ISOLATION AND CHARACTERISATION OF DNA SEQUENCES FROM THE HUMAN Y CHROMOSOME

Lina Florentin ©

Thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow, Faculty of Medicine

Department of Medical Genetics
Duncan Guthrie Institute
Yorkhill
Glasgow

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DECLARATION

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

Lina Florentin

To my parents,

Sam and Renée

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Abbreviations used

bp	base pairs
CSDWD	distilled, chelated, deionised, sterile water
cDNA	complementary or copy DNA (made from RNA)
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
FACS	fluorescence activated cell sorted
g	gram
kb	kilobase
l	litre
m	milli-
μ	micro-
n	nano-
O.D.	optical density
o/n	overnight
pfu	plaque forming units
poly(A)	poly riboadenylic acid
RNase	ribonuclease
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
Tris	tris (hydroxymethyl) aminomethane

Summary

3,000 clones were isolated from a Y-specific library, 103 of which were shown to hybridise with placental cDNA. Ten clones were chosen to be characterised, but only six recombinants were further isolated, characterised and mapped on the Y, the X and autosomes. Mapping was accomplished by (i) constructing a panel of man-mouse somatic cell hybrids bearing different parts of the X chromosomes; (ii) a panel of genomic DNAs from individuals bearing abnormalities of the Y chromosome (Y deletion panel) and by using material from flow sorted chromosomes and to a smaller extent a panel of somatic cell hybrids carrying different groups of autosomes.

Recombinants GMGY1, GMGY2 and GMGY11 detect homology between the long arm of the Y and autosomes. GMGY1 and GMGY2 were mapped to the region Yq11.23 to Yq11.12. GMGY11 was mapped to the region Ycen to Yq11.2. They were all mapped to certain autosomes with the use of somatic cell hybrids and DNA from flow sorted chromosomes. GMGY11 was shown to detect an autosomal polymorphism.

Recombinants GMGXY2 and GMGXY3 recognise homology with both the X and the Y chromosomes. GMGXY2 recognises homology also with a number of autosomes and was mapped to Ycen-Yp11.2 and to Xq13-Xq24. GMGXY3 is an X-Y specific probe which was mapped to Yq11.22 to Yq11.23 and to Xp22.3-Xpter. The X-specific fragments were mapped using a panel of somatic cell hybrids bearing different parts of the X chromosome and the Y-specific fragments were mapped using the Y deletion panel.

Clone Bi-4 was shown to contain a single-copy sequence of autosomal origin.

The degree of homology detected by GMGY1, GMGY2, GMGXY2 and GMGXY3 was examined by washing Southern blots at different levels of stringency.

GMGY1 was shown to be nonpolymorphic for ten restriction enzymes while GMGXY2 was nonpolymorphic for thirteen restriction enzymes.

Dosage studies were attempted for probes GMGY1 and GMGXY2 using panels of individuals with numerical abberations of the X and the Y chromosomes, but no conclusions could be drawn.

All recombinants hybridising to the Y chromosome were tested against a panel of eleven XX males, one true hermaphrodite and some of their relatives in order to detect any Y-specific fragments in the genome of these individuals. Probe GMGXY2 was shown to hybridise to five XX males. Southern analysis on DNA from flow sorted chromosomes from two XX males indicate that at least for these individuals the Y-specific fragment is carried by the fraction containing the X chromosome. The same probe was also shown to hybridise to three 46, XY females.

For the purposes of this study, several panels mentioned above were constructed, the majority of effort and time focussed on the following:

The first, a panel of twelve somatic cell hybrids bearing different parts of the human X chromosome, was characterised both with cytogenetic and molecular techniques. Fifteen well known X-specific probes were used in order to test this panel and by this means their location on the X chromosome was further confirmed. Nine hybrids were shown to contain a contiguous part of the X chromosome while three more hybrids were shown to contain noncontiguous parts of the X chromosomes and need to be further characterised in order to be used succesfully. The panel was then used to map the X-linked fragments detected by GMGXY2 and GMGXY3.

The application of molecular analysis of these hybrids helped to define the breakpoints of a translocation product involving the X chromosome retained in a hybrid, and

confirmed the presence of the part of the X chromosome in one known hybrid. They also indicated that deletion has occured involving only one locus in two separate hybrids and showed that one hybrid consisted of a mixed population of cells carrying two different polymorphic X chromosomes. These results emphasise the resolution capacity of molecular analysis and bring to the light the advantages and disadvantages of constructing and using a hybrid panel.

The second, a Y deletion panel was also constructed from individuals or somatic cell hybrids that have Y chromosome abnormalities and proved to be very useful in the mapping of the Y-specific fragments detected by the sequences isolated here. A panel of hybrids bearing different groups of autosomes was selected and used to map the autosomal fragments detected by some of the recombinants isolated in this study and to combine and compare the results obtained from mapping the same fragments with the use of flow sorted chromosomes.

The results presented here demonstrate the importance of such panels in the mapping of single-copy sequences isolated from chromosome-specific libraries.

The results are viewed in the light of all available data. Subjects for discussion are the molecular map of the Y chromosome, with special emphasis on its homology with the X chromosome and autosomes and subsequently its evolution. The presence of Y-specific fragments in the genome of XX males is discussed. The findings are consistent with the recently published data and build upon the new ideas about the Y chromosome and its homology with the X chromosome and further with the autosomes. These data could give an insight into the evolutionary steps that this chromosome has undergone and opens up new fields for research. Future work is fully discussed in this thesis.

In conclusion these results are believed to give a small but positive contribution to our knowledge of the Y chromosome and its relation to other chromosomes, to enrich our understanding of the evolutionary steps that the genome has undergone and to bring us a step closer to the full mapping of this chromosome (and ultimately the whole genome) and the isolation of the elusive testis determining factor(s). In this way one of the most important biological riddles, the mechanism(s) of primary sex determination, maybe a little closer to solution.

I N T R O D U C T I O N

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In the introductory chapter of this thesis the main steps of sex differentiation undergone by the human embryo are presented in order to introduce the reader to the subject of sex determination and differentiation and the role that the Y chromosome plays in it. The gene loci which are thought to be mapped on the Y chromosome are presented, followed by a discussion on the H-Y antigen which was considered until recently to be the testis-determining factor. In the subsequent four sections the X chromosome, X-inactivation and the two loci STS and Xg that are thought to escape inactivation are discussed. Against this background it is possible to discuss the subject of X-Y homology starting with information on the evolution of the sex chromosomes which is thought to establish the basis for this homology between the two chromosomes. Different mechanisms of sex determination amongst various species are presented. The Bkm sequences are discussed in this context and sex reversal in mice is studied in relation to Bkm sequences and X-Y interchange. X-Y homology revealed by different methods and the hypothesis of X-Y interchange are presented next. Several abnormalities of sex differentiation are listed accompanied by a discussion of the aetiology of XX males and XX true hermaphrodites. In the final part of the introduction the molecular structure of the Y chromosome is illustrated, as it has been revealed with the use of immunological and recombinant DNA techniques. The evolution of these chromosomes and their homology is further discussed in the light of this recent data. The information derived from data obtained by the use of recombinant DNA technology on the subject of XX males is presented.

1.1. General Initiation and Principals of Sex Differentiation

Sex determination and differentiation involves a series of sequential and orderly processes: establishment of genetic sex at fertilisation, translation of genetic sex into gonadal sex, and of gonadal sex into body sex (Jost 1970).

Though the sex of the human embryo is determined at the time of conception, sexual dimorphism does not appear before the seventh week. The indifferent gonad, which appears during the fifth week, has neither male nor female features and consists of surface epithelium (cortex) and undifferentiated central mesenchyme (medulla). At this stage, numerous primordial germ cells, from which the sperm or ova will arise, have migrated from the yolk sac to the indifferent gonad, and are found embedded in the epithelium. The structure thus formed is called the genital ridge. The primitive sex cords then appear in the mesenchyme of the indifferent gonad; in males these become the seminiferous tubules and in females they regress. Thus both ovarian and testicular development originate from the genital ridge.

Transformation of the indifferent gonad into the embryonic testis occurs at about the seventh week of gestation and is characterised by:

1) The embryonic testicular stage where the primary signal is said to be a masculinising determinant carried on the short arm of the Y chromosome.

2) The fetal testicular stage where embryonic testis become fetal with the appearance of fetal Leydig cells which are initially observed around the eighth week (Niemi **et al** 1967) and produce androstenedione and testosterone (ref. in Simpson 1976). Androgens are responsible for the

transformation of the indifferent external genitalia into male external genitalia and for the growth of the internal ducts which are derived from the mesonephric structures (Wolffian ducts). These are the vasa efferentia epididymis, vasa deferentia, seminal vesicles, ejaculatory ducts and the prostate.

The prenatal development of the testes influences the regression of the paramesonephric ducts (Müllerian ducts) by a locally diffusing hormone factor (inhibitor) which is secreted by the Sertoli cells, around the tenth week (Tran **et al** 1977). The Sertoli cells surround the germ cells assuming the contour of the developing tubules (Jost 1970). Additional factors may be required for completion of normal testicular differentiation and for completion of ductal and genital development. Paramesonephric degeneration and genital masculinisation occur during the third month. However within a few months after birth, the fetal Leydig cells disappear and the fetal testis becomes a resting prepubertal testis, devoid of obvious endocrine or reproductive function. During puberty however, the testis undergoes dramatic changes, spermatogenesis begins and hormone function is resumed.

In summary, testes secrete androgens (probably testosterone) that causes the Wolffian duct differentiation and genital virilisation and also a locally diffusing factor that causes Müllerian regression. Additional factors might be required for completion of normal testicular differentiation and the completion of ductal and genital development.

Ovarian development is characterised by:

1) The embryonic ovarian stage, when the primordial germ cells become incorporated into the epithelial cords and are then termed oogonia. These epithelial cords are later fragmented into small clusters, each composed of a spherical arrangement of cord cells surrounding each

individual oogonium. These clusters are called primordial follicles. Unlike testicular primordial germ cells, ovarian germ cells do not change their appearance during early ovarian differentiation. There is also no evidence that the embryonic ovary has any endocrine activity.

2) The formation of the early fetal ovary. At this stage the oocytes are in meiotic prophase which occurs only in the prenatal period. During this stage the oocytes enlarge. After completion of prophase, meiosis is interrupted and is completed when ovulation takes place 12-40 years later. Metaphase, anaphase and telophase of meiotic division I occur during preovulatory maturation of the oocyte and meiotic division II is completed after fertilisation.

3) The late fetal ovary is characterised by the presence of the primary follicles mentioned previously. The germinal cell population is at its maximum at 18-22 weeks with about 7×10^6 oogonia and oocytes. After birth the primary number of follicles decreases and at birth there are 2×10^6 germ cells half of which show signs of degeneration (Baker 1963). By the 7th year only 300,000 oocytes persist and of these only about 400 develop into oocytes that will ovulate during a woman's reproductive life (Block 1952, Block 1953, Baker 1963). The transformation from early to late fetal ovary is apparently controlled by genes. In 45,X foetuses, oocytes degenerate shortly after formation of the primary follicles, apparently because the follicular layer is incomplete (J.E. Jirasek unpublished data, ref. in Simpson 1976).

The final stage is the perinatal ovary, which is characterised by secondary and tertiary follicles but apart from those that ovulate, all the rest of the follicles become atretic. In the absence of the testis (as mentioned before) the paramesonephric structures (Müllerian ducts) develop into the Fallopian tubes and uterus and

contribute to the vagina, since they do not depend upon androgen production (Simpson 1976). Without androgens the mesonephric structures remain rudimentary.
(Note: The above information is taken mostly from the books of Simpson 1976 and Wachtel 1983).

1.2. The Y Chromosome

1.2.1. Theories on Human Sex Determination

It has long been accepted that the presence of a Y chromosome is necessary for human male differentiation. The main evidence was presented in 1959 when a 45,XO individual was found to be female (Ford **et al**), while a 47,XXY individual was found to be male (Jacobs and Strong). Since then numerous theories have been proposed to explain the role of the Y chromosome in sex determination. A summary of the main points of these theories is presented below. Further details can be obtained by consulting the review by Bühler (1980).

Ford (1970) offered two alternatives , either that the Y is the sole factor responsible for the initiation of male differentiation or that there are loci on other chromosomes which are repressed in the female and derepressed in the presence of the Y in the male.

Hamerton (1971) proposed the existence of a controlling centre on the Y which activates genes or their operators on the euchromatic X. They later in turn stimulate the medulla of the indifferent gonadal ridge to become testis while at the same time suppressing the development of the cortex so that the ovary cannot develop. According to the same author, autosomal modifying genes can influence the medullary stimulating genes in the same way as the controlling centre on the Y, thus male

differentiation could occur without the presence of the Y.

In Boczkowski's theory (1971), a testis differentiation factor (TDF) and a testis inhibition factor (TIF) are present on the X chromosome. The presence of the Y in the male causes the TIF to be repressed and testis differentiation occurs.

Mittwoch (1973) proposed that the Y only promotes the faster growth of the originally bipotent gonad to develop into testis while the primitive gonad grows slowly in the absence of the Y chromosome to develop into the ovary.

Ohno in 1976 proposed the existence of two regulatory genes necessary for sex determination in mammals, one for determination of the gonadal sex, not necessarily situated on the Y chromosome and another one for differentiation of phenotypic sex located on either the X or an autosome.

1.2.2. Loci on the Y Chromosome

i. Testis Determining Factor(s) and the Fertility Factor

Karyotype-phenotype correlations have been up to now the only way to map loci on the Y chromosome. The reviews of Bühler (1980), Davis (1981), and Fitch (1985) have been the main sources of information for the points presented below.

Cases of patients missing the fluorescent material, (ring Y chromosomes, Y;autosome translocations, and Y deletions), but still exhibiting all the male features and who are fertile, provide evidence that the TDF (testis determining factor(s)) are not on the distal part of the long arm of the Y chromosome. In addition, normal female development takes place when the brightly fluorescent portion of the Yq is present in otherwise normal female karyotypes such as Y;autosome or Y;X translocations (ref.

in Davis 1981).

Cases with ring Y chromosomes or carrying a minute Y chromosome of debatable material, provide evidence that the TDF are near the centromere on either the short or the long arm of the Y (German **et al** 1973, and ref. in Davis 1981).

Cases of patients who are not mosaic, carry an isoYq and are females, (Jacobs and Ross 1966 and ref. in Davis 1981) or who carry an isoYp and are males, suggest that the TDF are on the short arm of the Y (ref. in Davis 1981).

Cases without the distal short arm of the Y (dicYq, Davis 1981) which show development of the testes suggest that the TDF are not on the distal portion of the short arm of the Y.

However, two cases of Yq deletions in females with no evidence of masculinisation have been reported, which might suggest that the TDF are on Yq (Nuzzo **et al** 1967, Fraccaro **et al** 1966). In such cases mosaicism for XO cells can never be excluded and as these cases were reported before the development of banding techniques, the karyotype analysis is questionable.

Other cases of translocations involving the long arm of the Y associated with masculinisation also indicate that the testis determining factor(s) might be on the long arm of the Y chromosome. Ferguson-Smith (1969) argued that the TDF might be located on both the short and the long arm of the Y near the centromere. This was based on a case of i(Yq) with an XO cell line which showed a slight degree of masculinisation of external and internal sexual organs.

Evidence for a fertility factor (a factor involved in spermiogenesis) on the distal portion of the non-fluorescent segment of Yq is based on the finding of azoospermia in normal males with testes and deletion of the Yq in which the fluorescent and distal portion of the non-fluorescent segment have been lost (Ferguson-Smith 1965, Tiepolo and Zuffardi 1976).

From the above it is clear that the vital role of the Yp, especially the pericentromeric region, in testicular development is unquestionable. There seems, however, to be some argument concerning results from certain human cell lines, especially about the location of the 'initiation' and 'maturation' testis loci (Bühler 1980, Davis 1981).

Ohno (1979) and Wachtel (1977) favoured the existence of multiple copies of the H-Y structural gene (see next section) on the pericentromeric region of the Y chromosome. Ohno suggested in 1967 that there might be several kinds of male-determining factors on the mammalian Y but that each of these exist in multiple copies along the entire length of the Y. From all this controversy the explanation is that a factor or factors are present on the Yq in some subjects but not in others. It has been suggested that testis determinants on Yp could also be transferred to the Yq in an unknown percentage of subjects by means of a pericentric inversion (Koo 1977).

Relevant to the above, are observations on the H-Y antigen whose role in sex determination and the location of the gene(s) which code for it have been a recurring matter of dispute.

ii. The H-Y Antigen

The term H-Y antigen refers to a transplantion antigen that stimulates the rejection of male skin grafts by female mice of the same inbred strain. More precisely this rejection of male to female skin grafts is attributed to an antigenic cell surface component

thought to be determined by a gene or group of genes associated with the Y chromosome. The gene is called the H-Y (histocompatibility-Y) gene and the male specific cell surface component is called the H-Y antigen. The first

papers published on this subject were by Eichwald, Silmser and Wheeler (1957), Billingham and Silvers (1958), and Billingham and Silvers (1960).

In 1971 Goldberg and her colleagues discovered that male-grafted, female mice produce sera containing antibodies (H-Y antibodies) that are cytotoxic for sperm and male epidermal cells (Scheid **et al** 1972). Specificity for the reaction was demonstrated by the serological absorption technique developed in their laboratory. These absorption tests showed that the H-Y antigen is not restricted to sperm and male epidermal cells, but probably occurs in all male tissues. It was detected serologically in blood, brain, bone marrow, lymph nodes, liver, skin, spleen, testis, and thymus (Wachtel 1979).

Studies on the expression of the H-Y antigen in vertebrates revealed that the H-Y is not in fact specific for the male but for the heterogametic sex. In birds and certain species of amphibians, the female heterogamety of the ZZ/ZW type prevails and in these animals it is the female whose cells express the H-Y antigen. These studies revealed that this molecule is highly conserved in vertebrate evolution (Wachtel 1983). This widespread evolutionary conservation of a particular cell surface component indicated the invariant persistence of a specific function, and the hypothesis was that this specific function may be to direct the indifferent embryonic gonad to develop towards whichever mature gonad, testis or ovary, typifies the heterogametic sex of each species (testis in XY males, and ovary in ZW females) (Wachtel 1975). It was also suggested that the H-Y antigen was not testosterone-dependent (Bennet **et al** 1977).

The next step was then to locate this <u>H-Y</u> gene on the Y chromosome. It has been claimed as most likely to be on the short arm of the Y chromosome (Koo 1977, Faggiano 1980), where all the male determining gene(s) have been

assigned (as shown above), perhaps nearest to the centromere (Koo 1977). In 1975 Wachtel suggested that either the testis-determining gene and the H-Y locus are extremely closely linked or that the two are in fact one and the same.

Since the H-Y was thought to be the mediator of testicular differentiation, cases of intersexuality amongst mammals, including humans whose gonadal sex did not correspond with chromosomal or phenotypic sex were studied.

Wachtel in 1978 detected levels of H-Y antigen in the male and in two intersex goats (Saanen goat breed) but the results were inconclusive. Ghosh **et al**, also in 1978, found three cases of 46,XY pure gonadal dysgenesis to be H-Y negative, and three patients with Klinefelter's syndrome, two true hermaphrodites, and two individuals with familial male hermaphroditism to be H-Y positive. Controls for normal males and females were satisfactory, therefore supporting the findings. Fraccaro **et al** in 1979 found H-Y to be positive for two XX true hermaphrodite individuals who originated from the same family. Wolf **et al** in 1980 examined Turner's syndrome patients and found them to be H-Y positive with variations. They all proposed different models in order to explain the function of the H-Y antigen, and discussed the possibility of it being a regulator or being regulated by a gene or a group of genes situated on the X, or an autosome, or both. Wachtel in a review (1980) seemed to favour Ohno's proposal (1979), of multiple copies of the H-Y stuctural genes on the pericentromeric region of the Y chromosome.

Reports, however mentioned in the reviews of Silvers **et al** (1982), also Muller (1982), indicate that the male specific antigen(s) defined by graft rejection or by killer cell action are not identical to serologically defined H-Y antigen(s)*. Therefore Silvers **et al** (1982) recommended that the term H-Y antigen would be reserved for the male-

* Furthermore, different methodologies employed for the above H-Y antigen studies may account for some of the conflict in their findings.

specific transplantation antigen and accordingly the target of the serological assay would be referred to as the serologically detectable male (SDM) antigen. Evidence that these factors are different molecules was first provided by Melvold **et al** (1977). They identified a mutant male mouse that lacked the H-Y transplantation antigen but possessed the SDM antigen. A second dinstinction between the two antigens was demonstrated in the case of the XO mouse. Celada and Welshons (1963) showed that spleen cells from XO mice were unable to sensitise female recipients against cells from male donors of the same strain, indicating that XO mice lack the H-Y transplantation antigen. However, XO mice have been shown to be positive for the SDM antigen (Engel **et al** 1981). Patients with Turner's syndrome have also been shown to be SDM antigen positive (Wolf **et al** 1980). In 1981 Koo and Varano showed that a monoclonal antibody to the SDM antigen blocked the cytotoxic T-cell response to H-Y **in vitro**. However, the immune-response genes controlling the response to the H-Y transplantation antigen were different from those that control the cell-mediated cytotoxicity response (Hurme **et al** 1978). These results suggested that the H-Y transplantation antigen may be distinct from whatever is detected in both the serological and the **in vitro** assays.

Silvers **et al** (1982) reviewing the recent literature on H-Y antigen and SDM, concluded that since they are frequently associated with the heterosex, it is quite possible that they are in some way involved in sexual differentiation and as Muller (1982) proposed in his review, H-Y might be one important testicular differentiation factor but not necessarily the only primary inducer of the testis. From the available data, they concluded that neither the H-Y transplantation antigen, nor the SDM antigen is the primary cause of gonadal differentiation.

Finally in 1984 McLaren **et al** reported that H-Y was absent from exceptional, XX, sex-reversed, male mice that develop testes and are of indisputable male phenotype, because they carry the Sxr locus on the X chromosome (see section 1.5.). This finding has been regarded as very important because it clearly shows that the locus for the transplantation antigen was unlikely to be responsible for testis determination as it could be seperated from the testis determining factor. Burgoyne **et al** (1986) recently showed that these H-Y negative male mice, in losing the genetic information that encodes H-Y, have also lost the genetic information required for spermatogenesis. They proposed that a gene distinct from the testis-determining gene could be present on the mouse Y which would be necessary for spermatogenesis. The possibility that the product of this 'spermatogenesis gene' is the H-Y antigen was raised.

These two experiments however, will be further discussed in the section (1.5.) which deals with the condition of sex reversed mice (XX,Sxr male mice).

iii. Other Loci on the Y Chromosome

Other loci thought to be on the Y chromosome were also apparently localised by information obtained from karyotype-phenotype correlations:

1) Somatic development

Skeletal maturation seems to be influenced by a gene on the Y chromosome rather than by both X and Y chromosomal genes (Tanner **et al** 1959). The evidence comes from the fact that XXY individuals have the same skeletal age as boys, throughout the age range 6-18, while XO individuals have approximately the same skeletal age as the

girls. They concluded that the relative retardation of growth and maturation of boys versus girls of the same age is caused by genes on the Y chromosome. This is supported by the fact that the retardation starts in the 7th week of embryonic life (beginning of testicular development) and ends in puberty. Skeletal age retarding factors seem to be situated on Yq.

2) Body growth

This seems to be influenced by autosomal, X and Y chromosomal genes (Lindsten **et al** 1974 and Ryman **et al** 1975). Evidence comes from examining individuals with numerical and structural abberations of the X and the Y chromosome, such as XO gonadal dysgenesis (small stature), XXY Klinefelter's syndrome and XYY males all of which are on the average, taller then normal males (Tanner **et al** 1959, Nielsen 1974, De la Chapelle 1972). Growth promoting factors are thought to be located on both the long and the short arm of the Y chromosome.

3) Dental development

Lindsten **et al** (1974) concluded from their studies on Turner's syndrome that X as well as Y chromosomal factors influence tooth development. According to Barlow (1973) the quantity of gonosomal heterochromatin might influence dental development, while Alvesado (1975) talked of the direct influence of the Y chromosome in tooth size and of a regulatory role in the stimulation of growth (studies on 47,XYY males). It seems that the Y chromosomal genes influencing tooth size are not identical to the testis determining or the H-Y genes and are definately not included in the exchange or translocation theory of the origin of the XX male syndrome (Alvesado and de la Chapelle 1979).

4) Dermal Ridge Counts

Evidence suggests that the finger-tip pattern size, as measured by the total ridge-count, is an autosomal trait which is independently influenced by the sex-chromosome complement, normal or abnormal. In males, the presence of each X chromosome diminishes the total number of ridges in the patterns nearly three times more than the presence of each Y-chromosome. In females, the reduction of ridges, as the X-chromosome complement increases, is less regular (Penrose 1967).

Finally, the Y chromosome shows a normal distribution of heterochromatin in the long arm.

1.3. The X Chromosome

1.3.1. Some Loci on the X Chromosome

As with the Y chromosome, karyotype-phenotype correlations provided the first information on the location of genes on the X chromosome. A deletion involving most of the short arm of the X leads to a phenotype which fulfills the criteria for Turner's syndrome (multiple stigmata of Turner's syndrome), while deletion of most of the long arm of the X leads to a phenotype with streak gonads and no Turner's stigmata, a condition described as 'pure gonadal dysgenesis' (Ferguson-Smith **et al** 1964).

The same author suggested in 1965(a) that two X chromosomes are necessary for normal development of oogonia, as germ cells do not have inactive X chromosomes. Sarto **et al** (1973) proposed that the long arm of the X might contain a region that must be intact on the active X if ovarian development is to be normal. Later, Summit **et al** (1978) defined the region more precisely as from Xq13 to Xq27. Madan in 1983 confirmed the existence of this

critical region but her results also showed that there is a possibility of this segment consisting of two critical regions separated by a small segment in band Xq22. The existence however, of fertile females with deletion of a part or the whole of this critical segment suggests, according to Madan, that it is not the break in the critical segment, as was first thought, but rather the contact between loci in the critical segment and loci external to it that is responsible for the abnormal sexual phenotype. Skibsted **et al** (1984) supported the idea that when band Xq27(28) is intact, normal ovarian development may be expected. It has been also suggested that accessory factors necessary for the maintenance of oocytes might be situated on the short arm of the X between Xp21 and the centromere (Hodgson **et al** 1981).

1.3.2. X-inactivation

The hypothesis of X-inactivation (Lyon 1961) states that, in each cell of the early female mammalian embryo, one of the two X chromosomes becomes inactivated, the choice of which X is to be inactivated in any one cell is at random. For all subsequent descendents of that cell the same X is inactivated. The adult female is thus a mosaic for clones of cells each with the paternal or maternal X inactivated. Lyon based her hypothesis on the results she obtained from breeding mice with suitable mutants and this theory was later extended to all mammals including man.

The cycle undergone by the X chromosomes (Figure 1) is as follows: at conception both X chromosomes of a female embryo are active but at an early stage in development (blastocyst) one X becomes inactive and forms the sex-chromatin body. When this inactivation first occurs it takes place in the trophectoderm and in a non-random way

with the paternal X being preferentially inactivated (Takagi **et al** 1975, Ropers **et al** 1978). Random X inactivation, paternal or maternal, takes place later in the cells of the inner cell mass that will form the adult somatic cells of the female. The choice once made remains fixed in all descendants of a cell, so that an adult female is a mosaic of patches of cells with one or the other X active. X chromosomes in the germ line are inactivated in early embryogenesis, and then go through a reactivation step about the time of entry into meiosis (Gartler **et al** 1975, Kratzer and Chapman 1981). Ohno and Makino first showed in 1961 that both Xs are active in oogonia and oocytes in both rats and humans.

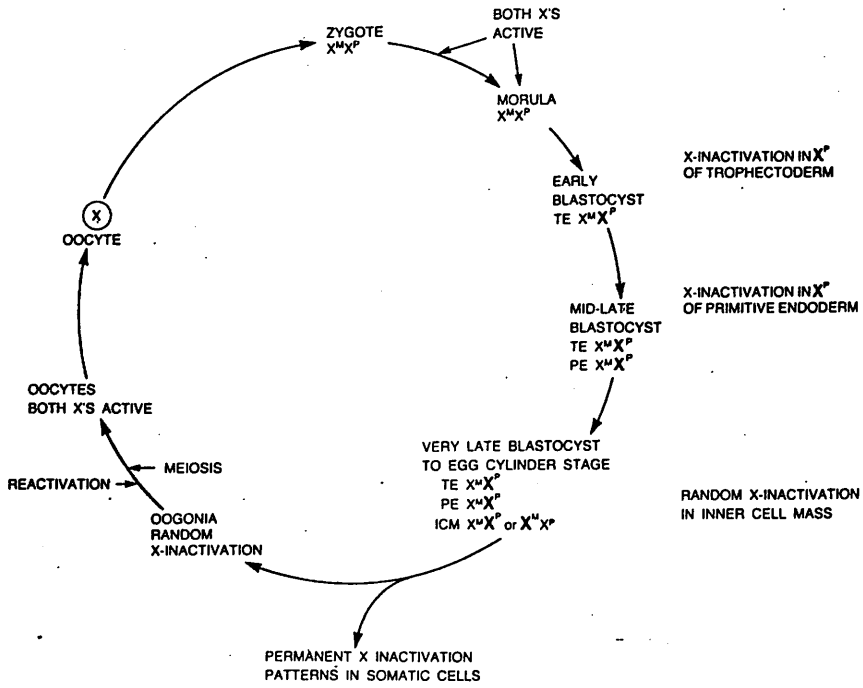


FIGURE 1: The X chromosome inactivation and reactivation cycle. X^P and X^M represent the paternal and maternal chromosomes respectively. The inactive state of a chromosome is indicated by bold print. TE is trophectoderm; PE is primitive endoderm; ICM is inner cell mass. (Information and diagram from Gartler and Riggs (1983).

In cases with multiple X chromosomes it was observed that the Barr bodies (Barr and Bertram 1949) always numbered one less than the number of multiple Xs present which clearly showed that no matter how many Xs there are in a cell, only one is finally active. At the eight cell embryo, prior to X-inactivation, there is no dosage compensation as mentioned above (Ferguson-Smith **et al** 1960). The absence of dosage compensation at these early developmental stages was not expected, because its presence would seem to be necessary to avoid an aneuploidy effect. It is likely as Gartler and Riggs (1983) suggest that this aneuploidy effect is avoided by the limited number of genes active at these early stages and by the brief period in which a dosage effect is present. Interestingly enough, at the onset of meiosis in the male, the single X chromosome undergoes a precocious condensation and inactivation, just the opposite of what occurs in meiosis in the female (Monesi 1971, Lifschytz **et al** 1974).

X-inactivation happens in a random way but after it has occurred, selection will promote the line in which the cells divide faster and/or are more viable. Usually but not invariably, the more successful line is the one that is better genetically balanced. In persons with an abnormal X chromosome, selection results in this chromosome being inactivated. In carriers of balanced reciprocal X-autosomal translocations, the normal X is usually inactivated also through selection. It seems that inactivation has a tendency to spread from the X to an attached autosomal segment and if the translocated X chromosomes were inactivated, a series of autosomal genes would become non-transcribing in these cells. On the other hand, in women who have an unbalanced X-autosomal chromosome translocation, the X derivative chromosome is usually inactivated, although the proportion inactivated may vary between individuals and between cells (Therman 1980).

Cattanach and Issacson in 1965 performed an experiment in order to test whether the choice of which X becomes genetically inactivated was under genetic control and whether autosomal material attached to the X becomes completely inactivated when that X is inactivated. The test material was the female mouse heterozygous for Cattanach's X-autosome translocation. These animals possess, in addition to the normal autosomal complement, a piece of autosome inserted into one X. This duplication (of part of linkage group I- shown to be part of chromosome 6) carries the wild-type alleles at two coat-colour loci, pink-eye (p) and chinchilla (c^{ch}). The results indicated that the X-inactivation is a random process and that autosomal material attached to the X is usually, but not always, inactivated and the probability of inactivation of each autosomal locus is dependent upon the proximity of the locus to the breakpoint. They proposed that this 'spread' could be controlled by a gene or genes located on the X chromosome. Such a gene would have the properties of the postulated 'inactivation centre' and could be the site from which the inactivation process would 'spread' along the X chromosome. Evidence for the spreading of inactivation into the autosomal segment in an inactive, human , X-autosome translocation comes also from Mohandas **et al** (1982). Mouse-human cell hybrid clones retaining an inactive translocated chromosome involving the X chromosome and chromosome 13 were isolated and esterase D_1, a marker on the segment of chromosome 13 translocated to the X was shown not to be expressed in these clones.

Many workers have proposed that there are multiple control centres of X-inactivation. However Eicher (1970) provided the first evidence using Cattanach's translocation. Both cytological and gene expression data showed that both X chromosome segments on either side of the autosomal insert can be inactivated, but at least one autosomal gene was not. Eicher and her colleagues and

others interpreted this data as indicating the existence of at least two inactivation centres. Much controversy seems to exist concerning the number and the location of this inactivation centre. More information can be obtained from the review of Gartler and Riggs (1983). The alternative view supporting a single X-inactivation control centre, however, started to emerge from observations on cases of human X-autosome translocations and other abnormal X chromosomes.

Therman and Patau (1974) reviewed such cases, the origin of these chromosomes, phenotypic effects and especially their inactivation. They proposed that the inactivation centre was located at the proximal part of the Xq arm, since abnormal chromosomes with two such centres tend to form bipartite Barr bodies and since they observed no evidence for the occurence of i(Xp) chromosomes in the literature. The clinical description from some such patients with this apparent karyotype clearly contradicted their having the i(Xp) chromosome and thus being monosomic for the whole Xq. Patients with the claimed i(Xp) chromosomes displayed fewer symptoms, usually only primary amenorrhea, than those having an Xq- chromosome (which resembles chromosome 18 in its morphology). It was thought therefore to be highly unlikely that the lack of the whole Xq would lead to a more normal phenotype than its partial deletion. It is now thought that the apparent i(Xq) cases are usually partial deletions of Xq. In 1979 Therman **et al** by examining three women with 46,XXq- in which the breakpoints were slightly different and by comparing these with relevant X-autosomal translocations, proposed that the centre of X-inactivation is localised near, but not at the border of the Q-band next to the centromere on the long arm.

Schempp **et al** (1983) identified three human X-chromosomal segments which replicated earlier than the rest of the X chromosome. The third, early replicating

segment was located on the long arm of the X at Xq13.1 and could, in the light of its position, be the location of the hypothetical X-inactivation centre.

Lyon in 1974 gave a more precise term of inactivation, as a lack of genetic transcription due to unresponsiveness. The gene or chromosome fails to respond to those agents which normally evoke transcription, whether those agents are extrinsic to the cell, such as hormones, or intrinsic, such as inducers or repressors produced by other genes.

Until now, scientists have been trying to explain X-inactivation at the molecular level, but experimental evidence in support of this theory is lacking. Mohandas **et al** (1981) present results which provide evidence that DNA methylation plays a role in inactivation of the X chromosome and that inactivation occurs on the human X in a segmental fashion. It is well established that DNA-protein interactions are altered when DNA is methylated (Razin and Riggs 1980). Such changes could account for the inactivation of the X-linked genes, and heterochromatinisation of the X chromosome. Wolf and Migeon (1982) however, compared the methylation patterns of DNA from cells with active and active and inactive X chromosomes by Southern blot analysis using cloned, X chromosome-specific probes and found no evidence to support any relationship between X-chromosome inactivity and degree or pattern of methylation.

It has been postulated for some time that a part of the X chromosome including the X-Y pairing segment (see section 1.6.) escapes inactivation, and from observations that have been made this seems to be at the tip of the short arm. Evidence came first from karyotype-phenotype correlations (Ferguson-Smith 1964, Ferguson-Smith 1965b). Phenotypic differences were observed between normal females and individuals with XO chromosome constitution or

deletions of the short arm of one of their Xs. Similarly it was observed that physical and mental disability of patients with Klinefelter's syndrome increased with the number of Xs. The fact that there are important phenotypic differences suggested that this is because of an abnormal dosage effect, either because both Xs are genetically active in the early embryo, or because inactivation does not involve part of the short arm of the X chromosome which is therefore monosomic or trisomic for loci in this region or because of both factors.

More recent evidence that the tip of the short arm of the X escapes inactivation came later from two loci, STS and Xg which have been mapped recently in this region, both close to the pairing segment.

i. STS Locus

Steroid sulphatase (STS), sterolsulphate sulphohydrolase, E.C. 3.1.6.2.) is a microsomal enzyme that catalyzes the hydrolysis of various 3,β-hydroxysteroid sulphates. It is present in many mammalian tissues and is found in particularly high concentrations in the placenta (Shapiro 1983). It has has been demonstrated to be present in liver, kidney, adrenals, testis, ovaries, fibroblasts, and lymphocytes but differing substrate specificities suggest that heterogeneity of the enzymes involved. Much controversy exists as to the number of steroid sulphatases and whether steroid sulphatase and the microsomal enzyme, arysulphatase C are one and the same thing. Arysulphatase C is one of a group of arylsulphatases (arylsulphate sulphohydrolases A, B and C), which catalyze the hydrolysis of a variety of arylsulphates.

Information on the arysulphatases and steroid sulphatase is taken from Dulaney and Moses (1978) and Shapiro (1983).

Deficiency of STS causes X-linked ichthyosis, an inborn error of metabolism inherited as an X-linked recessive trait. The disorder affects 1 in 6000 males and its major clinical consequence is ichthyosis. Corneal opacities may also be observed. The mechanism by which steroid sulphatase deficiency causes ichthyosis is not completely clear, but may be related to the accumulation of cholesterol sulphate in the skin. STS deficiency may be assayed in skin fibroblasts (Shapiro 1983). It has also been demonstrated in hair roots, which seem to give more consistent assay results, presumably reflecting the **in vivo** situation more accurately than cultured cells (Aitken **et al** 1981).

The genetic locus coding for STS is located on the short arm of the X chromosome in the region from Xp22 to Xpter (first assignment by Mohandas **et al** 1979). This was later confirmed (Muller **et al** 1980, Mohandas **et al** 1980) and more precisely located to the terminal band Xp22.3 (Tiepolo **et al** 1980). Independent studies on the pattern of inactivation of STS, performed by various groups concluded that the STS locus must escape inactivation (Tiepolo **et al** 1980, Madan **et al** 1981, Mohandas **et al** 1980). In fibroblasts, the observed ratio of STS activity of 1.7:1 between females and males approximates to expected value (Madan **et al** 1981).

Ropers **et al** in 1981 measured the activity of STS in fibroblasts with numerical and structural X chromosome aberrations and discussed the possibility of the STS gene being inactivated when carried on an abnormal X chromosome, a similar situation to that observed for the Xg locus, which is subjected to X-inactivation when carried on an abnormal X while remaining active on a structurally normal X (Polani **et al** 1970, see next section). This could mean that the rules for inactivation of Xg also apply to STS. In support of the idea that the STS locus is inactivated when carried on an abnormal X chromosome was the case of a

mother and her daughter who both had an identical X/13 translocation chromosome which carried the STS locus. In the mother who had a balanced (reciprocal) translocation, the STS levels were in the female range. In the daughter however, who had an unbalanced karyotype the abnormal X chromosome was inactivated, the STS levels were low (Ropers **et al** 1981).

Migeon **et al** in 1982 drew attention to the fact that the ratio of mean values for normal females to that of normal males is greater than 1:1 but less than the ratio of 2:1 expected if the STS loci on all X chromosomes were equally expressed. They suggested that maybe the STS locus on the inactive X is not equally expressed and attempted to prove this by testing two heterozygotes for X-linked STS deficiency, who were also heterozygous for the common electrophoretic variants of glucose-6-phosphate dehydrogenase (G6PD A and B). Both females carried the STS mutant on the same chromosome that carried the G6PD B allele. G6PD B clones were shown to have less STS activity than G6PD A clones and since the X chromosome carrying the mutant STS did not produce any STS, it was clear that the normal STS allele produces less enzyme when it is on an inactive X, than on the active X chromosome. They suggested that this reduced expression of the STS locus on the inactive X chromosome may reflect the influence of neighbouring genes that are subject to inactivation. Or on the other hand, differential expression at the STS locus could be due to other mechanisms regulating the expression of genes on the X chromosome.

Ropers **et al** (1981) reported STS values for three supernumerary X-chromosome fibroblast cultures (47,XXX, 49,XXXYY and 49,XXXXY) to be high compared to females. They reported however, significantly elevated levels of STS activities in one strain with supernumerary X chromosomes but they considered the possibility that this effect might be due to ageing of the used cultures. Females with

terminal deletions of Xp including the STS locus were shown to have STS values within the male range. The authors felt however that due to the limitations of the test and the biological variability it was not possible to conclude for the existence or not of a linear correlation of STS levels to the number of X chromosomes present.

The data of Chance and Gartler in 1983 indicated a dosage effect at the STS locus when comparing 1X vs multiple X strains. However, the STS activity appeared to plateau at the 2X level, as seen by comparison of 2X, 3X and 4X strains. It seems possible therefore, that there may be a limit to the intracellular levels of STS, perhaps due to control by an autosomal locus or some limitation inherent in rapidly dividing cultures. Lykkesfeldt **et al** (1984) finally presented a study which demonstrates that the human X-locus which contains the gene for STS enzyme, to some extent escapes inactivation. The STS gene situated on an inactive, heteropycnotic, X chromosome expresses approximately 45% of the activity originating from STS loci on active X chromosomes.

ii. Xg Blood Group Locus

The Xg blood group is a red cell surface antigen the locus for which is X-linked. The antigen is called Xg^a, the antibody anti-Xg^a, the phenotypes Xg(a+) and Xg(a-), the allele responsible for the antigen, Xg^a and that for its so far silent allele, Xg. Xg(a+) is dominant to Xg(a-).

The first evidence that Xg was not inactivated, was obtained in 1963 (see for review Race and Sanger 1975). Their chimaera case had Xg+ and Xg- lines, whereas mosaicism could not be demonstrated in Xg(a+) heterozygotes. By 1971 a lot of data supported the concept that Xg escapes inactivation. Almost certainly direct evidence that the allele Xg^a when carried on an inactive

late-labelling X can nevertheless produce its antigen, came from Buckton **et al** 1971. A mother and daughter had a balanced translocation t(Xp-;14q+), the short arm of one X being translocated on the long arm of chromosome 14. In both these female members of the family who had the balanced translocation it was the normal X which was late-labelling. The mother was Xg(a-); the father was Xg(a+), and his X was late-labelling in his daughter, yet it had made her Xg(a+).

This absence of inactivation applies to erythropoietic tissue (Race and Sanger 1975). In 1974 Fellous **et al** * detected Xg^a on cultured fibroblasts, even up to at least the twelfth passage and also in man/man, man/mouse and man/hamster hybrid lines. The antigen could also be detected on some cultured lymphoid cell lines, though not on fresh, peripheral blood lymphocytes. The same workers tried to show that the Xg locus of fibroblasts, like that of erythroblasts, is not subject to inactivation. Eleven clones were isolated from the fibroblast culture of a donor known to be heterozygous Xg^aXg and all these clones gave the reaction Xg(a+). Subsequently, Polani **et al** (1970) studied two groups of women with structurally abnormal X chromosomes. In one group, the short arm of the X was missing. These patients had isochromosomes for the long arm of the X or other short arm deletions. The other group had long arm deletions or short arm isochromosomes; therefore the long arm was missing. From the exceptional inheritance of Xg in the families of these people and together with the preferential DNA late labelling pattern of the structurally abnormal X chromosome under discussion, it was concluded that the Xg is not subject to inactivation when carried on a normal X and is probably inactivated when present on a deleted X. From similar studies Sanger **et al** (1971) came to similar conclusions as Polani **et al** (1970).

In order to map the Xg gene on the X chromosome, linkage data with other X-linked loci was used. The first

* Subsequently these findings were retracted by the authors.

indication that there was a measurable distance between the Xg and the X-linked form of ichthyosis came at around 1964 (reviewed in Race and Sanger 1975) and studies on families afterwards established the first data, θ=0.11 (Adam **et al** 1969, Went **et al** 1969). Xg was shown to be linked to ocular albinism and retinoschisis.

Since the Xg probably escaped inactivation and was linked to X-linked ichthyosis it was suspected to be located on the short arm of the X chromosome alongside the STS locus. In 1981 however, Boyd **et al** presented a case of a woman with an X-Y translocation, 46X,t(X:Y)(Xqter-Xp22.3:Yq11-Yqter), who gave birth to a child carrying the translocation and suffering from ichthyosis. The mother failed to inherit the Xg locus along with her father's X chromosome, which places the Xg in the region Xp22.3-Xpter. Also, her STS activity was in the male range, suggesting that she does not have the STS locus on that chromosome. Thus, there is now substantial evidence that STS and Xg do not become inactivated.

The Xg has been of particular interest to the studies of XX males and their families as will be shown in more detail later in this chapter.

1.4. Mechanisms of Sex Determination amongst Various Species and the Evolution of the Sex Chromosomes

The mechanism of sexual reproduction appeared very early in evolution and it can be found today even in bacteria. In **Escherichia coli**, for example, fertilisation occurs by passage of the single chromosome from the male into the female bacterium (Lederberg 1959). Gradually the mechanism became more complicated and took the form of bisexuality. The more sophisticated state of bisexuality was initiated by setting aside a particular pair of chromosomes for specialisation and making either the male or the female the heterogametic sex. Sex chromosomes as we now know them then appeared. Among surviving members of vertebrates, however, the truly differentiated X and Y chromosomes occur only in mammals, while female heterogamety with well differentiated Z and W chromosomes operates in avian and ophidian species (Ohno 1967).

When Ohno (1967) wrote his monograph on the evolution of the sex chromosomes, there was no evidence for morphologically recognisable sex chromosomes in reptiles, amphibians, fishes and lower vertebrates in general, with the exception of snakes (see below). This fact did not mean that sex chromosomes did not exist in these species but only that they were morphologically identical. However with the availability of new techniques a different picture was revealed. In the following paragraphs, a few examples of sex determination mechanisms studied in animals belonging to different places in the zoological scale will be presented, together with a few important observations with regard to the differentiation of their sex chromosomes. This information aims to help to understand the steps that the X and Y have undergone during evolution, the behavior of the XY pair and its consequences for male differentiation. Moreover it also aims to point out the

difficulties faced by many scientists who are trying to find a common model in order to explain the evolution of the sex determination mechanism.

1.4.1. The mechanisms

Invertebrates

Sex determination by haplodiploidy has been studied in several insects such as the honeybee and coccids. The male honeybee comes from an unfertilised egg, is haploid, has only maternal chromosomes and can therefore transmit only maternal chromosomes. In coccids, sex determination is in large part if not exclusively under maternal control. An XX female/XO male sex determining mechanism can be found in the more primitive coccids. True male haploidy (males from unfertilised eggs) occurs in a group of relatively primitive coccids, the iceryines. In specialised coccids such as mealybugs and eriococcids, fertilisation takes place, but during early development of the male embryos the paternal set of chromosomes becomes heterochromatised and is later eliminated at spermatogenesis. Therefore it was concluded that the heterochromatinisation of the coccid chromosomes indicates the genetic inactivation of a chromosome and that such a system could serve as intermediate between regular diploidy and true male haploidy. In the most specialised group of coccids, the armoured scale insects, most species have lost the system of facultative heterochromatinisation and have substituted instead simple elimination of the paternal chromosomal set during early embryogenesis (refs. for the above paragraphs on coccidae and honeybee sex determination system, to be found in Brown and Chandra 1977).

Caenorhabditis elegans is a small free-living nematode, about 1mm long as an adult, which occurs

naturally in garden soil. The two natural sexes of **C. elegans** are the self-fertilising hermaphrodite (XX) and the male (XO). They are both diploid with five pairs of autosomes. Females do not occur naturally in C. elegans but most other nematode species (including relatives of **C. elegans** such as **C. remanei**), have an XX female/XO male sex determination system (Hodgkin 1985).

Long ago it was thought that the ratio X/A (ratio of X chromosomes (X) to autosomes (A)) played the primary role in sex determination of this nematode. This was concluded from experiments on **Drosophila**. In this fly 1X:2A produces males, 2X:2A produces females and the intermediate ratio 2X:3A produces intersexuals. A polygenic 'balance' theory of sex determination was proposed in which sex was determined as the result of a balance between 'feminising' genes on the X and 'masculinising' genes on autosomes (Bridges 1925). It is clear now that for both **Drosophila** and **C. elegans** the sites on the X chromosome provide only a quantitative signal, which sets the state of a small number of major sex determining genes. It is these key control genes that determine sexual phenotype.

So far, in **Caenorhabditis elegans** seven sex determining genes have been identified by means of mutations that cause a variety of sexual transformations. All seven genes are located on the autosomes (her-1, tra-2, tra-3, fem-1, fem-2, fem-3, tra-1). The seven genes form a cascade of four steps, with negative regulatory interactions at each step. According to a proposed model, only the first gene in the pathway, her-1, responds directly to the X/A ratio. However, much more research needs to be done before this cascade of gene interaction will be well understood (Hodgkin 1985).

The female **Drosophila** is XX and the male XY. The Y chromosome however carries few fertility genes but no sex determining functions. Females have twice as many X chromosomes as males, and dosage compensation has evolved

to regulate the activity of the X chromosome in such a way that the single X of males is transcribed at twice the rate of an X in females. The X/A ratio acts as a primary signal that intergrates all processes related to sex by setting the state of activity of the X-linked Sxl gene. This in turn leads to a specific pattern of activity of a few autosomal regulatory genes that finally dictate the sexual pathway. When Sxl is on it dictates female phenotype and a low rate of transcription of the X chromosome. When it is off it dictates the male pathway and a high rate of transcription. Another ten loci (da, her, tra, tra-2, dsx, ix, mle, msl-1, msl-2, msl-3) have so far been found to be involved in **Drosophila** sex determination and mutations in some of these loci result in the development of intersexuals or transformation from males to females and **vice versa** (Baker **et al** 1983 and Nothinger **et al** 1985).

Vertebrates

In fish, synchronous and asynchronous hermaphroditism exists. Functional hermaphroditism persisted in teleost fish which suggests that very few and labile sex determining factors existed in the genome of ancestral vertebrates. Much of the data indicates that sex determination in teleosts seems to be polyfactorial with epistatic sex determining genes located on 'sex chromosomes'. Heteromorphic sex chromosomes have been found in this group of fish though very rarely for example in the eels **Anguilla anguilla** and **Anguilla rostrata** (ref. in Becak 1983). In these cases heterochromatinisation did not occur or the heterochromatin was eliminated during the process of reduction of the W. Most teleost fish are based on a XY/XX system but a ZZ/ZW system has also been found. Sex inversion can be achieved upon the administration of either estrogen or androgen via the rearing water or the diet, but there is no generally valid scheme for hormonal treatment

(from Reinboth 1983, and Becak 1983).

In amphibians and reptiles, functional and nonfunctional hermaphroditism is an exception. In both the primitive and the highly evolved groups of the **Urodela** and the **Anura** both sex-determining types XY/XX and ZW/ZZ exist. However, while in teleosts sex reversal by hormones occurs in both directions, in amphibians it occurs mainly in the homogametic sex. In these groups it is evident that heterochromatinisation is the first step to differentiation, for example **Rana clamitans** (refs. from Becak 1983 and Schmid 1983).

In reptiles, temperature-determined sex is common and is known in three of the four living orders: turtles, snakes and lizards, and crocodilians (Harvey **et al** 1982) for example the crocodile **Alligator mississippiensis** (Ferguson **et al** 1982). It is interesting that male producing and female producing temperatures in lizards are the reverse of those found in turtles. Higher temperatures produce males in lizards and females in chelonians (Bull 1980).

Morphologically distinguishable sex chromosomes are found in only two orders, the turtles and the snakes and lizards, but it is uncertain whether genotypic sex determination originated once or more then once. In turtles **Siebenrockiella** and **Staurotypus** of the XX/XY type sex chromosome differentiation is due to a pericentric inversion. In these species heterochromatinisation occurs only on the X but not on the Y chromosome (from Becak 1983).

Snakes, however, exhibit the Z and the W in three different stages of differentiation (Becak **et al** 1964). In **Boidae** the W and the Z are homomorphic. In many members of the **Colubridae** family, the Z and the W differ by a pericentric inversion and in poisonous snakes which are the most highly evolved of all reptiles, some members of the **Crotalidae** and **Viperidae** family exhibit a minute W, similar

to that of avian species (Kobel 1962).

Finally, avians are all of the type ZZ/ZW with the two sex chromosomes being well differentiated. Estrogen may produce partial and transient sex reversal of the homogametic animal (Becak 1983).

It seems that sex determination is more rigorous the more advanced the animal is in the zoological scale. The order in which the examples of sex determination in different species is presented here, is similar but not exact to the evolutionary path. In this context, information on **C. elegans** was presented together with **Drosophila** because their mechanisms have many similarities.

1.4.2. Models

Many researchers have tried to find common features in the sex determining mechanisms of different animals in order to create a common model that would explain the evolution of sex determination. It was hoped that data from studies in other species would help to form a model for the sex determination mechanism in mammals and particularly in man. A satisfactory model should also be able to explain all the existing abnormalities of sex determination caused by mutations which transform sex or result in sex-specific lethality. These were described above for **Drosophila** and **C. elegans** and a number of them found in mammals are listed below: a) X-linked mutation Tfm (testicular feminisation) in mouse causes XY males to transform to phenotypic infertile females (Lyon and Hawkes 1970), b) a mutation consistent with an autosomal recessive form of inheritance in Saanen goats causes pseudohermaphroditism and male infertility (Basrur **et al** 1964, Soller **et al** 1969), c) X-linked mutation which transforms XY males to fertile females in the Scandinavian wood lemming, **Myopus schisticolor** (Fredga 1983), d) a dominant autosomal

mutation Tas which causes sex reversal in XY individuals (Washburn and Eicher 1983).

The special sex chromosome constitution of the wood lemming for example provides an excellent opportunity to investigate the interaction between genes involved in sex determination (Fredga 1983). The study of such mutations could provide clues as to the sex determination mechanism operating in man, explain conditions such as XX males that do not seem to result from X-Y interchange (see section 1.7. and 1.8.7.) and lead to the isolation of the main human male determinant which has not yet been found despite years of research.

Chandra in two of his papers (1984 and 1985) proposed such a model for mammalian, male determination, based on a passive Y chromosome (1984) after studying mutations described above. The model consists of the following components:

1) a postulated X-linked gene which was called Tdx (testis-determining gene, on the X chromosome), whose product is considered essential for male determination,

2) an autosomal gene that synthesises a limited quantity of a repressor capable of binding to the Tdx gene to prevent transcription,

3) a large number of sites on the Y chromosome which do not code for any protein but which bind the autosomal repressor with very high affinity, and

4) RNA polymerase

It is assumed that the autosomal repressor has higher affinity for the multiple sites on the Y chromosome than for Tdx and that the affinity of this repressor for Tdx is greater than that of RNA polymerase. As a result, in XY cells, the Y effectively binds all available repressor, leaving RNA polymerase to bind to Tdx and initiate transcription. In XX cells, the Y-linked, high-affinity sites are absent, consequently Tdx is blocked by the repressor. According to this model, the function of the Y

chromosome in the determination of the male gonad, is to buffer the cell against the repressor so that transcription of Tdx can occur. Mutations occuring within this system would result in conditions like the ones described above.

In his 1985 paper, Chandra attempts to explain some of the sex determination mechanisms described above by applying modifications in his proposed model and thereafter traces evolutionary relationships between the different systems. The argument still exists whether sex has a polyphyletic or monophyletic origin especially with such a variaty of karyotypes. Chandra believes that if there is a homology between sex-determining mechanisms among eukaryotes, it should be possible to derive one sex-chromosome system from another with very few mutational steps or to demonstrate a close relationship.

1.4.3. Sex Chromosome Differentiation

With regard to the evolutionary steps that the sex chromosomes took during the process of differentiation and specialisation, Ohno (1967) proposed the following, based partly on observations in the ophidian group (described above):

1) Differentiation from the initially homomorphic and largely homologous sex pair happened exclusively at the expense of the element which was elected to accumulate factors governing the development of the heterogametic sex. Conversely the other member of the pair which had been elected to accumulate the determining factors of the homogametic sex remained inviolate and was completely conserved in its entirety.

2) The first morphologically identifiable step of differentiation undertaken by the W was a pericentric inversion.

Differential accumulation of opposing sex-determining

factors by two members of an originally homologous pair could only be possible if they remained isolated from each other during meiosis of the heterogametic sex. Free crossing over would bring male and female determining factors onto the same chromosome and that would be undesirable. Ohno writes that Bowen (1965) suspected inversions, deletions or supressor genes on the W chromosomes to be responsible for the isolation mechanism. The finding of a pericentric inversion in the **Colubridae** family of snakes supported this theory. A pericentric inversion would create a stronger need for the establishment of the permanent isolation mechanism and facilitate further differentiation of the sex chromosomes. Partial heterochromatinisation of a region of the W or the Y containing non-sex determining genes could occur. According to this hypothesis the Z was conserved but had to accommodate the hemizygous state, thus dosage compensation evolved (as in the **Drosophila** sex determination mechanism). However Ray-Chaudhuri **et al** (1971) suggested that heterochromatinisation occured as the first step in the differentiation of the snake sex chromosomes. They believe that allocycly in one of the two chromosomes can also reduce the frequency of crossing over between them. The examples of sex chromosome differentiation presented above tend to support this idea though they can not be conclusive because in some species (like turtles for example) heterochromatinisation does not seem to be the first step to differentiation.

In an attempt to understand the origins and significance of chromosome sex determination Jones and Singh started a comparative study of the DNA of snakes with and without morphologically distinct sex chromosomes. Their progress and the conclusions they made are presented in the following section. An alternative view to sex chromosome differentiation is also presented.

1.4.4. Bkm Sequences

DNA sequences comprising a quantitative, significant, structural component of the W sex-determining chromosome of female snakes was recovered as a sex-specific satellite DNA by comparative ultracentrifugation of the DNAs of males and females of the Indian banded krait (**Bundarus fasciatus**). This satellite DNA is a minor density component and is called Bkm (banded krait minor satellite DNA). Bkm related sequences are present in both sexes of many snake species, but always in quantitative excess by a factor of at least two in female DNA, which, as mentioned above is the heterogametic sex. It is clearly present throughout the W chromosome but is interspersed with other DNA, perhaps as a result of the evolutionary process. Bkm can be used to identify the W chromosome in other homologous species or heterologously over a very wide range of species throughout the suborder, **Ophidia**, by means of **in situ** hybridisation (Singh 1980). However, that does not apply in the cases of snakes belonging to the primitive species of **Boidae**, where there is no quantitative sex difference and considerably less Bkm-related DNA in general. The elaboration of the sex determining chromosome has thus been paralleled by the quantitative increase of Bkm related DNA in both sexes, and a change in the organisation of these sequences on the W chromosome, where they have become concentrated (Jones and Singh 1981).

The point should be made that Bkm-related DNA was shown to exist broadly in two types of arrangements. In autosomes and presumably also in the Z chromosome, the arrangement involves an interspersion pattern which prevents the sequences being recovered as a satellite fraction, presumably because they are arranged in a long period interspersion. In the W chromosome however, the Bkm sequence interspersion is relatively frequent so that the base sequence composition of W chromosome DNA is heavily

influenced by Bkm-related DNA, or alternatively Bkm DNA may exist in relatively long monotonous stretches on this particular chromosome (Jones 1983).

These Bkm sequences have also been assumed to play an important role in the elaboration of the sex determining mechanism as well as contributing to the structural changes exhibited by the W chromosome (Jones and Singh 1981). This hypothesis was tested by searching for related sequences in species other than snakes. Hybridisation of labelled Bkm to the DNA extracted from male and female birds shows a quantitative excess of hybrids in female DNA compared with male DNA. However, results from **in situ** hybridisation indicate that though the W contains the Bkm family, its contribution seems to be minor. Similar experiments with the DNAs from male and female mice showed that there are related minor DNA components in both species, but quantitatively, there is no significant differences between the DNAs of the sexes, indicating that the contribution of the Y, if any, is small. Digestion of the DNAs from the two sexes of mice with the restriction enzymes Alu I and Hae III identified differences between them with the male exhibiting extra bands.

Southern blot analysis and **in situ** hybridisation experiments showed that the Bkm-related sequences are present on the Y and especially on the pericentromeric region which is the sex determining region and present in the DNA of the XX,Sxr, male mice (for more information on XX,Sxr mice, see section 1.5.). Similar studies indicated that like mice there does not appear to be a quantitative sex difference between human male and female DNA (Jones and Singh 1981). Later experiments with **in situ** hybridisation showed that Bkm sequences do not hybridise significantly to the human Y chromosome but to chromosomal regions Xq21 and Xp21, 6q21, and 11q13 (Kiel-Metzger **et al** 1985). Similar experiments on the mouse demonstrate the presence of Y chromosome-related DNA sequences on proximal chromosome 17

in XX,Sxr male mice and normal mice. The paper argues for a gene(s) related to sex determination or differentiation within this region (Kiel-Metzger and Erickson 1984) based also on the report from Washburn and Eicher (1983) on sex reversal in XY mice, proposed to be caused by a dominant mutation on chromosome 17.

Singh **et al** in 1984 recovered a conserved, transcribed component of the snake, W chromosome, satellite DNA (Bkm) isolated from **Drosophila** and male mouse libraries. By nucleotide sequencing they showed that it consists of repeats of the tetranucleotide GATA that are concentrated in the sex-determining region of the Y chromosome of the mouse, on the W chromosome of the snakes, and in the proximal region of the X chromosome of **D. melanogaster** in the same patterns previously shown by using uncloned Bkm probe. It also appears to be transcribed in a sex-specific and developmentally regulated manner.

Using a GATA probe and an uncloned Bkm satellite DNA on Southern blots of human male and female DNA and on male human chromosomes **in situ**, Singh and Jones (1986) re-examined the question of whether there is a general connection between these sequences and sex determination. They found that these sequences are relatively abundant and frequently show polymorphic arrangements in human DNAs. Sex-related patterns were absent, presumably because in this species the Bkm sequences are composed of relatively short repeats interspersed through non-repeated sequences. **In situ** hybridisation supported this by showing that human acrocentric chromosomes, including the Y chromosome, appear to contain concentrations of Bkm DNA. The majority of grains was shown to be preferentially concentrated on the proximal region of chromosome Y as in the mouse, therefore, quite possible near the male-determining factor. The authors observed that the blot hybridisation signal was significantly enhanced when using Bkm probe as compared to GATA probe presumably because of the covalent association

of different sequences present in Bkm (unpublished data) being maintained in human DNA. They therefore used the Bkm probe for **in situ** hybridisation and proposed that perhaps Kiel-Metzger **et al** 1985 failed to detect Bkm sequences on the human Y because they used a GATA probe.

The fact that the avian W chromosome has a quantitative excess of the Bkm sequence family compared with other avian chromosomes is consistent with the hypothesis that the avian and ophidian sex chromosomes are related (Ohno 1967). The fact that the same sequence family is concentrated in the sex-determining region of the mouse Y chromosome is a demonstration of sequence conservation between sex determining chromosomes of mammals and reptiles and is consistent with the proposal by Ohno (1967) that the sex chromosomes in these separate lines have been preferentially selected as the same pair of homologous autosomes present in the common ancestor (Jones and Singh 1981).

Based on observations of snake sex chromosome behaviour in which the W chromosome, just like the mammalian X, seems to undergo a cycle of inactivation and reactivation, it was suggested that the primary event in W chromosome specialisation was chromosomal inactivation (Jones and Singh 1985). This, according to the authors, would explain both the accumulation of Bkm sequences and the subsequent rapid morphological evolution of this chromosome. A model was constructed which suggests that the evolution of W is triggered by mutations which control the process of mitotic W condensation to be modulated by the sex determinants. According to this model, the cycle of somatic inactivation and germ cell activation of the W chromosome signifies the cycle of sex gene expression. Depending on the expression cycle of the sex gene which it carries, the sex chromosome may be genetically silenced. Essentially it is proposed that the sex genes have, in effect, 'hijacked' the chromosome. They suggested that

probably X chromosome inactivation has a similar explanation. While the sex allele on the X chromosome causes it to decondence in time for normal synapsis and crossing over to occur and therefore is only somatically isolated and does not evolve, the sex allele on the W chromosome causes it to decondense after meiosis and therefore the W is genetically and somatically isolated. Consequently, sex determining genes apart, its DNA can evolve freely, with the most unstable sequence elements becoming quantitatively disproportionate (details from Jones and Singh 1985).

The Bkm sequences have been of great use to the investigation of the subject of XX,Sxr, sex-reversed male mice discussed in the following section.

1.5. Sxr, Sex Reversal in Mice

Cattanach **et al** (1971) described an autosomal dominant mutation named Sxr which was responsible for the transformation of XX mice into phenotypically normal males with small testes, which in the adult, are devoid of germ cells. During the fetal and early postnatal development of these XX male mice only a few spermatogonia and no meiotic divisions are seen. The 39,X Sxr mice are also phenotypically normal males, but although all stages of spermatogenesis are present including spermatozoa they are infertile. The authors found no evidence of a Y;autosome translocation and were unable to map the mutation to any of the autosomal linkage groups. They proposed that Sxr may be analogous to similar mutations in other mammals.

When Bennett **et al** (1977) found that XX,Sxr male mice are H-Y positive, they interpreted the data by saying that Sxr represents a cytologically undetected translocation between the Y chromosome and an autosome. They then proposed that the most likely origin of this translocation involved a rearrangement of the chromatid type in the father of the original male in which case the original male could have retained a normal Y chromosome plus a duplication of a region of the Y involved in the translocation. Winsdor **et al** (1978) supported the theory that Sxr involves a chromosomal rearrangement, rather than a single gene mutation, from observations they made when examining the chromosomes of Sxr, male carriers and XO,Sxr mice in the diakinesis stage of meiosis. They observed a significant increase in failure of XY pairing in Sxr carrier males and noted chromosome fragments which were interpreted as evidence of an unstable Y-autosome translocation. Since the condition seemed to be transmitted in an autosomal pattern, it appeared to the authors that Y chromosomal material was being incorporated into one of the autosomes.

In 1982, however, Singh and Jones used the satellite DNA (Bkm) from the W chromosome of the banded krait, (see section 1.4.4. for more details) which hybridises to sequences close to the centromere on the normal mouse Y chromosome (Jones and Singh 1981) to study sex reversed XX,Sxr male mice. Normal male mice have Bkm sequences close to the centromere of the Y chromosome as has been shown by **in situ** hybridisation (Singh and Jones 1982). In XX,Sxr mice (but not normal females) these sequences were shown to be present at the distal end of one X. In Sxr male carriers in place of a single location of hybridisation attributable to the Y chromosome, such as was found in normal males, it was clear that a proportion of somatic cells contained two such locations. These were associated with two very small chromosomes, each somewhat less then the size of the normal

Y chromosome, which were closely associated. This was taken as an aberrant, sex determining chromosome complex and was referred to as $Y\text{-}Y_1$. Regular transfer of the Y Bkm sequence to the X occur during male meiosis and this leads to sex reversal in 50% of XX offsprings.

These findings led Burgoyne (1982) to propose the X-Y crossover model which postulates that: 1) the pairing observed between the X and the Y chromosome at zygotene is a consequence of genetic homology, 2) that there is a single obligatory crossover between the X and the Y pairing segments, and 3) the segment of the X which pairs with the Y is protected from subsequent X inactivation. Genes distal to the proposed crossover ("pseudoautosomal genes") will appear in both male and female offspring. Burgoyne regarded Sxr as an example of "failure to map to an autosome", one of the suggested criteria for identifying a pseudoautosomal gene.

Burgoyne viewed Sxr as a duplication of the Y-linked testis-determining factor, which has been translocated to the distal tip of the Y chromosome and thought that the aberrant $Y\text{-}Y_1$ chromosome complex described by Jones and Singh was just two separate chromatids of the Y. During meiosis, when haploid sperm are generated by a carrier male, crossing over between the X and Y chromosomes creates four types of sperm: (i) an X carrying the translocated Bkm sequences from Y; (ii) a normal X; (iii) a Y carrying both Bkm sequences; and (iv) a normal Y created by the transfer of the distal, Bkm sequence bearing end of the carrier Y to the X. Assuming that each sperm has an equal chance to fertilise ova, this would produce the observed 1:1:1:1 ratio of XX,Sxr males, XX females, XY,Sxr carrier males, and XY noncarrier males. This is represented diagrammatically in Figure 2 below.

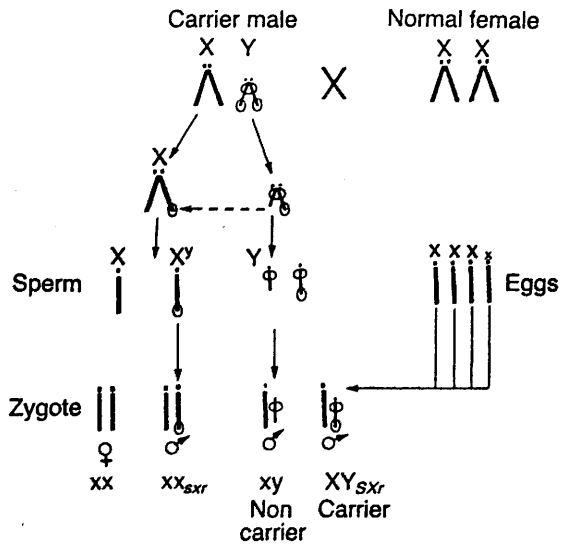


FIGURE 2: Transmission of the mouse Sxr factor (from Jones and Singh 1985).

Later findings by Evans **et al** (1982) showed that the aberrant Y chromosomal complex was just a single abnormal Y chromosome possessing an additional, terminal Bkm region and it was suggested that this rearrangement involves the transfer of all or most of this region from one Y chromatid and its attachment to the distal end of an X chromatid during male meiosis, therefore supporting the interpretation of Burgoyne. Furthermore, closer inspection of XY bivalents in mice not carrying Sxr suggests that crossing over is a normal feature of X-Y pairing, and may be a necessary requirement for the production of functional sperm.

Further experiments on XX,Sxr mice, were done by McLaren and Monk (1982) and Cattanach **et al** (1982) to test whether Sxr was located on one of the X chromosomes and is therefore perhaps subject to X-inactivation. They examined mice heterozygous for Searle's X-autosome translocation, T(X;16)H, such that the normal X chromosome postulated to carry Sxr would be preferentially inactivated (since it is

known that this translocated X remains always active (Lyon **et al** 1964). The data showed that some XX,Sxr mice develop as fertile females, some as males and some as intersexes. This was simply explained by assuming that the Sxr male-determining region is inactivated in some cells but not in others. Inactivation may spread to a variable extent from the inactive X chromatin into the attached Sxr fragment in an analogous fashion to the position effect variegation for autosomal genes translocated onto the inactive X chromosome.

As previously discussed for the H-Y antigen (section 1.1.2.), X/XSxr and T16H/XSxr mice (which in the absence of banded karyotyping cannot be distinguished from X/XSxr males) are positive for H-Y antigen, showing that the translocated region contains the gene controlling H-Y, whether or not this is identical with Tdy (male-determining locus for mice). It was shown by Simpson **et al** (1984) that T16H/XSxr females are also H-Y positive, whether tested by an **in vitro** cytological T-cell assay or by the proliferative responces of H-Y specific T-cell clones. This would be of no surprise since these Sxr-carrying females were mosaics, containing a cell population in which the Sxr sequences are expressed. It is interesting that the presence of H-Y antigen in no way interferes with female reproduction. In this experiment, however, one exceptional Sxr-carrying female out of 11 tested was negative for the H-Y antigen. To ensure that this was inherited and not the result of an extreme case of a mosaic distribution, in which virtually all cells had the expression of the Sxr region repressed by X inactivation, a series of experiments was done by McLaren **et al** (1984). The female was mated with a normal male and all her descendants were tested. Those who inherited her mate's Y chromosome were, as expected, H-Y positive; on the other hand, those who have received her Sxr region in the absence of a Y chromosome, that is, X/XSxr males and T16H/XSxr individuals (whether male or

female) were H-Y negative. Histological examination of the testes of three H-Y negative (T16H or X)/XSxr males suggests that they do not differ from those of H-Y positive (T16H or X)/XSxr males. It seemed therefore to the authors that they were dealing with a variant form of the Sxr region, which retains the testis-determining property but has lost the sequences controlling the expression of H-Y transplantation antigen. They called the new variant Sxr', with the constitution, Tdy^{+}, Hya^{-}, where Hya is the locus (structural or regulatory) controlling the expression of H-Y antigen. Tdy and Hya must therefore be two separate loci. The authors believe that though their data cannot allow them to determine whether Sxr' lacks the Hya locus altogether or retains it in a modified form (antigenity by which the molecule is determined has been lost), the separation of testis determination from expression of H-Y transplantation antigen eliminates the antigenic determinant as a candidate for the primary sex determinant factor.

Burgoyne **et al** (1986) produced XO,Sxr' mice and compared them with XO,Sxr mice to see if the loss of H-Y antigenicity is correlated with any additional defect in spermiogenesis. Each XO,Sxr was positive for H-Y antigen and each XO,Sxr' was negative for H-Y antigen. Histological analysis revealed that spermatogenesis is more severely affected in XO,Sxr' than XO,Sxr mice. There is almost total elimination of spermatogenic cells beyond the spermatogonial stage in adult XO,Sxr' testes, and this block is evident prepubertally, at the onset of meiosis. As the loss of spermatogenesis in XO,Sxr' mice is correlated with the loss of H-Y expression, the authors were tempted to conclude that H-Y antigen is the product of the spermatogenesis gene, thus reinstating a male-specific function for this evolutionary conserved male antigen. The same correlation of H-Y antigen negativity and spermatogenic failure was seen in the XO male described by

Melvold **et al** (1977). They found a mutant mouse which suffered germ-cell aplasia and bilateral Leydig cell tumors, while actively secreting androgens which maintained libido and accessory gland function at a high level. The mouse appeared to lack a Y chromosome but it could not be determined whether part of it was retained somewhere else in the genome. It was found to be negative for H-Y antigen but positive for the SDM antigen.

1.6. X-Y Homology and X-Y Interchange

One can assume from the studies mentioned above on the evolution of the mammalian sex chromosomes, that the X and the Y were originally a homologous pair. Further evidence for this comes from studies of karyotype-phenotype correlations. Ferguson-Smith in 1965 observed that short stature and Turner's stigmata may result from deletion of either a part of the short arm of the X or of part of the long arm of the Y chromosome. He also proposed that the X and the Y might contain X homologous loci at several points in both arms so that the short arm of the Y may occasionally associate and interchange non-homologous loci with the short arm of the X chromosome.

Müller **et al** 1982 applied a technique for replication studies on prometaphase human sex chromosomes and revealed a distinct early replicating segment on both distal Xp and Yp arms. These segments correspond to the high resolution bands Xp22.3 and Yp11.3 and their identical patterns of replication indicated functional homology of these two parts. It should be pointed out that the segment of the X chromosome involved here is the part of the X thought to escape inactivation and includes the loci for Xg and STS. These observations were compatible with Polani's view (1982) of homologous segments with similar genetic information on both sex chromosomes. He proposed that there

were loci homologous to both Xg and STS on the short arm of the Y chromosome. These 'allelic loci' could be assumed to be silent.

More information on the subject of X-Y homology was obtained from studies on the first meiotic division of human spermatocytes. During the zygotene stage of the first meiotic prophase, homologous chromosomes pair longitudinally with each other, a process known as synapsis. This meiotic pairing is the prerequisite for the regular anaphase separation of meiotic chromosomes. Synapsis probably occurs at homologous loci and when viewed under the electron microscope, structures called synaptonemal complexes, formed between the visibly single zygotene chromosomes, can be observed.

The X and the Y chromosomes were not generally believed to synapse. Up until 1969 there was some cytological evidence for a terminal association between the short arm of the X and the short arm of the Y (McIlree 1966) and a suggestion that the long arm of the Y was associated with the short arm of the X chromosome (Ferguson-Smith 1966, Ferguson-Smith and Path (1969), Húlten 1966) during diakinesis and first metaphase, when the XY bivalent is easily recognised. Chiasmata were not demonstrated with certainty between the X and the Y (Húlten 1966). However, the fact that the short arm of the X undergoes an end to end association with the short arm of the Y was a consistant finding in the studies of Chen and Falek (1969), but the definite evidence that the Yp and the X associate came from studies using Q-banding (Pearson and Bobrow 1970) and later from Chen and Falek (1971). Solari and Tres (1970) were able to observe the formation of a synaptonemal complex between the X and the Y during zygotene-pachytene and this was taken as strong evidence for the existence of homologous regions between the X and the Y. The authors further suggested that the end to end

association is actually a chiasma in the majority of mammals. If synapsis occurs in a limited region of the X-Y pair, the possibility exists that crossing over occurs in that region. This crossing over should be reflected in the partial sex linkage of genes located in this region of the X chromosome. No convincing data was available at the time though Ferguson-Smith in 1966 proposed that an accidental cross-over between the X and the Y could result in the male determinants being transferred from the Y to the X, thereby producing XX males (see next section for more information). This end to end association between the short arms of the X and the Y was also confirmed by Moses **et al** (1975). It followed that in so far as the synaptonemal complex reflects chromosome homology, the distal portion of the X chromosome short arm is homologous to the Y short arm. The attached end to end synaptonemal complex in the XY pair in diplotene and diakinesis implies synapsis and is consistent with the possibility that the tandem arrangement results from chiasma terminalisation. According to Solari (1980), in an extensive study of synaptonemal complexes of the XY bivalent, 30% of Xp and 90% of Yp undergo synapsis. A recent study however, on the nature and the extent of XY pairing at meiotic prophase in man, by Chandley **et al** (1984), provided strong circumstantial evidence for an XY synaptic zone at early pachytene, which involves not just the Yp but can extend across the Y centromere and into the proximal region of Yq. At its longest it may even involve all of the euchromatic portion of the Y chromosome. An association between the telomeric regions of Xq and Yq was observed in some of the spermatocytes. In one rare cell a distinct pairing zone appeared to have formed in the distal region of the long arms in addition to the pairing of the short arms.

Polani (1982) also suggested that perhaps an unequal crossing over occurs between the X and the Y, which would give rise to exceptions to normal X and Y-linked

inheritance. According to the same paper a region of homology does not mean that crossing-over has an equal probability of occuring at every point of the homologous segment nor equally in the two sexes.

In the same year Burgoyne put forward his 'X-Y crossover model' based on observations of the transmission of Sxr in mice as discussed already. The model was extended to humans and Figure 3 below illustrates the main features.

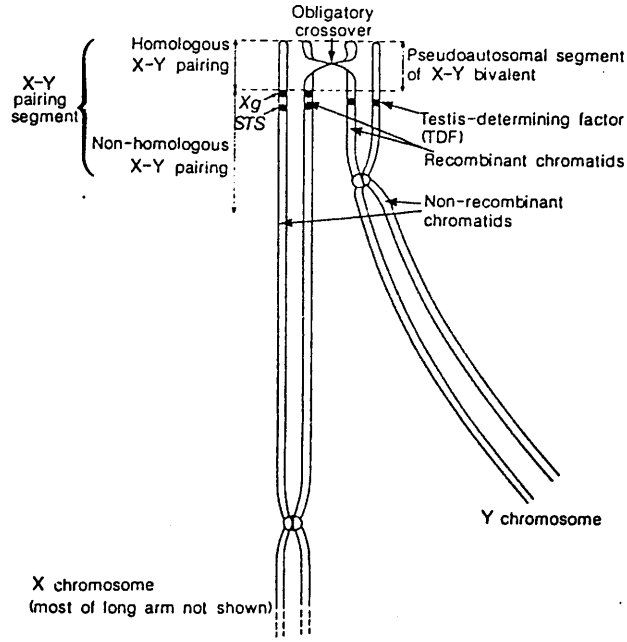


FIGURE 3: Model for crossing over between the X and the Y chromosomes (from Burgoyne 1986).

The chromosomes are split into the following regions:

1) the X differential segment carrying X-linked genes which are dosage compensated in females,

2) the Y differential segment carrying the Y-linked testis-determining factor (TDF),

3) the X pairing segment which is never X-inactivated and which is homologous to

4) the Y pairing segment.

The X and Y pairing segments are split into two regions by a single obligatory crossover. On the X, proximal to the crossover, there are such genes as Xg and STS, which are not dosage compensated and which may have near counterparts on the Y. Genes distal to the proposed crossover will appear to be autosomally transmitted and are therefore called 'pseudoautosomal genes'.

The first genetic evidence that crossing over is a regular feature of normal X and Y chromosomes in the mouse comes from the studies of Keitges **et al** (1985). They demonstrated that a mouse steroid sulphatase (STS) variant, which had seemed to be inherited autosomally, was in fact transmitted to XO offspring via the X chromosome. This observation, together with the existing pedigree data, proved that the STS gene was present and expressed on the X and Y chromosomes and that there must be an obligatory crossover proximal to the STS locus to account for the absence of either X or Y linkage of STS. This pseudoautosomal mode of inheritance of the mouse STS gene contrasts with that in human, where the STS gene is X-linked, although close to the pseudoautosomal region.

The application of recombinant DNA technology has begun at last to produce a lot of interesting information concerning the pseudoautosomal segment. This will be dealt with in section 1.8. where all the molecular data on the X-Y homology will be presented.

1.7. Abnormalities of Sex Differentiation in Humans

There are a great many different abnormalities of human sex differentiation, the study of which can provide invaluable information on the normal processes involved. Listed below are some of these disorders, all of which are associated with abnormalities of the internal and external

genitalia and failure to reproduce. Details on their clinical features can be found in Simpson (1976), Ferguson-Smith (1971), de la Chapelle (1972) and de la Chapelle (1981). More extensive information will be presented on XX males and true hermaphrodites followed by a discussion on how these conditions occur in the first place. This information again aims to explain certain aspects of sexual determination and differentiation.

Female Pseudohermaphroditism

They are 46,XX individuals whose external genitalia do not develop as expected for normal females. The etiology can be genetic (most common syndromes are autosomal recessive disorders) or teratogenic. Unlike other disorders fertility is possible in some of these cases.

Male Pseudohermaphroditism

These are individuals with a Y chromosome whose external genitalia does not develop as expected for normal males. The cytogenetic forms include individuals who display a variety of karyotypes, all of which carry a Y chromosome e.g. 45,X/46,XY, 45,X/47XYY, and 45,X/46,XY/47,XYY. The same group includes the testicular feminisation syndrome (androgen insensitivity syndrome) whose karyotype is 46,XY. These individuals have female external genitalia, bilateral testes, a blindly ending vagina, and no Mullerian derivatives. At puberty they undergo breast development and puberal feminisation as expected for normal females. Their psychosexual behavior is female. The gene (tfm) responsible for the trait is an X-linked recessive. The basic endocrine defect is end-organ insensitivity to androgen and the incidence is 1 in 10,000 males. The complete form appears to be due to a deficiency of androgen receptors.

Despite pubertal feminisation (breast development), some individuals with testicular feminisation may have clitoral enlargement and labioscrotal fusion. The term incomplete testicular feminisation then applies. Both syndromes have similar modes of inheritance but are distinct. The question rises then, whether these two conditions are due to genes in different loci or due to alleles at the same locus. Either condition may occur in a family but never both together.

Gonadal Dysgenesis

Gonadal dysgenesis is associated with monosomy of the X or structural rearrangements of the X and the Y but may also be associated with apparently normal male or female complements. When associated with short stature and certain somatic anomalies then the term Turner's syndrome is used.

When the condition is present with a normal female or male karyotype then it is referred to as XX or XY gonadal dysgenesis and may affect several members of the same family. Furthermore, in any given family each affected member has the same chromosomal complement, 46,XX or 46,XY. In some families, XY gonadal dysgenesis appears to be inherited as an X-linked recessive trait. A very rare form of this condition is associated with interstitial deletion of Yp.

Klinefelter's Syndrome

These are males with at least one Y and at least two Xs. One percent of mentally handicapped males have XXY Klinefelter's syndrome but relatively few XXY patients display somatic anomalies or are severly handicapped. 46,XY/47,XXY patients are even less severely affected than 47,XXY patients. By contrast, all reported 48,XXXY or 49,XXXXY individuals have been severely mentally retarded.

The frequency of somatic anomalies is also higher in 48,XXXY than in 47,XXY, and highest of all in 49,XXXXY.

Sex Reversal in Man

46,XX Males and True Hermaphrodites

46,XX males are phenotypic males with bilateral testes and an apparent female chromosomal complement. True hermaphrodites are individuals with testicular and ovarian tissue, they are mostly 46,XX, some are 46,XX/46,XY, or have other chromosomal complements. Both groups raise similar questions about the nature of the sex reversal but will initially be presented separately.

46,XX males: the incidence of this syndrome is around 1 in 20,000 (Evans **et al** 1979). Their general appearance is male with respect to muscularity, distribution of fat, and the general body proportions are normal. Associated malformations are rarely found. The mean height of this group is 168.2cm, which is smaller than that of 47,XXY individuals. Permanent tooth size seems to be smaller than those of normal males and similar in size to those of normal females. The incidence of gynecomastia in this group is similar to the 47,XXY group. They have male psychosexual orientation, normal to weak secondary sexual characteristics, penis and scrotum are usually normal. Testicular size is small. Hypospadias have been reported in some individuals. They are found to have abnormal testicular histology with azoospermia and normal to low androgen levels, features identical to Klinefelters' syndrome. Mild mental retardation, a common feature of the XXY syndrome, is not typical in XX males. The commonest reasons for referral are the same in both syndromes: infertility, small testes or an abnormality of the secondary sexual characteristics. However, despite similarities between XX males and 47,XXY individuals it is likely that they have different etiologies.

True hermaphrodites are defined as individuals who have: 1) histologically verified ovarian follicles or proof of their prior existence (e.g. corpora albicantia) and not simply fibrous stroma and 2) seminiferous tubules, not simply Leydig cells. The gonadal tissue may be distributed either.

1) bilaterally: both ovarian and testicular tissue present on each side usually in the form of ovotestes, or

2) unilaterally: both ovarian and testicular tissue present only on one side, or

3) alternate: ovarian tissue present on one side and testicular tissue present on the opposite side.

1.7.1. Hypotheses and Facts on the Etiology of XX Males

From the information presented above, it seems that in 46,XX males and 46,XX true hermaphrodites, testes develop contrary to the axiom that a Y chromosome is required for testicular differentiation. Possible explanations for this are: a) undetected 46,XX/46,XY chimaerism or 46,XX/47,XXY mosaicism, b) loss of the Y after initiation of testicular differentiation c) transfer of testis determinants from the Y to the X chromosome, d) translocation of testicular determinants from the Y to an autosome and e) a mutant gene(s). All these theories are extensively reviewed in Simpson (1976), Ferguson-Smith (1971), de la Chapelle (1972) and de la Chapelle (1981). De la Chapelle (1981) concluded that there is no unifying hypothesis which could explain all the known facts about XX males. While mosaicism appears very unlikely in most cases, Mendelian gene mutation, translocation, X-Y interchange, a minimal deletion, or preferential inactivation of an X chromosome might all be possibilities. De la Chapelle believed the most likely explanation to be that the etiology of the XX male condition is heterogeneous.

A lot of data, however, supports the hypothesis put forward by Ferguson-Smith in 1966 that an accidental crossing-over between the X and the Y is responsible for an X-Y interchange and the transfer of the male determinants from the Y to the X chromosome. This was based upon the observations that some patients had failed to inherit the paternal Xg allele and the short arms of the X and the Y are associated terminally during male meiotic prophase as already described. This terminal association is necessary for the segregation of the X and the Y to each of two secondary spermatocytes and the precocious condensation of the XY bivalent prevents or reduces crossing-over between the X and the Y. Failure to prevent this crossing-over between the X and the Y might result in XX males or true hermaphrodites. When the sperm carrying these abnormal X and Y sex chromosomes takes part in fertilisation, an XX zygote containing male determinants and an XY zygote lacking male deterninants will result. In the XX zygote, random X-inactivation during early embryogenesis may lead to mosaicism for two cell lines, one with the X which carries the male determinants on it, being inactivated, and the other with the normal X inactivated. Assuming that the Y loci transferred to the X will take part in the inactivation process, the two cell lines will be ovary-determining and testis-determining respectively. The situation is therefore analogous to the XX/XY individuals in whom sex differentiation may range from males with testes to females with ovaries and include cases of true hermaphroditism with both ovaries and testes.

In this theory of X-Y interchange, it is postulated that crossing over at a partially homologous region leads to the interchange of nonhomologous loci distal to the point of crossing over. However as Ferguson-Smith suggested the Y chromosome may contain X-homologous loci at several points in both arms so that the short arm of the Y may occasionally associate and interchange nonhomologous loci

with the short arm of the X. Any such interchange is likely to be reciprocal, hence nonhomologous X-linked loci might be transferred from the X to the Y with equal frequency. Since the Xg locus is on the short arm of the X it is conceivable that X-Y translocations may result in transfer of the Xg locus to the Y; this could explain the failure of some XX males to inherit their paternal Xg allele and the male distribution of Xg phenotypes found in 76,XX males (Race and Sanger 1975). Ferguson-Smith also suggested that this X-Y interchange could also explain other types of abnormal sex differentiation, for example, in women with streak gonads and an apparently normal XY karyotype, the Y chromosome may have lost its male determinants by interchange with the X during paternal meiosis.

Early studies by Wennström **et al** (1967) in an attempt to find a difference between the late replicating chromosome of the XX males and that of normal females, using autoradiography, were unsuccesful. Since then it has been shown in a number of patients and using different methods that X-Y interchange does occur. Madan (1976) found a significant difference between the length of the short arm of the two Xs in an XX male case and also that the short arms of the larger X were significantly different from those of the X chromosome derived from normal individuals. The significance lies specifically in the size of the terminal band of the short arm of the X. If this extra material was to come from the Y chromosome it would come from the euchromatic region since quinacrine staining was negative for that part of the X, and the size of the extra material indicated that it came from the short arm of the Y chromosome. Evans **et al** (1979) analysed 12 XX males and found a clear difference between the average centromeric index of X chromosomes of 46,XX males and those of normal males and females. This result is interpreted as a consequence of an increase in the length of the short arm

of one of the X chromosomes in these individuals. The amount of the additional material in the distal region of Xp in 46,XXp+ males varies considerably which could suggest that different pieces of Y material are transferred each time from the Y to the X. However when de la Chapelle **et al** (1979) tested their own group of XX males using the same method, they did not find similar results; only one XX male had a significantly higher centromeric index, suggesting an XXp+ phenomenon. They also discussed the possibility of an etiological heterogeneity of the XX male condition. In 1982 Magenis **et al** analysed G-banded prometaphase chromosomes from 3 XX males and showed clearly the existence of extra bands on the distal end of one X short arm. The bands were similar both in size and staining properties to the distal short arm of their father's Y chromosome (in the two cases examined) and also to other chromosomally normal males. The extra material on the X chromosome was not C or G11 positive in the two cases examined, suggesting that the proximal Y long arm was not present. This finding also provides support for the theory that the testis determinants are localised between bands p11.2 and pter of the Y chromosome.

Conclusive evidence of the transfer of Y-specific sequences was obtained in 1984, and this is discussed extensively later in section 1.8.7..

1.8. Recent Data on the Nature of the Y Chromosome, X-Y Homology, X-Y Interchange and the Evolution of the Sex Chromosomes

Much of the work so far on these issues has been done with cytogenetic and biochemical techniques and by phenotype-karyotype correlations. More recently, current modern techniques in molecular biology and immunogenetics have been used to investigate the subject and a wealth of information concerning the molecular structure of the Y chromosome has been accumulated. Y probes have been isolated from libraries constructed from human-rodent hybrid cell lines carrying the Y chromosomes as the only detectable human chromosome or from sorted Y chromosomes using the FACS machine and from genomic libraries. Methods such **in situ** hybridisation and Southern blot analysis were used to map these probes. In the following sections recent data obtained mostly within the last four years will be presented, organised into classes of sequences with similar natures.

1.8.1. Gene Sequences

i. MIC2 locus

12E7 is a monoclonal antibody raised against lymphocytes from a patient with a T-cell acute lymphocytic leukaemia, which reacts strongly with cortical thymocytes and to a lesser extent with other human cells (ref. from Goodfellow 1981). All mouse tissues tested lack the 12E7 antigen and the only species which show cell surface expression of this antigen are the higher primates: man, chimpanzee, and gorilla. The 12E7 antigen is expressed generally on human cell lines. Its expression has been tested on lymphoma, carcinoma and sarcoma cultured cell lines. Only a limited number of cells derived from normal

tissues have been tested and these include fibroblasts, thymocytes, peripheral blood lymphocytes, granulocytes, platelets, red blood cells and muscle. All these tissues are 12E7 positive. The only human cell line which has been shown to lack the 12E7 antigen is a Burkitt's lymphoma cell line (Goodfellow 1983).

The 12E7 antigen was shown to be polymorphic and has two levels of expression, termed high and low. All Xg(a+) individuals show high levels of 12E7 antigen expression but Xg(a-) individuals fall into two categories, showing either the high or low-level 12E7 phenotype. The probability that these results occurred by chance was very small, thus indicating a relationship between the Xg locus and the 12E7 polymorphism. Moreover, the data implied a sex limitation because although Xg(a-) males may be high or low-level expressors of 12E7, Xg(a-) females always have the low-level 12E7 phenotype. A hypothesis was therefore put forward that a locus, Yg, equivalent to Xg, is present on the Y chromosome. Like Xg, the Yg locus must have two alleles, Yg^a and Yg. It was postulated that Xg^a and Yg^a allow high-level expression of the 12E7 antigen, whereas Xg and Yg do not. The Xg therefore is acting as a controlling locus for the 12E7 antigen polymorphism. It should be noted that the gene frequency of the postulated Yg^a allele (0.68), is very similar to that of the Xg^a allele (0.66) when calculated from similar populations (Goodfellow and Tippett 1981).

The gene controlling the expression of 12E7 has been assigned to the X chromosome, using a panel of human-rodent hybrids. Hybrids which have a complete human X chromosome express the antigen, whereas hybrids which lack the X chromosome fail to express it. This X-linked gene was named MIC2 and later on MIC2X. The fact that the 12E7 antigen is not found in rodents suggested that the MIC2 gene is a structural gene for the 12E7 antigen. Also, since the precise relationship between the Xg locus and MIC2 is not

clear, it is possible that they are identical (Goodfellow 1983). Using a panel of rodent-human hybrids containing different fragments of the X chromosome, MIC2 was located on the short arm of the X between the breakpoint Xp22.3 and Xpter (Goodfellow **et al** 1983). This region, as mentioned previously, lies well within the X-Y pairing segment and is thought to escape inactivation. It also includes the loci for Xg and STS which are already shown to escape inactivation. Though it has been proposed that equivalent but silent loci might be on the tip of the short arm of the Y, there is no direct evidence to prove this. The location of MIC2X in this region prompted scientists i) to test whether the MIC2 gene escapes inactivation and ii) to look for expression of the 12E7 antigen deriving from the Y chromosome. The results showed that the MIC2X gene escapes inactivation when on an inactive X although the expression level is lower than normal*
(Goodfellow **et al** 1984). In view of the finding that MIC2X escapes X-inactivation, one would expect increased levels of 12E7 in cells with multiple, inactive X chromosomes. Preliminary studies do show that 12E7 is expressed at increasing levels in cells with increasing numbers of inactive human X chromosomes (Goodfellow **et al** 1984).

Using human-mouse hybrids which have retained the Y chromosome as the only detectable human chromosome, expression of the 12E7 antigen from the Y chromosome was shown. Furthermore a human-mouse cell line which carried only part of the Y from Yqter to Yq1.1 failed to express the antigen, therefore localising the gene coding for 12E7 on the Y, in the region from Yq1.1 to Ypter. The gene on the Y was later called MIC2Y and this can be regarded as the first and so far, only, demonstration of a functional gene being shared by both the X and the Y chromosomes. Furthermore, biochemical and genetical data confirmed that expression of the cell surface antigenic determinants is independently controlled by an X-linked gene MIC2X and a Y-

* It should be noted however that the lower expression levels for 12E7 were observed in hybrid clones carrying an inactive X chromosome. The authors made suggestions to explain these findings.

linked gene MIC2Y. By using a bacterial expression system the 12E7 antibody was used to select cDNA clones which encode the 12E7 antigen. A probe pSG1 was isolated and preliminary Southern blot analysis showed that the probe hybridises to the human X and Y chromosomes. The same probe was hybridised **in situ** to replication banded metaphase chromosome spreads derived from the lymphocytes of a normal male. Analysis of the silver grain distribution in cells revealed two major areas of probe hybridisation, Xp22.2-pter and distal Yp11.2-pter. The localisation of MIC2 sequences to the distal short arm of the Y chromosome and to the tip of the X chromosome is the first formal demonstration of sequence homology between expressed loci in the pairing region (Buckle **et al** 1985). The same probe was shown recently to detect multiple RFLPs which is a common feature of other pseudoautosomal sequences (for further details see section 1.8.6.). The inheritance of the MIC2 RFLP's was investigated in three large kindred. In a total of 46 informative male meiosis analysed, a single recombination event was observed between MIC2 and TDF. The individual who is a recombinant between MIC2 and TDF is a normal female individual. This result suggests that there is about 2% recombination between MIC2 and the sex-determining gene(s) TDF, and that this is the most proximal pseudoautosomal locus thus far described (Goodfellow **et al** 1986).

ii. Argininosuccinate Synthetase Gene

Daiger **et al** in 1982 used a probe (pAS-1) to the human urea cycle enzyme argininosuccinate synthetase (AS), to detect individual variation in restriction enzyme digests of human DNA from lymphocytes, fibroblasts and transformed cell lines. They found that at least 15 genomic fragments are detected with pAS-1 in DNA digested with restriction enzymes recognising 6-base sequences. This multiplicity of

fragments is explained in part by the observation that AS-like sequences are found on more than seven human chromosomes including 6,9 and X. The human structural AS gene is tentatively assigned to 9q34. Digestion with some enzymes produces one or two fragments always present in males but not in females thus showing that AS-like sequences are detected on the Y chromosome as well. The relationship between these sequences on the X and Y chromosomes and the AS structural locus is not yet known but as will be mentioned later in the discussion chapter they could be pseudogenes.

iii. Actin Sequences

Heilig **et al** (1984) demonstrated the presence of actin like sequences on the X and the Y chromosomes using cDNA clones which corresponded to human α skeletal actin or to a hamster (β or γ) cytoskeletal actin. These actin-like sequences showed more homology to the latter probe suggesting therefore that the X and Y linked actin fragments bear more resemblance more β or γ actin genes than the muscle α actin gene. The human genome contains more than 20 actin-related sequences (Engel **et al** 1981). At least six expressed genes code for the known muscle a actins and for the β or γ actins (Vandekerckhove and Weber 1979). Data deriving from **in situ** hybridisation experiments suggested that actin sequences are dispersed on many autosomes and that actin sequences might be present on the long arm of the X chromosome (Soriano **et al** 1982). The X-linked actin sequence was assigned to the centromeric region between Xp11 and Xq11. These sequences were absent from 4 XX males and 2 XX true hermaphrodites. The pattern of hybridisation obtained was reminiscent of the argininosuccinate synthetase cDNA probe. Koenig **et al** (1985) showed that the presence of actin sequences on both the X and the Y chromosomes is not related to an extended

homology between these two chromosomes since the sequences which flank the Y actin sequence were shown to be completely male specific in man.

1.8.2. Repeat Sequences

i. The 3.4kb and 2.1kb Tandem Repeats

Two tandemly repeated sequences which account for about 50%-70% of the human Y chromosome were isolated by using restriction enzyme analysis and nucleic acid hybridisation. Digestion of male and female DNA with the enzyme Hae III gives two distinct fragments specific for the male. Their sizes are 3.4kb and 2.1kb, respectively, and they are present in 4000 and 2000 copies per haploid genome (Cooke 1976). By the use of a different method involving extensive reassociation between tritiated DNA prepared from male and an excess of DNA from a female, radiolabelled, reiterated DNA specific for the Y chromosome was obtained. These highly purified sequences reassociate only with DNA from individuals carrying a Y chromosome and are called It-Y DNA (Kunkel **et al** 1976). This DNA represents an heterogeneous population of 15-30 reiterated DNA families that are confined to a single chromosome, each of which is present in 300-600 copies per family. It-Y DNA is located on the long arm of the Y and there is no evidence as yet that these sequences play any role in male determination (Kunkel **et al** 1977).

The It-Y DNA and the 3.4-2.1kb fragments isolated by Cooke (1976) are different DNA sequences. Exhaustive hybridisation with female DNA gives intermediately reiterated sequences (It-Y DNA) which do not cross-react to female DNA. Restriction enzyme purification gives a fast reassociating fraction (Fr-Y DNA) which does cross-reassociate to female DNA to give a poorly matched hybrid

(Cooke and McKay 1978). It was shown though that Fr-Y DNA, like the It-Y DNA, is derived from the brightly fluorescent region in the long arm (McKay **et al** 1978). The same authors also showed that: i) the amount of the specific repeated DNA sequence recognised by the first male specific band (Fr-Y 1, 3.4kb), is roughly proportional to the length of the fluorescent segment of the human Y chromosome for which man is polymorphic, ii) the amount of the sequence varies without any concurrent change in the sequence itself, iii) female DNA does contain sequences very similar to the polymorphic Y-linked sequence, at a much reduced level, and iv) the second Y-linked sequence recognised by the second male-specific band (Fr-Y 2, 2.1kb) evolves independently of the first.

Bostock **et al** (1978) showed that part or all of the 3.4kb repeated sequence between the Hae III recognition sites is sufficiently homologous to DNA sequences present on autosomes. Despite this homology, the 3.4kb spacing of the Hae III recognition site seems to be unique for the Y chromosome. Experiments on aberrant Y chromosomes (various Yq deletions) showed that the 3.4kb fragment is located in the distal region of the nonfluorescent portion of the long arm of the Y chromosome and in the most proximal region of the fluorescent portion (band Yq12). The same fragment was used as a probe against a panel of somatic cell hybrids containing chromosomes 7, 9, 14, 15, X or a 3/17 translocation as the only human chromosomes (Cooke and McKay 1978). It was shown to hybridise with each of these chromosomes, but the amount of hybridised material and the pattern of hybridisation for each of these chromosomes was different. This indicates that the arrangement of restriction sites in the related repeated sequences varies from chromosome to chromosome. It was suggested that such differences between chromosomes, could be caused by different arrangements of interspersed sequences or by changes in restriction sites and are apparent over much

shorter stretches of repeated sequences. Similarly, Szabo **et al** (1980) using **in situ** hybridisation localised the 3.4kb fragment along most of the long arm of the Y and showed that part of the 3.4kb is not specific to the Y and seems to hybridise to the centric regions of chromosomes 1, 9, and 16, and the D+G groups of chromosomes, 13, 14, 15, 21, and 22. This part of the 3.4 non-Y-specific fragment, therefore, seems to be partially homologous with at least two classes of highly reiterated sequences.

The 2.1kb fragment was shown to be concentrated in the distal portion of the heterochromatic block (Schmidtke **et al** 1980, Szabo **et al** 1980) and is interspersed with the 3.4kb fragment (Schmidtke **et al** 1980). Using **in situ** hybridisation the 2.1kb fragment (1.9 according to Szabo **et al** 1980) labelled all of the human chromosomes at a low level, although some regions seem to be more heavily labelled (e.g. the centromere of chromosome 1). The difference in the hybridisation pattern between the 3.4 and 2.1kb fragments suggests that they do not share common sequences.

The 2.1kb fragment accounts for about 20% of the Y chromosome. This Hae III fragment was cloned into a plasmid vector and was called pHY2.1. The insert of 2.12kb is not composed of any repeat sequences and gives a distinct 2.00kb band when hybridised with female DNA and two distinct bands of 2.12kb and of 2.00kb when hybridised with male DNA. There are about 2000 copies of the 2.12kb band and about 100 copies of 2.00kb band on the Y chromosome. Using the **in situ** hybridisation technique, the 2.1kb fragment was shown to be present on the tip of Yq1.2 as shown previously. Autosomal copies were often present on the telomeres of a variety of chromosomes: 2p, 3p, 5p, 6p, 7p, 10p, 11p, 13p, 14p and q, 15p, 18p, 21q, 22q, and also Xp and q. The sequence is widely distributed on chromosome 12 being absent only from the telomere of 12q, and showing maximum hybridisation in the middle of the same arm (Cooke

et al 1982).

In situ hybridisation experiments using the 3.4kb and 2.1kb fragments were also carried out in chimpanzee and gorilla, and similar results were obtained. The level of hybridisation to the Y chromosome was no higher than that for other chromosomes, the 2.1kb was more dispersed than the 3.4kb, but some preferential labelling of certain chromosomes was observed with the 2.1kb fragment (Szabo **et al** 1980). The pHY2.1 clone was also hybridised against the genomic DNAs of several primates: gorilla DNA gave hybridisation to a distinct size class of fragments, chimpanzee DNA showed a pattern typical of a dispersed repeat, with gibbon DNA there was no detectable hybridisation and chinese hamster and mouse DNA gave negative results. There was no detectable difference in the hybridisation pattern between male and female DNA from gorilla and chimpanzee confirming an autosomal location for the sequences in these species (Cooke **et al** 1982).

Kunkel and Smith (1982) used the same human male-specific 3.4kb Hae III restriction endonuclease fragments to explore the evolutionary history of man's Y chromosome and identified four sets of reiterated sequences on the basis of their relative sequence homology with autosomal DNA. These sequences which account for 40% of the human Y-chromosome, are interspersed within the same 3.4kb Hae III fragments, are heterogeneous and contain all reiterated DNA previously demonstrated to be specific for the Y chromosome (It-Y DNA).

Y-specific 3.4kb Hae III sequences do not reassociate with either human female or ape DNA under standard reassociation criteria. However, approximately half of It-Y DNA (cross reacting It-Y) reassociates with both human female and ape DNA at reduced reassociation criteria. The remaining half (Y-specific It-Y) retains its specificity for the human Y chromosome.

Non-Y-specific 3.4kb Hae III sequences reassociate

with both human female and ape DNA at standard reassociation criteria. One subset of non-Y-specific 3.4kb Hae III sequences forms stable duplexes with human Y chromosome DNA and with human and ape autosomal DNA. No detectable base-mismatch occurs among these homologues suggesting complete conservation of these sequences during primate evolution. The second subset of non-Y-specific Hae III sequences forms highly mismatched duplexes with human and ape autosomal DNA. The finding that homologues of 3.4kb Hae III sequences are not found within the Y chromosome of apes but are only present in autosomes suggests that 3.4kb Hae III sequences are largely autosomal in origin (Kunkel **et al** 1982).

It seems very unlikely that the 3.4kb and the 2.1kb repeats here are involved in sex determination. The reasons for this are 1) there is considerable variation in the amount of these sequences within the population of normal males and 2) Yq to autosomal translocations occur relatively frequently within the population and when present in a 46,XX individual have no effect on fertility (Cooke and Noël1979, Burk **et al** 1983). They can however be very useful in karyotyping and prenatal diagnosis as has been demonstrated already (Cooke and Noël 1979, Burk **et al** 1983, Lau **et al** 1984, 1985, Schmidtke **et al** 1985).

ii. Alphoid family of Sequences

The alphoid (or α-satellite) family of sequences has been found in every primate and is preferentially located at the centromeres (Singer 1982). Eco RI digestion of human DNA reveals major repeated DNA fragments of 340 and 680bp ("alpha dimer" or "alphoid" DNA). These have been found to hybridise preferentially to centromeric regions of many autosomes, weakly on the X and not detectably on the Y (Manuelidis 1978). Alphoid DNA in Bam HI-digested X chromosome is organised in a 2.0kb repeat, reflecting

cross-reaction with the X-specific, DXZ1, tandem repeat family. This repeat maps in the centromeric region and with about 6000 copies may represent 5 or 10% of the X chromosome (Willard **et al** 1983).

A cloned copy of this repeat was used by Wolfe **et al** (1985), to screen human Y chromosome cosmids. Several were found to cross-hybridise. Two were chosen (cosmid 84 and 97) in order to elucidate the chromosome centromeric repeats. Cosmid 84 was shown to hybridise strongly to a male specific 5.5kb Eco RI fragment. Cross-hybridisation to the Bam HI 2kb X fragment and to a 340bp ladder diagnostic of alphoid repeats was also observed. Clone 97 displayed similar hybridisation patterns to those found with cosmid 84. It strongly hybridises to the 5.5kb Y fragment and 340bp repeats of low intensity are visible. The 5.5kb fragment was localised to the pericentromeric region or the short arm of the Y chromosome. The degree of homology between the X and the Y sequences was about 70%. It was estimated that there are approximately 100 copies of the Eco RI 5.5kb repeat on the Y chromosome. Chimpanzee male and female DNA, and male orangutan DNA hybridised with the alphoid ladder but did not show the 5.5kb Eco RI repeat. Thus, the Y centromeric sequence arrangement appears to have changed rapidly amongst higher primates. It was concluded that the X and the Y chromosomal alphoid repeats were not closely related in sequence. Cross-hybridisation of the Y centromeric repeat was shown to be stronger to the centromeres of autosomes 13, 14, and 15 than it is to the X, while DXZ1 hybridises more strongly to the centromeres of chromosome 22 than it does to the Y (Yang et al 1982).

1.8.3. Sequences which Recognise Homology Between the Y and Autosomes

Recombinants which recognise homology between the Y and autosomes have been isolated by Bishop **et al** (1983 and 1984), Lucotte and Ngo (1985), Ngo **et al** (1986) and Affara **et al** (1986a). Probe p49f is a highly polymorphic probe (Lucotte and Ngo 1985, and Ngo **et al** 1986) which has been located to a sub-region of Yq11 and detects around 15 Taq I bands (18 bands under nonstrigent conditions), among which two bands were also present in female digests, whereas the remaining bands were Y-specific. The two bands were mapped on an autosome (their unpublished results). The probe detects a family of moderately repeated sequences present in restriction fragments of different sizes and spans a region of about 100kb. Five of these bands, each representing a single DNA fragment, can either be present, absent or variable in length and studies on 44 male individuals indicated that these five variable Taq I fragments detected by probe p49f can be considered as five independent allelic series. A total of 16 haplotypes, each defined by a different combination of the various forms of each of these five restriction fragment length polymorphisms, were observed among the 44 individuals scored. These Taq I restriction polymorphisms cannot be detected with other restriction digests and have been attributed to point mutations. A preliminary study showed that the common haplotypes are present in three racial groups sampled and no particular combination was specific to a particular race.

1.8.4. Sequences which Recognise Homology between the X and the Y Chromosomes

DXYS1 was the first site of single-copy DNA sequence homology to be found between the human X and Y chromosomes. A phage clone isolated from a human genomic library contained a 13.5kb human insert. From this insert several smaller fragments were purified. A 4.5kb Eco RI single-copy fragment hybridised to both the X and the Y chromosomes exclusively. The same probe hydridised to Southern blots of genomic DNAs digested with Taq I enzyme, revealed allelic restriction fragments 10.6, 11.8 which segregated with the X and 14.6kb which segregated with the Y chromosome. The X and Y linkage of these fragments was further confirmed with a study on 48 members of a single kindred. Experiments were carried out using different restriction enzymes to digest the DNAs and other sequences isolated from the initial phage clone as probes. The data derived from these experiments suggested that the Taq I RFLP is the result of base-pair substitution within the Taq I sites. The extent of sequence homology between the X and the Y was initially estimated at around 28kb (Page **et al** 1982) and later extended to 36kb (Page **et al** 1984). The single copy sequences in the human insert of the initial phage clone hybridised under high strigency conditions did not detect any homology with hamster or mouse DNA. It was also estimated that the X and Y chromosomes differ at 0.83+/-0.54% of their nucleotides in DXYS1. DXYS1 was localised on the X and the Y chromosomes using Southern blotting and **in situ** hybridisation techniques. It was mapped to Xq1-q22 and Ycen-pter. DXYS1 homologous sequences were found only on the X chromosome and not on the Y of chimpanzee, gorilla and orangutan. The explanation given for this is that DXYS1 homologous sequences were transposed from the X chromosome to the Y chromosome after the human line diverged.

Sequences with similar characteristics and mapping

position were later isolated by Bishop **et al** (1983) and (1984), Wolfe **et al** (1984a and 1984b), Geldwerth **et al** (1985), Koenig **et al** (1985) and Affara **et al** (1986a). Wolfe **et al** (1984a and 1984b) isolated probe p75/78 which was mapped to the Y chromosome and Xq13-qter and sister subclone p75/79 which recognises a male-specific band that mapped to Yq11-qter and three other bands, one of which was shown to derive from the Xq13-qter region and another one from Xq13-qter and the Y chromosome. Geldwerth's probes were more precisely mapped to the Ypter-Yq11 and Xq12-Xq22-24 regions and the homology between the X and Y sequences was estimated to be 97 to 99%. Most of these probes hybridised to the X chromosome of chimpanzee as was shown by Page **et al** (1984). Koenig's St25 probe, a 6.1kb Eco RI fragment was shown to contain a middle repetitive sequence homologous to a poly(A)+ 1.6kb long RNA species. The sequences flanking this repeat, specifically detecting the X and Y linked fragments, were subcloned into plasmids, termed St25/1 and St25/2 and then used for further experiments. The Y sequences corresponding to probe St25 were shown to be present in the DNA of 4 XX males out of 7. These XX males were shown to be positive for other X,Y probes. St25/1 probe which corresponds to a 2.1kb Taq I fragment detects a RFLP on the X chromosome with two allelic fragments 1.6 and 2.1kb long.

Koenig **et al** (1984) isolated a 2.1kb Eco RI/Pst I fragment the locus of which was named DXS31 and detected homology both on the X and the Y. The X fragment was localised in the region Xp22-Xpter and the Y fragment in Yq11-qter. The homology estimated between the X and the Y was about 80%. An indication for the existence of an X-linked RFLP detected with this probe was found only with the enzyme Hind III but further studies on a large number of females indicated that it seems to correspond to a rare variant in the caucasian population and therefore is not

useful in genetic analysis. DXS31 was tested against 4 XX males and 2 XX true hermaphrodites and was not found to be transferred, while the dosage of the X-linked fragments was identical to normal females.

The same probe tested against chimpanzee and lemur was shown to be present on the X and the Y chromosomes of chimpanzee and gave an identical Eco RI pattern as for humans but its location in lemurs seems to be autosomal. The presence of the same male-female differential pattern in chimpanzee and man indicates that the sequence was present in the Y chromosome before the divergence of the two species. The good conservation of the hybridisation signal between man and lemurs suggests that the DXS31 sequences are functionally important and could correspond to an expressed gene.

Cooke **et al** (1984) isolated a sequence fragment named pUC9H1 which was shown to be present on the Xq and Yq. The location of the X fragment was between Xq24-Xqter and the Y between Yq11-qter. Sequence analysis revealed no base changes in 1kb. This probe does not detect any homologous sequences in the mouse DNA. This result together with the sequence data, implies that the exchange of DNA sequences between the X and the Y chromosomes in the region of this sequence has occured relatively recently and has resulted in the sequence identity demonstrated in this paper.

Similarly Mueller **et al** (1983) while screening a human Y-enriched library for recombinants hybridising to both the X and the Y, isolated a clone which hybridised to a repeated 6.4kb Eco RI fragment from the Xq12-Xq21 and Ycen-Yq11 regions. A single-copy subclone was used for the isolation of homologous sequences from an X chromosome-specific library and a total genomic library. Restriction analysis and blotting experiments suggested that the sequences flanking this clone are different on the X and Y, respectively.

Probes which recognise homology between the X, the Y and autosomes were isolated by many groups of scientists and the homology they recognise between the X and the Y varies. The recombinants isolated by Bishop **et al** (1983) and (1984), and Affara **et al** (1986a) (probe GMGXY2 described in this thesis) recognise homology between the short arm of the Y and the long arm of the X in positions similar to the ones described for loci such as DXYS1 (Page **et al** 1984). Rappold **et al** (1984) mapped two probes using **in situ** hybridisation. Clone 2.8 was localised to Xq11, Xp2.2, Yp1.1 and Yq1.2 distal end. Clone YACG35 hybridised to the same X chromosomal subregions as clone 2.8, but on the Y chromosome only one binding site was found which was located on the long arm in the border region between the euchromatin and heterochromatin. Both probes hybridise to autosomes as well. Casanova **et al** (1985) isolated a human DNA sequence ($p12f_2$) which detects homology between the X, the Y and autosomes. The Y-specific DNA fragments detected by this probe were mapped to the region between Yq11.1 and Yq11.22 but the X and autosomally specific fragments were not mapped. In addition, this probe detected Y-specific polymorphisms with enzymes Eco RI (5.6/5.2/1.5kb and 5.6/3.2/1.5) and Taq I (10/8/4kb, and 8/8/4), thus belonging to another special category of recombinants which detect Y-specific polymorphisms. The data suggested that the size variations of the Y-specific Eco RI and Taq I DNA fragments detected, were generated by the same event, probably through an insertion/deletion mechanism involving a 2kb DNA sequence. The two invariant Eco RI fragments of 5.6 and 1.5kb and most of the autosomal fragments detected by probe $p12f_2$ were present in Eco RI digested DNA from four male chimpanzees and one male gorilla, thus suggesting the conservation of these sequences among primates. This result indicates that this insertion must have happened relatively recently (in evolutionary terms). The same group demonstrated the possibility of this probe being used in

estimating genetic distances within and between species.

1.8.5. Y-specific Sequences

A wealth of Y-specific probes have been isolated and located on either the short or the long arm of the Y chromosomes. Bishop **et al** (1983 and 1984) and Affara **et al** (1986a) isolated and mapped their probes to the short arm of the Y, while Wolfe **et al** (1984a and 1984b) mapped a Y-specific probe to Yq11-qter, and Burk **et al** (1985) mapped another probe between Yq11 and the Y chromosome subcentromeric region. The pattern and intensities obtained at high stringency by the last probe indicated that in humans this fragment is single copy. It was not detected in female apes, therefore being specific for the Y chromosome of this species as well. The hybridisation pattern obtained with DNA from the chimpanzee is identical to that in humans, although differences in intensity can be detected between some bands. The restriction pattern obtained with gorilla DNA suggested a shorter fragment whereas the restriction pattern obtained with orangutan suggested a longer fragment with homology for this human Y fragment. This could be due to insertion-deletion events. No homology was detected with male mouse or chinese hamster DNA at high or low stringencies. In Macaccus old monkey, equivalent hybridisation was seen with both male and female DNAs. The heterogeneous nature of its hybridisation suggested that the related sequences in this species are highly repeated.

Both Muller **et al** (1986) and Affara **et al** (1986a) isolated Y-specific probes which seem to be moderate repeats, are located on the short arm of the Y chromosome and seem to be present in the genome of some XX males. This point will be more extensively discussed in section 1.8.7.. These clustered repeats form a new class of sequences on the Y chromosome.

1.8.6. Pseudoautosomal Sequences

The sequences described below form a very special group with regard to their homology with the X and the Y chromosome, for which only recently information has been accumulated.

First Cooke **et al** (1985) isolated a human sequence present on the tips of the X and Y chromosomes, which is telomere-associated, and is repeated only a few times at each telomere. Telomeres are DNA sequences which ensure the integrity of linear chromosomes through meiosis and mitosis. The probe called 29C1 is a subclone from a cosmid, CY29 which was isolated from a cosmid library constructed from the DNA of 3E7 (a mouse-human hybrid carrying multiple Y chromosomes and used in this study as well). CY29 was isolated during a screen for CpG-rich sequences on the human Y chromosome. These sequences are frequently associated with genes (Bird **et al** 1985). Analysis was done to find out how polymorphic the sequence is and whether the X is homologous to this probe. The results showed that out of 23 unrelated individuals, digested with three different enzymes, there were no two individuals having identical patterns and since all the Pst fragments detected by this probe (which is itself a Pst fragment) were shown to be polymorphic this probe could not be flanking a polymorphic region but should itself be polymorphic. As these polymorphisms were detected by many enzymes they cannot be point mutations in restriction sites. The copy number of this sequence was estimated to be between 3 to 10 copies per haploid genome. The probe seems to define a boundary between the hypervariable and non-variable regions of the X and Y chromosomes. Family studies were done using this probe to further comfirm the localisation of the sequence on both the X and the Y chromosomes and showed X/Y recombination which is consistant with there being an obligate recombination event. Recombination was shown to be

proximal to the region detected by the probe in the families studied for this paper. The polymorphic DNA locus detected by 29C1 is now called DXYS14.

Simmler **et al** (1985) isolated a single-copy fragment, named 113D, from a Y chromosome derived clone and assigned it to both the X and the Y chromosomes. The restriction fragments detected by this and two more probes from the same clone cover more than 50 identical restriction sites. It thus appears that none of the fragments from this locus (now called DXYS15) display restriction sites occuring exclusively on only one of the sex chromosomes. A Taq I restriction fragment length polymorphism was observed in both male and female DNAs. Male homozygosity with a minimum of four allelic forms was observed indicating that these allelic forms are shared by both sex chromosomes. The presence of four allelic forms of one sequence with copies on both the X and the Y chromosomes, strongly supports ancient homology of these regions, which continues to behave as autosomal sequences (pseudoautosomal behaviour). The detection of two X/Y recombinants among five progeny was consistent with the 50% recombination required for a pseudoautosomal sequence, but the sample is still too small to rule out the possibility of partial sex linkage.

From the same clone another probe, 113F was isolated which detects a sequence repeated 15-20 times per haploid genome. This repeat, DXYZ2 was shown to map exclusively to the short arm, terminal region of each sex chromosome and appears to be pseudoautosomal. The repeats seem to be interspersed and were shown to hybridise to distantly related mammals such as cattle, but not rodents, under identical conditions of stringency. Such inter- and intraspecific sequence conservation suggests the possibility of some DXYZ2-associated functions in biological processes, for example: telomeric recognition, X non-inactivation or X/Y crossing-over (Simmler **et al** 1985).

Another pseudoautosomal locus DXYS17 was defined by a probe isolated by Rouyer **et al** (1986) and was shown to detect highly polymorphic DNA sequences. The same group used this probe and the other two (29C1 and 113D) presented just above to do family studies. They demonstrated several characteristics of pseudoautosomal sequences such as: 1) they can be exchanged between the X and the Y chromosomes by a crossing over mechanism, rather then by gene conversion, 2) they can exhibit partial sex linkage, the extend of the linkage being different for three independent pseudoautosomal loci and 3) the pseudoautosomal region is characterised by a high recombination activity (10 fold higher recombination frequency in males compared to females) and thus lends itself to extensive mapping. This mapping is facilitated by the occurence of a single crossing over per meiosis taking place at varying locations in the region. They estimated that it seems unlikely that the pseudoautosomal region streches more then 5,000kb.

Finally, Affara **et al** (1986a) isolated a probe named GMGXY1 which seems to acquire the properties of a pseudoautosomal sequence.

1.8.7. A Molecular Analysis of the XX Male Genome

In 1984 De la Chapelle **et al** presented the case of an XX male who expressed his father's 12E7, a Y-linked marker, but failed to express his father's allele for Xg, an X-linked marker. This finding suggested that an interchange had taken place between the X and the Y chromosome. The authors suggested that the breakpoints on the XX males might vary between these individuals. However the first demonstration at the molecular level of Y material being transferred from the Y to the X was performed by Guellaen **et al** in 1984. They studied the cases of four XX males with Y-specific probes and found that three of the four cases

were positive for these probes. They observed, however that not all the probes were present on every individual therefore suggesting genetic heterogeneity. This was also the first attempt to order the probes along the Y chromosome. Furthermore the mother of one of the XX males positive for one of the probes was herself negative for the same probe, therefore excluding maternal inheritance. The authors pointed out that although there was an XX male who was negative for these Y-specific probes, this does not exclude the possibility of Y chromosome material being present but not detected with the probes. In 1985 Page **et al** studied a family with three XX males that De la Chapelle **et al** presented in 1977 as evidence for autosomal recessive inheritance, using a series of Y-specific probes. The findings gave proof that this was not an autosomal recessive inheritance of maleness but that all three XX males carry Y-specific sequences as a result of new mutations. Moreover, none of the three XX males are positive for all of the Y-specific sequences tested here. The authors concluded that each of these XX males carry only a part of the Y chromosome. They also found no maternal contribution to the XX maleness in this family although the mother had previously been said to be H-Y positive. In a previous paper by Page and de la Chapelle (1984) both the maternal and paternal contributions were demonstrated with the use of X-linked RFLPs in at least five out of the seven families of XX males that were tested. In the other two families, paternal contributions was not determined but could not be ruled out either. Vergnaud **et al** (1986) tested more Y-specific probes on the genomes of the two XX males which are second cousins (from the same family discussed just above) and were still unable to differentiate between the portions of the Y chromosome that they possess. They were also unable to detect Y DNA sequences in the mothers of the two related XX males.

Muller **et al** (1986) isolated four Y-specific probes, which seem to be moderately repeated. Two of these probes were assigned to the middle of the short arm of the Y chromosome at band Yp11.2 by **in situ** hybridisation and were both detected in five out of seven XX males and were reduced in copy number in one out of two 46,XY gonadal dysgenesis patients. In addition, the hybridisation signal of one of these two probes that detects one single band, was weaker in the 46,XX males than in 46,XY control males. These findings suggest the close proximity of the two moderately repeated sequences to testis determining genes. Similarly Affara **et al** (1986a) isolated three probes which seem to be clustered repeats from the short arm of the Y chromosomes and showed that only some of the bands they detect were present on some XX males while the rest of them were absent. This served as valuable information for the localisation of these probes on the short arm of the Y chromosome.

Vergnaud **et al** (1986) analysed by hybridisation the genome of 19 XX males, two XX hermaphrodites and six persons with microscopically detectable anomalies of the Y chromosome for the presence or absence of 23 Y-specific DNA fragments many of which have been reported above. No Y-specific DNA sequences were detected in nine individuals (the two hermaphrodites and seven XX males) but the presence of one or more Y-specific restriction fragments were detected in the genome of the rest of the XX males and the individuals with deletions of the Y chromosome. In this way they constructed a deletion map of the Y-specific DNA sequences. Their data is consistent with the idea that, in all of these cases tested, only a single contiguous portion of the Y chromosome is present; that is, the Y-specific sequences can be ordered so that, in each of the patients tested, the Y sequences present are a single, uninterrupted cluster. They divided the Y chromosome into seven intervals which can be ordered in different ways, one of which is

consistent with each of the XX males studied having received a terminal, contiguous portion of the Y chromosome, that is one which includes a telomere. Following this terminal model and assuming that the XX males have testes because of the presence of a male-determining portion of the Y chromosome, they proposed that the male determinant can be mapped in interval 1 which in one of their models includes the telomere. However they proposed another internal model in which it is conceivable that these XX males have received internal, contiguous portions of the Y chromosome and in one of the variations of this model interval 1 was placed near the centromere. In summary, deletion intervals 1, 2, and 3 are clearly within Yp but cannot be definitively ordered with respect to each other. Interval 1 contains the testis-determining gene(s), interval 4 contains the centromere, although some probes classed in interval 4 could, in principle, be located distal to intervals 1, 2, and 3 on Yp. Intervals 5, 6, and 7 are on the Yq.

Affara **et al** (1986b) examined the genome of twelve XX males and their relatives with fourteen different Y probes some of which were Y long arm probes. They used flow sorted chromosomes to show that the Y probes were actually hybridising to the X chromosome and not to an autosome. The long arm probes were not present in the genome of these XX males therefore diminishing (but not excluding) the possibility that male determination in these individuals is due to some sort of mosaicism which involves a normal Y chromosome. Only two individuals were shown to lack all of the Y probes used in this study while the rest of the XX males were shown to carry some but not all of the probes. The mothers of some of the XX males were also tested with these probes but were all shown to be negative. Affara **et al** were able from these results to reorder some of the probes used here, in such a way that the interchange would always include the telomere of the Y chromosome. Following

this order of probes, only two XX males could not be explained with a single crossover because they were shown to transfer sequences from the proximal part of the short arm of the Y chromosome without transferring any of the other probes distal to these. The authors proposed that the order of sequences on the Y chromosome is not invariant, since the pressure of recombination in maintaining the colinearity of homologous chromosomes is not operative for the non-pairing region of the Y chromosome. In as much as rearrangements do not interfere with important functions, then variations in the order of sequences on the Y chromosome will be tolerated. It is suggested that inversion polymorphisms may occur in the Y chromosomes of normal males, and these rearrangements are revealed when either deleted Y chromosomes or XX males are examined with a series of probes. The authors proposed that an inversion is present in the Y chromosome of the fathers of these two XX males and were able to show, that given that this was true, only a single recombination event was necessary to generate the pattern of Yp probes observed in these XX males.

More recently Andersson **et al** (1986) published their results on a probe which detects a Y-specific family of DNA sequences. Most of the sequences homologous to this probe were shown to be localised on Yp (and are present in some XX males), while other homologous sequences are on Yq (Page unpublished results). Three XX males and two normal males were tested by **in situ** hybridisation for the presence and location of this probe. The location of this probe on the short and the long arm of the Y chromosome (Yp and Yq11) was confirmed and in all three XX males the grains were clustered on the terminal band Xp22. These results confirm that XX maleness in these cases arises through an X-Y interchange involving the testis determining factor, confirming the earlier results of Magenis **et al** (1984).

1.9. Hybrid Formation and Selective Systems Used in This Study

Fusion of human and mouse cells is achieved with the use of polyethyleneglycol (PEG). PEG is a nonionic, polymeric, surfactant which acts on the interacting cell membranes leading to the formation of heterokaryons (Pontecorvo 1976). These kind of cells tend to loose chromosomes and in the case of human-mouse hybrids it is the human chromosomes that preferably disappear, but the reason for this is unknown. However it is possible to select for three human chromosomes number 16, 17, and X by using certain selection systems based on the principles of purine and pyrimidine salvage pathways.*

Mammalian cells can synthesise purines and pyrimidines **de novo** but they also have the enzymes which are needed for the salvage of purines and pyrimidines from the degradation of nucleic acids and from food. The cells prefer to use purines and pyrimidines that derive from the outside environment rather than make their own but probably most cells utilise both pathways to a certain extent.

In this study the two important enzymes are HGPRT (hypoxanthine guanine phosphoribosyl transferase) and TK (thymidine kinase), the first is located on the long arm of chromosome X and the second one is located on chromosome 17. This makes it possible to select for these chromosomes using the HAT selection medium as will be explain directly below. Figures 4 and 5 illustrate the steps involved in the selection of hybrids carrying chromosomes X or 17 respectively.

Mouse cells which are $HGPRT^-$ are fused with human cells which are $HGPRT^+$ since they carry the X chromosome. In $HGPRT^-$ mouse cells the purine salvage pathway does not work therefore they need to synthesise purines **de novo**. However, after the fusion these cells are put in to the HAT selective medium together with ouabain. The use of ouabain,

* Further chromosomal selection systems are available e.g. Tunnacliffe **et al** 1983.

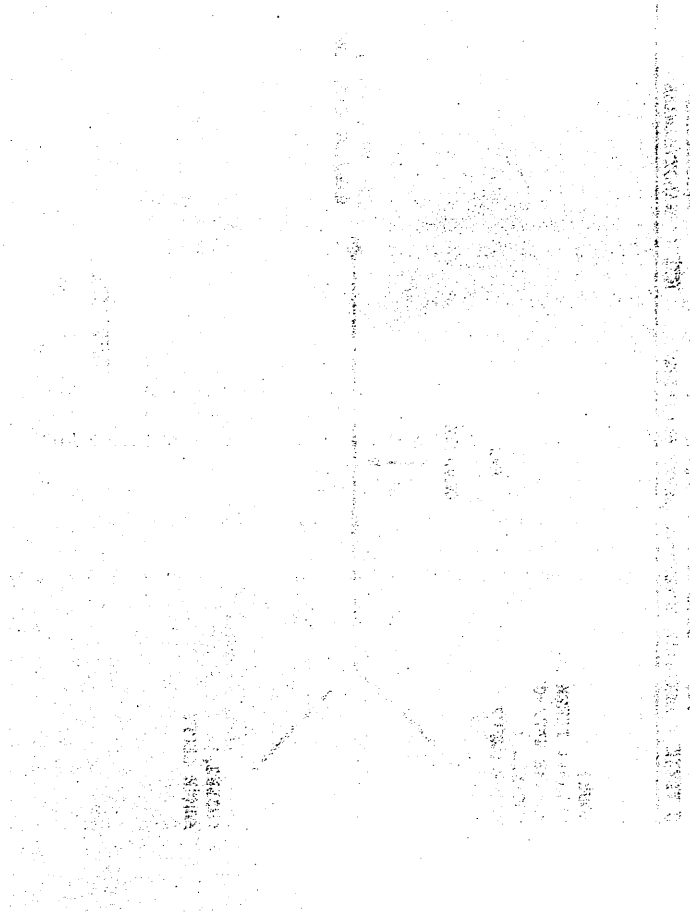
which is added to the medium in order to eliminate human parental cells, takes advantage of the differential toxicity of the drug. The drug inhibits plasma-membrane mediated, ATPase-dependent, K^+ intake and Na^+ output. Human cells are sensitive to ouabain concentrations ranging from $3x10^{-8}$ to 10^{-7}M while mouse L cells show sensitivity at 10^{-3}M (Chu and Powell 1976). The HAT selection system (which has hypoxanthine, aminopterin and thymidine) eliminates the $HGPRT^-$ deficient mouse parental cells. The antimetabolite aminopterin blocks the **de novo** synthesis of purines and the methylation of desoxyuridilic acid for the formation of thymidilic acid which is important in the selection of hybrids from TK^- cells as discussed below. The drug is a specific inhibitor of folic acid reductase. This enzyme catalyzes the synthesis of reduced folate which is required at several steps in purine and pyrimidine biosynthesis. Cells which grow in the presence of aminopterin depend therefore on exogenous sources of purines and thymidine. Hypoxanthine can act as an exogenous source of purines as long as there is HGPRT to convert it to inosinic acid. Therefore media which contain these three will only allow the growth of $HGPRT^+$ cells but not $HGPRT^-$. Mouse $HGPRT^-$ cells die and only hybrid cells which conserve the HGPRT locus (therefore the long arm of the X chromosome) survive.

As was explained before, cells in the presence of aminopterin depend on an exogenous source of thymidine which is also present in HAT allowing TK^+ cells to survive while TK^- cells die. Thus with the HAT selective system also it is possible to select for hybrids which retain chromosome 17.

If it is necessary to eliminate the normal X or that part of the X which carries the HGPRT gene then counterselection may be achieved using 6-thioguanine. The cells are left in non selective medium for some time and then medium with 6-thioguanine is added to the cultures. 6-thioguanine is a guanine analogue, therefore this

antimetabolite is allowed to enter into some guanine reactions. 6-thioguanine is then incoorporated into the DNA and HGPRT$^+$ cells which can utilise the purine salvage pathway die. Cells which are HGPRT$^-$ cannot use 6-thioguanine therefore can survive and grow (information taken from Chu and Powell 1976).

The HGPRT selection system described above was used in the construction of a somatic cell hybrid panel which contained different parts of the X chromosome. The TK selection system was used as an alternative method where it was not possible to select for HGPRT, as this locus was deleted on the rearranged chromosome.



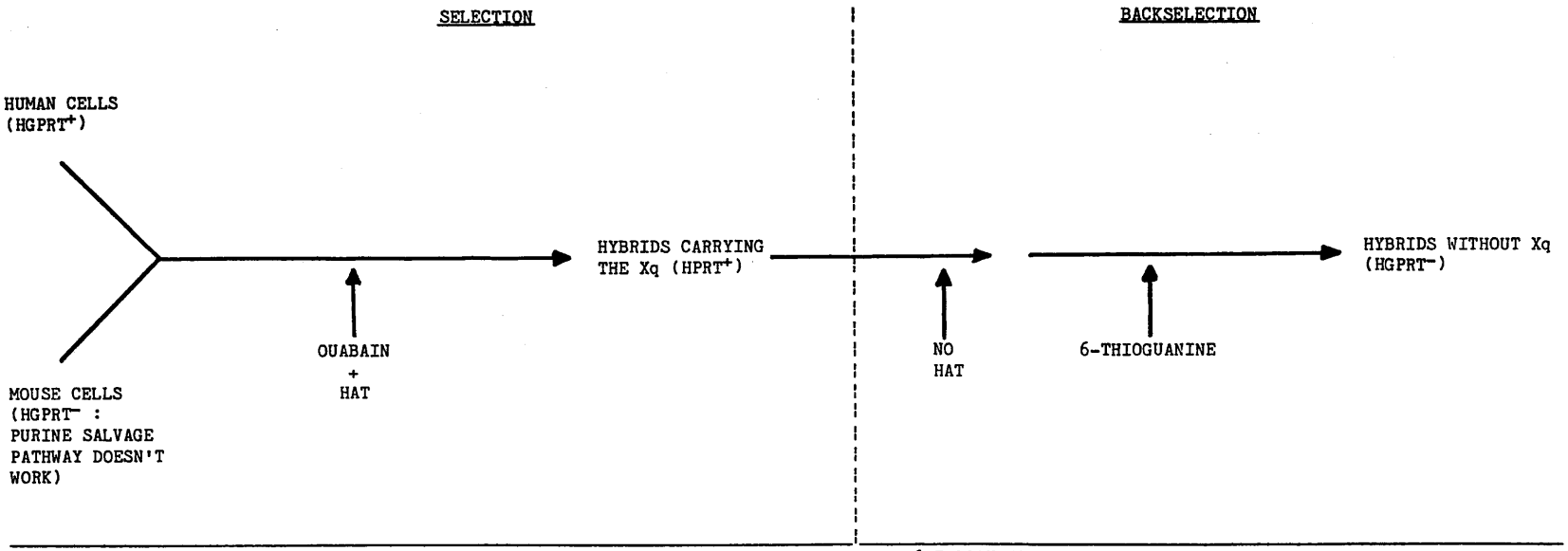
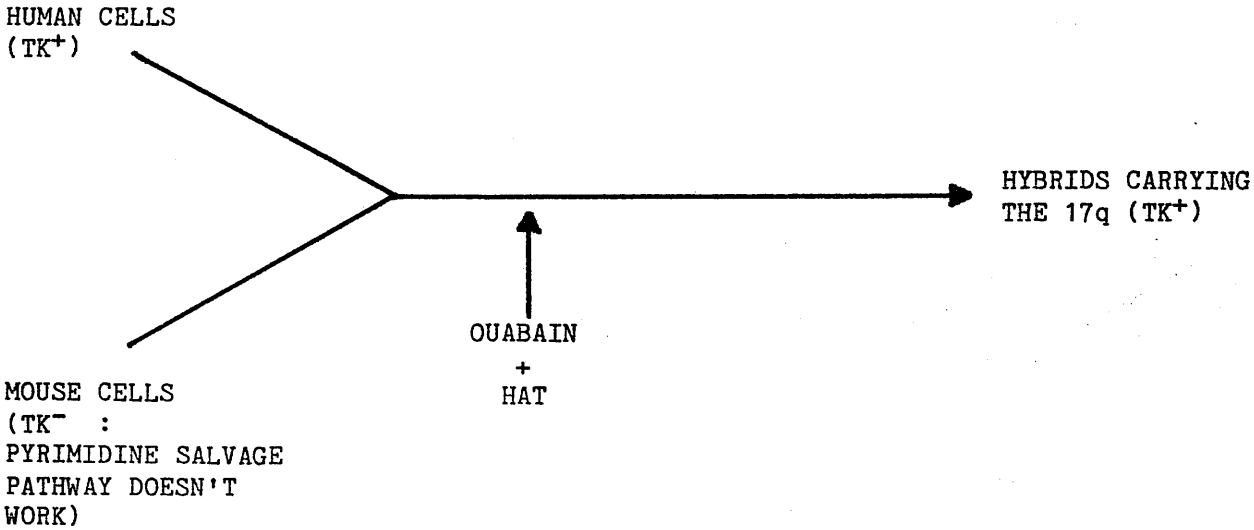


FIGURE 4 : DIAGRAMMATICAL REPRESENTATION OF THE HGPRT SELECTION/BACKSELECTION SYSTEM USED TO OBTAIN MOUSE/HUMAN HYBRID CELL LINES



OUABAIN : INHIBITS PLASMA-MEMBRANE MEDIATED, ATPase-DEPENDANT K^+ INTAKE AND Na^+ OUTPUT (KILLS HUMAN CELLS)	HAT : HYPOXANTHINE AMINOPTERIN THYMIDINE (SELECTS FOR TK^+ CELLS)

FIGURE 5 : DIAGRAMMATICAL REPRESENTATION OF THE TK SELECTION SYSTEM USED TO OBTAIN MOUSE/HUMAN HYBRID CELL LINES

The aims of this project

The aims of the thesis were set against a background of information dating from the beginning of 1983. These are:

a. To isolate and purify single-copy sequences from a Y-specific library, following a strategy of screening that would favor isolation of X-Y homologous sequences particularly those mapping to the tip of the short arm of both chromosomes.

b. To map these single-copy sequences to the X and Y chromosomes and to identify possible autosomal homologies.

c. To use these sequences to study a number of XX males in order to clarify the nature of this syndrome, and ultimately to map and identify testis determining factors.

In order to achieve the above aims a large part of this study was dedicated to the construction of three main panels:

1. a panel of hybrids bearing different parts of the X chromosome,
2. a panel of genomic DNAs from individuals bearing abnormalities of the Y chromosome, and
3. a panel of genomic DNAs from XX males and some of their relatives.

In addition DNA from flow sorted chromosomes and a panel of somatic cell hybrids bearing different group of autosomes were also used in order to map the autosomally linked fragments isolated in this study

It has to be appreciated that it would have been impossible for one individual to produce all of the material necessary for such an undertaking. Therefore the collaboration of a number of my colleagues was necessary, and this is fully acknowledged at the beginning of this thesis.

MATERIALS AND METHODS

2.1. General

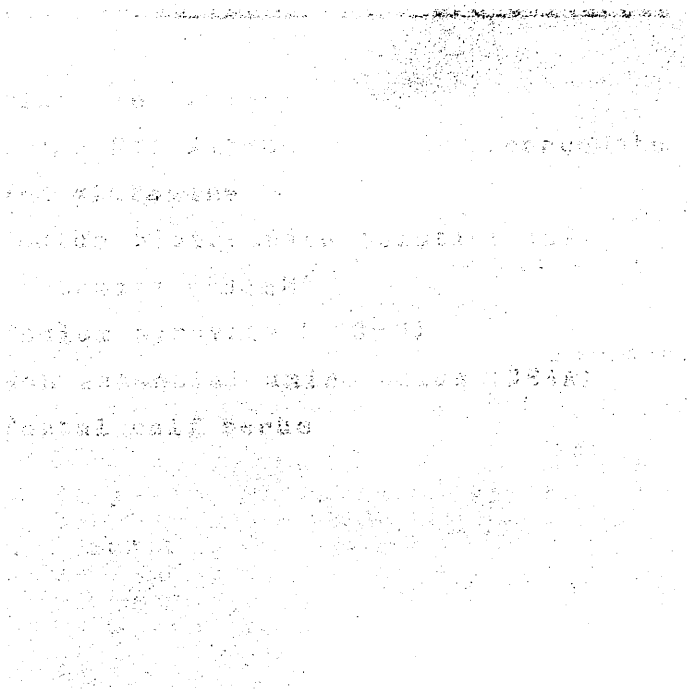
All aqueous solutions were prepared in chelated, sterile, deionised, distilled water (CSDWD), and unless otherwise stated in the text were sterilised by autoclaving in a pressure cooker.

All glassware, most plastics and all tips for micropipetting were sterilised by autoclaving.

Centrifugation was carried out on a Sorvall RC-5B Refrigerated Superspeed centrifuge, an Eppendorf or Damon type microcentrifuge, or on an MSE bench centrifuge using appropriate rotors as specified in the text.

All 15ml and 30ml tubes, also all Eppendorf tubes and tips for micropipetting are Sarstedt type.

Universals and bijoux are Sterilin 128B and 129C type.



2.2. Culture Media

All constituents were sterilised.

Ef (medium for culturing mouse cell lines)

150ml Distilled water
18ml MEM Eagle (Glasgow modification) without sodium bicarbonate and glutamine
10ml Sodium bicarbonate solution (5%)
2ml Glutamine (200mM)
20ml Foetal calf serum

PM (basal medium for the maintenance of somatic cell hybrids)

As Ef with the addition of:
2ml Non essential amino acids (Gibco 16-816)
2ml Sodium pyruvate (100mM)

F-12 (medium in which fused cells are cultured)

150ml Distilled water
18ml Ham's F12 without sodium bicarbonate and glutamine
6.5ml Sodium bicarbonate solution (5%)
2ml Glutamine (200mM)
2ml Sodium pyruvate (100mM)
2ml Non essential amino acids (NEAA)
20ml Foetal calf serum

F-12 Selection medium (for culture of fused hybrids)

For use as a selective medium for the culture of human/mouse fusion hybrids, the selective agents HAT and Ouabain (see below) were added to the normal F-12 medium described above.

HANKS balanced salt solution

$CaCl_2.2H_2O$	185.5mg/l
KCl	400mg/l
KH_2PO_4	600mg/l
$MgSO_4.7H_2O$	200mg/l
NaCl	8000mg/l
$NaHCO_3$	350mg/l
Na_2HPO_4	47.5mg/l
Glucose	1000mg/l
Sodium phenol red	17mg/l

Selective Agents

HAT

This was used for the selection of human chromosome 17 when the rodent line is TK- (LMTK) or human chromosome X when the rodent line is HPRT- (LA9).

The stock solutions which were stored at -20°C are the following:

Hypoxanthine	10^{-2}M
Aminopterin	10^{-3}M
Thymidine	10^{-2}M

Hypoxanthine and thymidine was first dissolved in a few mls of 0.2M NaOH. HAT was prepared from these stock solutions by adding 3.2ml thymidine and 1.6ml aminopterin to 20ml of hypoxanthine. This was filter sterilised and stored at 4°C.

Ouabain

This was used for the selection against human cells. The stock solution was 10^{-4}M (7.3mg/100ml) and was stored at 4°C, protected from the light. 2ml of this solution was added to 200ml of medium, to achieve a final concentration of 1µM.

6-thioguanine (Sigma A4882)

This was used for selection against human chromosome X. The stock solution was 200µg/ml and was stored at room temperature. 6ml of this was added to 200ml of medium, to achieve a final concentration of 6µg/ml. Only cells which have been grown in medium without selective agents for three weeks can be treated with 6-thioguanine.

2.3. Reagents

McIlvainie's buffer (pH 7.0 and 4.5)

0.1M citric acid was added to 16.47ml of 0.2M Na_2HPO_4 to obtain a final volume of 20ml. The pH was checked and adjusted to 7.0.

0.1M citric acid was added to 9.0ml of 0.2M Na_2HPO_4 to obtain a final volume of 20ml. The pH was checked and adjusted to 4.5.

Versene buffer solution

For 2 litres of 10x stock solution:

NaCl	160.0g
KCl	8.0g
Versene	4.0g
Phenol Red	0.4g

Phenol Red was dissolved in ~100ml of water before being added to the dissolved salts.

200ml of 10x stock solution was diluted in 1,900ml distilled water and sterilised by autoclaving. It was stored at 4°C. The pH was adjusted to 7.4 with sodium bicarbonate.

Trypsin versene buffer

To make a stock solution, 5g of trypsin was dissolved in 2,000ml of versene buffer (diluted form). This was sterilised by millipore filtration and stored at -20°C.

2ml of stock trypsin versene buffer was added to 30ml versene buffer solution. The pH was adjusted to 7.6 with sodium bicarbonate.

100xDenhardt's solution

2% Ficoll
2% Bovine serum albumin
2% Polyvinylpyrrolidone

20xSSC (per 5 litres)

441g	Tris.HCl (pH 7.2)
877.5ml	NaCl

'E' buffer

40mM Tris-acetate pH 8.0
20mM Sodium acetate or Potassium acetate
1mM Na EDTA

TE

10mM Tris pH.8
1mM EDTA pH.8

Phage Dilution Buffer

0.1M Tris.HCl pH.8
0.1M $MgSO_4$
0.1% gelatine w/v

L-broth (per litre)

10g Bacto-tryptone
5g Bacto-yeast
10g NaCl
1g Maltose
10mM $MgSO_4$

Maltose: 20% stock, filter sterilised.
Bottom agar: 15g of agar was added per litre of L-broth.
Top agar(ose): 7g of agar(ose) was added per litre of L-broth.
After autoclaving, the top agar was cooled to 47°C before use.

Ampicillin (Amp)

The concentration of the stock solution was 25mg/ml and was made by adding 0.5mg Amp to ~10ml H_2O, and by adding NaOH dropwise until the solution was clear. The volume was made up to 20ml with H_2O and stored at -20°C.

X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside)

The stock solution was made by dissolving 100mg X-Gal in 5ml dimethylformamide (D.M.F.), and was stored at -20°C.

Phenol

The phenol was distilled and 8-hydroxyquinoline was added to it to give a yellow colour. Changes in the colour indicate that the phenol has been oxidised and cannot subsequently be used for DNA extractions. To avoid oxidation of the phenol the container was wrapped in aluminium foil and kept in a dark place.

Formamide

Formamide was deionised by stirring at 4°C for 4-5 hours with 3-5g/100ml of Bio-Rad mixed bed resin (20-50 mesh). Once deionised it was stored at -20°C.

2.4. Cell Lines

Somatic cell hybrids were obtained by fusion of mouse LA9 or LMTK cells to human fibroblasts or lymphoblastoid cells. The LA9 mouse cell line is deficient in the HPRT and APRT locus and the LMTK cell line is deficient in the TK locus.

The human fibroblast or lymphoblastoid cell lines used for fusion in this study were obtained from individuals with abnormal karyotypes involving the X chromosome. Listed below are the human parental lines used, the mouse parental lines they were fused with, their karyotypes, and the derived hybrids for which cytogenetic and DNA analysis was done.

EH:	46,XX,t(X;11)(p22;p15)	EHA97II/VIIIt4(4(9))
*W5:	46,XX,t(1;X)(p32;Xp21)	W5A915IXt4(7)
*W2:	46,XX,t(X;8)(p21;q24)	W2A96It6(7)
LN:	46,XXp-	LNA94IRbXIIIt4(6(9))
*CE:	46,XXp-	CETK1aIVt4(7)
NE:	46,XX,t(X;11)(q13;p13)	NEA916IIt3(10)
		NEA921R_2bt11
FN:	46,XX,t(X;19)(q24;q13)	FNA98IXt4(3),
		FNA92bIIRa$_1$It6(13(3))
DH:	45,Xter rea(X;9)(q27;p23)	DHTK18a
WH:	46,Xdel(X)(q24)	WHTK17IIIt5(6)
HN:	46,Xdel(X)(q13)	HNTK6VII/It4(4(11))

(* denotes lymphoblastoid cell lines)

Diagrams of the rearranged chromosomes described above can be found in Figures 6 to 11.

Cell line W5 was reported by Lindenbaum **et al** (1979) and contains a paracentric inversion between Xp21 and Xp11.

Human parental lines CE, DH, WH, and HN were fused with mouse LMTK cell line, while the rest were fused with LA9 cell line.

NEA921R_2b and FNA92bIIRa$_1$I are backselection products from hybrid cell lines NEA921 and FNA92bII respectively (both lines isolated in this study).

Seven mouse-human hybrids produced during these fusions were selected because of their different autosomal contents. These are:

NEA915RBVIB$_{t(10)7}$
FNA95R2$_{t10}$
NEA918$_{t5}$
EHA97III$_{t4(6)}$
AMIR2XI$_{t10(6)}$
LNA94IRbXII$_{t4(6(5))}$
EHA97VIII$_{t4(6)}$

The following rodent-human hybrids which were also used in this study, were gifts and are described in Goodfellow **et al** (1982), Goodfellow **et al** (1983), Burk **et al** (1985).

AMIR2N contained the X derivative of an X:Y translocation with breakpoints in Xp22.3 and Yq11.21.

Horl9X contained the X as the only detectable human chromosome.

Hybrid cell lines 3E7 and 7631 contained the Y as the only detectable human chromosome. The former was isolated by Dr. M. Marcus and Dr. R. Voss from the hybrid described by Marcus **et al** (1976); the latter was described in Shalev **et al** (1977), and was a gift of Dr. J. Hamerton.

THYB133R contained chromosome 21 as the only human detectable chromosome. This hybrid is a derivative of THYB133 (Goodfellow **et al** 1980) and was obtained from Dr. C. Bostock.

The lymphoblastoid cell lines used carrying Y chromosome abnormalities were the following:

A1 cell line was derived from a adult male with 46,XYqh-.

DR, and RN cell lines was derived from normal fertile males with a particularly small distal long arm heterochromatic segment, evident on Q and C banding. The karyotype was described as 46,XYqh-.

SN cell line was derived from an infertile male with short stature, hypospadias and absence of the right testis. Testicular biopsy showed the absence of germ cells (Ferguson-Smith 1976). Chromosome analysis showed 45,X/46,Xdic(Y)(q11.23).

FF cell line was derived from an azoospermic patient with normal stature and genitalia. Testicular biopsy showed few germ cells which were arrested at the primary spermatocyte stage (Ferguson-Smith 1976). Chromosome analysis showed 45,X/46,Xdic(Y)(q11.23).

WC cell line was derived from an individual who was a male of normal stature and appearance, testicular biopsy, however, revealed the complete absence of germ cells. Chromosome analysis revealed the presence of a minute metacentric Y chromosome which was regarded as an isochromosome for the short arm. Only one centromere was apparent on C banding and there was no evidence of chromosomal mosaicism (Ferguson-Smith 1976). The karyotype was therefore 46,X,i(Yp)

ED cell line was derived from a female patient with secondary amenorrhea who was found on further investigation to have an abnormal karyotype with 48 chromosomes (unpublished). In addition to the two normal X chromosomes, there were two identical large Y chromosomes, interpreted as isodicentrics for the long arm and proximal short arm with a breakpoint at Yp11.2. The karyotype was 48,XX,dic(Y)(p11.2)dic(Y)(p11.2).

AMIR2N somatic cell hybrid line was described above. The human parental line from which this hybrid was derived came from a male patient (ME) with X-linked ichthyosis who was nullisomic at the Xg and steroid sulphatase loci (Ferguson-Smith **et al** 1982). His full karyotype was 46,Yt(X;Y)(p22.3;q11.2). The rearranged chromosome present in the mother of this patient is shown in Figure 7.

DNA was extracted from all of these hybrid and lymphoblastoid cell lines, from the mouse cell lines LA9 and LMTK and also from normal male and female individuals.

Two basic panels were constructed in order to map the probes isolated from the Y library, these were called the X panel and the Y panel.

The DNA samples used in the basic X panel were normal female, normal male, Horl9X, 3E7, AMIR2N, NEA9, FNA9, WC, ED, and A9. Variations of this panel were used and these are described in the Figures in the Results chapter. This panel was used in order to find out whether the isolated probes were X, Y or autosome derived and also whether they were repetitive or not. A more complete X panel was used in order to map X-linked probes. This panel included all the hybrids produced in this study as described above.

The Y panel included all the cell lines bearing abnormalities of the Y chromosome (described above), a normal male and female and the hybrids 3E7 and 7631.

A panel of DNAs was constructed to screen for restriction fragment length polymorphism (RFLP) and consisted of the DNAs from four normal males and one normal female. These DNAs were digested with 15 different enzymes as follows : Bam HI, Bgl I, Bgl II, Eco RI, Hind III, Pst I, Pvu II, Sma I, Xba I, Bst NI, Hae III, Hpa II, Msp I , Taq I, and Cfo I.

DNA was also isolated from blood samples of eleven XX males, one true hermaphrodite, 3 XY females and some of their relatives. Extensive chromosome analysis of the peripheral blood lymphocytes from these individuals was done in order to exclude XX/XY or XX/XXY mosaicism.

The lymphoblastoid cell lines from which chromosomes were sorted were:
CN a 46,XX individual with a deletion of part of the long arm of the X chromosome and a chromosome 1 centric polymorphism.
ST a 46,XY individual with a deletion of part of band Xp2.1 and a chromosome 1 centric polymorphism.
RH and HM both 46,XX males.

An X chromosome dosage panel was constructed from the DNA of individuals bearing different numbers of sex chromosomes: SL (48,XXXY), HN (48,XXYY), HS (47,XYY), BE (47,XYY), DE (45,XO), McN (47,XXY), McE (48,XXXX).

A list of the polymorphic X chromosome probes used in this study and a description of the RFLPs detected by them is given in Table 1 (information from HGM 8, 1985; Drayna and White, 1985; Davies 1985 and van Ommen **et al** 1986).

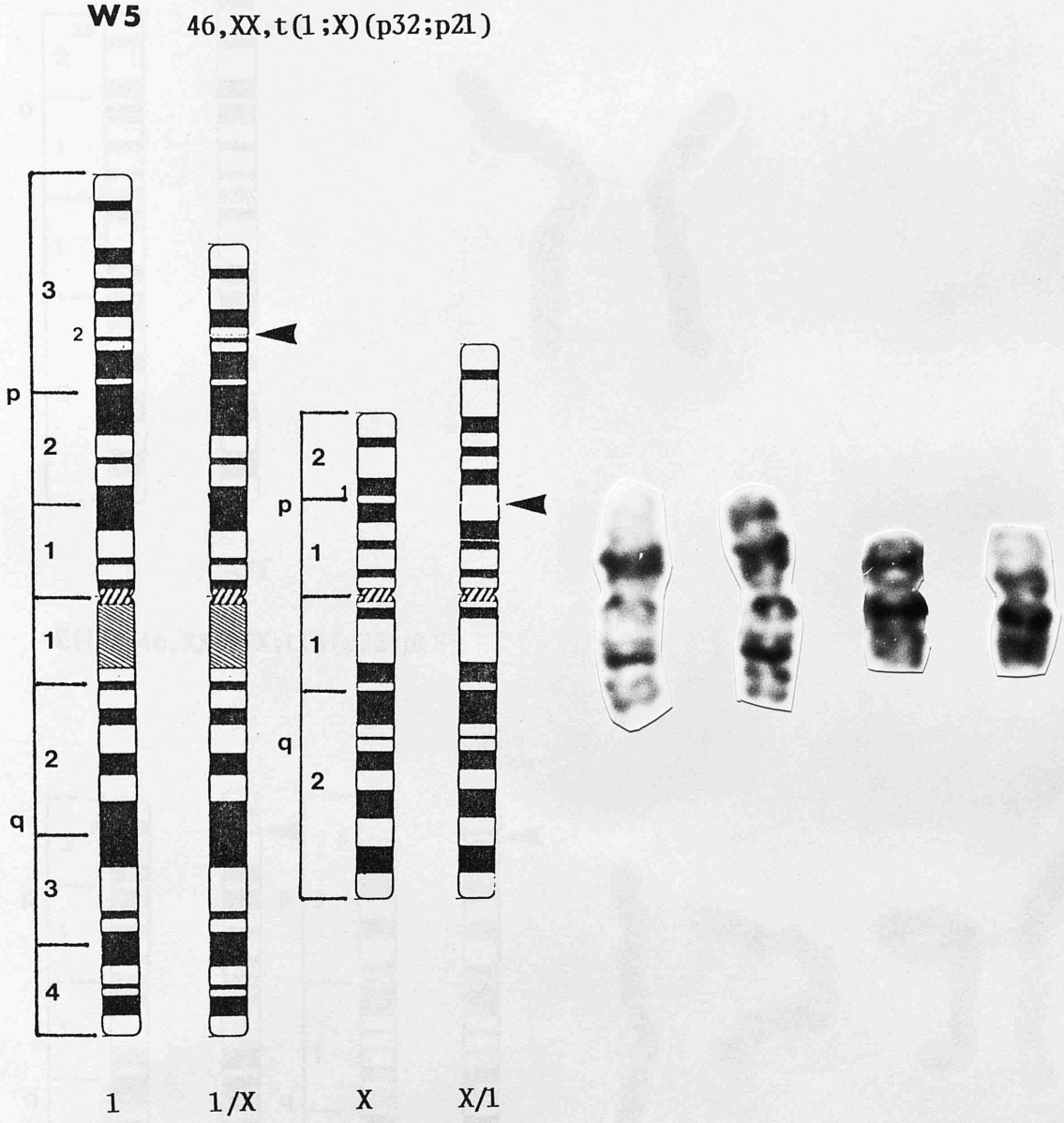
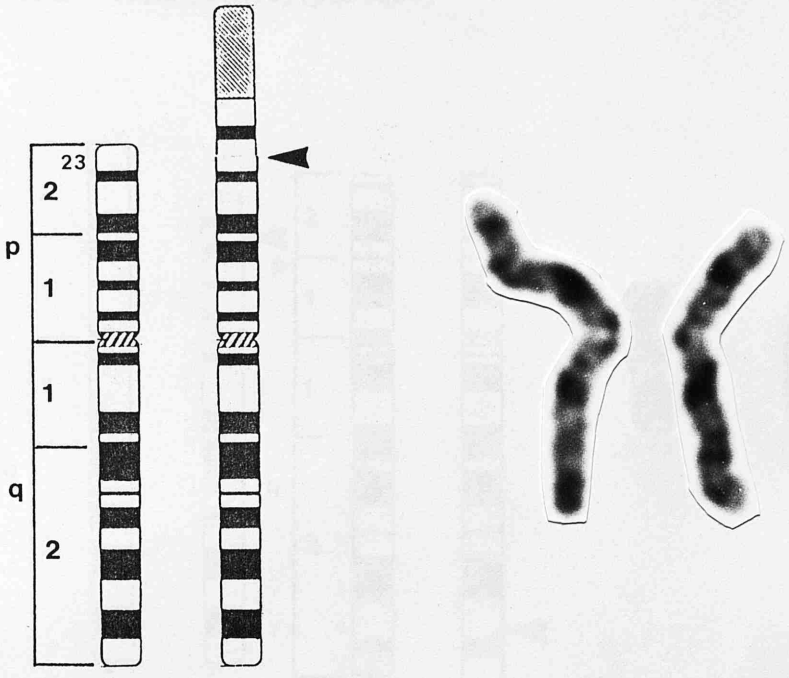


FIGURE 6: Rearranged chromosomes from patient W5. This case is reported in Lindenbaum **et al** (1979). There is an additional paracentric inversion between Xp21 to Xp11 on the X/1 chromosome.

ME 46,Xt(X;Y)(p22.3;q11.2)



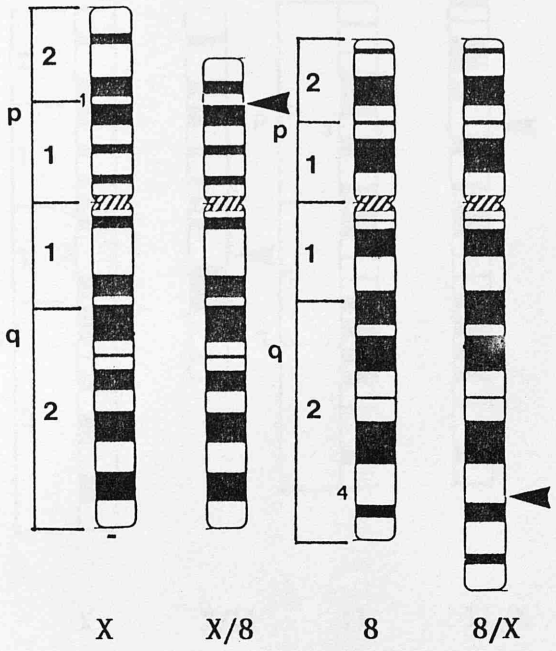
X X/Y

EH 46,XX,t(X;11)(p22;p15)

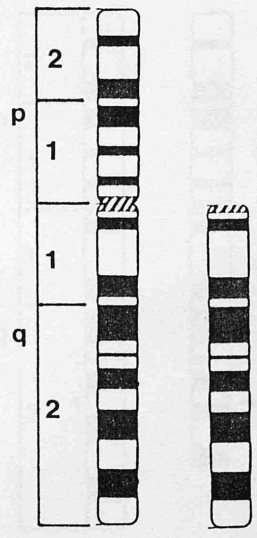
X X/11 11 11/X

FIGURE 7: Rearranged chromosomes from the mother of patient ME and from patient EH.

W2 46,XX,t(X;8)(p21;q24)



CE, LN 46,XXp-



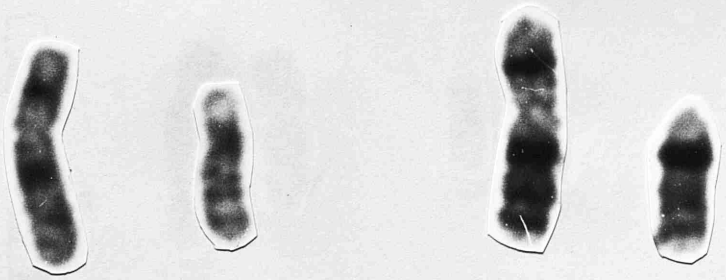
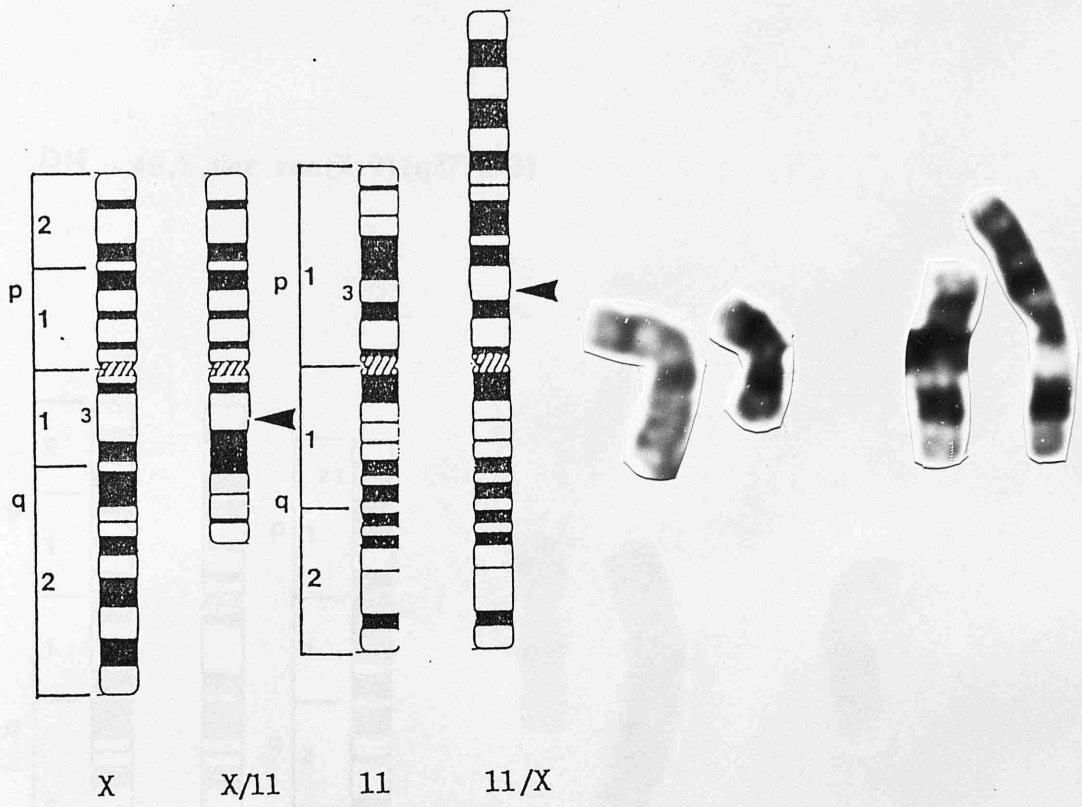


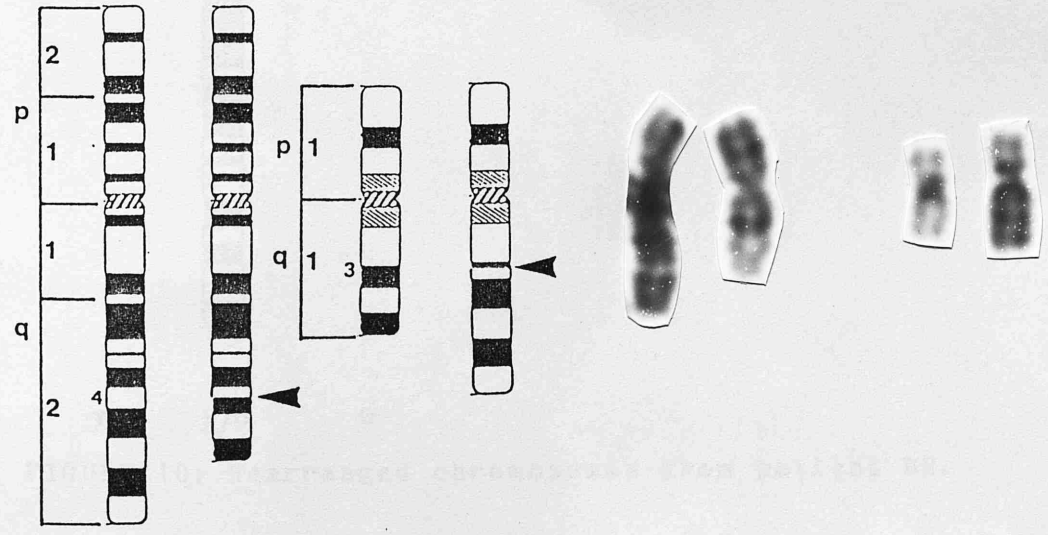
FIGURE 8: Rearranged chromosomes from patients W2, CE and LN.

NE 46,XX,t(X;11)(q13;p13)



X X/11 11 11/X

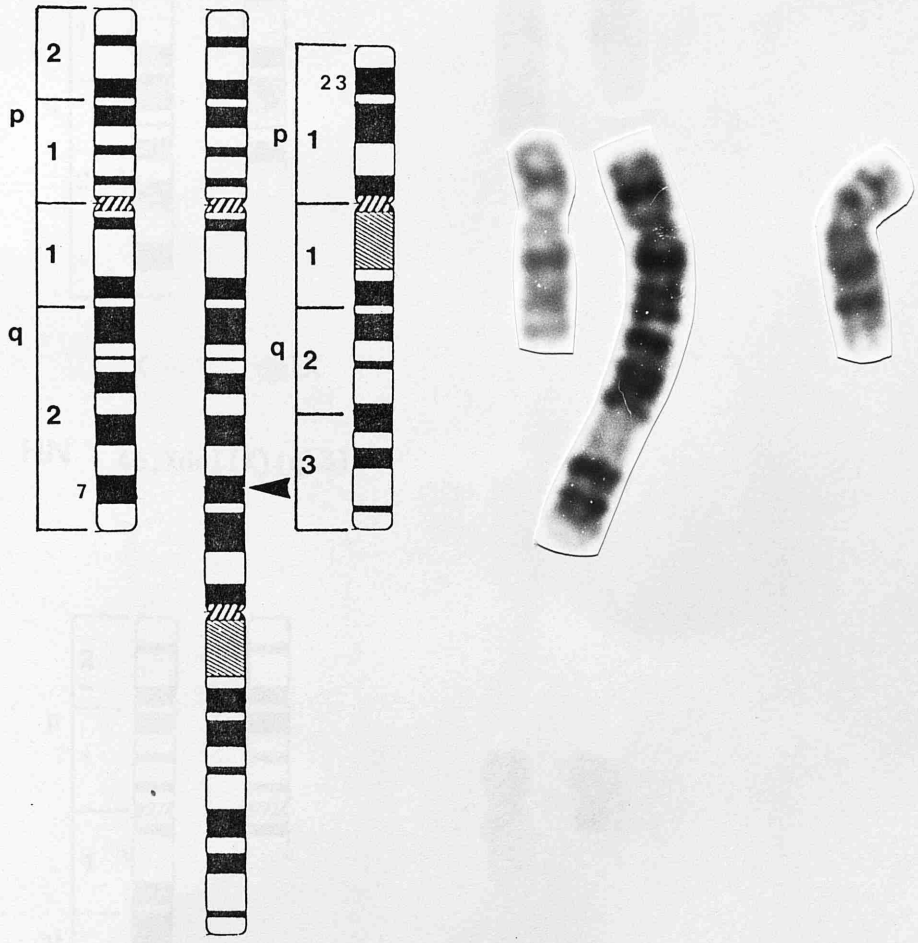
FN 46,XX,t(X;19)(q24;q13)



X X/19 19 19/X

FIGURE 9: Rearranged chromosomes from patients NE and FN

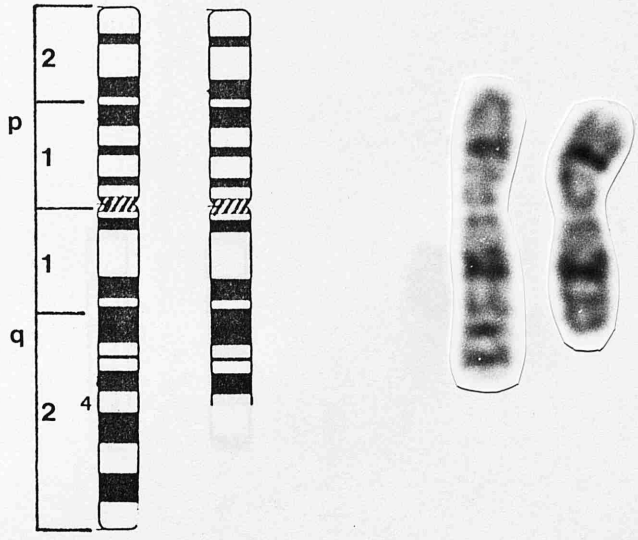
DH 46,X,ter rea(X;9)(q27;p23)



X X/9 9

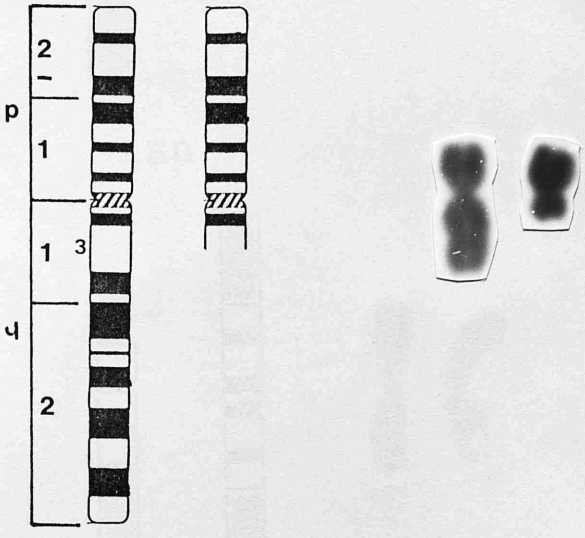
FIGURE 10: Rearranged chromosomes from patient DH.

WH 46,Xdel(X)(q24)



X delX

HN 46,Xdel(X)(q13)



X delX

FIGURE 11: Rearranged chromosomes from patients WH and HN.

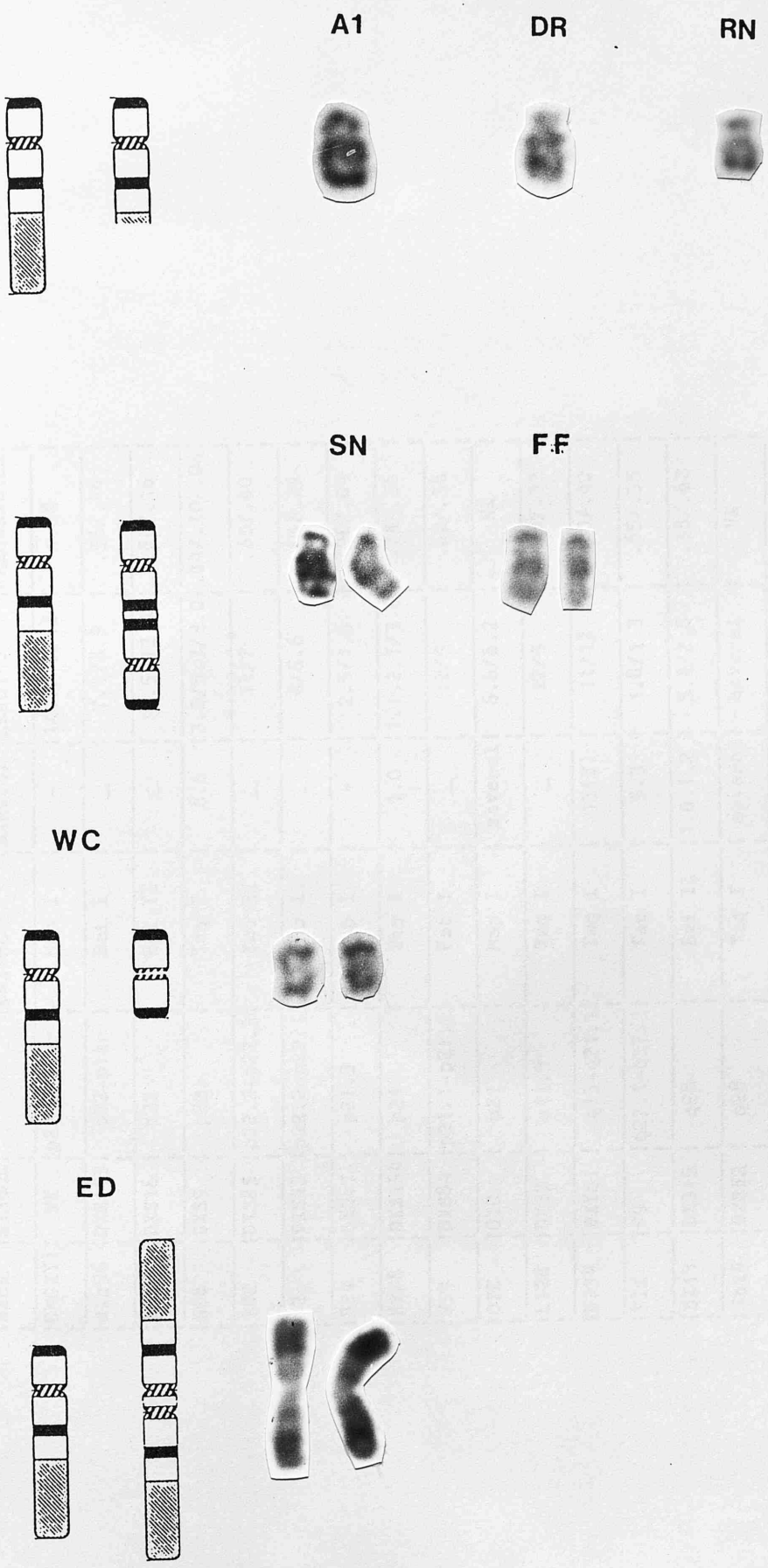


FIGURE 12: Rearranged Y chromosomes from individuals included in the Y deletion panel.

PROBE NAME	HGM SYMBOL	X REGION	POLYMORPHISM ENZYME	CONSTANT BAND(S)	ALLELE LENGTHS	ALLELE FREQUENCIES
GMGXY1	NK	p22.3-pter	Msp I	-	1/.7/.5/.3	NK
dic56	DXS143	p22-pter	Bcl I	_	7.4/8.9	.56/.44
pX23	DXS16	p22	Bgl II	_	17.5/12.5	.84/.16
RC8	DXS9	p22	Taq I	6.6	3.2/5.3/3.0	.84/.10/.06
782	DXS85	p22.2-p22.3	Eco RI	_	14/7	.60/.40
D2	DXS43	p22.2-p22.1	Pvu II	_	6/6.6	.71/.29
B24	DXS67	p21.3	Msp I	-	2.5/1.5	.96/.04
87/8	DXS164	p21	Taq I	1.0	1.1,2.7/3.8	.74/.26
754	DXS84	p21.1-p21.2	Pst I	_	12/9	.62/.38
OTC	OTC	p21	Msp I	several	6.6/6.2	NK
L128	DXS7	p11.3	Taq I	_	12/9	.68/.32
DP34	DXYS1	q13-q21.1	Taq I	15(Y)	11/12	.60/.40
FIX	F9	q27.1-q27.2	Taq I	5.3	1.8/1.3	.65/.35
DX13	DXS15	q28	Bgl II	1.0,1.2	5.4/2.8	.38/.62
St14	DXS52	q28	Taq I	several	several	NK

TABLE 1: LIST OF PROBES USED IN THE MOLECULAR ANALYSIS OF THE HYBRID PANEL CONTAINING DIFFERENT PARTS OF THE X CHROMOSOME

NOTE: NK - Not known

2.5. Methods Used in the Production of Human/Mouse Hybrids

2.5.1. Fusion Procedure for the Preparation of Human Fibroblasts/Mouse Fibroblast Hybrids

The mouse and human cells were trypsinised from their respective flasks, and the trypsin was inactivated with twice as much Ef or F10 (see general section) as appropriate. The number of cells was counted using a haemocytometer and 1 to 2 x 10^6 of each cell type were mixed together in a 30ml universal. The cells were spun down at 1000rpm for 10 minutes, taken up in 10ml Ef medium without serum, and spun down again. Using a pipette the cells were gently resuspended for 45 seconds in 1ml of PEG 6000 (50% in Ef without serum) without causing frothing. The PEG was allowed to drain from the pipette for another 15 seconds so that the cells were in PEG for a total of 1 minute. To the cell mixture 9ml of Ef without serum was added quickly and the resulting 10ml was split between two universals containing 15ml Ef without serum (The aim of this step is to maximise dilution of the PEG in the minimum time). The cells were spun down again and each pellet was taken up in 10ml F12 medium. The cells were seeded into 60mm Petri dishes (Nunc) each of which contained 4ml of F12. About 10^5 cells/dish was the usual aliquot, although this may be vary depending on cell size and speed of killing.

After 48 hours of incubation at 37°C in air + 5% CO_2, the medium was removed from the dishes and the cells were fed with selective medium (see general section). The cells were thereafter fed twice a week, always with selective medium. Colonies of hybrid cells started to appear around the third week but the time varied for each fusion.

The fusion procedure for the preparation of human lymphoblastoid cell/mouse fibroblast hybrids is identical

to that described above except that a 10:1 ratio of lymphoblastoid:mouse cells is used.

2.5.2. Picking Colonies

A small volume of versene buffer (few drops) was added to a well defined, active colony and left for 30 seconds. The colony was then picked up by gently scraping the cells from the dish using a Pasteur pipette which also contained a small volume of versene buffer. The cells were sucked up and placed in a 50ml tissue culture flask containing selective medium. The flask was incubated at 37°C, with 5% CO_2 in air. After the first passage, ouabain was excluded from the selective medium.

2.5.3. Subcloning

If chromosome analysis indicated that a cell line was useful then the cells were either subcloned in order to obtain a pure line or if a cell line was required with the Xp derivative, then selection against the Xq (see next section) was performed prior to subcloning.

The cells to be subcloned were trypsinised and the trypsin was inactivated by dilution with an excess of medium. The cells were counted and serial dilutions were carried out with medium, to obtain a cell suspension of approximately 5 cells/20mm-diameter well dish (Nunc). The multiple well plates were incubated at 37°C in 5% CO_2 and the medium was changed in the wells after 24 hours and twice a week thereafter. Colonies were picked as described above but only one was picked from each well, avoiding those with more than one colony growing in the early stages.

2.5.4. Backselection

Hybrid cell lines which carried the Xp derivative, but also the normal X or the Xq derivative, were grown for three weeks in PM medium (see general section) and then in PM supplemented with the counterselective agent 6-thioguanine at a final concentration of 6µg/ml. The human chromosome content was monitored cytogenetically until complete segregation of the normal X chromosome or the Xq derivative was observed. The counterselected cell population was then subcloned and subsequently expanded as described below.

2.5.5. Expanding a Hybrid Cell Line

Cells from well defined, active colonies were grown in small 50ml plastic bottles (25cm^2 growth area) until they reached confluency. The cells were trypsinised and transferred to a medium sized bottle (80cm^2). At this stage a medium sized bottle for cytogenetic analysis was set up. If the hybrid cell population proved to be informative for the purposes of the study, it was further expanded in large bottles (170cm^2) to the level of 10^7-10^8 cells (about 10^8 cells were needed for a DNA preparation). The cells were analysed both cytogenetically (a harvest was done at the final stage) and using X-chromosome specific DNA probes, to ensure that they contain the derivative X as required. Thus, all the results shown refer to the same transfer number of the cell cultures.

2.5.6. Freezing Cells

At each passage, cells were frozen by the following method: cells were fed the day before freezing, were in a

good condition and almost confluent. They were trypsinised, spun down and the pellet was resuspended in ~1ml of DMSO (10% dimethylsulphoxide solution in PM medium containing 20% serum) and transferred to a vial. The temperature was reduced gradually by placing the vials in a polyestyrene box at -70°C o/n and the cells were afterwards stored in liquid nitrogen.

2.5.7. Reconstitution of Frozen Cells

The cells were thawed in water at 37°C and were transferred to a universal containing 5ml of medium. The cells were spun down at 1000rpm for 5-10 minutes, the pellet was resuspended in 1ml of medium and transferred to a culture bottle containing medium and 5% CO_2 (the size of the bottle and the volume of medium added depended on the size of the culture before freezing). The medium was changed after 24 hours.

2.5.8. Trypsinisation

The following procedure was used to remove cells from the surface of the bottles. The medium was removed and versene buffer (4ml to a 260ml flask, or 2ml to a 50ml flask or dish) was added and allowed to act for 30 seconds. This loosens the cells slightly. The versene buffer (see general section) was then removed and the cells were detached in 4ml or 2ml of trypsin versene buffer during incubation at 37°C.

2.5.9. Mycoplasma testing (from Chen 1977)

Mycoplasma testing was performed regularly during the production of somatic cell hybrids and immediately before pelleting cells for DNA analysis.

The cells were grown in Petri dishes for at least four days and tested before they were confluent. Cells were fixed in situ with methanol/acetic acid (3:1 v/v). The staining solution consisted of 0.05µg/ml bisbenzimid-Hoechst 33258 in Dulbecco's phosphate buffered saline (Dulbecco and Vogt 1954). Fixed cells were stained for 10 minutes, washed three times with distilled water, mounted in McIlvainie's (phosphate/citrate) buffer pH 5.5, and observed immediately under U.V. light using a fluorescence microscope.

2.5.10. Harvesting Hybrid Monolayer Cultures for Chromosome Analysis

Harvesting was performed 24 hours after trypsinisation and passage of cells, when the number of cells was approximately 5×10^5. The cell cycle was arrested in metaphase with colchicine (0.2µg/ml) and incubated at 37°C for 1ρ hours. The cells were trypsinised, washed from the bottle with versene buffer, treated with about 10ml of prewarmed 0.075M KCl and incubated at 37°C for ~30 minutes. After centrifugation and removal of supernatant the cells were fixed in methanol/acetic acid (3:1) which was freshly prepared and cooled to 4°C before being added dropwise. The cells were then refrigerated at 4°C for at least 30 minutes. This step was repeated, without refrigeration, twice and the cell suspension was dropped onto wet glass slides which were then dried on a hot plate (adapted from Moorhead **et al** 1960). Throughout the procedure centrifugation was for 5 minutes at 1,000rpm. The slides

were examined under the phase microscope for quantity and quality of mitoses.

2.5.11. Staining Procedures

i. Trypsin/Leishman (modified from Seabright 1972)

Slides were soaked in 2xSSC for 2 hours at 60°C, rinsed with normal saline, then distilled water and dehydrated by soaking for 2 minutes in each of 50,70,90,95, and 100% ethanol and finally air dried. The slides were treated prior to staining with a trypsin solution (Difco Bacto-Trypsin) whose concentration, temperature and time of action depended on the cell type. A series of trials were performed to find the optimal time. The concentration of the trypsin was usually 0.25% and the time ranged from 10-90 seconds. The slides were removed from the trypsin, rinsed in saline and stained in a solution which contained 1 part Leishman's stain (BDH Poole, 0.15g powder stirred cold for 2 hours in 100ml methanol and then filtered) and 4 parts phosphate buffer, pH 6.8 (made with pH 6.8 tablets from BDH Gurr, code 33199). The slides were well covered with stain for 3 minutes and then rinsed with the phosphate buffer and dried on a hot plate. Slides were examined microscopically and if the chromosomes were poorly banded or bloated the trypsin time was increased or decreased respectively. If the bands were very pale or very dark then the staining time was increased or decreased accordingly. The slides were not mounted because in some cases it was necessary to destain the slides for fluorescent staining as described below.

ii. Quinacrine/Bisbenzimid - Hoechst 33258 (from Kucherlapati et al 1975)

When appropriate, slides were put in xylene for 5-10 minutes to remove the immersion oil, then dried and soaked in methanol until they were destained (usually o/n). They were then rehydrated by taking them through a series of ethanol dilutions (95%, 90%, 70%, 50%) for 2 minutes in each. They were then placed in McIlvainie's buffer pH 7.0 (see general section) and subsequently stained for 30 minutes in the dark with staining solution consisting of 50µg/ml quinacrine hydrochloride and 0.1µg/ml Hoechst 33258 in McIlvainie's buffer pH 7.0. Slides were washed 3 times in McIlvainie's buffer pH 7.0 and mounted in the same buffer of pH 4.5. Slides were examined under U.V. light in a fluorescence microscope immediately but remain fluorescent for 2 weeks if covered with aluminium foil and stored at 4°C. Under U.V. light the mouse chromosomes have a very bright fluorescent centromere and the human ones have a pale centromere with typical Q bands.

iii. G11 Stain (from Bobrow and Cross 1974)

The Fischer-Giemsa stain (G146 from Eastman-Kodak) was prepared by grinding 1g in a few ml of glycerol until fine (~15 minutes). Glycerol was added to a volume of 66ml and stirred at 60°C for 24 hours. It was then removed from the heat, 66ml of ethanol was added, and it was left to stir o/n at room temperature.

The Fisher-Giemsa stain was diluted 1 in 20 with a solution of 0.75ml of 0.7N NaOH in 50ml distilled water. The solution was mixed carefully with minimum shaking in order to prevent air bubbles (which cause the stain to precipitate). This was freshly made. The staining time varied between slides but a good starting time was 10-15 minutes. The stain was rinsed from the slides under running

water before being removed from the staining jar. This technique stains mouse chromosomes red with blue centromeres and human chromosomes pale blue with some specific regions stained red.

2.5.12. Microscopy and Photography

About 10 to 20 cells were analysed initially from each cell line in order to find one which is informative. The informative cell line was then subcloned (as described below). About 5 to 10 cells were analysed from the subclones and 40 more cells from the informative one and the best and/or critical chromosome spreads were photographed with an Exacta camera using Ilford PAN F film. All the cases which were not conclusively characterised by G-banding were also analysed with the differential stain and pictures were taken of some of these cells.

2.6. Recombinant DNA Technology

2.6.1. Culture and Preparation of Cells for DNA Extraction

i. Blood Samples (modified from Kunkel et al 1977)

5-25ml of whole peripheral blood was collected in 30ml sterile plastic universals (see general section) containing 2ml of a 4.5% EDTA/0.7% NaCl solution. The universals were inverted several times to ensure that the blood and EDTA were thoroughly mixed. Insufficient mixing leads to coagulation of the blood and the samples cannot be used for DNA extraction. If the blood samples were not used for DNA extraction immediately, they were stored at -20°C. The samples were thawed when necessary, by leaving them at room temperature for 2-3 hours. They were then mixed with 5 volumes of 4°C cold lysis mix, (0.32M sucrose, 10mM Tris.HCl, 5mM MgCl , 1% Triton X-100 (v/v) (BDH Chemical Ltd.), pH 7.5) and spun for 10 minutes at 6K in a Sorvall centrifuge, using a HS-4 rotor and precooled tubs. The supernatant was removed and the pelleted nuclei were used for DNA extraction.

ii. Lymphoblastoid Cell Lines

Established lymphoblastoid cell lines, transformed with Epstein-Barr Virus (EBV) were grown in HAM'S F10 (Gibco) + 10% fetal calf serum + 10% new born calf serum + 1% Penicillin/Streptomycin (Gibco). When the culture reached $\sim 10^8$ cells, it was spun down, and the cells were resuspended and washed in saline to remove any traces of serum.

iii. Fibroblast Cell Lines

Fibroblast cells were grown in culture bottles in HAM'S F10 (Gibco) + 10% fetal calf serum + 10% newborn calf serum + 1% Penicillin/Streptomycin (Gibco), until the monolayer was confluent. The cells were trypsinised following the same method as for hybrid cells (see section 2.5.10), centrifuged at 10,000rpm and washed three times in saline.

iv. Mouse or Hybrid Cell Lines

The medium in which the hybrid and mouse cells were grown and the trypsinisation conditions for these lines have been described in section 2.2. The cells were grown until the monolayer was confluent and after trypsinisation they were spun down, resuspended and washed three times in Dulbecco's phosphate buffered saline, part A, at 4°C.

Due to the relatively small volumes of these cultures the pelleting, washing and subsequent lysing of the cells (by the procedure described for blood samples) was done in 30ml sterile plastic universals and they were spun down at 1000rpm in a bench centrifuge (MSE) at room temperature.

The final pellets from the cell lines described above were either used immediately for DNA extraction or stored at -20°C for longer periods.

2.6.2. DNA Extraction

The pelleted nuclei from the blood samples or the washed pellets from the cell lines described above were resuspended in 4.5ml of 0.075M NaCl, 0.024M EDTA, pH 8.0, and 250µl of 10% SDS (w/v) (Sigma). Preboiled RNAse (Sigma chemical company) to a final concentration of 50µg/ml, was

added to the pellets from the cell lines. The mixture was incubated for 1-2 hours with shaking at 37°C o/n. 100µl of 10mg/ml proteinase K (Gibco BRL, Bethesda Research Laboratories) was added and the sample was incubated at 37°C o/n with gentle shaking. The sample was then transferred to a 13ml plastic tube and an equal volume of phenol/chloroform (1:1) equilibrated with 10mM Tris.HCl, pH 8.0 was added to it. The mixture of the sample with phenol/chloroform was mixed gently for 10 minutes and then centrifuged for 10 minutes at 10K in a Sorvall centrifuge using a SM-24 rotor. The aqueous layer was transferred into a clean tube and the phenol extraction was repeated until the interphase was clear. Usually 2-3 phenol extractions were enough to remove any impurities from the DNA. An equal volume of chloroform was then added to the aqueous layer, the sample was mixed for 10 minutes and then spun at 9K for 10 minutes. The aqueous layer was removed to a universal, then a 1/10 volume of 3M sodium acetate pH 7.5 and two volumes of ice-cold absolute ethanol were added to it. The universal was inverted several times until the DNA precipitated and was spooled out with a closed Pasteur pipette. The DNA was resuspended in 10mM Tris.HCl or TE buffer, (10mM Tris.HCl pH 8.0, 0.1mM EDTA) in a bijoux by shaking gently for 2-3 days at 4°C. The DNA was stored at 4°C.

At the ethanol step, DNA from blood samples was left o/n at -20°C so that the DNA would precipitate more efficiently. With samples other than blood, however, RNA precipitates along with the DNA. In order to avoid the collection of RNA, ethanol at room temperature was used and the DNA was immediately spooled out.

The concentration of the DNA samples was calculated by measuring the O.D. of the preparations at 260nm in a spectrophotometer (LKB Pye Unicam). 1µg of DNA was digested with Eco RI restriction enzyme o/n at 37°C, (for details about the digestions see below) in order to check the DNA

for digestability and presence of RNA and 1µg of DNA was mixed only with Eco RI buffer to check for degradation. They were both run on a gel.

2.6.3. Flow Sorting of Human Chromosomes and Preparation of DNA

Human chromosomes were flow sorted on a FACS440 from either PHA stimulated peripheral blood lymphocytes or lymphoblastoid cell lines, using the method described by Young **et al** 1981.

The sorted chromosome samples (in buffer with 1% sarcosine added) were pooled in large centrifuge tubes and the volumes were noted. Proteinase K was added to a final concentration of 100µg/ml and the samples were incubated at 37°C for approximately 3 hours. About 4ml of tRNA was added as a carrier (stored at -20°C at a concentration of 5mg/ml in H_2O). An equal volume of phenol/chloroform was added to each sample and mixed well by repeated inversions. The tubes were spun at 10,000rpm for 10 minutes at room temperature and the aqueous layers were removed. The phenol layers were re-extracted with approximately 1/5 volume of 0.5M Tris buffer and were added to those obtained from the first extraction. The volumes were reduced by adding an approximately equal volume of butanol, mixing well and spinning for 2 minutes at 5,000rpm at room temperature. This step was repeated until the volume was reduced to 0.1ml. In the later stages Eppendorf tubes were used and the spinning was done in an Eppendorf centrifuge. Approximately 2-2.5 volumes of cold ethanol was added and the samples were incubated at -20°C or -70°C. The DNA was spun down at 10,000rpm for 5-10 minutes and the pellet was resuspended in the appropriate volume of water for digestion (see section 2.6.6 for the method).

2.6.4. DNA and mRNA Preparation from Liver and Placental Tissues

The tissue was removed and frozen as soon as possible in liquid nitrogen to prevent degradation of the tissue and hence the nucleic acid molecules. The tissue was taken out of the liquid nitrogen and broken up into small pieces, then it was crushed to a powder and added to an appropriate volume (~500ml depending on the size of the sample) of guanidine thiocyanate (5M guanidine thiocyanate, 50mM Tris pH 7, 50mM EDTA, 5% β-mercaptoethanol, sterilised by filtration). The mixture was shaken gently to dissolve and 20% Sarcosyl was added to 0.2% final concentration. Both these chemical substances prevent degradation of the tissue. The RNA/DNA was then prepared by ultra-centrifugation (Sorvall OTP-65, Ultra Centrifuge, AH627 swing out rotor) for 48-72 hours at 25K in a CsCl gradient. The correct gradient was achieved by adding 4.3ml of CsCl suspension (5.7M CsCl, 50mM EDTA, pH 7) to 17ml pollyallomer tubes.

After centrifugation, the DNA was collected (top layer) from the CsCl gradient, using a plastic pipette and was transferred into 30ml tubes where it was precipitated with 4-5 volumes of cold 70% ethanol. The DNA was spooled out using a heat-sealed Pasteur pipette and was resuspended in 0.075M NaCl, 0.024M EDTA, pH 8.0, the volume depending on the size of the DNA pellet. 10% SDS was added to 0.5% concentration and proteinase K to 100μg/ml and this was incubated o/n at 37°C. The DNA was phenol and chloroform extracted (as described for blood samples) and precipitated with 0.3M sodium acetate and absolute ethanol. The pellet was washed in ethanol, resuspended in TE (volume depending on the size of pellet) and finally stored at 4°C.

The RNA formed a translucent pellet at the very bottom of the centrifuge tube. About 100µl of filtered 8M urea was added and the pellet was resuspended using a 200µl Pipetman (Gilson). More 8M urea was added if necessary. The suspension was transferred to Eppendorf tubes and 2 volumes of 4M LiCl was added to each tube. These were placed for a few days at 4°C to allow the RNA to precipitate. It was then pelleted and resuspended in sterile distilled water. Sodium acetate to a final concentration of 0.3M and 2 volumes of 100% ethanol was added to it and this was stored at -20°C.

Isolation of poly(A)$^+$ RNA

When ready to isolate poly(A)$^+$ RNA (i.e. mRNA), the RNA was pelleted, dried, dissolved in elution buffer (10mM Tris, pH 7.5, 0.2% SDS, 37°C warm). An oligo(dT) column was prepared by resuspending oligo(dT) cellulose in elution buffer and the column was poured in a Bio Rad glass column. This was washed with 10ml of elution buffer and equilibrated in about 10ml of binding buffer (elution buffer + 0.4M NaCl). NaCl was added to the RNA to a final concentration of 0.4M and the RNA was passed through the column. The effluent was collected and passed through the column twice more, to ensure that all the poly(A)$^+$ RNA was bound to the column, and finally collected in a poly(A)$^-$ tube. The column was washed with 3ml of binding buffer and collected into the poly(A)$^-$ tube. The poly(A)$^+$ RNA was eluted by passing 5x 1ml of warm (37°C) elution buffer through the column and collecting it in a poly(A)$^+$ tube. The column was washed with 3ml of binding buffer which was also collected in the poly(A)$^+$ tube. The poly(A)$^-$ was again added to the column and passed over three times, so that any remaining poly(A)$^+$ RNA would be bound to the column. This was eluted by washing the column with elution buffer. A tenth volume of 4M NaCl was added to the poly(A)$^+$ mixture

and the binding and elution procedure was repeated once more. The final elution was done with 8ml of elution buffer. The $O.D._{260}$ of a 1ml aliquot of the poly(A)$^+$ mixture was measured in order to find the concentration and then it was lyophilised to ~4ml final volume. It was then ethanol precipitated and stored at -20°C. When required, a small aliquot was resuspended for reverse transcription (as described in section 2.6.9).

2.6.5. Removal of Repeated Sequences from Hybridisation Probes (from Sealy **et al** 1985)

The large amounts of DNA isolated from tissues as described above was mainly used for preassociation experiments where the repeated sequences from radiolabelled probes were competed out by pre-reassociation with large amounts of sonicated genomic DNA. The DNA was subjected to 15 sonic bursts of 5 seconds, with 15 seconds intervals in between, using a Soniprep 150 MSE sonicator. The DNA was ethanol-sodium acetate precipitated as described previously and was left at -30°C o/n. The DNA was spun down to a pellet and resuspended in TE to a concentration of 10mg/ml. The following were added in a 1ml volume reaction: 125µl of 30x SSC, 5-7mg of the sonicated genomic DNA in a volume of 500µl and the radioactive probe. The volume was topped up to 1ml with TE. The radiolabelled probe was mixed very well with the sonicated DNA and the salt and was denatured in boiling water for 10 minutes. It was then cooled on ice for 1-2 minutes and then incubated for 20 minutes at 68°C in a waterbath. The probe was then added to the prewarmed hybridisation mixture and was applied to the filter. The rest of the hybridisation and washing conditions were as described later in section 2.6.10.

2.6.6. Restriction Enzyme Digestion

The basic digestion reaction was carried out in a 40µl volume which contained 4µl of salt buffer (see below), 2µl of 0.1M spermidine, 2µl of enzyme (20 units), and an appropriate volume of sterile distilled water, depending on the concentration of the DNA sample. Spermidine (Sigma Chemical Company) was used to improve the digestion of the DNA samples (see Bouche 1981). 5-7µg or very occasionally 10µg of DNA from genomic samples and 10-15µg of DNA from hybrid cell lines was digested each time.

Digests of genomic DNA samples and hybrid DNA samples were performed o/n at the required temperature for each enzyme (see below) in small Eppendorf tubes. Digests of plasmid or phage DNA were sometimes done for 3-4 hours but preferably it was done for longer periods like o/n. The reactions were terminated by the addition of 5µl of 'T' mix (50mM EDTA, 50% glycerol, 2% Ficoll, and 50µg/ml Orange G). The digests were either stored at -20°C this way until further use or were immediately loaded on the gel. The buffers used for DNA digestion were as follows (These were prepared at 10x the working concentration) :

Low: 10mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT
Medium: 50mM NaCl, 10mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT
High: 100mM NaCl, 50mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT

The enzyme SmaI does not work well in any of the above buffers so it needed a separate buffer (S) consisting of 20mM KCl, 10mM Tris.HCl (pH 8.0), 10mM $MgCl_2$, and 1mM DTT.

Listed below are the various enzymes used during the experiments, the buffers they work at and the sites they

recognise. Most of the enzymes work at 37°C with the exception of BclI (60°C), BstNI (60°C), and TaqI (65°C).

Enzyme	Recognition site	Buffer
Bam HI	G↓GATCC	MEDIUM
Bcl I	T↓GATCA	MEDIUM
Bgl I	GCCNNNN↓NGGC	MEDIUM
Bgl II	A↓GATCT	LOW
Bst NI	CC↓($\frac{A}{T}$)GG	LOW
Eco RI	G↓AATTC	HIGH
Eco RV	GAT↓ATC	HIGH
Hae III	GG↓CC	LOW
Hind III	A↓AGCTT	MEDIUM
Hinf I	G↓ANTC	MEDIUM
Hpa I	GTT↓AAC	LOW
Hpa II	C↓CGG	LOW
Hph I	GGTGANNNNNNNN↓	LOW
	CCACTNNNNNNN	
Kpn I	GGTAC↓C	LOW
Msp I	C↓CGG	LOW
	C↓CMEGG	
Pst I	CTCGA↓G	MEDIUM
Pvu II	CAG↓CTG	MEDIUM
Sac I, Sst I	GAGCT↓C	LOW
Sal I	G↓TCGAC	HIGH
Sma I, Xma I	CCC↓GGG	SMA
Taq I	T↓CGA	LOW
Xba I	T↓CTAGA	HIGH
Xho I	C↓TCGAG	HIGH

N represents any nucleotide. The arrow shows the point at which DNA is cleaved in the 5'-3' direction. In some cases core buffer (BRL) was used instead of the buffers described above, especially in double digests involving two enzymes which require different salt concentrations.
BstNI came from C.L.Laboratories, SacI from NBL. All others supplied by BRL.

2.6.7. Gel Electrophoresis (based on Sharp et.al. 1973)

Electrophoresis was carried out in 0.8% agarose (Sigma) gels in 'E' buffer (see general section). Samples were loaded into the sample wells and in certain gels a 1kb ladder was run along with the samples to provide molecular weight markers (Gibco BRL). The gels were then stained for 10 minutes in 'E' buffer containing ethidium bromide (Sigma) to a final concentration of 1µg/ml. The DNA was visualised on a U.V. transilluminator (UVP, INC) and photographed using a Polaroid CU-5 land camera fitted with a red filter, and a black and white Polaroid type 667 film.

2.6.8. Southern Blotting

After electrophoresis the gel was treated with 0.25M HCl for 15 minutes to cause partial depurination of the DNA (see Wahl **et al** 1979). This was followed by two successive 20 minute periods in denaturing solution (0.5M NaOH, 1.5M NaCl) and then two 20 minute washes in neutralising solution (0.5M Tris.HCl pH 7.4, 3M NaCl). The DNA was transferred to a nitrocellulose filter (Schleicher and Schull, BA85 0.45m no.401198) in 20xSSC, as described in Southern 1975. After 16-24 hours the filter was removed from the gel, washed in 2xSSC, blotted dry and baked in an oven at 80°C for 2 hours. Baking for more then 2 hours causes the filters to become brittle. The filters can be stored prior to hybridisation in heat-sealed polythene bags.

Hybond N nylon based transfer membrane (Amersham) was subsequently used instead of nitrocellulose filter. This is a hydrophilic membrane and does not require pre-wetting. The DNA was fixed to the transfer membrane by a 5 minute U.V. treatment instead of baking.

2.6.9. Preparation of Radiolabelled Probe

i. Nick Translation (from Rigby et al 1977)

DNA probes were labelled with α-[^{32}P]-dCTP by the nick translation method. The reaction was performed in a volume from 30 to 50µl. About 0.5µg of recombinant or total genomic DNA was radiolabelled to a specific activity ranging from 15 to 85x10^{6}cpm/µg. DNA was dissolved in 5x buffer (Amersham nick translation kit) and water, when necessary. 2µl of DNA polymerase (included in the same kit) and 30-50µCi of α-[^{32}P]-dCTP (Amersham) were added to each reaction. The reaction was incubated at 14-15^{o}C for one hour. Labelled DNA was separated from unincorporated nucleotides by passage through a Sephadex G-50 column (medium type from Pharmacia and glass columns from Bio Rad), equilibrated with 1xSSC, 0.1% SDS. The resultant fractions were counted using a hand-held G-M detector (Mini-Instruments Ltd., type 5-10) and an aliquot from the first peak was counted on an LKB 1215 Rackbeta liquid scintillation counter.

ii. Oligonucleotide Labelling (Feinberg and Vogelstein 1983)

The advantages of this method over nick translation are (i) it can be used to label DNAs which are impure or at low concentration, and (ii) it can be used to label DNA cut from low melting point gels without having to elute it beforehand.

DNA in agarose was boiled for 10 minutes, and then incubated at 37^{o}C for at least another 10 minutes. Routinely 20µl of the DNA/agarose mixture (50-100ng of DNA) was mixed with 10µl of reaction mix (see below), 2µl of nuclease-free BSA (10mg/ml from Sigma), 1µl of Klenow fragment (Amersham), 2µl of α-[^{32}P]-dCTP (Amersham) (10µCi/µl) and 5µl of sterile, distilled water. The

reaction was incubated at room temperature o/n, and then terminated by the addition of 50µl of stop mix (20mM NaCl, 20mM Tris.Cl pH7.5, 2mM EDTA, 0.25% SDS, 1µM cold dCTP). As with nick translation, the DNA was separated from the incorporated nucleotides on a G-50 Sephadex column.

Oligo reaction mix

Firstly, the following solutions are made up:

Solution A:
1.25M Tris.HCl pH8.0
0.125M $MgCl_2$

Solution B:
1ml Solution A
18µl β-mercapto-ethanol
5µl dATP,dGTP,dTTP
(each of these dissolved in TE at 100mM)

Solution C:
2M Hepes, pH6.6

Solution D:
Hexadeoxyribonucleotides (Pharmacia, PL No. 2166) at 90 O.D. units per ml.

Solutions B, C and D are then mixed in the ratio 100:250:150 to give 10x reaction mix.

iii. Reverse Transcription of mRNA to cDNA

For the preparation of radiolabelled cDNA, 4-5µg of mRNA in solution was used. The RNA was spun down in an Eppendorf centrifuge for 5 minutes at 10,000rpm in a microcentrifuge. The supernatant was discarded and the pellet was left to drain. It was then washed in 100% alcohol, spun down, the pellet was again left to drain and put in an 80°C oven for 1-2 minutes to dry. The RNA was then resuspended in 32µl of water and heat-denatured for 5 minutes at 70°C. It was cooled on ice and spun down briefly, before adding: 5µl 1M Tris.HCl pH.8.3, 1.3µl 3M

KCl, 10µl 0.1M $MgCl_2$, 4µl 0.25M mercaptoethanol (Sigma), 4µl of 1mg/ml oligo(dT) (P-L Biochemicals, Inc.) in H_2O, 4.8µl dA (20mM), 4.8µl dT (20mM), 4.8µl dG (20mM) all from Sigma, 25µl of 1mg/ml Actinomycin D (Sigma), 1µl RNAasin (30 units/µl) from PNL Biochemicals, which prevents RNA degradation and improves the yield of the reaction, 8µl 32P-dCTP (80µCi) from Amersham, and 2 µl of reverse transcriptase ~20-30 units (Anglian Biotechnology). These were mixed well and incubated for 1 hour at 37°C. The reaction was stopped with 40µl of 0.5M EDTA and 30µl of 2N NaOH, was mixed well and incubated for 2 hours at 42°C. This was run through a long G-50 Sephadex column equilibrated with 1xSSC, 0.1% SDS. The radiolabelled cDNA was collected and its specific activity measured as described above. This was usually around $12-15x10^6$ cpm/µg.

2.6.10. Hybridisation, Washing Conditions and Autoradiography

1. Prehybridisation

Filters (nitrocellulose or Hybond-N) were prehybridised in 50% formamide [deionised], 5x Denhardts, 5xSSC, 50mM sodium phosphate pH 6.8, 380µg/ml sonicated, denatured salmon sperm DNA, 10µg/ml Poly (A), 0.2% SDS. Nitrocellulose membranes were incubated at 42°C o/n, but Hybond-N membranes were sometimes prehybridised for shorter periods of time, usually 3-4 hours.

2. Hybridisation.

The radiolabelled probe was denatured by boiling for 10 minutes and then added to 5-10ml of hybridisation buffer (50% Formamide (McQuilken), 1x Denhardts (see general section), 5xSSC, 20mM sodium phosphate pH 6.8, 100µg/ml

salmon sperm DNA, 20µg/ml Poly (A) (Boeringer), 10% dextran sulphate (Sigma)). The prehybridisation buffer was discarded and the hybridisation buffer with the probe was added to the filter. The bag was heat-sealed again. The filters were incubated at 42°C o/n or for 2 nights.

3. Post-hybridisation washing

After hybridisation, the filter was removed from the bag and washed at room temperature in 2xSSC. Filters used for screening of the library with cDNA and genomic DNA were washed in 2xSSC, 0.1% SDS twice for 10 minutes at room temperature and afterwards in 1xSSC, 0.1% SDS for 20 minutes at 42°C. The filters were then monitored using a mini-monitor (GM Instruments) and if necessary, were washed down to 0.5xSSC, 0.1% SDS.

The filters used for Southern blotting of genomic or hybrid DNA were washed in 2xSSC, 0.1%SDS twice for 10 minutes at room temperature, and then in 0.5xSSC for 20-30 minutes at 65°C. Again, if the background sounded quite radioactive after monitoring, the filter was further washed down to 0.1xSSC, 0,1%SDS at 65°C. Occasionally, a 0.05xSSC, 0.1%SDS wash was required with certain probes.

4. Removal of the probe from filters

One of the advantages of using Hybond-N membrane for Southern blotting is that the radiolabelled probe can be removed very efficiently and the filter can be reprobed a number of times. The radiolabelled probe was removed by washing the filters in 0.4M NaOH for 30min at 42°C and for 30min in neutralising solution (as used for Southern blotting). Filters were rinsed in 1xSSC, 0.1% SDS and put in heat-sealed bags prior to further prehybridisation and hybridisation. The efficiency of the removal process was checked by exposing the stripped filters to X-ray film.

5. Autoradiography

Filters were placed between sheets of polythene and exposed to Kodak X-ray film between Dupont Cronex Lightning Plus intensifying screens, in X-ray cassettes. The films used were X-Omat S and X-Omat XAR-5. Exposure times varied from a few hours, to weeks. The films were developed in a Fuji RG II X-Ray Film Processor, using Kodak X-Omat developer and fixer.

2.6.11. Preparation of Plating Bacteria

The bacterial strain used was E.coli LE392. A single colony picked from an L.Agar plate or 10µl taken from a glycerol stock were, inoculated into 50ml of L-broth (see general section) in a 250ml conical flask. The culture was incubated by shaking o/n at 37°C in an orbital shaker (New Brunswick Scientific). 0.2% maltose was added to the culture medium to aid the absorbance of bacteriophage λ to the host bacteria. The cells were spun down in plastic universals at 2,500rpm for 15 minutes at room temperature. The supernatant was discarded and the cell pellet resuspended in sterile 10mM $MgSO_4$ to give an O.D.600 of 2, which represents ~1.5 x 10^9 cells/ml. The bacterial suspension was stored at 4°C and could be used for up to three weeks. However, the highest plating efficiency was obtained with fresh cells. Glycerol stocks were made following the simple procedure described in section 3.6.20 for plasmid glycerol stocks.

2.6.12. Plating and titration of the library

In order to determine the titre of the library, 10-fold serial dilutions were prepared in phage dilution buffer (0.1M Tris.HCl pH 8.0, 0.1M $MgSO_4$, 0.1% gelatine w/v) in a final volume of 1ml in 1.5ml Eppendorf tubes. The

suspension was thoroughly mixed before 0.1ml of each dilution to be assayed was dispensed into sterile bijouxs containing 100µl of plating bacteria. The mixture was vortexed and incubated for 20 minutes at 37°C to allow the bacteriophage particles to adsorb. 3-4ml of melted top agar (42°C), (prepared as described in the general section) was added to each of the bijouxs which were then inverted a couple of times to mix the contents, and immediately poured onto labelled plates containing 25-30ml of bottom agar (see general section). The plates were left standing half covered. When the top agar had set, the plates were inverted and incubated o/n at 37°C. The number of plaques on each dilution plate was noted, and the titre of the original suspension was calculated. The library used in this study was found to be 4.6×10^{10} pfu/ml.

2.6.13. Screening the library (from Benton and Davis 1977)

The Y-specific library used here was obtained from Dr. Kirby Smith, and was prepared from a human/hamster hybrid cell line, 7631, in which the only detectable human chromosome is the Y. The library was made by partial digestion of genomic DNA using the Eco RI sites of a Charon 4A vector. For initial screening, a number of plaques was plated out, sufficient to cover the entire mouse genome plus the Y chromosome, onto 250mm plates (Nunc). For screening and further purification of bacteriophage, small 100mm round plates (Sterilin or Nunc) were used. The same procedure was used for plating of the phage as was used for titrating the library. After o/n incubation the plates were put at 4°C for 10-15 minutes to harden the top agar. This prevents the top agar peeling off when it comes in contact with the nitrocellulose filter. The nitrocellulose filter (Schleicher and Schull, BA85 0.45m no.401116) was placed carefully on the surface of the

plate so that it came into direct contact with the plaques, which quickly transferred onto the filter. Both filter and plate were marked at the same locations using an ink marker (Pentel). After 30-60 seconds the filter was peeled off using blunt-ended forceps and immersed, DNA side up, in a shallow tray of denaturing solution (1.5M NaCl, 0.5M NaOH) for 30-60 seconds. The filter was then transferred to neutralising solution (1.5M NaCl, 0.5M Tris.HCl [pH 8.0]) for 5 minutes. The filter was finally rinsed twice in 2xSSC and placed on Whatman 3MM paper to dry. Subsequent impressions of the same plate were left on the plate about 30 seconds longer, or until the filter was completely wet. After all the filters were dry, they were wrapped between sheets of Whatman 3MM paper and the DNA was fixed to the filter by baking for two hours at 80°C in a vacuum oven. Overbaking was avoided since this would have caused the filters to become brittle.

2.6.14. Prewashing and Washing conditions

After baking, the filters were placed in heat-sealed plastic bags containing ~100ml of prewashing solution (50mM Tris.HCl, pH 8.0, 1M NaCl, 1mM EDTA, 0.1% SDS). The filters were incubated with gentle shaking, for 2 hours at 42°C. The prewashing solution removes from the filters any fragments of agarose or bacterial debris.
The prehybridisation, hybridisation and post-hybridisation conditions were as described for gel filters.

After the hybridisation mixture was discarded, the filters were ready to be washed as has already been described in section 2.6.10.

2.6.15. Selection and picking plaques

After the films had been exposed and developed, they were aligned with the plates, and individual plaques were picked.

Bacteriophage can diffuse considerable distances through the layer of top agar, and so to avoid cross-contamination between neighbouring plaques, a low density of bacteriophage was plated out for such purification steps. Also, this procedure was carried out as quickly as possible after plating. An effort was made to choose plaques that were well separated from each other. The plaques were picked using a Pasteur pipette with a rubber bulb. The fragments of agar were washed into 400µl of phage dilution buffer (described in general section) which was put in a 500µl Eppendorf tube and a drop or two of chloroform was added to it. The tubes were left to stand at room temperature to allow the phage particles to diffuse from the agar. The tubes were then stored at 4°C indefinitely. The procedure was repeated twice more.

2.6.16. Plate Lysate Stocks

High titre stocks were made from those phage to be studied further. They were plated out at a sufficiently high density to give confluent lysis, and therefore maximum yield of phage. After o/n incubation, 5ml of SM (per liter: 5.8g NaCl, 2.0g $MgSO_4.7H_2O$, 50ml of 1M Tris.HCl pH 7.5, 5.0ml 2% gelatin) was added to each plate. The plates were then shaken gently at 4°C for several hours. As much as possible of the SM was recovered with a plastic pipette and put into a 13ml tube. A further 1ml of SM was added to the plate to rinse the surface, and the plate was stored for 15 minutes in a tilted position to allow all the fluid to drain into one area. The SM was again collected and added

to the first harvest. The plates were discarded and 0.1ml of chloroform was added to each tube. These were then vortexed briefly and centrifuged for 10 minutes at 4K and at 4°C. The supernatant was transferred to a fresh tube, chloroform was added to 0.3% and after mixing the suspension very well the stocks were stored at 4°C.

2.6.17. Phage DNA Preparation

Two different methods were used to isolate DNA from phage:

1. Plate Lysate Method

For each bacteriophage, four plates were prepared as described above to achieve confluent lysis. Agarose was used instead of agar for the top agar because the latter often contains potent inhibitors of restriction endonucleases. After incubation phage were recovered from the plates as for the high titre stocks, with the addition of 5ml SM and incubation at room temperature for a couple of hours with constant, gentle shaking. The SM from all four plates was then pooled in a 30ml plastic tube and the bacterial debris was removed by centrifugation at 8K in a Sorvall (RC 5B) centrifuge, using the SM 24 rotor, for 10 minutes at 4°C. The supernatant was then recovered, RNAse A (Sigma) and DNAse (Boehringer Mannheim GmbH) was added it to a final concentration of 25µg/ml and 10µg/ml respectively and it was incubated for 30 minutes at 37°C. To this solution an equal volume of cold PEG/NaCl (20% PEG (polyethyleneglycol) w/v, 2.5M NaCl) was added, the mixture was vortexed and incubated for one hour at 0°C (ice water). The precipitated bacteriophage particles were recovered by centrifugation at 10K for 20 minutes at 4°C. The supernatant was discarded by aspiration and the tubes were

left to stand inverted on a paper towel to allow all the fluid to drain away. To the pellet 20ml of SM was added, the phage particles were resuspended by vortexing and the mixture was centrifuged for 2 minutes at 10K and 4°C to remove debris. The supernatant was transferred to a 13ml plastic tube, 5µl of 10% SDS and 5µl of 0.5M EDTA (pH 8.0) was added and the solution was incubated at 68°C for 15 minutes. The mixture was then phenol extracted (spun at 8-10K for 10 minutes each time) until the interphase was clear and then it was extracted once with chloroform. To the final aqueous phase an equal volume of isopropanol was added and this was stored at -70°C for 20 minutes or at -20°C o/n. Later it was centrifuged at 9K for 10 minutes and the pellet was taken up in 1ml of TE. Half a volume of ammonium acetate and two volumes of cold absolute ethanol were added to it and the mixture was stored at -70°C for 30-40 minutes. It was then spun down again at 9K for 10 minutes. This step was repeated twice more. The third time the same procedure was repeated in a smaller volume of 300µl of TE. This was vortexed and stored again for 30-40 minutes at -70°C and was spun in a microcentrifuge (5412 Eppendorf centrifuge, or Damon/IEC) for 10 minutes at 15K at room temperature. The pellet was washed in 95% ethanol then vacuum dried (HETOSICC Freeze Dryer type CD 13-3). The pellet was finally resuspended in 300µl of TE and stored at -20°C.

2. Plate Lysate Method by Scraping Top Agar

The protocol described below is for one plate, however four plates were used for each phage preparation. The phage was plated as described above, to achieve confluent lysis. This method required only 3ml of top agar made up with 0.5% agarose to be poured on the agar plate. Before pouring the top agar the plate was dried for no more than 10 minutes. The plate was incubated o/n without inversion. When

confluent lysis was achieved, the soft top agar was gently scraped into a centrifuge tube using a sterile, bent Pasteur pipette. 5ml of SM and 0.1ml of chloroform were added to the tube which was left to shake for 30 minutes at 37°C. The tube then was centrifuged at 8K for 10 minutes at 4°C in a Sorval HB4 rotor. The supernatant was transferred to a 13ml polypropylene centrifuge tube and was centrifuged once more at 8K for 10 minutes at 4°C in order to remove bacterial debris. The supernatant was recovered and RNAse A and DNAse I were added to it to a final concentration of 25µg/ml and 10µg/ml respectively. This was incubated for one hour at 37°C. An equal volume of PEG/NaCl was then added and the mixture was incubated o/n at 0°C (ice water). The rest of the protocol is the same as for the first method.

Once the phage DNA was ready, two small aliquots were run on a mini gel, one undigested and one digested, to check for degradation, bacterial DNA contamination and digestability. The enzyme used was Eco RI since this was the enzyme used to construct the library.

2.6.18. Subcloning into plasmid pUC13

1. Production of Competent Cells

The strain of bacteria used for transformation was E.coli JM83 . An aliquot of 10µl from a glycerol stock of these bacteria was inoculated into 5ml of prewarmed (37°C) L-broth in a 30ml sterile universal. The culture was incubated with shaking o/n at 37°C. 1ml of the o/n culture was inoculated into 100ml of prewarmed L-broth and was grown to an O.D of 0.6. This was reached in ~2 hours and represents about ~6×10^8 cells/ml. The cells were then spun down in chilled 30ml sterile plastic universals in a bench

centrifuge (IEC CENTRA-7R refrigerated centrifuge), at 3,500rpm for 10 minutes at 4°C. The supernatant was poured off and each of the pellets was gently resuspended in 25ml of ice-cold 10mM $MgSO_4$ and kept on ice for 30 minutes. The cells were spun down as above and each of the pellets was resuspended in 12.5ml of ice-cold 50mM $CaCl_2$ and kept on ice for 15 minutes. The cells were finally spun down as above and each pellet was resuspended in 2ml of ice-cold 50mM $CaCl_2$. The pellets were pooled in one universal and stored at 4°C. The cells were used the day after they were made competent because their efficiency increased six fold after being kept in the cold room o/n. After this their efficiency decreased to the original level.

2. Preparation of recombinant fragments for cloning

About 6µg of each of the phage DNAs containing fragments to be subcloned into a plasmid vector were digested in a 40µl volume (as described in section 2.6.6). The reaction was stopped with the addition of 5µl of EDTA and the reactions were incubated at 70°C for 5 minutes. 20µl of this reaction was run on a mini-gel to check whether the DNA had been digested. If the DNA had been digested the remainder of the digestion was diluted to 50µl with sterile distilled water. Sodium acetate was added to 0.2M. This was then extracted with 25µl of phenol/chloroform until no interphase was seen. The solution was not extracted with chloroform. After the phenol extraction, 3 volumes of cold absolute ethanol was added to the aqueous phase and this was incubated at -30°C for 30-45 minutes and then at -70°C for another 1-1.5 hours. The DNA was spun down and the pellet washed with 100% of cold absolute ethanol. The pellet was finally taken up in 20µl of water. The DNA concentration was approximately 150ng/µl. If not used immediately for ligation it was stored at -20°C.

3. Ligation

Plasmid pUC13 (uncut and cut with Eco RI) was commercially available from P and S Biochemicals. Ligations were done in a volume of 10µl containing 1µl 10x ligase reaction buffer, 1µl of 0.1 T4 ligase both available from the Amersham M13 cloning kit, 1µl 10mM ATP, 1µl 50mM DTT, (both from Sigma) and a volume of vector pUC13 and digested DNA that would give approximately the same number of plasmid particles to recombinants. The reaction took place o/n, at 14°C.

4. Transformation

Flat bottomed sterile bijoux were chilled on ice before use. Keeping the bijoux on ice, the ligation was mixed with ice-cold DNA buffer (10mM Tris.HCl pH 7.5, 10mM $CaCl_2$, 10mM $MgCl_2$, filter sterilized) to a final volume of 100µl. The buffer was then mixed with 200µl of competent cells and the mixture was kept on ice for 25 minutes, heat-shocked at 42°C for 2-3 minutes, kept at room temperature for 10 minutes and then 0.7ml of prewarmed L-broth was mixed with the above and incubated with shaking for 1 hour at 37°C in the orbital shaker. About 100µl of this mixture was spread on an Amp/X-Gal plate. The plates were left to incubate for ~16 hours and after incubation they were stored at 4°C until colonies were picked.

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

Three controls were carried out along with every transformation:

(i) Competent cells were transformed with cut and religated pUC13 DNA to check if the plasmid self-ligated.

(ii) Competent cells were transformed with uncut pUC13 DNA to estimate the maximum transformation efficiency obtainable.

(iii) Competent cells were taken through the transformation procedure with no transforming DNA added to check whether they were contaminated.

5. Recombinant selection

The bacterial host strain JM83, mentioned above, lacks part of the β-galactosidase gene called lac Z and cannot synthesise this enzyme. This defect is complemented, however, by the presence of a lac Z' gene on the pUC series of vectors, including pUC13 as used here. Cloning into such plasmids involves insertional inactivation of the lac Z' gene and recombinants are distinguished by their inability to synthesise β-galactosidase. This is assayed during transformation by adding a lactose analogue, Xgal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), which is broken down by β-galactosidase to give a product which is deep blue in colour. If Xgal is added to the agar, along with ampicillin and an inducer of β-galactosidase, such as isopropyl-thiogalactoside (IPTG)* , then non-recombinant colonies, the cells of which will synthesise β-galactosidase, will be coloured blue, whereas recombinants with a disrupted lac Z' gene and unable to make synthesise β-galactosidase will be white.

(* - E.coli strain JM83 are constitutive producers of IPTG, so there is no need to add this to the agar).

6. Picking Colonies

The white colonies, among the blue, were picked using sterile toothpicks and inoculated into 3ml of L-broth Amp in a sterile plastic universal. The cultures were incubated with shaking at 37°C o/n, in an orbital shaker. From these cultures, 1.5ml was transferred into Eppendorf tubes and spun down in a microcentrifuge for 10 minutes at room temperature. The supernatant was discarded and the cells were washed in 1.5ml of TE with vortexing, to remove any traces of L-broth. The cells were spun down again and resuspended in 700µl of cold STET buffer (50mM Tris pH 8.0, 50mM EDTA, 8% sucrose, 5% Triton X-100). To this, 50µl of fresh, cold lysozyme (10mg/ml) was added and the tubes were left on ice for 5 minutes. They were then placed in boiling water for 45-60 seconds and then spun in the microcentrifuge for 20 minutes. The supernatant was transferred to a fresh Eppendorf tube and extracted with an equal volume of buffered saturated phenol until no interphase could be seen. To the aqueous layer, sodium acetate to a final concentration of 0.2M and 0.6ml of isopropanol were added. The tubes were then left for 30 minutes at -70°C, or o/n at -20°C. It was then spun down and the pellet was washed in 95% cold ethanol. The tubes were inverted and left to drain on paper towel and the DNA pellet was resuspended in 40µl of TE. Half of this was digested in a 40µl reaction volume using the restriction enzyme Eco RI, to check whether the ligation had worked and the recombinant of interest had been subcloned.

7. Large scale preparation of plasmid DNA

When the desired recombinant was subcloned a larger amount of DNA was prepared. For this purpose the method described above was scaled up. About 300ml of culture was used for each preparation of DNA. One extra step was added

to this method by adding preboiled RNAse to a concentration of 25µg/ml and the mixture was left for 60 minutes in a 37°C waterbath. This was followed by phenol extraction.

2.6.19. Elution Of DNA From Low Melting Point Agarose Gels

In order to isolate the insert from the plasmid DNA the following procedure was used: About 40µg of DNA was digested with Eco RI in a ~800µl reaction volume. The digestion was run on a 1% low melting point gel (BRL) The gel was stained with ethidium bromide, destained and photographed. The required band was cut out with a scalpel and placed in a preweighed 30ml plastic tube. This was reweighed and the weight of the gel slice was calculated. An equal weight of water was added and this was heated at 70°C to melt the gel. This was incubated for at least 10 minutes at 37°C. Immediately after the tube was removed from 37°C ice-cold phenol (saturated with 10mM Tris.HCl) was added to it and vortexed. The mixture was spun in the Sorvall centrifuge using the SA 600 rotor, at 10K for 10 minutes. The supernatant was transferred to a fresh tube and the bottom layer was re-extracted with an equal amount of water. The new aqueous phase was then added to that from the first extraction. This was re-extracted with an equal volume of room temperature phenol until the interface was clear, to remove residual agarose. The aqueous phase was extracted with sequential equal volumes of butan-2-ol to remove phenol and to reduce the volume to approximately 100-200µl. This was transferred to an Eppendorf tube, 1/2 a volume of 7.5 ammonium acetate and 3 volumes of cold absolute ethanol were added. The DNA was allowed to precipitate at -20°C o/n. The precipitate was spun down at 10K for 10 minutes. The supernatant was removed and the pellet was washed with 100% cold ethanol, drained and the traces of ethanol were allowed to evaporate. The pellet was

taken up in 50-150µl of water depending on the size of the pellet. 5µl of the DNA were run on a gel to check for purity and concentration. The remainder of the DNA was stored at -20°C.

2.6.20. Storage conditions for plasmid recombinants

From the 3ml o/n culture 1.5ml was transferred to a sterile plastic bijoux, 1.5ml of glycerol was added, and they were inverted a couple of times to mix the contents. These glycerol stocks were stored at -20°C and were used to inoculate new cultures. Stabs were also made, using the following procedure: L-broth was made as usual and 6% agarose was added to it. This was melted and ampicillin was added to it as described above. About 2-3ml of the medium was poured into sterile small glass vials and was left to solidify. An o/n culture was grown by inoculating 10µl from the glycerol stock into 1ml of L-broth and incubating o/n at 37°C. A loopful was taken from this and stabbed into the medium in the vials. The stabs were incubated at 37°C o/n and subsequently stored at room temperature, in a dark place.

2.7. Strategy Followed to Select for Single Copy or Expressed Sequences

The library was initially screened with male genomic DNA in order to select for human sequences and eliminate the mouse sequences. The clones which hybridised to the probe were picked, then replated and re-screened with cDNA made from male placenta, in an attempt to select expressed sequences. Two nitrocellulose filter replicates were made for each plate. One of the filters was prehybridised in normal solution containing 1mg of sonicated DNA per ml of

prehybridisation solution in order to compete out clones which were completely repetitive, and also to compete out any repetitive sequences contained within clones that carried single copy sequences. The other filter was prehybridised as normal and both were hybridised with the cDNA. Clones which hybridised to cDNA were picked. The autoradiographs from the two replicas were compared, and clones which seemed to have the same intensity in both photographs, despite the competition were singled out, on the basis that they probably carry very little, if any, repetitive sequences. From these, ten clones were selected for further study. These were further purified using the same method of competition described above. Four purifications were carried out to ensure that the clones isolated were derived from one bacteriophage. DNA was made from all of them.

These DNAs were then digested with the restriction enzymes: Eco RI, Eco RI/Hind III, Eco RI/Sst I, and Eco RI/Xba I and were run on gels. Two gels of the same kind were prepared. These were stained with ethidium bromide, photographed, and after appropriate treatment were blotted onto nitrocellulose filters. One filter was probed with male genomic DNA, and the other with cDNA. The results were compared with the photographs of the gels to determine 1) bands which hybridised when probed with cDNA but did not hybridise with genomic DNA - suggesting that these might be expressed sequences, and 2) those that were present on the actual U.V. pictures but did not hybridise to the genomic DNA - suggesting that they might be single copy sequences.

The fragments which were chosen because they fullfilled either of the above requirements were subcloned into the Eco RI sites of the plasmid pUC13. DNA was isolated and further digested with Eco RI, before running on a low melting point gel in order to separate the insert from the plasmid DNA. Some of the subcloned fragments were

eluted from the gel, purified and nick translated. Other subcloned fragments were cut out from the gel and directly radiolabelled with the oligonucleotide labelling method. They were then used as probes against the X panel in order to find out whether they were single copy sequences and hybridised to the human Y, X or autosomes.

Those clones which did not appear to contain any obvious single copy sequences after the screening procedure described above, and had only two Eco RI sites, were digested with Eco RI and run on a low melting point gel. When the insert was well separated from the phage arms, it was cut out from the gel, and radiolabelled with the oligonucleotide labelling method. The probe was then preassociated in order to compete out the repetitive sequences, and hybridised to an Eco RI digested X panel (described in section 2.4). If the result indicated that there were some single copy sequences within these clones then these fragments were subcloned into the Eco RI sites of pUC13. DNA was isolated and double digested with Eco RI and fifteen other enzymes (Bam HI, Bgl I, Bgl II, Hind III, Pst I, Pvu II, Sac I, Bst NI, Hae III, Hpa II, Msp I, Taq I, Cfo I, Kpn I and Sma I). The DNA was run on a gel, stained and photographed, blotted and hybridised with genomic DNA probe to identify sequences that did not hybridise at all. These fragments were then cut out from a low melting point gel and were directly used as probes against an X panel to test whether they were Y, X or autosomal specific.

R E S U L T S

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3.1. Cytogenetic and Molecular Characterisation of Human/Mouse Somatic Cell Hybrids

Ten human cell lines carrying X-autosome translocations or deletions of the X chromosome were fused with mouse cell lines deficient either in HGPRT (A9) or TK (LMTK) (details in Materials and Methods).

Table 2 shows the number of clones and subclones obtained and analysed cytogenetically from each successful fusion, and the number of useful hybrids obtained after cytogenetic and molecular analyses.

The results from the cytogenetic analysis of these hybrids is shown in Tables 3 and 4. The results shown in these tables are important to estimate the amount of DNA which would be detected by a human probe.

CE, HN and DH human cell lines were fused to both LA9 and LMTK mouse cells. Clones in which the abnormal X was retained without the presence of the normal X were produced only with LMTK mouse cells. WH was only fused to LMTK cells and useful clones were obtained.

In all fusions between LN and A9, clones were produced in which the delX (Xp-) always segregated with the normal X chromosome, presumably because the normal X was active and was therefore selected (see Discussion for further details). In an attempt to get rid of the normal X, clones in which only a minority of cells contained the delX were backselected against the normal X using 6-thioguanine (described in Material and Methods). Cytogenetic analysis indicated that nine clones were potentially useful. These clones were then screened using a probe from the distal part of the short arm of the X (dic56) and a probe from the distal part of the long arm of the X chromosome (DX13). DIC56 probe was absent from the genome of all nine hybrids

HYBRID	NUMBER OF CLONES OBTAINED	NUMBER OF CLONES ANALYSED CYTOGENETICALLY	NUMBER OF SUBCLONES OBTAINED	NUMBER OF SUBCLONES ANALYSED CYTOGENETICALLY	NUMBER OF USEFUL CLONES AFTER CYTOGENETIC ANALYSIS	NUMBER OF USEFUL CLONES AFTER MOLECULAR ANALYSIS
EHA9	17	6	19	19	1	1
W5A9	17	16	18	7	2	2
W2A9	2	2	24	5	1	1
LNA9	4	4	64	62	-	-
LNA9R	2	2	32	24	9	9
CHTK	10	10	22	8	2	2
NEA9	30	6	12	7	1	1
NEA9R	11	11	20	20	1	1?
FNA9	19	7	16	5	1	1
FNA9R	3	3	12	5	2	1?
DHTK	35	28	-	-	1	1
WHTK	15	15	3	3	2	1?
HNTK	19	7	21	14	3	1
TOTAL	184	117	263	179	26	22

TABLE 2 : CELL LINES OBTAINED AFTER FUSION/SELECTION - CYTOGENETIC AND MOLECULAR ANALYSIS

NOTES : 1. ? - Inconclusive result due to discordance of cytogenetic and molecular analyses
2. R - Denotes backselected product

HYBRID	X REGION	NUMBER OF CELLS ANALYSED	NUMBER OF HUMAN CHROMOSOMES IN CELLS ANALYSED																									
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	DERX	DERAUT
AMIR2N	p22.3-qter	13	.	1	2 1	8	7	.	.	.	1	.	12 1	.	1	2 1	.	.	11	12	1	10	5 2	2	.	11 2	
EHA97II/VIII	p22-qter	50	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	19 5	
W5A915IX	p21-qter	50	.	31 6	.	42	.	38 3	42	.	.	39 4	41 3	.	3 18	1 2	13 8	10 35	43 2	2	.	29 5	30 4	2	.	42	
W2A96I	p21-qter	50	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	31	.	.	.	.	.	.	45 2	
LNA94IRbXIII	cen-qter	52	.	.	7 6	.	.	32 1	63	.	.	.	1	109	3 10	.	.	.	36 1	35	.	37	70	13		15 36	
CETK1aIV	cen-qter	50	.	.	8 3	.	.	.	2 1	37 1	.	2	.	.	.	.	.	.	41	6	2 3	39	.	1	.	40 5	
NEA916II	q13-qter	60	17 5	15 3	2 2	1	1	26 1	50	.	.	.	.	.	.	12 1	.	.	.	1	.	50	.	.	.	.	50 12	DER11
FNA98IX	q24-qter	60	.	.	.	47 2	1	.	54 1	.	.	2 1	.	.	.	.	1 1	.	.	.	.	.	.	.	.	.	52 5	DER19
DHTK18a	pter-q27	50	.	12 1	4 1	25	20	56	.	.	.	30	28	19	.	.	.	.	30	35	6	.	27	17	.	26 3	
WHTK17III	pter-q24	50	.	3 2	2	21	.	2	33 2	36 50	1	1 1	1	32 1	1	.	.	2 3	86 4	.	4 2	23	5	37 1	.	30 7	
FHA92bRa$_1$I	pter-q24	60	.	.	.	.	.	.	76 4	22 2	1 7	36 1	.	.	4	.	6	.	27	.	.	2	44	.	2	46	2	DER19
NEA921R$_2$b	pter-q13	24	.	4 1	.	1	17	.	2	7 1	.	.	14	11	.	.	1	.	15	1	1 1	16	19	.	.	9 1	
HNTK6VII/I	pter-q13	49	.	1	.	22 6	1	.	5 2	36 7	.	1	1	1	.	.	.	.	69 3	41 2	15 2	13 1	29	25 1	.	25 8	

TABLE 3 : CYTOGENETIC ANALYSIS OF A PANEL OF HYBRIDS BEARING DIFFERENT PARTS OF THE X CHROMOSOME

NOTES : 1. In each row the upper figure represents definite identification of the desired chromosome, while the lower figure denotes tentative identification
2. DERX - derivative X chromosome; DERAUT - derivative autosome

HYBRID	X REGION	NUMBER OF CELLS ANALYSED	% OF CELLS WITH X REGION	COPIES OF X REGION PER CELL	NUMBER OF HUMAN CHROMOSOMES PER CELL	NUMBER OF MOUSE CHROMOSOMES PER CELL
AMIR2N	p22.3-qter	13	77	0-2 AVE. 0.85	1-12 AVE. 6.5	79-92 AVE. 87
EHA97II/VIII	p22-qter	50	36	0-2 AVE. 0.38	0-2 AVE. 0.38	117-130 AVE. 122
W5A915IX	p21-qter	50	82	0-2 AVE. 0.84	5-20 AVE. 10	87-94 AVE. 91.3
W2A96I	p21-qter	50	86	0-2 AVE. 0.9	0-3 AVE. 1.6	79-142 AVE. 110.5
LNA94IRbXIII	cen-qter	52	25-77	0-2 AVE. 0.29 (0-3 AVE. 0.98)	4-16 AVE. 9.2	69-100 AVE. 87
CETK1aIV	cen-qter	50	80	0-2 AVE. 0.8	1-8 AVE. 3.8	115-125 AVE. 119
NEA916II	q13-qter	20	75	0-2 AVE. 0.8	1-6 AVE. 3.6	77-110 AVE. 93
FNA98IX	q24-qter	20	Not estimated	Not estimated	Not estimated	Not estimated
DHTK18a	pter-q27	50	50	0-2 AVE. 5.2	1-13 AVE. 6.8	90-180 AVE. 135
WHTK17III	pter-q24	50	50	0-1 AVE. 0.6	2-13 AVE. 7.6	110-130 AVE. 123
FNA92bRa$_1$I	pter-q24	60	68	0-2 AVE. 0.76	0-7 AVE. 4.7	130-139 AVE. 135
NEA921R$_2$b	pter-q13	24	37.5	0-1 AVE. 0.375	2-8 AVE. 5.125	60-75 AVE. 70
HNTK6VII/I	pter-q1.3	49	44	0-2 AVE. 0.5	1-11 AVE. 6.7	101-106 AVE. 106

TABLE 4 : ANALYSIS OF THE CHROMOSOME CONSTITUTION OF THE MOUSE/HUMAN HYBRID CELL LINES

NOTES : 1. 25% of cells in hybrid LNA94IRbXIII contain positively identified region of the X chromosome, an additional 77% possibly contain the X region.
2. AVE. - Average

while DX13 probe was present, confirming the presence of only the long arm of the X.

Cytogenetic analysis of WHTK clones gave two potentially useful clones : WHTK17I and WHTK17III, but a preliminary DNA analysis using a few probes from the short and the long arm of the X showed that only WHTK17III had the delX without contamination of a normal X chromosome. It should be pointed out that out of 19 cells that were analysed in WHTK17I, one cell seemed to have an X chromosome, and that was later confirmed to be human by fluorescent staining.

Similarly, cytogenetic analysis of HNTK clones gave three potentially useful clones: HNTK6VII/I, HNTK6VII/VI and HNTK6VII/IX. The only line free of Xq sequences, according to preliminary DNA analysis, was HNTKII/I. Cytogenetic analysis of the other two lines, using both the Giemsa stain and the fluorescent stain did not indicate the presence of a normal X (see Discussion chapter), while DNA analysis detected long arm sequences.

Hybrid clones of NEA9 and FNA9 which were shown by cytogenetic analysis to carry the derivative X (X/11 and X/19 respectively) in a small percentage of their cells, were backselected.

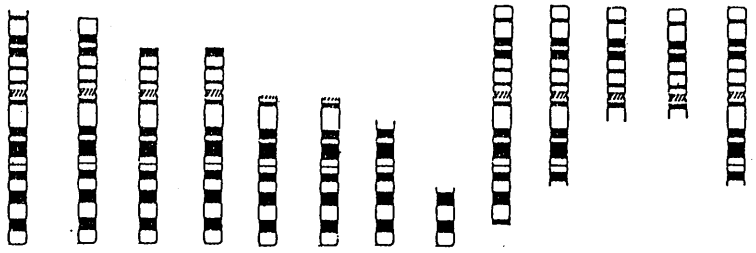
FNA9 backselected line, FNA9bRa$_1$VI was shown after extensive cytogenetic analysis to be missing a normal X or the reciprocal translocation which carries the long arm of the X (19/X). Preliminary DNA analysis, though, showed the existence of the long arm sequences of the X chromosome. Another pellet was made from the same line to exclude the possibility of contamination, and its cytogenetic analysis again did not reveal the presence of the long arm of the X. Analysis of other subclones was performed to find another

useful line but FNA2bIIRa$_1$I was the only other potential candidate. Its cytogenetic analysis with both Giemsa and fluorescent stains revealed the presence of the long arm of the X (19/X) in a very small percentage of cells (2 out of 60). Both clones, however, were submitted to DNA analysis which again confirmed the presence of the long arm of the X.

NEA9 backselected line NEA921R$_2$b was the only hybrid cell line which seemed to have retained the reciprocal translocation involving the short arm of the X on its own (X/11), after extensive cytogenetic analysis with both stains mentioned above. The DNA analysis, however did not agree with these results and indicated the presence of long arm sequences.

Table 5 summarises the results obtained from the molecular analysis of these hybrids (examples of Southern blots are shown in Figure 13), using fifteen X specific DNA probes, distributed along the short and the long arm of the X chromosome (Table 1 in Materials and Methods). AMIR2N hybrid is included in the screening as well. The inconsistencies mentioned above can be seen in this table and will be discussed in the next chapter. The results obtained from the molecular analysis of WHTK17III and DHTK18a are interesting in view of the fact that the cytogenetic analysis could not give accurate breakpoints for this rearrangement.

Horl9X was subcloned and some of the subclones together with the original clone were tested by Southern blot analysis using a few of the probes from both the short (GMGXY1, dic56, PX13, 782) and long arm of the X chromosome (FIX, DX13). Probes DIC56 (7.4kb/8.9kb) and 782 (14kb/7kb) produced some interesting results. Two of the subclones : Horl9X-I and Horl9X-V- have the 8.9kb dic56 allele while Horl9X-VI, Horl9X-VII and the original clone Hor91X have



HYBRID	1	2	3	4	5	6	7	8	9	10	11	12	13
	p22.3	p22	p21	p21	cen	cen	q13	q24	q27	q24	q13	q13	q24
PROBE													
GMGXY1	-	-	/	/	/	/	-	-	/	/	/	+	+
DIC56	+	+	-	-	-	-	-	-	+	+	+	+	+
PX23	+	+	-	-	-	-	-	-	+	+	+	+	+
RC8	+	+	-	-	-	-	-	-	+	+	+	+	+
782	+	+	-	-	-	-	-	-	+	+	+	+	+
D2	+	+	-	-	-	-	-	-	+	+	+	+	+
B24	+	+	-	-	-	-	-	-	/	-	+	+	+
87/8	+	+	-	-	-	-	-	-	+	+	+	+	?-
754	+	+	+	+	-	-	-	-	+	+	+	+	-
OTC	+	+	+	+	-	-	-	-	+	+	+	+	?+
L128	+	+	+	+	-	-	-	-	+	-	+	-	+
DP34	+	+	+	+	+	+	+	-	+	+	-	-	-
F9	+	+	+	+	+	+	+	+	+	+	+	-	+
DX13	+	+	+	+	+	+	+	+	-	?	?	-	-
ST14	/	/	/	/	/	/	/	/	-	/	/	/	/

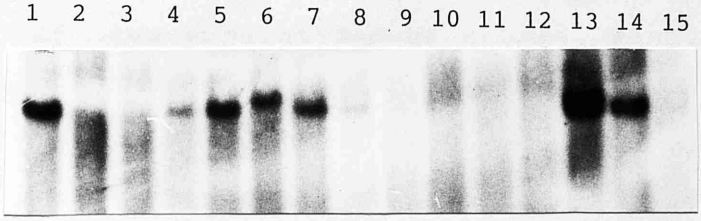
TABLE 5: DISTRIBUTION OF X-SPECIFIC PROBES ON THE HYBRID PANEL BEARING DIFFERENT PARTS OF THE X CHROMOSOME

KEY :

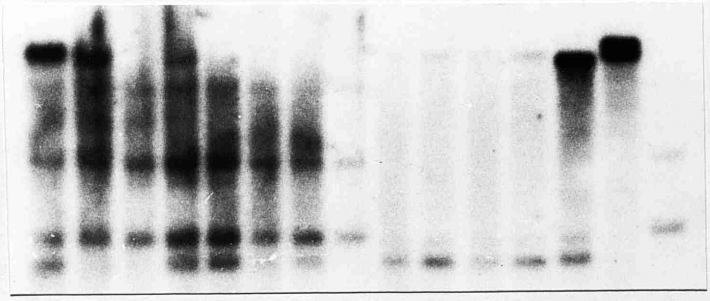
1. AMIR2N
2. EHA97II/VIII
3. W5A915IX
4. W2A96I
5. LNA94IRbXIII
6. CETK1aIV
7. NEA916II
8. FNA98IX
9. DHTK18a
10. FNA92bRa$_1$I
11. NEA921R$_2$b
12. HNTK6VII/I
13. WHTK17III

\+ positive hybridisation
\- negative hybridisation
? inconclusive result
/ not tested

NOTE : Breakpoints on the X are shown below the chromosome diagrams



D2 probe/PVU II digest



DP34 probe/TAQ I digest

FIGURE 13: Southern analysis of the hybrid panel probed with D2 and DP34. The panel consists of:

1. AMIR2N
2. NEA916II
3. FNA98IX
4. EHA97II/VIII
5. WHTK17III
6. NEA921R_2b
7. HNTK6VII/I
8. FNA92bRa_1I
9. W2A96I
10. W5A915IX
11. CETK1aIV
12. LNA94IRbXIII
13. Normal female
14. Normal male
15. A9 mouse cell line

the 7.4kb allele. Similarly Horl9X-I and Horl9X-V were shown to have the 14kb 782 allele while Horl9X-VI has the 7kb allele. This suggests that the original population is a mixture of two populations of cells, each containing a different X chromosome. The fact that only one allele of dic56 is present in the original clone would suggest that possibly in this culture one population overgrew the other.

Horl9X and some of its subclones were also probed with 87/8 and the initial results showed that the hybrids were negative for this probe. This remains to be confirmed again in another experiment.*

Another thirteen hybrids which retained different groups of autosomes were further analysed and used in an attempt to localise the autosomal fragments recognised by the Y probes isolated in this study. Table 6 summarises the results from the cytogenetic analysis performed and Figure 17 shows the Southern blots probed with the three Y-autosome specific fragments isolated in this study and described in the second part of the results.

* In view of the inconsistencies which were present after testing this hybrid, data from this cell line had to be interpreted with caution, e.g. when used for the regional mapping of probes.

HYBRID	NUMBER OF CELLS ANALYSED	NUMBER OF HUMAN CHROMOSOMES IN CELLS ANALYSED																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	DERX	
NEA915RBVIB	15	.	.		.	.	.	.	.	.		.		.	.		.	15	.	.	.	.	.	.	.	
				5							1		1			4										
FNA95R2	10	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	
															4											
NEA918	20	.	.	.	.	.	10	3	.	.	8	2	.	.			.	.		6	1	.	.	.	.	
							4								1	2										
EHA97III	21	.	9	.	19	.	.	1	.	.	10	.	.	.	2		.	14		.	.	.	.	.	.	
AMIR2XI	15		.	14	13	.	8	17	7		1	30	1	1				8	12	11	11	5	3	.	5	X/Y
		6		4	5			2	3	2	1	3		2	1	1	1	2	5	1	1	1			3	
LNA94IRbXII	8	.	.	.	.	.	5	6	.	.	.	.	10	.	.	.	.	6	.	.	11	10	2	.	5	Xp-
																									1	
FNA92bIIRA$_1$VI	25	.	.		.	.			.	.	.	16	.			.	.	.	.	.	.	17	2	.	25	X/19
				3			1	1						3	1											
FNA92bIIRa$_1$I	19		.	.	.	.	.	1	1	.	.	12	2	.	.	.	.	.	.	.	.	10	.	.	12	X/19
		1						1				2													3	
WHTK17III	50	.	3		21	.		33	36		1	1	32		.	.	2	86	.	4	23	5	37	.	30	delX
			2	2			2	2	50	1	1		1	1			3	4		2			1		7	
CETK1aIV	50	.	.	8	.	.	.	2	37	.		.	.	.	.	.	.	41	6	2	39	.	1	.	40	Xp-
				3				1	1		2									3					5	
EHA97VIII	21	.	30	.	17	.	.	18	.	.	1		.	.	.	.	.	15	.	.	.	.	.	.	.	
			5					3			1	1														
W2A96I	50	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	31	.	.	.	.	.	.	45	X/8
											1														2	
THYB133R	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	.	.	.	

TABLE 6 : CYTOGENETIC ANALYSIS OF A PANEL OF HYBRIDS CONTAINING DIFFERENT GROUPS OF AUTOSOMES

NOTES : 1. In each row the upper figure represents definite identification of the desired chromosome, while the lower figure denotes tentative identification
2. DERX - derivative X chromosome

3.2. Isolation and Characterisation of Sequences from a Y-Specific Library

3.2.1. Isolation of probes

From the initial screening of the library with total human male genomic DNA as a probe, about 3,000 clones were positive and these were picked. From the screening of these clones with cDNA only 103 clones hybridised with the probe, and these were picked and stored. Ten clones were selected for further study because they complied with the criteria for selecting single copy or expressed sequences (see section 2.7. in Materials and Methods). These were purified and their DNA digested, blotted and probed with cDNA and total male genomic DNA, but only six clones (Bi, Cii, Dii, Ei, Bi-2 and Bii-7) were successfully characterised. From the other four one, was shown to be repetitive, one was shown to detect a Y-specific fragment but was not further purified and characterised, and the last two seemed to contain some single-copy sequences but this needs to be further confirmed.

The information given below is derived from digestion of the DNA clones with the enzyme Eco RI. Most of the clones produced more than one fragment because they have been isolated from a partially digested library.

Bi clone produced at least three fragments (4.5kb, 4kb and 2.1kb). The 4kb fragment seemed to be positive for the cDNA probe.

Dii clone produced at least three fragments (6.1kb, 4kb and 1.6kb). The 1.6kb fragment seemed to be positive for the cDNA probe and did not hybridise with total male genomic DNA.

Ei clone produced at least four fragments (9kb, 3.5kb, 3kb and 1.8kb) and the 3.5kb fragment was negative for the total male genomic DNA probe. The 1.8kb fragment also seemed to be negative for the same probe but was not further characterised.

Bi-2 clone produced five fragments (6.4kb, 5.3kb, 2.2kb, 1.4kb and 0.8kb) of which the 0.8kb fragment was negative for the total male genomic DNA probe.

Cii, an intact 17kb fragment, and Bii-7, an intact 7kb fragment, were not shown to contain any obvious single-copy sequences after digestion with the enzymes mentioned in section 2.7. and after being probed with genomic DNA and cDNA probes. They were thus preassociated with genomic DNA to compete out any repetitive sequences, and used as probes against an Eco RI digested X panel. Both were shown to contain single-copy sequences.

All these fragments were subcloned into the Eco RI site of the plasmid vector pUC13.

3.2.3. Characterisation of Probes

i. A Recombinant of Autosomal Origin

Bi-4

The 4kb fragment (Bi-4) derived from clone Bi was found to be highly repetitive. DNA from this fragment was digested with Eco RI (in order to separate the insert from the plasmid DNA) and a variety of enzymes such as Bam HI, Hind III, Hpa I, Pvu II, Sac I, Sal I, Sma I, Xba I, Xho I for which Bi-4 does not have any sites and Bgl II, Hinf I, Hph I, Kpn I, and Pst I which cut the 4kb fragment into different size fragments. The digests were run on gels, blotted and probed with total male genomic DNA in an attempt to process out the repetitive sequences and detect single copy sequences that might be present. Two double digests Eco RI/Kpn I (three fragments of 2.1kb, 1kb, and 0.9kb) and Eco RI/Pst I (two fragments of 2.8kb and 1.2kb) seemed to produce fragments that did not hybridise to the genomic DNA and thus might be single-copy sequences. A potential single-copy Pst I fragment of 1.2kb was shown to contain highly repetitive sequences when used as a probe against a simple panel containing DNA from a male, a female, Hor19X (X only hybrid), 3E7 (Y only hybrid) and mouse A9 DNA. The Kpn I 2.1kb fragment which was also negative for the male genomic probe was not further tested.

Later on, using the method of preassociating the probe with an excess of sonicated human genomic DNA before hybridisation, as described in Materials and Methods, a single-copy sequence was detected, but was shown to be of human autosomal origin because it hybridised to the genomic DNA of both male and female individuals and did not hybridise to the DNA of Hor19X (X only hybrid), 3E7 (Y only hybrid) and mouse A9 DNA.

ii. Recombinants which Recognise Homology Between the Y and Autosomes

a. GMGY1 Probe

GMGY1, an 800bp probe was isolated from clone Bi-2. The probe was tested against the X panel of genomic and hybrid DNAs (as described in Materials and Methods, section 2.7., and shown in Figure 15), which had been digested with Eco RI. This probe was shown to be derived from the Y chromosome because it hybridised to the DNA from the hybrid carrying the Y as the only detectable human chromosome (3E7), and not to the hybrid which contained only the X (Horl9X). It also hybridised to both male and female genomic DNA. This suggested that the probe also detects some autosomal sequences, in addition to those on the Y. In order to separate the autosomal from the Y sequences, the panel of DNAs was digested with the enzymes Msp I and Taq I (Figure 15), which have been found to be more efficient for detection of polymorphisms (Knowlton **et al** 1985). Different band patterns were observed with both enzymes and the autosomal bands were well separated from the Y band. Msp I was chosen as the most suitable restriction enzyme to be used for further experimentation since the pattern it produced was very clear. The sizes of the autosomally linked fragments in the Msp I digest are 3 and 1.7kb, while the Y-linked fragment is 2.4kb.

The next step involved localisation of the autosomal and Y derived sequences. Initially with the use of the mouse/human hybrids AMIR2N, NEA919II and FNA98IX (described in Materials and Methods and section 3.1. from Results) which contain different groups of human chromosomes, together with part of the X chromosome (see Table 7), the autosomal hybridisation was shown to occur on one or more of chromosomes 1, 2, and 6. Figure 15 shows GMGY1

hybridising to the DNA from hybrid NEA916II but not to that of hybrids AMIR2N or FNA98IX. Table 7 shows the content of chromosomes in each of these hybrids and the percentage of their occurrence. It can be seen that only hybrid NEA916II was shown by cytogenetic analysis to contain these chromosomes.

Subsequently, the DNA from another thirteen hybrids, which were chosen because they bear different groups of autosomes (described in section 3.1. of Results), was digested with the restriction enzyme Msp I enzyme and probed with GMGY1 probe in an attempt to localise more precisely the autosomally-linked fragment. The probe was shown to hybridise to four additional hybrids (FNA95R2, AMIR2XI, LNA94IRbXII, and FNA92bIIRa$_1$I) with a variety of intensities, presumably related to the number of copies of the autosome in question present in these lines (Table 7 shows the percentage of copies of each chromosome present in each hybrid). The autosome complement of each of hybrid lines to which this probe hjybridises was examined, and by combining the analyses of all the hybrids, the consensus of data showed that the most probable candidate would be chromosome 1 although in two of the hybrids, FNA95R2 and LNA94IRbXII, to which GMGY1 hybridised this chromosome was not detected cytogenetically. Possible explanations for this might be (i) that a small number of cells has been examined in these hybrids (see Table 6) and the particular chromosome has gone undetected or (ii) quite probably a rearrangement has occurred between chromosome 1 and one or more mouse chromosomes and therefore it has not been detected (to be further discussed later).

The autosomal fragments were more accurately assigned when the probe was hybridised to Msp I digested DNA from human chromosomes purified by flow cytometry from an individual with a chromosome 1 centric polymorphism and a deletion of Xp21. Figure 18A and B show the flow karyotype of this individual and the autoradiograph of GMGY1

hybridised to these DNAs, respectively. Lane 3 which contains the polymorphic chromosome 1, and lane 4, containing the normal chromosome 1 together with chromosome 2, both show a positive signal for the autosomally-linked fragments, supporting the localisation of these fragments to chromosome 1. Lane 10, which contains chromosomes 21, 22 and the Y shows the presence of the Y linked band, confirming the assignment of the 2.4kb fragment to this chromosome.

In an attempt to analyse the strength of homology between these sequences, a simple panel was constructed containing the genomic DNA from two normal females, two normal males, and from the (Y only) 3E7 hybrid and the (X only) Hor19X hybrid. The DNAs were digested with Msp I enzyme, blotted on filters, hybridised with the probe and washed down to different stringencies, of 0.5xSSC, 0.1xSSC and 0.05xSSC respectively (Figure 20A). In 0.5xSSC all three bands can be detected, in 0.1xSSC the lower autosomal band disappears, and in 0.05xSSC the higher autosomal band becomes very faint, indicating that the homology between the probe and these sequences is not exact.

The probe was then tested against the Y panel which includes individuals with deletions, translocations and other abnormalities of the Y chromosome and has been described in Materials and Methods section 2.7. Figure 21D shows the DNAs digested with Msp I enzyme, probed with GMGY1 and washed down to 0.1xSSC. The probe was mapped in this way to the region from Yq11.23 to Yq11.12. The Y-linked fragment was present in all individuals who lack most of the heterochromatin of the long arm of the Y chromosome (panel members 5, 6, 7). It was also present in panel members 11 and 12 who have a complete Y long arm and part of the long arm of the Y up to Yq11.21, respectively. The Y-linked fragment was however, absent from panel member

number 10, which carries an isochromosome for the short arm of the Y, thereby excluding its location on the short arm of the Y chromosome. No hybridisation was detected in panel members 8 and 9 which both have deletions of part of the Y chromosome from Yq11.23 to the Yqter. Table 9 summarises the results obtained with all five probes on this panel.

The RFLP panel described in Materials and Methods section 2.7. was probed with GMGY1. Digests with the restriction enzymes Bst NI, Bam HI, Eco RI, Hae III, Hind III, Msp I, Pst I, , Pvu II, Tha I, and Xba I did not detect any polymorphisms.

Of the enzymes used in this study only Bam HI, Msp I, Pvu II, Taq I and Xba I separated the Y-linked and autosomally-linked fragments. Pvu II and Xba I produced identical patterns.

Genomic DNA from individuals bearing one or two copies of the Y chromosome as well as one or multiple copies of the X chromosome (normal male (46,XY), SL (48, XXXY), HN (48,XXYY), HS (47, XYY), BE (47,XYY)) were probed with GMGY1 in order to do dosage studies but the results were inconclusive.

b. GMGY2 Probe

GMGY2 is a 1.1kb fragment isolated from clone Bii-7 (a 6.8-7kb fragment which was subcloned into pUC13) after an Eco RI/Hind III digestion.

Clone Bii-7 was digested with Eco RI (to separate the insert from the plasmid DNA) and various other enzymes listed in section 2.7. Enzymes Bam HI, Bgl I, Hpa II, Kpn I, Msp I, Sac I (and, of course, Eco RI) did not have any sites within the 7kb insert. The following enzymes cut the Bii-7 fragment into various size fragments: Cfo I (4.8kb, 1.5kb, 0.7kb), Hind III (1.1kb, 5.9kb), Pst I (6.1kb, 0.9kb), Pvu II (1.2kb, 5.8kb) and Hae III which produced

many small fragments. All digests were run on gels, blotted and probed with genomic DNA. The 1.1kb Eco RI/Hind III fragment was isolated because it did not hybridise with total male genomic DNA.

The fragment was used as a probe against the Eco RI digested X panel, which was subsequently digested with Msp I and Taq I enzymes. Only Y and autosomally-linked fragments were detected. The Taq I digest was chosen to be used for further experimentation because the main Y band was well separated from the autosomal bands, although at least one more of the Y fragments it detects has the same size as an autosomal one. Figure 14 shows the Southern blot analysis of the X panel probed with GMGY2. The 4.2kb fragment is shown to be Y and autosomally linked, the 3.6kb and 0.7kb fragments are autosomally linked only, and the 2kb fragment is Y linked only. The 5.5kb fragment is present in the genomic DNAs of both male and female, present in the Y only hybrid 7631 (although the band is much more faint than the 2kb Y-linked fragment), and absent from the 3E7 Y only hybrid. It is possible that this fragment is Y and autosome specific but this sequence is missing from the Y chromosome present in the 3E7 hybrid. Alternatively, this fragment could be autosomally-linked, since the 7631 hybrid has been shown to contain autosomal sequences which have not been detected cytogenetically (Burk **et al** 1985). This hybrid will be discussed later.

Localisation of the autosomally linked fragments was achieved using initially hybrids AMIR2N, NEA916II and FNA98IX. Only AMIR2N seemed to hybridise with GMGY2 (Figure 14) and was shown to have present the complete range of fragments detected on both male and female genomic DNA. By comparing the chromosome contents of these hybrids (Table 7) it was shown that the probe shares homology with one or more of chromosomes 11pter-p1.3, 13, 17, 18, 19q1.3 to qter, 21, and 22 and perhaps chromosomes 3, 5 or 9.

The probe was further hybridised to the DNA from the additional thirteen hybrids described in section 3.1 which were used to map the autosomally-linked fragment detected by **GMGY1**. Bands of 5.5kb and 4.2kb seemed to segregate together and were present on hybrids NEA915RBVIB, FNA95R2, LNA94IRbXII, FNA92bIIRa$_1$V$_1$, FNA92bIIRa$_1$I, WHTK17III, ThyB133R, 7631 and, as mentioned above, on hybrid AMIR2N. Since these bands are present on the ThyB133R hybrid which contains 21 as the only human chromosome these fragments were mapped on chromosome 21. However cytogenetic analysis of NEA915RBVIB and FNA95R2 did not detect any chromosome 21 therefore suggesting that perhaps these fragments can derive from the hybridisation of this probe to other chromosomes as well. By combining the analyses of these hybrids, possible chromosomes were 5, 9, 12, 13, 15, 16 and 22. The Y-only hybrid 7631 was shown to contain parts of chromosomes 4, 12, and 14 (Burk **et al** 1985). The 3.6kb fragment was present on hybrids WHTK17III, perhaps (very faint) on FNA92bIIRa$_1$I and on AMIR2N. The possible location was concluded to be on chromosome 5, 9, 13, 16 and 22. The 0.7kb autosomally-linked fragment was present again on hybrids WHTK17III and AMIR2N and perhaps on AMIR2XI and it was concluded that it hybridises to the same set of autosomes as the one before. Finally one more fragment above the 5.5kb one was detected and was shown to be present on FNA95R2, WHTK17III, AMIR2N and perhaps very faintly on FNA92bIIRa$_1$VI and FNA9R2bIIRa$_1$I thus again hybridises to one or more of the chromosomes mentioned above (see Figure 17 and Table 7).

GMGY2 was then hybridised to the same blot of sorted chromosomal DNA digested with Msp I used above for GMGY1 (Figure 18C flow karyotype), in order to localise the autosomal sequences more precisely. Figure 18C shows the autoradiographs from this experiment, to facilitate the interpretation of the blot results, the autosomal bands were numbered from 1 to 4. Bands No A1, A3, and A4 were

present on lane 7 which carries the DNA from chromosomes 13 to 16. The Y band, A2 and A4 are present on lane 10 which carries the DNA from chromosomes Y, 21, and 22. The presence of the Y linked band in this lane further confirms its localisation to the Y chromosome. It seems also that band A2 might be present on lane 6 which carries the DNA from chromosomes 9 to 12. The results obtained both from the hybrids and the sorted chromosomal DNA show that this probe shares homology with chromosome 21 and one or more of chromosomes 13, 15, 16, 22 and perhaps 9, 12 and 11pter-p1.3.

Washing the probe at different stringencies, as was done for GMGY1, did not seem to make any difference, which suggests that homology is quite precise.

The probe was localised to the region between the Yq11.12 and Yq11.23, using the same Y panel as for GMGY1 (see Figure 21A). The probe was present or absent from the same individuals as probe GMGY1 (Table 8).

c. <u>GMGY11</u>

This probe is a 2.1kb fragment isolated from clone Cii (a 17kb fragment which was subcloned into pUC13) after an Eco RI/Hind III digestion. Clone Cii was digested with Eco RI and a range of enzymes, as described for clone Bii-7, run on a gel, blotted and probed with male genomic DNA to find sequences that did not hybridise to it. The 2.1kb fragment did not hybridise to genomic DNA. The fragments produced by the Eco RI/Hind III digest were 4.8kb, 3kb, 2.1kb, 1.8kb, 1.6kb, 1.4kb, 1.2kb, and 1.1kb.

The fragment was tested against an Eco RI digested X panel and appeared to hybridise to the Y chromosome and some autosomes but not to the X chromosome. However it hybridised to the same sized fragments on both male and

female DNA. In an attempt to separate the Y and autosomally-linked fragments, the X panel was digested with Msp I and Taq I enzymes. Different patterns were observed with each enzyme (Figure 14), but in both cases the Y band was well separated from the autosomal bands. Taq I enzyme was chosen for further experimentation because it generated two fragments for the Y chromosome and detected an autosomally linked polymorphism (Figure 14). The sizes of the Y bands generated by the Taq I digest are 8 and 5.5kb, while the autosomal bands are 4, 3.8 and 2.3kb.

The autosomally-linked fragments were present only in the DNA of the NEA916II hybrid, therefore suggesting that this probe recognises homologous sequences with one of chromosomes 1, 2, or 6 (Table 7). DNA from the thirteen hybrids which bear different groups of autosomes were digested with Taq I and probed with GMGY11. The results were not very clear yet the probe was shown to hybridise to FNA92bRa$_1$I in addition to NEA916II mentioned above and perhaps very faintly to LNA94IRbXII (Figure 17, Table 7). A clear conclusion cannot be drawn from this result but it seems that the probe hybridises to chromosome 1 and perhaps to 6, 15 and 16.

The blot with the sorted chromosomal DNA, previously used to map the other two probes, was again probed with GMGY11 but the hybridisation was not very succesful, probably because the filter had been used twice already. A weak hybridisation however, could be detected in lane 3 (autosomal band A3), which carries DNA from the polymorphic chromosome 1, and in lane 4, which carries DNA from normal chromosomes 1 and 2. The results from the hybrids and from the sorted chromosome DNA blot together, would tend to indicate that this probe is homologous to sequences on chromosome 1 (Figure 18D). It was noticed that these autosomally-linked fragments are polymorphic with the 3.8kb fragment being constant, and the 4kb and 2.3kb fragments

being allelic.

GMGY11 was tested against the Y panel in order to localise the Y-linked fragment. It was shown to be present on the DNA of all the panel members except the normal female, individual WC (panel member number 10) who has a deletion of the long arm of the Y chromosome and AMIR2N (number 12) which carries part of the long arm of the Y from Yq11.2-qter. This result maps the sequence to Ycen-Yq11.2 (Figure 21B and Table 8).

iii. A Recombinant which Recognises Homology between X, Y and Autosomes

GMGXY2

GMGXY2, a 3.5kb fragment, derived from phage clone Ei. With an Eco RI digested panel, the probe was shown to hybridise to the X, Y and autosomes. Digestion of the X panel with Msp I and Taq I enzymes did not give any satisfactory pattern of hybridisation (no difference was detected between male and female DNA, Figure 15), and so the Eco RI digest was used for further experiments (Figure 16). More than one X-linked and Y-linked fragments can be detected but most of them cannot be distinguished from autosomally linked bands of the same size. The size of the main (distinguishable) X-linked fragment detected with the Eco RI digest is 4.7kb and that of the Y-linked fragment is 3.5kb.

An attempt was then made to remove the autosomal sequences from this probe. After a series of experiments which included using different enzymes (Bal III, Bam HI, Hind III, Hinf I, Hpa II, Kpn I, Msp I, Pst I, Pvu II, Sal I, Sma I, Sac I, and Xho I) to try and cut the probe in the middle, the probe was finally cut with Eco RI/Pvu II and Eco RI/Bam HI. The restriction enzyme Bam HI cut the insert

into two fragments of 1.7kb and 1.8kb which were not further used. Pvu II restriction enzyme cut the insert into three fragments of 1.9kb, 0.9kb and 0.7kb. The two smaller fragments 0.9kb and 0.7kb were used as probes against a simple panel made with DNA from a normal male, normal female, Y only hybrid, X only hybrid and mouse A9. The pattern they produced was identical (results not shown) and similar to the one obtained by GMGXY2 when washed at higher stringencies as described in the paragraph below.

Washing the simple X panel at the same levels of stringencies as used for the other probes, gave an even more striking result, because quite a few bands actually disappeared when the stringency was raised, and a simpler pattern emerged (Figure 20B).

Sorted DNA from individual CN (see flow karyotype Figure 19A) was probed with GMGXY2 in order to localise the autosomal sequences. Several of the autosomal fragments detected by this probe were present on chromosome 1 but homologous sequences were detected also on chromosome 2 and a range of other autosomes. The sequences therefore recognised by this probe seem to be dispersed throughout the genome.

In order to localise the X-linked fragment, a more complete panel was used containing the mouse/human hybrids described earlier on in this study, which carry different parts of the X chromosome (Tables 3 and 5). Using this panel, the main X sequence was shown to be present in the hybrids Hor19X, AMIR2N, EHA97II/VIII, NEA916II, and FNA92bIIRa$_1$I (Figure 16. It was shown to be absent from hybrids FNA98IX and WHTK17III and possibly from HNTK6VII/I and NEA921R$_2$b, thus the probe was mapped to the region Xq13-Xq24. The interpretation of this Southern analysis was difficult because the main X band cannot be clearly visualised due to obstruction from two or more autosomal

bands present in the same area. To exclude the possibility that the band present on FNA98IX, just above the position of the main X band is not an X polymorphism detected by this probe, the DNA from the parental lines of the hybrids FNA98IX (FN), NEA916II (NE), AMIR2N (McE) and fifteen normal females was digested with Eco RI restriction enzyme and probed with GMGXY2. The results did not reveal any polymorphism.

In order to achieve a better separation of the fragments detected by this probe and distinguish the X-specific bands from the autosomal ones, DNA from normal males and females was digested with Eco RI and run on gels containing different percentages of agarose (0.4%, 0.6%, 0.8%, 1.0% and 1.2%). The gels were also run under reduced current but a better separation could not be achieved.

To localise the probe on the Y chromosome, the Y panel was used. The result showed that GMGXY2 is absent only from AMIR2N hybrid (panel member number 12) which carries an X/Y translocation with the Yq11.2 to Yqter part of the chromosome. This excludes its localisation on that part of the long arm of the Y chromosome. DNA however from ED (panel member number 11) and WC (number 10) hybridised with GMGXY2. The only part of the Y that is common in both these individuals is Ycen to Yp11.2 which maps GMGXY2 in this area and further supports the cytogenetic analysis which detected the presence of two dicY in the ED individual (Figure 21E and Table 8). Localisation of GMGXY2 is further supported with the result from the panel of XX males (see next section).

The RLFP screening panel was probed with GMGXY2. Enzymes Bst NI, Bam HI, Bgl I, Bgl II, Eco RI, Hind III, Hae III, Hpa II, Msp I, Pst I, Pvu II, Taq I, and Xba I did not reveal any polymorphisms. Sma I and Cfo I digestions will need to be repeated because they did not digest the

samples. Of all these enzymes only Eco RI produced a male specific pattern.

A dosage panel constructed from individuals with multiple copies of the X or the Y chromosome was probed with GMGXY2 in order to detect a dosage effect for the main X and Y bands. The panel was composed of a 46,XO (DE), 46,XX (normal female), 47,XXX (McN), 48,XXXX (McE), 46,XY (normal male), 48,XXXY (SL), 48,XXYY (HN), 47,XYY (HS) and 47,XYY (BE). The results however were not conclusive and the experiment should be repeated.

Finally an attempt was made to look for expression of this probe by doing a Northern blot but the RNA used here proved to be of poor quality, therefore such an experiment should be repeated.

iv. A Recombinant which Recognises Homology between the X and the Y

GMGXY3

From phage clone Dii, a 1.6kb Eco RI fragment was subcloned into pUC13 and used as a probe against the X panel. The result indicated that this probe hybridised to the Y chromosome but that it still contained some repetitive sequence elements. The fragment was further screened for single-copy sequences as has been described in Materials and Methods and was shown not to have sites for the restriction enzymesBam HI, Bgl I, Bgl II, Pst I, Pvu II, Hae III, Cfo I. . The following enzymes cut the 1.6kb fragment as follows: Sac I (1.4kb, 0.2kb), Hpa II and Msp I (1.1kb, 0.5kb). An Eco RI/Hind III digest (1kb, 0.6kb) produced a 600bp fragment that did not hybridise to genomic DNA. This fragment (GMGXY3) was used to probe the X panel digested with Eco RI. The result showed that the repetitive

element had been succesfully removed, but the probe detected the same size fragment on both the X and the Y chromosomes (Figure 14). The panel was digested with Msp I and Taq I and both digests showed separation of the X and Y specific bands. Taq I enzyme was again chosen for further experiments. The X band detected in this digest was 23kb and the Y band 2.3kb.

The X band was present on the hybrid which carries a complete X (Horl9X) but absent from the hybrid which carries a deletion of the X chromosome from Xp22.3 to Xp terminal (AMIR2N), thereby assigning the X-linked fragment to this region (Figure 14).

With the use of the Y panel, GMGXY3 was localised to the region Yq11.22 to Yq11.23 (Figure 21C and Table 8). It was absent from the DNA of WC (panel member number 10) with an isoYp thus excluding the localisation of this probe on the short arm of the Y chromosome. However, GMGXY3 hybridised to all the other members of the panel, including AMIR2N (number 12) which has the region of the Y chromosome from Yq11.2 to Yqter and to DNA from individuals SN and FF (numbers 8 and 9 respectively) both of whom have a deletion of the Y chromosome from q11.23 to qter.

Washing the simple X panels at increasing stringencies (0.5, 0.1, and 0.05 x SSC) after having probed them with GMGXY3 showed a gradual disappearance of the X-linked band (Figure 20C).

Table 9 summarises the information obtained on the five probes described in the above sections.

GMGY2 /X PANEL

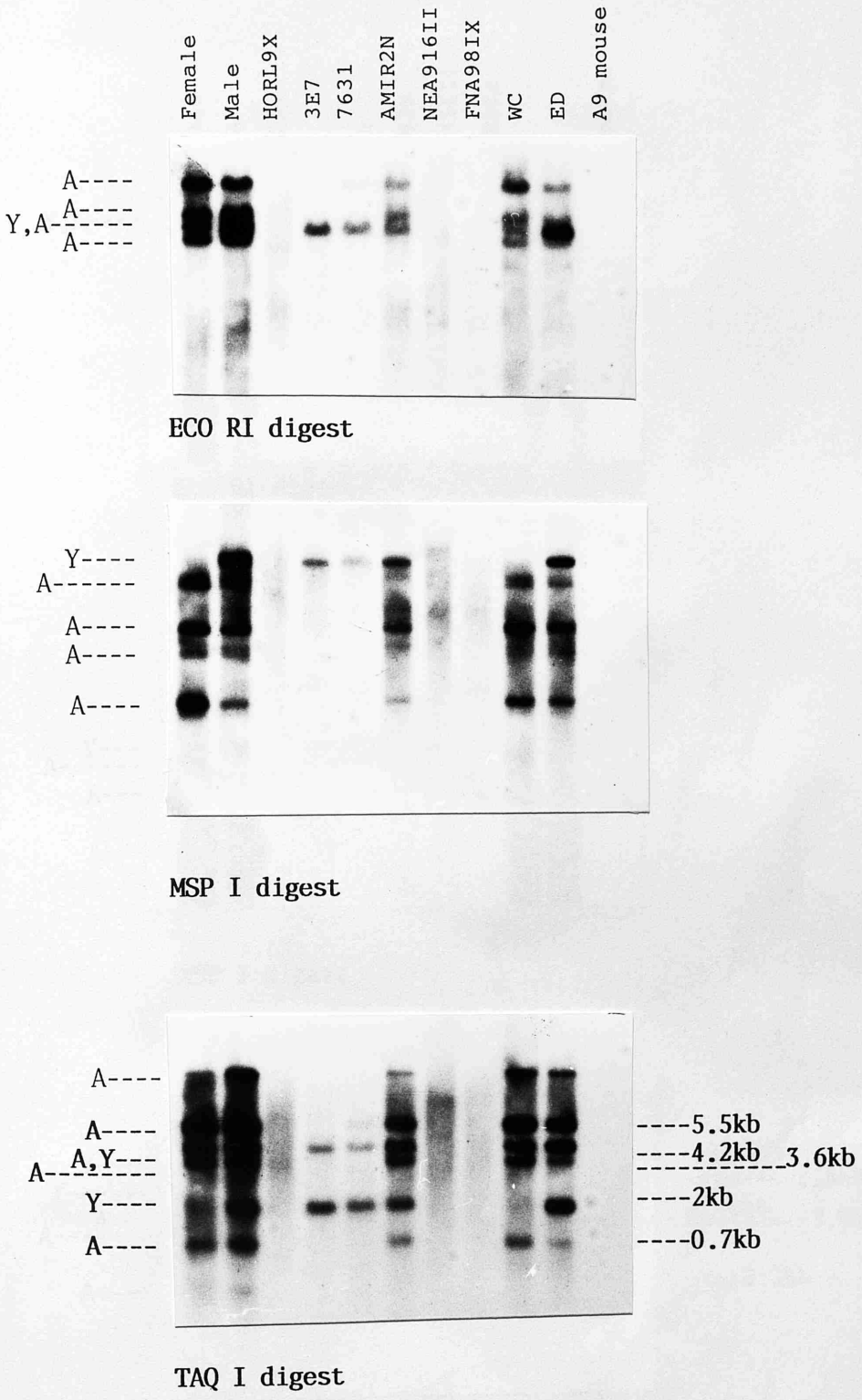
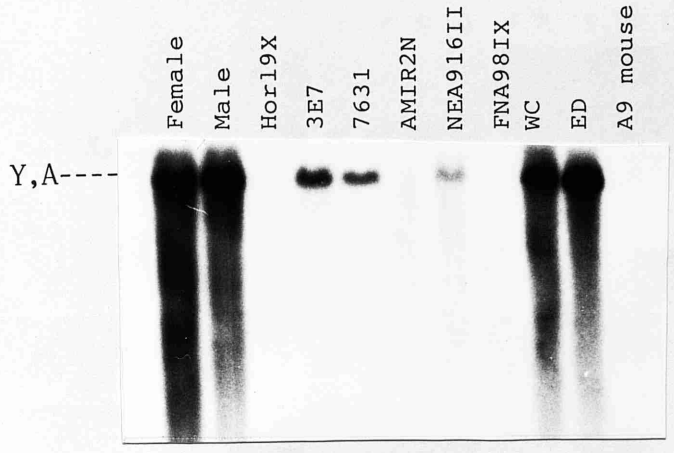


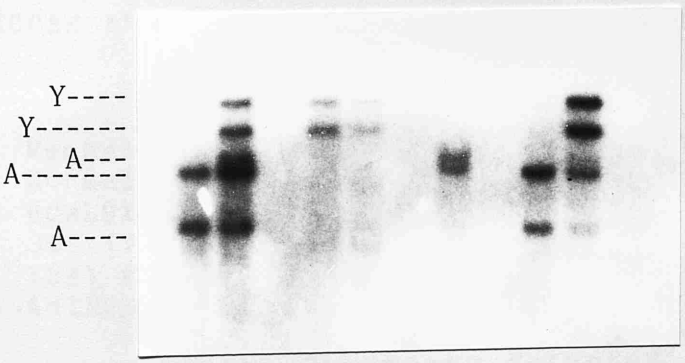
FIGURE 14 : See legend on following page

GMGY11/X PANEL



ECO RI digest

MSP I digest



TAQ I digest

FIGURE 14 : See legend on following page

GMGXY3/X PANEL

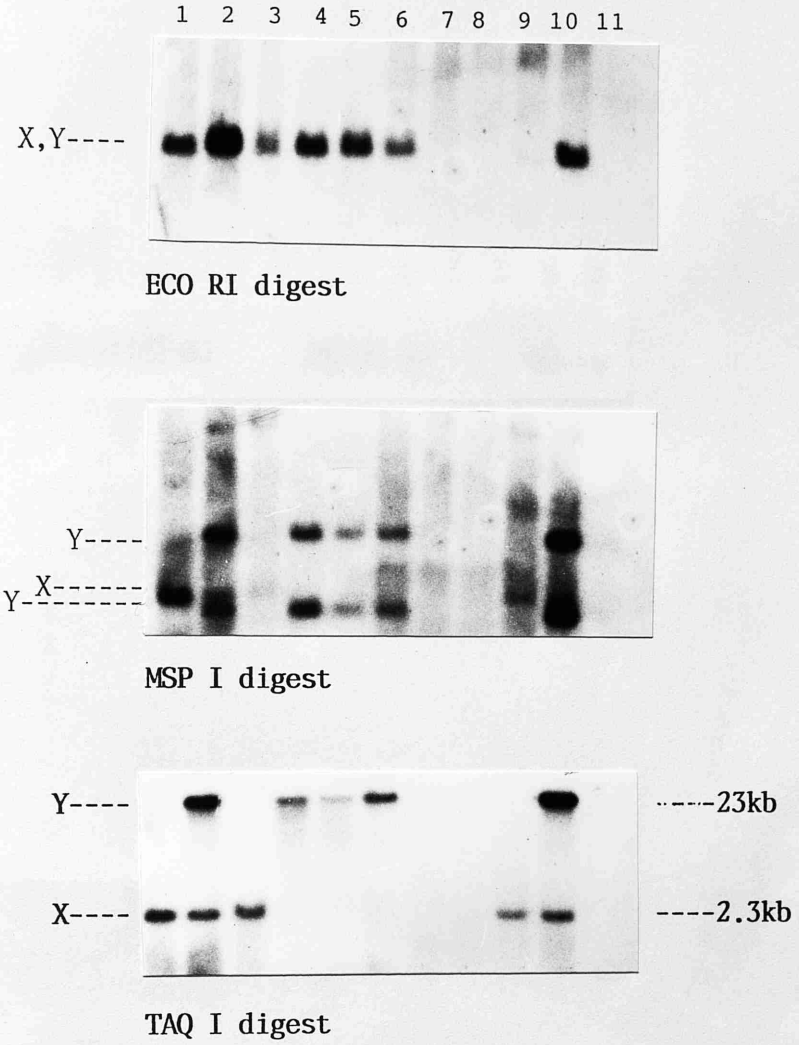
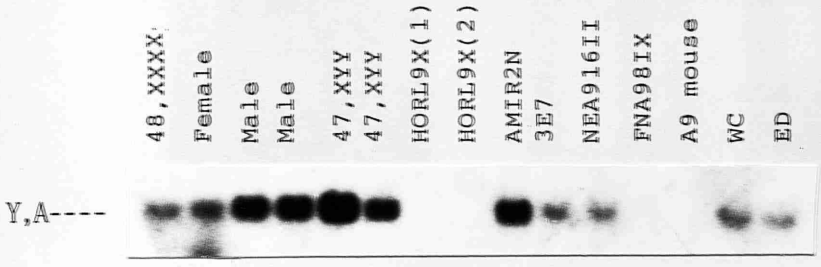


FIGURE 14: Southern analysis of X panel with probes GMGY2, GMGY11, and GMGXY3. The members of the panel are:

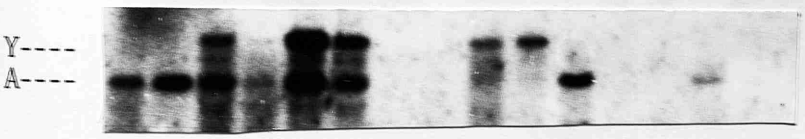
1. Normal female
2. Normal male
3. HORL9X (X only) hybrid
4. 3E7 (Y only) hybrid
5. 7631 (Y only) hybrid
6. AMIR2N hybrid
7. NEA916II hybrid
8. FNA98IX hybrid
9. WC male with an iso Yp
10. ED female with a dic Yq
11. A9 mouse cell line

The size of the fragments detected is indicated to the right of the photograph. X, Y or autosomal linkage are denoted by the letters X, Y or A respectively. The filters were washed at 0.5xSSC.

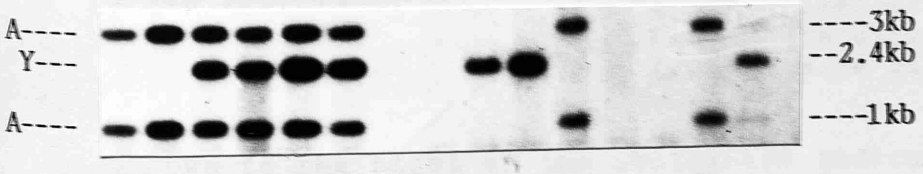
GMGY1/X PANEL



ECO RI digest



TAQ I digest



MSP I digest

FIGURE 15 : See legend on following page

GMGXY2/X PANEL

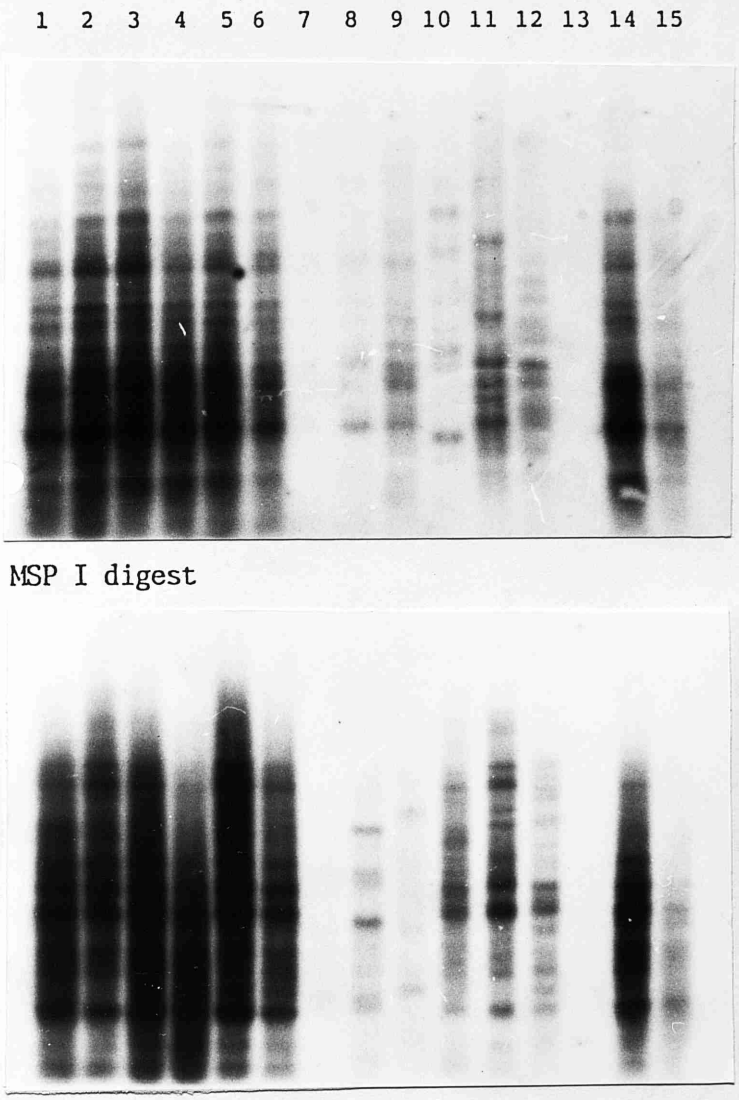


FIGURE 15: Southern analysis of the X panel with probes GMGY1, and GMGXY2.
The members of the panel are:

1. 48,XXXX individual
2. Normal female
3. Normal male
4. Normal male
5. 47,XYY individual
6. 47,XYY individual
7. HORL9X (X only) hybrid
8. HORL9X (X only) hybrid
9. AMIR2N hybrid
10. 3E7 (Y only) hybrid
11. NEA916II hybrid
12. FNA98IX hybrid
13. A9 mouse cell line
14. WC Male with an iso Yp
15. ED Female with a dic Yq

The arrows to the right of the photograph indicate the size of the fragments detected. Y or autosomal linkage are indicated by the letters Y or A respectively. Lane 8 contains twice the amount of DNA as lane 7. The filters were washed at 0.5xSSC.

GMGXY2/X PANEL

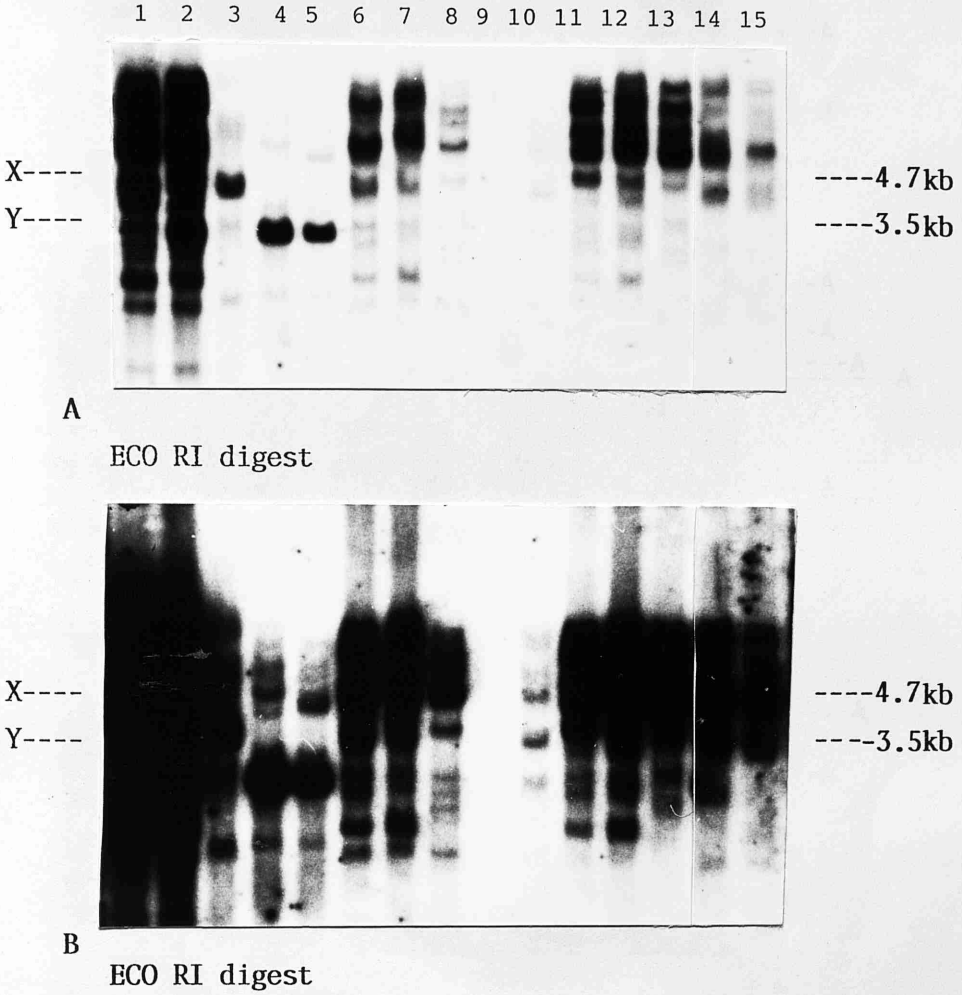


FIGURE 16: Southern analysis of the X panel with probe GMGXY2. The members of the panel are:

1. Normal female
2. Normal male
3. HORL9X (X only) hybrid
4. 3E7 (Y only) hybrid
5. 7631 (Y only) hybrid
6. AMIR2N hybrid
7. NEA916II hybrid
8. FNA98IX hybrid
9. A9 mouse cell line
10. EHA97II/VIII hybrid
11. WHTK17III hybrid
12. NEA921R_2b hybrid
13. HNTK6VII/I hybrid
14. FNA92bIIRa$_1$VI hybrid
15. FNA92bIIRa$_1$I hybrid

X or Y linkage are indicated by the letters X or Y, respectively. In FIGURE 16A the autoradiograph has been exposed for 20 hours and in FIGURE 16B for 4 days. The filter was washed down to 0.5xSSC.

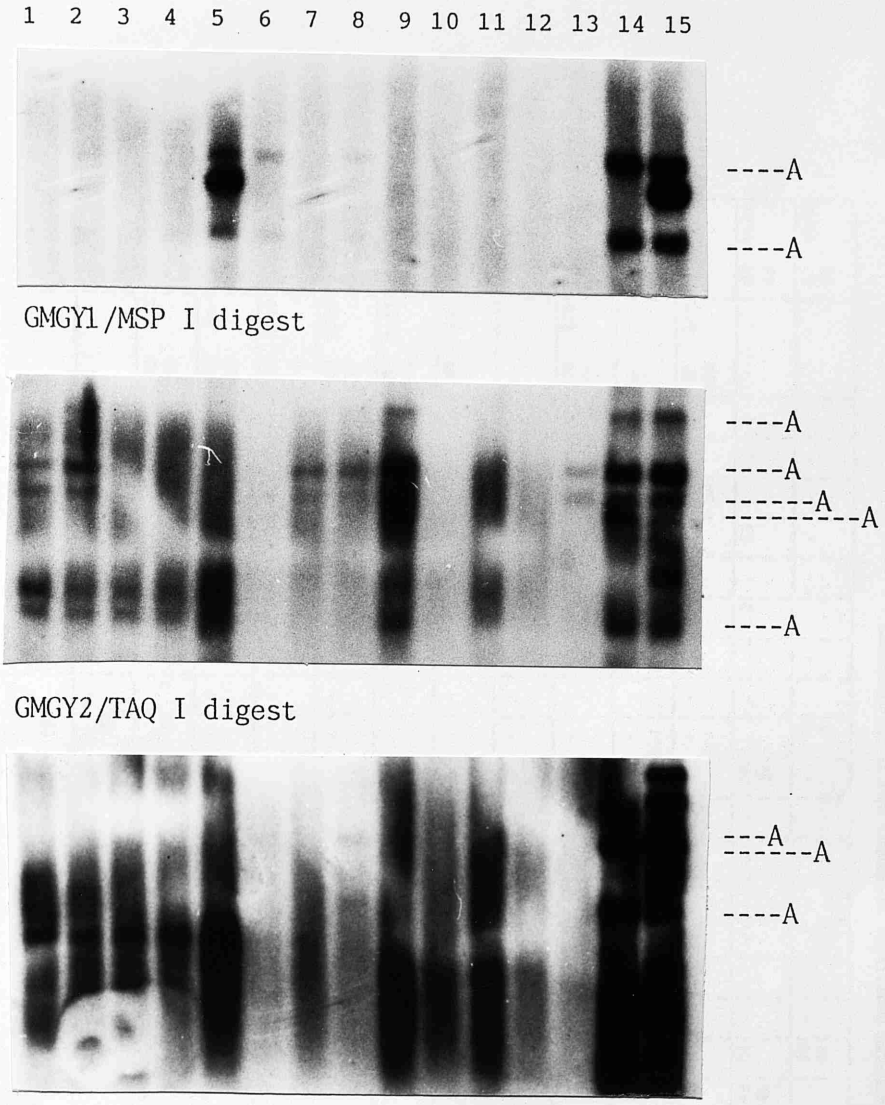


FIGURE 17: Southern analysis of the hybrid panel bearing different groups of autosomes probed with GMGY1, GMGY2 and GMGY11. The panel consists of:

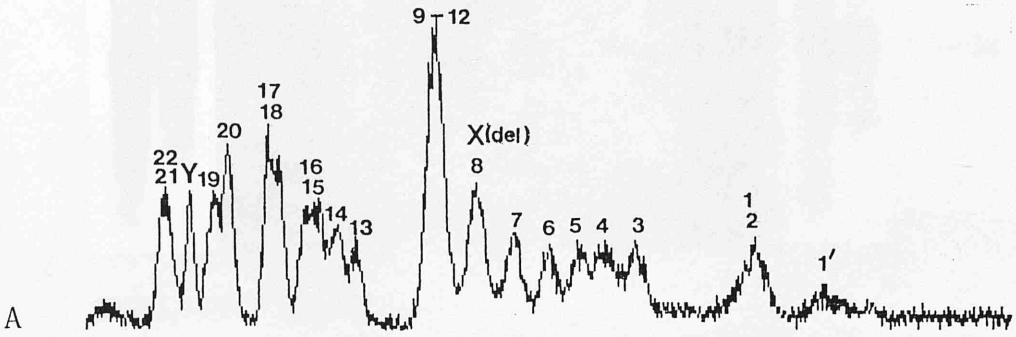
1. NEA915RBVIB
2. FNA95R2
3. NEA918
4. EHA97III
5. AMIR2XI
6. LNA94IRbXII
7. FNA92bIIRa$_1$V1
8. FNA92bIIRa$_1$I
9. WHTK17III
10. CETKaIV
11. EHA97VIII
12. W2A96I
13. THYB133R
14. Normal female
15. Normal male

The autosomally-linked bands are indicated by the letter A to the right of the photographs. The filters were washed down to 0.5xSSC.

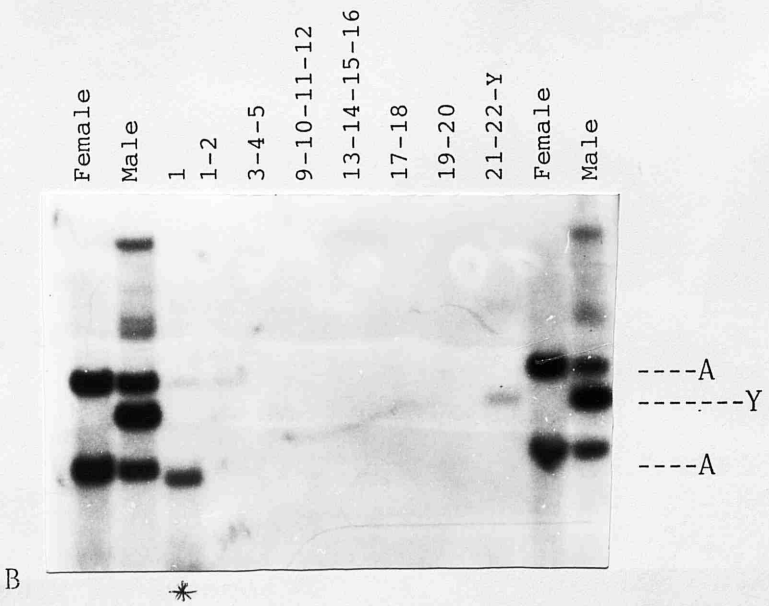
HYBRID	NUMBER OF CELLS ANALYSED	PERCENTAGE OF ANALYSED CELLS CONTAINING DENOTED HUMAN CHROMOSOMES																							DERX	DERAUT
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X		
NEA915RBVIB	15	.	.	33	.	.	.	.	.	.	10	.	10	.	.	27	.	100	.	.	.	.	.	.	.	
FNA95R2	10	.	.	.	.	.	.	.	.	.	.	.	.	.	40	.	.	.	.	.	.	.	.	.	.	
NEA918	20	.	.	.	.	.	50 70	15	.	.	40	10	.	.	5	10	.	.	.	30	5	.	.	.	.	
EHA97III	21	.	43 48	.	90	.	.	5	.	.	48	.	.	.	10 20	5	.	67 90	5	.	.	.	.	.	.	
AMIR2XI	15	40	.	93 120	87 120	.	53	113 127	47 67	13	7 14	200 220	7	7 20	7	7	7	53 67	80 113	73 80	73 80	33 7	20	.	33 53	X/Y
LNA94IRbXII	8	.	.	.	.	.	63	75	.	.	.	.	125	.	.	.	.	75	.	.	138	125	25	.	63 75	Xp-
FNA92bIIRA$_1$VI	25	.	.	12	.	.	4	4	.	.	.	64	.	12	4	.	.	.	.	.	.	68	8	.	100 .	X/19
FNA92bIIRa$_1$I	19	5	.	.	.	.	.	5 10	5	.	.	63 74	11	.	.	.	.	.	.	.	.	53	.	.	63 79	X/19
WHTK17III	50	.	6 10	4	42	.	4	66 70	72 172	2	2 4	2	64 66	2	.	.	4 10	172 180	.	8 12	46	10	74 76	.	60 74	delX
CETK1aIV	50	.	.	16 22	.	.	.	4 6	74 76	.	4	.	.	.	.	.	.	82	12	4 10	78	.	2	.	80 90	Xp-
EHA97VIII	21	.	143 167	.	81	.	.	86 100	.	.	5 10	5	.	.	.	.	.	71	.	.	.	.	.	.	.	
W2A96I	50	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	62	.	.	.	.	.	.	90 94	X/8
THYB133R	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	40	.	.	.	
AMIR2N	13	.	8	15 23	62	54	.	.	.	8	.	92 100	.	8	15 23	.	.	85	92	8	77	38 54	15	.	85 100	X/Y
NEA916II	60	28 37	25 30	3 7	2	2	43 45	83	.	.	.	.	.	.	20 22	.	.	.	2	.	83	.	.	.		83 85	11/X
FNA98IX	60	.	.	.	78 82	7	.	90 92	.	.	3 5	.	.	.	.	2 4	.	.	.	.	.	.	.	.		87 95	19/X

TABLE 7 : CYTOGENETIC ANALYSIS OF A PANEL OF HYBRIDS CONTAINING DIFFERENT GROUPS OF AUTOSOMES - PERCENTAGE OF ANALYSED CELLS CONTAINING DENOTED HUMAN CHROMOSOME

NOTES : 1. In each row the upper figure represents definite identification of the desired chromosome, while the lower figure denotes tentative identification
2. DERX - derivative X chromosome; DERAUT - derivative autosome



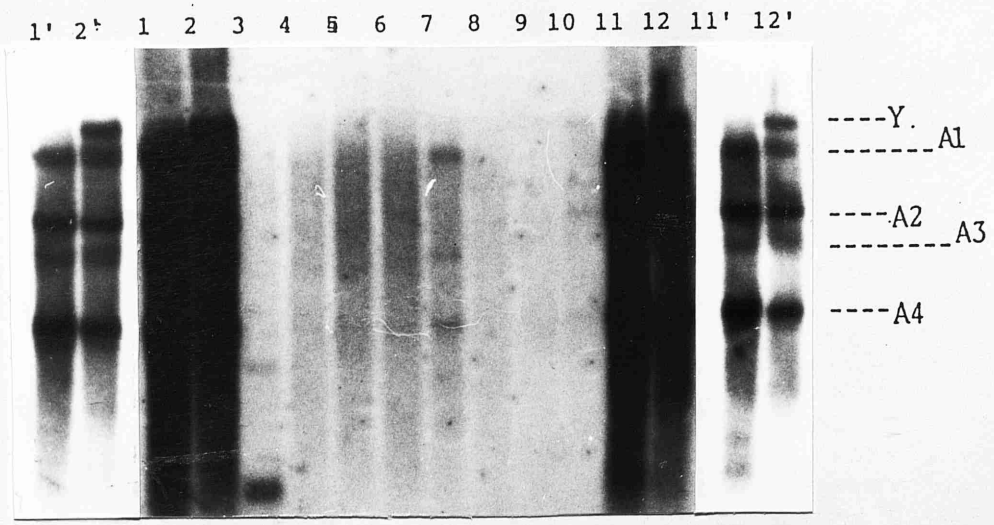
ST individual



GMGY1/MSP I digest

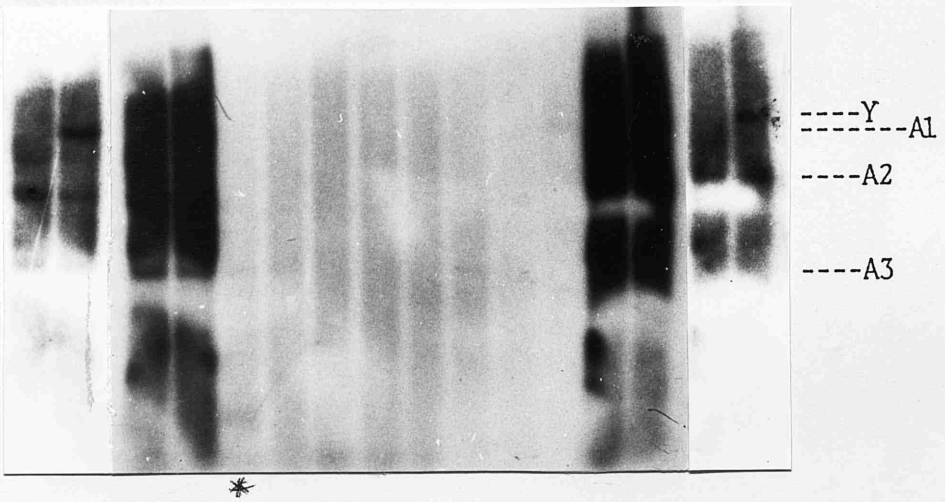
FIGURE 18 : See legend on following page

* The lower band on chromosome 1 lane is suspected to represent plasmid contamination.



C

GMGY2/MSP I digest



D

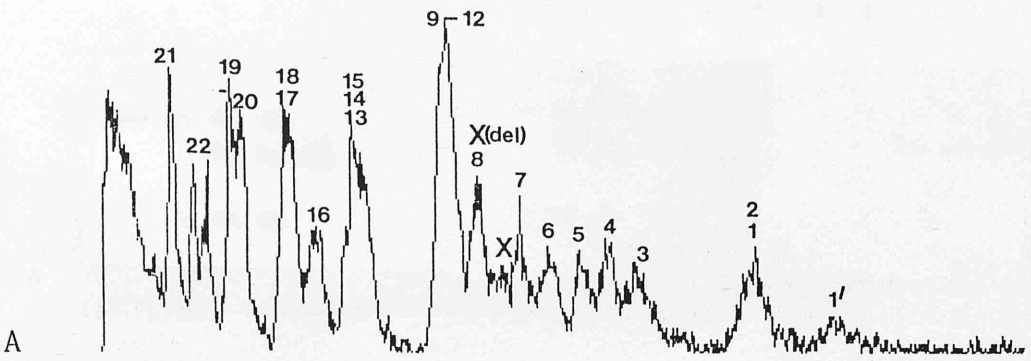
GMGY11/MSP I digest

FIGURE 18A: Flow karyotype from individual ST with a polymorphic chromosome 1 and a deletion of Xp2.1.

FIGURE 18B, C and D: Southern blot analyses of the sorted chromosomes from this individual using probes GMGY1, GMGY2, and GMGY11 respectively.
The order of the DNA samples is as follows:

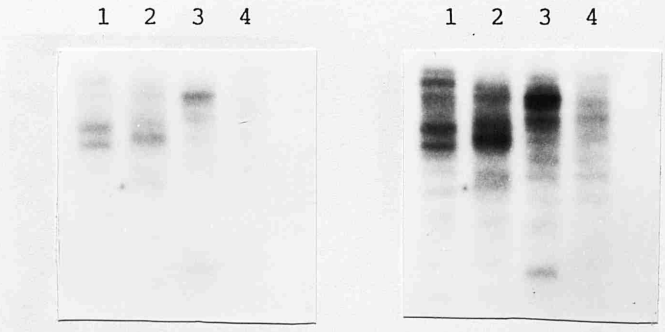
1. Normal female
2. Normal male
3. Polymorphic chromosome 1
4. Chromosomes 1 and 2
5. Chromosomes 3, 4 and 5
6. Chromosomes 9 to 12
7. Chromosomes 13 to 16
8. Chromosomes 17 and 18
9. Chromosomes 19 and 20
10. Chromosomes 21, 22 and Y
11. Normal female
12. Normal male

Lanes 1', 2', 11', and 12' are identical to lanes 1, 2, 11 and 12, except that the exposure of the photograph has been adjusted in order that the bands can be visualised. The filters were washed at 0.5xSSC.



A

CN individual



B C

GMGXY2/ECO RI digest

FIGURE 19A: Flow karyotype from CN individual with a polymorphic chromosome 1 and deletion of part of the long arm of the X.

FIGURE 19B,C: Southern analysis of sorted chromosomes from this individual using probe GMGXY2. Figures B and C are identical except that the autoradiograph in B has been exposed for less time. The order of DNA samples is as follows:

1. Chromosomes 13 to 18
2. Chromosomes 6, 7, 8, X and delX
3. Chromosomes 1 and 2
4. Polymorphic chromosome 1

The filter has been washed at 0.5xSSC.

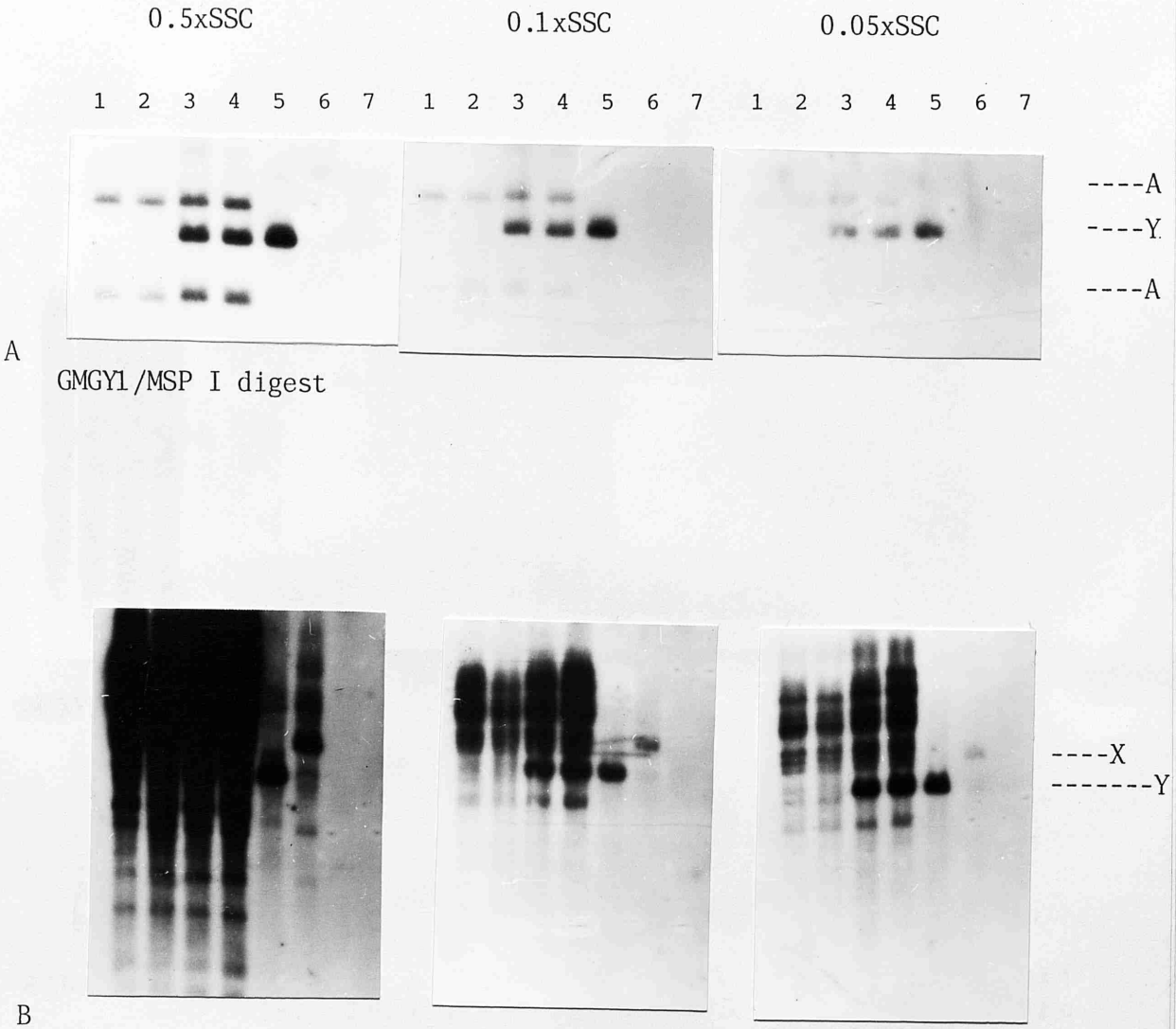
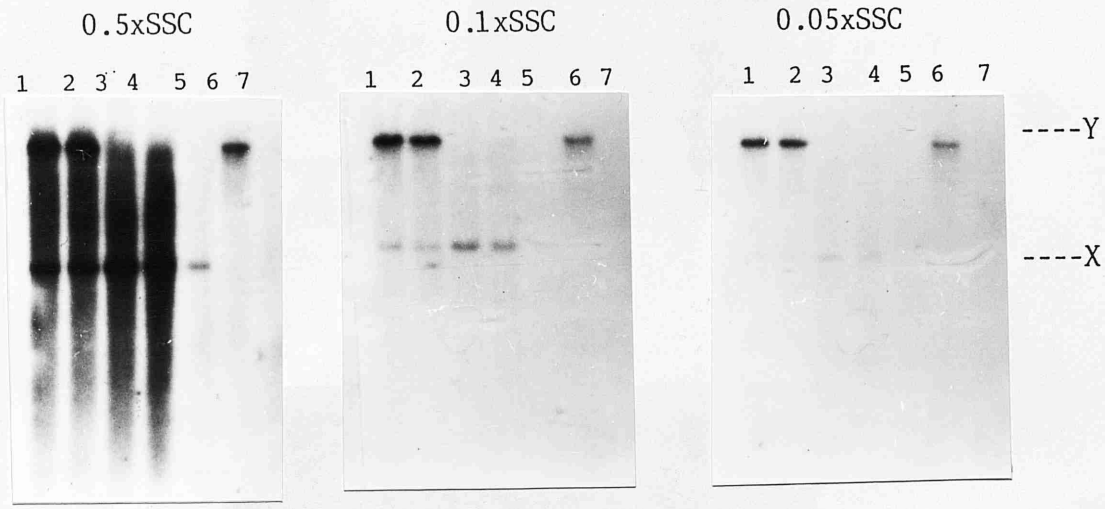


FIGURE 20: See legend on following page



C

GMGXY3/TAQ I digest

FIGURE 20: Southern analysis of panel probed with GMGY1 (A), GMGXY2 (B) and GMGXY3 (C). Filters were washed at 0.5xSSC, 0.1xSSC and 0.05xSSC.

For A and B the order is:

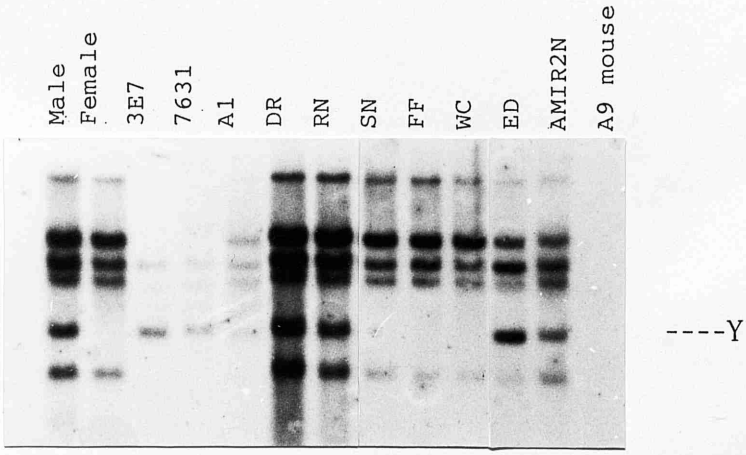
1. Normal female
2. Normal female
3. Normal male
4. Normal male
5. 3E7 (Y only) hybrid
6. HORL9X (X only) hybrid
7. A9 mouse cell line

For C the order is:

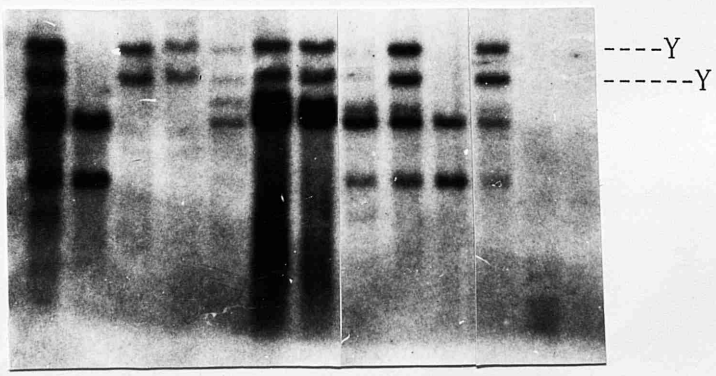
1. Normal male
2. Normal male
3. Normal female
4. Normal female
5. HORL9X (X only) hybrid
6. 3E7 (Y only) hybrid
7. A9 mouse cell line

X or Y-linked bands are indicated by the letters X and Y to the right of the photographs.

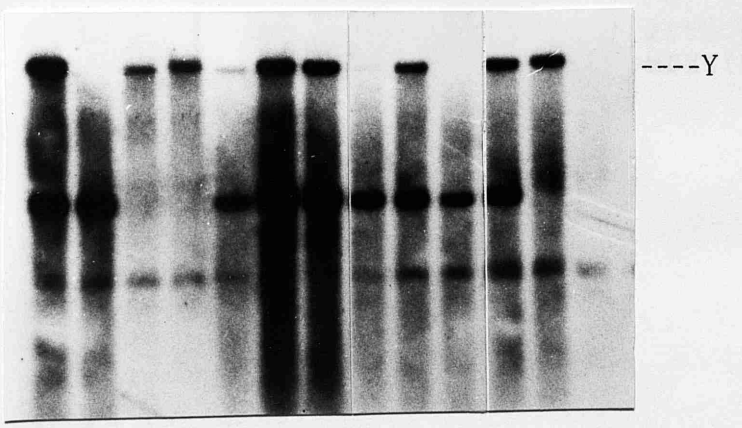
Y PANEL



GMGY2/TAQ I digest



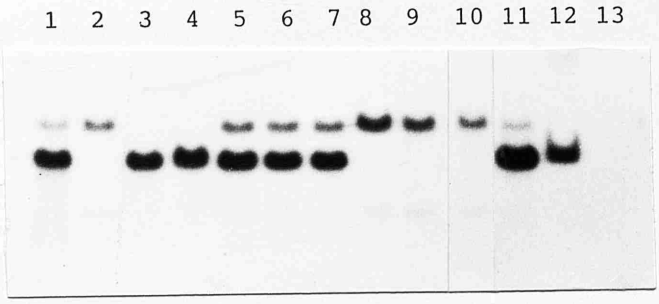
GMGY11/TAQ I digest



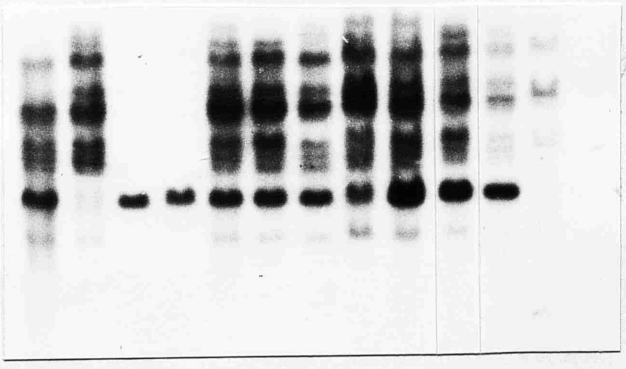
GMGXY3/TAQ I digest

FIGURE 21 : See legend on following page

Y PANEL



GMGY1/MSP I digest

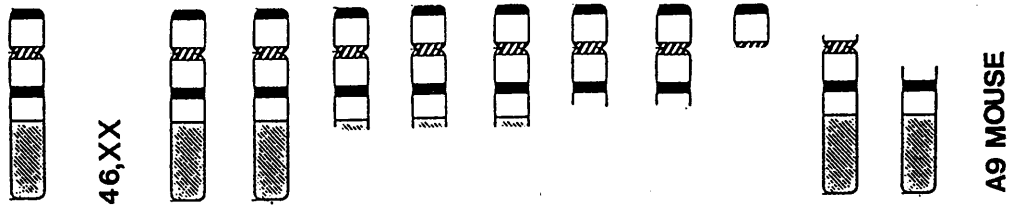


GMGXY2/ECO RI digest

FIGURE 21: Southern analysis of the Y panel with probes GMGY2(A), GMGY11(B), GMGXY3(C), GMGY1(D), and GMGXY2(E). The panel consists of:

1. Normal male
2. Normal female
3. 3E7 (Y only) hybrid
4. 7631 (Y only) hybrid
5. A1 (46,XYqh-)
6. DR (46,XYqh-)
7. RN (46,XYqh-)
8. SN (45,X/46,Xdic(Y)(q11.23))
9. FN (45,X/46,Xdic(Y)(q11.23))
10. WC (46,Xi(Yp))
11. ED (48,XX dic(Y)(p11.2) dic(Y)(p11.2))
12. AMIR2N hybrid (containing Yq11.21-qter)
13. A9 mouse cell line

The Y-linked bands are indicated by the letter Y to the right of the photographs. The filters were washed at 0.1xSSC.



PROBE													
GMGXY2	+	-	+	+	+	+	+	+	+	+	+	-	-
GMGXY3	+	-	+	+	+	+	+	+	+	-	+	+	-
GMGY11	+	-	+	+	+	+	+	+	+	-	+	-	-
GMGY1	+	-	+	+	+	+	+	-	-	-	+	+	-
GMGY2	+	-	+	+	+	+	+	-	-	-	+	+	-

TABLE 8 : DISTRIBUTION OF Y-SPECIFIC SEQUENCES ON THE HUMAN AND HYBRID PANEL BEARING DIFFERENT PARTS OF THE Y CHROMOSOME.

KEY : + positive hybridisation - negative hybridisation

PROBE	SIZE	ENZYME	FRAGMENTS			Y LOCALISATION	X LOCALISATION
			Y-LINKED	X-LINKED	AUTOSOMAL		
GMGXY2	3.5	Eco RI	3.5	4.7	Numerous	Ycen - Yp11.2	Xq13 - Xq24
GMGXY3	0.6	Taq I	23	2.3	-	Yq11.2 - Yq11.23	Xp22.3 - Xpter
GMGY11	2.1	Taq I	8, 5.5	-	4, 3.8, 2.3	Ycen - Yq11.2	-
GMGY1	0.8	Msp I	2.4	-	3, 1.7	Yq11.3 - Yq11.12	-
GMGY2	1.1	Taq I	2, 4.2	-	5.5, 4.2, 3.6, 0.7	Yq11.3 - Yq11.12	-

TABLE 9 INSERT SIZES AND FRAGMENTS DETECTED BY Y CHROMOSOME PROBES

NOTE : All sizes in kilobases

3.2.3. Molecular analysis of XX males

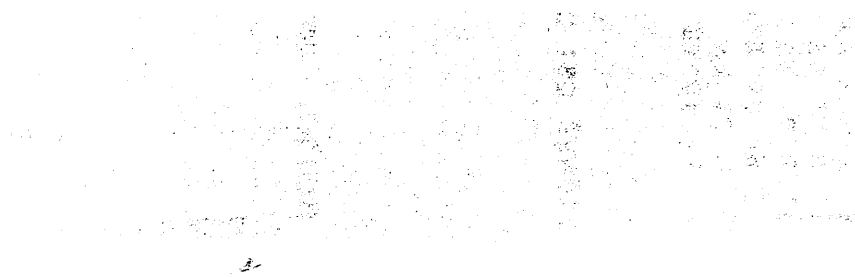
All the probes described above were used to examine the genomic DNA of 11 46,XX males and one 46,XX true hermaphrodite and some of their relatives for the presence of Y-specific sequences. The results are shown in Figures 22 to 26; probes GMGY1, GMGY2, and GMGY11 did not hybridise to the DNA of any of these XX males or their female relatives, while they did hybridise to their male relatives. This result confirmed that at least in the peripheral blood lymphocytes of these individuals there is no sign of mosaicism involving a complete Y chromosome. Hybridisation with GMGXY3 produced the same results as with the three probes mentioned above and in addition there was no sign of a dosage effect with the X specific band, expected from loss of one X allele. The Y-specific band of GMGXY2, however, was present in the genomes of XX males HM, JM, RH, TA and ST and in the male relatives of all these individuals. It was absent from the genomes of the rest of the XX males and from all the female relatives.

Figure 27A shows the flow karyotype of RH and the hybridisation pattern of GMGXY2 to chromosomal DNA sorted from this individual (Figure 27C and D). It can be seen that the Y-specific band is present in the fraction containing chromosomes X and 7. The possibility that this signal represents hybridisation to chromosome 7 cannot be completely excluded, but the most likely explanation is that the Y-specific band is hybridising to one of the X chromosomes of this individual. It is also interesting to note that there is a difference in size between the two X chromosomes in this individual. This is shown by the presence of only one X chromosome in the peak just to the left of the 7 peak, and an additional chromosome in the 7 peak. This shows that one of the two X chromosomes is slightly larger than the other. Ferguson-Smith **et al** (1985)

did DNA measurements of the interchange chromosome by flow cytometry in this and other cases and demonstrated the existance of two distinct classes of XX male, some with one X chromosome approximately 3.8% larger than normal and others in whom both X chromosomes were normal in size. This correlated with the number of the Y-specific sequences present in the DNA of these patients. Cytogenetic studies done by the same group showed transfer of Yp11.3 to the X in five out of seven cases studied, including RH. These results are consistant with the theory that XX males arise via the transfer of Y chromosome DNA to the X chromosome.

Figure 27B shows the flow karyotype of HM, another of the XX males in which the Y-specific sequence of GMGXY2 is present. There is no detectable difference between the X chromosomes of this individual as determined by cytogenetic techniques and flow analysis but Southern analysis of the sorted chromosomes from this individual also showed the Y sequence to be located in the X-7 fraction (Affara **et al** 1986b).

GMGXY2 was shown to hybridise to the DNA of three 46,XY females - VA, SM and DM (Figure 26).



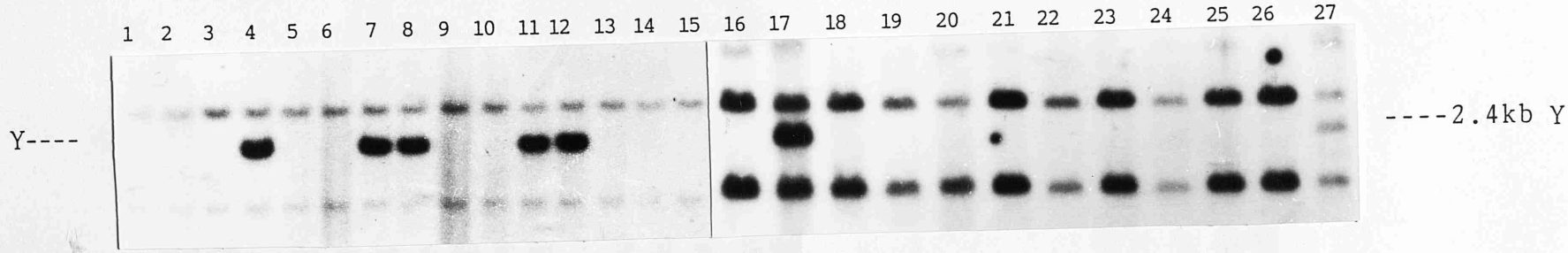
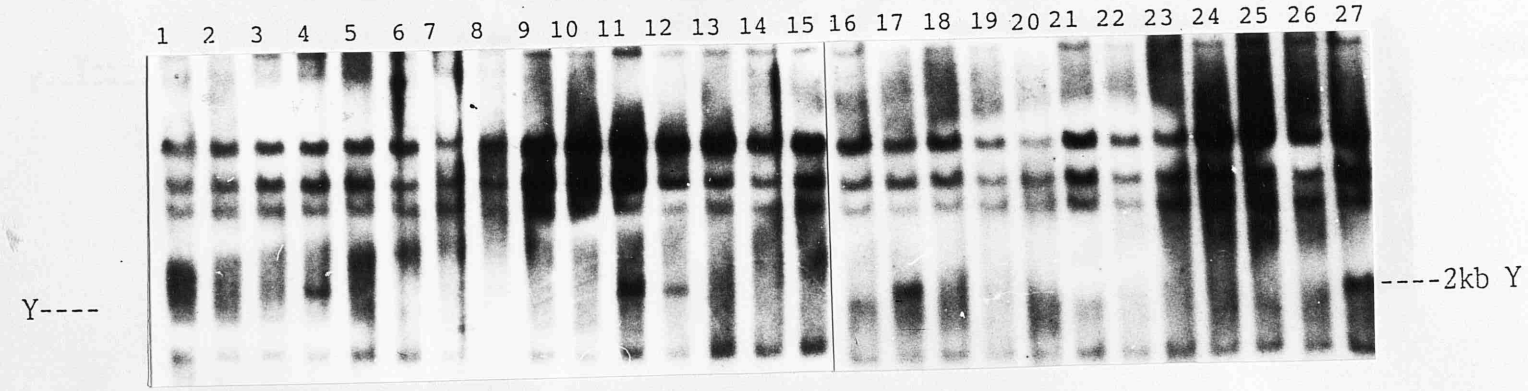


FIGURE 22: Southern analysis of XX males, one true hermaphrodite and some their relatives using GMGY1 probe. The order of the genomic DNAs is:

1. PP 46,XX male
2. M of PP
3. RV 46,XX*
4. F of RV
5. M of RV
6. HM 46,XX male
7. U of HM
8. F of HM
9. M of HM
10. JM 46,XX male
11. B of JM
12. B of JM
13. S of JM
14. RH 46,XX male
15. S of RH
16. AP 46,XX male
17. F of AP
18. TA 46,XX male
19. KS 46,XX male
20. S of KS
21. M of KS
22. RT 46,XX male
23. MM 46,XX male
24. ST 46,XX male
25. AN 46,XX male
26. M of AN
27. F of AN

M=mother, F=father, U=uncle, S-sister, B=brother
* denotes true hermaphrodite
The filter was washed at 0.1xSSC.

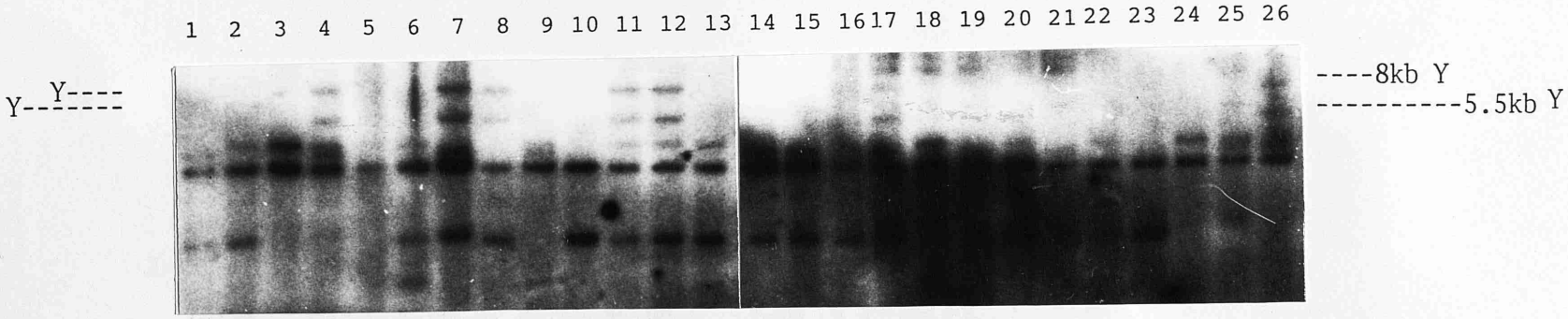


GMGY2/TAQ I digest

FIGURE 23: Southern analysis of XX males, one true hermaphrodite and some their relatives using GMGY2 probe. The order of the genomic DNAs is:

1. PP 46,XX male
2. M of PP
3. RV 46,XX*
4. F of RV
5. M of RV
6. HM 46,XX male
7. U of HM
8. F of HM
9. M of HM
10. JM 46,XX male
11. B of JM
12. B of JM
13. S of JM
14. RH 46,XX male
15. S of RH
16. AP 46,XX male
17. F of AP
18. TA 46,XX male
19. KS 46,XX male
20. S of KS
21. M of KS
22. RT 46,XX male
23. MM 46,XX male
24. ST 46,XX male
25. AN 46,XX male
26. M of AN
27. F of AN

M=mother, F=father, U=uncle, S-sister, B=brother
* denotes true hermaphrodite
The filter was washed at 0.1xSSC.



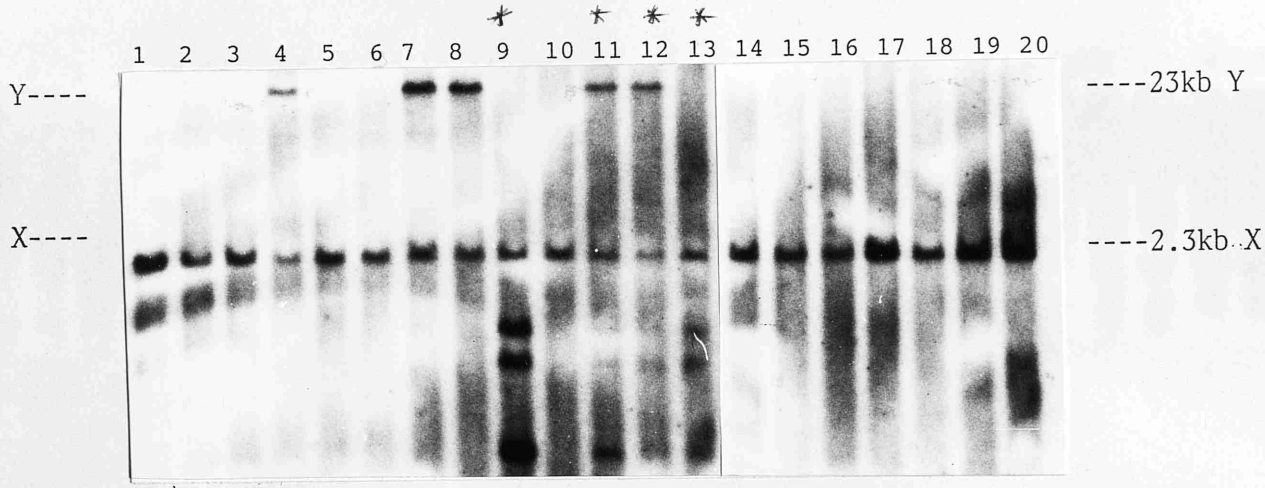
GMGY11/TAQ I digest

FIGURE24: Southern analysis of XX males,one true hermaphrodite and some of their relatives using GMGY11 probe. The order of genomic DNAs is:

1. PP 46,XX male
2. M of PP
3. RV 46,XX*
4. F of RV
5. M of RV
6. HM 46,XX male
7. U of HM
8. F of HM
9. M of HM
10. JM 46,XX male
11. B of JM
12. B of JM
13. S of JM
14. RH 46,XX male
15. S of RH
16. AP 46,XX male
17. F of AP
18. TA 46,XX male
19. KS 46,XX male
20. S of KS
21. RT 46,XX male
22. MM 46,XX male
23. ST 46,XX male
24. AN 46,XX male
25. M of AN
26. F of AN

M=mother, F=father, U=uncle, S-sister, B=brother
* denotes true hermaphrodite
The filter was washed at 0.1xSSC.

* The bands below the X band in lanes 9, 11, 12 and 13 are suspected to represent plasmid contamination.

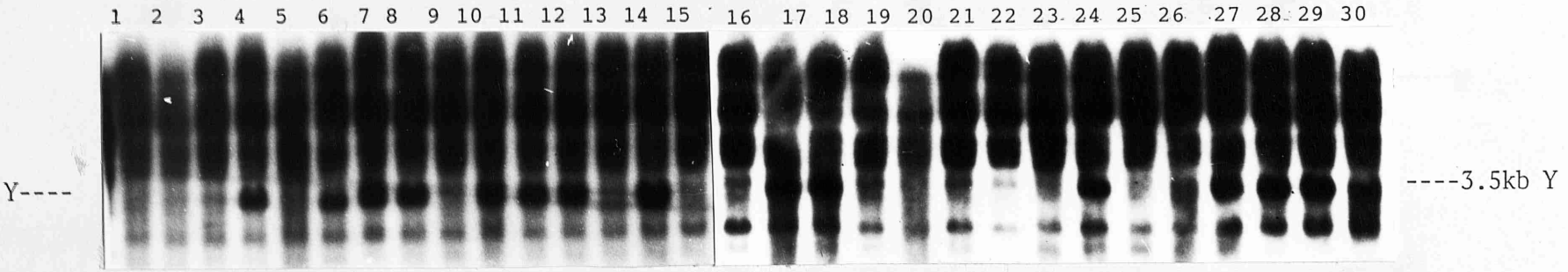


GMGXY3/TAQ I digest

FIGURE 25: Southern analysis of XX males, one true hermaphrodite and some their relatives using GMGXY3 probe. The order of genomic DNAs is:

1. PP 46,XX male
2. M of PP
3. RV 46,XX*
4. F of RV
5. M of RV
6. HM 46,XX male
7. U of HM
8. F of HM
9. M of HM
10. JM 46,XX male
11. B of JM
12. B of JM
13. S of JM
14. TA 46,XX male
15. KS 46,XX male
16. S of KS
17. M of KS
18. RT 46,XX male
19. MM 46,XX male
20. ST 46,XX male

M=mother, F=father, U=uncle, S-sister, B=brother
* denotes a true hermaphrodite
The filter was washed at 0.1xSSC.

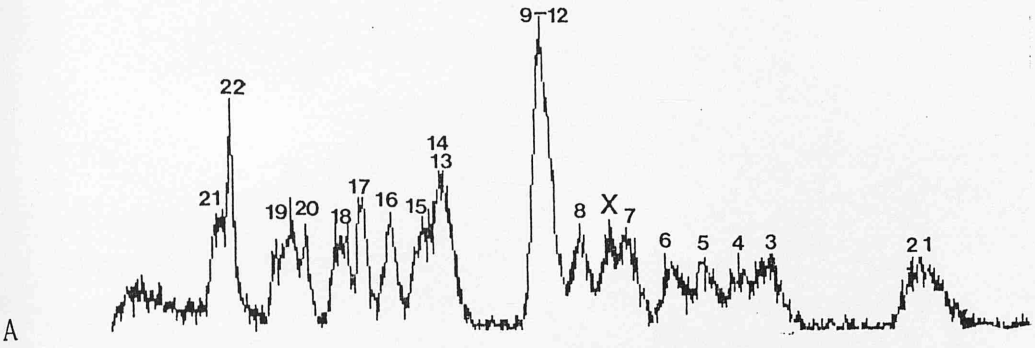


GMGXY2/ECO RI digest

FIGURE 26: Southern analysis of XX males, one true hermaphrodite and some their relatives using GMGXY2 probe. The order of genomic DNAs is:

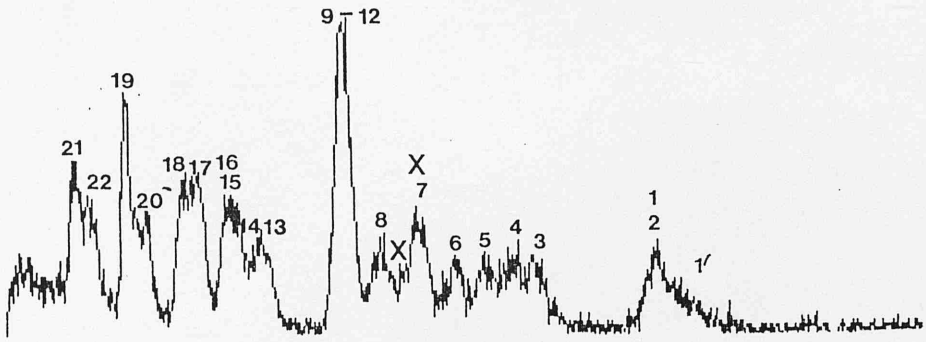
1. PP 46,XX male
2. M of PP
3. RV 46,XX*
4. F of RV
5. M of RV
6. HM 46,XX male
7. U of HM
8. F of HM
9. M of HM
10. JM 46,XX male
11. B of JM
12. B of JM
13. S of JM
14. RH 46,XX male
15. S of RH
16. AP 46,XX male
17. F of AP
18. TA 46,XX male
19. KS 46,XX male
20. S of KS
21. M of KS
22. RT 46,XX male
23. MM 46,XX male
24. ST 46,XX male
25. AN 46,XX male
26. M of AN
27. F of AN
28. VA 46,XY female
29. SM 46,XY female
30. DM 46,XY female

M=mother, F=father, U=uncle, S-sister, B=brother
* denotes a true hermaphrodite
The filter was washed at 0.1xSSC.



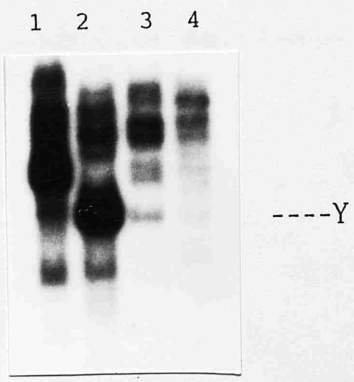
A

HM - 46,XX male

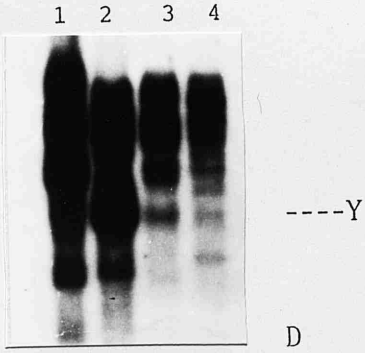


B

RH - 46,XX male



C



D

GMGXY2/ECO RI digest

FIGURE 27A and B: Flow karyotypes from individuals HM and RH, respectively.

FIGURE 27C and D: Southern analysis, of two fractions of sorted chromosomes from RH individual probed with GMGXY2. The order of the DNA samples is as follows:

1. HORL9X (X only) hybrid
2. 3E7 (Y only) hybrid
3. Chromosomes 7 and X
4. Chromosomes 1 and 2

The autoradiographs in 27A and 27B are identical except that the former was exposed for less time. The filter was washed at 0.5xSSC

D I S C U S S I O N

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The first part of this chapter will be spent discussing the nature of the homologies detected by the Y chromosome sequences described earlier and their significance to the evolution of the human genome. The use of Y chromosome probes in the investigation of XX males will also be considered. In the second part, the screening method used to isolate these recombinants and the construction of the somatic cell hybrid panels used to map the fragments detected by them will be put into perspective.

4.1. Analysis of the Homology Observed between the Y Chromosome and the Rest of the Chromosomes

It becomes apparent when looking at the mapping positions of the five recombinants isolated in this study that there is a great deal of sequence homology between the Y chromosome, the X chromosome and the autosomes. This implies that a high level of DNA rearrangement involving these chromosomes must have occurred during the course of evolution.

4.1.1. Focus on the Homology Detected between the Y Chromosome and Autosomes

This is the first report in the literature to describe the localisation of the autosomal fragments detected by Y chromosome DNA probes using somatic cell hybrids and flow sorted chromosomes. The only other sequences to have been assigned to autosomes in a similar way are the 3.4 and 2.1kb tandem repeats (Bostock **et al** 1978, Szabo **et al** 1980, Cooke and Mackay 1983). These, however, represent a different class of Y chromosome sequences. Other probes which detect Y-autosome homology have been described by

Bishop **et al** (1983) and Ngo **et al** (1986) but the autosomes with which their probes shared homology were not defined.

Probes GMGY1, GMGY2, and GMGY11 are derived from the long arm of the Y chromosome and all three have been shown to recognise homologous sequences on one or more autosomes as follows: GMGY1 and GMGY2 are situated close to the heterochromatin in the region between Yq11.23 and Yq11.12. GMGY1 shows homology with chromosome 1, while GMGY2 hybridises to chromosome 21 and at least one other autosome. GMGY11 has a more proximal location on the Y, in the region Yq11.12 to Yq11.23 and may also hybridise to chromosome 1. In addition to these three probes, Affara **et al** (1986a) reported three more probes which recognise Y-autosome homology. Two were localised on the short arm of the Y chromosome and the other was localised on the long arm of the Y in the same area as GMGY1 and GMGY2. These results clearly demonstrate that there has been a series of interchanges involving different parts of the Y chromosome and a variety of autosomes during the course of evolution.

The sequences mentioned above have been shown to be interspersed with other sequences which are Y-specific and X-Y or X-Y-autosome sequences (Affara **et al** 1986a). One such sequence which recognises autosomal homology in addition to X-Y homology is GMGXY2 (isolated in this study) which has been localised on the short arm of the Y chromosome and in the region Xq13-q24; it has also been shown to recognise homologous sequences with many autosomes including chromosomes 1 and 2. This recombinant seems to belong to a different group of sequences which is discussed more extensively below because of its homology with the X and a larger range of autosomes. It should still be considered in this part of the discussion, however, because of the homology it shows with the autosomes.

The Y-autosome homologous sequences cannot be regarded

as a separate group from the X-Y-autosome homologous sequences or those showing X-Y and X-autosome homology because it is possible that they originated as part of the same process of DNA rearrangement. Bishop **et al** (1984) proposed that Y-autosome sequences could have initially been present on the X, Y and autosomes but the X-located fragment was lost during the course of evolution. As an example they cited Balazs **et al** (1983) who showed the existence of X-autosome homology, in which case, one could envisage that the Y located fragment has been lost.

Other anonymous DNA sequences which demonstrate X-Y-autosome homology have been reported by Rappold **et al** (1984), Bishop **et al** (1983, 1984), and Casanova **et al** (1985). Probe, pAS-1 a cDNA probe for the human urea cycle, enzyme argininosuccinate synthetase (Daiger **et al** 1982), the actin-like family of sequences (Heilig **et al** 1984) and the alphoid family of sequences (Willard **et al** 1983, Willard 1984, Wolfe **et al** 1985) also seem to be dispersed on many autosomes as well as the sex chromosomes. These sequences show homology between different parts of the X, the Y and a wide range of autosomes.

Homology between the Y Chromosome and Chromosome 1

The localisation of the autosomal fragments detected by the Y chromosome probes isolated in this study allow a clearer insight into the evolutionary steps undergone by the human genome by providing information about which chromosomes the Y has exchanged material with.

By combining the results presented here with those from the literature it was noted that the consensus of data indicates that significant homology exists between the Y chromosome and chromosome 1. The 3.4kb and the 2.1kb Hae III fragments, as mentioned in the introduction (section 1.8.2.), detect homology with several chromosomes but they also show specific hybridisation with the centromere of

chromosome 1 (Szabo **et al** 1980). A number of other probes which detect sequence homology with different parts of the Y chromosome (shown in parentheses) hybridise to chromosome 1: GMGY1 (Yq1.23), GMGY11 (Yq11.12-Yq11.23), YACG35 (Yq11.12-q11.23) (Rappold **et al** 1984) and GMGXY2 (Yp11.2-Ycen).

A possible explanation for this homology with chromosome 1 could be due to the fact that this is the largest chromosome and would therefore be more available for recombination. The homology between chromosome 1 and Y recognised by probe GMGY1 was tested to determine how closely related these sequences are. It was observed that the chromosome 1-linked bands are gradually lost when the stringency of post-hybridisation washing is raised. This implies that the homology is not exact and that these sequences have diverged from each other. In contrast, the homology detected by probe GMGY2 was unaffected by the same treatment, which implies a closer degree of homology and limited divergence between these Y-autosome sequences. This suggests that the recombination between chromosome 1 and the Y preceded that between the Y and one or more of the chromosomes recognised by GMGY2.

These findings raise important questions regarding the mechanisms by which the interchange between the chromosomes occurred, the direction in which the sequences moved and when these events took place. It seems possible that the Y chromosome may have arisen partly as a mosaic of autosomal sequences constructed through one or more mechanisms such as DNA transposition, viral intergration events, unequal crossover leading to amplification of certain sequences, or simply translocations, duplications and deletions.

Kunkel and Smith (1982), for example, showed that homologues of the 3.4kb Hae III sequences are not found on the Y chromosome of apes but are only present in the autosomes, suggesting that the 3.4kb Hae III sequences are

largely autosomal in origin. Cooke **et al** (1983) proposed that because very few genes are known to be situated on the Y chromosome, this might mean that the DNA for these functions, and associated intragenic DNA, is insufficient to support other chromosomal functions, particularly segregation and so there has been selection for this chromosome to acquire extra DNA. The simplest model to explain this accumulation would be by the amplification of tandemly repeated sequences to provide large blocks of DNA by unequal crossing over. The separation of sequences from chromosome to chromosome would allow the divergence of Y chromosome sequences from the autosomal sequences.

These homologies however could also be the result of transpositions or viral intergrations. Philips **et al** (1982) showed the presence of many copies of C-I retrovirus (M720)-related sequences on the Y chromosome of some Mus species and discussed the possibility of interchromosomal recombination of viral DNA leading to the amplification of Y-chromosome sequences. They suggested that markers such as these virus-related sequences offer the means to study the evolution of this chromosome. The mammalian Y chromosome could accumulate such fragments because it exists in a permanent monosomic state with very limited opportunities for recombination with the X chromosome.

One should ask whether only the Y chromosome appears to be a patchwork of different parts of the genome, or if this is the case amongst autosomes as well but this has not yet been demonstrated. Was it just a natural progression for the Y chromosome to recombine so often with autosomes, or was it because during evolution it has taken to the task of eliminating most of its functions to reach a minimum, of mainly sex determination? It could be that as a result of the DNA rearrangements mentioned above, the Y chromosome has dispersed all the genes or sequences not directly involved in sex determination to other chromosomes.

These findings support Ohno's (1967) observation that the enormous array of karyotypes reveals the extent to which the original autosomal linkage groups of a common ancestor have been shuffled around while the autosomes have broken and reunited many times.

Probes such as GMGY1, GMGY2, GMGY11 and perhaps GMGXY2 provide useful tools with which to examine these theories. The evolutionary aspects of the Y chromosome and of the genome in general can be studied using these recombinants against the genomic DNA not only from primates but also from animals belonging to the lower zoological scale. This would provide information about how the species evolved and how recent the homology between the Y and other chromosomes is. It would be interesting to analyse the nature of Y-autosome and X-Y-autosome homology by using these probes to isolate the homologous sequences from autosomal libraries - for example from a library of chromosome 1. The argininosuccinate synthetase probe pAS-1 is a probe for a functional gene whose source is other than a library for the Y or the X chromosome but detects homologous sequences on both sex chromosomes. Characterisation of such sequences isolated from an autosomal library could provide information as to whether these sequences moved from the autosomes to the Y or from the Y to the autosomes or both. The degree of homology between these closely related sequences could be also investigated.

4.1.2. Focus on the Homology between the X and the Y Chromosomes

i. GMGXY2 Recombinant/Xq-Yp-Autosomal Homology

Probe GMGXY2, as mentioned earlier, recognises homologous sequences on the X, the Y and the autosomes. The pattern it produces is reminiscent to the pattern obtained

by the human argininosuccinate synthetase gene probe pAS-1 and the hamster cytoskeletal actin sequences (Daiger **et al** 1982, Heilig **et al** 1984). Daiger **et al** (1982) mentioned that the relationship between the AS structural locus and the AS-like sequences on the X and Y is not yet known. The sequences are short relative to the structural locus but they contain sequences homologous to both the 3' and 5' portions of pAS-1. This arrangement is compatible with that of pseudogenes that lack introns. The situation is similar (Heilig **et al** 1984) for the actin-like sequences that are present on many autosomes and possibly on the long arm of the X (Soriano et al 1982). Some of these sequences also correspond to pseudogenes as found in other gene families (Jeffreys 1981).

It is very early to decide whether GMGXY2 is member of a similar family of sequences. Two subfragments derived from the 3.5kb GMGXY2 fragment were shown to produce the same pattern when used as probes against genomic DNAs which implies that these two units might be the result of a duplication and further supports the possibility that GMGXY2 might be a member of a family of pseudogenes. While the main X and Y bands recognised by GMGXY2 have been localised, the two other probes discussed above have not been mapped very accurately on the X or Y chromosomes. The actin probe has been shown to derive from the centromeric region between Xp1.1 and Xq11, but its location on the Y chromosome has not been mapped as yet. The exact location of PAS-1 probe on the X and the Y should also give more information on these families.

While GMGXY2 has been shown to be transferred in some XX males, this was not the case with the actin probe (Heilig **et al** 1984). More XX males should be tested using the actin probe before one can be sure they do not transfer to the genome of XX males. PAS-1 probe should also be tested against XX males.

Daiger **et al** (1982) found that some restriction enzymes did not produce an obvious male-specific fragment with the argininosuccinate synthetase probe pAS-1 and explained it by saying that the pattern was obscured by the multiplicity of bands. In a similar way for GMGXY2, Eco RI has been the only one from a number of enzymes tested here to generate a male specific band well separated from the rest of the bands.

Other probes which detect homology between the X, the Y and the autosomes have been isolated (Bishop **et al** 1983, 1984, Rappold **et al** 1984, Casanova **et al** 1985). Interestingly enough the Y-specific DNA fragments detected by a probe ($p12f_2$) isolated by the last group were mapped on the long arm of the Y chromosome, unlike GMGXY2.

The homology that the GMGXY2 probe detects between the X and the Y can be further discussed. The main X and Y bands detected by this probe have been mapped to the region Ycen to Yp11.2 and at the X chromosome from Xq13-Xq24. This type of homology has also been detected by other probes: DXYS1 (Page **et al** 1982) was localised to the region from Ycen to Ypter and from Xq1-q22 and similarly Bishop **et al** (1983,1984) found probes present on the euchromatic part of the Y and the long arm of the X, also Wolfe **et al** 1984 a,b, Geldwerth **et al** (1985), Koenig **et al** (1985), and Affara **et al** (1986a).

One difference, however, between these probes and GMGXY2 is that although all these probes map to the same regions of the X and the Y chromosomes, they do not recognise homology with autosomes. A possible explanation for this is that these represent two different but neighbouring groups of sequences on the Y, one that detects homology between the X, Y and autosomes and one that detects homology only between the X and the Y. In support of the fact that these sites must be close to each other is that GMGXY2, and X-Y probes isolated by Page **et al** (1984,

1985) and Koenig **et al** (1985) are shown to be present in the genomic DNA of some XX males, implying that they come from regions of the Y chromosome that are very close together.

The indicated homology between the short arm of the Y and the long arm of the X is interesting since this part of the Y is involved in normal testis development and the part of the X which is homologous to this region is important for normal ovarian development (see Introduction).

GMGXY2 should be further investigated in order to find out whether it is really a member of a pseudogene family. It would also be interesting to use this probe to select and further characterise sequences from autosomal libraries Moreover its order and relationship to the other X-Y probes should be determined with the use of pulsed field gel electrophoresis (Anand 1986).

ii. GMGXY3 Recombinant/Xp-Yq Homology

GMGXY3 is another probe that seems to detect an interesting homology. It was shown to be situated on the short arm of the X above Xp22 and on the Y long arm between Yq11.22 and Yq11.23. This type of homology was originally suggested by Ferguson-Smith (1965) based on phenotype-karyotype correlations. A similar site has been found by Koenig **et al** (1984) though they were not able to localise their sequence as precisely as GMGXY3; they mapped it between Yq11 and Yqter. GMGXY3 was tested against XX males and was not found to be transferred, while the dosage of the X linked fragments appeared to be the same as for normal females, just as Koenig **et al** (1984) found for their own probe. By **in situ** hybridisation, Rappold **et al** (1984) showed a similar region of X-Y homology with their probes. As discussed earlier, however, their probes also detect homology with autosomes. The probe isolated by Koenig **et al** (1984) detected a rare X-linked polymorphism as has been

extensively discussed in section 1.8.4. of the introduction. Probe GMGXY3 was not tested for polymorphisms in this study therefore it would be interesting to do RFLP studies using this probe in order to compare these results with those of Koenig **et al.**

GMGXY3 does not have an exact homology with the X as shown after subsequent washes in higher stringencies. When DXS31 was washed in higher stringency conditions the Y specific bands disappeared (Koenig **et al** 1984).

GMGXY3 occupies an interesting position on both the X and the Y chromosomes, in view of the recent report by Yen **et al** (1986) in which they seem to have isolated an cDNA probe for the STS gene which maps to the region from Xp22.3 to Xpter and to the long arm of the Y chromosome. GMGXY3 was shown to hybridise to the cDNA probe used in the library screening procedure. It would therefore be interesting to test GMGXY3 for expression, using Northern blotting and also by screening a cDNA library for homologous sequences. Other experiments which should be done with this probe include the analysis of individuals with X/Y translocations and families with STS deficiency - these would provide information about the precise location of GMGXY3 with respect to the STS gene and whether it actually represents part of the gene itself. This probe could also eventually be used as a diagnostic tool to detect breakpoint differences in patients with deletions of the short arm of the X. Another possible use for GMGXY3 would be to screen an X-chromosome specific library to pick up more sequences around this area and perhaps approach the STS locus by walking or jumping along the chromosome (Collins and Weissman 1984). A Y-specific library could also be screened, in order to investigate the extent of the homology between the two chromosomes in this region.

4.1.3. More on the Evolution of the Sex Chromosomes

Probes that are homologous to the short arm of the Y chromosome and the long arm of the X chromosome, and are therefore similar to GMGXY2, have been shown to be present only on the X chromosome of chimpanzee, gorilla and orangutan (Page **et al** 1984, Geldwerth **et al** 1985). Page **et al** (1984) proposed and Geldwerth's results agreed that the DXYS1 homologous sequences were transposed from the X chromosome to the Y chromosome after the human line diverged. It was estimated that the homology between the X and the Y chromosomes is about 97-99% and at the locus DXYS1 the extend of the homology is about 36kb. It is interesting that after GMGXY2 has been washed to high stringency, the main X and Y bands have not been washed away, therefore supporting the theory that the X and Y are closely homologous in these regions.

The homology detected by Koenig's **et al** (1984) probe between the short arm of the X and the long arm of the Y, similar to GMGXY3, was estimated to be around 80%. It was shown to be present on the X and the Y chromosomes of chimpanzee and gave an identical pattern as for humans but its location in lemurs seems to be autosomal or perhaps pseodoautosomal (Koenig **et al** 1984). According to the same authors, this result suggests that the DXS31 sequences are functionally important and could correspond to a gene. DXS31 and GMGXY3 lie in that region of the X chromosome which escapes inactivation and carries many important genes, this, together with the fact that the GMGXY3 fragment hybridised to placental cDNA , reinforces the necessity to find out if this sequence is expressed. GMGXY3 should also be used as a probe against primates and other animals in order to find out if it has been conserved during the course of evolution.

Burk **et al** (1985) isolated a probe similar to GMGY11 but which does not detect any autosomal homology and was

shown to be confined to the Y chromosome of the apes. By interpreting the restriction fragments obtained by gorilla and orangutan DNA they proposed that this event could be due to insertion-deletion. In the study presented here it has not been possible to study the DNAs of primates with the probes isolated to see if these sequences have been also transposed recently. At the moment it is only possible to speculate from their chromosomal locations that they belong to the same group of sequences as other probes characterised up to now by other workers.

Significant progress has been made since the hypothesis proposed by Ohno twenty years ago, when he suggested that the X and the Y chromosome originated as a homologous pair which diverged from each other through evolution, the Y chromosome taking the role of the male sex determinant while the X chromosome did not undergo major changes. A large number of sequences detecting homologies between the X and the Y chromosome have been isolated but a very small percentage of them are confined to the short arm of both sex chromosomes (pseudoautosomal region - Cooke **et al** 1985, Simmler **et al** 1985, Rouyer **et al** 1986, Affara **et al** 1986a). Instead, most of these sequences, including the sequences described in this thesis, recognise homology between the short arm of the Y and the long arm of the X and between the long arm of the Y and the short arm of the X, therefore reinforcing a particular point raised by Ashley (1984), that synapsis between the X and Y chromosomes is not perhaps the result of sequence homology.

These observed homologies between the X and the Y could have been regarded as remnants from the pericentric inversion undergone by the Y chromosome early in evolution. However, as can be seen from the literature, work on primates has shown that at least the Yp-Xq homology is very recent having arisen most probably by means of a transposition before the divergence of the man from apes.

Moreover Cooke **et al** (1984) showed the presence of a homologous region on the long arm of both sex chromosomes and suggested that exchange between the two chromosomes must have also happened relatively recently.

More studies on animals other than primates should be able to provide the answers concerning the evolutionary steps that the sex chromosomes underwent. An important point however to be kept in mind is that perhaps the origin of species might be polyphyletic rather than monophyletic in which case homologies would be difficult to trace among animals other than primates and man for whom there is enough evidence that they derived from each other (Seuãnez 1979).

4.1.4. Two probes Mapped near the Heterochromatin of the Y Chromosome

It is interesting that both GMGY1 and GMGY2 are situated in a region which is missing from two azoospermic patients. This region has been claimed, by Ferguson-Smith (1965) and Tiepolo and Zuffardi (1976) to contain a potential spermiogenesis gene. Additional work carried out by colleagues in this department, using a more detailed Y deletion panel and a wider range of Y long arm probes, showed that these probes are the most proximal to the heterochromatin (to be published). These two probes could be parts of the actual gene, or could be linked to it, and might therefore lead us to the gene by walking the chromosome. If this was to be done, a DNA or cDNA library made from adult testis or sperm should be used because there is a possibility that these tissues would be enriched for spermiogenesis messenger sequences. They should also be used in Northern blots to find out if they are themselves expressed, and if this is the case, the structure of the gene could be analysed by sequencing, for example.

Probes such as GMGY1, GMGY2 and GMGY11 can be used as diagnostic tools to detect the presence of Yq sequences in patients with sex chromosome abnormalities which are undetected cytogenetically. Another possible use for these probes is for antenatal sex determination* and if they are linked to the spermiogenesis gene, they may also provide some information about the molecular defect in cases of infertility.

Furthermore it might be possible to perfect DNA dosage studies in order that numerical or other abnormalities of the Y chromosome can be detected with these probes.

From a different angle these probes can be also used to see if the Y-autosome homology detected by them is the same amongst different ethnic groups. A complete RFLP search should also be performed, again with different racial groups, to test for the existence of population-specific polymorphisms which could then be used for ethnological studies (for example to study the movements of populations).

4.1.5. Presence of a Y-chromosome Sequence in XX Males

In another part of this study a panel of 11 XX males and one true hermaphrodite was probed with five Y-specific probes to look for Y-specific sequences in the genome of these inwdividuals.

GMGXY2 was shown to be present in the genomic DNA of five XX males and was the only probe isolated in this study to hybridise to the DNA of such individuals. Further experiments done by fellow workers in this department (unpublished data) show this probe to be present in nine XX males out of 18 studied so far (including the number of XX males presented here), while DXYS1 (Page **et al** 1982) was shown to be present in only one XX male . This suggest that GMGXY2 must be more proximal to the testis determinant factor(s) than DXYS1. Additional data from another parallel

* after DNA digestion and Southern blotting. Dot-blotting would be inappropriate in view of autosomal

study in this department using more probes from the short arm of the Y chromosome (Affara **et al** 1986b) placed the GMGXY2 probe more distal to the location defined using the Y deletion map and showed that this is only one of two probes present in one XX male named HM. This further suggests that GMGXY2 might be very near the testis determining factor(s). The fact that this fragment contains two smaller sequences which produce the same pattern when they hybridise to genomic DNAs is very intriguing. One could ask whether the testis determinant locus or the region around it consists of multiple copies as proposed by Ohno (1979).

The main Y-specific band of GMGXY2 was shown to be present in the fraction containing chromosomes X and 7 which had been flow sorted from each of the individual XX males RH and HM. Although one cannot exclude the possibility that the probe hybridised to the 7 and not to the X chromosome it is most probable that the Y-specific sequence is present on the X thus confirming that these XX males have arisen as a result of X-Y interchange. This is further confirmed in the case of RH by flow cytometry studies which showed that one of the X chromosomes is slightly larger than the other and the detailed cytogenetic analysis which showed the presence of the tip of the Y chromosome on one of the X chromosomes. On the other hand there was no detectable difference between the X chromosomes of XX male HM and cytogenetic analysis failed to show the presence of Y chromosome material on the tip of either X chromosome. This would tend to agree with the molecular data which suggests that only a small part of the Y short arm has been transferred in HM. More probes are present in the DNA of RH as shown by Affara **et al** (1986b). These results support the hypothesis that at least some of the XX males occur because of interchange between the X and the Y chromosomes in the paternal meiosis and that the amount of Y material transferred each time is variable.

This has been further confirmed by many groups of scientists such as Guellaen **et al** (1984), De la Chapelle **et al** (1977), Page **et al** (1985), Muller **et al** (1986), Vergnaud **et al** (1986).

Probes GMGY1, GMGY2, GMGY11 and GMGXY3 were not present on the genome of any of the XX males tested, which provides evidence against the possibility that male determination in these XX males is due to some form of sex chromosome mosaicism.

Vergnaud **et al** (1986) and Affara **et al** (1986b) used many probes against a number of XX males and tried to order them on the Y chromosome in such a way that each time an interchange would occur between the X and the Y chromosome a single contiguous portion of the Y chromosome would be transferred and a single crossover would occur. Vergnaud **et al** (1986) divided the short arm of the Y chromosome into three intervals and constructed two models - one in which the testis determining locus is near the telomere, and in the other model it is close to the centromere (for further information, see section 1.8.7. of the introduction). The latter model, however, in order to explain the transfer of the testis determining factor(s) without the more distal Yp sequences, requires the presence of a double crossover, in the parental meiosis, which is an unlikely event in the first place. Affara **et al** (1986b), in order to explain the transfer of Y-specific probes from the middle of the Y short arm without transferring any probes distal to them in two of their XX males (including the one tested here, HM), proposed that a paracentric inversion has occured in the fathers of these individuals which brought these Y sequences nearer to the telomere. They suggested that as long as rearrangements do not interfere with important functions, then variations in the order of sequences on the Y chromosome can be tolerated.

Inversions that do not involve the centromere are difficult to detect on the Y chromosome because of its

small size and therefore have not been studied extensively, though they have been shown to occur (Berstein **et al** 1986). It is not possible therefore to estimate the frequency with which they occur, neither it is possible, as yet, to estimate the frequency with which double recombination occurs between the X and the Y chromosomes. One cannot conclude as to which event causes this abnormal order of probes on the X chromosome of some of these XX males. It should be pointed out, however, as Affara **et al** (1986b) mentioned, that the genesis of an XX male is an unusual event and may itself be accompanied by complex rearrangements and interstitial deletions resulting in anomalous patterns of transfer. This should be kept in mind when trying to order probes on the Y short arm using breakpoints from XX males.

Due to the important position that GMGXY2 occupies it could be used to screen a cDNA or genomic library made from fetal testis of eight weeks old (when the testis determinant is presumably expressed), and then walk or jump the chromosome to approach the testis determinant(s) and investigate this region.

4.2.1. Comments on the Screening of the Y-Specific Library

The Y-specific library used here is deficient in some known tandemly repeated Y sequences, such as the Y-3.4kb and Y-2.1kb Hae III repeated fragments (Burk **et al** 1985). The library was constructed from Eco RI partially digested DNA and it was therefore likely that the large inserts produced would contain single copy sequences interspersed with repetitive elements. For this reason it was decided that screening this library with both genomic and hamster DNA and looking for sequences that did not hybridise at all would not produce single copy clones. An alternative procedure was used which would theoretically screen directly for single-copy, expressed sequences. Initially the library was screened with total human DNA to select the human sequences and leave behind the chinese-hamster sequences. In the second round, placental tissue was used as a source of cDNA since as mentioned in the introduction contains particularly high concentrations of STS. It is therefore quite probable that a lot of the mRNA produced in this tissue, encodes this protein and a lot of cDNA complementary to the STS region on the X chromosome could be produced. At the time that this research commenced, it was still believed that an STS locus, silent or not, was present on the tip of the short arm of the Y chromosome which was homologous to that on the tip of the short arm of the X and therefore by following this method of screening the possibility of picking sequences shared by both the X and Y short arms was increased. In the event that this mRNA was not greatly enriched for STS messengers, it was still possible to pick up other expressed sequences on the Y about which little was known.

None of the sequences isolated in this study recognise homology with the short arms of both the X and Y chromosomes. This presumably is due to the fact that very

few sequences are actually shared between these regions as demonstrated in the current literature, which shows that the nature of the homology between the X and the Y is not confined only to these areas, as was previously thought. It should be mentioned though, that one more probe, GMGY3, (described in Affara **et al** 1986a) has been isolated using this method. GMGY3 has been shown to be the most distal of the Y-specific short arm sequences isolated in this department and has been shown to be transferred to nine of twelve XX males (Affara **et al** 1986b).

With regard to the attempt to isolate DNA probes homologous to cDNA for the STS gene, it is not possible to assess the effectiveness of this technique until the probes can be tested for expression, as mentioned previously. Evidence in support of this method, however, comes from the fact that during the screening procedure only 103 clones from a total of 3,000 which were positive for male genomic DNA hybridised to the placental cDNA.

The possibility however that expressed sequences related to the testis determining locus would be isolated was very low since this locus on the Y chromosome is thought to be expressed only very early in development. A different source of mRNA, such as testis from aborted embryos of 7-9 weeks could give a better probe to screen with for these particular sequences because it is probably the only time in development that the testis determining locus is expressed. Other sources of mRNA which may perhaps produce useful probes, could be adult testes or testis from an individual during puberty (when testis undergoes changes - see introduction) or from sperm itself. The mRNA produced from such material could be related to the testis determining factor(s) on the short arm of the Y, if this is active again during puberty, or to the postulated spermiogenesis locus on the long arm of the Y chromosome near the heterochromatin. It is possible that the placental

mRNA used in this study carried remnants of these signals or mRNA related to other loci on the Y chromosome.

Ten clones which were assumed to contain little or no repetitive DNA were chosen to be studied further. It became obvious, however, when the clones were digested, blotted and probed with both genomic and cDNA, that they did in fact contain quite a lot of repetitive sequences. This suggested that the method followed here for screening by competition to isolate single copy sequences was not successful.

4.2.2. Isolation of a Human Autosomal Sequence from a Y-specific Library

Bi-4 a 4kb fragment was shown to contain a human autosomal single-copy sequence. The fact that a human autosomal sequence was pulled out from this Y-specific library is not surprising. Extensive isoenzyme analysis of the Y hybrid cell line 7631 from which this library was constructed, revealed the presence of the human isozyme of nucleoside phosphorylase in all assays and occasionally phosphoglucomutase-2 and aromatic alpha-ketoacid reductase. These enzymes are present on chromosomes 14 (14q13.1), 4 (4p14-q12) and 12 (12p) respectively, suggesting that fragments of these chromosomes are present in this hybrid cell line though they are cytogenetically undetectable (G-, G-11, and quinacrine banding) (Burk **et al** 1985). It is likely, therefore, that the Bi-4 autosomal sequence derives from one of these chromosomes and since the 4kb fragment has been shown to hybridise with cDNA it might be part of a structural autosomal gene. It is unfortunate that because this has not been part of the thesis this probe was not studied further to find out which chromosome it derives from, and whether it is indeed expressed.

4.2.3. Construction of a Hybrid Panel Comments on the Selection Strategies

A important part of this project, as already mentioned, involved the construction and characterisation of a human-mouse hybrid panel containing different parts of the X chromosome. The panel was constructed for use in the mapping of X-linked fragments detected by the recombinants isolated during this study. By applying different strategies, twelve hybrid clones bearing different parts of the X chromosome were obtained and these will be discussed in the following sections.

In cell line HN, the HGPRT locus has been deleted as a result of the deletion breakpoint being above this locus at Xq1.3, and thus selection for the delX could not be carried out in this line. Cytogenetic analysis of WH and DH cell lines failed to show whether the region of the X that includes the HGPRT locus was present on the abnormal chromosomes. Finally in cell lines, CE and LH the delX was assumed to be preferentially inactivated and selection for the HGPRT locus in this case was not possible. As a result, the above human cell lines also were fused with the LMTK mouse cell line, and selection was made for the TK locus on human chromosome 17. It was hoped that the derivative X would be retained randomly in some of the clones without being accompanied by the normal X chromosome. Further subcloning eliminated any contamination with the normal X chromosome.

Unfortunately the retention of the derX chromosome in these cell lines is essentially a random event. Also, a large number of clones had to be analysed cytogenetically to confirm these findings, a costly and time-consuming approach. Given these potential disadvantages however, the method was successful in the cases of HN, DH and CE as

confirmed by the DNA analysis of these hybrids. WH seems to be an exceptional case which will be discussed later.

In the case of CE and LN however, another approach might have been more productive. A large number of clones and subclones were analysed and found to contain either the normal X chromosome alone or both the normal and the derX (Xp-). The fact that the delX was never found on its own supported the notion that the delX is inactivated and therefore cannot be selected. In this situation it would have been interesting to try to reactivate the derivative X using 5-azacytidine (Mohandas **et al** 1980), thus allowing this chromosome an equal chance to be selected as the normal active X. Once this chromosome was reactivated, the cells could then be grown normally in HAT medium and from then on the procedure would be similar to the other hybrids. Mohandas **et al** 1980 used this technique to successfully reactivate a structurally normal, inactive human X chromosome retained in a mouse-human hybrid in order to study the role of DNA methylation in X inactivation.

Lines FNA9 and NEA9 had to be backselected in order to obtain the non-selectable X. As can be seen from the Materials and Methods and Table 2, this was an extremely time-consuming method.

4.2.4. Cytogenetic versus Molecular Analysis

The molecular analysis of these hybrids by using X-linked probes mapped along the X chromosome produced very intriguing (DHTK18a) and occasionally conflicting results (FNA92bRa$_1$I, NEA921R$_2$b, WHTK17III, HNTK6VII/I, Hor19X). In hybrids AMIR2N, EHA97II/VIII, W5A95IX, W2A96I, CETK1aIV, LNA94IRbXIII, NEA916II, and FNA98IX the cytogenetic and the molecular analyses agreed very well with each other.

i. DHTK18a Hybrid

In the case of DHTK18a, the problem, as mentioned above, was that the routine cytogenetic analysis of the parental line was unable to specify whether the breakpoint lay above or below the HGPRT locus at band Xq26-q27.2 (McKusick 1986). It was observed that the fusion between DH and A9 (results not shown) produced clones in which the derX (X/9) always segregated together with the normal X and never on its own. This fact would suggest that either the abnormal chromosome was inactivated, or possibly lacked the HGPRT locus and hence could not be selected. A different approach was therefore used, in which DH was fused to LMTK mouse cells and DHTK18a was produced containing the derX without a normal X. The molecular analysis of this hybrid line however showed some interesting results. All the probes from the tip of the short arm down to and including FIX, were present on the chromosome, while DX13 and St14 that are distal to FIX were negative. Given that FIX has been assigned to Xq27.1-q27.2 distal to HGPRT and proximal to Xq27 (Boyd **et al** 1984, Buckle **et al** 1984, McKusick 1986) and since DX13 and St14 are in Xq28 (Harper **et al** 1984, Oberlé **et al** 1985) the breakpoint of this translocation must be between the FIX and DX13. St14 sites in the region Xq27.1-q27.2 to Xq28 and the HGPRT locus is present on the derX.

This experiment demonstrates the usefulness of hybrids and of DNA analysis over the cytogenetic analysis. DNA analysis using probes that are already mapped can be very useful to define breakpoints in cases of patients which cannot be resolved cytogenetically due to technical limitations, such as DH. However molecular analysis of the genomic DNA of such cases can be obstructed by the presence of a normal X in the individual in question, and dosage studies should be performed. Alternatively if this derivative was present in a hybrid without the normal X, then the analysis would be much more succesfull.

ii. W5A915IX Hybrid

The case of W5A915IX should be mentioned. This derX (1/X) also involves also a paracentric inversion of part of the short arm of the X chromosome between Xp2107 and Xp1106 (Lindenbaum **et al** 1979 described in Materials and Methods). While Southern analysis can detect the absence of the Xp21 to Xpter region, it cannot detect a rearrangement such as an inversion because it does not involve (as far as the probes used in this study showed) any deletion of sequences. This demonstrates one of the weaknesses of DNA analysis compared to routine cytogenetic analysis and perhaps points out the advantage of **in situ** hybridisation which seems to be the link between the two techniques at the moment. In the near future the advantages of pulsed field electrophoresis should also help to bridge the gap between DNA and cytogenetic analysis (Anand 1986).

iii. FNA92bIIRa$_1$I and NEA921R$_2$b Hybrids

The DNA hybridisation probe results and the cytogenetic results for FNA92bIIRa$_1$I and NEA921R$_2$b do not correspond. Ideally for FNA92bIIRa$_1$I all the probes from the tip of the short arm of the X down to and including DP34 and for NEA921R$_2$b down to and including L128 should be positive. However, B24 is missing from the DNA of FNA92bIIRa$_1$I, while the three probes just proximal to it (754 and OTC) are present. L128 is negative, DP34 gives a weak positive signal, FIX is definitely positive, it was not possible to conclude for DX13 (Table 5).

NEA921R$_2$b is positive for all the probes down to L128 as expected, negative for the next probe DP34, and positive for FIX. DX13 was again inconclusive. These inconsistencies highlight the problems faced when working with hybrid cell lines and may suggest that either:

a) a normal X or the reciprocal translocation might be present in these hybrid lines at such a low level that it was undetected cytogenetically, but sufficient to cause

distortion of the DNA probe results or;
b) Parts of the X chromosome that have not been selected in the first place are present due to complex rearrangements between the X chromosomes and rodent chromosomes.

The possibility of contamination from the normal X or the reciprocal translocation cannot be ruled out, although the clones were further subcloned to eliminate such contamination and a large number of cells was examined. Thus extensive cytogenetic analysis finally revealed the presence of a normal X or the derX in a very small number of cells (about 5%) in one of the potentially useful hybrids of the FNA9 backselection (FNA92bIIRa$_1$I) and in one of the WH to LMTK fusions (WHTK17I). Extensive cytogenetic analysis, however, of FNA92bRa$_1$VI and NEA921R$_2$b did not reveal the presence of a normal X or the derivative autosome. Two of the potentially useful pellets from the fusion between HN and LMTK, HNTK6VII/VI and HNTKVII/IX had to be eliminated after an initial DNA screen with a few probes from the short and the long arm of the X chromosome - although the cytogenetic analysis did not show the presence of a normal X chromosome. In these cases it can only be assumed that the cytogenetic analysis failed to detect the undesired chromosomes or that the second possibility mentioned above can explain this result.

Rearrangements between mouse and human chromosomes occur frequently in hybrid cell lines and have been observed by other groups (Wieaker **et al** 1984, Oberlé **et al** 1986). They cannot be detected with G-banding but can be recognised by the G-11 banding. The technique was applied in this study on a small scale, and the initial results revealed that a certain degree of interchange occurs in every hybrid and that the extent of rearrangement varies from line to line. The G-11 banding however, has the disadvantage that it can be applied only on slides that have not been stained before with the G-banding stain. It is therefore extremely difficult to

identify the chromosomes and hence to decide whether the chromosome of interest is rearranged or not.

An additional problem when trying to interpret the results obtained from the molecular analysis of these hybrids is that some of these probes detect homologous sequences in the mouse genome which interfere with the results.

Probes FIX, DX13 and St14 which are crucial in the detection of the X chromosome long arm sequences present in the hybrids, also detect DNA sequences on the mouse genome. Some of these bands have the same size as the polymorphic bands detected in human DNA. Mouse homologies for FIX and St14 have also been reported in the literature (Boyd **et al** 1984, Oberlé **et al** 1985) and this is to be expected when dealing with structural genes (Brennard **et al** 1982). Boyd **et al** 1984 used Eco RI enzyme to digest the DNA of hybrids, and probed with FIX which revealed mouse and human fragments of different sizes. Their results indicated that there is a locus homologous to the human factor IX in the mouse but which diverges in sequence sufficiently to substantially reduce the amount of probe bound under the hybridisation conditions used. In the study presented here the restriction enzyme Taq I was used which reveals in the mouse a band of the same size as the human 1.3kb allele (Gianelli **et al** 1984). Raising the stringency of the post-hybridisation washing reduced the intensity of the mouse DNA signal and in some cases this made the interpretation of the results easier. The hybridisation pattern with DX13 (Harper **et al** 1984) was more complicated however. In this situation it would have been better to look for an enzyme which generates bands of different sizes for mouse and human DNA, as mentioned above, but this was not done. It would have also facilitated the analysis of these results if the DNA of the parental lines had been digested with the same enzymes to identify the polymorphisms present in their

DNA. If the parental line was homozygous for a specific allele, it would at least give information concerning the size of the band one would expect to find in the DNA of the hybrids. If the parental line was heterozygous, it would be possible to define whether both X chromosomes were present or only one, depending on which and how many alleles (bands) were present in the genome of these hybrids.

The possibility is however that not all the probes would be homozygous for both X chromosomes in each parental cell line. Based on this idea all the hybrids and especially FNA92bRa$_1$I and NEA21R$_2$b were checked to see if they revealed heterozygosity for any of these probes. None of these hybrids were shown to be heterozygous for at least 8 of these probes with reasonable frequences of alleles in the population (see Table 1). This observation reduces the probability that a normal X is present and distorts the results. This, together with the inconsistent presence or absence of X-specific in the DNA of these two hybrids reinforces the idea that a complex rearrangement has occured between parts of the X chromosome(s) and mouse chromosomes in these lines. It is worth mentioning afterall that these backselected lines were subjected to a variety of culturing conditions. The extended time these cell lines were maintained in culture might have caused more breakage than normal among the chromosomes and thereby increasing the possibility for rearrangements to occur.

iv. HNTK6VII/I and Horl9X Hybrids

The molecular analysis of cell line HNTK6VII/I agrees with the cytogenetic analysis apart from one probe, L128, for which the line should be positive but gives a negative result. This could be explained by the presence of a cytogenetically undetected deletion in the derX that includes the L128 locus. Oberlé **et al** (1986) came across similar problems and on one occasion they proposed that this could happen due to a deletion polymorphism present in

the parental human chromosome. This reinforces the necessity to examine all the parental lines as well, which so far has not been possible as mentioned above.

Horl9X is another similar example where probe 87/8 seems to be absent from the genome when the rest of the probes are all present. An event similar to the one above could have happened or in the extreme of possibilities the parent of this cell line could be a carrier for a deletion in the DMD (Duchenne muscular dystrophy) area. This requires confirmation with other PERT probes (Kunkel **et al** 1985).

v. WHTK17III Hybrid

The case of WHTK17III perhaps is a different one. It is thought to involve a deletion of part of the long arm of the X, but the possibility that WH might derive from an interstitial deletion that leaves present the tip of the long arm of the X is proposed by cytologists. In the particular case of WHTK17III, the distribution of the probes and their presence or absence in such an unusual pattern (see Table 5) suggests that the delX has derived from a complex type of rearrangement of the X chromosome present in the parental line. Alternatively, as discussed above an undetected normal X could be present in a very small proportion of the cells or more likely a complex rearrangement between the X chromosome(s) and the mouse chromosome(s) has occurred in this hybrid.

4.2.5. Somatic Cell Hybrids versus Flow Sorted Chromosomes

In an attempt to compare the mapping with hybrids against flow sorted chromosomes, thirteen different hybrids were selected because they retain different groups of autosomes (Tables 6 and 7). These hybrids were obtained while trying to isolate the hybrids discussed above. The lines were probed with probes GMGY1, GMGY3, and GMGY11. The

hybridisation patterns (Figure 17) were compared to the groups of autosomes retained in these hybrids and these results were compared to those obtained by probing filters containing groups of sorted chromosomes (Figure 18).

The results obtained by the two methods did agree to a large extent, though in certain cases such as with LNA94IRbXII and FNA95R2 for example, GMGY1 detected the presence of chromosome 1 sequences while the cytogenetic analysis did not. In this case, as in the ones discussed above, it is possible that chromosome 1 is involved in a rearrangement with a mouse chromosome and therefore cannot be detected cytogenetically. Alternatively, the fact that this chromosome was not detected could be due to the fact that very few were cells analysed in this line.

It has become obvious from the discussion so far and the experiments described above that hybrid cell lines are difficult to work with. An extensive cytogenetic analysis is necessary using a variety of banding techniques, while the scientist has always to take into consideration the fact that rearrangements may occur between mouse and human chromosomes. However once hybrids are established, that is once they have reached the level where they become stable and have been well characterised in terms of human chromosomes, (and as far as possible in terms of mouse/human rearrangements), they can be very useful tools in molecular analysis. They can be grown very easily numerous times and produce much greater amounts of DNA than it is possible to produce from flow sorted chromosomes. Charts in which the percentage of the X chromosome DNA in the hybrid DNA, for example, has been estimated would be very useful in order to allow the researcher to include enough DNA in the analysis to represent the chromosome of interest. It should be possible to calculate this from figures like the ones presented in Tables 3 and 4. In this study about double the amount of DNA usually added to a

reaction was used so the human DNA content would be well represented. The use of chromosome sorting for mapping purposes also on the other hand has its advantages and disadvantages. As Ruddle points out (1982) one of the drawbacks of the chromosome sorting techniques is the long sorting times required for recovery of sufficiently large samples and the high cost of the procedure. The amount of DNA recovered is not very large and the filter can be re-used only a limited number of times due to the fact that there is not much DNA on it in the first place and every time it is washed some of the DNA comes off. Though there might be some contamination between different groups of sorted chromosomes, this can be easily monitored, and by using different polymorphisms such as those used in this study more chromosomes could be sorted individually. Both methods seem to have advantages and disadvantages and perhaps for the moment both should be used in conjunction for mapping.

Many groups of researchers have used hybrids to map probes succesfully on sex chromosomes such as Murray **et al** 1982, Oberlé **et al** 1986, Wieaker **et al** 1984 and Boyd **et al** 1984). All of these researchers used a variety of different X fragments, some with similar breakpoints to those described here. One hopes that though similar, these breakpoints are not identical and therefore contribute to the finer mapping of probes. One could envisage an international bank of well-characterised hybrids containing different chromosomes either singly or in small groups, or with different parts of the same chromosome in a range of hybrids as was done here. This would be of great use for the mapping of any new probes. These could be prepared in different laboratories using a variety of selection systems such as the one proposed by Tunnacliffe **et al** (1983).

One method that perhaps would be useful to define human chromosomes within hybrids would be **in situ**

hybridisation, for example with a probe such as the centromere-specific alphoid sequences (Willard 1984).

4.2.6. Evaluation of the Usefulness of Somatic Cell Hybrids in this Study and in Molecular Genetic Analysis in General

a) The presence of a contiguous part of the X chromosome was confirmed in nine of the hybrids obtained during this study and their importance was immediately demonstrated by using them to localise the X-linked fragment recognised by two probes isolated in another part of this study. The confirmation of the mapping positions of the previously assigned X-specific probes further demonstrates the usefulness of somatic cell hybrids in the mapping of DNA sequences.

b) The molecular analysis of three other hybrids did not agree with the cytogenetic analysis and revealed the presence of noncontiguous parts of the X chromosome. This illustrates the difficulties faced when working with hybrids. These lines however can be used as effectively as the ones mentioned above, by accurately defining the parts of the X chromosome present in these lines using more X-specific probes. In fact they might provide different breakpoints which have not been found in other individuals.

c) The breakpoint on the X chromosome in a translocation product retained in a hybrid was more accurately defined. It had not been possible to be ascertain this by cytogenetic techniques and demonstrates the resolving power that can be achieved by Southern blot analysis over routine cytogenetic analysis. The importance of using hybrids rather then genomic DNAs in cases such as this is highlighted, since the same analysis

in genomic DNA would have be obstructed by the presence of a normal X

d) A panel of hybrids bearing different groups of autosomes was used in order to map the autosomally-linked fragments detected by probes isolated in this study and to compare these results with those obtained by using flow sorted chromosomes to map the same sequences. The data were largely in agreement and the experiment showed that both methods should be used to assign single-copy sequences to chromosomes.

e) In order to ensure that there is no contamination by an undesired chromosome, subcloning of the particular hybrid clone should be performed and a large number of cells should be analysed to minimise the possibility that a chromosome present in a very low number of cells will not be detected. In addition to this both G-banding and differential staining should be used to enhance the cytological analysis.

f) Human/mouse rearrangements seem to occur frequently in interspecific hybrids and the frequency of their occurrence should be estimated with the use of G-11 banding and should be taken into consideration. This study also showed that deletions of very small regions can occur and the scientist should be aware of this when using hybrids or genomic DNAs to localise new sequences. Also demonstrated is the importance of a detailed characterisation of hybrids before they are further used to map other sequences.

g) The use of probes to confirm the presence or absence of a chromosome or a part of a chromosome as done here for the X chromosome, proved to be very successful and as can be seen from the results, it is the most sensitive method of screening. However the researcher should not be

inclined to use this method as a replacement for cytogenetic analysis, but should rather combine both techniques to improve the speed and accuracy of analysis.

h) Certain probes recognise homology with mouse DNA which tends to make the interpretation of the results difficult if not impossible. For this reason probes that do not detect such homologies should be chosen or enzymes which generate different size bands for man and mouse should be used.

i) The same probes should be initially used to test the DNA from the parental lines of the hybrids in order to know which polymorphisms to expect for the particular chromosome, in the DNA of the hybrids and in order to detect any abnormalities or deletions.

Conclusions

A number of conclusions can be drawn from this study regarding:

1. The nature of the recombinants obtained:

a) The isolation of three sequences which detect homology between the long arm of the Y chromosome and various autosomes (GMGY1, GMGY2, GMGY11) reinforces the idea that a lot of recombination has occured during evolution between at least the long arm of the Y chromosome and autosomes.

b) One more sequence (GMGXY2) which recognises homology between the Y short arm, the X chromosome and autosomes suggests that the short arm of the Y chromosome has been involved in recombination with other chromosomes as well.

c) The localisation of the autosomal fragments detected by these probes give an insight into the evolutionary steps, undergone by the human genome and opens new fields of research.

d) The degree of divergence between the homologous sequences was shown to vary, which indicates that recombination between different chromosomes must have happened more then once during evolution and at different times.

e) The isolation of two sequences, one which detects homology between the short arm of the X and the long arm of the Y (GMGXY3), and one which detects homology between the long arm of the X and the short arm of the Y and also a variety of autosomes (GMGXY2) , agrees with the recent data

that homology between the X and the Y chromosomes is not confined only to their short arms.

f) GMGXY2 is regarded as a potential member of a family of pseudogenes and its relationship and position to the Xq-Yp group of sequences is considered.

2. The nature of the XX male syndrome

a) The presence of GMGXY2 Y-specific fragment in the DNA of five XX males and the demonstration of its presence on the X chromosome of two of them, supports the hypothesis that at least some XX males derive from X-Y interchange in parental meiosis. This result combined with data from this laboratory shows that the amount of Y material transferred in XX males is variable.

a) The absence of four Y-long arm fragments from the genome of 11 XX males excludes the possibility of mosaicism for at least the long arm of the Y chromosome in this cases, therefore suggesting that these XX males are not the result of mosaicism.

The homologies reported here between the X and the Y chromosomes comply very well with the newly constructed picture of the Y chromosome and its homology with the X and further with autosomes.

The results presented here do not deal directly with the evolution of the Y chromosome since this would only be possible if one would study these sequences in connection with primates and other animals. They give however an insight into the evolutionary steps that the Y chromosome and generally the genome has undergone, by showing homology of this chromosome with the X and equally important with other autosomes as well. It also raises important questions regarding the nature and importance of these homologies.

One would hope that further isolation and characterisation of sequences from the Y chromosome will make it the first chromosome to be fully characterised at the molecular level. Furthermore due to its unique function, the Y chromosome provides important information as to the evolution of the genome and species. Its relation to the X chromosome and the autosomes should provide us eventually with information regarding the evolution of the sex determining mechanism and bring us nearer to testis determinant factor(s).

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