficult to identify each chromosome separately,since they are not banded. The only identifiable chromosomes are chromosomes 1 ,2 ,3 ,9 ,16 and the marker. The arrow indicates the marker chromosome.

Fig.3.4.7b

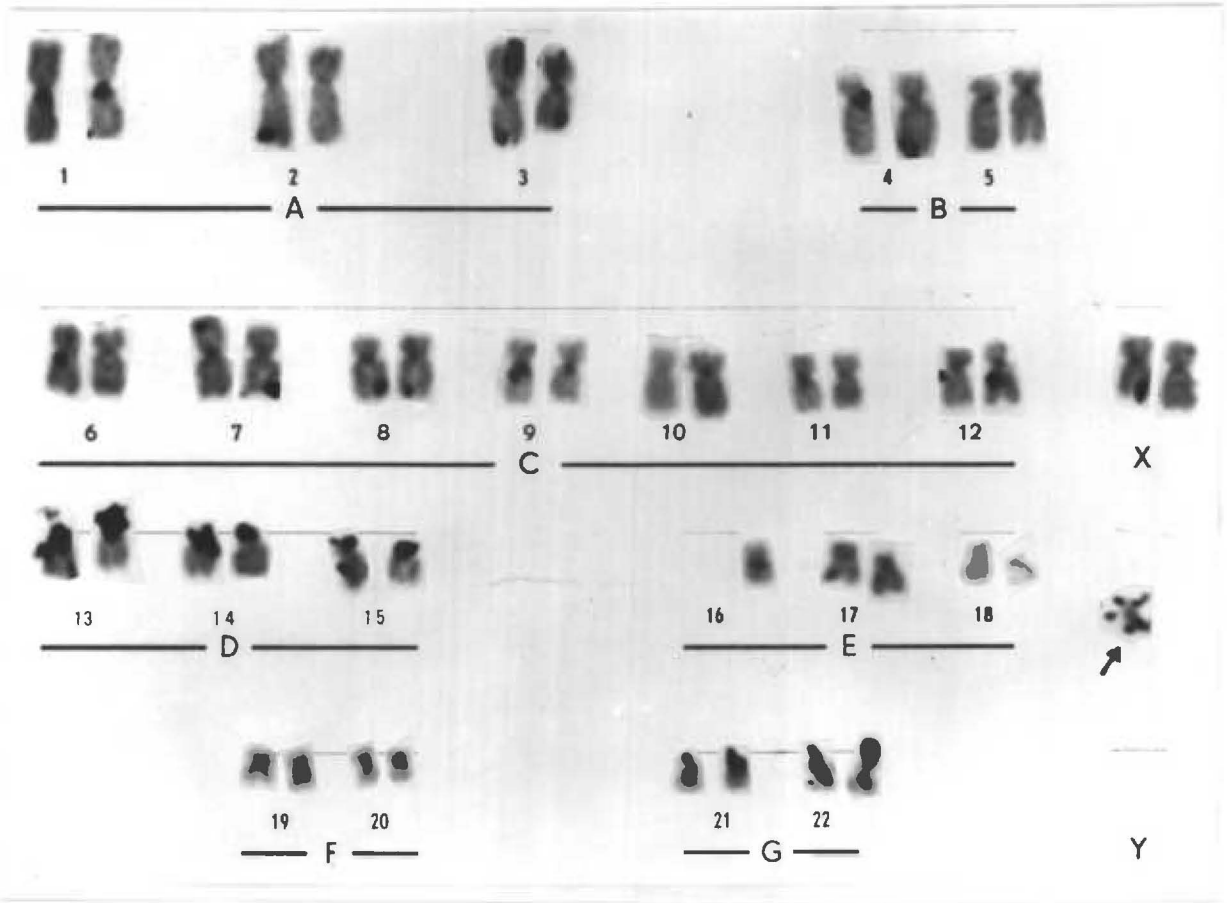


Fig. 3.4.8a

In situ hybridization of (^{3}H)Xlrl01 plasmid (specific activity 10^{7} dpm/µg DNA) to peripheral lymphocyte chromosome preparations of Mrs.D (47,XX + marker). For each slide 150 ng of (^{3}H)plasmid in 4 µl of formamide hybridization buffer was applied. Time of exposure ,at 4°C varied from 1-3 weeks (one week for the slide photographed). Note the distribution of the silver grains on both ends of the marker chromosome (indicated by the arrow).

Fig.3.4.8a

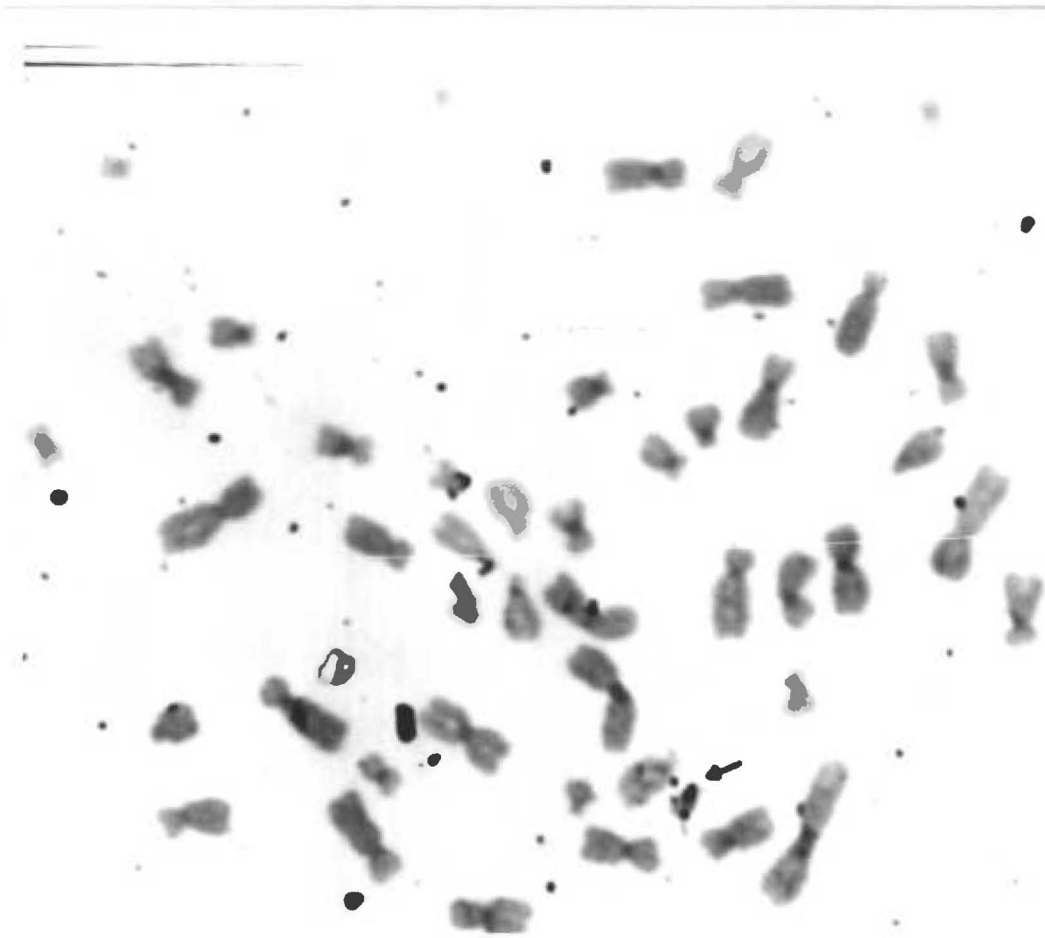
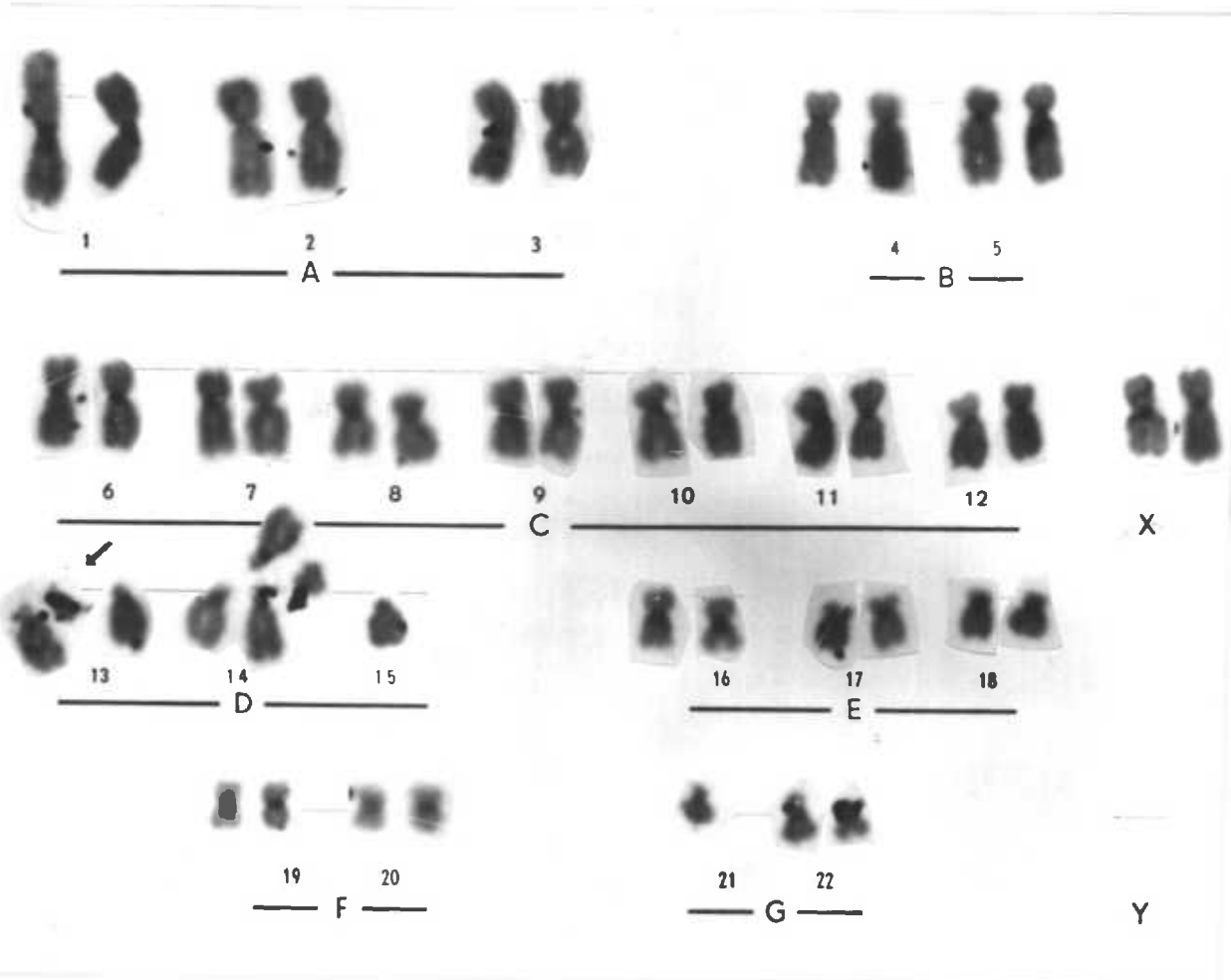


Fig. 3.4.8b

Karyotype of Fig.3.4.8a .
Arrow indicates the marker chromosome. The marker is in satellite association with a group D chromosome. Note the silver grains on some of the chromosomes from groups D and G.

Fig. 3.4.8b



groups and the marker chromosomes in both cases studied are shown in Figures 3.4.5 and 3.4.6. There were specifically more grains on the marker chromosome and the satellite regions of D and G groups, which are known to be the sites of 28S and 18S ribosomal genes (Henderson et al., 1972; Evans et al., 1974), than on any other chromosomal groups (Fig. 3.4.5, 3.4.6). Table 3.4.1 shows the distribution of the silver grains per NOR region on the D & G groups compared to a background value calculated by dividing the number of grains distributed on the chromosomal groups (other than D and G groups and the marker) by their added relative lengths. The marker chromosome is nearly four times more heavily labelled than the short arms and satellite regions of the average D and G group chromosomes in both cases studied. It was not possible to distinguish between the two ends of the marker chromosomes after in situ hybridization but grains were observed over both satellite regions of the marker in 50% of the cells scored for Mrs. A.and in 39% of the cells of Mrs. D. (Table 3.4.2). This strongly suggests that NORs are present on both ends of the extra marker chromosome of Mrs. A. and Mrs. D., and that each of the two NORs of each marker carries on average double the number of ribosomal genes present on the average D and G group chromosome.

3.4.5. RESULTS OBTAINED FROM STUDYING THE PHENOMENON OF SATELLITE ASSOCIATION OF THE CHROMOSOMES:

The frequency of satellite association of the marker chromosome to other satellited chromosomes of D and G groups was compared to the frequency of satellite association of individual member of the satellited acrocentric chromsomes with each other. The marker chromosome was found to be greatly involved in satellite association as a two-NOR unit compared with the rest of D and G groups (Table 3.4.3a). It is shown in Table 3.4.3b

Table 3.4.1

Distribution of the silver grains per NOR per cell in the marker and G and D groups of chromosomes for Mrs.A. and Mrs.D.

Table 3.4.1

	Mrs.A.	Mrs.D.
Grains/marker chromosme/cell	3.9	3.8
Grains/NOR/cell in the marker*	1.94	1.9
Grains/short arm/cell in group D	0.91	1.04
Grains/short arm/cell in group G	1	1.4
Background †	0.04	0.03

* The marker chromosome has two NOR regions (two satellite regions).

† Background was measured by dividing number of the grains distributed on the chromosomes other than the marker and groups D and G by their added relative lengths.

Table 3.4.2

case	number of cells with silver grains on one satellite region	number of cells with silver grains on both satellite regions
Mrs.A.	4 (50/)	4 (50/)
Mrs.D.	11 (61/)	7 (39/)

Comparison of the distribution of the silver grains on both satellite regions of the marker chromosome for both Mrs.A. and Mrs.D.

Table 3.4.3a

case	frequency of involvement in satellite association of D group	G group	marker	total number of cells scored
Mrs.A.	43	37	24	30
Mrs.D.	129	39	46	52

Frequency of involvement, in satellite association, of D and G groups of chromosomes and the marker chromosome in Mrs.A. and Mrs.D.

Table 3.4.3b

Case	frequency of involvement in satellite association per NOR of D group*	G group†	marker‡
Mrs.A.	7.16	9.25	12
Mrs.D.	21.5	9.75	23

---

* figures from table 3.4.3a divided by 6

† figures from table 3.4.3a divided by 4

‡ figures from table 3.4.3a divided by 2.

Frequency of involvement ,in satellite associations ,of the D and G groups of chromosomes and the marker chromosome in Mrs.A. and Mrs.D. adjusted per NOR region.

that the frequency of satellite association of each NOR region of the marker is 1.5 times higher than the frequency of satellite association of an average NOR region of D and G groups in both cases studied. Therefore the marker chromosome shows 3 times higher frequency of satellite association than an average NOR of D and G group chromosomes in both cases studied as the marker is bisatellited and has two NOR regions each actively involved in satellite association.

It was found that the more heavily silver-stained satellite region of Mrs. D.'s marker is involved in 80% of the satellite associations involving the marker chromosome, as scored from cells stained with silver.

CHAPTER FOUR

DISCUSSION

Section I

4.1 THE ISOLATION AND LOCALIZATION OF HUMAN Y CHROMOSOME - SPECIFIC GENE SEQUENCES.

4.1-1

Sexual determination in man and animals is believed to be controlled by the Y chromosome. The presence of a Y chromosome determines maleness (the heterogametic sex), no matter how many X chromosomes may be present. The mechanism is believed to involve the expression of a single gene product, a cell surface antigen (H-Y antigen). The evidence for this come from studies done by Wachtel et al. (1975), Koo et al. (1977), Silvers and Wachtel (1977) and others . Other models have been proposed as a result of studies on animals such as mouse, rat and guinea pig (Gasser and Silvers, 1972; Wachtel et al., 1974). No other gene product has been localized to the Y chromosome although the condition known as hairy-ear was described as being Y-linked (Slatis and Apelbaum, 1963; Dronamarju, 1965). Therefore it may be anticipated that the Y chromosome will contain little, if any, single copy DNA sequences coding for proteins.

Direct comparison of the DNA sequences in males and females should provide information on the DNA content of the Y chromosome and exhaustive competitive hybridization between male and female DNAs should allow purification of sequences, if any, specific to the Y chromosome.

In addition to the DNA of the sex chromosomes there will be other differences between the gene content of individuals and races which may affect the DNA sequences obtained experimentally.

It was shown by Gosden et al. (1975b) by in situ hybridization, that the four human satellite DNAs were mainly localized in the C-bands of chromosomes 9 and Y (the centromeres and the fluorescent segment of the Y chromosome) as well as the C-bands of chromosomes 1,5,7,10,12,13,14,15,17,

20,21, and 22.

A different approach to the study of highly repeated sequences, using restriction enzymes, was used by Cooke (1976). He described a highly repeated DNA, unique to human males and amounting to 50% of the Y chromosome, by Hae III digestion. Later Cooke and McKay (1978) showed that these sequences (of size 3.4 kb) were present in about 3000 copies, and that they cross-hybridize with female DNA in solution to give a poorly matched duplex. They also concluded that these sequences have been derived by divergence from human satellite III sequences; a finding which was confirmed by Bostock et al. (1978) by in situ hybridization of (^{3}H)RNA complementary to the 3.5 kb fragment of a Hae III digest of male satellite III DNA. It seems that the same sequences were originally on the Y and other chromosomes but changes during evolution had eliminated the Hae III cleavage site, so that cross-hybridization still occurred but no specific fragments after Hae III restriction are found.

Further studies on the Y chromosome-specific gene sequences using restriction enzyme analyses were performed by McKay et al. (1978) and Kinross et al. (1978) and have been discussed in the introduction (Section 1.2.7).

Kunkel et al. (1976) developed a novel approach for the isolation of the Y chromosome-specific gene sequences, by cross-hybridizati to saturation between male and excess female DNA. They demonstrated that the male-enriched sequences obtained are Y chromosome-specific by showing them to hybridize twice as fast to DNA from a 47,XYY male as to DNA from a 46,XY male. This method of analysis would not detect the sequences described by others (Cooke, 1976; Cooke and McKay, 1978; Kinross et al., 1978) for two reasons:

1. Highly-repeated sequences would have self-annealed under the hybridization conditions used even in a 10^{4} - fold female DNA excess.

2. The Y-specific satellite sequences would be removed by the cross-hybridization to excess female DNA as described above.

However, Kunkel et al. (1976) found that these sequences represented between 7 and 11 percent of the human Y chromosome DNA. Later, Kunkel et al. (1977), using DNA from persons of abnormal karyotypes, showed that the isolated sequences (it-Y-DNA) are not associated with male determination and that they appeared to be localized on the Yq12 region. They also showed that these sequences are a collection of 15-30 different families each of which is of reiteration frequency from 300 to 600 copies. The human X and Y chromosomes may show terminal association during meiosis but side to side pairing, as shown by other chromosomes, does not occur (McKusick, 1968). This suggests that the X and Y chromosomes may have homologous sequences at the ends but that crossing-over between the two chromosomes does not occur. None of the genes on the X chromosome have yet been found on the Y (Bodmer and Cavalli-Sforza, 1976). If any homologous segment did exist, genes carried on it would produce a trait with a characteristic pattern of inheritance in families, so-called partial sex-linked. However, there is no evidence for partial sex-linkage in man, although this may occur in certain animals. The purpose of the enrichment procedure was to determine to what extent this procedure would provide a purification of chromosome-specific gene sequences, despite other differences in the genome of the individuals, and to use in situ hybridization to test the enrichment and to analyze some variants or abnormal karyotypes with the chromosome-enriched material.

The Y-enriched DNA isolated at Cot 10^4 after three cycles contains sequences that are Y-specific. This is shown by the reannealing curves (Figs. 3.1.7 and 3.1.8) of the Y-enriched probe to excess unlabelled male and female DNA, where there were sequences that hybridized to the male but not to the female DNA.

Cytogenetically the Y chromosome is the only one where there is a difference between male and female. Other genomic difference cannot be excluded, but cannot be detected cytologically. However, as

no two individuals are exactly the same in their inherited constitutions except monozygotic twins (Harris, 1975), it is not possible to exclude the other genomic differences (apart from the Y) between any individual male and female subjects by cycles of hybridization to saturation between their two genomes. These genetic differences have many implications for individuals and for society, ranging from rare genetic diseases to common differences such as eye colour. Genetic factors influence behavioural attributes, susceptibility to diseases and in fact most human characteristics, however they are defined. Perhaps the best studied of the common differences are the ABO blood groups. Many sets of genetic differences similar to the ABO blood groups are now known; these alone give rise to something like 1,000,000 different genetic types, and yet this is only a small proportion of the total of genetic differences that must exist between people (Bodmer and Cavalli-Sforza, 1976). Amongst the other genetic differences are the polymorphisms observed in the enzymes of which a good example is lactate dehydrogenase, LDH (Harris, 1975). Other polymorphisms are noticed in cytogenetic studies, including polymorphisms in the pericentric heterochromatin of chromosomes 1 and 9, as well as polymorphic chromosome 16 and a common variation in the size of the distal fluorescent segment of the Y chromosome, two variants of which have been studied in this work. The genetic variations between individuals can be associated with national or racial groups, as in the inheritance of certain diseases. A common example is sickle cell anaemia, which is mainly found among blacks of West African origin.

The two reannealing curves (Figs. 3.1.7 and 3.1.8) to determine the Y chromosome specificity of the sequences isolated did not go to completion, and the final hybridization at Cot 10^4 of the Y-enriched probe to excess male DNA was 40-42% as compared to 29-30% for reannealing to excess female DNA. This could be due to the presence of high amounts of degraded labelled DNA in the probe, as suggested by the size of the

Y enriched DNA (Fig. 3.1.6). Such short sequences are incapable of hybridizing either to themselves or to other DNA sequences in the mixture and, therefore, will not form stable duplexes and will be assayed as single stranded. However, they were present in the probe when hybridized to both excess male and female DNA and hence their effect will be equal for both reannealing curves, and will not affect the estimation of the Y-specific gene sequences. These short sequences could have been removed either by running the Y-enriched DNA on sucrose gradients and isolating the material sedimenting rapidly or by hybridizing the Y-enriched probe to unlabelled male DNA to remove the unhybridized single stranded DNA and retain the stable duplexes which would be assayed for Y chromosome specificity. Much of the degraded material will not compete with the Y chromosome-specific DNA in reannealing to chromosomal DNA as it will not hybridize to any DNA nor to itself. Its effect on the kinetics of the in situ hybridization process (if any) is unknown. However, during HAP analysis the HAP column was washed first with 0.03M PB, in order to remove any very short non-hybridized stretches of DNA. The exact size cut-off with respect to HAP binding is not known relative to solution hybridization under conditions of varying stringency.

4.1-2 The nature of the Y chromosome-specific gene sequences:

From the Kinetic analysis of the three successive cycles of hybridization of the first enrichment experiment, it is seen that the non-hybridized single strand DNA isolated in the first cycle of hybridization was five times enriched in the second cycle and hence ran to Cot 5 and not Cot 1, as did the rest of the labelled male DNA. The unlabelled female DNA hybridized to it was always kept at 10^4-fold excess and run to Cot 10^4. The same holds for the single stranded material isolated from the second cycle hybridization reaction and entering the third cycle. This was enriched two-fold in relation to the second cycle and therefor ten-fold enriched in relation to the original material. Therefore, the male enriched gene sequences isolated after the third cycle of hybridization

were those sequences remaining non-annealed at Cot=10. The $Cot_{\frac{1}{2}}$ for the self-reannealing of non-repeated DNA sequences (Figs. 3.1.4 and 3.1.5) was found to be around $9 \times 10^2 - 10^3$. This finding agrees with the previous value found by Ottolenghi et al. (1974). Any sequences failing to anneal at Cot 10 have a repetition frequency of less than approximately 100 copies (as Cot = concentration of nucleotides per litre X seconds during the process of reannealing). Hence these sequences are intermediate-repetitive and low-repetitive in nature (and will also include less than 100 copies of any highly repetitive Y chromosome- specific gene sequences). Since the human diploid nucleus contains 3.5×10^{12} daltons of DNA (Kimura, 1973), and the Y chromosome-specific gene sequences were found to account for 0.79% - 89% of the total genome, then those sequences will be of $4.2 \times 10^4 - 4.65 \times 10^4$ kb (molecular weight of $2.8 \times 10^{10} - 3.1 \times 10^{10}$ daltons). For the same reason the Y-chromosome enriched sequences isolated from the second and third experiments were those failing to anneal after Cot 10.5 and 8.3 (repeated 95 and 120 times and less respectively). The in situ hybridization of Y-specific sequences to the "normal" male 46, XY chromosomes (Section 3.1.1) did not reveal any specific hybridization to the centromeres of any chromosome except for two grains found in one occasion only on a chromosome from the C group which was thought to be an X (Figs. 3.2.2. and 3.2.3). In general a random distribution of grains on the other chromosomes and a low background were found. These findings suggest the absence of satellite DNA in the Y chromosome-enriched DNA that hybridizes to the C-bands of the chromosomes other than the Y.

From Fig. 3.1.7. one notes that at Cot 10^{-1} the hybridization percentage of the Y-enriched sequences isolated from the first enrichment experiment to excess unlabelled male DNA was 5%. This figure, which seems high and which should have been removed during the process of enrichment, is almost one sixth of the percentage reannealing (30%) at Cot 10^{-1} of the labelled total male DNA to excess unlabelled male DNA

(Fig.3.1.4). As expected it seems that almost all the high repetitive sequences were removed during the enrichment processes as evidenced by

1. The kinetic analysis as already described; the Y-enriched sequences being of Cot 10 and more.
2. The reannealing curves (Figs. 3.1.7 and 3.1.8).
3. By in situ hybridization results. The 10% and 5% hybridization at Cot 10^{-1} may have originated from:-

a) The intrastrand base paring due to palindromic sequences which occurs at very low Cot values (Wilson and Thomas, 1974). A figure of 3% was found for the eukaryotic DNAs studied by Davidson and Britten (1973) when they were of sizes of 450 base pairs.

b) Contamination with small amounts of high repetitive sequences during the process of enriching. The DNA annealing does not go to completion during reassociation and unhybridized sequences will be partly removed by elution with 0.03M PB and partly enriched during each step of enriching. Those DNA stretches will, therefore, be eluted with the male-enriched DNA and will give rise to a higher proportion of non-annealing material at low Cot values.

4.1-3 Localization of the human Y chromosome - specific gene sequences by in situ hybridization:

The chromosomal specificity of the Y chromosome-enriched sequences was further tested by in situ hybridization. All the evidence from in situ hybridization of Y chromosome-enriched gene sequences to normal male chromosomes showed specific localization of the silver grains on the Y chromosome, and more specifically on the Yq12 region (81% of the grains on the Y). Less hybridization was observed to the Yq11 region and least to Yp region. The random distribution of the silver grains on the rest of the chromosomes and lack of grains at the centromeres showed that the method had not only enriched for the Y chromosome-specific gene sequences but also removed highly repetitive sequences.

The distribution of the grains over the Y chromosome, as compared to the background, was shown to be statistically significant ($p < 0.01$).

The results from solution hybridization indicated an average enrichment of 118-fold in the Y chromosome-specific gene sequences (after allowing for the sequences which did not reanneal at all as the Y specific sequences represent an average of 0.84% of the total male genome).

Naturally, the exact nature of the unlabelled DNA sequences remaining in the Y-enriched probe is not known; as shown above, it will also include non-Y-specific sequences which have not hybridized, low molecular weight DNA, and any nucleotide material which has not been removed during purification steps besides 4×10^3 - fold excess of single stranded unlabelled female DNA. However, it is interesting to consider the specificity of the in situ hybridization making alternative assumptions about the unlabelled material.

If one assumes, as appears likely from the lack of hybridization to E. coli DNA (Figs. 3.1.7 and 3.1.8), that the labelled probe cannot hybridize to the unlabelled female-derived DNA fragments remaining, then this material also cannot compete in the cell for hybridization sites on the chromosomes with the labelled sequences during in situ hybridization. It is also impossible for the (^{3}H)DNA, that hybridizes to excess total female DNA and included in the Y chromosome-enriched probe, to hybridize in situ to the male chromosomal DNA as such labelled sequences are mostly low repetitive and the kinetics of in situ hybridization shows reannealing to a lower Cot value (See 4.5-4). The in situ hybridization of the labelled Y chromosome-specific gene sequences to normal male chromosomes has probably gone to Cot = 0.9, since the reannealing rate of the in situ has been shown to be 20 times slower than molecular hybridization in solution (See 4.5-4). In solution, the molecular hybridization of the total labelled Y-enriched DNA to chromosomal

DNA is anticipated to go to a Cot = 0.15 (as 3.6 ng of the labelled Y chromosome-enriched gene sequences was dissolved in 4µl of the formamide hybridization buffer and hybridized for 16 hours). Since the Y chromosome-specific sequences were 118-fold enriched (as they represent only 0.84% of the total male genone), the reannealing of such Y chromosome-specific gene sequences will go to Cot = 18. This represented the reannealing Cot of the intermediate repetitive sequences. These results, therefore, are in general agreement with the finding of Kunkel et al. (1977) that the it-Y-DNA is derived from the fluorescent segment of the Y chromosome. This method can therefore be used to study chromosome anomalies associated with the fluorescent segment of the Y chromosome (such as translocations or deletions), even with an only partially purified probe.

4.1-4 Assay of the Y chromosome-specific gene sequences.

The human Y chromosome genome was found to be around 2.01% of the total (46, XY) male genome (Korenberg and Engels, 1978). In this work the Y-chromosome specific gene sequences estimated were found to be between 0.79% and 0.89% (average 0.84%) of the total male genome (See 3.1.2.10.) or 40-45% of the total Y genome . Therefore, it is possible that these sequences are totally (or mostly) located on the Y chromosome. This assumes the absence of any other sequence-specific genomic differences between the male and female genomes studied other than for the Y chromosome. This is excluded by evidence from in situ hybridization which located those sequences specifically on the Y chromosome. As these sequences represent mid-repetitive and low-repetitive sequences of 100 repeats and less, the rest of the Y chromosome genome will include primarily other mid-repetitive and highly repetitive sequences. Previous work demonstrated the occurrence of highly repetitive chromosome-specific gene sequences by restriction enzyme analysis (Cooke, 1976; Kinross et al., 1978; McKay et al., 1978; Bostock et al., 1978) and of mid and high-repetitive sequences by extensive hybridization procedures (Kunkel et al., 1976 and 1977). The Y chromosome-

specific intermediate repetitive sequences (it-Y-DNA) isolated by Kunkel et al.(1976) were found to occupy 10% of the total Y genome. As reported by Cooke (1976) the tandemly repeated Y chromosome specific gene sequence was shown to amount to 50% of the Y genome (about 1% of the total male genome according to estimation of Korenberg and Engels, 1978).

4.1-5 Final Conclusion

There exist mid-repetitive and low-repetitive gene sequences which are specific to the human Y chromosome and which are up to 100 times repeated. They are mainly derived from the distal fluorescent segment of the Y chromosome.

The method followed for extraction of those sequences did, in the final analysis, isolate sequences which consist of the followings:-

1. Radiolabelled Y chromosome-specific gene sequences of the order of 90-100 copies repetition and less, which hybridize to male DNA but not to female DNA.
2. Radiolabelled non-Y chromosome specific sequences that hybridize to female DNA.
3. Degraded unlabelled and labelled DNAs that do not hybridize to any DNA.
4. Low-repetitive unlabelled female DNA.

The DNA isolated does not hybridize to other than the Y-chromosomal DNA as no specific hybridization in situ occurred on any chromosome apart from the Y. This proves that the major (and perhaps the only) difference between the male and female genomes in DNA sequences present at a repetition frequency of the order of 100 copies or less resides in the Y chromosome. There are also Y chromosome-specific gene sequences in the highly repetitive and mid-repetitive classes (Kunkel et al.,1976 and 1977 Cooke,1976; Cooke and McKay,1978; and McKay et al.,1978)

From the above it is concluded that the Y chromosome includes the major genomic differences between a male and a female and that almost all the genomic sequences derived from the Y chromosome are Y-specific.

Section II

4.2 THE APPLICATION OF THE Y CHROMOSOME-SPECIFIC GENE SEQUENCES IN STUDYING VARIANTS OF THE Y CHROMOSOME

4.2-1 Studying the Y chromosome in a male with 46,XYq+

The objective of studying this particular case was to look for the distribution of the Y chromosome-specific gene sequences of the order of 100 copies repetition frequency and less, on the extended Y-chromosomal long arm from a male with a 46,XYq+. Differences in the size of the Y chromosome can occur as a normal variation in a random population (Unnerus et al., 1967). The results of in situ hybridization of the Y chromosome-enriched gene sequences to peripheral lymphocyte chromosome preparation from a 46,XYq+ male, (Fig. 3.3.3. and 3.3.4.) showed that those sequences were localized on the Yq12 region; the region which coincides with the fluorescent segment of the long arm of the Y chromosome when stained with quinacrine. The Y chromosome-specific gene sequences on the Yq+ chromosome (Fig. 3.3.5) were spread throughout the fluorescent portion as also observed for the "normal" Y chromosome in a 46,XY male (Fig. 3.2.5). Such a finding coincides with the observation of Bobrow et al. (1971) and Schnedl (1971) who noted that the variation in the length of the Y chromosome is entirely due to variation in the amount of fluorescent material in the Yq12 region.

It is concluded, therefore, that region Yq12 of a Yq+ chromosome contains mid-repetitive and low-

repetitive gene sequences of the order of 100 copies repeat and less, similar to a "normal" Y chromosome, which are specific to the Y chromosome and which do not occur in females. It therefore seems that the Yq12, in this case, is a normal variation of the Y chromosome regarding this type of gene sequence.

Geraedts et al. (1975) showed that big Y chromosomes with a large fluorescent segment were characterized by an increase in the amount of chromosomal DNA. Such a suggestion may not mean an augmentation, in a Yq+ chromosome, of the dosage of the single-copy gene sequences which are active in mRNA synthesis because such an increase could cause abnormal function (Krone and Wolf, 1977). It is, therefore, possible that much of the extra chromosomal DNA present in a Yq+ chromosome consists of mid-repetitive sequences. Other studies have shown that the highly repeated (rapidly reannealing) Hae III-digested DNA (Fr-Y-DNA) of the length 3.41 kb (rather than the 2.1 kb fraction) varied in amount according to the size of the Y chromosome being higher in cases of Yq+ without any variation in the sequences itself (quantitative rather than qualitative variation). (McKay et al., 1978).

Quantitative variations in the amount of mid and low-repetitive gene sequences cannot be estimated by our techniques. The presence of an extra Y chromosome in 47,XYY males does not, in general, cause major phenotypic abnormalities, in spite of the increase in gene dosage (See 4.2-2). This may be due to the very small number of structural genes situated on the Y chromosome; only the gene for the H-Y antigen has been assigned

there definitely (Wachtel et al., 1975). In the case we have studied, 46,XYq+ male, this gene would not be present in increased amount as it has been localized on the short arm rather than the long arm of the Y chromosome (Koo et al., 1977).

4.2-2 Studying the nature of the Y chromosomes in a 48,XYYY male

Statistical analysis of the results of *in situ* hybridization of the Y chromosome-enriched gene sequences isolated as in section I (Chapter III) to chromosome preparations from the peripheral lymphocytes of the male with 48,XYYY confirmed previous findings as their Y chromosome localization. There was equal distribution of the silver grains on all the Y chromosomes. This indicates that all the Y chromosomes in this particular case had almost equal concentration of the Y chromosome-specific gene sequences present at the order of 100 copies repeat and less.

As the *in situ* hybridization process did not detect the single-copy gene sequences, it is impossible to measure the distribution of this class of gene on the Y chromosomes in this case of 48,XYYY and this will remain unsolved until *in situ* hybridization process has been modified to improve the sensitivity of detection of sequences present at very low frequencies.

It was not possible to differentiate between any of the three Y chromosomes, although in the study of Ridler *et al.* (1973) it was found that one of the three chromosomes often seemed to differ in overall morphology

from the rest. It was difficult to state the real difference and in what sense (Ridler, personal communication). However, that difference was not primarily one of size for the measurments showed a unimodal distribution (Ridler *et al.*, 1973). This finding coincides with my finding in that no relevant difference in the lengths of the Y chromosomes was observed.

Neither with the process of *in situ* hybridization nor with staining techniques, was it possible to tell whether there was a change in the dosage of a gene causing the clinical abnormality in this particular patient (mainly mental retardation with 81% intelligent quotient, hypotrophic testes and aggressive personality) reported by Ridler *et al.* (1973).

Cases of 48,XYYY males are extremely rare and in the few cases reported there were no clinical abnormalities of the internal organs; the only effects were on IQ (intelligence quotient) or the personality (Townes *et al.*, 1965; Pozsonyi and Sergovich, 1971; Schopflin and Centerwall, 1972; Ridler *et al.*, 1973).

Correlation of the clinical signs with the abnormal karyotype remains, therefore, obscure. It was stated by Levitan and Montague (1977) that generally any addition to the normal number of sex chromosomes, whether X or Y, tends to produce physical and mental disabilities which increase with the number of additional sex chromosome; but such a statement is controversial with respect to the Y in the view of cases of 47,XYY males. Such individuals with 47,XYY karyotypes are, for the most part, normal males (Stenchever and Macintyre, 1969). This may, perhaps be due to the relatively small amount of genetic material of the usual trait-determining type carried by the Y chromosome. However, some abnormalities of the internal organs, such as undescended testicles, hypogonadism with seminiferous tubules and dysgenesis have been reported in males with 47,XYY karyotypes. Some 47,XYY males may be characterized by aggressive personality (See Levitan and Montague, 1977).

The 48,XYYY male case studied here did not show any abnormalities of the internal organs apart from the hypotrophic testes; yet it seems that the additional Y chromosomes in this case have some role in the mental abnormality and aggressive personality noted in this patient.

It would be beneficial to study the highly repetitive gene sequences in this case of 48,XYYY by applying restriction enzyme digestion as has been done previously on a "normal" Y from 46,XY male and in 46,XYq+ (McKay et al., 1978) and for other different conditions associated with structural abnormalities of the Y chromosome (Kinross et al., 1978).

Obviously both the extra Ys in addition to the original Y chromosome have come from the father, who was a normal 46,XY individual (Ridler, personal communication). Therefore, the extra chromosomes could have originated from non-disjunction at the second meiotic division of the two paternal germ cells. Such a chromosomal aberration may have also resulted from mitotic non-disjunction in early zygotic cleavage, but in such an event an XO/XY/XYY/XYYY mosaicism would have resulted. The 48,XYYY case studied here was not a mosaic. However, there have been four reports of mosaics with XYYY (Fraccaro et al., 1962; Cox and Berry, 1967; Weiner et al., 1968), and one mosaic with XYYYY karyotype (Berghe et al., 1968).

Section III

4.3 THE ISOLATION OF HUMAN X CHROMOSOME-SPECIFIC GENE SEQUENCES

The isolation of the X chromosome-specific gene sequences followed the same principle as the isolation of the Y chromosome-specific sequences, through two cycles of extensive hybridization.

The reannealing curves of the X-enriched probe to excess unlabelled parent mouse and HORL9.X DNAs (Fig. 3.3 .11) revealed the existence of sequences that hybridized to the HORL9.X DNA but not to the parent mouse DNA. Those sequences, therefore, must be human X chromosome-specific, as the only genomic difference between the parent mouse and the HORL9.X DNA is the presence of the human X chromosome. The finding that at Cot 10^4 (Fig.3.1.12) the hybridization to the HORL9.X DNA does not go beyond 33% can be attributed to highly degraded labelled and unlabelled DNA as discussed previously (See 4.1).

The low percentage of reannealing of the X-enriched probe to the HORL9.X and mouse DNAs at Cot 10^{-1} shows that most of the highly repetitive sequences from the parent mouse DNA as well as the HORL9.X DNA had disappeared after two cycles of enriching for the X genome. The reannealing still noted at Cot 10^{-1} can be explained as background due to intrastrand base pairing of the presence of palindromes (Davidson and Britten,1973) as described previously.

The two reannealing curves in Fig. 3.1.12 start to diverge after Cot 10^2 with a very marked difference

after Cot 10^3. A maximum difference is noted at Cot 10^4. Thus, the X-enriched sequences contain DNA that partially reanneals to the mouse DNA before Cot 10^3. Only the difference between the two curves represents X-specific sequences. Since the ratio of the parent mouse DNA/HORL9.X DNA in each cycle of the hybridization was 10^4, the HORL9.X DNA will go to Cot 1 as the mouse DNA goes to Cot 10^4 in the first cycle. The non-hybridized material isolated from the first cycle of hybridization, entering the second cycle, was only 28% of the original input (since 72% of the labelled DNA has reannealed to the unlabelled DNA in the first cycle). This means that those sequences are enriched during the second cycle at a proportion of (100/28 =) 3.6 times. Therefore, such sequences will go to Cot (3.6 X 1 =) 3.6 when the unlabelled parent mouse DNA goes to Cot 10^4 in the second cycle. It is obvious that the X chromosome-specific gene sequences are those sequences annealing at greater than Cot 3.6. As the $Cot_{\frac{1}{2}}$ for annealing HORL9.X DNA to mouse DNA was found to be 10^3 (Fig. 3.1.11), the human X chromosome-specific gene sequences isolated after Cot 3.6 are sequences that are repeated approximately 300 times and less.

The invention of a unique dosage compensation mechanism in a common ancestor was used to preserve the original X chromosome <u>in toto</u> despite extensive speciation which followed (Ohno, 1969). This is apparent from the fact that the X chromosome, in a great majority of mammals, is of the same size comprising about 5% of the genome, and also the extensive homology of X-linked genes among diverse mammalian species.

If 100% homology exists between the loci on

the mouse X chromosome and those on the human X chromosome this will, probably, lead to cross-hybridization between the two genomic sets; hence they will form stable duplexes and will not be enriched in the non-hybridized fractions. The recognized human X loci which are homologous to those on the mouse X are α-galactosidase, hypophosphataemia, HGPRT, Menkes syndrome, phosphoglycerate kinase and testicular feminization (McKusick, 1975). There are other suspected homologous sites between the human and mouse X chromosomes such as glycogen storage disease, icthyosis, Keratosis follicularis spinulosa decalvans cum ophiasi and Pelizaeus-Merzbacher disease (McKusick, 1975).

Although the same proteins are coded for on the human and mouse X chromosomes, cross-hybridization may be incomplete as the protein sequences will have diverged to some extent and the third base of each triplet codon may also have diverged.

It was concluded by King and Jukes (1969) that most proteins contain regions where substitutions of many amino acids can be made without producing appreciable changes in protein function. This conclusion is of importance to the work has been done here as homologous loci between human and mouse X chromosomes may be different in their gene sequences while the corresponding protein synthesized fulfils the same function. There is a difference of 17 amino acids between the human and the mouse α-globin, which consists of 141 amino acid (King and Jukes, 1969). Gummerson and Williamson (1974) found that only 30% of the mouse globin gene is homologous to that of the human. An example of a highly changeable protein (i.e. highly diverged or highly non-conserved) is Fibrinopeptide B (King and Jukes, 1969).

With the current information vailable it may not be possible to tell how much a single copy gene sequence on the human X chromosome and the mouse are conserved or diverged. For this reason the human X chromosome-specific gene sequences isolated here will not include recognized homologous genes (assuming the DNA sequences have been conserved) and may be devoid of many others. Therefore, it is anticipated that such human X chromosome-specific gene sequences will not hybridize to the sites of the "homologous" loci, if they are used as probes in situ to a normal female chromosome Karyotype. This will, therefore, be a technical limitation in the isolation of human X chromosome-specific gene sequences by the method employed here. However, this will not produce any difference in the rate of reannealing to either the total mouse DNA or the HORL9.X DNA (Fig.3.1.12). This is because the X chromosome-enriched gene sequences, which are used as a tracer, do not contain the shared sequences in either hybridization. The highly repetitive gene sequences on the human X chromosome, have self reannealed and removed on HAP.

Human X chromosome-enriched gene sequences could be used as probes for in situ hybridization to chromosome preparations from HORL9.X cells to test for contamination, and also to chromosome preparations from normal male and female Karyotypes to study the location of any repetitive sex-chromosome sequences on the autosomes. They could also be used, as in the case of the Y chromosome-enriched sequences, to study special genetical cases associated with abnormalities of the X chromosome, such as translocation of regions of the X chromosome to other chromosomes, by in situ hybridization.

The success in isolation of human X chromosome-specific gene sequences, as judged by solution hybridization, somatic cell (mouse-human) hybrids, should make it possible to isolate gene sequences related to other human chromosome when it was possible to preserve them in similar hybrids in the presence of suitable selective medium. Cross-hybridization between "homologous" gene loci on the human and mouse chromosomes should always be considered when attempting such procedures.

Section IV

4.4 Study of two cases of extra bisatellited marker chromosome by *in situ* hybridization of ribosomal genes, silver staining of the nucleolus organizers, and satellite association of the chromosomes.

4.4-1 Nature of the satellites of the marker chromosomes:-

The evidence from the trypsin-Giemsa staining and C-banding suggests that the marker chromosomes are dervied from the acrocentric chromosomes (either the D or G group). This was confirmed by *in situ* hybridization and silver staining. The *in situ* hybridization showed that the 28S and 18S ribosomal genes were present on both ends of the marker chromosomes. The staining of the satellite regions of the marker chromosomes in both cases indicated sites for rRNA synthesis on both ends, as Miller *et al.* (1976 a and b) in studies on rodent-human somatic cell hybrids have shown that only the NORs of the species producing rRNA are stained by the silver staining technique, and it has been assumed that this stain is specific for active NORs. There is a high frequency of involvement of the marker, as a two-NOR unit, in satellite association as compared to the frequency of satellite association of other acrocentric chromosomes (Table 3.4.3 a and b). Satellite association has long been considered as a measure of activity in production of rRNA as it is thought to involve those acrocentric chromosomes whose NORs were involved in nucleolus formation in the previous interphase (Ferguson-Smith , 1964). By these criteria, the marker chromosome is active in ribosomal RNA synthesis at both NORs in both cases studied.

Miller *et al.* (1977) have shown a positive

correlation between the frequency of satellite association of human normal acrocentric chromosomes and the amount of silver staining of the NOR regions, and this is confirmed by these results.

The finding that the more heavily silver-stained satellite region of Mrs. D.'s marker chromosome is involved in 80% of the satellite associations as scored from cells stained with silver supports this view. It was not possible to correlate this with ribosomal gene content due to the difficulty in distinguishing the two ends of the marker following *in situ* hybridization.

4.4-2 Ribosomal gene content of the marker NORs and its relation to chromosomal aberration:-

Since more silver grains per NOR were detected on both marker chromosomes after *in situ* hybridization as compared to those on the D and G chromosomes, (Table 3.4.1) it is concluded that there are more ribosomal genes on the marker chromosomes than on the other satellited chromosomes. At present there are conflicting reports as to whether the frequency of satellite association is correlated to the number of rRNA genes present on a chromosome as shown by *in situ* hybridization or to other factors. Warburton *et al.* (1976) and Henderson and Atwood (1976) have demonstrated a positive correlation between the number of rRNA genes and frequency of involvement in satellite association. This is contrary to results obtained by Evans *et al.* (1974) who found the chromosomes with large numbers of ribosomal genes are not preferentially involved in satellite association. These results show an increased frequency of satellite association with a large ribosomal gene number.

There have been reports on an increased risk of chromosome abnormalities (such as translocation) in the progeny when there is an increase in heterochromatic chromosomal regions such as the occurrence of a long satellite stalk in an acrocentric chromosome in the parents, as this excess heterochromatin may interfere with meiosis or mitosis during early development (Nielsen and Sillesen, 1975; Friedrich and Nielsen, 1974; Ferguson-Smith and Handmaker, 1963; Bauchinger and Schmid, 1970). It has, also been suggested that participation of acrocentric chromosomes in satellite association may predispose them to breakage and translocation. (Ferguson-Smith and Handmaker 1961 and 1963; Bishum, 1966; Ford, 1968; Galsperine, 1968; Nakagome, 1973; Hannson and Mikkelsen, 1974 a and b). It is noteworthy that at least one of the NOR regions of the marker chromosome of each case studied, is particularly active in satellite association as compared to the D or G chromosomes (Table 3.4.2).

The NORs of the marker chromosomes have introduced an extra dose of ribosomal genes of 40% (Mrs. A.) and 30% (Mrs. D.) as judged by in situ hybridization. However, both Mrs. A. and Mrs. D. are phenotypically normal and developing normally in spite of the extra bisatellited marker chromosome. Both Mrs. A. and Mrs. D. have extra functionally active NORs.

Mikkelsaar et al., (1977) showed a consistent pattern of silver-positive NORs in all the acrocentric chromosomes. They found that 90% of individuals have a modal number of 8-10 silver-positive NORs per cell. In Mrs. D. 70-80% of the NOR regions of the D and G chromosomes are functionally active and therefore it seems that there is a negligible degree of compensation for the high dose of functionally active NORs introduced by the marker.

In Mrs. A., on the other hand, a certain degree of compensation is introduced for the marker as only 50-60% of the NOR regions of the acrocentric chromosomes are functionally active. It has been suggested that the NORs are a system showing a remarkably low sensitivity to alterations of the gene dose. (Krone and Wolf, 1977). In Xenopus, for example, the loss of up to 75% of the rDNA is not lethal (Krone and Wolf, 1977). The lower limit of the ribosomal gene number that allows normal development in humans is unknown.

4.4-3 The origin of the extra marker chromosomes:

It has not been possible to determine from which of the acrocentric chromosomes the extra metacentrics have been derived. A direct comparison of the specific features of each NOR with those of the parental acrocentrics is unlikely to be informative in cases such as those reported here, where the marker is familial. The parental chromosomes of origin could have been lost by normal meiotic segregation in as short a time as two generations. Such an opportunity exists only for those extra metacentrics that have arisen de novo. However, certain conclusions can be drawn about the mechanisms of origin of the extra metacentrics from these findings. The two main possibilities for the origin of the aberration are isochromosome formation and centric fusion translocation. Both resulting aberrations can be dicentric, the former as a result of isochromatid breakage and fusion in the proximal part of the long arm of an acrocentric chromosome. However, isochromosome formation appears to be unlikely in either case as the two arms of each metacentric are not entirely symmetrical. In Mrs. A. the major difference appears to be in the satellites, only one of whicch is readily distinguished in the majority of cells; no differences are observed in silver

staining, in situ hybridization or satellite association. In Mrs. D. the two arms are more asymmetrical in length, and one consistently shows heavier silver staining with more frequent involvement in satellite association.

Schrek et al., (1977) were able to show that six out of twelve cases of extra chromosomal anomalies, they studied, were derived from chromosome 15 by employing anti-5 methylcytidine antibodies which specifically bound normally to chromosome 15.

4.4-4 Final Conclusion:

It is possible for extra functionally active 28S and 18S ribosomal genes to be associated with phenotypically normal features and not to be associated with any clinical abnormalities. In antenatal diagnoses, therefore, the discovery of an extra dosage of functionally active ribosomal genes represented by bisatellited chromosomes does not necessarily reflect an abnormality in development and should be assumed to be a "normal" event or a non-clinical variant, particularly if either of the phenotypically-normal parents carried such a chromosome. This also indicates that the clinical abnormalities seen in trisomy 21 and in trisomy 13 are not due to the extra NOR on these chromosomes, but to extra dosage of other 21 or 13-specific gene sequences. Further studies on similar cases with more information about the pattern of the inheritance are suggested.

Section V

4.5 ASSESSMENT OF IN SITU HYBRIDIZATION:

As many of the conclusions in the preceeding sections are based on the method of *in situ* hybridization , it is important to consider the specificity and quantitation of the method as well as the technical problems associated with it.

4.5-1 General View:

In the last eight years the method of *in situ* hybridization has proved to be useful in localizing repetitive gene sequences such as the ribosomal genes. Attempts to localize single copy gene sequences have failed because of the difficulty of obtaining sufficient grain counts from such small lengths of hybridizing DNA, however highly labelled.

In situ hybridization involves four main steps:

1) Fixation of the target cells.
2) Denaturation of chromosomal DNA.
3) Molecular hybridization with the radio-labelled nucleic acid probe.
4) Autoradiography.

The final result of the *in situ* hybridization reaction is highly dependent on the approach selected to achieve each of these steps. The main consideration is to increase the signal whilst maintaining good chromosome morphology.

Fixation of the target cells:

This is usually performed by treating the preparations several times with absolute methanol: glacial acetic

acid (3:1).

Denaturation of chromosomal DNA:

Many methods have been attempted in order to achieve a high efficiency and to minimise any effect on chromosome morphology. These methods include treatment with NaOH (Gall and Pardue, 1969), HCl (Jones, 1973; Szabo et al., 1977), heat (Macgregor and Kezer, 1971) and acetic acid (shapiro et al., 1978). Denaturation by NaOH may remove a lot of chromosomal DNA. (Shapiro et al., 1978). It was found that while HCl preserves chromosomal architecture, it limits the denaturation of DNA to A-T sequences (Singh et al., 1977). However, incubation of the chromosome preparations at 65°C for $2\frac{1}{2}$ hours, in 95% formamide in 0.05% SSC, was found to give satisfactory morphology with efficient hybridization. Shapiro et al. (1978) found that denaturation of the DNA with acetic acid during in situ hybridization also preserves the chromosomal morphology. Without pre-banding it is impossible to recognize each individual chromosome after in situ hybridization; only chromosomes 1,2,3, 9, 16 and Y are always identifiable. Pre-banding either with trypsin (Henderson and Atwood, 1978) or quinacrine (Evans et al., 1974) has been used with in situ hybridization. Pre-banding was unnecessary in this work as the target chromosome was the Y or the marker chromosomes which were readily recognizable.

4.5-2 Scoring the grains and their relation to gene dosage:

Silver grains were scored on photographic prints and compared with direct microscopic viewing where necessary.

Consideration was paid to the size of the grains; one grain was easily counted. Groups of grains were

counted as individuals if they were discrete while if they were coalesced a relative comparison was made by measurement and sight estimation. For example, four grains were scored on the long arm of the Y chromosome in Fig. 3.2.3, while two grains were regarded to be on the centromere of the X in the same figure. Similarly five grains are scored on the marker chromosome in Fig. 3.4.7b while one grain was on one of the chromosomes 21 and five on the other.

Although this introduces an element of arbitrariness into the estimation, it is the only way to estimate several grains; in general, slides were used where few grains per chromosome were visible.

In this work the high density of silver grains on the NORs of the marker chromosomes in both cases (Table 3.4.1) as compared to those on an average D or G chromosome, was regarded as an indication of a higher ribosomal gene dosage of 40% in Mrs. A. and 30% in Mrs. D. Previously, some authors used the number of silver grains as an indication of the gene dosage. In studying the localization of the rDNA in small nucleolus-like structure, in human diplotene oocyte nuclei, Wolgemuth-Jarashow et al. (1977) were able to calculate the approximate number of the rRNA genes contained in the micronucleoli from the percentage of the grains distributed over the micronucleoli. The calculated number was found to be 42 genes per micronucleolus.

4.5-3 Background:

One of the problems with the application of this method is excessive background. Background could be due to:

1) Non-specific base pairing.

2) Failure to remove completely the non-hybridized labelled nucleic acids.

3) Access to random light sources.

4) Mechanical damage to the photographic emulsion.

The second factor is difficult to eliminate completely; prolonged incubation at high temperature, in order to reduce this factor, will damage the chromosome architecture. It can be reduced by either:-

1) Prolonged washing in buffer (2 X SCC).

2) By enzymatic treatment, such as with S1 nuclease.

3) By scoring the background and comparing it to the grains over the target (as carried out in this work).

The background will, obviously, be higher with probes of higher specific activity and is particularly high when (^{125}I) is used to radiolabel the nucleic acid. The background in my work was considered in relation to the relative lengths of the chromosomes other than those which displayed specific labelling with the silver grains, i.e. the Ys in the "normal" 46, XY male (3.2 and 3.3), or the markers and G and D groups in Mrs. A. and Mrs. D's cases (3.4). Statistical analyses (X^2 test) comparing the background in the 3 male cases studied, to the spread of the grains on the Ys (i.e. grain/relative length), revealed a highly significant difference between the two densities ($p < 0.01$ in each case). This meant that the hybridization to the Y chromosomes is significantl over background.

Another factor which increases the background is the long exposure time required with low specific activity probes. Shortening of the exposure time could be achieved by using plasmids containing specific gene sequences (Malcolm et al., 1977).

This was observed in the two cases of 47,XX + marker, studied in 3.4, when results were obtained after one week's exposure (Fig. 3.4. 8a). An eight-week exposure using the same probe is shown in Fig. 3.4. 7a.

4.5-4 Kinetics of *in situ* hybridization:

Szabo *et al.* (1977), studying the *in situ* hybridization of ^{125}I-labelled 5S, 18S + 28S ribosomal RNAs to polytene chromosomes of *Drosophila melanogaster*, found that the rate of *in situ* hybridization reaction is three to five times slower than the corresponding filter hybridization reaction. They showed that the properties of the *in situ* hybrids were similar to those of filter hybrids. It was also shown by them that the rate of the reassociation process is inversely proportional to the complexity of the nucleic acid probe and that the presence of proteins on the slide does not seem to greatly influence the reaction. It was shown that the rate of filter hybridization is five times slower than that of solution hybridization. (Bishop, 1970). If the rate of *in situ* hybridization is assumed to be four times (on average) slower than that of filter hybridization, then it will be twenty times slower than the rate of solution hybridization.

4.5-5 Efficiency of *in situ* hybridization:

The efficiency is the ratio of site-associated grains to calculated disintegrations for the amount of probe that would be bound to a specific chromosomal site (Henderson *et al.*, 1978). The efficiency is calculated as

$$E = \frac{C}{SWD} .$$

Where C = constant (Avogadro's number divided by the number of minutes per day times 10^6) Therefore C = 4.18×10^4.

S = Specific activity (dpm/ug) of the probe used.

W = Molecular weight equivalent of the chromosomal site to be detected.

D = Time of exposure in days per site-associated grain.

The efficiency of <u>in situ</u> hybridization and detection of the (^{3}H) Y chromosome-enriched gene sequences (specific activity 1.98×10^7 dpm/μg DNA) to chromosome preparations from a normal 46, XY male with a "normal" Y chromosome was calculated.

$$E = \frac{4.18 \times 10^{14}}{1.98 \times 10^7 \times 0.8\% \times 3.5 \times 10^{12} \times 23.3} = 0.004\%$$

The Y chromosome-specific gene sequence was 0.8% of the total human diploid genome which is 3.5×10^{12} daltons per cell (Kimura, 1973).

Time of exposure in days per site-associated grain = 23.3 (41 grains scored on the Y chromosome from 17 cells; time of exposure = 56 days). A corresponding calculation yields an efficiency of 0.03% in the 46, XYq+ case (69 grains on 15 chromosomes in 56 days) and 0.04% in the case of 48,XYYY male (269 grains on 81 chromosomes in 56 days).

On the other hand, in cases of localization of a specific locus, the molecular weight of the target site will be smaller and this will lead to a higher efficiency. This was obvious when the efficiency of <u>in situ</u> hybridization of the

(^{3}H)Xlr101 plasmid containing Xenopus laevis 28S and 18S ribosomal genes (specific activity 10^7 dpm/μg DNA) to chromosome preparations from Mrs. D. was calculated:

$$E = \frac{4.18 \times 10^{14}}{10^7 \times 5 \times 10^6 \times 130 \times 1.5} = 7.5\%$$

As there is a minimum of 50 copies of the human ribosomal genes per haploid cell (Young et al., 1976); the total copies of the ribosomal genes will, therefore, be 65-70 or(130-140 per diploid cell) assuming that the marker chromosome contributes an extra 30-40% of ribosomal genes (See 4.4-2). Since only the structural portion of the 28S and 18S genes of the Xenopus plasmid will cross-hybridize to the human ribosomal genes, the total molecular weight of the targets will be $5 \times 10^6 \times 130$ daltons as the m.w. of a single 18S and 28S ribosomal gene repeat is 5×10^6 dalton (Wellauer and Daivd, 1973).

The average efficiency of in situ hybridization for Mrs. A. was found to be 3.6%. From the above comparison of the efficiencies, it is concluded that with the use of recombinant plasmids as probes in in situ hybridization, much higher efficiencies can be obtained.

In assigning single copy gene sequences by in situ hybridization an efficiency of more than 10% with cDNA or mRNA probes is considered anomalous (Henderson et al., 1978). This, obviously, does not hold when plasmids are used as probes. In abnormally high efficiency, a chromosomal assignment can be erroneous, as this would indicate that either the radioactivity had been bound by some mechanism other than hybridization or that conatminants had hybridized to a larger or more highly iterated

sequence than any for which the probe was intended (Henderson et al., 1978). The contaminants include other nucleic acids in the probe used for in situ hybridization. Such contaminants could be completely eliminated by incorporating the probes into plasmids. In the case of hybridization of (^{3}H) Y chromosome-specific gene sequences to chromosome preparations from various male cases studied, the probe was not purely Y chromosome-specific, as was demonstrated by Figs. 3.1.7 and 3.1.8. Such a probe was only Y chromosome-enriched as it contained other gene sequences as contaminants. Besides eliminating the contaminants a high increase in the efficiency of in situ hybridization can be achieved by using recombinant plasmids containing specific gene sequences (Malcolm et al., 1977). This is mainly due to shortening in the time of exposure as was noted in section 4 (Chapter 3) when the slides were exposed to photographic emulsion for 1-3 weeks and compared to results obtained after a week's exposure during in situ hybridization with (^{3}H)Xlr101 plasmid containing Xenopus laevis ribosomal genes. With plasmid both strands of the gene insert hybridize to strands of the chromosomal DNA, and hence the entire plasmid and insert sequences can contribute to the signal (Malcom et al., 1977). In order to allow for the self-annealing of the complementary strands of the plasmid DNA which will compete with their hybridization to chromosomal DNA and also to drive the reaction, an excess of radioactive probe is used. 150 ng of the (^{3}H)Xlr101 plasmid containing 19.2 ng of ribosomal gene was used per slide, while only 4-6 ng of (^{3}H) Y chromosome-enriched gene sequences was used for each slide, only part of which was Y chromosome-specific.

4.5-6 The use of *in situ* hybridization for localizing single copy gene sequences:

There have been many attempts to localize single copy gene sequences on the chromosomes by *in situ* hybridization. It is necessary in such cases to use highly purified probe labelled at high specific activity to obtain sufficient grain counts on the target sites.

With few exceptions (*e.g.* globin genes) it has not been possible to purify single copy structural genes from complex eukaryotic genomes using conventional biochemical methods.

In order to achieve the necessary Cot value, within the limitations in the kinetics of *in situ* hybridization as compared to the rate of molecular hybridization in solution (See 4.5-4), a high probe concentration in the hybridizing buffer with a long incubation time must be used. However, high probe concentrations lead to a high background, and prolonged incubation can be deleterious to chromosome architecture. Henderson and Atwood (1978) incubated trypsin-prebanded mouse chromosome preparation with rabbit mRNA as tracer, for 72 hours, trying to localize mouse globin genes. They suggested that *in situ* hybridization can not be used to localize unknown single copy gene loci despite sufficient labelling of the globin gene probe due to the presence of contaminants that hybridize to recognized chromosomal regions. These might have been removed by cloning the probe in a recombinant plasmid.

It was suggested by Deisseroth *et al*, (1977) that *in situ* hybridization, as a technique for mapping the human globin gene, is less successful than other techniques. These authors localized the human α-globin structural gene to chromosome 16 in human-mouse somatic cell hybrids by molecular hybridization of purified human α-globin cDNA to DNA extracted from the hybrid cells. By employing

a similar technique, Deisseroth et al., (1978) localized the human β-globin gene loci on human chromosome 11 in somatic cell hybrids. Those two findings were contrary to previous findings of Price et al. (1972) and Price and Hirschhorn (1975) who, by in situ hybridization of rabbit globin mRNA to metaphase spreads of human chromosomes, localized the human globin genes on chromosome 2 and one of the B group chromosomes. However, failure of such attempts might have been due to impurity of the probes and failure to achieve an appropriate Cot value similar to that obtained with solution hybridization.

Section VI

GENERAL DISCUSSION

Isolated gene sequences are useful tools for studying the nature of chromosomal aberrations. Their application in the field of medical genetics is gradually becoming a sophisticated technique to confirm a provisional diagnosis already made by the traditional staining techniques, or occasionally for primary diagnosis. The development of recombinant plasmid technology allows the easy preparation of such gene sequences as they can be grown in a bacterial host in pure form and in large amounts. Consequently, it is now relatively simple to study the structure of such gene sequences, and to apply them as probes for *in situ* hybridization.

The advantages of the use of recombinant plasmids in *in situ* hybridization have been discussed already. Purification of the gene sequences by other techniques is still problematic, especially for those present only in single copies. Further development in this area depends upon the availability of specific sequence probes of greater purity. The introduction of restriction enzymes has allowed the isolation of structural gene sequences with surrounding DNA regions, and examples of the isolation of Y chromosome-specific gene sequences have been described (Cooke, 1976; McKay *et al.*, 1978; Bostock *et al.*, 1978; Cooke and McKay, 1978). DNA sequences cleaved by restriction enzymes can be sub-divided into elements, defined in terms of the distance between two adjacent recognition sites for a particular restriction endonuclease.

The isolation of chromosome-specific gene

sequences by excess hybridization between two genomic sources that differ by a single chromosome (e.g. male and female) did not allow purification of gene sequences specific to a chromosome free from contaminants, including degraded nucleic acid oligomers incapable of hybridizing to themselves or to added DNA sequences. It may have been possible to remove such contaminants by running the chromosome-enriched gene sequences on sucrose gradients and discarding low molecular weight DNA, or hybridizing it with total male DNA and retaining the stable duplexes. Such procedures were not performed due to the low final yield and the expected further loss by those techniques, as the purpose of isolating those sequences was to use them as probes for in situ hybridization. Screening such chromosome-enriched gene sequences (whether Y or X or any other chromosome) to a library of immobilized plasmid-human DNA recombinants would allow the radiolabelled chromosome-specific gene sequences to hybridize to their complementary strands. It would be possible to isolate them on filters, and to clone them in recombinant plasmids for further use in quantity. Maniatis et al., (1978) were able to isolate structural genes directly from large eukaryotic genomes by screening libraries of DNA fragments cloned in phage λ.

Isolating and studying single copy gene sequences is, in general, difficult. The globin gene could be studied because it was possible to isolate globin mRNA and hence to make cDNA to it which could then be used to study the structures of the globin genes in normal and various clinical conditions associated with the globin gene system such as thalassaemia (Williamson, 1976). However, it was possible to locate the Y chromosome specific gene sequences, present at 100

copies repeat and less, specifically on the Yq12 regions in a "normal" Y from a 46,XY male and on Yq+ from 46,XYq+ and 48,XYYY males. The distribution of gene sequences on the Y chromosomes from these three different individuals was similar.

This approach could also be useful in the study of cases such as 46,XYq- (deletion of the long arm), and of translocation of the Y chromosome to other chromosomes. The Y chromosome-specific gene sequences may also be used to study males with a 46,XX karyotype, to find whether hybridization occurs on any chromosomal region. Applying the same principle to isolating chromosome-specific gene sequences (Section I, Chapter III), one would be able to isolate sequences related to any chromosome after obtaining human-mouse hybrid cells containing that specific chromosome to purify specific probes. It would also be possible to isolate the chromosome-specific gene sequences at any Cot value (i.e. of any number of sequence copies) by altering the ratio of concentrations between the two DNAs in hybridization reaction.

Examples of the application of cloned gene sequences to study the nature of extra chromosomes has been discussed. Comparing the results described here to those obtained from traditional banding and silver staining techniques as well as the phenomenon of satellite association, shows that recombinant gene sequences used as probes are very useful in <u>in situ</u> hybridization as they can be visualized with high efficiency as compared to staining methods. Although the results of <u>in situ</u> hybridization in Mrs. A. and Mrs. D. could not assay the functional activity of the NORs on the acrocentric or the marker chromosomes, they still give a clue about the change in the gene

dosage and the proportion of ribosomal DNA sequences in relation to the total grain counts observed on the rest of the NORs. With the development of further recombinants containing other specific genomic sequences for single copy genes, such plasmids could be used for gene mapping and to confirm an already assigned gene position on a chromosome. The short time of exposure (which can be as little as a week) might make such a process a "routine" molecular cytogenetic technique in all the aspects of cytogenetics and antenatal diagnoses to study special cases of deletion and translocation.

No evidence has been presented in this thesis relating to the nature of the DNA isolated as Y or X specific. It is assumed that these DNAs are mixtures of "coding" and"structural" DNA sequences.For the sake of clarity the isolated DNA has been referred to as gene sequences. This is not meant to imply any functional origin.

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ABBREVIATIONS

BSA	bovine serum albumin
BSS	balanced salt solution
cpm	counts per minute
DNA	deoxyribonucleic acid
rDNA	ribosomal DNA
cDNA	complementary DNA
DNase	deoxyribonuclease
dpm	disintegration per minute
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
dCTP	deoxycytidine triphosphate
E.coli	Escherechia coli
EDTA	Ethylene diamine tetraacetic acid
^{3}H	tritium
HAP	hydroxylapatite
Hepes	N-2-hydroxyethyl piperazine-n-2-ethane sulphonic acid
i.u.	International unit
kb	kilobase
m.w.	molecular weight
NOR	nucleolus organizer
PB	phosphate buffer
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
RNase	ribonuclease
rpm	rotation per minute

SA	satellite association
SDS	sodium dodecyl sulphate
SSC	sodium saline citrate (0.15M NaCl, 0.015M trisodium citrate)
TCA	trichloroacetic acid
Tris	2-amino-2(hydroxymethyl)-propane-1,3-diol