MOLECULAR ANALYSIS OF DNA SEQUENCES FROM THE HUMAN Y CHROMOSOME

WENDY A. BICKMORE

PhD
University of Edinburgh
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ABSTRACT

Models of mammalian sex chromosome evolution and behaviour during meiosis, have predicted that the X and Y chromosomes evolved from an homologous pair of chromosomes, homology being maintained only in the region of chromosome pairing at meiosis as a result of crossing over between the chromosomes. I have demonstrated that the human X and Y chromosomes share homology outside of the meiotic pairing segment, and estimate this homology, to be 100%, on the basis of restriction enzyme mapping and DNA sequencing. This high degree of homology is not the result of conservation or correction mechanisms operating upon these sequences.

Related sequences in the great apes have been shown to be located only on the X chromosome. It is therefore suggested that a transposition event has occurred between the long arm of the X and the Y chromosome very recently, since the divergence of humans from the great apes. This observation is consistent with those of several other workers, who have identified similar transpositions between the X and Y chromosomes. I have used cosmid cloning and pulsed-field gel electrophoresis techniques to analyse the extent of the homology between the human X and Y chromosomes. These results indicate that the transposition was a large scale event spanning at least 50, and possibly several hundred kb. Such a transposition may be a reflection of the close association of the X and Y chromosomes in the sex vesicle during meiosis in the male.

The human Y chromosome is intensively studied, its small size, approximately 3 x 10^4kb, making it the most amenable human chromosome for molecular mapping studies. It also has a unique

biological role in directing indifferent embryonic gonads to differentiate into testes, thus determining the male sex. The human Y chromosome is, therefore, the focus for the search for sex determining genes. Only a small amount of DNA $<5 \times 10^3$kb may be specific only to the Y. Amongst this DNA we might expect to find the sex determining genes. Y specific DNA sequences are recovered at a low efficiency by conventional cloning techniques, e.g. constructing cosmid libraries from somatic cell hybrids. A majority of human sequences isolated in this manner are found to be homologous to loci elsewhere in the genome. I have isolated human Y chromosome specific sequences at a high frequency using the phenol-enhanced reassociation technique (PERT). Digested male DNA and a 1000x excess of sonicated female DNA are denatured, then allowed to reassociate (the rate of reassociation enhanced by the formation of an emulsion with phenol). Only DNA present in the male DNA alone should reassociate back to a double-stranded DNA molecule with clonable ends. Clones recovered by this method are then used to probe Southern blots of male and female DNA to identify sequences located on the Y chromosome alone.

ABBREVIATIONS

A	Adenine
ATP	Adenosine triphosphate
β-me	β mercaptoethanol
bp	base pairs
BSA	Bovine serum albumin
C	Cytosine
CAIP	Calf alkaline intestinal phosphatase
cM	Centimorgans
Cot	Initial single stranded DNA concentration x time(s)
cpm	Counts per minute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanidine triphosphate
dTTP	Deoxythymidine triphosphate
ddNTP	Dideoxynucleotide triphosphate
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreiotol
EDTA	Ethylenediaminetetracetic acid
euc	Euchromatin
FACS	Fluorescence-activated cell sorter
FTL	Freeze thaw lysate
G	Guanine
HAT	Hypoxanthine aminopterin thymidine

HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid
HPRT	Hypoxanthine guanine phosphoribosyl transferase
IPTG	Isopropyl-1-thio-β-D-galactoside
K	1000 revolutions per minute
kb	Kilobase pairs
mins	Minutes
OD	Optical density
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PERT	Phenol emulsion reassociation technique
pfu	Plaque forming units
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SE	Sonic extract
SM	Storage medium
Spi	Sensitive to P2 interference
ss	Single stranded
SSC	Sodium saline citrate
Sxr	Sex reversal
T	Thymidine
TCA	Trichloroacetic acid
TE	10mM Tris, 1mM EDTA
TEMED	N,N,N',N'-tetramethylene diamine
Tris	Tris (hydroxymethyl) aminomethan
UV	Ultraviolet
vol	Volume

w/v	Weight/volume
XGal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION

1.1. Motives for studying the mammalian X and Y chromosomes

The mammalian X and Y chromosomes are intensively studied in two contrasting contexts:-

a) as highly specialised chromosomes with specific functions in the determination of sex. The mammalian Y chromosome determines the male sex.

b) as models for the study of general chromosome structure and behaviour.

1.1.1. The use of the Y chromosomes as a model for molecular mapping techniques

Conventional genetic analysis of the human Y chromosome is difficult due to a lack of known functional genetic loci. However, it is the smallest of the human chromosomes consisting of ~3 x 10^4kb of DNA. Its size thus makes it the chromosome most amenable to molecular analysis.

Cytogenetic methods enable chromosomes to be studied at a resolution of 5 x 10^3kb, this being approximately the amount of DNA in half of a chromosome band. The standard techniques of molecular biology, allow DNA of up to 10^2kb to be examined in detail. The ability to study chromosomes at the resolution of 10^2kb to 10^4kb, would yield important information on large-scale aspects of the genome such as the organisation of genes with respect to controlling elements, or other genes, and the structure of chromosome features such as centromeres and satellite DNA. Techniques are currently being developed for this sort of approach to studying the genome. Chromosome mediated gene transfer can be used to create somatic cell hybrids containing only small portions of human chromosomes

(Pritchard and Goodfellow, 1986; Porteous et al, 1986). Regions of chromosomes may be isolated for molecular cloning by physically microdissecting the chromosome in question (Rohme et al, 1984; Fisher et al, 1985). Pulsed field gel electrophoresis (Carle and Olson, 1986) and its associated technology enables long stretches of DNA to be manipulated and resolved. The power of this latter technique would be greatly enhanced by the ability to clone DNA sequences in one step, which are separated from each other by a large distance in the genome (chromosome jumping) (Collins and Weissman, 1984; Poustka and Lehrach, 1986).

It seems likely that the human Y chromosome will be the first mammalian chromosome to be mapped at a molecular level, using the sort of approaches described above. Much of the information obtained however, will be applicable to chromosomes in general.

1.1.2. The genetic map of the X chromosome

In contrast to the dearth of genetic loci mapped on the Y chromosome, the X is the human chromosome best characterised genetically. Many disease loci were assigned to the X, prior to the advent of recombinant DNA technology, due to the manifestation of recessive mutations on this chromosome in the hemizygous male. Indeed the current genetic map of the human X chromosome has largely been formed by the search for restriction fragment length polymorphisms (RFLPs) linked to such diseases as Becker and Duchenne muscular dystrophies (Monaco et al, 1985; Ray et al, 1985), retinitis pigmentosa (Battacharya et al, 1984) and haemophilia A (Harper et al, 1984). The close linkage of RFLPs to a disease locus makes prenatal diagnosis for the defect possible, using foetal

trophoblast DNA, in families informative for the RFLP (Weatherall, 1985).

The isolation of random X chromosome specific probes has been facilitated by the construction of libraries enriched for X chromosomal material. This can be achieved by the use of somatic cell hybrids in which the X is the only detectable human DNA, as the source of cloning material. Clones containing human sequences can then be identified by their hybridisation to human specific repeats (Gusella et al, 1980). A more direct approach is to sort chromosomes prior to cloning. Chromosomes are stained with an intercalating dye and sorted on a fluorescence-activated cell sorter (FACS), on the basis of their fluorescence. DNA can then be cloned from a fraction enriched for a particular chromosome (Davies et al, 1981).

Random probes may be mapped to regions of the X chromosome by in situ hybridisation (Hartley et al, 1984), or by Southern hybridisation to panels of somatic cell hybrids containing cytogenetically defined portions of the human X chromosome (Murray et al, 1982). The resolution of these techniques however, is limited (see preceding section). A more detailed map of the X chromosome has been constructed based on the linkage of RFLPs (Drayna and White, 1985). The resulting genetic map of the human X chromosome spans approximately 200 recombination (cM) units with markers spaced along its length such that any X linked disease will map within 10 cM of at least two markers (see Fig. 1.1). It must be emphasised however, that this is a genetic map, based on meiotic recombination frequencies, it does not indicate the physical separation of markers on the chromosome. Indeed it is well known

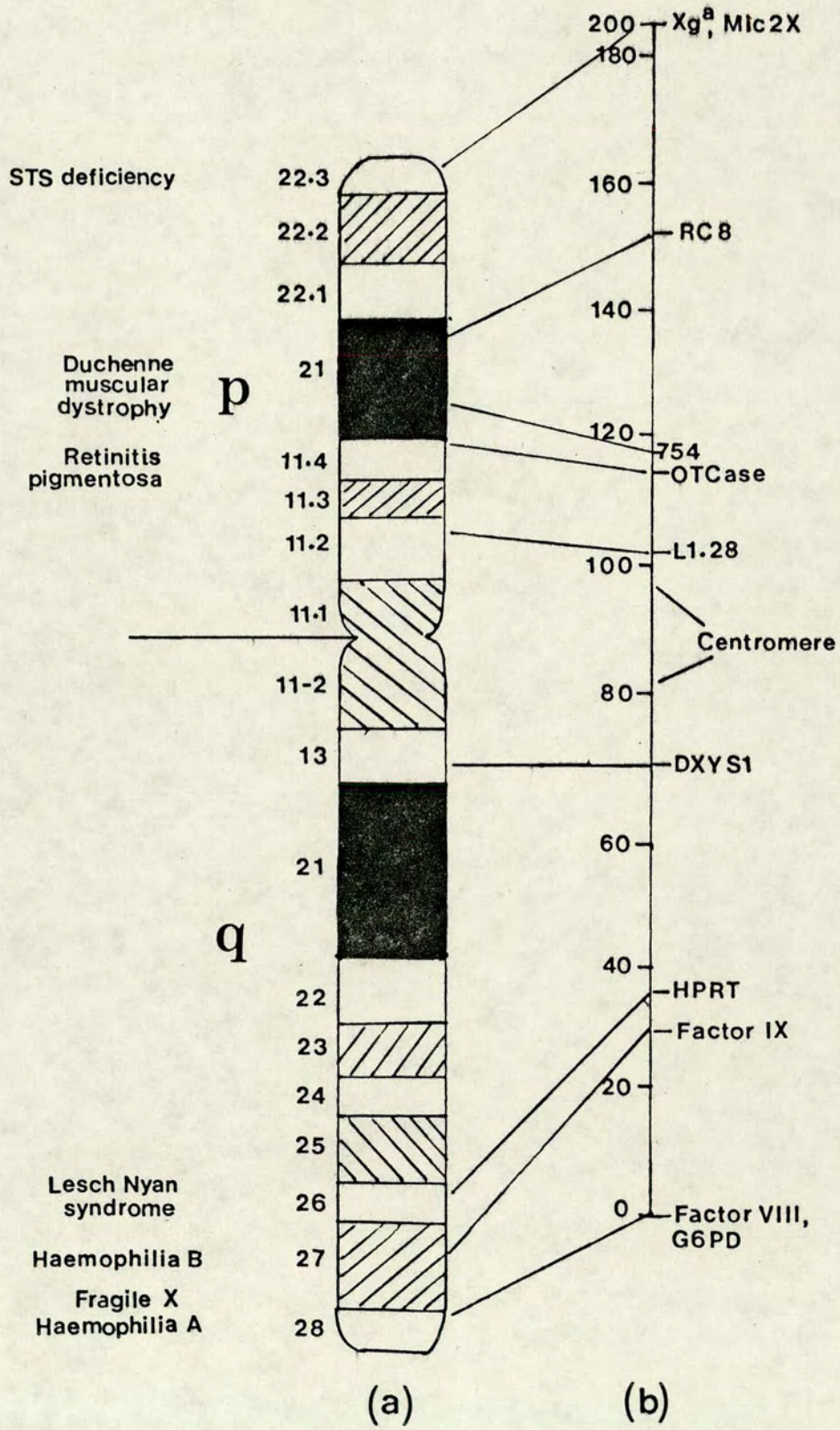
FIGURE 1.1

Map of the human X chromosome

a) Physical map of the human X chromosome showing the location of disease loci and DNA markers.

b) Genetic map of the human X chromosome in centimorgans, based on linkage analysis of DNA markers (Drayna and White, 1985).

Comparison of the relative positions of markers on the physical and genetic maps shows the increased recombination towards the telomeres of the chromosome relative to the centromeric region.


STS deficiency
Duchenne muscular dystrophy
Retinitis pigmentosa
Lesch Nyan syndrome
Haemophilia B
Fragile X
Haemophilia A
p
q
22·3
22·2
22·1
21
11·4
11·3
11·2
11·1
11·2
13
21
22
23
24
25
26
27
28
200
180
160
140
120
100
80
60
40
20
0
Xg^a, Mic2X
RC8
754
OTCase
L1.28
Centromere
DXYS1
HPRT
Factor IX
Factor VIII, G6PD
(a)
(b)

that there is an increase in recombination towards the telomeres of chromosomes (Hultén, 1982; Laurie, 1982; Hartley, 1984). This tends to expand the genetic map relative to actual physical size towards the ends of chromosomes.

It is hoped that the genetic mapping approaches which have been applied to the human X chromosome can be used to construct a linkage map of the entire human genome.

1.1.3. X chromosome inactivation

In mammals the male is the heterogametic sex (XY). Thus in the homogametic (XX) female, X-linked genes are present at twice the dosage found in males. Potentially this situation could lead to an imbalance in the expression of such genes between the sexes. The severe effects of aneuploidy observed in mice and humans (Epstein, 1985) suggests that such a disparity of gene expression may be lethal in mammals. This is at odds however with the phenomenon of dominance. Kacser and Burns (1981) have shown that metabolic fluxes are only slightly affected by changes in the concentrations of enzymes involved in them. The presence of only one locus for X-linked enzymes in males, as opposed to two in females would therefore not be expected to be detrimental to either sex (Chandra, 1986). It may, however, be important that some gene products, such as those involved in switching on batteries of other genes during development, are maintained at a certain level, thus necessitating some form of dosage of compensation between the sexes.

Lyon (1961) proposed that the phenomenon of X chromosome inactivation occurred in female mammals. The reasons for inactivation might be to ensure that females and males expressed X-

linked genes at equal levels. How such a chromosome inactivation is established and maintained from one cell to its progeny, is of importance to studies on gene control. Investigation of individuals heterozygous for electrophoretic variants of X-linked proteins e.g. phosphoglycerate kinase (PGK) have shown that, except in extra-embryonic tissue where preferential inactivation of the paternally derived X occurs, X chromosome inactivation in somatic cells during early embryogenesis is random (McMahon et al, 1981). Adult somatic tissue consequently, is a mosaic of clonally derived paternal and maternal inactive X containing cells.

X chromosome inactivation is thought to initiate at a single genetic locus, which in mice is termed the X controlling element (Xce) (Cattanach, 1975). An equivalent locus in humans is thought to be located on Xq (Therman et al, 1979) and from here inactivation spreads out to the rest of the chromosome except the tip of Xp, where several loci, e.g. Xg (a blood group marker), steriod sulphatase (STS) and MIC2X, escape inactivation, at least partially (Race and Sanger, 1975; Shapiro et al, 1979; Migeon et al, 1982; Goodfellow et al, 1984).

Once established in somatic cells, the pattern of X inactivation is maintained throughout replication and cell division. Experimental evidence suggests that methylation of cytosine residues in DNA at 5'CpG sites plays a role in silencing expression from the inactive X. The X linked enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT) is part of the salvage pathway of purine synthesis, and enables mammalian cells in culture to grow in media containing hypoxanthine, aminopterin and thymidine (HAT). Aminopterin interferes with folate metabolism, thus blocking de novo nucleotide

synthesis. Utilisation of the salvage pathway then becomes essential for cell survival. DNA from a hybrid cell containing an active human X chromosome can transform $HPRT^-$ cells to HAT resistance. DNA from a cell containing an inactive X is inert in such a transformation, except after treatment with 5-azacytidine (Venolia et al, 1982). 5-azacytidine, a cytosine analogue, becomes incorporated into DNA during replication, and inhibits DNA methylation (Friedman, 1981). Reactivation of other X-linked markers e.g. PGK and glucose-6-phosphate dehydrogenase (G6PD) is often concomitant with the restoration of HPRT activity by 5-azacytidine. Inhibition of DNA methylation therefore appears to release genes on the inactive X from their inactivation mechanisms in hybrid cells.

The restriction endonucleases HpaII and MspI are isoschizomers, cleaving at the DNA sequence $^{5'}C/CGG$. HpaII, however, will not cut if either C residue is methylated, whereas MspI is only sensitive to methylation of the 5'C (Walder et al, 1983). This pair of enzymes can therefore be used to assay methylation of DNA, although they provide information on only 1/16th of the total methylatable sites in the genome. Wolf and Migeon (1982) detected no differences in X methylation of random DNA clones between males and females using this assay. Methylation of DNA on the inactive X will probably only occur at certain critical sites, and these may have been refractory to the analysis used in the above experiment. Wolf et al (1982), on the other hand, were able to demonstrate methylation differences between human HPRT genes on active and inactive X chromosomes. Active alleles in both males and females were not methylated at their 5' ends, although the body of the gene may have been

methylated. Inactive HPRT alleles in contrast showed increased methylation of these 5' regions. Removal of this methylation occurred upon 5-azacytidine induced reactivation of the HPRT gene.

The possible role of DNA methylation in regulating gene expression has been hotly debated. During vertebrate evolution methylation has spread through the genome, and its general consequence seems to be the depression of transcription. This effect might be overcome in tissue specific and developmentally regulated genes by the involvement of specific transcription factors and enhancer sequences. Genes involved in basic metabolism, expressed in all cells ('housekeeping' genes) may not have such a means of augmenting their transcription to overcome any deleterious effects of methylation. It may therefore be necessary to maintain the 5' ends of this latter class of genes free from methylation. Indeed, Bird et al (1985) have found 'HTF (HpaII tiny fragment) islands' of unmethylated DNA in vertebrates, and all housekeeping genes so far examined are associated with such islands at their 5' ends (Bird, 1986). These islands do not show the CpG deficiency manifest in the genome as a whole, probably as a consequence of their non-methylated state. 5-methyl cytosine is a hot spot for mutation, it being readily deaminated to a T residue (McCelland and Ivarie, 1982).

X inactivation in the germ line, in contrast to the soma, is reversible, reactivation occurring in oogenesis prior to entry into meiosis (Gartler and Riggs, 1983). Evidence suggests (Sandford et al, 1985) that the inactive X in the germ line and extra-embryonic cells, is not methylated, the HPRT gene on the inactive X from these sources being active in transformation experiments. It is plausible

that an initial event, such as chromosome condensation, brings about X inactivation. Methylation of the chromosome then follows in cells where the inactivation is to be permanent. Methylation in this context is merely serving to further imprint a pattern of gene expression on a cell, which has already been established. It is worth noting that Wolf and Migeon (1982) were unable to reactivate HPRT on the inactive X from human fibroblasts using 5-azacytidine, in contrast to the work of Venolia et al (1982) in hybrid cells. This suggests that the inactivity of the X is more stable in normal cells than when it is in the foreign environment of a hybrid. Removal of methylation in the former case is insufficient to release the chromosome from inactivation, indicating an underlying inactivation mechanism independent of DNA methylation. Evidence from other systems also indicates that methylation follows as a consequence of gene inactivity, rather than being the primary cause of it (Bird, 1986). Methylation of active genes may be prevented sterically by the presence of bound factors at the 5' ends of such genes. Only when these genes are no longer being expressed, and bound factors presumably not present, may DNA methylase be able to gain access to the DNA to bring about methylation.

Further studies on the phenomenon of X inactivation will continue to provide clues as to mechanisms operating during early embryogenesis, and the methods that cells use to regulate their gene expression. It will also be interesting to establish how the inactivation mechanism is transmitted over almost the entire length of a chromosome ($\sim 10^5$kb). The functional significance of X inactivation will be further discussed in the following section.

1.2. Evolution of sex chromosomes

The mammalian sex chromosomes are thought to have evolved from an homologous pair of chromosomes differing at a single locus involved in sex determination. They have since diverged and become morphologically distinct and functionally specialised. Much work on sex chromosome evolution has centred on studies in snakes, as living species of these animals are thought to provide examples of the major evolutionary steps involved.

In contrast to the mammalian XY system, female snakes are heterogametic (ZW), males being ZZ. In primitive snakes, e.g. Boidae, the sex chromosomes are morphologically identical. The more advanced Colubridae have sex chromosomes of comparable size, but distinguished by the position of the centromeres. Poisonous snakes of the families Crotalidae, Elapidae and Viperidae, on the other hand, are regarded as the most highly evolved snakes and their sex chromosomes are easily distinguished, the W being small and condensed. Chromosomal sex determination has therefore evolved since the emergence of the snake order (Ohno, 1967).

The evolutionary appearance of differentiated sex chromosomes is accompanied by a quantitative excess of a repeated DNA sequence family (Bkm satellite) on the W as compared to the other chromosomes (Singh et al, 1980). Indeed heterochromatin is a common feature of sex determining chromosomes in vertebrates; its appearance preceding the major structural divergence of the W/Z and X/Y chromosomes, and the genetic degeneration of the W and Y.

Many speculative hypotheses have been put forward to explain the evolution of differentiated sex chromosomes. Müller suggested that a cessation of crossing over between the XY or ZW pairs, in

order to protect the integrity of a sex determining locus was the primary event leading to the structural and genetic divergence of the sex bivalents (Charlesworth, 1978). The hemizygous Y and W chromosomes thus accumulated mutations and became largely functionless, losing many of their genetic loci, leading to the structural changes observed in these chromosomes. This view of sex chromosome evolution predicts that it was only after the structural changes in the Y and W leading to inactivation, that heterochromatin was able to spread through these chromosomes. This is at odds with the presence of Bkm satellite DNA on the W of snakes with apparently homomorphic sex bivalents. The X and Z chromosomes in contrast are proposed to have been genetically conserved during vertebrate evolution (Ohno, 1967) possibly via the operation of gene conversion mechanisms in the homogametic sex.

Recently Jones (1983) has proposed that W and Y inactivation, and thus loss of selection pressure, was the driving force behind the divergence of the sex chromosomes. His hypothesis is that sex determining genes on the W and Y were brought into proximity of, and assumed control over, a locus controlling chromosome condensation such that when the sex determining genes are switched off, the chromosome condenses and is thus inactivated. If transcription of such sex determining genes occurs only during very early embryogenesis, all other genes on these chromosomes, not expressed in early embryonic development, will be under no selection pressure, so will mutate rapidly to functionlessness, or be completely lost. Heterochromatin will also spread rapidly through the largely inactive chromosomes.

Such a hypothesis demands a re-examination of the phenomenon of

X inactivation. The viability of mammalian female early embryonic cells and undifferentiated cell lines, in which both X chromosomes are active (Monk and Harper, 1979; McBurney and Strutt, 1980) and the apparent absence of dosage compensation in the ZW sex determination system (Jones, 1983) questions the absolute requirement for X chromosome inactivation in mammals. A scenario can be envisaged in which the X, like the Y, evolved such that expression of genes upon it, involved in sex determination, also controlled chromosome condensation, possibly via an Xce-like locus. The switching off of one copy of these genes at the time of cellular differentiation would consequently result in chromosome inactivation. Genes upon the X however, unlike the Y, are still subject to selection pressure, at least one copy of the chromosome being active in all cells. Even if it is believed that the phenomenon of X chromosome inactivation arose 'accidentally' the transcription from two X chromosomes in differentiated mammalian cells may now be a lethal condition.

1.3. The XY pair at meiosis

Despite the obvious structural differences between the sex bivalents, they must still behave as if an homologous pair of chromosomes at meiosis in the male.

Meiosis is a complex process of cell division in which homologous chromosomes pair, exchange genetic material, and orientate themselves on the meiotic spindle apparatus to allow for the correct segregation of chromosomes into daughter cells. This process could potentially pose problems for the differentiated sex

bivalents.

During the zygotene stage of meiosis, autosomes begin to synapse from their termini (telomeres). It is at this time that the sex vesicle appears in males, as a darkly staining body containing the X and Y chromosomes, which can often be seen attached at their telomeres, with irregular small excrescences along their axes. Spermatocytes undergo structural and morphological change during pachytene, which lasts about sixteen days in man. During early pachytene XY pairing is at its most extensive, covering the distal third of Xp and capable of extending over 3/4 of the length of the Y, i.e. all of Yp and across the centromere into Yq in humans (Chandley, 1984). Large excrescences are visible on the chromosome axes outside of the pairing region. As pachytene proceeds there is a shortening of the XY synaptonemal complex and its eventual disappearance, together with a splitting of the chromosome axes. By diplotene the X and Y chromosomes have separated.

It has been assumed that homologous chromosomes recognise and pair with each other during meiosis as a result of their common DNA sequences. In the early stages of meiosis however, many non-homologous chromosome associations can be observed, primarily at telomeres. It is generally considered that chromosome pairing initiates at telomeres, and subsequently spreads to the rest of the chromosome. Initial association of chromosomes at telomeres may occur at random, but only homologous pairings being subsequently stable (Chandley, 1984).

Koller and Darlington (1934) were the first to postulate the presence of homologous material in the XY pairing segments. This is supported by much indirect evidence. The presence of loci on Xp

which escape X chromosome inactivation (e.g. Xg and STS) infers that there are homologous loci on Yp , which removes the necessity for dosage compensation of these genes. The somatic effects in XO humans (Simpson, 1982) also indicate a requirement for transcription from two X chromosomes, or one X together with homologous loci on the Y, in order for normal development to occur (Polani, 1982). However, as Chandley's work clearly demonstrates, XY pairing is capable of extending beyond any possible region of DNA homology, the Y centromere being seen to pair with Xp in some meioses.

Burgoyne (1982) has outlined a model, whereby a single obligate crossover occurs between the X and Y in the pairing region, to allow for correct segregation of the chromosomes at meiosis. It has long been thought that chiasmata have an important role in disjunction of chromosomes during the first meiotic metaphase, and their presence has been demonstrated in autosomal bivalents (Hultén, 1974). The frequency of chiasmata observed on the autosomes, suggests that, in the relatively small XY pairing segment only one chiasma will be formed. Solari (1980) observed 'dense bars' in synaptonemal complexes of human spermatocytes at pachytene, under the electron microscope. The frequency and telomeric bias in the position of these bars suggests that they represent chiasmata. The distribution of these sites on the X and Y indicates that crossing over occurs mainly in the distal part of the pairing segment. End to end association of X and Y at diakenesis in mammals is presumed to result from terminalisation of chiasmata.

Genetic evidence for obligatory crossing over between the X and Y chromosomes has come from investigation of the (Sxr) (sex reversal) mutation in Mus musculus (Singh and Jones, 1982). XY_{Sxr}

males carry a single aberrant Y chromosome (Evans et al, 1982) on which a paracentric region of the chromosome, presumably containing sex determining genes, is duplicated onto the distal end of the Y, as evidenced by its hybridisation to Bkm sequences. In the progeny of a cross between a normal female and an XY_{Sxr} male, half of the expected females are phenotypically male and have an XX_{Sxr} karyotype. This pattern of inheritance is that expected for an autosomal locus, and can only be explained for loci on sex chromosomes if exchange of genetic material occurs between the X and Y at meiosis. The 'pseudoautosomal' pattern of inheritance of Sxr was thus the first concrete evidence for crossing over between sex bivalents in mammals, provided that the transfer of Sxr from the aberrant Y of XY_{Sxr} males to the paternal X was merely a consequence of normal recombination in this region at male meiosis.

Models of crossing over have been largely based on studies in bacteria and fungi. The model which best satisfies the available experimental evidence was first devised by Holliday (1964). This predicts that for every Holliday junction occurring between a pair of DNA molecules, the resulting half-chiasma resolve symmetrically, giving rise to either recombinant or non-recombinant products (Dressler and Potter, 1983). Figure 1.2 illustrates this model in the context of the crossing over between the mouse X and Y_{Sxr} chromosomes.

FIGURE 1.2

Model for crossing over between the mouse X and Ysxr chromosomes via a Holliday recombination mechanism

The Sxr mutation results from a duplication of a region (Sxr) of the mouse Y chromosome carrying sex determining genes onto the distal end of that chromosome, which is involved in crossing over with the X chromosome at meiosis. In this way sex determining genes are transferred onto the X chromosome and result in XX_{sxr} progeny which are phenotypically male.

a) At male meiosis the X and Y chromosomes pair. A nick is introduced into one DNA strand of each chromatid.

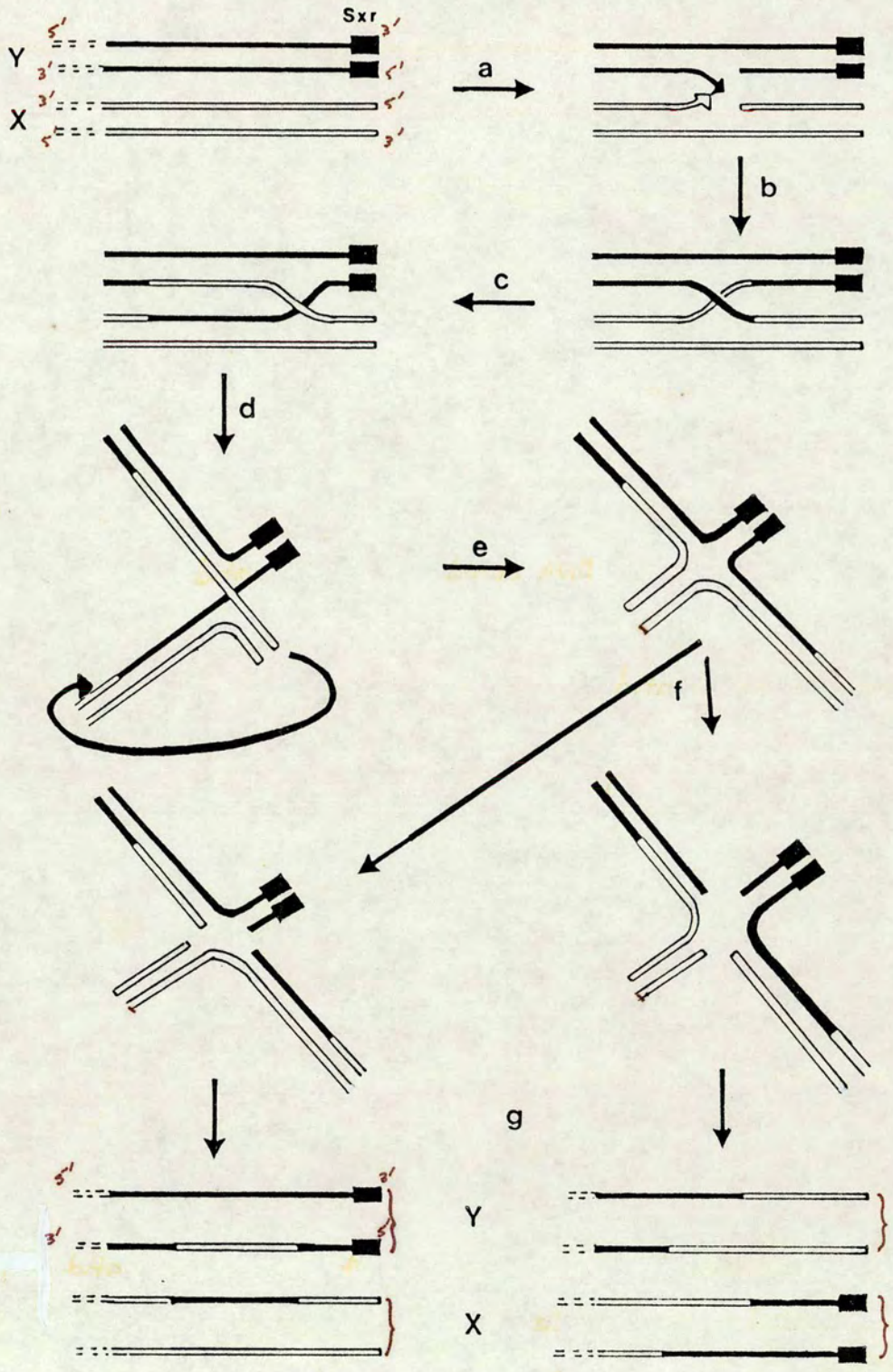
b) The free DNA ends at these nicks become associated with the opposite chromatid. This reciprocal strand exchange is stabilised by covalent bond formation to form the Holliday intermediate.

c) The Holliday intermediate is fluid. Reciprocal strand exchange can continue thus moving the cross over point. This can give rise to regions of heteroduplex DNA.

d-e) If the Holliday intermediate is rotated its symmetrical properties can be easily seen.

f) Cleavage to resolve the structure can occur symmetrically on either an east-west or north-south axis.

g) Repair of these molecules produces 4 types of chromatid. 1 Y chromatid still carries the Sxr mutation whilst the other has lost it. Of the 2 X chromosomes 1 has acquired the Sxr mutation and will thus give rise to XX_{sxr} progeny which are phenotypically male.


Sxr
Y
X
a
b
c
d
e
f
g
Y
X

Further evidence for genetic exchange between the X and Y at meiosis in the mouse has come from genetic analysis of the STS locus (Keitges et al, 1985). This locus, like that in humans, is shown to be X-located, but exhibits a pseudoautosomal pattern of inheritance consistent with the presence of a functional Y-located allele.

It has recently been directly demonstrated that the human X and Y pairing regions hold sequences in common, at least one of which is a functional gene (Cooke et al, 1985; Simmler et al, 1985; Darling et al, 1986). Such sequences as expected show pseudoautosomal inheritance consistent with their exchange between the sex chromosomes at meiosis. These sequences will be discussed in more detail in subsequent chapters.

1.4. The role of the Y chromosome in determining sex, and the predicted nature of genes and other important sequences upon it

An appreciation of the genetic nature of the mechanisms underlying sex determination came initially from observations in insects of a chromosome which showed differential staining and lack of synchrony with other chromosomes, during cell division in spermatogenesis. This led to the suggestion that this chromosome might be concerned with the determination of the sex of the organism (McClung, 1902).

In the presence of the mammalian Y chromosome, irrespective of the number of X chromosomes present, male differentiation normally results (Jacobs and Strong, 1959; Ford et al, 1959; Welshons and Russell, 1959; Russell and Chu, 1961). The Y chromosome is thus the focal point in the search for genes controlling mammalian sex determination.

Historically genetics has generally directed a flow of information from phenotype through to the DNA level. The dearth of Y-linked functional genetic loci has meant however, that analysis of the human Y chromosome has preceded largely in the reverse direction. Many random DNA sequences have now been assigned to the chromosome. Their function, if any, is as yet unknown.

Cytological observations of Y chromosomes, and the phenotype of individuals with Y structural abnormalities enables us to make predictions as to the molecular structure of the chromosome, and the location of important genes upon it. The specialised features of a sex determining chromosome must be considered together with the basic functions which are required of all chromosomes. These include replication and stable segregation into daughter cells at cell division and maintenance of integrity in the cell as a linear DNA molecule.

The human Yp is thought to encode a locus which directs the indifferent embryonic gonads to differentiate into testes, as well as other genes involved in male sex determination. Such a locus may not necessarily be a structural gene, the possibility has been raised (Chandra, 1985) that it involves non-coding DNA which binds regulatory molecules. A Yp localisation for the human testis determining factor has been inferred from the documentation of the

sexual phenotype of males with cytogenetically defined structural anomalies of the Y chromosome; see Davis (1981) for a critical review. Conclusions drawn from this type of analysis are often conflicting and must be treated with a certain amount of caution. Cytogenetically, it is very difficult to distinguish Yp and Yq euchromatin, and small translocations and deletions of Y material can easily go undetected. Many cases of sex chromosome abnormalities are also accompanied by mosaicism, which can confuse the interpretation of results.

Most cytogenetic data however, is consistent with a testis determining factor being located on Yp in the pericentric region, but not involving the centromeric heterochromatin. Cases of 46,X,i(Yq) in which the Y is present as an isochromosome with deletion of the entire short arm and duplication of Yq, show no signs of masculinisation. In contrast cases of dicentric Yq chromosomes with duplication of the Y long arm plus the short arm pericentric region are males with testes, but they are defective in spermatogenesis (Davis, 1981).

Further evidence in support of a Yp localisation for a testis determining gene or genes comes from the analysis of males with an XX chromosome constitution (XX males), and Swyer's syndrome females who have an XY karyotype. Ferguson-Smith (1966) suggested that the paternally derived X of some XX males carries a testis determining fragment transferred from the Y to the X by erroneous crossing over at meiosis in the father. This idea was primarily based on the failure of XX males to inherit their father's Xg allele, suggesting that the tip of the paternal Xp had been replaced, possibly by Y chromosomal material. Magenis et al (1984) have demonstrated

cytogenetically a translocation of Yp11.2-pter (not including any centromeric heterochromatin) onto one X chromosome in one XX male, and a deletion of this region in one XY female. Y chromosomal specific DNA sequences have been found in several XX males; 46 XY gonadal dysgenesis females in contrast show deletions of Y chromosomal material (Rosefeld et al, 1979; Guellan et al, 1984; Page et al, 1984; Müller et al, 1986).

Other genes involved in gonadal differentiation may be present on the Y. Differences in the extent of gonadal development in males with incomplete Y chromosomes had led Buhler (1980) to propose that there are separate initiation and maturation factors required for testicular development. Evidence for the presence of testis determining factors on Yq is ambiguous. Deletions of Yq material have been associated with cases of azoospermia in normal males with testes. The large number of cases reported (Davis, 1981) strongly suggests that genes involved in spermatogenesis may be located on the euchromatic part of Yq. Other loci, not involved in sex determination, have also been postulated to lie on Yq (Buhler, 1980).

A locus or loci which many have predicted to be located on the Y chromosome and to be involved in testis determination, is that coding for the evolutionarily conserved H-Y antigen (Wachtel, 1983). H-Y antigen was first detected as a transplantation antigen in mice, as a result of graft rejection of male cells by syngeneic females (Eichwald and Silmser, 1955). Serological methods of defining H-Y were also developed. It now seems unlikely however, (Silvers et al, 1982) that the H-Y antigen as defined by T-cell mediated transplantation tests and cytotoxic T cell assays (tH-Y) is the same

molecule recognised serologically (sd H-Y).

XY female mice, with ovaries, have been shown to be tH-Y +ve, and conversely many XX males, both mouse and human, are tH-Y-ve. This indicates a Y localisation for the tH-Y gene but suggests that its presence is not required for testis determination. Burgoyne et al (1986) have used the mouse Sxr mutation described in the preceding section, to study the relationship between tH-Y expression and testicular development. Most XX_{Sxr} mice are tH-Y positive, but McLaren et al (1984) isolated a variant of Sxr (Sxr') which still resulted in the production of testes, but was tH-Y-ve. This infers that the tH-Y and testis determining loci are not one and the same. XX_{Sxr} mice do not show any degree of spermatogenesis, presumably due to lethal effects of two X chromosomes in the male germ cells. XO_{Sxr} mice, however do exhibit spermatogenesis, although they are sterile due to the absence of XY pairing at pachytene. $XO_{Sxr'}$ (tH-Y-ve) mice on the other hand are arrested early in spermatogenesis. tH-Y may therefore be the product of a gene involved in spermatogenesis and thus may have a Yq localisation in humans.

In contrast sd H-Y is found in the absence of Y chromosomal material, being present in XO mice (Engel et al, 1981), and in females in which the distal Xp is deleted from one X chromosome (Haseltine and Ohno, 1981). Additionally some normal XY males with testes are sd H-Y-ve (Silvers et al, 1982). Evidence linking sd H-Y antigen with sex determination is therefore weak, and the gene controlling its expression is almost certainly not Y-linked.

As discussed in Section 1.3, it has now been directly demonstrated that the tip of the human Y short arm is homologous to, and crosses over with sequences at Xpter. Genetic evidence first

suggested that genes (encoding the 12E7 antigen) will be found on both the human X and Y chromosomes (Goodfellow et al, 1983). 12E7 is a widely distributed cell surface antigen defined by the monoclonal antibody 12E7. 12E7 antigen expression has been shown to segregate with the human X and Y chromosomes in somatic cell hybrids. The X and Y located loci controlling 12E7 expression, MIC2X and MIC2Y, have been localised to Xp22.32-pter and the euchromatic part of the Y (Ypter-q11), respectively. MIC2X escapes X inactivation (Goodfellow et al, 1984). Biochemical analysis has been used to study the proteins controlled by the MIC2 loci. SDS polyacrylamide gel electrophoresis and isoelectric focusing, coupled to Western blotting with the 12E7 antibody has failed to distinguish between the MIC2X and MIC2Y encoded polypeptides (Banting et al, 1985).

Genetic investigations of the 12E7 antigen have implied the presence of another Y-linked locus termed Yg (Goodfellow and Tippett, 1981). Polymorphism in the level of 12E7 expression is found on red blood cells, but not nucleated cells, and is related to polymorphism at the Xg locus (Goodfellow et al, 1985). Homozygous and heterozygous females positive for Xg antigen (Xg^a/Xg^a or Xg^a/Xg) are always 12E7 antigen high expressors. Homozygotes negative for Xg antigen (Xg/Xg) are low expressors. In contrast, males negative for Xg antigen may express high or low levels of 12E7. This variation is presumed to reflect the existence of a Y-linked locus (Yg) controlling 12E7 expression on red blood cells in a similar manner to Xg. Xg/Yg^a males are thus 12E7 high expressors and Xg/Yg results in low expression of 12E7. Xg antigen positive males (Xg^a/Yg^a or Xg^a/Yg) are always high expressors. High levels of 12E7

expression on red blood cells therefore correlates with the presence of either Xg^a and/or Yg^a alleles. It must be noted here that the Yg locus does not encode the Xg antigen. 1-5% of sons do not match their father's Yg type. This may be as a result of XY crossing over at meiosis.

In humans the terminal two-thirds of Yq is heterochromatic, exhibiting intense staining with quinacrine. This DNA is thought to be transcriptionally inactive and functionless. Normal males show extensive variation in the length of this region (McKay et al, 1978). Several normal females have also been shown to contain these sequences translocated onto their autosomes or X chromosomes (Goodfellow et al, 1985). Models of sex chromosome evolution predict that the XY pairing segment will be the only remaining relic of ancestral homology conserved between the sex chromosomes in mammals. Crossing over between the sex bivalents within this region is no doubt the mechanism by which this homology is maintained. The presumed absence of crossing over between other regions of the X and Y chromosome has resulted in the divergence of sequences located here, which may have been held in common originally prior to the structural and genetic differentiation of these chromosomes. Many loci present on an ancestral Y chromosome will also have been lost, through lack of selection pressure on the largely inactive chromosome.

We would therefore expect sequences, isolated at random from the human Y chromosome, to fall mainly into three categories:-

1) Repetitive sequences, concentrated and tandemly repeated on the Y chromosome.

2) Sequences (single or low copy number) specific to the Y

chromosome.

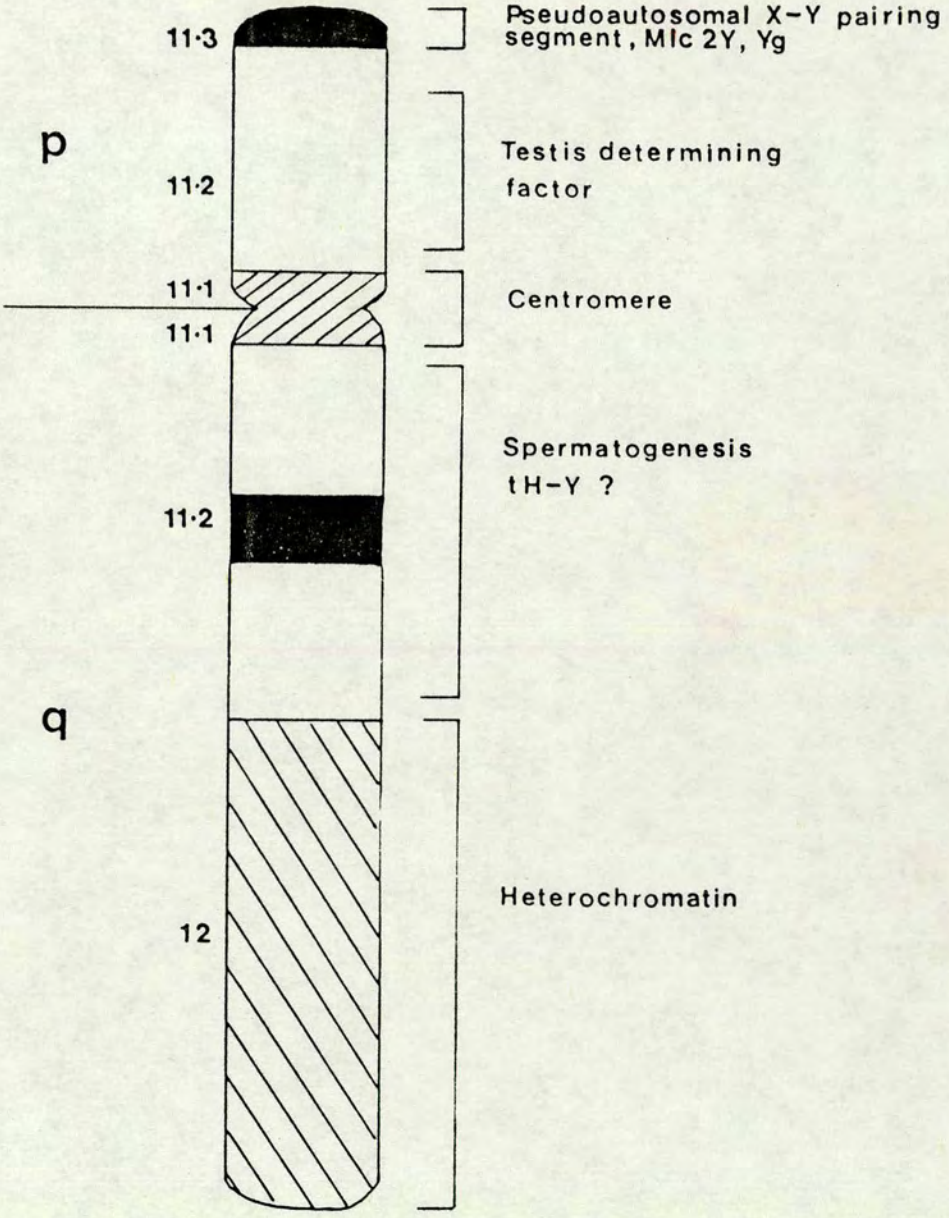
3) Sequences located on both the X and Y chromosomes, and consequently presumed to originate from the meiotic pairing regions.

Figure 1.3. illustrates features of the speculative model of Y chromosome structure described above. This will be elaborated on in the light of current molecular studies of the Y chromosome, in relevant chapters of this thesis. The work described here primarily involves the investigation of XY homologous sequences in humans, shown to reside outside of the meiotic pairing region; and their evolutionary origins.

FIGURE 1.3

Speculative model of the structure of the human Y chromosome and the location of important genes upon it

(See Section 1.4).


11·3
p
11·2
11·1
11·1
11·2
q
12
Pseudoautosomal X–Y pairing segment, Mic 2Y, Yg
Testis determining factor
Centromere
Spermatogenesis
tH–Y ?
Heterochromatin

CHAPTER 2

MATERIALS AND METHODS

2.1. Mammalian cell culture

2.1.1. Maintenance of cells and cell lines in culture

Cells were grown in Eagle's modified Dulbecco's or RPMI 1640 medium (Flow Laboratories), supplemented with 10% foetal calf serum (Gibco Bio-cult) which had been heat inactivated at 56°C for 30 mins. Penicillin and streptomycin (Gibco Bio-cult) were added to cultures at 100 units/ml and 100µg/ml respectively. All cultures were grown at 37°C in 25, 80 or 175cm^2 plastic flasks (Nunc), in a 10% CO_2 atmosphere.

Monolayer cultures were split when confluent by trypsinising. Cells were washed twice with ~3mls trypsin (trypsin 0.5g glucose 1g, EDTA 0.1g, 50ml saline D concentrate/litre). Saline D concentrate consists of 0.24g Phenol Red, 160g NaCl, 8.0g KCl, 0.9g Na_2HPO_4, 0.6g KH_2PO_4/litre. The cells were then detached by a final incubation with trypsin.

0.5ml batches of cells, containing ~1 x 10^6 cells, were stored in freezing medium (5% DMSO, 95% foetal calf serum) in liquid nitrogen.

2.1.2. Karyotyping of cells

Metaphase spreads of cell lines were prepared in order to confirm their karyotypes. Colchicine was added to cells in culture at 10^{-6}M for 1 hour. Cells were pelleted at 800 rpm for 5 mins, resuspended in 10ml 0.075M KCl and left for 15 mins before repelleting. The cells were then resuspended in 5ml of fix (3 vols methanol:1 vol. acetic acid) and repelleted. This latter procedure was repeated three times. The cells were finally resuspended in a small volume of fix and dropped onto microscope slides.

The presence of the human Y chromosome was ascertained by

staining spreads with 0.5% w/v quinacrine for 10 mins. After washing, the spreads were mounted under coverslips, sealed and visualised under UV light (Neofluar 40 lens, Zeiss Universal microscope). The heterochromatin on the distal long arm of the human Y chromosome fluoresces brightly with quinacrine (Sumner et al, 1971).

The presence of human chromosomes in human x mouse hybrids was detected by differential G-11 staining. Metaphase spreads were placed in 2 x SSC for 5 mins, then transferred to a staining pot containing 0.5ml Giemsa (Fischer) and 15ml of 14mM NaOH for 15 mins. Human chromosomes stain much lighter than mouse chromosomes using this procedure.

2.1.3. Origins of mammalian cells and cell lines

OX: A human male lymphoid line containing 4 Y chromosomes/diploid cell (Sirota et al, 1981). Grown as a suspension culture in RPMI 1640 medium (gift from P. Goodfellow, London).

TilI and REN2: Human lymphoid cells (XXXXY). Grown as suspension cultures in RPMI 1640. (Gift from M. Steel, Edinburgh).

ThyB-1-33-C112: Mouse x human hybrid containing the human X as the only cytologically detectable human material (Lund et al, 1983). Grown as a suspension culture in RPMI 1640 supplemented with HAT (hypoxanthine, aminopterin, thymidine) to select for cells carrying the human X-linked HPRT gene (the mouse parent was $HPRT^-$).

3E7: Hybrid cell line between a human male lymphocyte and the

mouse cell line RAG. The Y chromosome is the only cytologically detectable human material (Marcus et al, 1976). This line was grown as a monolayer in Eagle's modified Dulbecco's medium.

853: Chinese hamster x human hybrid containing the Y chromosome as the only cytologically detectable human material (Burk et al, 1985). Grown as a monolayer in Eagle's modified Dulbecco's medium.

Buttons: ♂ Chimpanzee (Pan troglodytes)

Sampson: ♂ Gorilla (Gorilla gorilla)

Delilah: ♀ Gorilla (Gorilla gorilla)

Primary fibroblasts (a gift from J. Delhanty, London) grown in Eagle's modified Dulbecco's medium supplemented with 10mM HEPES pH7.0.

♂ Gorilla: (Gorilla gorilla):

Primary fibroblasts grown in Eagle's modified Dulbecco's medium. A gift from K. Smith (Baltimore, USA).

2.2. Preparation of DNA from mammalian cells

The number of cells used for preparation of DNA was estimated using a Coulter cell counter or Neubauer haemocytometer.

2.2.1. Preparation of DNA from cells grown in culture, for cloning and conventional gel electrophoresis

Cells were pelleted at 800rpm for 5 mins, washed twice with phosphate buffered saline solution (PBS) containing 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4 (pH7.3)/litre, then resuspended in

PBS at ~10^8 cells/ml. 1ml of cells were added dropwise to 20ml urea lysis buffer (7M urea, 2% SDS in TE) whilst continuously swirling the lysate. 20ml of phenol (buffered with 0.1M Tris HCl pH8.0, and containing 0.1% 8-hydroxyquinoline, 0.2% β-me, pH7.4) were added, followed by chloroform/octan-2-ol (24:1). After gentle mixing, the lysate was spun at 2K, 5 mins to separate the organic and aqueous phases. The latter, including any flocullence at the interface, was removed. This and all subsequent manipulations of the DNA were performed using wide bore pipettes to minimise shearing of the DNA. The DNA was extracted twice with 1:1 phenol/chloroform then with chloroform alone, and precipitated at 0°C by the addition of sodium acetate to 0.3M, and two vols of 96% ethanol. DNA was pelleted at 2.5K, 20 mins, at 4°C, washed in 70% ethanol and resuspended in TE. DNase free RNase was added to the DNA at 50μg/ml; after 1 hour at 37°C, proteinase K (Boehringer Mannheim GmbH) was added at 50μg/ml and incubation continued for 1 hour. The DNA was then re-extracted twice with phenol/chloroform, and dialysed overnight versus TE at 4°C. The yield of DNA was estimated by measurements of the optical density (OD) at 260nm (DNA at a concentration of 50μg/ml has an $OD_{260nm} = 1$).

2.2.2. Preparation of DNA from white blood cells

Blood (~10ml) was collected into EDTA (1mg/ml) to prevent clotting. 3 vols 155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA pH7.4 were added and the blood left at 4°C for 15 mins with gentle mixing to lyse the red blood cells. The white blood cells were pelleted at 1K, 10 mins then resuspended in 10ml of SE (75mM NaCl, 25mM EDTA pH8.0) and 100μg/ml proteinase K added. SDS was added to 1% and the

lysate incubated at 37°C for 2 hours. After three phenol/chloroform extractions the DNA was precipitated with isopropanol, washed in 70% ethanol and resuspended in TE (G. van Ommen, per. comm.).

2.2.3. Preparation of high molecular weight DNA in agarose plugs for pulsed field gel electrophoresis

The methods of DNA preparation described in 2.2.1. and 2.2.2 can yield DNA of up to 600kb in length. In order to obtain molecules larger than this, the DNA must be protected from shearing forces during its preparation. This can be achieved by embedding cells prior to lysis in a block of agarose (Schwartz and Cantor, 1984).

Cells grown in culture or white blood cells prepared from freshly collected blood, as described in the previous section, were washed well in PBS and resuspended to a concentration of 2.5×10^7 cells/ml. The cells were warmed to 40°C whilst 1% low gelling temperature agarose (Sea Plaque) in PBS was melted and cooled to 50°C. Cells were added to an equal volume of molten agarose, mixed and aliquoted into perspex moulds (1 x 1 x 0.2cm). When set, the blocks of agarose were transferred to NDS (0.5M EDTA, 1% lauryl sarcosine, 10mM Tris-HCl, pH9.5) to lyse the cells. Proteinase K was added (0.5mg/ml) and the blocks incubated at 50°C for 24 hours. The NDS and proteinase K were then replaced and incubation continued for a further 48 hours. After rinsing with several changes of NDS, the blocks were stored at 4°C in NDS (R. Anand, pers. comm.; Schwartz and Cantor, 1984).

2.3. Bacterial Culture

2.3.1. Media used for the culture of E.coli

L-Broth: 10g tryptone (Difco)
5g yeast extract (Difco)
10g NaCl
2.46g $MgSO_4$ /litre pH7.2

L-agar contains in addition 15g agar/litre and 6.5g/l for top agar.

C-Y agar: 10g casamino acids (Difco)
5g yeast extract
3g NaCl
10g agar /litre pH7.2

top CY agar contains 6.5g/l agarose in place of the agar

T-Broth 10g tryptone
8g NaCl /litre pH7.0

SOB: 20g yeast extract
0.59g NaCl
0.19g KCl /litre pH7.0

After autoclaving sterile $MgCl_2$ and $MgSO_4$ were added to a final concentration of 10mM each.

H agar: 10g tryptone
8g NaCl
12g agar /litre pH7.0

This agar reduces the number of satellite colonies when plating out bacterial transformations. H-top agar contains only 8g agar/litre.

2 x TY broth:	16g	Na_2HPO_4	
	10g	yeast extract	
	5g	NaCl	/litre pH7.0
M9 salts	6g	Na_2HPO_4	
	3g	KH_2PO_4	
	1g	NH_4Cl	/litre
	0.5g	NaCl	
glucose	1 litre M9 salts		
minimal agar:	15g	agar	
	1ml	1M $MgSo_4$	
	1ml	0.1M $CaCl_2$	
	1ml	1M thiamine HCl	
	10ml	20% glucose	

Reagents were sterilised separately then mixed when cool.

Where appropriate, antibiotics were added to media and agar. Most bacteria were grown at 37°C, with good aeration for liquid cultures. Stocks of bacteria were stored at -70°C in 20% glycerol. Cultures which were to be infected with bacteriophage lambda were grown in the presence of 0.4% maltose. This induces the synthesis of the maltose transport protein, required for absorption of λ phage onto the surface of E.coli cells. These cells were stored at two fold concentration in 10mM $MgSO_4$ at 4°C for up to 1 week.

2.3.2. E.coli strains used

BHB 2688: N205 recA$^-$, containing a λ lysogen (imm^{434}, cIts, b2, red$^-$, Eam, Sam)

BHB 2690 N205 recA$^-$, containing a λ lysogen (imm^{434}, cIts, b2, red$^-$, Dam, Sam).

These strains were used for the preparation of *in vitro* packaging extracts (Hohn, 1979). Both contain λ lysogens under the control of a temperature sensitive cI repressor. Upon induction packaging proteins are produced but no intact virions are released since excision of the lysogen is prevented by the b2 deletion.

LE392: hsdR-, supE, supF (Kaiser and Murray, 1985).

This strain is used for the propogation of λ vectors. As with other strains used for cloning DNA it is an *E.coli* K12 derivative, but is deficient in restriction ($hsdR^-$) to protect the introduced DNA. SupE and F suppress most λ amber mutations.

Q358: $hsdR^-$, supE

Q359: $hsdR^-$, supE, P2

This strain carries a P2 lysogen, so can be used to detect spi^- recombinants of λ phage (Karn *et al*, 1983).

ED8767: supE, supF, met^-, $recA^-$

$RecA^-$ host used for plating and growth of cosmids (Murray *et al*, 1977).

JM83: ara, Δlac-pro, strA, thi, Ø80d lacZΔM15 (Vieira and Messing, 1982). A K-12 host for pUC plasmids, carrying the lacZΔM15 on a Ø80 integrated into the chromosome.

JM101: Δlac pro, thi, supE, F'traD36, pro AB, lac I^qZ M15. Strain used for the propogation of M13. Since JM101 contains the $lacI^q$ mutation which overproduces the lac repressor, isoproyl-1-thio-β-D-galactoside (IPTG) must be added to induce β-galactosidase synthesis (Müller-Hill *et al*, 1968).

2.4. Vectors used for cloning in bacterial cells

All vectors used in this thesis are summarised in Fig. 2.1.

2.4.1. Vectors based upon bacteriophage lambda

λgt WES.λB: Wam 403, Eam 1100, Sam 100, cI857 ts. A replacement vector for cloning 2-15kb EcoRI fragments. Requires a SupE, SupF host such as LE392 for growth (Leder et al, 1977).

EMBL4: A replacement vector for cloning 9-22kb fragments into BamHI, EcoRI or SalI sites of a polylinker (Kaiser and Murray, 1985). The central replaceable fragment contains a functional gam gene which renders the phage unable to grow on a P2 lysogen (sensitive to P2 interference, Spi^+) such as Q359. Thus only recombinant phage in which the gam gene has been replaced (Spi^-) can grow on this host. Such red^- gam^- phage are dependent on host recombination systems for plaque formation. This is enhanced by Chi sequences present in the vector arms of EMBL4.

Charon 36: Replacement vector for cloning 7.9-21kb fragments into a polylinker (F. Blattner, pers. comm.).

2.4.2. Plasmid vectors pUC 8/9 and 18/19

The pUC series of plasmid vectors are based upon pBR322 (Bolivar et al, 1977) retaining both a high copy number, and the ampicillin resistance gene (β-lactamase) (Vieira and Messing, 1982; Norrander et al, 1983). All of the pUC vectors contain a polylinker (see Fig.2.1) inserted into the β-galactosidase gene (lacZ). Non-

FIGURE 2.1

Vectors for cloning into E.coli

a) Replacement vectors based upon bacteriophage λ.

The maps of the vectors λgt WES, Charon 36 and EMBL4 are shown relative to the genome of wild type λ. Filled-in sections represent replaceable fragments and gaps are deletions created in the construction of these vectors. Polylinker restriction sites are shown above each map. B = BamHI, E = EcoRI, H = HindIII, K = KpnI, S = SalI, Ss = SstII, X = XbaI.

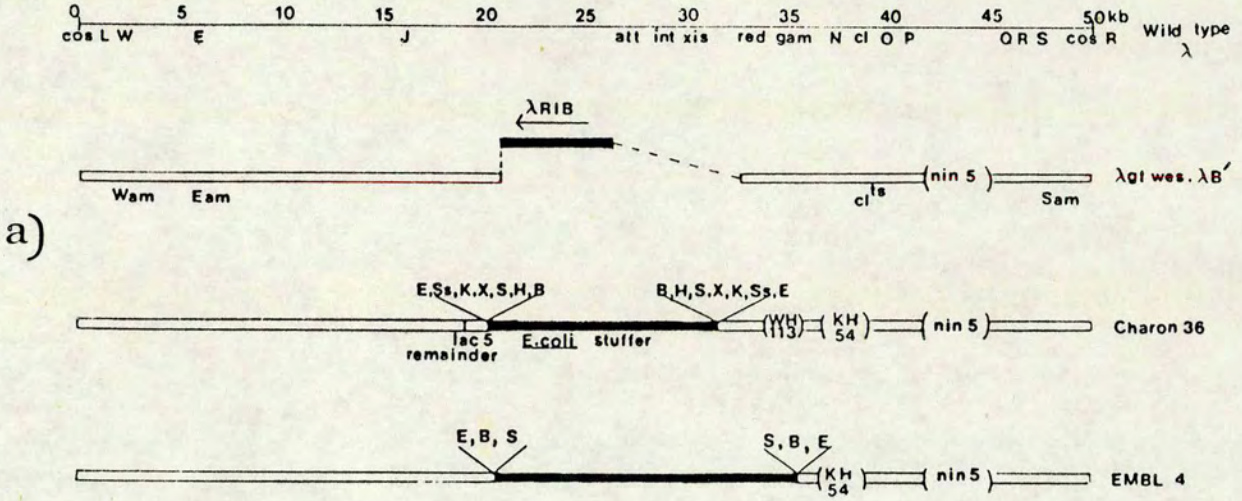
b) The pUC series of plasmid vectors and their polylinkers

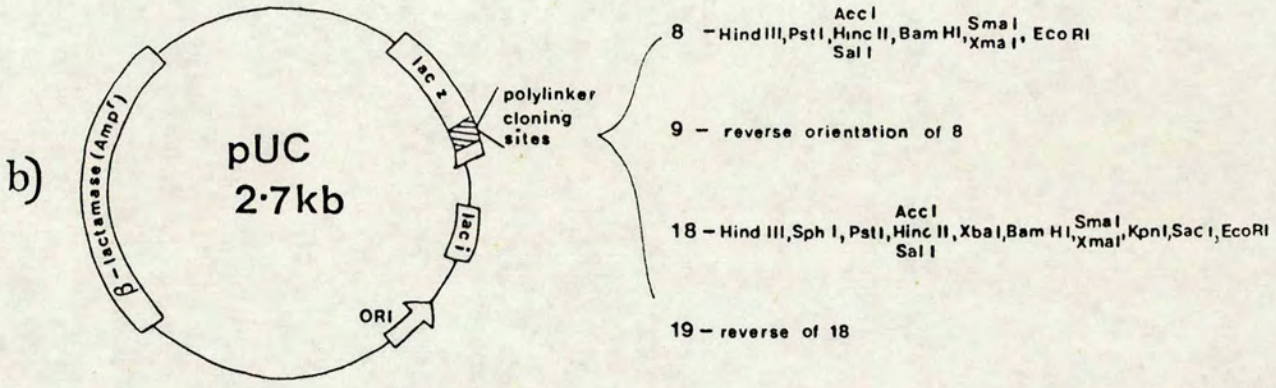
ORI = origin of replication.

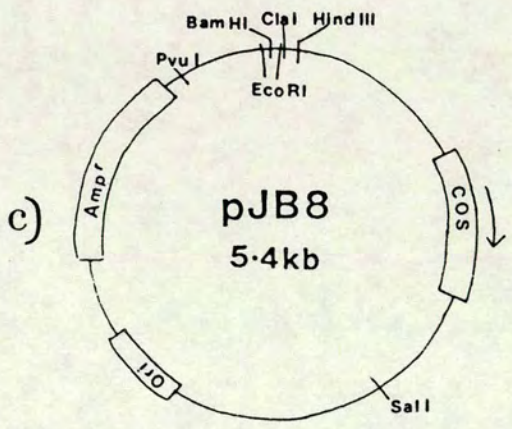
c) pJB8 cosmid vector.

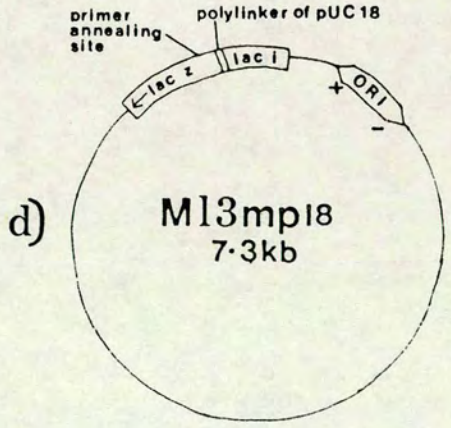
d) M13 mp18 sequencing vector. + and - indicate the direction of synthesis of + and - strands of the phage.

See Section 2.4 for further details.


0 5 10 15 20 25 30 35 40 45 50kb
cos L W E J att int xis red gam N cI O P Q R S cos R
Wild type λ
λRIB
Wam Eam
cI ts
nin 5
λgt wes. λB′
Sam
a)
E,Ss,K,X,S,H,B
B,H,S,X,K,Ss,E
lac 5 remainder
E.coli stuffer
WH 113
KH 54
nin 5
Charon 36
E, B, S
S, B, E
KH 54
nin 5
EMBL 4


b)
pUC
2·7kb
lac z
polylinker cloning sites
lac i
ORI
β-lactamase (Ampr)
8 – Hind III, Pst I, Acc I, Hinc II, Sal I, Bam HI, Sma I, Xma I, Eco RI
9 – reverse orientation of 8
18 – Hind III, Sph I, Pst I, Acc I, Hinc II, Sal I, Xba I, Bam HI, Sma I, Xma I, Kpn I, Sac I, EcoRI
19 – reverse of 18


c)
pJB8
5·4kb
Bam HI
Cla I
Hind III
Pvu I
Eco RI
Ampr
COS
ori
Sal I


d)
primer annealing site
polylinker of pUC 18
lac z
lac i
ORI
+
–
M13mp18
7·3kb

recombinant plasmids are able to synthesise this enzyme, which breaks down 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal), to release a blue indolyl derivative. In recombinant plasmids the lacZ gene is interrupted by foreign DNA, thus the colonies remain colourless.

2.4.3. pJB8

Packaging of λ concatmers is dependent on the cos site of the λ genome (Hohn, 1979). The cosmid vector pJB8 is based upon pBR322 and contains the λ cos site (Ish-Horowicz and Burke, 1981). This vector enables DNA of 32-47kb to be cloned, recombinant molecules being packaged in vitro into λ particles. This allows for very efficient transfection of host cells, which are then selectable by their resistance to ampicillin.

2.4.4. Vectors based on the single-stranded DNA coliphage M13

M13, a single stranded (ss) DNA filamentous phage of E.coli infects cells via the F-pilus. Replications occurs via a double-stranded (ds) replicative form (RF) giving rise to ss progeny virions. M13 has been developed as a cloning vector for DNA sequencing (Messing, 1983; Norrander et al, 1983). Foreign DNA is cloned into the polylinker of the M13 RF (Fig.2.1). Annealing of an oligonucleotide (17mer) next to the polylinker of the ss form allows inserts to be copied by Klenow fragment of E.coli DNA polymerase I. As for the pUC plasmid vectors, recombinant molecules can be identified by the disruption of the lac Z gene using the X Gal assay.

2.5. Manipulation of bacteriophage λ DNA

2.5.1. In vitro packaging of λ DNA

E.coli strains BHB2688 and 2690 (Section 2.3.2) harbour λ lysogens under the control of a temperature sensitive cI repressor. Upon induction they accumulate viral proteins. BHB2688 synthesises components of the viral capsid but is unable to assemble them due to an amber mutation in the E gene. BHB2690 is defective in the D protein required for mature head formation and packaging of λ DNA. Extracts of these two strains complement each other functionally, allowing λ DNA to be packaged into infectious virions (Hohn, 1979).

Prior to the preparation of in vitro packaging extracts, the strains were tested for the presence of a temperature-sensitive cI repressor by their reduced growth at 42°C as compared to 30°C.

Freeze thaw lysate (FTL): 3 x 500ml of L-Broth were inoculated with BHB2688 and grown at 30°C until OD_{580nm} = 0.3. Cultures were then induced by maintaining them at 45°C for 15 mins, followed by vigorous aeration at 37°C for 2 hours. Successful induction was assayed by the ability of chloroform to lyse a sample of the culture. The cells were harvested at 9K, 4°C for 10 mins then resuspended in 3ml cold 10% sucrose, 50mM Tris-HCl pH7.5. The cell suspension was dispensed into 2 x 10ml Oakridge centrifuge tubes and 75µl of fresh lysozyme solution (2mg/ml in 0.25M Tris-HCl pH7.5) gently mixed into each. The tubes were quick frozen in liquid nitrogen and stored at -70°C overnight. After thawing 75µl of buffer M1 (10mM Tris-HCl pH7.5, 30mM spermidine and 60mM putrescine neutralised with Tris base, 18mM $MgCl_2$, 15mM ATP neutralised with NaOH, 0.2% β-me) were added to each tube. The lysate was spun at 35K, 4°C for 35 mins in a fixed angle rotor. 20µl aliquots of the

supernatant were dispensed into pre-cooled plastic straws, quick-frozen and stored in liquid nitrogen.

Sonic Extract (SE): A 500ml culture of BHB 2690 was grown and induced as for BHB2688. After harvesting the cells were resuspended in 3.6ml buffer A (20mM Tris-HCl pH8.0, 3mM $MgCl_2$, 1mM EDTA, 0.5% β-me) and sonicated in short blasts on ice, without foaming, until no longer viscous. Cell debris was pelleted at 6K, 4°C for 6 minutes and 30µl aliquots of the supernatant were dispensed, quick-frozen and stored as for FTL.

Packaging Reaction: FTL and SE aliquots were thawed immediately prior to use. 0.5µg DNA in a volume of 2µl were added to 7µl buffer A and 2µl buffer M1. 6µl SE and 10µl FTL were added and the reactions incubated at 28°C for 1 hour. After packaging the phage were diluted with storage medium (SM) containing 5.8g NaCl, 2g$MgSO_4$, 50ml 1M Tris-HCl pH7.5 and 5ml 2% gelatin/litre, and stored at 4°C with a few drops of chloroform added to inhibit bacterial growth. Phage particles were added to plating cells (Section 2.3.1) and incubated at 37°C for 20 mins to allow phage to adsorb to the cells. The cells were then plated out in top agar. Efficiencies of up to 2 x 10^8 pfu/µg input DNA were obtained using this method.

2.5.2. Small scale isolation of λ DNA

Phage were adsorbed to plating cells at ratios ranging from 1:200 to 1:1 (H. Cooke, pers. comm.), then used to inoculate 5ml L-Broth and cultures grown overnight. Successful lysis was indicated by the presence of bacterial debris. Cells and cell debris were pelleted at 2.5K, 4°C for 10 mins and RNase and DNase I added to the supernatant, each to a concentration of 1µg/µl. After incubation at

37°C for 30 mins, an equal vol of 20% PEG 6000, 2M NaCl in SM was added and the phage left to precipitate out at 4°C for 1 hour. The phage particles were pelleted at 10K, 4°C for 20 mins, resuspended in 0.5ml SM, and burst open by incubation at 68°C for 15 mins with 5µl 10% SDS, 5µl 0.5M EDTA pH8.0. The λ DNA was then purified by a series of phenol, phenol/chloroform and chloroform extractions before ethanol precipitation.

2.5.3. Large scale isolation of λ DNA (Yamamoto et al, 1970)

250µl bacteria were infected with phage as described in the preceding section, and used to inoculate 500ml L-Broth. After overnight culture 10ml of chloroform were added and the cell debris removed by centrifugation in stainless steel bottles at 5K, 4°C, for 15 mins. RNase and DNase I, at 1µg/ml each, were incubated with the supernatant for 30 mins at room temperature. Phage were then precipitated by the addition of 70g/l PEG 6000 and NaCl to 0.5M, followed by incubation at 4°C for 1 hour. Phage were pelleted at 5K, 4°C for 15 mins and resuspended in 5ml SM. After chloroform extraction CsCl was added to the phage suspension at 0.75g/ml and the phage banded in a fixed angle rotor at 35K, 15°C overnight. The opaque phage band was collected with a syringe and the CsCl removed by dialysis against TE for 1 hour at room temperature. Phage DNA was purified by phenol and chloroform extraction followed by ethanol precipitation.

2.5.4. Concentration of recombinant phage libraries

The phage particles constituting a genomic library of clones contain inserts of differing lengths, thus have different densities.

Such a collection of phage will therefore not band tightly on a continuous CsCl gradient as described in the preceding section, but can be concentrated by sedimentation through a preformed CsCl step gradient (Maniatis et al, 1982).

After removal of cellular debris by pelleting at 5K, 4°C for 5 mins, CsCl was added to the phage suspensions at a concentration of 0.5g/ml. The volume was made up to 8ml with SM containing 0.5g/ml CsCl. A step gradient of 3 x 1.6ml steps (1.7, 1.5 and 1.45g/ml CsCl in SM) was formed in a 14ml polycarbonate ultracentrifuge tube, and the phage loaded on top. The gradient was spun at 32K, 4°C for 2 hours in a swing-out rotor. Phage particles collect at the interface between the 1.5 and 1.45g/ml steps. The gradient was fractionated and the phage located by spotting aliquots of each fraction onto a lawn of appropriate bacteria.

2.6. Manipulations of plasmid DNA

2.6.1. Preparation of E.coli competent for transformation by plasmid DNA

The conditions under which E.coli cells will take up DNA have been well studied (Hanahan, 1985). JM83 cells were made highly competent to take up plasmid DNA and transformed by the method of Hanahan (1983), with special care being taken to ensure that all glassware used was free of detergent.

100ml SOB were inoculated with a single colony of JM83 and grown at 37°C in a 175cm^2 plastic tissue culture flask until OD_{560nm} = 0.48. The cells were cooled on ice for 10 minutes and all subsequent steps performed at 4°C. After pelleting at 2.5K, 4°C for

10 mins the cells were gently resuspended in 33ml of cold filter sterilised FSB (10mM $CaCl_2$, 10mM CH_3COOK, 100mM RbCl, 45nM $MnCl_2$, 3mM hexamine cobalt chloride 10% glycerol, pH6.4). After 10 mins the cells were repelleted, resuspended in 8ml FSB and 280µl DMSO (spectroscopic grade) added. 10 mins later another aliquot of DMSO was added and 200µl aliquots of the cells quick frozen and stored in liquid nitrogen. Efficiencies of up to 4×10^7 colonies/µg plasmid DNA were obtained using this method.

2.6.2. Transformation of competent JM83

An aliquot of competent cells was thawed at room temperature then left on ice for 10 mins before adding plasmid DNA (<10µl). After 30 mins on ice the cells were heat-shocked at 42°C for 90 seconds then returned to ice for a few mins. 800µl SOC (SOB + 20mM glucose) were added and the cells incubated at 37°C for 1 hour to allow for expression of β-lactamase. Prior to plating, cells were resuspended in SOB to remove DMSO, then spread on H agar containing 50µg/ml ampicillin. 30µl XGal (2% in dimethylformamide) were added to top agar if discrimination between recombinant and non-recombinant plasmid-containing colonies was required (Section 2.4.2).

2.6.3. Small scale isolation of plasmid DNA (Wilimzig, 1985)

A plasmid-containing colony was grown overnight in 5ml L-Broth containing 25µg/ml ampicillin. After harvesting the cells were resuspended in 200µl TELT buffer (50mM Tris-HCl pH7.5, 62.5mM EDTA, 0.4% Triton X100, 2.5M LiCl). 20µl fresh 10mg/ml lysozyme (Sigma) were added and the cells placed in a boiling water bath for 1 min.

rRNA and protein were then allowed to precipitate out, due to the presence of LiCl, on ice for 5 mins and removed by centrifugation for 8 mins. The supernatant was extracted with phenol/chloroform and the plasmid DNA collected by ethanol precipitation.

2.6.4. Large scale isolation of plasmid DNA

A method based on that of Birnboim and Doly (1979) was used to prepare large amounts of plasmid DNA.

500ml L-Broth containing 25µg/ml ampicillin were inoculated with a single colony, and the culture grown overnight. After harvesting, the cells were resuspended in 18ml solution I (50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA) and 2ml solution I containing 5mg/ml lysozyme. After 10 minutes 40ml fresh 0.2M NaOH, 1% SDS were added and the lysate left on ice for 5 mins, after which time 20ml cold 3M CH_3COOK, 2M CH_3COOH were added and incubation continued for 15 mins. Cell debris was pelleted at 8K for 5 mins and the supernatant strained through muslin. DNA was precipitated by the addition of 0.6 vols of propan-2-ol and collected by pelleting at 8K for 5 mins. The pellet was washed with 70% ethanol, and resuspended to 13ml with TE. 1.58g CsCl and 1.5ml of 10mg/ml ethidium bromide were added such that the refractive index of the resulting solution was 1.393. The plasmid DNA was banded by centrifugation in an 18ml polyallomer tube at 40K, 20°C overnight in a vertical rotor (which allows the CsCl gradient to reach equilibrium rapidly). Plasmid DNA is more dense than the bacterial chromosomal DNA, due to plasmid supercoiling constraining the amount of EtBr which can be intercalated into the molecule. The plasmid band was collected under UV light (300nm) with a syringe and the

EtBre removed by a series of extractions with butan-2-ol until no colour remained in the aqueous phase. The DNA was then precipitated with 3 vols of 70% ethanol.

2.7. Manipulation of cosmid DNA

pJB8 vector DNA was prepared as described in the preceding section..

Cosmids were packaged *in vitro* using λ packaging extracts, and adsorbed onto ED8767 plating cells grown in the presence of maltose. Cells were then incubated in L-Broth at 37°C for 1 hour prior to plating on H-agar containing 50µg/ml ampicillin.

Recombinant cosmids were prepared for analysis on a small scale using the TELT method (Section 2.6.3).

2.8. Manipulation of M13 DNA

2.8.1. Preparation of JM101 cells competent for transformation by M13 DNA

JM101 cells were streaked onto glucose minimal medium plates. This selects for cells carrying a plasmid encoding a gene involved in proline synthesis (the host cell has a deletion in this gene). The same plasmid is needed for synthesis of the F-pilus required for infection by M13.

1ml of an overnight culture of JM101 was used to inoculate 100ml 2 x TY and the cells grown to OD_{550nm} = 0.4. After harvesting the cells were resuspended in 50ml cold 50mM $CaCl_2$ and left on ice for 20 mins. The cells were then repelleted and resuspended in 10ml

of the $CaCl_2$ solution, and stored at 4°C for up to 4 days.

2.8.2. Transformation of JM101 by M13 DNA

1ng ds or 10ng ss M13 DNA were added to 300µl of competent cells. After 40 mins on ice the cells were heat-shocked at 42°C for 3 mins then returned to ice. 200µl of a fresh exponential culture of JM101 were added and the mixture plated in top H-agar. If identification of recombinant phage-containing plaques was required, 40µl 2% XGal and 40µl fresh 100mM IPTG were incorporated into the top agar.

2.8.3. Preparation of single-stranded M13 DNA

100ml 2 x TY were inoculated with 1ml of an overnight culture of JM101. An isolated M13 plaque was added to 1.5ml of these cells and grown at 37°C for 5 hours. The cells were harvested and retained for preparation of the ds form of M13. The supernatant was respun to ensure no bacterial cells were remaining, then the phage particles precipitated by the addition of 200µl of 20% PEG 6000 in 2.5M NaCl. After 15 mins on ice the phage were collected by centrifugation and resuspended in 100µl TE. After phenol/ chloroform and chloroform extractions the ss M13 DNA was ethanol precipitated.

2.8.4. Preparation of double-stranded M13 DNA

The cells harvested in the previous section were used to prepare ds M13 DNA by the TELT method (section 2.6.3).

2.9. Manipulation of DNA by enzymes

2.9.1. Restriction endonuclease digestion

Restriction endonucleases were purchased from: Anglian Biotechnology Ltd., Amersham International plc, Bethesda Research Laboratories, Boehringer Mannheim GmbH and New England Biolabs. Digests were carried out according to the manufacturers' specifications or in standard high, medium and low salt buffers (Maniatis et al, 1982). Gelatin was added to all digests at 0.1mg/ml and spermidine at 5mM to digests in high salt buffers. Reactions were stopped by heat inactivation, freezing at -20°C or by the addition of EDTA to 10mM.

2.9.2. Dephosphorylation of DNA

To prevent unwanted ligation of certain DNA fragments, 5'-phosphate groups were removed by calf alkaline intestinal phosphatase (CAIP) (Chaconas and van de Sande, 1980). 5 units of CAIP (Boehringer Mannheim GmbH) were incubated with digested DNA at 37°C for 1 hour. The reaction was stopped by heat inactivation at 68°C for 10 mins in the presence of 10mM EDTA, 0.1% SDS, and the CAIP removed by phenol, phenol/chloroform and chloroform extractions.

2.9.3. Ligation of DNA molecules

The enzyme ligase, isolated from E.coli infected with bacteriophage T4, catalyses the formation of a phosphodiester bond between 3'-OH and 5' phosphate groups of DNA (Weiss et al, 1968). This enzyme can therefore be used to join complementary cohesive termini of ds DNA molecules. Ligations were carried out overnight

at 15-20°C with 0.5 units T4 ligase in 50mM Tris-HCl pH7.4, 10mM $MgCl_2$, 1mM spermidine, 100µg/ml BSA, 1mM ATP, 10mM DTT.

2.10. Separation of DNA molecules by centrifugation through a sucrose density gradient (Maniatis et al, 1982)

A 10-40% sucrose gradient was prepared in a 36ml polyallomer tube, using 10% and 40% sucrose solutions made up in 1M NaCl, 20mM Tris-HCl pH8.0, 5mM EDTA. Prior to loading, DNA (~200µg) was heated to 68°C for 10 mins. The gradient was spun in a vertical rotor at 43K, 15°C for 2.75 hours, then fractionated into 500µl aliquots for analysis by gel electrophoresis. Fractions containing DNA of the desired size were pooled and dialysed against TE at 4°C overnight. The DNA was extracted with butan-2-ol, until the volume of DNA was <5ml, then precipitated with ethanol.

2.11. Separation of DNA molecules by electrophoresis

General techniques of electrophoresis were as described by Sealey and Southern (1982).

2.11.1. Agarose gels for the analysis of DNA of 0.5 to 50kb in length

Vertical agarose gel electrophoresis was used to analyse DNA fragments of between 0.5 and 50kb. Gels were 3mm thick, 9 or 18cm wide and 12 or 18cm long. Agarose (Sigma, Type II) concentrations of 0.4 to 2% were used, depending on the size range to be resolved, and gels were made up and run in E buffer (26mM Na_2HPO_4, 3.3mM

NaH_2PO_4, 1mM EDTA pH7.5). DNA samples were loaded after adding a 1/10th vol of loading buffer (15% Ficoll, 0.25% Orange G, 0.25M EDTA in 10 x E buffer). The Orange G serves as a visible marker for the migration front of small (200-500bp) DNA fragments. Gels were run at voltage gradients of up to 10V/cm, then stained for 20 mins with a few drops of ethidium bromide (10mg/ml). Gels were photographed by overhead illumination with short wave (254nm) UV lamps (Mineralight). Kodak Technical Pan film was used together with a Polaroid MP4 land camera fitted with a red filter (Hoya 25A), with an exposure time of 2 minutes. Films were developed for up to 10 mins in Ilford Microphen developer, stopped with 5% acetic acid and fixed in Amfix (May and Baker).

Agarose gels containing radiolabelled DNA were fixed in 7% TCA for 30 mins, then dried down overnight under paper towels prior to autoradiography.

2.11.2. Preparative agarose gel electrophoresis

For the isolation of specific DNA fragments, horizontal agarose gels were run, TAE buffer (1.04M Tris-acetate, 0.002M EDTA) being used in place of E buffer. To visualise the DNA after staining a long wave (300nm) UV transilluminator was used, to minimise UV induced damage to the DNA. A cut was made in the gel adjacent and at 90° to the DNA fragment of interest, and a small piece of DEAE membrane (Schleicher and Schuell) inserted into it. The gel was replaced in the electrophoresis tank at 90° to the original orientation and the DNA electrophoresed onto the DEAE paper. After washing with TE, the DNA was eluted from the paper by incubation in 2M NaCl, 68°C for 2 hours. The DNA was then precipitated from

solution by ethanol precipitation, 100µg/ml dextran being added as a carrier to aid DNA recovery.

2.11.3. Pulsed field gel electrophoresis

Conventional electrophoresis of DNA through agarose, is unable to resolve DNA molecules of >50kb effectively. Schwartz and Cantor (1984) and Carle and Olson (1984) initially described electrophoretic apparati which enable DNA molecules of up to several thousand kb to be resolved, using orthogonal electric fields. The apparatus used for the work in this thesis was designed and constructed by D. Fletcher, and employs a system based on that of Carle and Olson (1984) in which DNA is separated by alternately applying two electric fields which are approximately at right angles to each other, termed orthogonal-field-alternation gel electrophoresis (OFAGE).

A 20 x 20cm 1% agarose gel was cast and run in 0.5 x TAE at 300V/200mA for 24-30 hours. The switching time of the two pairs of electrodes was varied according to the size range to be resolved, longer pulse times resulting in separation of larger DNA molecules. To maintain the buffer at a constant temperature of 10-20°C, buffer was recirculated through a cooling system.

High molecular weight DNA was prepared in agarose blocks as described in Section 2.2.3. To digest DNA embedded in agarose, the blocks were washed in 5ml TE, 4°C for 30 mins, three times, followed by a wash in 1 x restriction buffer. Each plug was then divided into three and each piece placed in a separate Eppendorf tube. After a further wash in 1 x restriction buffer, each piece was incubated in 50µl of appropriate buffer together with 100µg/µl

gelatin and 5mM spermidine if required, at room temperature for 10 mins. Enzyme was then added (10-40 units) and digestion carried out at the appropriate temperature for a maximum of four hours. For each digest, one piece of agarose was incubated without enzyme, as a control for non-specific degradation of the DNA.

Oligomers of λ DNA and yeast chromosomes were used as size markers for pulsed field gel electrophoresis. λ phage were prepared by the induction of a cI857 lysogen and the DNA purified by standard techniques (2.5.3). λ DNA was oligomerised via the cos site, by incubation at 37°C for 30 mins at a DNA concentration of 0.2µg/µl in 1 x gel loading buffer together with either 2 x SSC or 10mM $MgCl_2$.

Yeast chromosomes were prepared by the method of Carle and Olson (1985). Yeast strains were grown at 30°C to late log phase (10^8 cells/ml) in 100ml YEP (1g yeast extract, 2g glucose, 2g bactopeptone, 0.004g adenine, 0.004g uracil/100ml). After washing twice in 0.05M EDTA pH7.5, the cells were resuspended in 3ml 0.05M EDTA and mixed with 5ml of molten 1% low gelling temperature agarose (prepared in 0.125M EDTA pH7.5, and cooled to 42°C), 1ml of solution I (10ml 1M sorbitol, 0.1M sodium citrate, 0.06M EDTA pH7, 0.5ml β-me) and 10mg Zymolyase 60,000 (Miles) were added to the cell/agarose mixture which was then aliquoted into perspex moulds and allowed to set. The blocks of agarose were then incubated overnight at 37°C in 10ml 0.45M EDTA pH9.0, 10mM Tris pH8.0, 7.5% β-me, to produce yeast spheroplasts. The blocks were then incubated overnight at 50°C in NDS and 1mg/ml proteinase K and stored at 4°C in NDS.

2.11.4. Polyacrylamide gel electrophoresis

Analysis and purification of DNA fragments of <1kb was carried out by electrophoresis through polyacrylamide gels (Maniatis et al, 1982). A 29:1 mix of acrylamide:N,N-methylenebisacrylamide (BDH Chemicals Ltd.), at concentrations ranging from 3.5 to 7%, was used depending on the size range of DNA fragments to be resolved. Gels, poured between siliconised glass plates, were 0.5mm thick, 10cm wide and 20cm long, and were made up and run in TBE (54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA pH8.0/litre). Fresh 3% ammonium persulphate was added to the acrylamide solution (2.1ml/100ml solution) which was then degassed prior to adding TEMED (N,N,N'N'-tetramethylene diamine) at 60µl/100ml of solution. Samples were loaded together with a 1/10th volume of marker dye (5% glycerol, 2.5% bromophenol blue, 2.5% xylene cyanol), and gels run at 10V/cm. Staining and photography of polyacrylamide gels was as for agarose. Gels loaded with radiolabelled DNA were fixed in 10% acetic acid, 10% methanol for 10 mins prior to drying down and autoradiography. DNA was recovered from polyacrylamide gel slices by mashing slices in TE, and allowing DNA to diffuse out overnight at 4°C.

2.12. Immobilisation of DNA onto membranes

2.12.1. Immobilisation of bacterial colonies onto nitrocellulose

A method based on that of Grunstein and Hogness (1975) was used to bind DNA from bacterial colonies onto nitrocellulose filters (Schleicher and Schuell). Bacterial colonies were grown on nitrocellulose filters placed on top of agar plates. Replica filters were taken by placing a fresh sheet of nitrocellulose on top

of the colonies and pressing this 'sandwich' together to transfer colonies. The filters were then placed, colony side up, on a sheet of Whatman 3MM chromatography paper saturated with denaturing solution (0.5M NaOH, 1.5M NaCl) for 5 mins. Filters were neutralised on paper soaked in neutralising solution (0.5M Tris HCl, pH5.0, 3.0M NaCl) for a further 5 mins before rinsing in 2 x SSC, 0.1% SDS to remove bacterial debris. Nitrocellulose filters were then dried at room temperature, and baked at 80°C under vacuum for 2 hours to fix the DNA.

2.12.2. Immobilisation of phage plaques onto nitrocellulose

DNA from phage plaques was immobilised onto nitrocellulose by the method of Benton and Davis (1977).

A nitrocellulose filter was carefully lowered onto a lawn of plaques. To minimise the risk of peeling away of the top layer of the plates, agarose was used in place of agar and the plates well dried before plating of bacteria. The nitrocellulose filter was oriented on the plate by marking both with ink, then the filter peeled off and floated, DNA side up, on denaturing solution for a few minutes, followed by neutralising solution and 2 x SSC. Filters were then dried and baked.

2.12.3. Southern transfer of DNA from agarose gels onto nitrocellulose

DNA can be transferred onto nitrocellulose from agarose gels by the method of Southern (1975).

UV illumination of DNA, during the photographing of agarose gels, introduces ss nicks which facilitate the transfer of DNA

molecules out of agarose. However, for DNA fragments of >50kb, depurination of DNA in 0.25M HCl for 10-20 mins is necessary prior to denaturing, to allow efficient transfer of these large molecules.

Gels were denatured by shaking in denaturing solution for up to 1 hour, then neutralised for a further hour. A gel was then placed onto the wick of a blotting tray containing 20 x SSC (88.2g tri-sodium citrate, 170.3g NaCl/litre), and overlaid with a nitrocellulose filter pre-wetted in 2 x SSC, care being taken to avoid trapping air bubbles inbetween. Several layers of Whatman 3MM paper and a thick stack of paper towels were then placed on top, to draw liquid up through the gel. Nescofilm was placed around the gel to prevent short-circuiting of liquid between the wick and paper towels. After transfer overnight the nitrocellulose was rinsed in 2 x SSC, dried, then baked.

To obtain duplicate filters of an agarose gel, the gel was sandwiched between two sheets of nitrocellulose with paper towels either side. The only liquid available in this case, to bring about transfer of DNA, comes from within the gel itself, therefore prior to transfer both the gel and the nitrocellulose filters were soaked in 10 x SSC for 15 mins. Transfer is completed in 2 hours.

2.12.4. Southern transfer onto re-usable nylon membranes

DNA binds to nitrocellulose by hydrophobic interactions and consequently conditions under which radiolabelled probes can be stripped from the membrane, also result in the loss of the DNA bound to the nitrocellulose. If repeated hybridisations are to be carried out, it is therefore desirable to bind DNA more permanently to a

membrane. Various nylon membranes are available for this purpose. Three membranes were used in the course of this thesis, 'Zetaprobe' (Bio-Rad), 'Genescreen plus' (New England Nuclear) and 'Hybond N' (Amersham), each used according to the manufacturers' specifications. 'Zetaprobe' was used as for nitrocellulose. Transfer onto 'Genescreen plus' was carried out in 10 x SSC, then the membrane immersed in 0.4M NaOH for 1 minute after transfer to ensure complete denaturation of the transferred DNA. After neutralising in 0.2M Tris-HCl pH7.5, 2 x SSC for 5 mins, the membrane was dried at room temperature. Transfer onto 'Hybond N' was carried out in 0.025M sodium phosphate buffer pH6.5. After drying at room temperature, DNA was cross-linked to the nylon by irradiating the filter DNA side down, on a UV transilluminator (300nm) for 5 mins.

Probe was removed from nylon membranes by washing in 0.4M NaOH, 45°C for 30 mins following by neutralising in 0.1 x SSC, 0.1% SDS, 0.2M Tris-HCl pH7.5, 45°C for another 30 mins. Probe could also be stripped from 'Hybond N' by incubation in 2mM EDTA, 0.1% SDS at 80°C for 30 mins. It was important that nylon membranes were kept moist to prevent radiolabelled probes becoming fixed onto the membrane.

2.13. Radiolabelling of DNA

2.13.1. Nick translation

E.coli DNA polymerase I catalyses the addition of nucleotide triphosphates onto the free 3'OH of nicks in a ds DNA molecule. By virtue of the enzyme's 5'-3' exonuclease activity, nucleotides at the 5' side of a nick are removed, and in this manner (by a series of excision and addition reactions) nicks are moved along the DNA

(nick translation). DNA polymerase I can therefore be used to incorporate radioactive nucleotides into DNA (Rigby et al, 1977).

<100ng of DNA was used in each reaction, in the presence of 50mM Tris-HCl pH7.2, 10mM $MgSO_4$, 0.1mM DTT, 50µg/ml BSA, 10µM dATP, 10µM dGTP, 10µ,M dTTP. 4 units of E.coli DNA polymerase I, and 1µl of 0.1µg/ml DNaseI were added together with 20µCi α-^{32}PdCTP (3000Ci/mmol, 10mCi/ml, Amersham). After incubation at 15°C for 40-60 mins the percentage of radioactive nucleotide incorporated into the DNA was estimated from the proportion of counts precipitated onto a Whatman GF/A filter by 10% TCA, which quantitatively precipitates oligonucleotides of >20 bases. The specific activity of probes obtained by this method was $>10^8$ cpm/µg.

Removal of proteins and unincorporated nucleotides from radiolabelled probes reduces background during hybridisation. This may be achieved by passing the probe down a Sephadex G-50 (fine) column, DNA being excluded from the gel matrix (Maniatis et al, 1982). Alternatively, probes may be extracted with phenol/chloroform and precipitated at -70°C by the addition of ammonium acetate to a concentration of 2M, and 2.5 vols of ethanol.

2.13.2. Random-primer labelling

If clones, in which the insert size is small compared to the vector, are to be used as hybridisation probes, a higher signal to noise ratio is obtained if the insert is purified away from the vector sequences. This was achieved by appropriate restriction enzyme digestion, electrophoresis through low-gelling temperature agarose or polyacrylamide and excision of relevant bands from the gels. DNA in this condition is an inefficient template for E.coli

DNA polymerase I which is sensitive to contaminants such as those found in agarose. However, using hexamers derived from DNaseI digestion of calf thymus DNA (Pharmacia) as primers, the Klenow (large) fragment of DNA polymerase I is able to copy the template DNA. Klenow fragment retains the 5'-3' polymerase and 3' 5' exonuclease activities of the intact enzyme but lacks the 5'-3' exonuclease. This enables labellings using Klenow fragment to be carried out overnight if necessary, without consequent degradation of the product (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984).

Low-gelling temperature agarose slices containing DNA to be labelled were placed in pre-weighed Eppendorf tubes, and water added (3ml/g of gel). The agarose was then boiled for 7 mins to melt the gel slice and denature the DNA. Polyacrylamide gel slices were mashed in TE, and the DNA allowed to diffuse out.

Just prior to labelling, the DNA was boiled for 5 mins, cooled and 17µl added to 1µl 10mg/ml BSA, 25µCi α^{32}P dCTP, 1 unit Klenow fragment and 5µl oligolabelling buffer. This latter solution contains a 1:25:1.5 ratio of solution A: 2M HEPES pH6:hexadeoxy-ribonucleotides in TE at 90 OD units/ml. Solution A consists of 1ml 1.25M Tris-HCl pH8.0, 0.125M $MgCl_2$, 18µl β-me, and 5µl each of 0.1M dATP, dTTP, dGTP in 3mM Tris-HCl, 0.2M EDTA pH7.0. After incubation at 37°C for 2-15 hours the incorporation of radioactive nucleotides was assayed as for nick translations, and the probe cleaned up as described in the preceding section. Because of the small amounts of DNA used in each oligolabelling reaction, the specific activity of probes generated by this method was high, generally $>10^9$cpm/µg.

2.13.3. Labelling the 5' ends of DNA with T4 polynucleotide kinase

T4 polynucleotide kinase catalyses the transfer of the γ-phosphate of ATP to a 5'OH terminus of DNA or RNA (Maniatis et al, 1982). 1-50 pmoles of dephosphorylated DNA were incubated at 37°C for 30 mins with 10 units T4 polynucleotide kinase, 100µCi γ-^{32}P ATP (Amersham), 50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine and 0.1mM EDTA, in a total volume of 50µl. The reaction was stopped by the addition of 2µl of 0.5M EDTA, and the DNA extracted with phenol/chloroform.

2.14. Hybridisation of radiolabelled DNA to DNA immobilised on membranes

Filters were hybridised in either sealed plastic bags, or hybridisation tubes. Nitrocellulose filters were wetted in 6 x SSC prior to hybridisation. Prehybridisation and hybridisation were carried out at 68°C in 10% dextran sulphate, 0.1% sodium pyrophosphate, 0.1% SDS, 5 x SET/Denhardt's solution (20 x SET/Denhardt's contains 0.4M Tris-HCl pH7.8, 3M NaCl, 20mM EDTA, 0.4% Ficoll, 0.4% polyvinylpyrrolidine and 0.4% BSA). Sonicated salmon sperm DNA, denatured by boiling, was added at 100µg/ml as a competitor. For Hybond N membranes the SDS concentration was raised to 0.5% and for Genescreen plus to 1%. Prehybridisation was allowed to continue for 1-4 hours, labelled probe was then denatured by boiling for 10 mins and added to the mix at a concentration of <10ng/ml. After hybridising for at least 18 hours, filters were washed to remove probe non-specifically bound to the membranes. All

washing solutions contained 0.1% sodium pyrophosphate, 0.1% SDS. The first wash was carried out at room temperature in 2 x SSC, followed by a 65°C wash in 1 x SSC. The final wash was carried out in 0.1 x SSC at 55-68°C depending on the degree of stringency required; then the filter wrapped in Cling-film prior to autoradiography.

2.15. Autoradiography

Autoradiography was carried out using X-ray film (Fuji, RXNIF). In order to obtain quantitative signal and high sensitivity, films were pre-flashed (to an OD_{540} of 0.15), and exposed at -70°C together with an Ilford fast tungstate intensifying screen (Laskey and Mills, 1977). If maximum resolution were required pre-flashing and use of an intensifying screen were omitted and exposure carried out at room temperature. X-ray films were developed with an Agfa-Gevaert automatic X-ray film processor, G153 developer and E353 fixative (Agfa-Gevaert).

2.16. Sequencing of DNA

DNA was sequenced using the Sanger dideoxy method (Sanger et al, 1977), after cloning of the appropriate DNA fragments into one of the M13 series of vectors (see Section 2.4.4).

To prime the DNA synthesis reaction a universal primer (17 mer) was annealed to ss M13 adjacent to the cloning site. Klenow fragment can then be used to extend this primer, using the DNA fragment cloned into the M13 vector as template. 4 separate

sequencing reactions are carried out, each supplied with all four dNTPs, but each one containing a different dideoxy NTP (ddNTP). Incorporation of a ddNTP into the newly synthesised DNA strand results in chain termination as no 3'-OH group is available to form the next phosphodiester bond. By controlling the ratio of dNTP:ddNTP a series of DNA fragments is generated, all originating at the primer, and terminating at the sites of incorporation of the appropriate ddNTP. If one of the dNTPs is radioactively labelled, the fragments can be resolved on high definition thin polyacrylamide gels (Sanger and Coulson, 1978) and detected by autoradiography.

The following solutions of dNTPs and ddNTPs were made up:

A° Equal vols of TE, 0.5mM dCTP, 0.5M dGTP, 0.5mM dTTP.

C° Equal vols of TE, 0.5mM dGTP, 0.5mM dTTP, 1/20th vol 0.5mM dCTP.

G° Equal vols of TE, 0.5mM dCTP, 0.5mM dTTP, 1/20th vol 0.5mM dGTP.

T° Equal vols of TE, 0.5mM dCTP, 0.5mM dGTP, 1/20th vol 0.5mM dTTP.

A mix: equal vols A° and 0.1mM ddATP

C mix: equal vols C° and 0.1mM ddCTP

G mix: equal vols G° and 0.1mM ddGTP

T mix: equal vols T° and 0.1mM ddTTP

5µl of ss DNA template were annealed with 2µl M13 primer (Amersham) in 10mM Tris-HCl pH8.0, 5mM $MgCl_2$, in a total volume of 10µl, at 60°C for 2 hours. After quenching on ice, 2 units of Klenow fragment and 10µCi of α-^{35}S dATPαS (>400Ci/mmol) were added and well mixed. 2.5µl of this mix were aliquoted into Eppendorf tubes marked A, T, G and C and 2µl of the relevant dNTP/ddNTP mix

placed just inside the rim of each tube. Reactions were then started simultaneously in each tube by briefly spinning down the dNTP/ddNTP mixes. After 20 mins at room temperature 2µl of chase solution (0.5mM mix of all dNTPs in 10mM Tris-HCl pH8.0) were added to each tube, and left for 15 mins. 4µl formamide dye mix (100ml formamide, 0.03g xylene cyanol, 0.03g bromophenol blue, 0.75g Na_2 EDTA) were then added and the tubes placed in a 95°C water bath for three mins immediately prior to loading onto a sequencing gel.

Sequencing gels were poured between clean, siliconised glass plates (40 x 20 x 0.04cm). Buffer gradient gels were used, in order to achieve more even spacing of bands over the entire length of the gel; made up and run in TBE (10.8g Tris base, 5.5g boric acid, 0.93g EDTA, pH8.3/litre). A 40% stock solution of acrylamide (19:1 acrylamide:NN'-methylenebisacrylamide) was made up and de-ionised by mixing with Amberlite MB1 resin (5g/100ml) for 30 mins, then filtered. 80ml of light solution (36.8g urea, 8ml 10 x TBE, 12ml 40% acrylamide) and 20ml of heavy solution (9.2g urea, 5ml 10 x TBE, 3ml 40% acrylamide, 1g sucrose, 1mg bromophenol blue) were prepared. 80µl and 20µl of fresh 25% ammonium persulphate were added to the light and heavy solution respectively. The light solution was then degassed before the addition of TEMED (80µl to light solution, 20µl to heavy solution). 12.5ml of light solution followed by 12.5ml heavy solution were taken up in a 25ml pipette and poured between the glass plates, followed by the rest of the light solution, then the comb inserted. When set, the gel was clamped to a Bio-Rad sequencing apparatus connected to an LKB (2297 Macrodrive 5) power supply. The bottom buffer tank was filled with 2 litres of running buffer at room temperature, and the upper reservoir with buffer at

50°C. The gel was then prerun for 1 hour at 1600V, 0.46mA to enable the gel to reach a temperature high enough to prevent renaturation of the loaded DNA. 1-2µl of denatured DNA were loaded/slot with a drawn out capillary tube, and the gel run until the bromophenol blue marker had run off the bottom. After allowing to cool, the glass plates were prised apart, the gel fixed for 10 mins in 10% acetic acid, 10% methanol, then transferred from the glass plate onto a sheet of Whatman 3MM paper., After covering with Saran-wrap, the gel was dried down under vacuum at 80°C until no longer tacky. The Saran wrap was then removed and the gel placed in a cassette directly in contact with X-ray film (no pre-flashing) and exposed without intensification, at room temperature.

2.17. Determination of DNA restriction fragment length using a digital microdensitometer

The mobility of DNA fragments during electrophoresis bears a logarithmic relationship to their size, smaller molecules migrating faster than larger ones. The length of a DNA restriction fragment can therefore be calculated by comparing its mobility to that of fragments of known length (Elder and Southern, 1983).

4" x 5" negatives of ethidium bromide stained gels, containing DNA fragments of unknown size together with standards of known length were scanned. This was carried out by Dr. John Elder (MRC, CAPC), using hardware described fully by Elder et al, (1986). After scanning, the optical density profile of each track was displayed, so that unwanted peaks could be removed (such as those resulting from partial digest products, and scratches on the negative), and

multiple peaks unresolved by the computer resolved manually. The length (kb) of known standards of DNA were assigned to the relevant peaks and the origins of all tracks on the gel normalised, to account for any distortions in the gel.

A computer print out of data obtained from the gel included:

i) The mobility and assigned length of standard DNA fragments.

ii) Raw and normalised mobility values for unknown fragments.

iii) A 'local' estimate of the length of unknown fragments, based on comparison of mobility to that of standards closest to the unknown fragment.

iv) A 'global' estimate of the length of unknown fragments, using all standards to assign size.

CHAPTER 3

ISOLATION AND LOCALISATION OF A SINGLE-COPY DNA SEQUENCE FROM THE HUMAN Y CHROMOSOME

Construction of the sorted chromosome library and isolation of λY2:13, described in this Chapter, was the work of H.J. Cooke.

The mammalian Y chromosome provides a system in which DNA sequences from a specific chromosome can be readily identified and studied, since this chromosome is present only in the heterogametic (♂) sex.

The initial identification of DNA sequences derived from the human Y chromosome arose from a comparison of restriction enzyme digestion patterns of male and female DNAs (Cooke, 1976), and from reassociation of male DNA with excess female DNA to generate a probe specific for the Y chromosome (Kunkel et al, 1976). Both of these approaches demonstrated the presence of repeated sequences associated specifically with the human Y chromosome.

In order to isolate non-repeated DNA sequences from the human Y chromosome, a library of EcoRI fragments was constructed in the λgt WES vector, using DNA enriched for the Y chromosome by fluorescence activated cell sorting (FACS) (Cooke et al, 1983). As the source of cloning material, an individual was chosen in whom the Y was well resolved from the other chromosomes in the flow-karyotype. Cytological observation of the sorted material suggested that the Y chromosome represented 80% of the chromosomes in the enriched fraction. This was supported by molecular analysis of this fraction for Y chromosome repeats. Given the small size of the Y, as compared to other human chromosomes, sequences from the Y chromosome would represent <80% of the DNA extracted from this sorted chromosome fraction.

From this library clones hybridising strongly to nick-translated total human female DNA were eliminated, as these

contained DNA sequences present in the female genome at a high copy number. The remaining clones were used to probe Southern transfers of DNA from the somatic cell hybrid 3E7, which contains a human Y chromosome in a mouse background. In this way clones containing sequences originating from the human Y chromosome could be identified.

3.1. Isolation of a single-copy probe hybridising to the human Y chromosome

One of the clones thus showing hybridisation to 3E7 DNA was termed λY2:13. When used as a probe to digests of total human genomic DNA, however, repeated sequences present within the clone largely obscured any signal originating from sequences present at a single-copy level in the genome.

λY2:13 was therefore subcloned as a partial MboI digest into the BamHI site of pUC9. Colonies of JM83 containing recombinant molecules (Section 2.4.2) were spotted onto a nitrocellulose grid. After regrowing the colonies for a few hours, replicas of this filter were taken by placing fresh nitrocellulose filters onto the colonies and applying pressure to ensure transfer of bacteria. One replica was probed with nick-translated total human female DNA, the other with the 8.5kb EcoRI insert of λY2:13 purified away from the arms by electroelution from a gel slice prior to radiolabelling.

Plasmids which showed no hybridisation to total genomic DNA, i.e. were free of repeated sequences, but did hybridise with the λY2:13 insert, were isolated. Those containing inserts of >1kb were then nick-translated and assayed for their effectiveness as

hybridisation probes in detecting a single-copy 8.5kb EcoRI fragment in Southern transfers of human male DNA.

One clone, pG15, was identified as a good probe by the above criteria. In order to optimise signal to noise ratio when using pG15 as a hybridisation probe, the 3kb insert was purified away from plasmid sequences using SalI and SmaI. These enzymes have sites in the pUC9 polylinker, flanking the BamHI site, but do not cut within the insert of pG15. BamHI itself cannot be used to release the insert of pG15 as ligation of an MboI fragment into a BamHI site generally results in loss of the latter. In the case of pG15 one flanking Bam site has been lost and one retained.

3.2. Chromosomal localisation of G15-related sequences

As expected, G15 (the insert of the plasmid pG15) detects a single 8.5kb EcoRI fragment in 3E7 and human male DNA suggesting that λY2:13 was derived from the human Y chromosome enriched by FACS. However the same band is also present in female DNA (Fig. 3.1), which of course lacks a Y chromosome.

The simplest explanation of this observation is that λY2:13 originates from another human chromosome contaminating the FACS fraction. Hybridisation to 3E7 DNA would then result from the presence either of an homologous sequence in the mouse genome, or non-Y derived human DNA in the 3E7 cell line. Indeed it is known that the 3E7 cell line does contain cytologically undetectable human DNA of non-Y origin (G. Rappold, pers. comm.; Bishop et al, 1984).

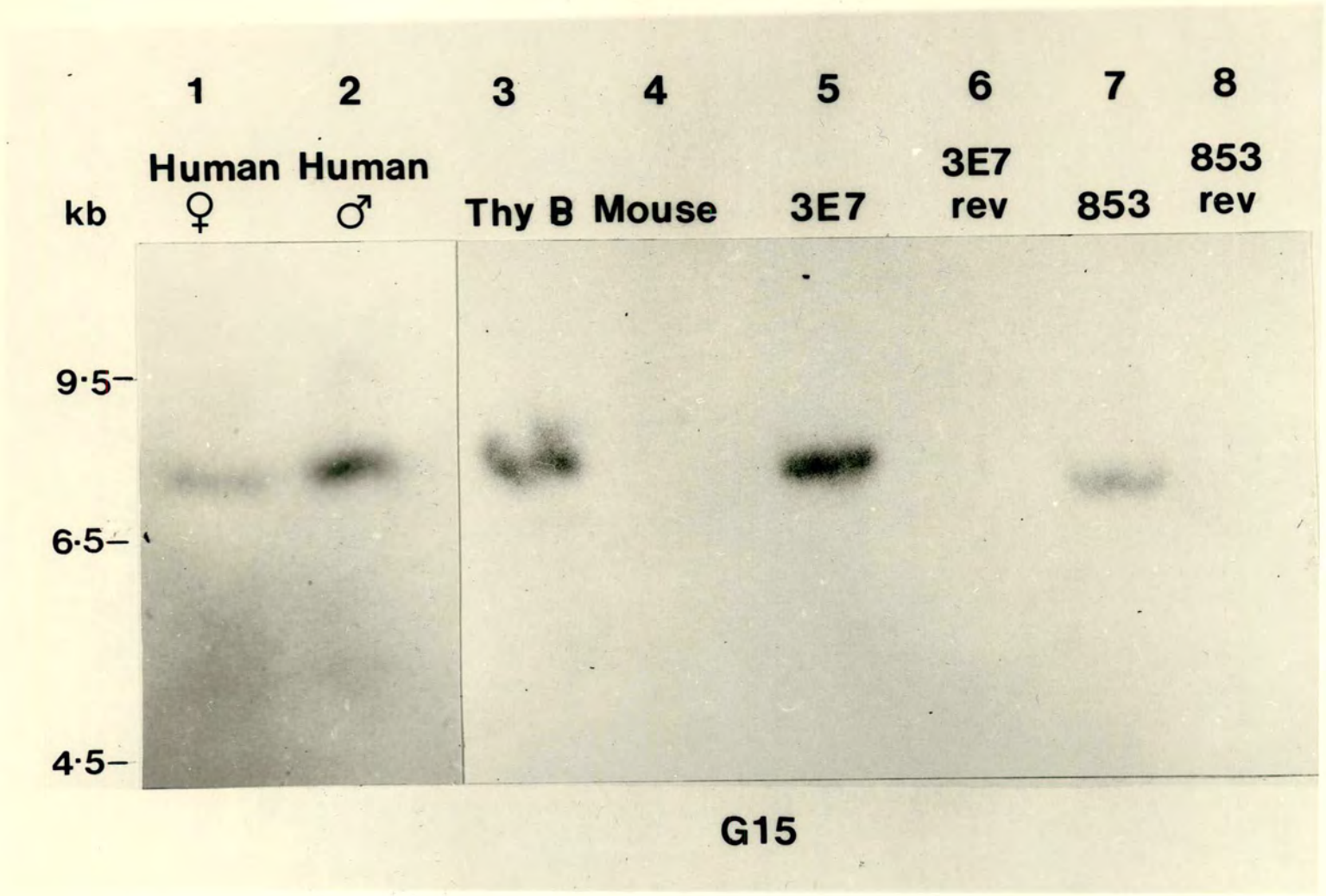
G15 does not hybridise to a revertant of 3E7 which has lost the human Y chromosome. Therefore there are no sequences homologous to

FIGURE 3.1

Chromosomal localisation of G15

DNAs were digested with EcoRI, run on agarose gels, transferred onto Hybond N and hybridised with G15 (the insert of pG15). The filters were washed in 0.1 x SSC, 68°C prior to autoradiography

1) Human female
2) Human male
3) ThyB1 - a mouse/human hybrid retaining the X chromosome as its only detectable human material.
4) Mouse
5) 3E7 - a mouse/human hybrid retaining the Y chromosome as its only detectable human material.
6) A revertant of 3E7 which has lost the human Y chromosome.
7) 853 - a Chinese hamster/human hybrid retaining the Y chromosome as its only detectable human material.
8) A revertant of 853 which has lost the human Y chromosome.


1
2
3
4
5
6
7
8
Human ♀
Human ♂
Thy B
Mouse
3E7
3E7 rev
853
853 rev
kb
9·5
6·5
4·5
G15

G15 in the mouse genome. G15 related sequences are also present in 853, a Chinese hamster/human hybrid containing the Y chromosome as the only visible human DNA, but not in a revertant of this line which has lost the Y (Fig. 3.1). These results indicate that G15 is indeed located upon the human Y chromosome.

Hybridisation of G15 to female DNA must therefore result from the presence of another copy of the sequence located elsewhere in the human genome, aside from the Y chromosome.

G15 hybridises to ThyB1, a mouse/human hybrid retaining the human X chromosome by HAT selection. It therefore seems probable that G15-related sequences are located on both of the human sex chromosomes, thus resulting in a 2 copy/diploid cell level of hybridisation to G15 of both male (XY) and female (XX) DNA.

3.3. Regional localisation of G15-related sequences

Demonstration of homology between the X and Y chromosomes is not of itself surprising. Indeed, as discussed in Chapter 1, XY homology has been predicted from models of sex chromosome evolution and their pairing at meiosis, such homology being maintained by crossing over between the chromosomes at this stage of cell division.

If the DNA sequences on the human X and Y chromosomes, detected by G15, are a reflection of the common ancestry and meiotic pairing behaviour of the mammalian sex chromosomes, we would expect these sequences to be located towards the termini of the short arms of these chromosomes.

Two techniques are available for localising DNA sequences to

regions of chromosomes: Southern hybridisation to somatic cell hybrids containing cytogenetically defined portions of chromosomes, and *in situ* hybridisation to metaphase chromosomes. Both of these approaches have been used to localise Y2:13 related sequences upon the human X and Y chromosomes.

For localisation of sequences upon the human X chromosome, DNAs from somatic cell hybrids carrying X-autosome translocations, with different break points on the X, were used (Wieacker *et al*, 1984). Figure 3.2. illustrates that G15 hybridises to all of these hybrids in a Southern hybridisation, except for the cell line containing only the qter-q28 region of the X. The region of the X held in common by those hybrids containing G15 related sequences is qter-q26, thus pointing to an Xq26-27 localisation for this DNA sequence.

A similar use of somatic cell hybrids to localise G15 on the human Y chromosome is complicated by the presence, in the hybrids available, of an X chromosome in addition to the relevant part of the Y. Iso Yp is a cell line containing an intact human X chromosome, and a Y isochromosome in which two short arms are fused together at the centromere, and from which Yq is absent. The somatic cell hybrid 445 x 393 carries a human X-Y translocation chromosome, Xqter-p22.3:Yq11:1-qter (Goodfellow *et al*, 1983). Southern hybridisation of G15 to DNA from both of these cell lines (Fig. 3.2) results in a signal level equivalent to <1 copy/diploid cell as compared to total human DNA. This was ascertained by densitometer scanning of gel photographs and autoradiographs. This signal is presumably due to the presence of Xq in both of these cell lines at less than 1 copy/diploid complement of autosomes. G15-related sequences therefore would appear to be absent from Y

FIGURE 3.2

Regional localisation of G15 upon the human X and Y chromosomes

DNAs were digested with EcoRI, run on agarose gels, transferred onto Hybond N, and hybridised with G15. Filters were washed in 0.1 x SSC, 68°C prior to autoradiography.

a) X-localisation:

1 - ThyB1 - mouse/human hybrid retaining an intact human X chromosome. Selected on HAT.

2 - 697x175 - mouse/human hybrid containing human Xqter-p21. Selected on HAT. This DNA was partly degraded prior to digestion, thus giving poor hybridisation.

3 - 676x175 - mouse/human hybrid containing human Xqter-q21:3. Selected on HAT.

4- 750 - mouse/human hybrid containing human Xqter-q26. Selected on HAT.

5 - 367 - Chinese hamster/human hybrid containing human Xqter-q28.

G15 hybridised to all human X chromosomes containing the Xq26-27 region.

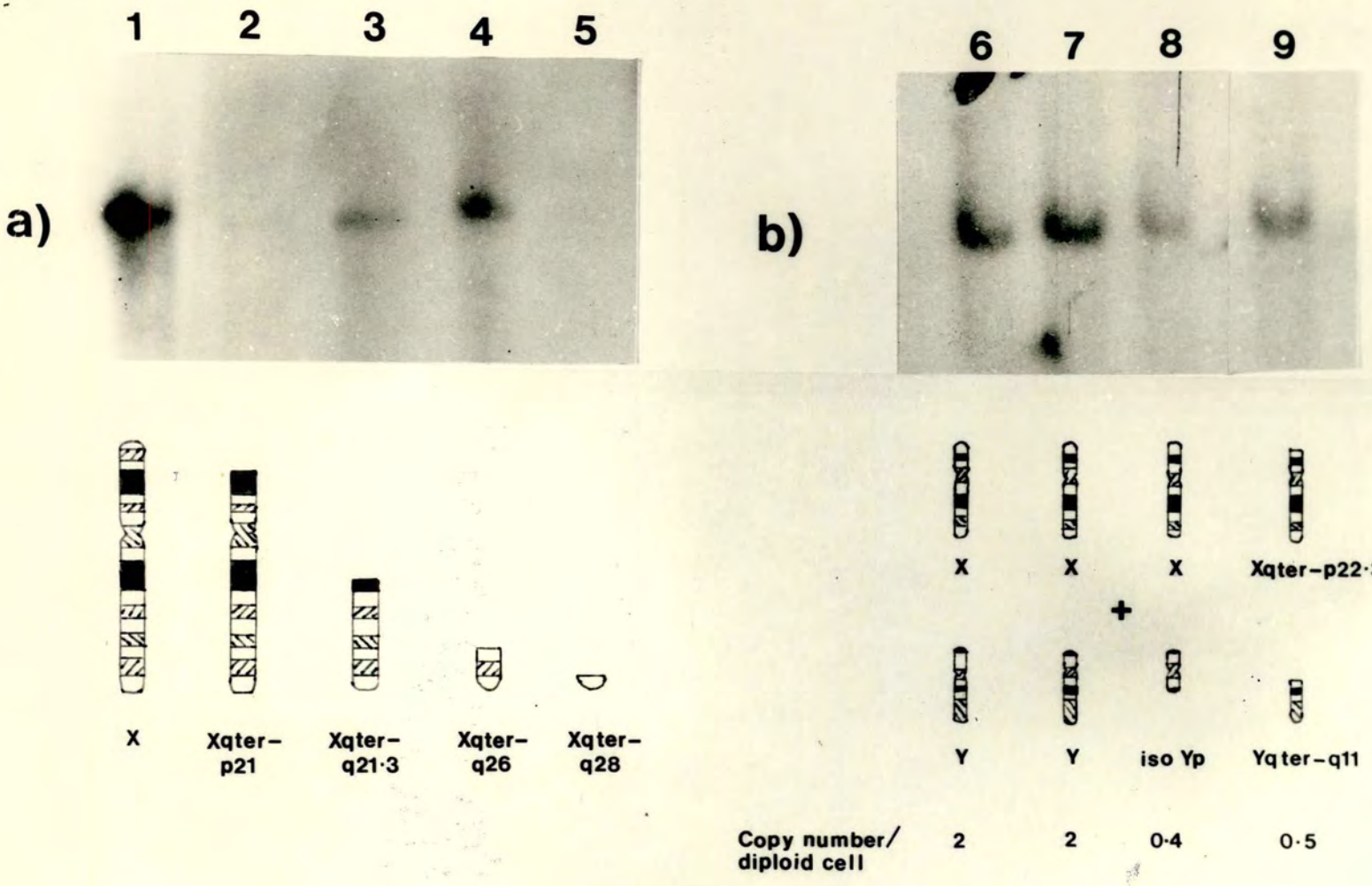
b) Y-localisation:

6 and 7 - normal males.

8 - IsoYp - cell line containing a normal X chromosome and an isoYp chromosome consisting of two Y short arms fused at the centromere - no Yq.

9 - 445x393 - mouse/human hybrid containing the translocation chromosome Xqter-p22.3 Yq11.1-qter. No Yq11.1-pter Selected on HAT.

The signal from IsoYp and 445x393 as ascertained by densitometer scanning of the gel photograph and autoradiograph is equivalent to <1copy/diploid cell compared to normal male DNA. This signal is due to the presence of Xq26-27 in both of these cell lines. G15 is therefore not present on Yp or Yq11.1-qter.


a)
1
2
3
4
5
X
Xqter–p21
Xqter–q21·3
Xqter–q26
Xqter–q28
b)
6
7
8
9
X
X
X
Xqter–p22·3
+
Y
Y
iso Yp
Yqter–q11
Copy number/ diploid cell
2
2
0·4
0·5

chromosomes containing an entire short arm inclusive of the centromere, and Yq11:1-qter but excluding the centromere. Sequences homologous to G15 may therefore be located on the long arm of the human Y chromosome just below the centromere in a part of the chromosome absent from the isoYp and 445 x 393 somatic cell hybrids.

In situ hybridisation of λY2:13 to metaphase chromosomes was carried out by G. Rappold (Cooke et al, 1984) using a HindIII fragment of the λY2:13 insert subcloned into pUC9 (pUC9H1) as a probe. 21.3% of grains were located on the human Xq24-qter in 10 metaphase spreads of 445 x 393 chromosomes. This is significantly above the background of 11.3% obtained with control probes, even though the number of spreads analysed was low. These in situ hybridisation data are therefore consistent with the Xq26-q27 localisation for 2:13 sequences from Southern hybridisation to somatic cell hybrids.

4.8% of grains on the same metaphase spreads were located on Yq of the X-Y translocation chromosome of 445 x 393. This is as compared with a background of 7.2% with control probes, and therefore does not represent significant hybridisation to the human Y chromosome in this cell line. Hybridisation of pUC9H1 to 90 metaphase spreads of a Chinese hamster hybrid carrying an intact human Y chromosome, resulted in 13% of grains being located on Yq as opposed to a background of 8.9%.

In situ and Southern hybridisation both indicate therefore that 2:13 related sequences are present on the long arm of the human Y chromosome close to the centromere, in a part of the chromosome absent from the X-Y translocation chromosome of 445 x 393. This is contrary to the claim of Cooke et al (1984), who conclude that 2:13-

related sequences are present on the Y chromosome of 445 x 393, possibly embedded in the heterochromatin of Yq. I would suggest that such a conclusion is not supported by their own in situ hybridisation data.

A sequence therefore has been isolated from the long arm of the human Y chromosome, probably located close to the centromere. This locus is homologous to sequences on the human X chromosome (Xq26-q27). These regions of the human sex chromosomes do not form a synaptonemal complex together at meiosis, thus it is unlikely that the X/Y homology defined by λY2:13 is the result of, or is involved in the regular pairing and exchange of genetic material between the sex bivalents at meiosis.

CHAPTER 4

ANALYSIS OF THE DEGREE OF HOMOLOGY BETWEEN THE HUMAN X AND Y CHROMOSOMES AT THE LOCI DETECTED BY λY2:13

Homology between the human sex chromosomes, residing outside of the meiotic pairing region could be envisaged as arising from several origins. Firstly, it may be a reflection of the common ancestry of the mammalian sex chromosome pair. Clock rates for DNA sequence divergence were initially derived from analysis of non-transcribed DNA such as that in the globin gene cluster of man and primates (Barrie et al, 1981) and were calculated to be about 0.2-0.4% per million years (see Section 5.1). Assuming that it is reasonable to extrapolate such data to other loci, it can be predicted that, since the divergence of mammals over 80 million years ago, the extent of homology remaining between the human X and Y chromosomes, outside of the pairing region, would be <70%. This level of homology would not be detected by the high stringency washing conditions (0.1 x SSC, 68°C) used in Fig. 3.1.

Homology >70% might be expected to be found if the loci defined by λY2:13 were functional, such that selection pressure was operating to conserve their sequences. However in this case homologous sequences might be expected to be found in the mouse genome. As discussed in the previous chapter, sequences hybridising with G15 are not present in mouse DNA. G15 also does not detect any transcripts in poly A^+ RNA from a variety of human tissues (a gift from G. Rappold, Heidelberg), data not shown.

Gene conversion type mechanisms operating between regions of the human sex chromosomes could be one means whereby a high degree of homology could be maintained between sex bivalents.

Detailed analyses were carried out on the 2:13 related X and Y loci using restriction enzyme mapping and DNA sequencing to more precisely define the level of, and perhaps throw light on the

origins of, this homology.

4.1. Restriction enzyme mapping of the X and Y loci defined by λY2:13

In order to determine the restriction maps of the X and Y chromosome loci hybridising with λY2:13, the X homologue of λY2:13 was cloned. A partial MboI digest of ThyB1 DNA (Section 2.1.3) was cloned into the BamHI site of EMBL4, recombinant phage being selected for by Spi selection on the P2 lysogen Q359 (Section 2.4.1). 10^6 recombinant phage were screened with G15, and two hybridising clones, λTB116 and 117 isolated (H. Cooke, pers. comm.).

Restriction maps of λY2:13, TB116 and 117 were generated by a combination of two approaches. Firstly, phage DNAs were digested with multiple restriction enzymes, and the size of the resulting restriction fragments determined. Secondly EcoRI and HindIII fragments in the phage DNAs were ordered relative to the cohesive ends of λ by annealing radiolabelled oligomers complementary to the left and right cohesive ends, to partial digests of phage DNA (Whittaker and Southern, 1986).

λY2:13, TB116 and 117 DNAs were digested to completion with the restriction enzymes: BamHI, EcoRI, HindIII, PstI and XbaI, either singly or in various combinations. All digests were carried out in the shortest time required for complete digestion to minimise non-specific degradation of the DNA. Where DNA was being digested with more than one enzyme, the DNA was extracted with phenol/chloroform and ethanol precipitated between each digest and the DNA resuspended in a buffer appropriate for the next enzyme to be used. All digests

were run on vertical 18 x 18cm, 0.8% agarose gels with size standards of λcI857 DNA digested with AvaI and HindIII, low voltage gradients being employed to minimise streaking and maximise resolution of the DNA fragments. Gels were photographed (Fig. 4.1) and the 4" x 5" negatives scanned on a microdensitometer (Section 2.17) to determine the length of restriction fragments. To aid in the interpretation of this data and consequent construction of restriction maps, all gels were transferred onto nitrocellulose and hybridised with G15 (Fig. 4.1).

Determination of unambiguous restriction maps, from the data obtained above, is simplified if the order in which certain restriction fragments appear in the phage DNA can be ascertained. DNA can be asymmetrically labelled using radioactive oligonucleotides cos L (5' - CCCGCCGCTGGA - 3') and cos R (5 - GGGCGGCGACCT -3') (synthesized by Cruachem, Livingstone) which are complementary to the left and right cohesive ends of λ, respectively (Rachwitz et al, 1984). Partial digest restriction fragments of λ DNA containing either the left or right cohesive end can therefore be identified selectively. Partial digestion of DNA is normally achieved by limiting either the digestion time or the amount of enzyme. Such partial digests, however, are irreproducible due to variations between batches of enzyme and DNA. This difficulty can be overcome by the introduction of thymidine dimers into DNA by UV irradiation. Restriction enzymes which contain TT in their recognition sequence such as EcoRI (5' - GAATTC -3') and HindIII (5' - AAGCTT -3') are unable to cleave at sequences containing thymidine dimers, so that partial digests are obtained by limit-digestion of UV irradiated DNA.

FIGURE 4.1

Restriction mapping of λ2:13, TB116 and 117

A. Phage DNAs were digested with restriction enzymes and run on agarose gels as described in the text.

B. The gels were transferred to nitrocellulose and probed with G15.

Track a = λ2:13 (human Y chromosome clone in λgt WES).

b = λTB116 (human X chromosome clone in EMBL4)

c = λTB117 (human X chromosome clone in EMBL4)

1 = digest with EcoRI

2 = digest with HindIII

3 = digest with EcoRI + BamHI

4 = digest with EcoRI + HindIII

5 = digest with EcoRI + XbaI

6 = digest with EcoRI + PstI

7 = digest with EcoRI + BamHI + HindIII

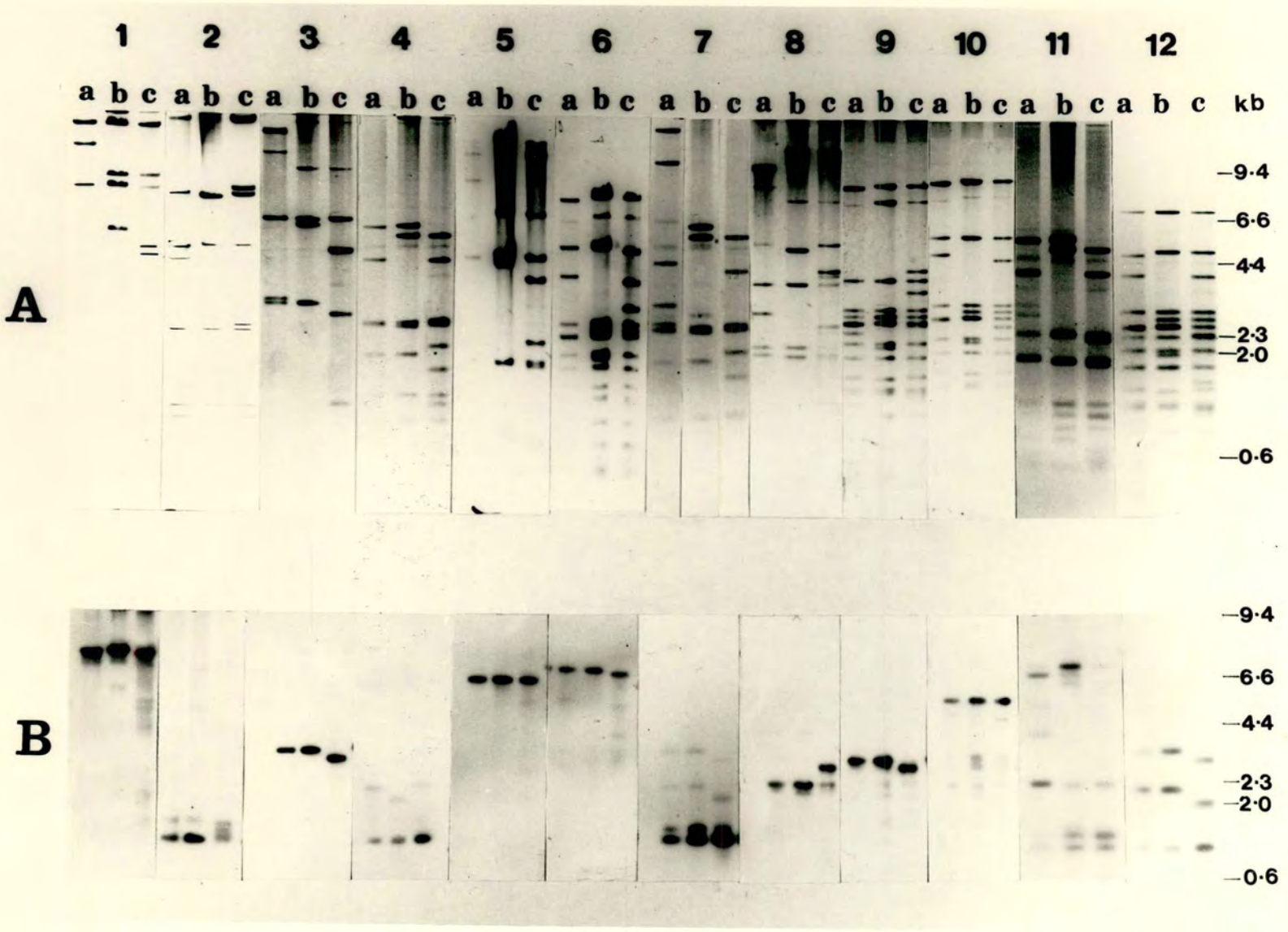
8 = digest with EcoRI + BamHI + XbaI

9 = digest with EcoRI + BamHI + PstI

10 = digest with EcoRI + XbaI + PstI

11 = digest with EcoRI + XbaI + HindIII

12 = digest with EcoRI + PstI = HindIII


1 2 3 4 5 6 7 8 9 10 11 12
a b c a b c a b c a b c a b c a b c a b c a b c a b c a b c a b c a b c
kb
9·4
6·6
4·4
2·3
2·0
0·6
A
B

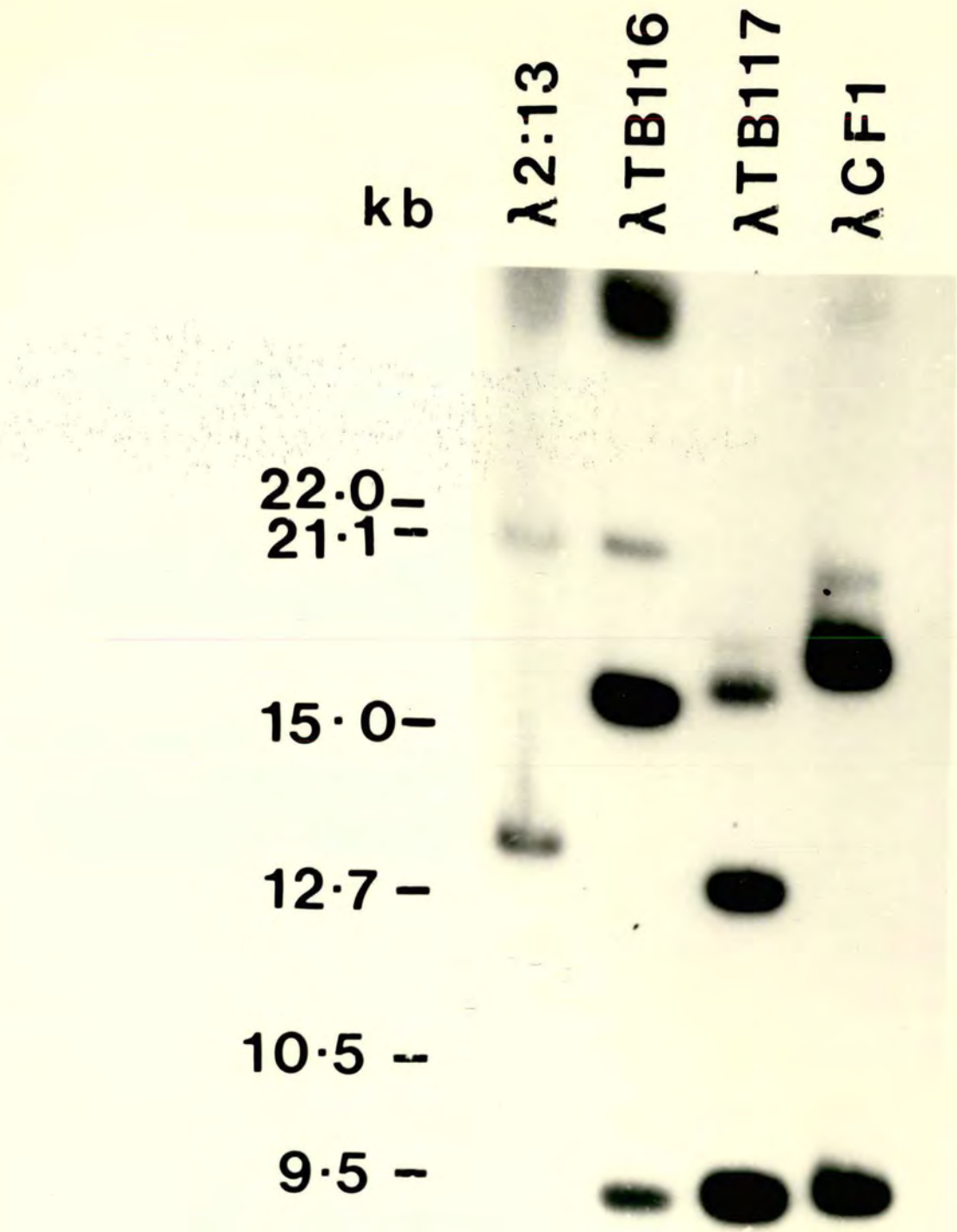
Phage DNAs in restriction buffer (Mg^{2+} is needed to prevent degradation of DNA during UV irradiation) were irradiated for 30 mins in open-topped Eppendorf tubes, 6cm away from a germicidal lamp (intensity maximum 245nm, fluence rate $30W/m^2$). DNAs were then limit-digested with the appropriate enzymes. 0.1-1µg of digested DNA in medium or high salt buffer was then heated to 70°C for 10 mins with a 4 molar excess of end-labelled cos L or R oligonucleotide (Section 2.13.3). The DNAs were then allowed to anneal at 45°C for 30 mins prior to loading onto a gel. The electrophoretic mobility of UV irradiated DNA is greater than that of unirradiated DNA fragments (Whittaker and Southern, 1986). DNA size standards were therefore UV irradiated to allow the length of unknown restriction fragments to be more accurately determined. DNAs were run on 0.5% vertical agarose gels at low voltage gradients, photographed and autoradiographed, and the size of the bands on the autoradiograph determined.

λTB116 and 117 are EMBL4 recombinants. The left arm of this cloning vector is 20.3kb in length. All partial digests of EMBL4 clones annealed to cosL will therefore be >20.3kb in length and thus poorly resolved by conventional agarose gel electrophoresis. Cos R is therefore the only appropriate oligonucleotide for use with EMBL4 (Fig. 4.2). The usefulness of this mapping technique could be improved by the design of λ vectors containing the cos site closer to the DNA insertion site. Band intensity in HindIII digests of UV irradiated DNA was more irregular than that seen with EcoRI (data not shown). This is most likely due to the sequence arrangement of the HindIII site which has two adjacent T residues at the 3' end. Some HindIII restriction sites can therefore form additional

FIGURE 4.2

Restriction mapping of λ2:13, TB116, TB117 and CF1 using cosR oligonucleotide

Phage DNAs were partially digested with EcoRI, annealed to end-labelled cosR oligonucleotide and run on a 0.5% agarose gel. The gel was then autoradiographed to identify those partial products containing the right hand cos site of λ.


kb
λ2:13
λTB116
λTB117
λCF1
22·0
21·1
15·0
12·7
10·5
9·5

thymidine dimers if a T residue abutts the 3' end of the recognition sequence. This phenomenon makes HindIII a less useful enzyme than EcoRI for this type of mapping approach.

Using the data obtained from the two techniques described above, restriction maps of λY2:13, TB116 and 117 were constructed. These are illustrated in Fig. 5.3. The localisation of G15 within these clones was ascertained by the pattern of hybridisation of G15 to the restriction digests of the phage DNAs (Fig. 4.1). As can be seen (in Fig. 5.3) the restriction map of the X and Y derived clones are identical over a minimum distance of 8.3kb; this therefore indicates a high degree of homology exists between the X and Y chromosomes at the loci detected by λY2:13.

4.2. DNA sequence analysis of the X and Y loci hybridising with 2:13

The restriction maps of λY2:13, TB116 and 117, although indicating that the inserts in these clones are very similar, give information on only a small percentage (~1%) of the bases in the clones. A more complete picture of the relationship between λY2:13, TB116 and 117, can be obtained by examining the DNA sequence of the clones.

To this end, the 2kb Bam-Xba fragments from λY2:13 and TB116, which hybridised strongly with G15 (marked as A in Fig. 5.3) were cloned into M13 mp18 (Fig.2.1). These subclones were then sequenced by the Sanger dideoxy method. By varying the length of time for which the sequencing gels were run, ~200 nucleotides of DNA sequence in from the Xba site of the M13 clones was determined, and

is illustrated in Fig. 5.4. As can be seen no base differences between the clones derived from the human X and Y chromosomes were detected. The long arms of human sex chromosomes are therefore >99.5% homologous at the loci defined by the clone λY2:13.

This data suggests that there has been recent exchange of genetic material between the X and Y chromosomes outside of the region thought to be involved in meiotic recombination. This may have occurred by RNA-mediated gene conversion operating upon an ancient homology.* Alternatively 2:13 sequences may have been present on both the X and Y chromosomes for only a relatively short period of time. If 2:13 sequences on the sex bivalents are diverging at ~0.2-0.4% per million years, this puts a maximum limit of 1.2-2.5 million years for the period during which the sequences have been present on both sex chromosomes, in order for >99.5% homology to be retained between them. This latter possibility is investigated in the next chapter.

* (Kourilsky, 1986)

CHAPTER 5

INVESTIGATION OF 2:13 SEQUENCES IN GREAT APES

5.1. Southern hybridisation of chimpanzee and gorilla DNAs to G15

Molecular phylogeny has generated a series of molecular clocks which have been used to place different species on the evolutionary tree and to date their divergence from each other. The most complete study (on birds and mammals) has been undertaken by Sibley and Ahlquist. To investigate the phylogeny of the hominoid primates Sibley and Ahlquist (1984) used DNA-DNA interspecies hybridisation to compare the single copy DNA of one species with that of another. The difference in temperature at which the homo and heteroduplexes are 50% dissociated gives a measure of the difference in nucleotide sequences between the two species and is termed the $\Delta T_{50}H$. One $\Delta T_{50}H$ represents a 1% difference in nucleotide sequence.

This approach resolves much of the criticism levelled at molecular clocks which are based upon comparisons of specific genes, proteins, or untranscribed DNA sequences. Rates of change may vary from one gene/protein or DNA sequence to another, but by dealing with the entire complement of single copy DNA in the genome as a whole, an average rate of change is obtained. Using DNA-DNA hybridisation Sibley and Ahlquist (1983) were able to demonstrate that the rate of evolution, at the DNA level, was uniform in more than 20,000 pairwise comparisons among bird species. Using estimates for the timing of biogeographical events such as the separation of landmasses which resulted in the physical isolation of various species they have calculated that one $\Delta T_{50}H$ is equivalent to an evolutionary distance of ~4.5 million years, though this time scale is questioned by many. Application of such time calibration to the hominoid primate DNA hybridisation data, places the divergence of the human and chimpanzee lines at 6-8 million years

ago, 2 million years after the divergence of the gorilla lineage from the human/chimpanzee ancestor. Previous attempts to resolve the branching order for the gorilla, chimpanzee and human lines, such as those based upon studies of rapidly evolving mitochondrial DNA, have failed (Ferries et al, 1981).

Extrapolating Sibley and Ahlquist's data to the 2:13 sequence XY homology in humans which is >99.5%, the two loci would have been diverging for not more than 2.2 million years, well after the human lineage split from that of the chimpanzees.

If the homology between the human sex chromosome pair as defined by 2:13, is the result of the relatively recent appearance of these sequences upon both the X and Y chromosomes, then, no such homology should exist between the sex chromosomes of the great apes, as the human homology postdates the divergence of the human species from its closest ape relatives.

To investigate the nature and chromosomal location of any sequences homologous to the human clone λY2:13, in the great apes, digests of male and female human, chimpanzee and gorilla DNAs were probed with G15. DNAs were obtained from blood or cells in culture by the methods described in Sections 2.2.1 and 2.2.2 and digested with EcoRI. After transfer onto nylon membranes, blots were hybridised with G15 and washed at a moderate stringency (0.1 x SSC, 60°C) to allow detection of sequences with partial homology.

As is shown in Fig. 5.1 there are indeed 2:13 related sequences in both the chimpanzee and gorilla genomes. In the chimpanzee, however, the hybridising fragments in both male and female EcoRI digested DNAs are 10.5kb in length, as opposed to 8.5kb in human DNA. In the gorilla DNAs examined the female exhibits both the 10.5

FIGURE 5.1

Investigation of 2:13 sequences in chimpanzee and gorilla

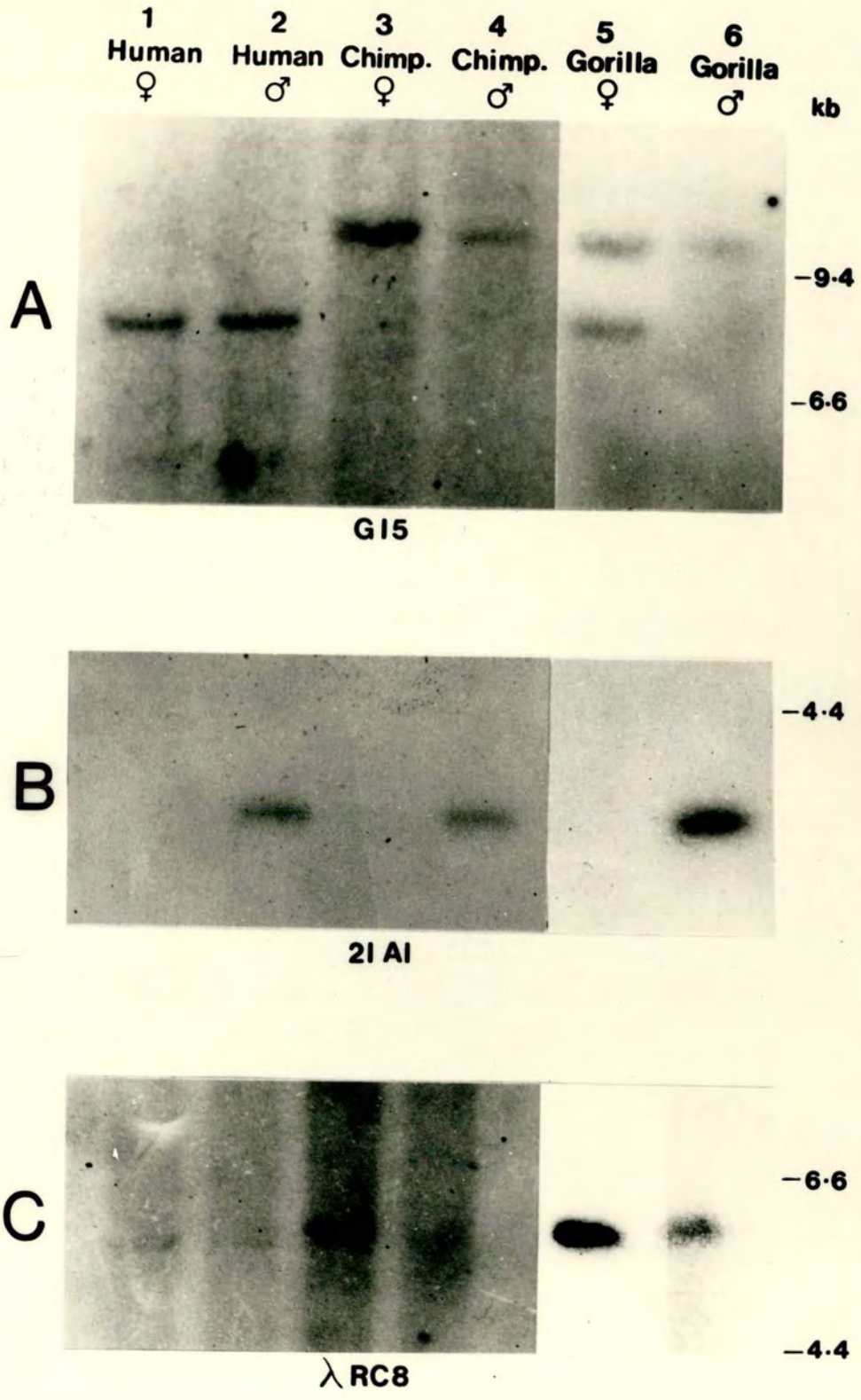
Male and female human, chimpanzee and gorilla DNAs were digested with EcoRI, run on agarose gels and transferred onto nylon membranes. Filters were washed in 0.1 x SSC, 60°C.

A: Southern hybridisation with G15.

B: Southern hybridisation with 21A1, the 3kb EcoRI insert of p21A1, a Y specific clone.

C: Southern hybridisation with λRC8, an X-linked clone.

All autoradiographs were scanned on a densitometer to determine the dosage of G15 in male and female chimpanzees and gorillas. The result of this are shown in Table 5.1.


1
Human
♀
2
Human
♂
3
Chimp.
♀
4
Chimp.
♂
5
Gorilla
♀
6
Gorilla
♂
kb
A
−9·4
−6·6
G15
B
−4·4
21 A1
C
−6·6
−4·4
λ RC8

and 8.5kb EcoRI fragments whereas the male has only the larger fragment.

The hybridisation signal from G15 in male and female human DNA is equivalent to a copy number of 2/diploid cell, 2:13 sequences being located on both the X and Y chromosomes, as discussed in Chapter 1. To establish the copy number of 2:13 related sequences present in the genomes of the chimpanzee and gorilla, the hybridisation signal to G15 of the latter species was compared to that of human DNA. Differences in the amounts of DNA in each track were compensated for by densitometer scanning (Joyce-Loebl) of the 5" x 4" negatives of the ethidium bromide stained gels, and measuring the peak intensities of the hybridising bands obtained when blots were probed with G15, as compared with those obtained with an X chromosome specific probe λRC8 (Davies et al, 1981) and the insert of a Y chromosome specific probe p21A1 (a gift from B. Smith) as reference standards (Fig. 5.1). Peak intensities were taken to be proportional to the areas under the curves obtained by densitometer scanning of autoradiographs, and were therefore compared by cutting out the shapes of peaks on pieces of paper and weighing them.

The results of these measurements are shown in Table 5.1. They demonstrate that 2:13 related sequences in chimpanzee and gorilla genomes exhibit sex-linked dosage, there being the equivalent of 2 copies/diploid cell in females, but only one copy/diploid cell in males. Therefore, in contrast to the XY homology observed in humans, 2:13 sequences are present on the X chromosome alone in chimpanzee and gorilla.

A trivial explanation for this observation might be that the Y

TABLE 5.1

Dosage of G15 sequences in male and female human, chimpanzee and gorilla DNAs

DNA	Ratios of DNA loadings on gels	Ratios of Peak intensities			Ratios of peak intensities corrected for loadings			copy number/ diploid cell		
		G15	21A1	λRC8	G15	21A1	λRC8	G15	21A1	λ RC8
Human ♀	1.00	1.00	-	1.00	1.00	-	1.00	2.0	-	2.0
Human ♂	0.80	0.97	1.00	0.44	1.21	1.0	0.55	2.4	1.0	1.1
Chimpanzee ♀	1.58	1.42	-	1.69	0.90	-	1.07	1.8	-	2.1
Chimpanzee ♂	0.99	0.43	1.02	0.52	0.43	0.82	0.53	0.9	0.8	1.1
Gorilla ♀	0.90	0.52 + 0.50	-	0.91	1.11	-	1.01	2.2	-	2.0
Gorilla ♂	1.00	0.47	0.88	0.48	0.49	0.85	0.48	1.0	0.9	1.0

copy of the 2:13 sequence has been lost from the lineages giving rise to the chimpanzee and gorilla. If however it is believed that the human and chimpanzee lines shared a period of common ancestry for 2 million years after the gorilla line diverged from them, such an explanation would demand that 2:13 sequence loss from the Y chromosome had occurred independently twice during evolution.

A simpler explanation, consistent with the very high level of homology of 2:13 sequences on the human sex chromosome, is that a transposition event has occurred between the X and Y chromosomes since the divergence of the human line from its closest relative, the chimpanzees. This has resulted in 2:13 sequences located on the X chromosome alone in the great apes, being duplicated onto the Y chromosome within the last few million years of human evolution.

5.2. Restriction enzyme and DNA sequence analysis of the chimpanzee 2:13 homologue

A functional constraint on human XY 2:13 sequence divergence can be further discounted if it can be shown that the 2:13 sequences in great apes are diverged from the human sequence. The 2:13 homologous sequence detected in the chimpanzee genome by Southern hybridisation, is contained on a 10.5kb EcoRI fragment, the human equivalent being 8.5kb in length (Fig. 5.1). Thus even at the restriction map level the chimpanzee 2:13 related sequence appears to be diverged from the human X and Y linked alleles.

To obtain further information on the relationship between the human and chimpanzee 2:13 sequences, the latter was cloned, restriction mapped and a small stretch of the DNA sequence compared

to that of the human sequence. Female chimpanzee DNA used for the Southern analysis in Fig. 5.1 was chosen as the starting material for cloning in preference to male DNA, as the latter contains only half the copy number of 2:13 related sequences as does the female (Table 5.1). The female chimpanzee DNA (gift of N.Hastie) was not in prime condition for cloning. When run undigested on a gel, some smearing was observed down the gel track, indicating that the DNA was partly degraded. This resulted in rather low cloning efficiencies.

λEMBL4 DNA was digested with BamHI to use as a cloning vector, Q359 being used as the plating strain to allow for genetic selection against non-recombinant phage. The chimpanzee genomic DNA was digested partially with MboI under conditions which generate the maximum number of molecules in the 15-25kb size range. This was established by digesting constant amounts of DNA under conditions of constant DNA concentration, digestion time and temperature, with serial dilutions of enzyme. Aliquots of these partial digests were then run on a 0.4% agarose gel to establish the conditions resulting in optimum ethidium bromide staining in the required size range. EtBr staining intensity is proportional to mass of DNA present, not the number of fragments, for example, a given number of 20kb fragments will exhibit twice the intensity of staining as the same number of 10kb fragments. Therefore in scaling up the partial digests the number of units of enzyme added/μg DNA was half that found to give maximum staining intensity in the desired size range. Insufficient chimpanzee DNA was available to be able to fractionate the partially digested DNA on a sucrose density gradient. Therefore after digestion and removal of MboI, the DNA was dephosphorylated

and ligated directly to vector DNA at a 1:4 molar ratio. An efficiency of 1.2×10^5 recombinant pfu/μg was obtained. This rather low efficiency was most probably due to the poor condition of the chimpanzee DNA, and resulted in the presence of too large a quantity of packaging extract to allow direct plating of phage at a high density (large concentrations of packaging extracts are toxic to bacteria). The phage were therefore concentrated on a CsCl step gradient, and the fractions titred on Q359. The phage were found to be present at a high enough concentration in some fractions to avoid the need for removal of CsCl by dialysis prior to large scale plating. It is advantageous to avoid this step if possible as loss of phage particles occurs due to their absorption to dialysis membranes.

The concentrated phage were plated out and screened in duplicate with G15 at moderate stringency. One positive hybridising plaque was purified after several rounds of screening and termed λCF1.

λCF1 DNA was prepared and mapped by the same series of approaches as used for λY2:13, TB116 and 117. Partial restriction mapping of λCF1 with the cosR oligonucleotide is shown alongside λY2:13, TB116 and 117 in Fig. 4.3. Restriction digests of λCF1 and their hybridisation to G15 are shown in Fig. 5.2. Comparing this data with that for the human X and Y derived clones shows that there are many similarities between these clones, as expected. The restriction map of λCF1 deduced from the mapping data is shown in Fig. 5.3 alongside those of λY2:13 and TB116/117. The chimpanzee derived clone has the same restriction map as the human clones around the region hybridising to G15, but contains additional

FIGURE 5.2

Restriction mapping of λCF1 and λGF1

λCF1 (a female chimpanzee clone in EMBL4) and λGF1 (a female gorilla clone in λgtWES) DNAs were digested with restriction enzymes, run on agarose gels and photographed. Gels were then transferred onto nitrocellulose and hybridised with G15.

Track	1	DNA	digested	with	EcoRI,	track	14	-	hybridisation to G15
"	2	"	"	"	BamHI	"	15	-	" "
"	3	"	"	"	HindIII	"	16	-	" "
"	4	"	"	"	EcoRI + BamHI		17	-	" "
"	5	"	"	"	EcoRI + HindIII		18	-	" "
"	6	"	"	"	EcoRI + PstI		19	-	" "
"	7	"	"	"	EcoRI + XbaI		20	-	" "
"	8	"	"	"	EcoRI + BamHI + HindIII		21	-	" "
"	9	"	"	"	EcoRI + BamHI + PstI		22	-	" "
"	10	"	"	"	EcoRI + BamHI + XbaI		23	-	" "
"	11	"	"	"	EcoRI + HindIII + PstI		24	-	" "
"	12	"	"	"	EcoRI + HindIII + XbaI		25	-	" "
"	13	"	"	"	EcoRI + PstI + XbaI		26	-	" "

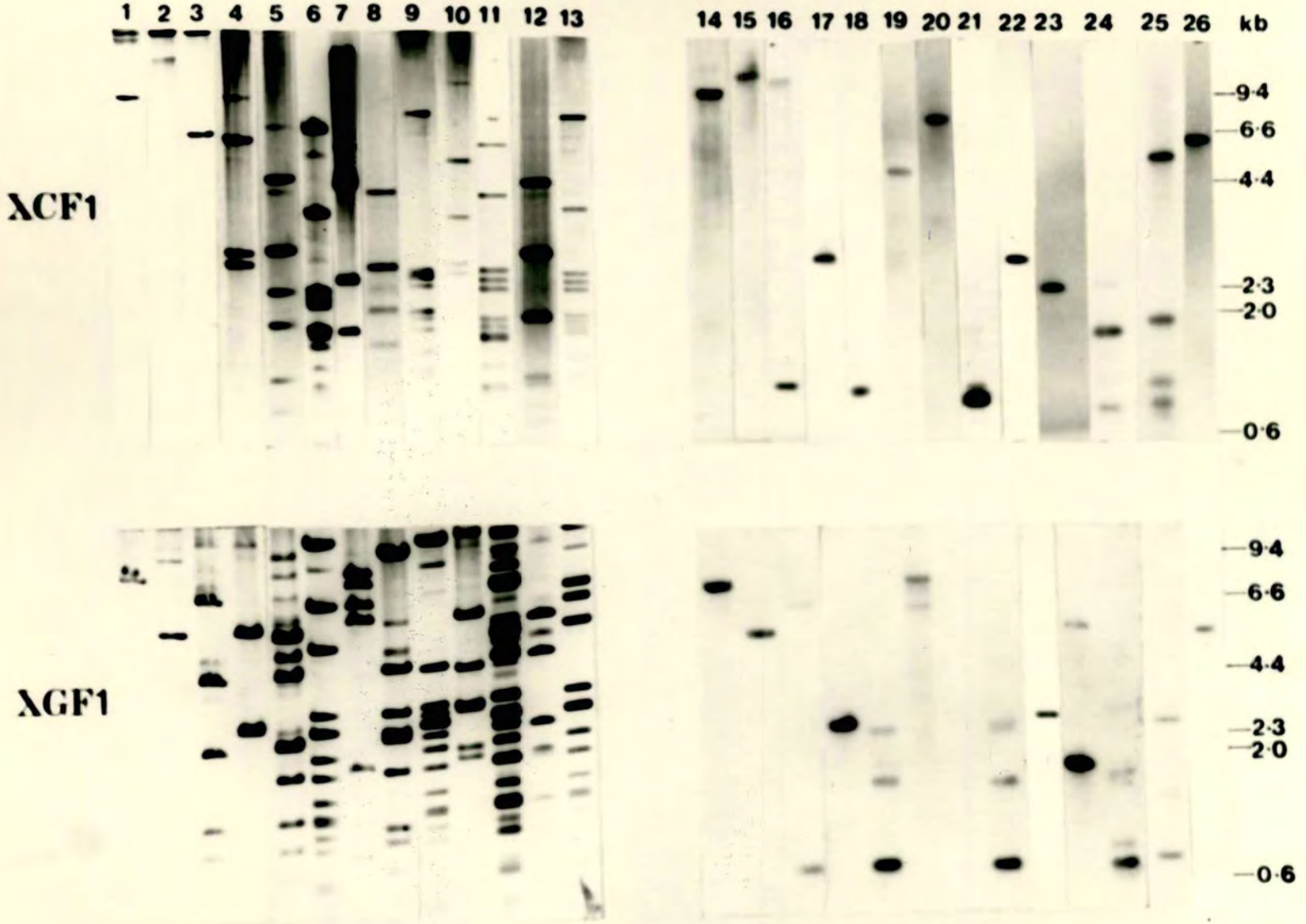

1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
kb
9·4
6·6
4·4
2·3
2·0
0·6
λCF1
λGF1

FIGURE 5.3

Restriction maps of 2:13 homologous clones, from the human X and Y chromosomes and the gorilla and chimpanzee X chromosomes

Restriction maps of inserts from λ clones:

λ2:13 (derived from the human Y chromosome)

λTB116 and λTB117 (derived from the human X chromosome)

λGF1 (derived from a gorilla X chromosome)

λCF1 (derived from a chimpanzee X chromosome)

The positions of G15, and fragment A used for sequencing studies, relative to these clones is indicated.

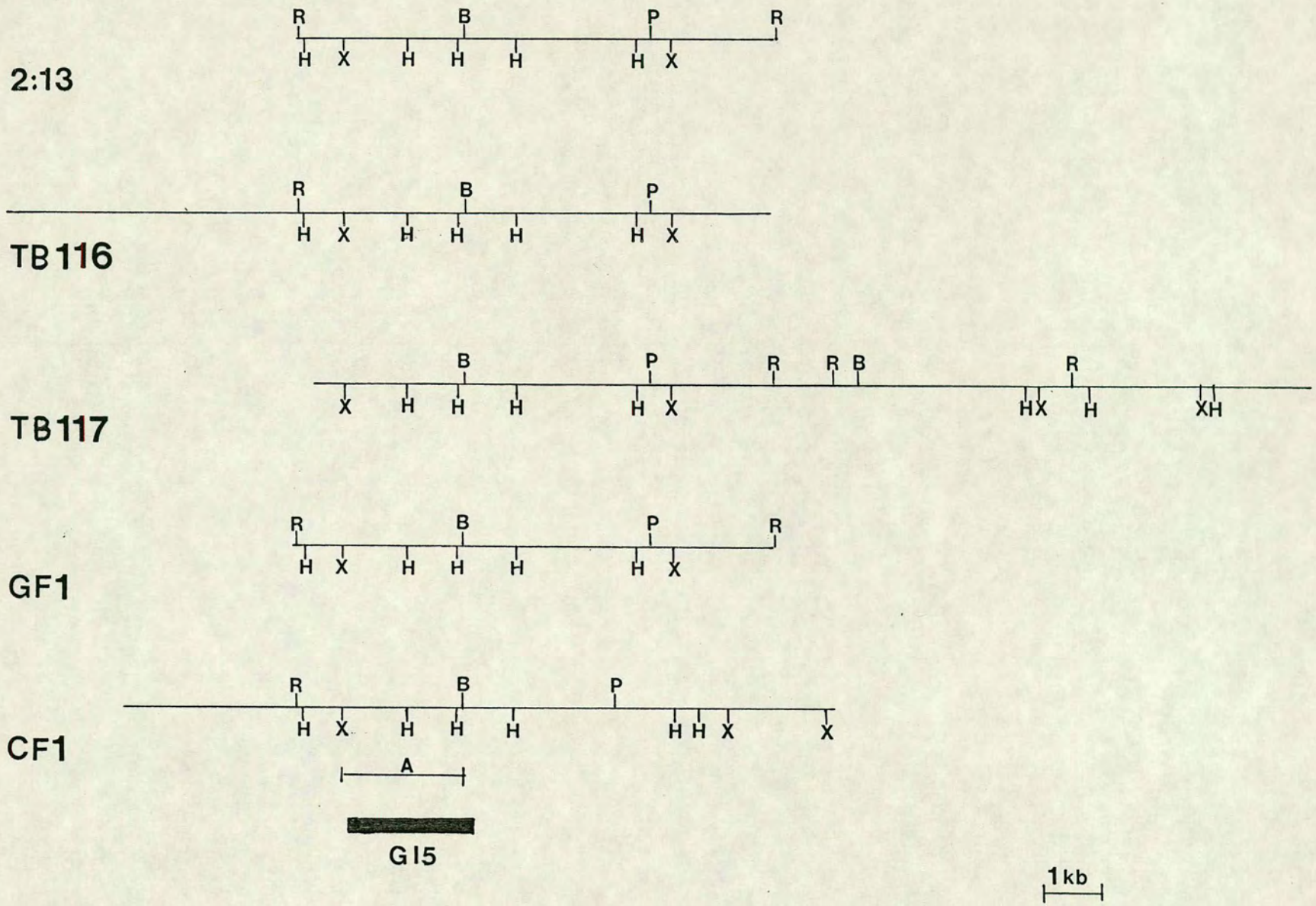
B = BamHI

H = HindIII

P = PstI

R = EcoRI

X = XbaI


2:13
R
B
P
R
H
X
H
H
H
H
X
TB116
R
B
P
H
X
H
H
H
H
X
TB117
B
P
R
R
B
R
X
H
H
H
H
X
HX
H
XH
GF1
R
B
P
R
H
X
H
H
H
H
X
CF1
R
B
P
H
X
H
H
H
H
H
X
X
A
GI5
1kb

HindIII fragments towards the right hand end. These are the source of the greater fragment size observed in chimpanzee DNA as compared to human DNA, when EcoRI digests are hybridised with G15 (Fig. 5.1).

The 2kb Bam-Xba fragment (labelled A in Fig. 5.3) held in common between the chimpanzee and human clones, was subcloned into M13, mp18 and sequenced in from the Xba site to compare the DNA sequence with that derived from the human clones (Fig. 5.4). ~200bp of sequence were determined. In contrast to the 100% sequence homology observed between the human X and Y 2:13 sequences, 2% sequence divergence was found between the chimpanzee and human clones. This is the average divergence which might be expected between a human and chimpanzee sequence based upon Sibley and Ahlquist's studies. The sequence and restriction site differences seen between the chimpanzee and human 2:13 related sequences support the idea that the high level of homology seen between the human X and Y loci is not due to any functional constraint on sequence divergence, the chimpanzee and human homologues having undergone significant divergence from each other. Rather the high levels of human XY homology at this locus are a reflection of the short period of time during which these sequences have been present on both human sex chromosomes.

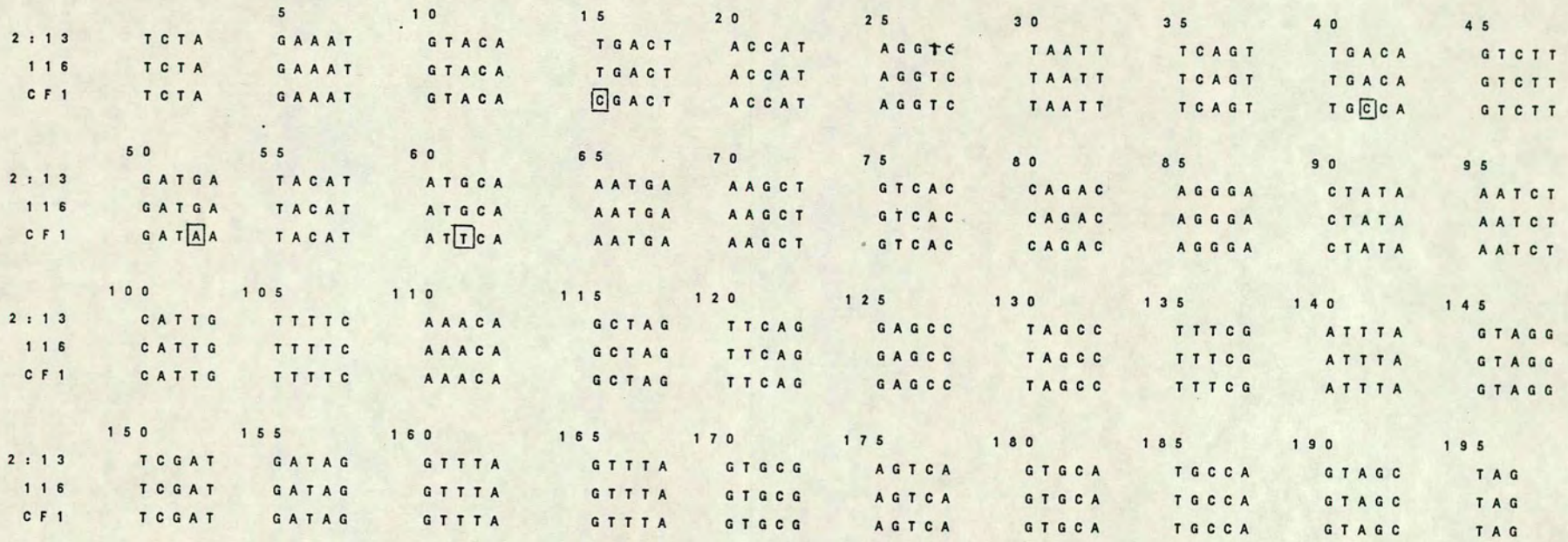
5.3. Investigation of a 2:13 gorilla homologue

2:13 homologous sequences are present in gorilla genomes, visualised as 8.5 and 10.5kb EcoRI fragments in Southern hybridisation (Fig. 5.1). To clone these two fragments, which are probably allelic, the same female gorilla DNA was used as for Southern analysis, since this individual carries both the 8.5 and

FIGURE 5.4.

Comparison of 2:13 related sequences from the human X and Y chromosomes and the chimpanzee X chromosomes

The 2kb Bam-Xba fragments (labelled as A in Fig. 5.3) from clones λY2:13, TB116 and CF1 were subcloned into M13 mp18 and sequenced in from the XbaI site. ~200 nucleotides of sequences are shown. No base differences are detected between the sequences deriving from the human X and Y chromosomes. The chimpanzee clone shows 2% divergence from the human sequence.


5 10 15 20 25 30 35 40 45
2:13 TCTA GAAAT GTACA TGACT ACCAT AGGTC TAATT TCAGT TGACA GTCTT
116 TCTA GAAAT GTACA TGACT ACCAT AGGTC TAATT TCAGT TGACA GTCTT
CF1 TCTA GAAAT GTACA CGACT ACCAT AGGTC TAATT TCAGT TGCCA GTCTT
50 55 60 65 70 75 80 85 90 95
2:13 GATGA TACAT ATGCA AATGA AAGCT GTCAC CAGAC AGGGA CTATA AATCT
116 GATGA TACAT ATGCA AATGA AAGCT GTCAC CAGAC AGGGA CTATA AATCT
CF1 GATAA TACAT ATTCA AATGA AAGCT GTCAC CAGAC AGGGA CTATA AATCT
100 105 110 115 120 125 130 135 140 145
2:13 CATTG TTTTC AAACA GCTAG TTCAG GAGCC TAGCC TTTCG ATTTA GTAGG
116 CATTG TTTTC AAACA GCTAG TTCAG GAGCC TAGCC TTTCG ATTTA GTAGG
CF1 CATTG TTTTC AAACA GCTAG TTCAG GAGCC TAGCC TTTCG ATTTA GTAGG
150 155 160 165 170 175 180 185 190 195
2:13 TCGAT GATAG GTTTA GTTTA GTGCG AGTCA GTGCA TGCCA GTAGC TAG
116 TCGAT GATAG GTTTA GTTTA GTGCG AGTCA GTGCA TGCCA GTAGC TAG
CF1 TCGAT GATAG GTTTA GTTTA GTGCG AGTCA GTGCA TGCCA GTAGC TAG

10.5kb fragments.

Female gorilla DNA was digested with EcoRI and 8-12kb fragments purified by gel electrophoresis and elution of the desired size range of DNA onto DEAE paper. The size selected DNA was then cloned into EcoRI digested, dephoshorylated λgt WES and packaged. 5×10^5 recombinant phage plaques were screened with G15 at moderate stringency and 5 hybridising plaques purified. The DNAs from these phage were analysed with EcoRI. It was expected that both the 8.5 and 10.5kb EcoRI fragments homologous to G15 in the gorilla genome, would be represented amongst these 5 clones since these 2 alleles are present at the same copy number in the female gorilla DNA. However, all 5 were found to contain 8.5kb EcoRI inserts. The 10.5kb EcoRI fragment may have been too large for cloning into λgt WES, although in theory the cloning capacity of this vector extends up to 13kb. Male gorilla DNA containing only the 10.5kb allele was therefore digested with EcoRI and cloned into Charon 36 and EMBL4 vectors (cloning capacities 7-22kb, and 9-25kb respectively). 6×10^5 recombinants from each library were screened with G15, but no hybridising plaques were identified. Thus it seems that in contrast to the ease with which the 8.5kb fragment in gorilla DNA homologous to G15, was cloned, the 10.5kb fragment appeared to be refractory to the cloning strategies employed here. This could be due to features of the DNA sequence which might make it poisonous to E.coli cells, or susceptible to rearrangement or deletion during cloning.

The gorilla clone homologous to G15 and containing an 8.5kb EcoRI insert in λgt WES, was termed λGF1. This clone was restriction mapped by multiple enzyme digestion and hybridisation of the digests to G15 (Fig. 5.2). The restriction map compiled from

this data is shown in Fig. 5.3. As can be seen, at this level of analysis the gorilla sequences homologous to G15 and contained within an 8.5kb EcoRI fragment appear to be identical to the human X and Y G15 related sequences. It was considered that no further insight into 2:13 evolution was to be gained by sequencing of λGF1.

A scenario can now be compiled tracing the chromosomal location and nature of 2:13 sequences through the evolution of the gorilla, chimpanzee and human species from a common ancestor about 10 million years ago, based upon Sibley and Ahlquist's branching order for these species.

In a common ancestor to the hominoid primates 2:13 sequences would have been present on the X chromosome in at least two polymorphic forms represented by 8.5 and 10.5kb EcoRI fragments. When the lineage giving rise to the gorilla branched off, both polymorphisms were carried on the X chromosomes giving rise to present day gorillas. Not enough individuals have been examined to determine the frequency of each polymorphic form in gorillas, but of 4 gorilla X chromosomes examined by Southern hybridisation 3 carry the 10.5kb EcoRI fragment hybridising to G15. 6-8 million years ago the human and chimpanzee lineages diverged. Of 4 chimpanzee X chromosomes examined, all carry the 10.5kb EcoRI fragment. The 8.5kb allele may be present in the present day chimpanzee population but was not detected in these studies.

Within the last few million years, in the lineage giving rise to the human species, 2:13 sequences on the long arm of the X chromosome were duplicated onto the Y. This transposition event seems to have involved only the 8.5kb allele of this sequence as out of a total of 28 human X chromosomes and 13 human Y chromosomes

examined during the course of this work, by Southern hybridisation, all show only 8.5kb EcoRI fragments hybridising to G15.

The presence of 2:13 sequence XY homology in all human males examined (probably all Caucasian) has implications concerning the origin of the human species. DNA which is not segregated randomly at meiosis is useful for the study of population genetics. Human mitochondrial DNA, which is maternally inherited, and has a higher rate of evolution than nuclear DNA, has therefore been used for tracing the origins of various ethnic groups. Stoneking et al (1986) have estimated that all known human mitochondrial DNA lineages can be traced back to one ancestral woman in existence 140,000-300,000 years ago, probably in Africa.

The human Y chromosome (excluding the pseudoautosomal region) is analogous to mitochondrial DNA in its behaviour at meiosis, it being transmitted to all male progeny only, and is monosomic thus preventing transfer of genetic material between Y chromosomes. Therefore unless the transposition event transferring 2:13 sequences onto the Y chromosome occurred independently several times during the course of human evolution, all present day human Y chromosomes must be derived from one ancestral chromosome onto which 2:13 sequences had been transposed from the X chromosome. Thus the human population may be descended ultimately from one ancestral man and woman, the so-called Adam and Eve hypothesis.

CHAPTER 6

STUDIES ON THE SCALE OF HOMOLOGY OBSERVED BETWEEN THE HUMAN SEX CHROMOSOMES AT THE 2:13 LOCUS

Speculation as to the nature of the mechanism which brought about transposition of 2:13 sequences from the X to the Y chromosomes, would be assisted by knowledge about the scale of the event. Clearly mechanisms for transposing small stretches of DNA about the genome will differ from those involving much larger amounts of chromosomes.

Prokaryotes and eukaryotes contain many DNA sequences which are able to move around the genome. In mammals these sequences include endogenous retroviruses and retroposons. This latter class (Rogers, 1985) represents mRNAs which are thought to have been reverse transcribed in the germ line and inserted back into the genome at a new location. Retroposons are characterised by the presence of poly A^+ tails, precise excision of introns from the original gene, and are flanked by short direct repeats of the insertion site. Examples of retroposons include processed pseudogenes for RNA polymerase II transcribed genes, such as human methallothionein II (Karin and Richards, 1982) and the moderately dispersed repeats such as the human Alu/mouse B1 and human Kpn/mouse MIF sequences. Retroposons derived from RNA polymerase II genes (with 5' upstream promoters) lose their promoters upon retrotransposition. The dispersed repeats however contain internal RNA polymerase III promoter sequences, thus are able to go through multiple rounds of retrotransposition, leading to their high copy number and dispersed nature in mammalian genomes (Rogers, 1985).

It is known that the human sex chromosomes do contain RNA polymerase II processed pseudogenes such as those for arginosuccinate synthetase (Daiger et al, 1982) and cytoskeletal actin (Heilig et al, 1984). In both of these cases both the X and Y

linked sequences are pseudogenes and, like most RNA polymerase II transcribed processed pseudogenes, are part of multigene families. Many restriction site differences are also detectable between the X and Y loci, and the homology at these loci extends for <50kb. These types of XY homologous sequences do not therefore resemble the type of homology observed at the 2:13 loci.

Mechanisms which might bring about transposition of large stretches of DNA between chromosomes most likely involve some form of recombination. In this case the region transferred may extend for many thousands of kilobases.

6.1. Cosmid cloning of 2:13 sequences from the human X chromosome

To analyse the scale of XY homology around 2:13 on a larger scale than that found by cloning into phage λ vectors, a cosmid library of ThyB1 cell line DNA was made in pJB8, thus allowing 30-45kb inserts to be cloned.

Steps were taken in the preparation of this library to prevent a) vector to vector ligation which may give rise to cosmids containing multiple vector and insert sequences and, b) self ligation of target DNA giving rise to cosmids containing non-contiguous pieces of genomic DNA.

Ish-Horovicz and Burke (1981) have described a method of preparing pJB8 vector to prevent possibility a). pJB8 DNA (Fig. 2.1) was digested with HindIII and SalI and the digests dephosphorylated. These digests were then cut with BamHI and the fragment from each digest containing the cos site was purified from a gel using DEAE paper. This results in left and right hand vector

fragments which cannot individually ligate together but can ligate either to each other or to target DNA. Only molecules with 2 cos sites separated by 35-50kb of target DNA can be packaged however. At all stages of vector preparation controls were carried out to ensure complete digestion and dephosphorylation had occurred where appropriate and that fragments were ligatable where this was desired.

ThyB1 cells (Section 2.1.1) were grown in culture, under HAT selection, harvested and the cells immediately used for DNA preparation as described in Section 2.2.1, care being taken to minimise shearing of the DNA. DNA was digested with MboI at a concentration of 0.1mg/ml at 37°C for 20 minutes, using 0.0005, 0.001, 0.003 and 0.005 units of enzyme/µg of DNA.

These partial digests together with undigested DNA and λ/H3, λ/SalI and λ oligomers as size standards, were run on a pulsed field gradient gel employing a short pulse time (12s) in order to ascertain the conditions resulting in optimal numbers of partial digest fragments in the 35-50kb size range (data not shown). 200µg of DNA were then digested under the above conditions with 0.001 units of enzyme/µg DNA and the digest size-fractionated on a sucrose density gradient. Fractions containing DNA of the appropriate size range were pooled, dialysed and resuspended in 100µl TE. This DNA was then dephosphorylated. Both the size fractionation and dephosphorylation procedures ensure that non-contiguous fragments of target DNA do not become ligated into the same cosmid and packaged, a situation which is obviously to be avoided.

pJB8 arms prepared as described and the size selected ThyB1 DNA were ligated together at a 4:1 molar ratio, packaged into λ <u>in vitro</u>

packaging extracts and plated on ED8767. 10^6 recombinant cosmids were obtained /µg of DNA packaged. 2×10^5 recombinants were plated onto nitrocellulose on four 20 x 20cm H-ampicillin plates using a suction device to ensure even spreading of cells. Prior to plating, the nitrocellulose filters were washed three times in boiling water to remove detergent which would otherwise reduce the plating efficiency. The cosmids were grown for 10 hours at 37°C until very small colonies appeared, then duplicate replica filters taken as described in Section 2.12.1. These filters were hybridised with G15. Even though G15 consists of the SalI-SmaI insert of pG15 (Section 3.1) purified away from pUC9 sequences, enough plasmid DNA is present to allow for cross-hybridisation to pJB8 sequences in all the cosmids. Therefore after radiolabelling G15, 1µg of BamHI digested pUC9 was added together with 25µl of 20 x SSC, and TE added to bring the volume up to 100µl. The DNA was then denatured by boiling for 10 minutes, cooled on ice and allowed to reassociate for 10 minutes at 68°C prior to adding to the hybridisations. This procedure allows signal due to vector DNA to be competed out by the large excess of 'cold' pUC9 DNA whilst preserving the signal from the insert (Sealey et al, 1985).

Two cosmids termed XA1 and XB1, hybridising strongly to G15, were isolated and their DNA prepared by standard procedures. EcoRI digests of these cosmids (Fig. 6.1A) revealed that they overlapped by >82%, only 2 fragments being different in each case. The cosmids were mapped by linearising the DNAs with PvuI followed by UV induced partial digestion with EcoRI (Section 4.1). These partial digests were blotted and probed with either the large or small PvuI/EcoRI fragment of pJB8 (see Fig. 2.1). This data (Fig. 6.1.B) in

FIGURE 6.1

Restriction mapping of cosmids XA1 and XB1

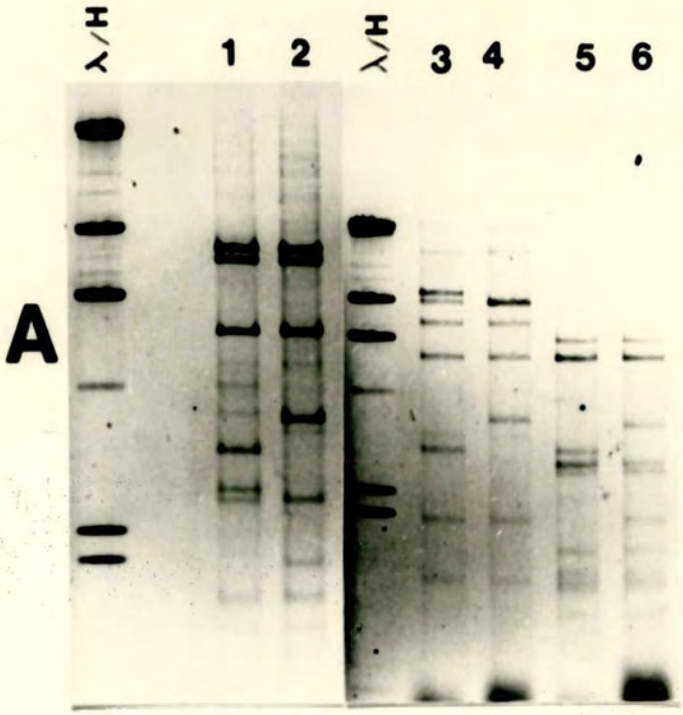
A Digests of XA1 and XB1 DNAs.

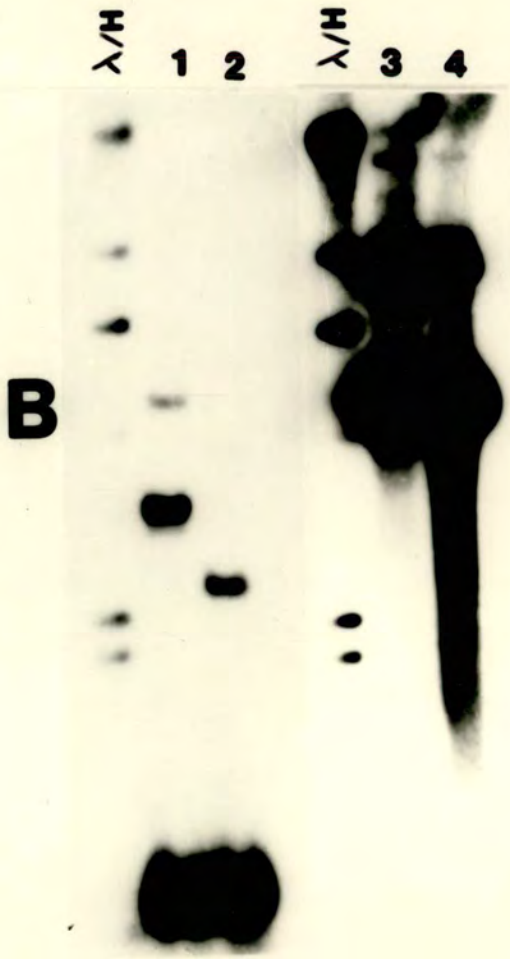
Track 1 XA1 digested with EcoRI
" 2 XB1 digested with EcoRI
" 3 XA1 digested with BamHI
" 4 XB1 digested with BamHI
" 5 XA1 digested with EcoRI + BamHI
" 6 XB1 digested with EcoRI + BamHI

B XA1 and XB1 DNAs were digested completely with PvuI then partially with EcoRI, transferred onto nitrocellulose and hybridised to either the small (0.5kb) or large (5kb) PvuI/EcoRI fragment of pJB8.

Track 1 XA1 hybridised to the 0.5kb pJB8 PvuI/EcoRI fragment
2 XB1 hybridised to the 0.5kb pJB8 PvuI/EcoRI fragment
3 XA1 hybridised to the 5kb pJB8 PvuI/EcoRI fragment
4 XB1 hybridised to the 5kb pJB8 PvuI/EcoRI fragment

λ /HindIII markers are shown (23.5, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6kb).


λ/H
1
2
λ/H
3
4
5
6
A


λ/H
1
2
λ/H
3
4
B

combination with the size of fragments generated by complete digestion of XA1 and XB1 with EcoRI, BamHI and EcoRI + BamHI allow a restriction map of the region surrounding the 2:13 locus on the human X chromosome to be constructed, extending for 32kb. This is extended to 37kb by λTB117 which extends 4kb beyond the region covered by XA1/B1 (Fig. 6.2).

To establish whether homology to the human Y chromosome is detected by the 2 and 4kb EcoRI fragments at the extreme ends of the insert in XB1 (corresponding to the 2.75 and 3.75kb EcoRI fragments at the ends of XA1 respectively) these fragments were cut out of a low gelling temperature agarose gel and oligolabelled to use as probes to human male and female DNA and to DNA from hybrids containing the human sex chromosomes. It was found, however, that low copy number signal was obscured from these probes by repeated DNA sequences within them. The radiolabelled probes were therefore reassociated with 1mg of sonicated human DNA, to Cot 10 before adding to the hybridisation mix, to strip out signal from the repeated components of the probes (Sealey et al, 1985). Figure 6.3 illustrates that XB1 2kb and 4kb EcoRI fragments detect 12.0 and 4.0kb EcoRI fragments respectively in human DNA with no sex-linked dosage which might be expected of a sequence from the X chromosome. These fragments are also detected in hybrid cell lines containing either the human X or Y chromosomes as their only cytologically detectable human material. XY homology therefore extends for at least 47kb surrounding the 2:13 sequence. (The terminal 4kb fragment of λTB117 could not be used as a probe as it is not resolvable from the adjacent 4kb fragment in the clone, so cannot be isolated from a gel.) The two terminal EcoRI fragments of XB1 were

FIGURE 6.2

Restriction map of cosmids XA1 and XB1

Restriction map, showing BamHI and EcoRI sites, of overlapping cosmids XA1 and XB1 derived from the human X chromosome, together with the relationship of these cosmids to the clones λ2:13, TB116 and 117 and pG15.

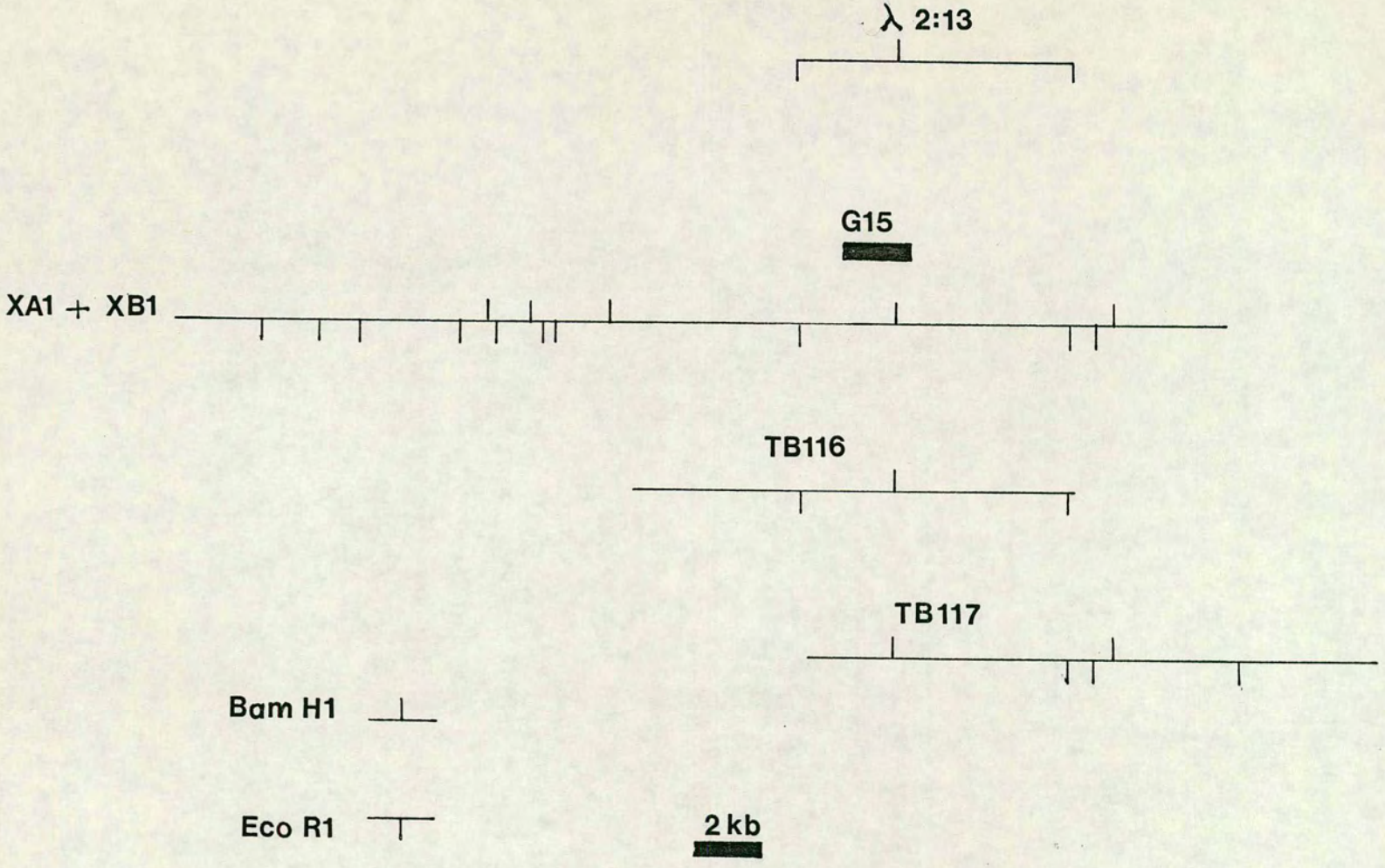
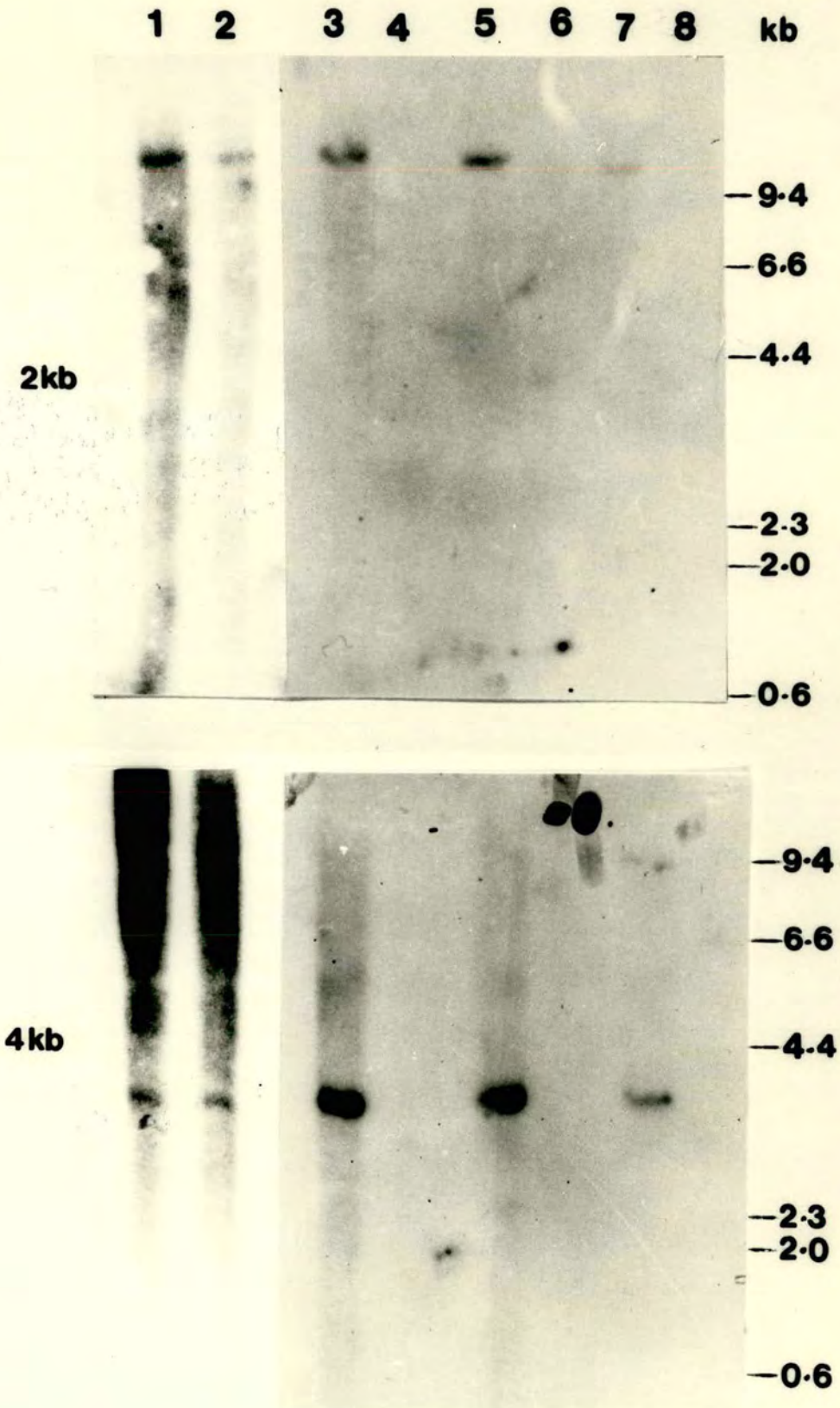

λ 2:13
G15
XA1 + XB1
TB116
TB117
Bam H1
Eco R1
2 kb

FIGURE 6.3

Hybridisation of the terminal EcoRI fragments of cosmid XB1 to the human X and Y chromosomes

DNAs were digested with EcoRI, transferred onto a nylon membrane and hybridised with the 2kb and 4kb terminal EcoRI fragments of cosmid XB1. Probes were stripped prior to hybridisation to remove signal from repeated sequences within these fragments.

Track 1 ♀ DNA
2 ♂ DNA
3 ThyB1 (mouse/human hybrid retaining the human X chromosome)
4 Mouse DNA
5 3E7 (mouse/human hybrid retaining the human Y chromosome)
6 3E7 rev (Revertant of 3E7 which has lost the human Y chromosome)
7 853 (Chinese hamster/human hybrid retaining the human Y chromosome)
8 853 rev (Revertant of 853 which has lost the human Y chromosome).


1 2 3 4 5 6 7 8 kb
9·4
6·6
4·4
2kb
2·3
2·0
0·6
9·4
6·6
4kb
4·4
2·3
2·0
0·6

used to reprobe the ThyB1 cosmid replica filters used above. No positive colonies were identified except for those containing XA1 and XB1 cosmids. At this stage it was considered unproductive to try to 'walk' (Bender et al, 1983) using cosmids, further out from the 2:13 locus, for several reasons. Chromosome walking is a slow and tedious process. In theory each step may progress the walk by ~30kb but in practice ~15kb is probably nearer the mark. It was surprising to find such a high degree of overlap between the 2:13 homologous cosmids XA1 and XB1. These cosmids were derived from DNA partially digested with the 4 base pair recognition site enzyme MboI. This enzyme should therefore generate essentially random fragments but I consider it likely that certain sites in the genome are cleaved preferentially over others, indeed this is known to be the case for some enzymes (New England Biolabs, 1985). Another factor influencing the representation of genomic libraries may be that certain DNA sequences are unclonable in E.coli, or incompatible with vectors used (P. Little, pers. comm.).

Walking out from the 2:13 locus may enable 100-200kb of DNA to be examined for XY homology. This is not a significantly larger scale of magnitude than 50kb. Pulsed field gradient gel electrophoresis has therefore been employed to try to extend the analysis over many hundreds of kilobases.

6.2. Long range analysis of 2:13 sequence XY homology using pulsed field gradient gel electrophoresis

Pulsed field gradient gel electrophoresis enables large DNA molecules to be resolved (Carle and Olson, 1984; Schwartz and

Cantor, 1984). In turn mammalian genomic DNA can be digested into large fragments by infrequently cutting enzymes, thus allowing Southern hybridisation of DNA to reveal hybridising bands containing many hundreds of kilobase pairs of DNA. This approach has been used to analyse the size of large restriction fragments hybridising to G15 from human X and Y chromosomes, in order to assess whether these chromosomes are still homologous at the 2:13 loci, on the basis of restriction fragment size, over a long stretch of DNA.

Sites for enzymes with a 6bp recognition sequence containing one or more CpG dinucleotides are rare in the mammalian genome (see Section 1.1.3). CpG residues however are clustered in 'HTF islands' (Bird, 1986) and are generally unmethylated at these sites. Cleavable sites for methylation sensitive CG enzymes should therefore be located primarily in these islands. Rare inter-island sites may be methylated and therefore not cleaved. Indeed it has been estimated that three-quarters of sites for SacII (5' CCGCGG) in the mammalian genome are located in 'HTF islands' (Brown and Bird, 1986).

No sites for NaeI, PvuI, SacII or SmaI (all enzymes recognising CpG) were found in XB1. Thus it was not possible to map out from 2:13 sequences to the next sites for these enzymes. Instead only bands hybridising to G15 could be detected without knowing the relative position of the probe within those fragments.

ThyB1 and 3E7 cell hybrid DNAs in blocks of agarose (see Section 2.2.3) were digested with SacII. This enzyme was chosen primarily because such a high proportion of its genomic cleavage sites are thought to be located in islands. I therefore considered that the use of this enzyme might reduce any possible confusion in

interpretation of the data, due to the differential methylation of the human X and Y chromosomes in the cell lines used. This is assuming that HTF islands are not methylated on the X and Y chromosomes in cell hybrids. SacII was also found to be of reproducible quality from one batch to another; this was not true of other enzymes tried such as Sfi which either did not cleave, or degraded the DNA. Digested DNA was then subject to orthogonal field alternating gel electrophoresis (OFAGE), transferred onto nylon membranes and hybridised with G15. Fig. 6.4 illustrates the results of these experiments. Gels which resolve DNA up to 650kb result in G15 hybridising bands in ThyB and 3E7 DNAs located at the top of the gel in a size range of DNA molecules which is not resolved under the pulse regime employed (Fig. 6.4A). The hybridisation signal is not the result of non-specific hybridisation due to the large amounts of DNA at the top of the gel, as no hybridisation is found in the control tracks containing DNA incubated in the absence of enzyme. To try to resolve these large bands ThyB SacII digested DNA was run on OFAGE using longer pulse times which increase the upper limit of resolution of the DNA. Fig. 6.4B illustrates that on a gel in which DNA of up to ~1000kb is resolved, two bands hybridising to G15 are present in ThyB DNA, one of 700kb and the other ~1000kb. This latter band could be due to the digests being partial, or to partial methylation of SacII sites in the DNA. When ThyB and 3E7 SacII digested DNAs were run together (Fig. 6.4C) using the same pulse time as in Fig. 6.4B, the upper limit of resolution of the gel was found to be lower than that in Fig. 6.4B. In general the size range of DNA resolved on gels under conditions of constant pulse times, was very irreproducible from run

FIGURE 6.4

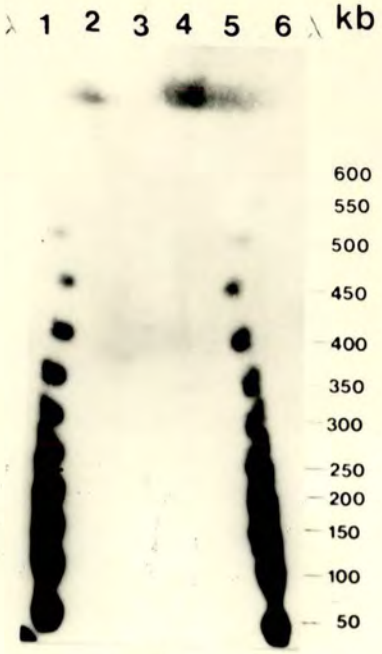
Analysis of the human X and Y 2:13 loci by pulsed field gradient gel electrophoresis

Human x mouse cell hybrid DNAs 3E7 and ThyB1 (which carry the human Y and X chromosome respectively) were digested in agarose blocks with SacII and run on OFAGE with various pulse times for 24 hrs. Gels were transferred onto nylon membranes and hybridised with G15 and a small amount of radiolabelled λ DNA to allow visualisation of λ oligomer size markers.

A: Track 1 3E7 control (no enzyme)
2 3E7 digested with SacII for 2 hrs.
3 blank
4 ThyB1 digested with SacII for 4 hrs.
5 ThyB1 digested with SacII for 2 hrs.
6 ThyB1 control (no enzyme)
pulse time 35s.

B: Track 1 ThyB1 digested with SacII for 2 hrs.
2 ThyB1 digested with SacII for 4 hrs.
pulse time 40s.

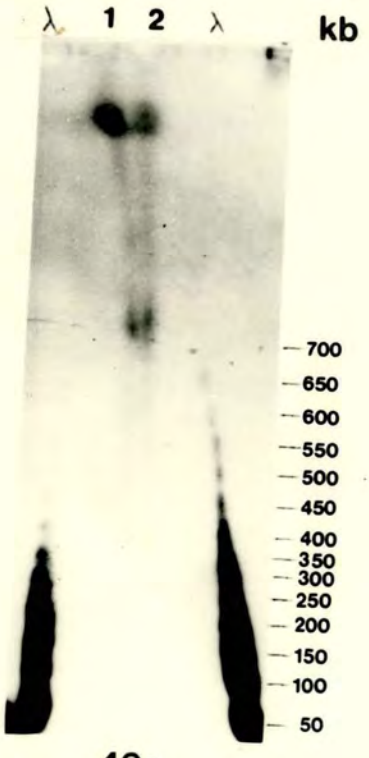
C; Track 1 3E7 control (no enzyme)
2 3E7 digested with SacII for 2 hrs.
3 3E7 digested with SacII for 4 hrs.
4 ThyB1 control (no enzyme)
5 ThyB1 digested with SacII for 2 hrs.
6 ThyB1 digested with SacII for 4 hrs.
pulse time 40s.



A

35s

B



40s

C

40s

to run. Parameters such as buffer temperature seem to have very profound effects on the results obtained. Nevertheless the limit of resolution of Fig. 6.4C is sufficiently large for differences to be seen in the hybridisation signal from ThyB and 3E7 DNA. Hybridisation to 3E7 DNA is seen only in the unresolved DNA, no 750kb hybridising band is visible. Provided this difference in restriction fragment size between the human X and Y chromosomes in hybrid cells, is not due to differences in the methylation of SacII sites in the two cell lines used, XY homology must break down within 750kb of 2:13. More extensive study would be needed to establish whether homology broke down on both sides of 2:13 sequences, or on only one side, homology extending further along the chromosomes on the other side.

It was slightly surprising to find that the SacII fragments from the human X and Y chromosomes, hybridising to G15, were so large. HTF islands have been correlated with the 5' ends of many genes particularly those coding for 'housekeeping' functions of which it is estimated there may be 20,000-30,000 in the genome. This would suggest that the average spacing of HTF islands in the genome, and therefore sites of cleavage for CG enzymes should be 100kb (Bird et al, 1985). Indeed when random mouse HTF islands were mapped (Brown and Bird, 1986) the average distance between islands was 109kb. It is possible, however, that 'islands' are not distributed evenly throughout the genome, but are clustered together, therefore leaving regions of chromosomes barren of 'islands' (and therefore genes?). The human X and Y chromosomes may be interesting in this respect. In a search for HTF islands on the human X chromosome the average incidence of sites for CG enzymes

such as SacII was every 250kb (S. Lindsay, pers. comm.) and a study of HTF islands around the Duchenne muscular dystrophy locus (Fig. 1.1) revealed none within 500kb of the probe PERT87 (Monaco et al, 1985; R. Anand, pers. comm.). The human Y chromosome seems to be a more extreme case. Screening of human cosmids derived from the 3E7 cell line showed that enzyme sites diagnostic for HTF islands were found at a frequency of <1/1,500kb (G. Rappold, pers. comm.). If HTF islands are associated with all housekeeping genes it may not be surprising to find few located on the Y chromosome as this chromosome is condensed and may therefore be largely inactive in somatic cells.

Given that 2:13 sequence homology on the human X and Y chromosomes extends for at least 50kb and possibly several hundred kb, I consider that the most likely mechanism for the transposition of 2:13 sequences from the X to the Y chromosome during recent evolutionary time was a sporadic crossing over event in the sex vesicle at meiosis , possibly mediated via dispersed repeats on both chromosomes. I have suggested here that XY homology does not extend for >750kb in at least one direction from 2:13. Indeed 2:13 has been localised to the same X chromosomal region (Xq26-q27) (see Section 3.3) as X specific DNA such as the HPRT gene. 2:13 sequences must therefore be within 5,000-10,000kb of the breakdown of XY homology on this basis alone.

CHAPTER 7

THE 'PSEUDOAUTOSOMAL' REGION OF THE HUMAN SEX CHROMOSOMES

I have demonstrated that extensive homology exists between the long arms of the human X and Y chromosomes. This is not in the region thought to be involved in pairing and crossing over of these chromosomes at meiosis (see Section 1.3). Sequences located in the pairing segment and distal to the site of any obligate cross-over, should be transmitted to offspring in an autosomal-like manner (i.e. not sex linked) termed 'pseudoautosomal' (Burgoyne, 1982). The presence of such pseudoautosomal sequences at the tips of the short arms of the human sex chromosomes has now been directly demonstrated (Cooke et al, 1985; Simmler et al, 1985). These groups have isolated clones which map to the tips of the short arms of both the human X and Y chromosomes. Probes derived from these clones (29C1, 113D and 601), are highly polymorphic and define pseudoautosomal loci DXYS14, 15 and 17 respectively. Probe 29C1 (Cooke et al, 1985) detects a smear of 18-25kb in hybridisations to male and female blood DNA digested with BamHI. This heterogeneous hybridisation signal is sensitive to degradation by Bal31 exonuclease, indicating that 29C1 is detecting a natural end of a chromosome (the telomere).

Studies on the inheritance of alleles of 29C1, 113D and 601, in families, shows that they are not transmitted in a sex-linked manner but undergo exchange between the sex chromosomes at meiosis. For example a father does not transmit his Y chromosome alleles to all of his sons, some sons inherit their father's X chromosome alleles. Conversely, some daughters inherit alleles from the fathers' Y chromosome.

In order to determine the frequency with which the pseudoautosomal probes recombine, RFLPs detected by these probes were studied in eight large families (Rouyer et al, 1986). DXYS14

was found to recombine most frequently showing 50% recombination and therefore no sex linkage. DXYS15 showed 32% recombination and therefore partial sex linkage. A recombination involving this locus always involved DXYS14 as well. Similarly DXYS17 showed 14% recombination, both DXYS14 and 15 loci co-segregating with all DXYS17 recombinants. Therefore these pseudoautosomal loci do not recombine independently at meiosis. Their co-segregation is incompatible with recombination operating via gene conversion, which is thought to operate on short stretches of DNA and would thus lead to independent segregation of each locus.

The genetic studies on DXYS14, 15 and 17 are consistent with a single obligate X/Y cross over at meiosis occurring at different locations in each meiosis but nearly always proximal to DXYS14. No double recombinants were found and recombination frequencies in the region added up without distortion, indicating the absence of multiple cross overs per meiosis.

The recombination frequency between DXYS14 and 17 in male meiosis is nine times greater than in female meiosis. The pseudo-autosomal region in male meiosis is thus a recombinational hotspot. This is probably due to the small chromosomal region available for crossing over between the X and Y chromosomes. Although the human X and Y chromosomes have been observed to pair over much of the length of the Y chromosome, the pseudoautosomal region in which recombination must take place is thought to extend for ~5,000kb (Rouyer et al, 1986). If crossing over were to take place outside of this region this could lead to transfer of Y-specific material onto the X chromosome or to acentric or dicentric chromosomes. The former case may explain the sporadic appearance of phenotypic males with an XX karyotype (see Chapter 9). In female meiosis crossing over may occur anywhere over the entire length of the chromosome ~120,000kb.

Male and female gorilla DNAs were probed with 29C1, the pseudo-autosomal probe detecting the DXYS14 locus close to the human X and Y short arm telomeres, and with 29A1, a probe from the same cosmid as 29C1 but more proximal. Fig. 7.1 shows the results of these experiments. 29C1 detects two weakly hybridising polymorphic bands in EcoRI digests of male and female gorilla DNAs. 29A1 detects a band, smear or both in human EcoRI digested DNAs. The smear in this case is not sensitive to Bal31 digestion and the molecular basis of this heterogeneity is not understood (H.J. Cooke, unpublished data). 29A1 detects 6 weak bands in an EcoRI digest of DNA from a female gorilla and 2 bands in a male gorilla. Thus like the loci detected by these probes in human DNA the gorilla sequences appear to be

FIGURE 7.1

Hybridisation of human pseudoautosomal probes to human and gorilla DNA

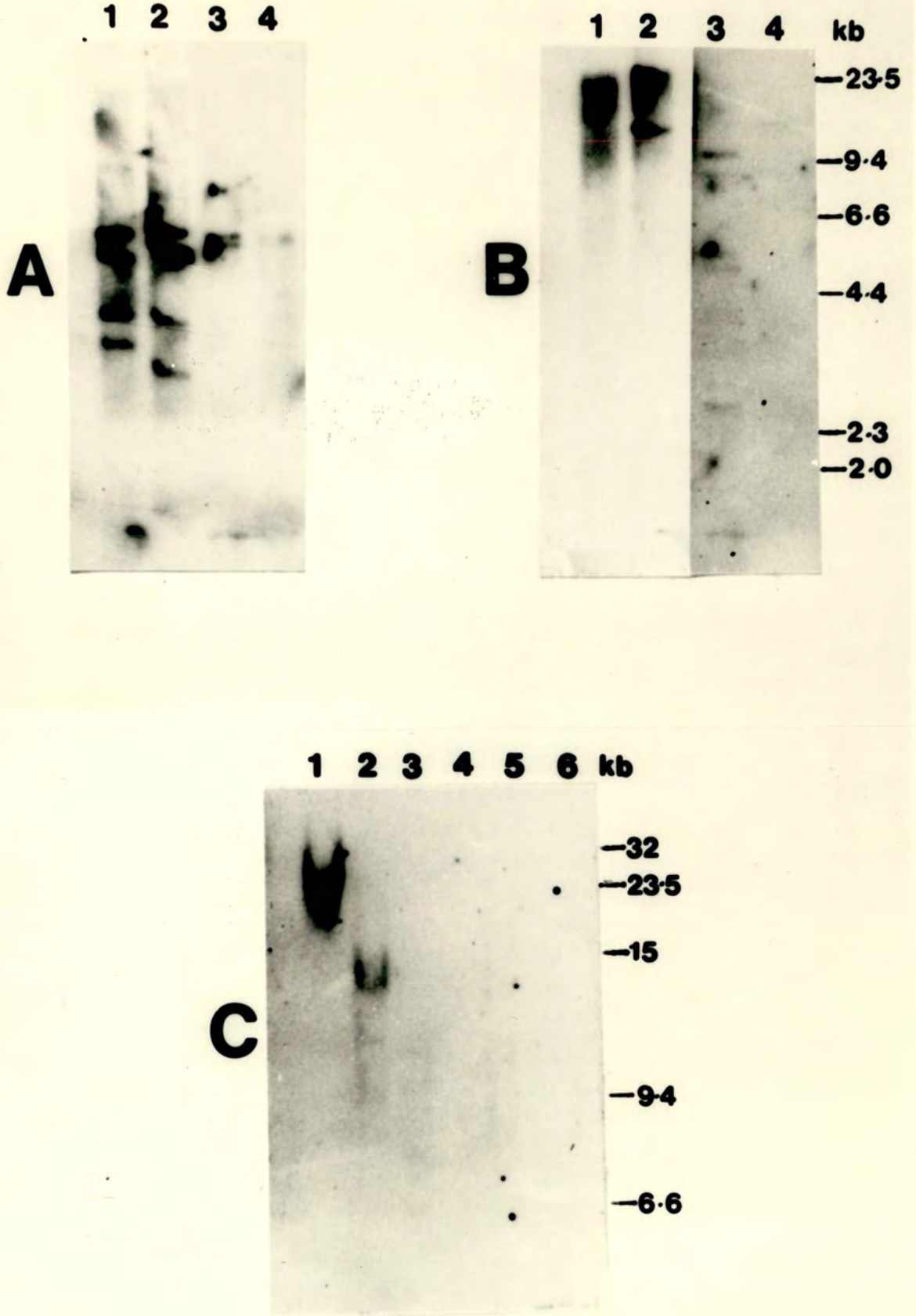
A and B: DNAs were digested with EcoRI, run on a 0.8% agarose gel, transferred to a nylon membrane and hybridised with A: 29C1 and B: 29A1. Filters were washed in 0.1 x SSC, 60°C.

Track 1: Human ♀
2: Human ♂
3: Gorilla ♀
4: Gorilla ♂

C:

Track 1: Human ♂ DNA digested with BamHI
4: Gorilla ♂ DNA digested with BamHI
2: Human ♂ DNA digested with Bal31 (0.2u/µg) for 0.5 hr followed by BamHl digestion
5: Gorilla ♂ DNA digested with Bal31 (0.2u/µg) for 0.5 hr followed by BamHI digestion
3: Human ♂ DNA digested with Bal31 (0.2u/µg) for 1.5 hr followed by BamHI digestion
7: Gorilla ♂ DNA digested with Bal31 (0.2u/µg) for 1.5 hr followed by BamHI digestion

DNAs were run on a 0.5% agarose gel, transferred to nitrocellulose and hybridised with 29C1. Filters were washed in 0.1 x SSC, 60°C.


A
1 2 3 4
B
1 2 3 4 kb
23·5
9·4
6·6
4·4
2·3
2·0
C
1 2 3 4 5 6 kb
32
23·5
15
9·4
6·6

highly polymorphic. When male human and gorilla DNAs are digested with Bal31 exonuclease followed by BamHI, a Bal31 sensitive smear is detected in human DNA (Fig. 7.1C). No signal is detectable in gorilla DNA. In Fig. 7.1A and B hybridisation of human pseudo-autosomal probes to gorilla DNA was very weak. This suggests that the gorilla sequences being detected by these probes are quite highly diverged from their human counterparts. Thus if as in human DNA, gorilla sequences homologous to 29C1 are heterogeneous in size after BamHI digestion, then they may not be detectable in a hybridisation due to their distribution over a broad size range of DNA.

In the absence of the availability of DNA from gorilla families or hybrid cell lines containing individual gorilla chromosomes, 29C1 sequences cannot be definitely assigned to the pseudoautosomal region of the sex chromosomes in this species. I consider it likely however, that the pseudoautosomal region of the human sex chromosomes is the remnant of homology shared by ancestral sex chromosomes prior to the evolution of morphologically and genetically differentiated sex bivalents. Sequences in primates, homologous to the human pseudoautosomal probes are therefore likely to be also located in the pseudoautosomal region of the sex chromosomes in these animals. The gorilla sequences detected by 29C1 and 29A1, are like the human sequences, polymorphic. Pseudo-autosomal sequences might be expected to have a high poly morphism level.

Meiotic recombination between two allelic sequences will generate a new hybrid allele, therefore pseudoautosomal sequences will evolve rapidly, crossing over at meiosis constantly shuffling

the DNA sequences in this region. This would explain the weak hybridisation of 29C1 and 29A1 to gorilla DNA.

Thus in contrast to the homology observed at the 2:13 locus on the long arms of the human X and Y chromosomes, which appears to be a recent evolutionary phenomenon, the pseudoautosomal region of the human sex chromosomes is probably an ancient homology predating the evolution of differentiated sex bivalents. The pseudoautosomal homology is necessary for chromosome pairing at meiosis, and is maintained by recombination at this stage of cell division. Such crossing over may be obligatory in order for correct disjunction of the sex chromosomes to occur at meiosis.

CHAPTER 8

CLONING OF HUMAN DNA SEQUENCES SPECIFIC ONLY TO THE Y CHROMOSOME

8.1. Increasing the rate of DNA-DNA reassociation by phenol-emulsion reassociation technique (PERT)

The work described in this thesis to date has been concerned with DNA sequences which the human Y chromosome holds in common with the X. Despite the insight which these types of sequence have been able to give us into Y chromosome evolution and the behaviour of the sex chromosomes at meiosis, it is perhaps fair to say that many of the most interesting sequences on the human Y chromosome are likely to be those low copy number sequences specific to that chromosome alone, with no homology to autosomes or the X chromosome. Such sequences may encode many important developmental genes such as those concerned with testis determination in the embryo, and spermatogenesis (see Section 1.4).

The human Y chromosome is ~30,000kb in length. At least 50% of the chromosome is composed of two repeated sequence families, DYZ1 and 2, defined by 3.4 and 2.1kb HaeIII bands in male genomic DNA (Cooke et al, 1983). These repeats are concentrated in the heterochromatic region of Yq and appear to vary in copy number from individual to individual, and presumably serve no purpose.

Of the remaining 15,000kb of Y chromosome DNA, approximately 5,000kb (Rouyer et al, 1986) is thought to comprise the pseudoautosomal region at Ypter and is therefore identical to sequences at human Xpter (see preceding chapter). Any sequences specific to the Y chromosome are thus to be found within 10,000kb of DNA.

Standard techniques of cloning human Y chromosomal DNA sequences fall into two main categories. Firstly, the construction of libraries from cell hybrids in which the Y is the only

cytological detectable human material, allows sequences derived from the Y chromosome to be isolated by screening for those clones hybridising to human DNA (Bishop et al, 1983). Secondly, libraries can be made from fractions of flow-sorted human chromosomes, enriched for the Y chromosome (Cooke et al, 1983). In the first instance human recombinants originating from non -Y DNA derive from the presence of invisible amounts of other human material in the hybrid lines, and from the presence in the mouse genome (in cases where the mouse is the genetic background of the cell line) of sequences homologous to the human Alu repeat, at a copy number of ~10,000 (R. Meehan, pers. comm.). Low recoveries of Y-derived clones in the second approach are a reflection of the purity of the sorted chromosome fraction employed in the construction of the library. Many groups (Bishop et al, 1983; Cooke et al, 1983; Wolfe et al, 1984; Burk et al, 1985; Cooke et al, 1985; Affara et al, 1986) have applied these approaches to isolate clones from the human Y chromosome. A striking feature of these studies is the low frequency with which Y specific DNA is isolated, a majority of Y derived clones sharing varying degrees of homology with the X chromosome and/or autosomes. This is shown in Table 8.1. Thus a large proportion of the human Y chromosome may be homologous to other chromosomes, particularly the X chromosome. <5,000kb of DNA may be specific to the Y chromosome alone. Isolation of human Y specific DNA is therefore rather a slow and inefficient process, clones identified as containing human DNA having to be subcloned to give single copy probes, then those probes used against panels of male and female DNAs to identify clones deriving from DNA specific to the human Y chromosome. For example, Bishop et al (1983)

TABLE 8.1. Analysis of clones derived from the human Y chromosome

Reference	Total number of clones	Number of clones detecting Y-only fragments	Number of clones detecting Y-X and/or Y-autosome homologies
Bishop et al (1984)	26	9	12 - Y-X 4 - Y-aut 1 - Y-X+aut
Affara et al (1986)	17	5	6 - Y-X 6 - Y-aut
Müller et al (1986)	15	4	11
Total number of clones in each category	58	18	40
% of total	100	31	69

screened 50,000-10,000 cosmids made from the 3E7 cell hybrid which contains the human Y chromosome on a mouse background. 248 clones were identified as containing human-derived inserts. Of these, 21 contained copies of the tandem repeats which constitute the Y heterochromatin. These cosmids were generally deleted and rearranged; tandem repeats being unstable even in recA$^-$ host (Brutlag et al, 1977). 30 cosmids were subcloned into pBR322 to generate single copy probes which were then hybridised to OX (XYYYY), male and female DNA. Only 6 probes identified bands specific to male DNA, and therefore present only on the Y chromosome. The remaining 24 probes detected sequences present on the Y chromosome, as evidenced by the increased signal intensity of OX DNA over that of normal male DNA, but bands were also present in female DNA. Thus most of the clones isolated from the human Y chromosome in this study detected Y chromosome sequences which were homologous to sequences loca ted elsewhere in the genome.

Kunkel et al (1985) very elegantly demonstrated the feasibility of a cloning technique whereby DNA absent from a small chromosomal deletion ~5,000kb (in this case involving the Duchenne muscular dystrophy locus on the human X chromosome, Fig. 1.1) can be enriched for by competitive DNA reassociation (deletion enrichment cloning). In this context normal female (XX) DNA can be envisaged as possessing a deletion of sequences specific to the Y chromosome, present in male DNA (XY). This excludes any sequence which the Y chromosome has in common with any other chromosomes. I therefore considered that competitive DNA reassociation could be applied to cloning single copy human Y specific sequences.

The basis for this approach was realised by Kunkel et al (1976)

who produced a hybridisation probe detecting Y specific repeated sequences by competing radiolabelled male DNA with an excess of female DNA. Lamar and Palmer (1984) extended this idea by digesting male mouse DNA ('tracer') from an inbred strain with MboI to generate small fragments compatible with the BamHI site of a cloning vector, sonicating a 100-fold excess of female DNA ('driver') from the same inbred stain, denaturing the mixture and allowing the DNA to reanneal to a Cot 1320 at 68°C in 2M $(NH_4)_2SO_4$, 50mM sodium phosphate buffer (pH 6.8). This, they claimed, increases the rate of reassociation 50-fold over that in standard 0.14M sodium phosphate buffer. They estimated that 95% of the DNA was reassociated by this technique. However Y chromosome specific DNA is present at a concentration 100-fold lower than that of the rest of the genome (which is also present in the female driver DNA) and thus only reaches a Cot of ~10. Only highly repeated sequences specific to the Y chromosome will therefore reassociate under these conditions (Britten and Kohne, 1968). After cloning into the BamHI site of pBR322, 13% of clones were found to contain mouse Y chromosomal DNA. Three clones were examined at random and found to detect repeated sequences present on the mouse Y chromosome, two of these however also detected bands in female mouse DNA and so represent Y-X or Y-autosome homolog ies which have not been competed out by the technique, presumably due to an insufficiently large excess of female DNA.

In order to be able to obtain single-copy DNA sequences from deletion enrichment cloning, DNA must be reassociated to 100%, in order that low copy number sequences in the tracer DNA, which is at low concentration, can reassociate. To bring this latter DNA to a

Cot of 10^3-10^4 will require reassociating the driver DNA to a Cot of 10^5-10^6. Using standard techniques of reassociation would therefore require unacceptably long periods of reassociation (several months). DNA remaining after such a period might be of dubious quality for cloning!

The rate of DNA-DNA hybridisation is dramatically increased (up to 10,000-fold) compared to standard conditions of 0.18 Na^+ 60°C, by a technique known as the phenol emulsion reassociation technique (PERT) devised by Kohne et al (1977). The rate of reassociation in this method is dependent on the formation of an emulsion with phenol, the type and concentration of ions present, temperature, concentration of DNA and the manner of agitation of the emulsion. Unlike aqueous systems where the rate of reassociation increases with the DNA concentration, PERT works better at low DNA concentrations. DNA is denatured at a concentration <100µg/ml, then allowed to reassociate at room temperature in the presence of 6-12% phenol and high concentrations of a chaotrophic ion. This mixture is agitated continuously to allow the phenol to form an emulsion. The manner by which PERT allows for rapid DNA reassociation at room temperature is unknown but some guesses can be made.

As well as increasing the rate of DNA reassociation, the phenol emulsion allows reassociation to proceed at room temperature. Under standard aqueous conditions the optimum rate of DNA reassociation occurs 25°C below the Tm (melting temperature) of the double stranded DNA i.e. ~60°C (Wetmur and Davidson, 1968). Phenol is very effective at lowering the Tm of DNA (Massie and Simm, 1965). In parts of the emulsion the Tm may be 40-50°C thus allowing reassociation to proceed at room temperature.

There seems to be two possible means whereby the presence of a phenol emulsion might increase the rate of reassociation, both involving creating regions of local high DNA concentration. Firstly, single-stranded DNA may concentrate at the phenol:aqueous interface with the bases facing into the phenol and the phosphate groups into the aqueous solution. Alternatively, DNA may be excluded from the interface of the emulsion. Due to the large number of water molecules tied up with forming the emulsion with phenol (water molecules will be ordered around each small drop of phenol), very little water will be available for solvating the DNA. Thus the DNA will form local high concentrations or semi-precipitates in the aqueous solution where DNA reassociation will occur - the volume exclusion principle (Wetmur, 1975). This idea is supported by the milky white appearance of the phenol emulsion in the presence of high DNA concentrations compared to the light grey appearance in the absence of DNA.

Reassociation is also prompted (especially at high DNA concentrations) by chaotrophic ions which disrupt hydrophobic bonds and thus may help aggregates of DNA to be dispersed. DNA must probably undergo many rounds of aggregation/dispersion to allow complementary DNA strands to collide with each other prior to their reassociation. Both the presence of chaotrophic ions and agitation of the emulsion will increase the rate of this. The inhibition of reassociation in the PERT by high DNA concentrations probably is due to the inability to disperse the aggregated DNA properly under these conditions.

8.2. Competitive reassociation of male DNA by female DNA

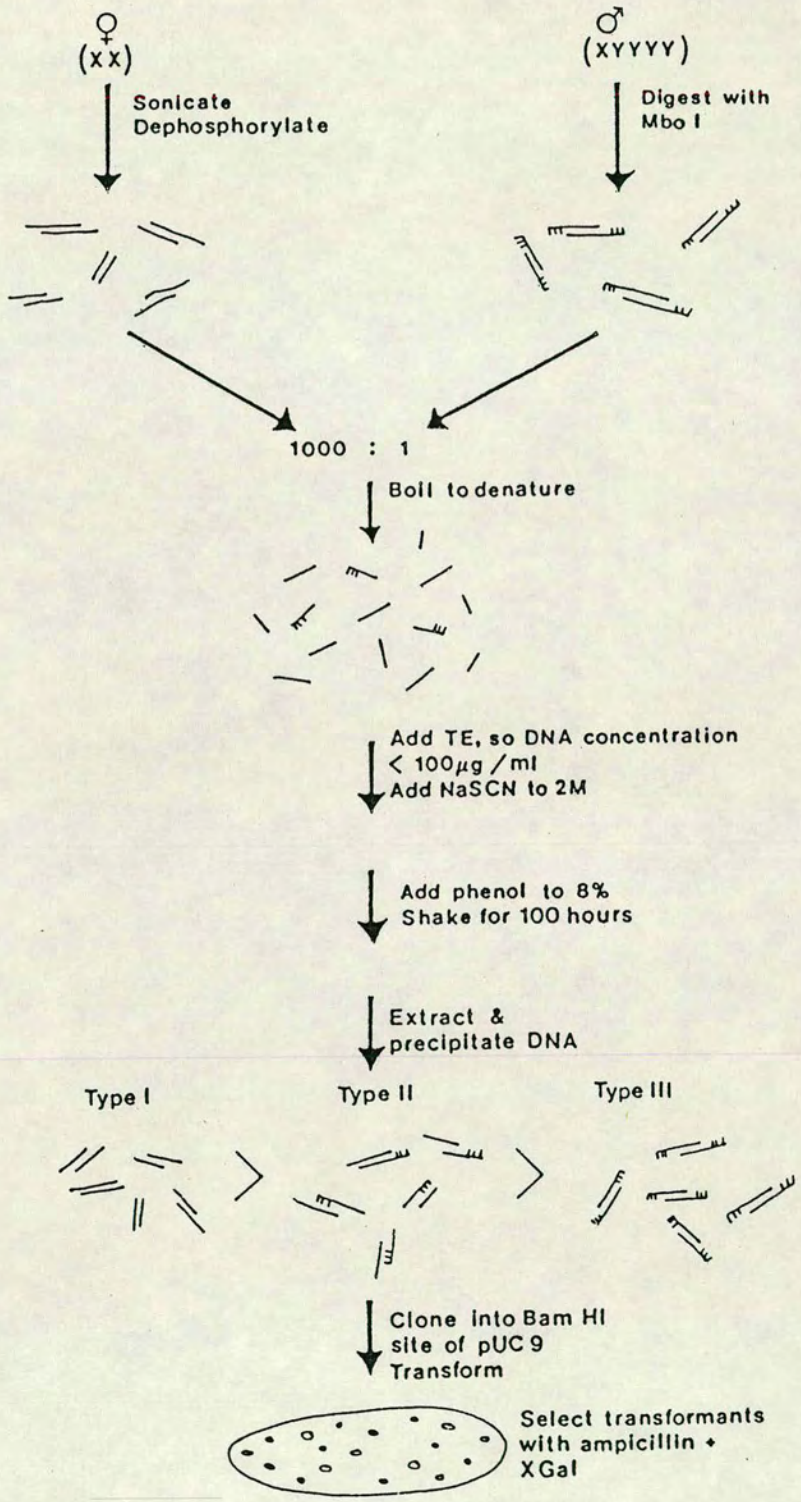
Using the extensive studies on PERT by Kohne et al (1977) conditions were chosen in which to perform competitive reassociation of male versus female DNA. The male DNA used was from the human lymphoblastoid cell line OX (Section 2.1.3). This cell line was ascertained by quinacrine staining to contain 3.8 Y chromosomes/ diploid cell complement. The use of a cell line containing multiple Y chromosomes optimises chances of cloning Y specific DNA. The OX DNA was digested with MboI to generate fragments of mean size ~500bp for cloning into the BamHI site of pUC9. The driver DNA for competing against male DNA was obtained from female placentas of several individuals. This ensures that any small deletions within the genome of one individual will not represent a significant proportion of the driver DNA, and will not therefore lead to enrichment of these sequences in the cloning technique. The driver DNA was sonicated with a 12mm probe (Dawes sonicator) fifteen times for 5 seconds each; then run on an agarose gel alongside the MboI digested male DNA, to ensure that the sonicated material was of the same size range as the digested DNA.

The general experimental strategy used for deletion enrichment cloning of human Y specific DNA is illustrated in Fig. 8.1. In the first experiment a 1000:1 ratio of female:male DNA was used. 250ng of MboI-digested male DNA and 250µg of sonicated female DNA in 4ml of TE was denatured by boiling for 10 minutes, cooled on ice, then transferred to a glass scintillation vial. Solid sodium thiocyanate (NaSCN) was added such that the final concentration was 2M. Thiocyanate is the chaotrophic ion most effective at promoting rapid reassociation in the emulsion system (Kohne et al, 1977). Fresh

FIGURE 8.1

Strategy of PERT-mediated competitive reassociation technique for cloning Y chromosome specific sequences

See text for details.


♀
(XX)
Sonicate
Dephosphorylate
♂
(XYYYY)
Digest with
Mbo I
1000 : 1
Boil to denature
Add TE, so DNA concentration
< 100μg / ml
Add NaSCN to 2M
Add phenol to 8%
Shake for 100 hours
Extract &
precipitate DNA
Type I
Type II
Type III
Clone into Bam HI
site of pUC 9
Transform
Select transformants
with ampicillin +
XGal

distilled phenol was then added to a final concentration of 8% and the mixture shaken to establish an emulsion. The emulsion immediately took on a milky white appearance and was maintained by shaking at room temperature on a platform attached to a Whirlimxer, for 100 hours. The phenol emulsion was then extracted twice with chloroform to remove the phenol and the DNA precipitated with ethanol, 100µg/ml dextran being added as a carrier to aid recovery of the DNA. The DNA was ligated to 1µg of BamHI digested, dephosphorylated pUC9, and used to transform competent JM83 cells which were plated out on H-ampicillin plates with X-Gal incorporated in order to identify recombinant colonies (see Section 2.4.2).

Three types of double stranded DNA molecules should result from the reassociation described above (Fig. 8.1). A majority of molecules (Type 1) will contain 2 strands each derived from the sonicated driver DNA which constitutes the bulk of the DNA. These molecules should not be clonable into the vector. Type II molecules will contain one strand of MboI digested male DNA with the complementary strand deriving from the excess driver (female) DNA. As these molecules contain one strand of sonicated DNA they also should not ligate to the BamHI digested pUC9. The third and least abundant type of molecule will result from the reassociation of MboI digested male DNA strands which have no complementary sequences within the driver DNA i.e. Y-specific sequences. These molecules (Type III) will be rare (<1 in 1.5×10^5) but are the only products of the reassociation which should be clonable into the BamHI site of pUC9 (Fig. 8.1).

From the ligation and cloning of half of the DNA in the first reassociation experiment, 38 white colonies were obtained and their

DNA analysed by linearisation with HindIII. 9 clones appeared to be smaller than pUC9, thus have deleted sequences. The cause of this is unknown but these plasmids may have been carrying unstable inserts. 20 clones contained very small inserts (<100bp) which were thought to be too small to be useful as hybridisation probes. The remaining 9 clones contained inserts of 100-500bp in size. This is below the mean size of the MboI digested male DNA and is a reflection of the preference of pUC9 for accepting small inserts over larger ones.

The 9 clones containing sizeable inserts were digested with EcoRI and HindIII to release the inserts together with ~30bp of flanking vector DNA. As discussed in Section 3.1 BamHI cannot be used to release MboI fragments ligated into a BamHI site. Inserts were separated from vector sequences on 7% polyacrylamide gels, radiolabelled by random-primer labelling and used to probe blots of female, male and OX DNAs to determine if any of the clones contained Y specific DNA. 2 clones detected repeated sequences and 7 unique sequences but none were specific to male DNA or showed dosage between male and OX DNA (data not shown), so were not Y-specific. The reassociation did therefore not appear to have resulted in a large enrichment of Y specific DNA which would be expected at a frequency of 1/300 clones on a random basis without competitive hybridisation. It was decided to check that the clones used did indeed derive from MboI digested fragments of DNA, i.e. contained the sequence 5' GATC at either end of the insert. MboI cannot be used to digest the cloned DNAs. Most strains of E.coli contain the enzyme dam methylase which introduces methyl groups at the A residue in the sequence $^{5'}$GATC, the recognition sequence of MboI (Hattman et al, 1978). MboI will not cleave the sequence $^{5'}$ G^{me}ATC but the

isochizomer of MboI, Sau3A1 is insensitive to methylation of this sequence. 5 of the clones used as hybridisation probes, and pUC9. were digested with Sau3A1 and run on a polyacrylamide gel. 4/5 of the clones were found to have lost one or other of the Sau3A1 sites flanking the inserts (data not shown). This indicates that these clones do not originate from type III molecules with MboI sites at either end. Sonicated DNA (type I molecules) produced no recombinant colonies when ligated to pUC9. I therefore think it likely that a majority of clones from this reassociation derive from type II molecules which are present in larger numbers than type III molecules. In the designing of the protocol for the competitive reassociation technique I originally thought that type III molecules would be the only DNA duplexes capable of cloning into the BamHI site of pUC9. It seems however that type II molecules are also clonable, albeit at a low efficiency. As one strand of type II molecules originates from MboI digested DNA, ligation to the vector will regenerate the sequence $^{5'}$GATC at one end of the insert, at the other end the DNA strand originating from sonicated material may terminate in residues at least partially complementary to the BamHI site of the vector in some molecules. Alternatively blunt end ligation may occur. The sequence GATC will not be regenerated at this end of the clone, thus the Sau3A1 site will be lost.

A second reassociation experiment was set up in which the sonicated driver DNA was dephosphorylated to prevent any further cloning of type II molecules. 100ng of MboI digested OX DNA and 100µg of sonicated dephosphorylated female DNA in 2ml TE were reassociated as in the first experiment (in 2M NaSCN, 8% phenol for 100 hours). The DNA was ligated to 0.5µg of vector DNA and used to

transform competent JM83 cells. 29 white colonies were obtained, all of which contained inserts. 21 clones had inserts of >100bp and were therefore usable as hybridisation probes. These clones were digested with Sau3A1 and run on a polyacrylamide gel. 19/21 clones retained Sau3A1 sites at either side of their inserts suggesting that they derived from type III molecules. Thus dephosphorylating the driver DNA has greatly reduced (80% down to 10%) the number of clones obtained with defective Sau3A1 sites which were thought to have originated from type II hybrid duplexes.

Clones having the correct restriction pattern with Sau3A1 were digested with EcoRI and HindIII to release their inserts, which were then cut from polyacrylamide gels. Each insert was then used as a hybridisation probe to female, male and OX DNAs to identify any clones containing Y specific DNA. Table 8.2 summarises the results of these hybridisations. 15.8% of clones (3/19) detected Y specific sequences or sequences present in OX DNA but absent from female DNA. No clones were found which detected Y-X or Y-autosome homologous sequences which represent a majority of sequences isolated from the human Y chromosome by other methods (Section 8.1). If Y-specific DNA sequences are indeed restricted to <5,000kb of DNA in the male genome (20,000kb in OX DNA) then on a random basis 1/300 clones from OX DNA would be expected to detect low-copy DNA sequences specific to male DNA. In the PERT-mediated competitive reassociation described above 1/6 clones are specific to the human Y chromosome. This represents a 50-fold enrichment for these sequences in the reassociation. The hybridisation patterns of the three OX-specific clones (pERT OXY 1, 2 and 3) to male, female, OX and somatic cell hybrid DNAs are shown in Fig. 8.2.

TABLE 8.2.

Summary of hybridisation patterns of clones resulting from PERT-mediated competitive reassociation of OX DNA with female DNA

	No of probes	% of total no. of probes tested
Clones resulting from reassociation	29	-
Clones tested as hybridisation probes	19	100
No signal	4	21
Repeated sequences	2	10.5
X or autosomal low copy number sequences	10	52.6
Low copy number sequences present in OX DNA but absent from female DNA (Y-specific)	3	15.8
X-Y or autosome Y homologous sequences*	0	0

*These sequences would be identified by their hybridisation to female, male and OX DNAs but with dosage between the signal from male and OX DNAs.

pERT OXY1 detects a 6kb male specific EcoRI fragment showing dosage between male and OX DNAs. This probe has a small repeat component in it, which can be removed prior to hybridisation by stripping (Sealey et al, 1985). The 6kb EcoRI fragment is also present in somatic cell hybrids carrying a human Y chromosome. Hybridisation of pERT OXY1 to somatic cell hybrids containing various parts of the Y chromosome (Section 3.3) localises pERT OXY1 to the short arm of the chromosome, signal being seen in the isoYp cell line from which Yq material is absent. No signal is detected in the 445 x 393 cell line which carries an X-Y translocation chromosome containing Yq11.1-qter (data not shown). pERT OXY1 therefore maps to the same region of the Y chromosome as the putative testis determining gene. However, no hybridisation is observed to two XX males which are presumed to carry a small region of the Y chromosome involved in testis determination (see Chapter 9).

pERT OXY2 detects a 0.6kb EcoRI fragment and a 10kb HindIII fragment in digests of OX DNA. No fragments are detectable however in male, female or hybrid DNAs which contain human X and Y chromosomes. pERT OXY2 is therefore derived from a type III molecule in that it is present in the tracer (OX) DNA but absent from the driver (female) DNA. However, pERT OXY2 cannot represent a sequence present on all Y chromosomes since it is absent from normal male DNA and from hybrid cell lines containing the human Y chromosome. One interesting possibility which will require further investigation is that pERT OXY2 represents a sequence present on some but not all Y chromosomes. As discussed in Chapter 5, Y chromosome sequences cannot be homogenised in a population as the

FIGURE 8.2

Hybridisation of pERT OXY1, 2 and 3 to human and hybrid cell line DNAs

A: Hybridisation of insert of pERT OXY1 to EcoR1 digests of

1 - Human ♀

2 - Human ♀

3 - OX (XYYYY)

4 - Human ♂

5 - Human ♂

6 - ThyB1 (mouse/human hybrid containing the human X chromosome)

7 - Mouse

8 - 3E7 (mouse/human hybrid containing the human Y chromosome)

9 - 3E7 rev (revertant of 3E7 which has lost the Y chromosome)

10 - 853 (Chinese hamster/human hybrid containing the human Y chromosome)

11 - 853 rev (revertant of 853 which has lost the Y chromosome)

B: Hybridisation of insert of pERT OXY 2 to:

Human ♀ DNA digested with 1 - EcoRI and 4 - HindIII

Human ♂ DNA digested with 2 - EcoRI and 5 - HindIII

OX DNA digested with 3 - EcoRI and 6 - HindIII

C: Hybridisation of insert of pERT OXY 3 to EcoRI digests of

1 - Human ♀

2 - Human ♂

3 - OX

4 - ThyB1

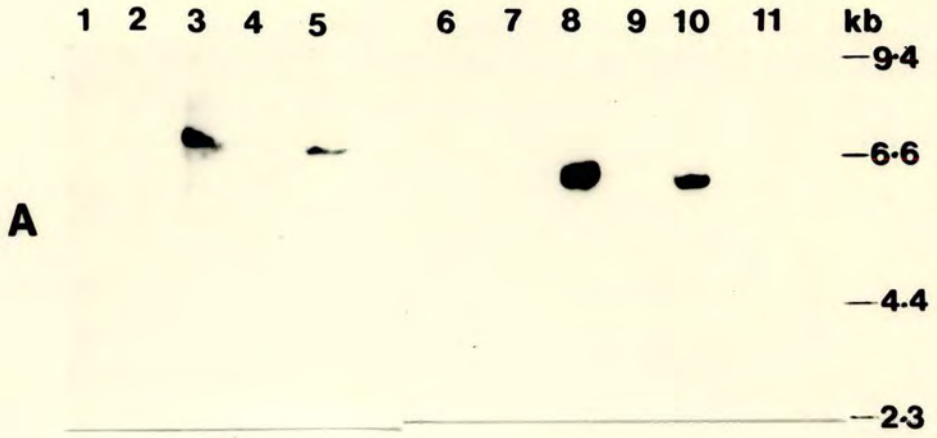
5 - Mouse

6 - 3E7

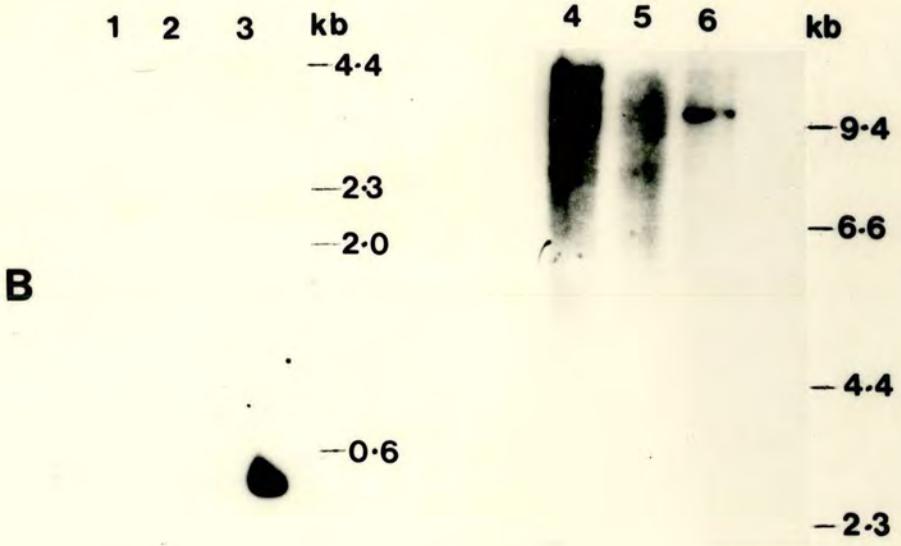
7 - 3E7 rev

8 - 853

9 - 853 rev


1 2 3 4 5 6 7 8 9 10 11 kb
9·4
6·6
4·4
2·3

A


1 2 3 kb
4·4
2·3
2·0
0·6
4 5 6 kb
9·4
6·6
4·4
2·3

B


1 2 3 4 5 6 7 8 9 kb
23·5
9·4
6·6

C

chromosome is always monosomic. A more trivial explanation might be that pERT OXY2 might represent DNA from mycoplasma which is known to contaminate the OX cell line though this would probably be present at a very high copy number. OX is also an Epstein-Barr virus (EBV) transformed cell line so pERT OXY2 may represent EBV sequences.

pERT OXY3 detects a single Y specific 9kb band in HindIII digests of male DNA with dosage between OX and male DNA. In EcoRI digests of male, OX and somatic cell hybrid DNAs, a large Y specific hybridising band is seen. This probe has not yet been regionally mapped on the Y chromosome but does not detect any bands in the two XX males previously examined.

The PERT-mediated competitive reassociation described here, has apparently resulted in a 50-fold enrichment for DNA sequences specific to the human Y chromosome, eliminating those sequences which the Y holds in common with autosomes and the X chromosome. I shall continue to further investigate this technique to improve the frequency at which Y specific DNA is cloned and to further study any such sequences present in XX males. As Kunkel et al (1985) have demonstrated the PERT DNA reassociation technique is applicable to other situations involving small chromosomal deletions. This group achieved a 10-fold enrichment (1/30 clones) for sequences absent (5,000kb) from the X chromosome of a boy with Duchenne muscular dystrophy by using his DNA as driver DNA against DNA from an XXXXY human lymphoid cell line. In this case only a 200-fold excess of driver over tracer DNA was used. The 10-fold enrichment of sequences observed in this case is therefore consistent with the 50-fold enrichment achieved in the reassociation described in this thesis in which a 1000:1 ratio of driver:tracer DNA was used. If

PERT mediated competitive reassociation (deletion enrichment cloning) is to be applied to deletions involving autosomal loci, the additional step must be taken of segregating the chromosome in question (deleted and non-deleted) into somatic cell hybrids. This is not necessary in the case of the X or Y chromosomes as each is monosomic in normal male DNA.

CHAPTER 9

DISCUSSION

RECENT EVENTS DURING THE EVOLUTION OF THE HUMAN Y CHROMOSOME AND THEIR BEARING UPON THE DNA SEQUENCE CONTENT OF THIS CHROMOSOME

Chapters 3-6 of this thesis describe the investigation of homology shared between the human sex chromosomes. This homology unexpectedly was found to be located at Xq26-27 and the centromeric region of Yq, well outside the region of these chromosomes involved in normal meiotic recombination. On the basis of restriction mapping and sequencing studies the extent of homology between the human X and Y chromosomes at this locus (2:13) appears to be ~100%. For other chromosome pairs this level of homology could be maintained by crossing over at meiosis. However, recombination between the human sex chromosomes outside of the pseudoautosomal region, at Xp and Yp ter, would lead to disast rous consequences due to the different structural and genetic makeups of the chromosomes, and is thus thought not to occur. Gene conversion operating at the 2:13 locus upon an ancient homology (dating from the time when X and Y chromosomes were an homologous pair) might serve to maintain such a high degree of homology. XY homology at this locus, however extends for at least 50kb and probably for several hundred kb. Gene conversion is not known to operate on this scale (Maniatis et al, 1980). A functional constraint on the 2:13 sequence may have prevented the divergence of X and Y copies since the time when these chromosomes were very similar in genetic content. The absence of a mouse DNA sequence and human polyA$^+$ RNA hybridising to 2:13 and the significant divergence of the chimpanzee homologue of 2:13 from the human equivalent, argues against this possibility.

The examination of the nature of 2:13 sequences in higher primates shows the sequence to be located on the X chromosome alone. The data is therefore consistent with a large transposition event having occurred in an ancestor to the present day human species

which has resulted in a region of the long arm of the X chromosome being duplicated on to the long arm of the Y. This transposition has occurred so recently in evolutionary time that no base differences have yet been detected at the 2:13 locus between the human X and Y chromosomes, in the 200bp sequenced in this thesis or in a further 1kb sequenced by Cooke et al, (1984).

The increasing number of studies of sequences derived from the human Y chromosome, by various groups in recent years, has revealed, against all expectations, that X-Y homologous sequences (excluding those from the pseudoautosomal region) represent a large proportion of the sequences isolated from the chromosome. Aside from this study of the 2:13 locus, the other most complete investigation of such a homology was carried out by Page et al (1984) on the DXYS1 locus, mapping to Xq13.2-q21.2 and Yp cent-p11.2. This probe detects a TaqI RFLP with 11 and 12kb X-linked alleles and a male specific 15kb fragment. Thus unlike 2:13 with which no base pair differences could be detected between the human X and Y chromosomes, DXYS1 X and Y loci are distinguishable. An important aside here is that no meiotic recombination was seen between the DXYS1 X and Y loci (the number of meioses examined was not stated) thus supporting the supposition that the X and Y chromosomes do not recombine outside of the pseuodoautosomal region. Using a rather complex calculation (Nei and Tajima, 1983) based upon the restriction site differences between the DXYS1 X and Y loci detected by Southern blotting and the number of base pairs detected by these restriction enzymes, Page et al, estimate that the human X and Y chromosomes share 99.2% homology at the DXYS1 locus which extends for >36kb. The DXYS1 locus therefore resembles the characteristics of the 2:13

locus in its high level of XY homology and the large extent of that homology. Hybridisation of DXYS1 to male and female chimpanzee, gorilla and orangutan DNAs reveals that like 2:13, DXYS1 is X-linked only, in these higher primates. This locus has therefore also been involved in a recent evolutionary transposition from the X to the Y chromosome.

The apparently random collection of human X-Y homologous sequences which have now been isolated, can be put into three main categories which are summarised in Tables 9.1, 9.2 and 9.3 and depicted in Fig. 9.1. The first and largest of these categories, which includes 2:13 and DXYS1 described above, represents sequences showing a high degree of homology between the X and Y chromosomes under conditions of high stringency (Table 9.1). For many XY homologous loci which show Y specific restriction fragments (indicated in the table), the method of Nei and Tajima (1983), based upon restriction site differences, has been used to estimate the level of homology between the two chromosomes and is found to be 97-99%. All of the category I sequences have been localised to the euchromatic region of the Y chromosome and to the long arm of the X chromosome. For only 2:13 and DXYS1 is it known whether XY homology extends over a long stretch of DNA and what the chromosomal location of the sequence is in higher primates. Thus it can only be speculated as to whether the high level of XY homology shown by all category I probes is due to recent transposition events from the X chromosome to the Y, as is the case for 2:13 and DXYS1.

Cosmid 75 (DXYS25) (Wolfe et al, 1984) localises to Xq21 and Yp (Goodfellow et al, 1985). One subclone of this cosmid p75/78 detects identical EcoRI fragments in male and female DNA under

TABLE 9.1

CATEGORY I HUMAN XY HOMOLOGOUS SEQUENCES

Probe	X localisation	Y localisation	Level of homology	Extent of homology	Localisation in higher primates
2:13 (this thesis)	Xq26-27	Yqcen-q11.1	High >99.4%	>50kb	X only
DXYS1 (Page et al, 1984)	Xq13-21.2	Ypcen-p11.2	High 99.2% Y specific Taq RFLP	>36kb	X only
DXYS25 (probe cos 75) (Wolfe et al, 1984; Goodfellow et al, 1985)	Xq21	Yp	High Y specific EcoR RFLP	>8.4kb	-
DXYS2 (probe 7b)	Xq13-q22	Y euc	High Y spec Msp RFLP	>6.5kb	-
DXYS4 (probe 1)	Xq12-q22	Y euc	High Y spec Taq RFLP	>13kb	-
DXYS5 (probe 47)	Xq12-q22	Yp11.2-pter	High Y spec Taq RFLP	>8kb	-
DXYS6 (probe 16)	Xq12-q22	Yp	High Y spec Taq RFLP 97-99%	-	-
DXYS7 (probe 13d)	Xq13-q22	Yp11.2-pter	High Y spec Taq RFLP 99%	-	-
DXYS8 (probe 115)	Xq13-q22	Yp11.2-pter	High Y spec Taq RFLP	>7kb	-
DXYS9 (probe 8j)	Xq13-q22	Y euc	High Y spec Msp RFLP	-	-
DXYS10 (probe 41a) (Bishop et al, 1984; Geldwerth et al, 1985)	Xq22-q28	Y euc	High		
p22b	Xq13-q24	Y euc	High	-	-
p17 (Geldwerth et al, 1985)	Xq13-q24	Y euc	High	-	-
DXYS12 (probe St25) (Koenig et al, 1985)	Xq13-q22	Ypter-Yq11.1	High 98% Y spec EcoRI and and Taq RFLPs	>7kb	X only in in macaques
DXYS 21 (GmGXYZ2)	Xq13-q24	Yp11.2-pcen	High	-	-
GMG XY4	Xq13-q24	Yp11.2-pter	High	-	-
GMG XY5	Xq13-q24	Yp11.2-pter	High	-	-
GMG XY6	Xq13-q24	Yp11.2-pter	High	-	-
p2F2 (Affara et al, 1986)	Xq13-q24	Yp11.2-pter	High	-	-
DXYS 27 (probe cY59) (Buckle et al, 1985)	Xq21	Yp	High	-	-
DXYS 13 (probe pTAK2) (Ahrens et al, 1985)	Xq22-q26	Y	High Y specific RFLPs	- -	-

fairly high stringency washing conditions (0.2 x SSC, 65°C). Another subclone p75/79 detects 3 EcoRI fragments common to male and female DNA, plus one additional male specific fragment.

In the study of Bishop et al (1984) of sequences derived from the human Y chromosome (see section 8.1), 13/26 probes detected sequences on the X chromosome in addition to the Y. In a more detailed analysis Geldwerth et al (1985) have localised 10 of these latter probes which detect XY homologies under high stringency washing conditions. 9 of these probes map to Xq12-q24 and one (DXYS10) to Xq22-q28. Their Y localisation is in the euchromatic region, with 3 probes being more finely localised to Yp. DXYS2 and 4-9 detect MspI or TaqI polymorphisms showing Y specific fragments, and the level of XY homology at these loci is estimated to be 97-99%.

Koenig et al (1985) have described an XY homologous probe. St25 (isolated from an X chromosome library) detecting X and Y loci DXYS12 at Xq13-q22 and the euchromatic region of the Y chromosome. The level of XY homology at DXYS12 was estimated to be 98% and St25 was found to be X-linked only in various macaque species. Thus like 2:13 and DXYS1, DXYS12 may have been involved in a recent transposition of sequences from the X to the Y chromosome. It is not known whether St25 is X-linked in the gorilla and chimpanzee, but the level of XY homology detected by St25 in humans, suggests that any transposition event may have occurred ~9 million years ago (see Section 5.1). This is after the divergence of the gorilla line from the human ancestor, but prior to the divergence of the human and chimpanzee lineages. Thus provided that the estimate of 98% homology at DXYS12 is accurate, St25 might be expected to detect XY

homologous sequences in chimpanzees, but be X-linked only in gorillas.

Affara, Florentin et al (1986) in an examination of 20 probes isolated from the human Y chromosome, found 7 detecting XY homologous sequences. Of these, 5 fall into category I. GMGXY2, 4, 5, 6 and p2F2 all localise to Xq13-q24, the latter 4 probes being located also to Yp11.2-pter. GMGXY2 is located at Yp11.2-pcen and detects in addition an autosomal sequence. This group also refined the localisation of probes 47, 115 and 13d (DXYS 5, 8 and 7 respectively in Table 9.1) to Yp11.2-pter.

Buckle et al (1985) have also briefly described the isolation of probes, detecting XY homologous loci DXYS27 and 13 respectively, which appear to fall into category I. This is based upon the detection of XY homology under conditions of high stringency and their localisation to the long arm of the X chromosome.

Category II XY homologous sequences (Table 9.2) are those with which the homology between the human sex chromosomes is detected only at low stringency. Thus the X and Y copies of these sequences are diverged from each other, so may represent more ancient transposition events than category I sequences. In contrast to the X localisation of category I probes to the long arm exclusively, category II sequences localise with one exception to Xp21-pter and to the euchromatic part of Yq.

DXS31, isolated by Koenig et al (1984) from an X chromosome library, detects only X specific bands in human DNA at high stringency. However if the stringency is dropped (1 x SSC, 60°C) Y-linked fragments are seen. DXS31 localises to Xp22.3-pter and Yq11-qter but proximal to the heterochromatic region. The extent of

TABLE 9.2 Category II Human XY Homologous Sequences

Probe	X localisation	Y locsalisation	Level of homology	Extent of homology	Localisation in higher primates
DXS31 (Koenig et al, 1984)	Xp22.3-pter	Yq11 - qter (Euc)	80%	-	XY homologous sequences in chimpanzees. X only in macaques. Autosomal in lemurs.
DXS69 (probe 71-7A) (Kunkel et al, 1983)	Xp21-pter	Yq	Y specific HindIII RFLP detects weak autosomal fragment also	-	-
DXYS11 (probe 52d) (Bishop et al, 1984	Yq22-q28	Yp	Low (2 x SSC, 68°C) Y specific EcoRI and TaqI RFLPs	-	-
GMGXY3 (Affara et al, 1986)	Xp22.3-pter	Yp + Yq11.1-q11.2	Low (0.5 x SSC, 65°C) Y specific TaqI RFLP	-	-
DXYS18 (probe pJA36D)	X	Yq	Low	<40kb	-
DXYS19 (probe pJA1165) (Arnemann et al, 1985)	X	Yq in hetero-chromatin	Low	<40kb	-

homology between the human X and Y chromosomes at DXS31 is estimated to be 80%, suggesting that the X and Y loci have been genetically separated for some time. This is confirmed by the detection of X and Y specific fragments hybridising to DXS31 in chimpanzees. In macaques however, which are separated from the human species by ~35 million years, DXS31 sequences are X-linked only. Furthermore, in the more distant lemurs DXS31 sequences appear to be autosomal. The ability of DXS31 to detect sequences in the lemur genome under conditions of moderate stringency (1 x SSC, 60°C) may indicate some functional role for this sequence. DXS31 therefore has been transposed from the X to the Y chromosome during primate evolution (since the divergence of the macaque lineage from that giving rise to the great apes and man). Unlike category I sequences however, this transposition predates the divergence of the human, chimpanzee and probably gorilla species, thus giving rise to lower levels of homology between the human X and Y loci than that observed for category I sequences. Prior to the transposition of DXS31 sequences from the X to the Y chromosome there appears to have been movement of these sequences from autosomes onto the X chromosome.

Kunkel et al (1983) isolated an expressed DNA sequence (71-7A) from the human X chromosome which also detects a Y-specific fragment. 71-7A maps to Xp21-pter and to Yq possibly in the heterochromatic region.

One of the probes isolated by Bishop et al (1982), 52d (DXYS11) appears to fall into category II in that at high stringency this probe detects multiple Y loci, but at reduced stringency (2 x SSC, 68°C) an X-linked fragment is also visible. However, the localisation of 52d to Xq22-28 and Yp is more characteristic of a

category I sequence.

Affara, Florentin et al (1986) also isolated a category II sequence GMGXY3 which detects XY homologous loci at low stringency only (0.5 x SSC, 65°C), and maps to Xp22.3-pter and to two loci on the human Y chromosome, one at Yq11.1-q11.2 and the other at Yp.

Finally, Arnemann et al (1985) have described probes from two Y-derived cosmids (11 and 36) which detect weak XY homologies. pJA36D (DXYS18) from cosmid 36, localises to Yq, its X localisation being unknown at present. An important feature of this probe is that pJA36B, another subclone from cosmid 36 is Y chromosome specific. pJA1165 (DXYS19) from cosmid 11 also defines a weak homology between the X and Yq. This cosmid also contains sequences homologous to the 3.4kb HaeIII repeat of the Y heterochromatin (Cook et al, 1983). Thus both of these loci (DXYS18 and 19) have only a short region (<40kb) held in common between the X and Y chromosomes.

The last category of human XY homologous loci (Table 9.3), and the last to be identified at the molecular level, are those found within the meiotic pairing region of these chromosomes (Xpter-Ypter). This was in fact the only class of human XY homology predicted to exist, on the basis of theories of sex chromosome evolution, the behaviour of the X and Y chromosomes at meiosis, and genetic evidence for shared sequences between the sex chromosomes (see Section 1.4).

DXYS14, 15 and 17 have already been discussed in detail in Chapter 7. These sequences are located at the distal ends of the short arms of the X and Y chromosomes and they are exchanged during meiosis. Their pattern of inheritance is therefore that which would be expected of an autosomal sequence, which has earned them the

TABLE 9.3 Category III Human XY Homologous Sequences

Probe	X localisation	Y localisation	Level of homology	Extent of homology	Localisation in higher primates
DXYS14 (probe 29C1) (Cooke et al, 1986)	Xp22.3 - pter	Ypter	100%	<5,000kb	Probably pseudoautosomal also in gorillas (this thesis)
DXYS15 (probe 113D) (Simmler et al, 1986)	Xp22.3 - pter	Ypter	100%	<5,000kb	
DXYS17 (probe 601) (Rouyer et al, 1986)	Xp22.3 - pter	Ypter	100% (pseudoautosomal)	<5,000kb (Rouyer et al, 1986)	
DXYS20 (D.Page - H.G.M.8)	Xp22.3 - pter	Ypter	100%	-	-
MIC2X/Y (probe pSG1) (Darling et al, 1986)	Xp22.3 - pter	Yp11-pter	>99.66%	-	-
GMGXY1 (Affara et al, 1986)	Xp22.3 - pter	Yp	High, no XY differences - detected at 23 restriction sites	-	-

FIGURE 9.1

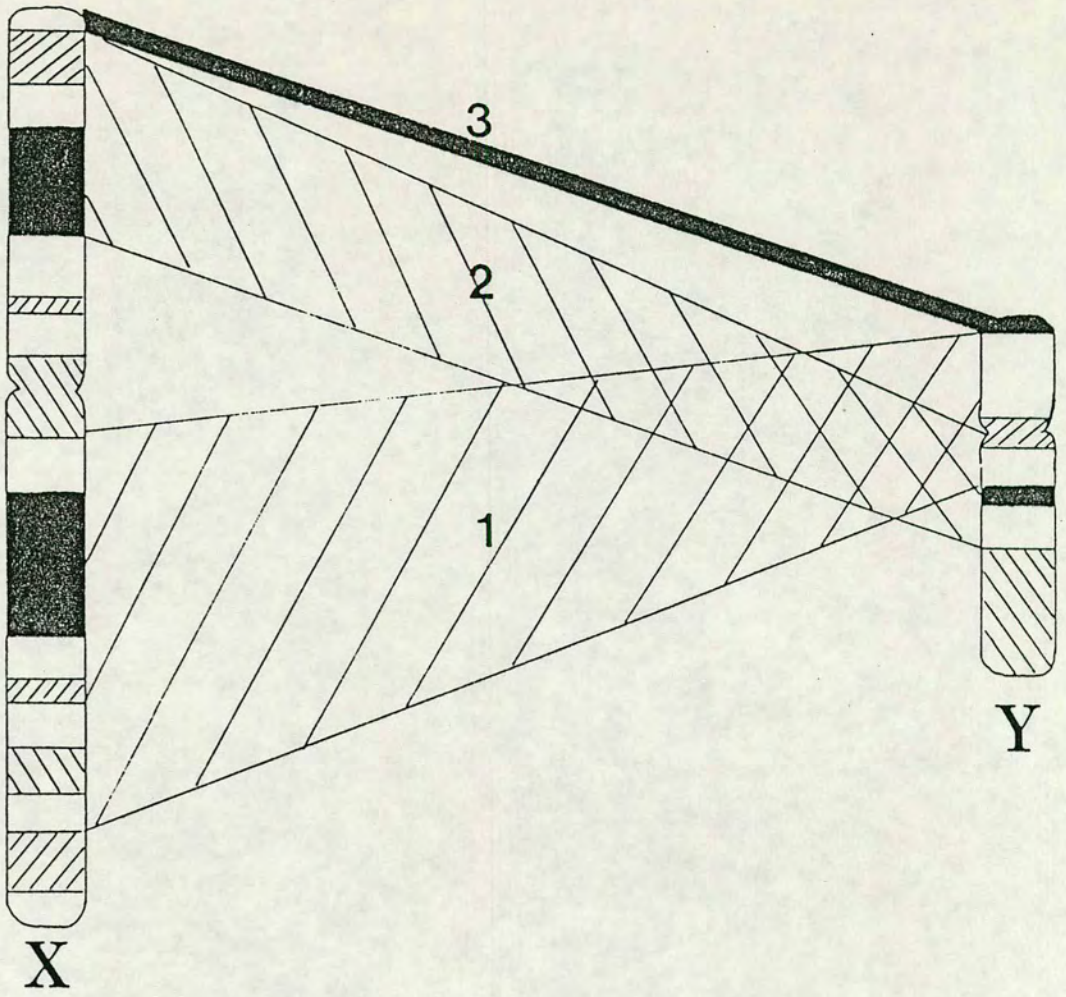
Regions of homology between the human X and Y chromosomes

Diagram of the human X and Y chromosomes showing the location of the 3 regions of homology between these chromosomes defined in the text and in Tables 9.1, 9.2 and 9.3.

1 = Category I XY homologous sequences, thought to be the result of a recent transposition event between the X and Y chromosomes.

2 = Category II XY homologous sequences, the result of a more ancient transposition event between the X and Y chromosomes.

3 = The pseudoautosomal region where crossing over between the human sex chromosomes occurs at meiosis.


3
2
1
X
Y

title 'pseudoautosomal'. Due to their recombination at meiosis, the X and Y loci of DXYS14, 15 and 17 are interchangeable, so effectively represent 100% sequence homology between the sex chromosomes. In gorillas I have shown (at least in the case of DXYS14) that these sequences are highly diverged from their human counterparts. This is consistent with the high recombination rate at this locus and is indicative that sequences in gorilla homologous to DXYS14 are also located in the pseudoautosomal segment of the sex chromosomes of this species. Rouyer et al (1986) have set an upper limit of 5,000kb on the extent of XY homology in the pseudoautosomal region.

D. Page at the 8th International Workshop on Human Gene Mapping has also reported the isolation of a pseudoautosomal probe DXYS20, mapping to Xpter and Ypter and involved in meiotic recombination.

As discussed in Section 1.4, genetic evidence first suggested that loci (MIC2X and MIC2Y) encoding the 12E7 antigen, would be located on the short arms of both of the human sex chromosomes. This has now been demonstrated to be the case, the cDNA clone corresponding to a MIC2 locus having been isolated (Darling et al, 1986). This probe pSG1, maps to the meiotic pairing region of the sex chromosomes Xp22.3-pter and Yp11-pter. No restriction site differences between the two chromosomes have been detected by this probe at 53 restriction sites setting a lower limit of 99.6% for the degree of homology between the X and Y chromosomes at this locus.

One of the XY homologous sequences isolated by Affara, Florentin et al (1986) may fall into category III. GMGXY1 maps to Xp22.3-pter and Yp with no restriction site differences between the X and Y chromosomes being detected at 23 sites of this locus.

The study of XY homologous sequences in this thesis and by other groups described above, has indicated that the human Y chromosome has undergone extensive change in its DNA sequence content and organisation in recent evolutionary time. Not all of these changes however, have involved the translocation of single copy sequences from the X chromosome.

DYZ1 and Z2 repeated sequences (defined by 3.4 and 2.1kb HaeIII bands in male DNA) constitute the bulk of the heterochromatic region of the long arm of the Y chromosome. Although in humans these sequences are present in a dispersed nature elsewhere in the genome, it is only on the Y that they are concentrated and present in tandem arrays. DYZ2 detects a discrete size class of fragment in gorilla DNA but there are no differences in the hybridisation patterns of male and female DNAs. In chimpanzees hybridisation is not to a discrete size class but is of a dispersed nature with again no sex-specific hybridisation pattern (Cooke et al, 1982). It is therefore possible that in a common ancestor to humans, chimpanzees and gorillas DYZ2 sequences were present in a form similar to the present day gorilla sequence giving a discrete size fragment with HaeIII, but not concentrated upon the Y chromosome. In the lineage giving rise to chimpanzees there was loss of some sequences resulting in the absence of a discrete size fragment with HaeIII. In the lineage giving rise to the human species DZY2 sequences have been amplified in tandem array upon the Y chromosome. The most likely mechanism for such an amplification is unequal crossover (Smith, 1976). In the case of the monosomic Y chromosome, this must occur via sister chromatid exchange at mitosis. The evolutionary changes to the human Y chromosome involving these repeated sequences

are thus probably distinct from those events resulting in the translocation of low copy number sequences from the X chromosome. I consider that the most likely mechanism for the latter is sporadic recombination between the X and Y outside of the pseudoautosomal region when the two chromosomes are in close association in the sex vesicle at male meiosis. The following is a possible story-line for the recent evolution of the human Y chromosome based upon the nature of the DNA sequences discussed in this chapter. Firstly, after the divergence of the old world monkeys (e.g. macaques) from the lineage giving rise to the higher primates, category II XY homologous sequences (with the possible exception of 52d) were translocated from Xp onto Yq - the structure of the X chromosome is thought to be largely conserved through evolution (Ohno, 1969). The organisation of these sequences on Yq was then probably disrupted by rearrangements accompanying the amplification on the long arm of the Y chromosome of the DYZ1 and Z2 repeats which now constitute the human Yq heterochromatin, after the divergence of the closest ape relative, the chimpanzee, from the human line. Thus category II sequences probably no longer detect long contiguous stretches of XY homology in present day humans. Then at some point since the divergence of humans and chimpanzees, possibly within the last few million years, in an individual whose chromosome has given rise to all the present day human Y chromosomes examined in this thesis (Section 5.3), category I XY homologous sequences were translocated from Xq to primarily Yp. Most probably not all category I sequences were transferred in one block, as their localisation spans the centromere; 2:13 sequences being located on Yq, unless there has been subsequent rearrangement of these sequences following a single

transposition event. Nevertheless, the scale of XY homology detected by category I sequences is likely to be large, of the order of hundreds of kilobases, as indicated by the studies on the 2:13 sequence in Chapter 6.

Thus much of the human Y chromosome appears to be a recent evolutionary collage. This is not, however, likely to be a phenomenon applicable to other chromosomes for unlike autosomes and the X, the Y chromosome appears to be largely inactive in somatic cells and is thus subject to little selection pressure.

Parts of the human Y chromosome which are proposed not to be of recent evolutionary origin are those sequences specific to the Y chromosome, including genes involved in sex determination and spermatogenesis. Chapter 8 of this thesis has described a method which can be used for specifically isolating these sequences from male DNA. Many groups are trying to track down sequences important for male sex determination by analysing the presence of Y chromosome sequences in the genomes of phenotypic males with an XX karyotype. Two hypotheses have been proposed to explain the existence of such sterile males a) an autosomal or X-linked mutation involving genes required for primary sex determination or b) the presence of small amounts of Y chromosome DNA including the sex determining locus on the paternal X chromosome due to abnormal XY crossing over at meiosis. This latter hypothesis was first suggested by Ferguson-Smith (1966). The two hypotheses are not mutually exclusive - de la Chapelle (1981) has argued for a heterogeneous aetiology for XX males, describing a family where the XX male phenomenon is transmitted through females thus ruling out any involvement of the Y chromosome. At least one

of these XX males has inherited both X chromosomes from his mother. Wolf (1981) proposed a model for sex determination whereby testis differentiation is prevented by an X-linked repressor locus, neutralised in the presence of the sex determining locus on the Y chromosome. Burgoyne (1984) has suggested that in the de la Chapelle pedigree both maternal X chromosomes carry recessive mutations of this X-linked repressor locus, thus resulting in gonadal differentiation into testes. Most sporadic (the majority) rather than familial causes of XX maleness however are most likely to result from the transfer of varying and probably contiguous amounts of the short arm of the Y chromosome, all including the testis determining locus, onto the paternal X by an over-extension of normal XY meiotic recombination (Chapter 7). Several groups (Guellaen et al, 1984; Müller et al, 1986; Affara, Ferguson-Smith et al, 1986; Vergnaud et al, 1986) have demonstrated the presence of Yp sequences in some XX males. These individuals are found to be heterogeneous in the amounts of Y material they carry but no correlation has been found between the amount of Y material present, and phenotypic character. Anderson et al (1986) have shown by in situ hybridisation that one such sequence, pD105, is present on the tip of the short arm of one X chromosome in each of three XX males, thus supporting the meiotic recombination hypothesis. Vergnaud et al (1986), Affara, Florentin et al (1986) and Affara, Ferguson-Smith et al (1986) have used XX males, XY females and other anomalies involving the human Y chromosome to try and construct a deletion map of that chromosome. Vergnaud et al's study is consistent with XX males carrying a single contiguous portion of the short arm of the Y chromosome, though the possibility of more complicated rearrange-

ments cannot be excluded. They have assigned each of 23 Y-specific restriction fragments to one of seven deletion intervals whose order, presupposing that XX males acquire a terminal contiguous portion of Yp, i.e. the telomere plus variable amounts of DNA extending toward the centromere, is shown in Fig. 9.2A. The testis determining locus maps to interval 1 of this map in agreement with the karyotype-sex phenotype correlations discussed in section 1.3. Probe 47a (Geldwerth et al, 1985) a category I XY homologous sequence is the sequence most frequently present in the XX males tested and is thus the probe closest to and proximal to the testis determining locus. These studies throw light on the organisation of the Y chromosome. Some probes, 50f, 118 (Guellaen et al, 1984) and 52d (Bishop et al, 1984) detect multiple Y-specific restriction fragments at high stringency. Not all of the fragments detected by one probe are found within a single deletion interval. Many category I XY homologous sequences, e.g. DXYS7 and 8 (Geldwerth et al, 1985) are clustered in interval I. DXYS1 (Page et al, 1984) however is in the centromeric interval 4. 2:13 will probably be located in intervals 4 or 5. Therefore category I XY homologous sequences have either been involved in multiple Xq-Yp transpositions (Yq in the case of 2:13) in recent evolution, or they have subsequently been rearranged since the occurrence of a single transposition event.

Numerous Y-specific sequences are found in different deletion intervals on Yp; thus XY meiotic pairing, often seen to extend the whole length of Yp (section 1.3) cannot be a reflection of contiguous DNA sequence homology along Xp and Yp. Pairing is likely to initiate via DNA sequence homology in the pseudoautosomal segment

FIGURE 9.2

Deletion maps of the human Y chromosome

A. Deletion map containing 7 intervals, constructed by Vergnaud et al (1986).

B. Deletion map containing 6 intervals constructed by Affara, Ferguson-Smith et al (1986) consistent with 8 XX males.

C. Deletion map with inversion of some markers as compared to B based upon 2 XX males. Deletion intervals 3-6 are identical for B and C.

These maps assume that the XX males carry a contiguous, terminal portion of Yp.

a) Probes detecting XY homologous sequences:

DXYS5, 7 and 8 (Geldwerth et al, 1985)

DXYS1 (Page et al, 1984)

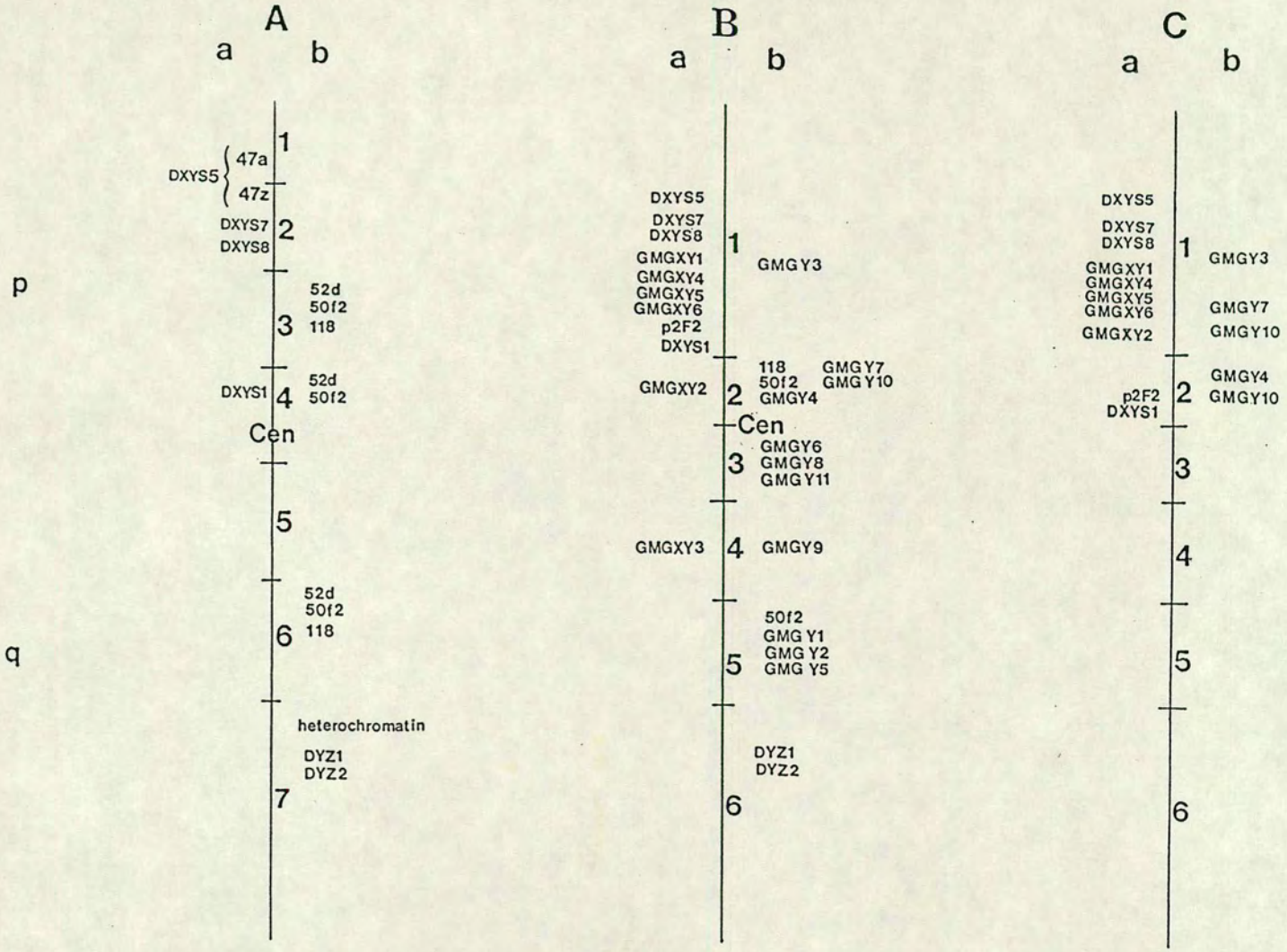
GMGXY1-6, p2F2 (Affara, Florentin et al, 1986)

b) Probes detecting Y specific sequences at high stringency:

52d (Bishop et al, 1984)

50f2,118 (Guellaen et al, 1984)

GMGY1-10 (Affara et al, 1986)


A
a
b
p
q
DXYS5
47a
47z
DXYS7
DXYS8
52d
50f2
118
DXYS1
52d
50f2
Cen
52d
50f2
118
heterochromatin
DYZ1
DYZ2
1
2
3
4
5
6
7
B
a
b
DXYS5
DXYS7
DXYS8
GMGXY1
GMGXY4
GMGXY5
GMGXY6
p2F2
DXYS1
GMGY3
GMGXY2
118
50f2
GMGY4
GMGY7
GMGY10
Cen
GMGY6
GMGY8
GMGY11
GMGXY3
GMGY9
50f2
GMGY1
GMGY2
GMGY5
DYZ1
DYZ2
1
2
3
4
5
6
C
a
b
DXYS5
DXYS7
DXYS8
GMGXY1
GMGXY4
GMGXY5
GMGXY6
GMGXY2
GMGY3
GMGY7
GMGY10
GMGY4
p2F2
DXYS1
GMGY10
1
2
3
4
5
6

of the sex chromosomes, then extend along the chromosomes possibly via protein-protein interactions.

Affara's studies on Y sequences in XX males are not consistent with a terminal transfer of Y chromosome DNA in all individuals (Fig. 9.2B and C). In some cases proximal Yp sequences are present in the absence of more distal sequences. Multiple recombination events may be responsible for the presence of Y chromosome material in these XX males. Alternatively, and more interestingly, the order of sequences upon the Y chromosome may vary in the population, in the form of inversion polymorphisms.

Our view of the human Y chromosome in molecular terms is advancing rapidly. This thesis has shown that sequences have been transposed from the X chromosome to the Y chromosome in recent evolution, which are located outside of the region of these chromosomes involved in meiotic recombination. This is consistent with the findings of other groups, summarised in Table 9.1. The human Y chromosome appears to be a patchwork of Y specific and X-Y homologous sequences. The sex determining locus is located on Yp probably in deletion interval 1 (Figure 9.2A). It can only be a matter of time before a sequence deriving from this locus is cloned, perhaps from an XX male via a method such as that described in Chapter 8. Alternatively a chromosome walk may be instigated from probe 47a which appears to be the closest sequence to the sex determining locus, isolated to date. Spin offs from the intensity of study of the Y chromosome have already included, isolation of a sequence (DXYS14) in close proximity to the natural end of a chromosome; well illustrating that despite its unique role in the human genome, the Y chromosome is able to continue to expand our

understanding of general chromosome structure and function.

REFERENCES

Affara, N.A., Ferguson-Smith, M.A., Tolmie, J., Kwok, K., Mitchell, M., Jamieson, D., Cooke, A. and Florentin, L. (1986) Variable transfer of Y-specific sequences in XX males. Nucl. Acids Res. 14, 5375-5387.

Affara, N.A., Florentin, L., Morrison, N., Kwok, K., Mitchell, M., Cook, A., Jamieson, D., Glasgow, L., Meredith, L., Boyd, E. and Ferguson-Smith, M.A. (1986) Regional assignment of Y-linked DNA probes by deletion mapping and their homology with X-chromosome and autosomal sequences. Nucl. Acids Res. 14, 5353-5373.

Ahrens, P., Albertson, H., Riis Vestergaasd, S., Bolund, L. and Kruse, T.A. (1985) Human gene mapping 8: Anonymous X-chromosomal probes revealing DNA polymorphisms one of which is a deletion of more than 3kb. Cyt. and Cell. Genet. 40, 567.

Anderson, M., Page, D.C. and de la Chapelle, A. (1986) Chromosome Y-specific DNA is transferred to the short arm of X chromosome in human XX males. Science 233, 786-788.

Arnemann, J., Cooke, H.J., Jakubiczba, S. and Schmidtke, J. (1985) Human gene mapping 8: Human Y-chromosome derived cloned DNA sequences. Cyt. and Cell. Genet. 40, 571.

Banting, G.S., Pym, B. and Goodfellow, P.N. (1985) Biochemical analysis of an antigen produced by both human sex chromosomes. EMBO J. 4, 1967-1972.

Barrie, P.A.,. Jeffreys, A.J. and Scott, A.F. (1981) Evolution of the β-globin gene cluster in man and the primates. J. Mol. Biol. 149, 319-336.

Bender, W., Spierer, P. and Hogness, D.S. (1983) Chromosomal walking and jumping to isolate DNA from the Ace and rosy loci and the bithorax complex in Drosophila melanogaster. J. Mol. Biol. 168, 17-33.

Benton, W.D. and Davis, R.W. (1977) Screening λgt recombinant clones by hybridisation to single plaques in situ. Science 196, 180-182.

Bhattacharya, S.S., Wright, A.F., Clayton, J.F., Price, W.M., Phillips, C.I., McKeown, C.M.E., Jay, M., Bird, A.C., Pearson, P.L., Southern, E.M. and Evans, H.J. (1984) Close genetic linkage between X-linked retinitis pigmentosa and a restriction fragment length polymorphism identified by recombinant DNA probe L1.28. Nature 309, 253-255.

Bird, A.P. (1986) CpG rich islands and the function of DNA methylation. Nature 321, 209-213.

Bird, A.P., Taggart, M., Frommer, M., Miller, O.J. and Macleod, D. (1985) A fraction of the mouse genome that is derived from islands of non-methylated CpG rich DNA. Cell 40, 91-99.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid Res. 7, 1513-1523.

Bishop, C.E., Guellaen, G., Geldwerth, D., Voss, R., Fellous, M. and Weissenbach, J. (1983) Single copy DNA sequences specific for the human Y chromosome. Nature 303, 831-832.

Bishop, C., Guellaen, G., Geldwerth, D., Fellous, M. and Weissenbach, J. (1984) Extensive sequence homologies between Y and other human chromosomes. J. Mol. Biol. 173, 403-417.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Construction and characterisation of new cloning vehicles II. A multipurpose cloning system. Gene 2, 95-113.

Britten, R.J. and Kohne, D.E. (1968) Repeated sequences in DNA. Science 161, 529-540.

Brown, W.R.A. and Bird, A.P. (1986) Long-range restriction site mapping of mammalian genomic DNA. Nature. In press.

Brutlag, D., Fry, K., Nelson, T. and Hung, P. (1977) Synthesis of hybrid bacterial plasmids containing highly repeated satellite DNA. Cell 10, 509-519.

Buckle, V., Boyd, Y., Craig, I.W., Fraser, N., Goodfellow, P.N. and Wolfe, J. (1985) Human gene mapping 8: Localisation of Y chromosomal sequences in normal and 'XX' males. Cyt. and Cell. Genet. 40, 593.

Buhler, E.M. (1980) A synopsis of the human Y chromosome. Hum. Genet. 55, 145-175.

Burgoyne, P.S. (1982) Genetic homology and crossing over in the X and Y chromosomes of mammals. Hum. Genet. 61, 85-90.

Burgoyne, P. (1984) The origins of men with two X chromosomes. Nature 307, 109.

Burgoyne, P.S., Levy, E.R. and McLaren, A. (1986) Spermatogenic failure in male mice lacking H-Y antigen. Nature 320, 170-172.

Burk, R.D., Ma, P. and Smith, K.D. (1985) Characterisation and evolution of a single-copy sequence from the human Y chromosome. Mol. and Cell. Biol. 5, 576-581.

Carle, G.F., Frank, M. and Olson, M.V. (1986) Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. Science 232, 65-68.

Carle, G.F. and Olson, M.V. (1984) Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. Nucl. Acids Res. 12, 5647-5664.

Carle, G.F. and Olson, M.V. (1985) An electrophoretic karyotype for yeast. Proc. Natl. Acad. Sci. USA 82, 3756-3760.

Cattanach, B.M. (1975) Control of chromosome inactivation. Ann. Rev. Genet. 9, 1-18.

Charconas, G. and van de Sande, J.M. (1980) 5'-^{32}P-labelling of RNA and DNA restriction fragments. Methods. Enzymol. 65, 75-85.

Chandley, A.C., Goetz, P., Hargreave, T.B., Joseph, A.M. and Speed, R.M. (1984) On the nature and extent of XY pairing at meiotic prophase in man. Cytogenet. Cell Genet. 38, 241-247.

Chandra, H.S. (1985) Sex determination: A hypothesis based on non-coding DNA. Proc. Natl. Acad. Sci. USA 82, 1165-1169.

Chandra, H.S. (1986) X chromosomes and dosage compensation. Nature 319, 18.

de la Chapelle, A. (1981) The aetiology of maleness in XX men. Hum. Genet. 58, 105-116.

Charlesworth, B. (1978)) Model for evolution of Y chromosomes and dosage compensation. Proc. Natl. Acad. Sci. USA 75, 5618-5622.

Collins, F.S. and Weissman, S.M. (1984) Directional cloning of DNA fragments at a large distance from an initial probe: A circularization method. Proc. Natl. Acad. Sci. USA 81, 6812-6816.

Cooke, H. J. (1976) Repeated sequence specific to human males. Nature 262, 182-186.

Cooke, H.J., Brown, W.R.A. and Rappold, G.A. (1984) Closely related sequences on human X and Y chromosomes outside the pairing region. Nature 311. 259-261.

Cooke, H.J., Brown, W.R.A. and Rappold, G.A. (1985) Hypervariable telomeric sequences from the human sex chromosomes are pseudoautosomal. Nature 317, 687-692.

Cooke, H.J., Fantes, J. and Green, D. (1983) Structure and evolution of human Y chromosomal DNA. Differentiation 23(S), 48-55.

Cooke, H.J., Schmidtke, J. and Gosden, J.R. (1982) Characterisation of a human Y chromosome repeated sequence and related sequences in higher primates. Chromosoma 87, 491-502.

Darling, S.M., Banting, G.S., Pym, B., Wolfe, J. and Goodfellow, P.N. (1986) Cloning an expressed gene shared by the human sex chromosomes. Proc. Natl. Acad. Sci. USA 83, 135-139.

Daiger, S.P., Wildin, R.S. and Su, T-S. (1982) Sequences on the human Y chromosome homologous to the autosomal gene for arginosuccinate synthetase. Nature 298, 682-684.

Davies, K.E., Young, B.D., Elles, R.G., Hill, M.E. and Williamson, R. (1981) Cloning of a representative genomic library of the human X chromosome after sorting by flow cytometry. Nature 293, 374-376.

Davis, R.M. (1981) Localisation of male determining factors in man: a thorough review of structural anomalies of the Y chromosome. J. Med. Genet. 18, 161-195.

Drayna, D. and White, R. (1985) The genetic linkage map of the human X chromosome. Science 230, 753-758.

Dressler, D. and Potter, H. (1982) Molecular mechanisms in genetic recombination. Ann. Rev. Biochem. 51, 727-761.

Eichwald, E.J. and Silmser, C.R. (1955) Untitled communication. Transplantation Bull 2, 154-155.

Elder, J.K., Green, D.K. and Southern, E.M. (1986) Automatic reading of DNA sequencing gel autoradiographs using a large format digital scanner. Nucl. Acids Res. 14, 417-424.

Elder, J.K and Southern, E.M. (1983) Measurement of DNA length by gel electrophoresis II: Comparison of methods for relating mobility to fragment length. Anal. Bioc. 128, 227-231.

Engel, W., Klemme, B. and Ebrecht, A. (1981) Serological evidence for H-Y antigen in XO female mice. Hum. Genet. 57, 68-70.

Epstein, C.J. (1985) Mouse monosomies and trisomies as experimental systems for studying mammalian aneuploidy. Trends in Genetics 1, 129-134.

Evans, E.P., Burtenshaw, M.D. and Cattanach, B.M. (1982) Meiotic crossing-over between the X and Y chromosomes of male mice carrying the sex-reversing (Sxr) factor. Nature 300, 443-445.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Bio. 132, 6-13.

Feinberg, A.P. and Vogelstein, B. (1984) Addenum: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Bioc. 137, 266-267.

Ferguson-Smith, M.A. (1966) X-Y chromosomal interchange in the aetiology of true hermaphrodtism and of XX Klinefelter's syndrome. Lancet ii, 475-476.

Ferris, S.D., Wilson, A.C. and Brown, W.M. (1981) Evolutionary tree for apes and humans based on cleavage maps of mitochondrial DNA. Proc. Natl. Acad. Sci. USA 78, 2432-2436.

Fisher, E.M.C., Cavanna, J.S. and Brown, S.D.M. (1985) Microdissection and microcloning of the mouse X chromosome. Proc. Natl. Acad. Sci. USA 82, 5846-5849.

Ford, C.E., Jones, K.W., Polani, P.E., de Almeida, J.C. and Briggs, J.H. (1959) A sex-chromosome anomaly in a case of gonadal dysgenesis (Turners syndrome). Lancet i, 711-713.

Friedman, S. (1981) The inhibition of DNA (cytosine-5) methylases by 5-azacytidine. Mol. Pharmacol. 19, 314-320.

Gartler, S.M. and Riggs, A.D. (1983) Mammalian X chromosome inactivation. Ann. Rev. Genet. 17, 155-190.

Geldwerth, D., Bishop, C., Guellaen, G., Koening, M., Vergnaud, G., Mandel, J-L. and Weissenbach, J. (1985) Extensive DNA sequence homologies between the human Y and the long arm of the X chromosome. EMBO J. 4, 1739-1743.

Goodfellow, P., Banting, G., Sheer, D., Ropers, H.H., Caine, A., Ferguson-Smith, M.A., Povey, S. and Voss, R. (1983) Genetic evidence that a Y-linked gene in man is homologous to a gene on the X chromosome. Nature 302, 346-349.

Goodfellow, P., Darling, S. and Wolfe, J. (1985) The human Y chromosome. J. Med. Genet. 22, 329-344.

Goodfellow, P., Pym, B., Mohandas, T. and Shapiro, L.J. (1984) The cell surface antigen locus, MIC2X, escapes X inactivation. Am. J. Hum. Genet. 36, 777-782.

Goodfellow, P.N. and Tippet, P. (1981) A human quantitative polymorphism related to Xg blood groups. Nature 289, 404-405.

Grunstein, M. and Hogness, D.S. (1975) Colony hybridisation: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA 72, 3961-3965.

Guellaen, G., Casanova, M., Bishop, C., Geldwerth, D., Andre, G., Fellous, M. and Weissenbach, J. (1984) Human XX males with Y single-copy DNA fragments. Nature 307, 172-173.

Gusella, J.F., Keys, C., Varsanyi-Breiner, A., Kao, F., Jones, C., Puck, T.T. and Housman, D.(1980) Isolation and characterisation of DNA segments from specific human chromosomes. Proc. Natl. Acad. Sci. USA 77, 2829-2833.

Hanahan, D. (1983) Studies on transformation of E.coli with plasmids. J. Mol. Biol. 166, 557-580.

Hanahan, D. (1985) Techniques for transformation of E.coli. In DNA cloning vol. I. pp. 109-135 (Ed. Glover, D.M.). IRL.

Harper, K. Winter, R.M., Pembrey, M.E., Hartley, D., Davies, K.E. and Tuddenham, E.G.D. (1984) A clinically useful DNA probe closely linked to Haemophila A. Lancet ii, 6-8.

Hartley, D.A., Davies, K.E., Drayna, D., White, R.L. and Williamson R. (1984) A cytological map of the human X chromosome - evidence for non-random recombination. Nucl. Acids Res. 12, 5277-5285.

Haseltine, F.P. and Ohno, S. (1981) Mechanisms of gonadal differentiation. Science 211, 1272-1278.

Hattman, S., Brooks, J.E. and Masurekar, M. (1978) Sequence specificity of the P1 modification methylase (M.Eco P1) and the DNA methylase (m.Eco dam) controlled by the Escherichia coli dam gene. J. Mol. Biol. 126, 367-380.

Heilig, R., Hanauer, A., Grzeschik, K.H., Hors-Cayla, H.C. and Mandel, J.L. (1984) Actin-like sequences are present on human X and Y chromosomes. EMBO J. 3, 1803-1807.

Hohn, B. (1979) In vitro packaging of and cosmid DNA. Methods Enzymol. 68, 299-309.

Holliday, R. (1964) A mechanism for gene conversion in fungi. Genet. Res. 5, 282-303.

Hultén, M. (1974) Chiasma distribution at diakenesis in the normal human male. Hereditas 76, 55-78.

Hultén, M.A., Palmer, R.W. and Laurie, D.A. (1982) Chiasma derived genetic maps and recombination fractions: Chromosome I. Ann. Hum. Genet. 46, 167-175.

Human Gene Mapping 8. (1985) 8th International Workshop on Human gene mapping. Cyt. and Cell. Genet. 40.

Ish-Horowicz, D. and Burke, J.F. (1981) Rapid and efficient cosmid cloning. Nucl. Acids. Res. 9, 2989-2998.

Jacobs, P.A. and Strong, J.A. (1959) A case of human intersexuality having a possible XXY sex- determining mechanism. Nature 183, 302-303.

Jones, K. W. (1983) Evolution of sex chromosomes. In development in Mammals (Johnson, M.M. ed) pp. 297-320. Elsevier.

Kacser, H. and Burns, J.A. (1981) The molecular basis of dominance. Genetics 97, 639-666.

Kaiser, K. and Murray, N. (1985) The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. In DNA cloning. Vol. I, pp 1-47. Ed. Glover, D.M. IRL

Karin, M. and Richards, R.I. (1982) Human metallothionein genes - primary structure of the metallothionein II gene and a related processed gene. Nature 299, 797-802.

Karn, J., Brenner, S. and Barnett, L. (1983) New bacteriophage lambda vectors with positive selection for clones inserts. Methods Enzymol. 101, 3-19.

Keitges, E., Rivest, M., Siniscalco, M. and Gartler, S.M. (1985) X-linkage of steriod sulphatase in the mouse is evidence for a functional Y-linked allele. Nature 315, 226-227.

Koenig, M., Camerino, G., Heilig, R. and Mandel, J-L (1984) A DNA fragment from the human X chromosome short arm which detects a partially homologous sequence on the Y chromosomes long arm. Nucl. Acids Res. 12, 4097-4109.

Koenig, M., Moisan, J.P., Heilig, R. and Mandel, J-L (1985) Homologies between X and Y chromosomes detected by DNA probes: localisation and evolution. Nucl. Acids Res. 13, 5485-5501.

Kohne, D.E., Levison, S.A. and Byers, M.J. (1977) Room temperature method for increasing the rate of DNA reassociation by many thousandfold: The phenol emulsion reassociation technique. Biochemistry 16, 5329-5341.

Koller, P.C. and Darlington, C.D. (1934) The genetical and mechanical properties of the sex chromosomes: Rattus norvegicus, ♂. J. Genet. 29, 159-173.

Kourilsky, P. (1986) Molecular mechanisms for gene conversion in higher cells. T.I.G. 2, 60-63

Kunkel, L.M., Monaco, A.P., Middlesworth, W., Ochs, H.D. and Latt, S.A. (1985) Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. Proc. Natl. Acad. Sci. USA 82, 4778-4782.

Kunkel, L.M., Smith, K.D. and Boyer, S.H. (1976) Human Y-chromosome-specific reiterated DNA. Science 191, 1189-1190.

Kunkel, L.M., Tantravahi, U., Kurnit, D.M., Eisenhard, M., Bruns, G.P. and Latt, S.A. (1983) Identification and isolation of transcribed human X chromosome DNA sequences. Nucl. Acids Res. 11, 7961-7978.

Lamar, E.E. and Palmer, E. (1984) Y-encoded, species specific DNA in mice: evidence that the Y chromosome exists in two polymorphic forms in inbred strains. Cell 37, 171-177.

Laskey, R.A. and Mills, A.D. (1977) Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. FEBS Letters 82, 314-316.,

Laurie, D.A., Palmer, R.W. and Hultén, M.A. (1982) Chiasma derived genetic lengths and recombination fractions: Chromosomes 2 and 9. Ann. Hum. Genet. 46, 233-244.

Leder, P., Tiemeier, D. and Enquist, L. (1977) EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: The λgt WES system. Science 196, 175-177.

Lund, E., Bostock, C., Robertson, M., Christie, S., Mitchen, J.L. and Dahlberg, J.E. (1983) U1 small nuclear RNA genes are located on human chromosome I and are expressed in mouse-human hybrid cells. Mol. and Cell. Biol. 3, 2211-2220.

Lyon, M.F. (1961) Gene action in the X-chromosome of the mouse (mus musculus L). Nature 190, 372-373.

McBurney, M.W. and Strutt, B.J. (1980) Genetic activity of X chromosomes in pluripotent female teratocarinoma cells and their differentiated progeny. Cell 21, 357-364.

McClelland, M. and Ivarie, R. (1982) Asymmetrical distribution of CpG in an 'average' mammalian gene. Nucl. Acid Res. 10, 7865-7877.

McClung, C.E. (1902) Spermatocyte divisions of the Locustidae. Kansas Univ. Sci. Bull. 1, 185-238.

McKay, R.D.G., Bobrow, M. and Cooke, H.J. (1978) The identification of a repeated DNA sequence involved in the karyotype polymorphism of the human Y chromosome. Cytogenet. Cell. Genet. 21, 19-32.

McLaren, A., Simpson, E., Tomorari, K., Chandler, P. and Hogg, H. (1984) Male sexual differentiation in mice lacking H-Y antigen. Nature 312, 552-555.

McMahon, A., Fosten, M. and Monk, M. (1981) Random-X-chromosome inactivation in female primordial germ cells in the mouse. J. Embryol. Exp. Morph. 64, 251-258.

Magenis, R.E., Tomar, D., Brown, M.G., Allen, L., Kammer, H., Van Dyke, D.L., Webb, M.J. and Tochen, M. (1984) Human gene mapping 7: Localisation of male determining factors to the Y short arm, bands p11.2-pter. Cytogenet. and Cell. Genet. 37, 529.

Maniatis, T., Fritsch, E.F., Lauer, J. and Lawn, R.M. (1980) The molecular genetics of human haemoglobins. Ann. Rev. Genetics 14, 145-178.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A laboratory manual. Cold Spring Harbor, N.Y.

Marcus, M., Tantravahi, R., Vaithilingam, G.D., Miller, D.A. and Miller, O.J. (1976) Human-mouse cell hybrid with human multiple, Y chromosomes. Nature 262, 63-65.

Massie, H.R. and Zimm, B.H. (1965) The use of hot phenol in preparing DNA. Proc. Natl. Acad. Sci. USA 54, 1641-1643

Messing, J. (1983) New M13 vectors for cloning. Methods. Enzymol 101, 20-78.

Migeon, B.R., Shapiro, L.J., Norum, R.A., Mohandas, T., Axelman, J. and Dabora, R.L. (1982) Differential expression of steroid sulphatase locus on active and inactive human X chromosome. Nature 299, 838-840.

Monaco, A.P., Bertelson, C.J., Middlesworth, W., Colletti, C., Aldridge, J., Fischbeck, K.H., Bartlett, R., Pericak-Vance, M,.A., Roses, A.D., and Kunkel. L.M. (1985) Detection of deletions spanning the Duchenne Muscular Dystrophy locus using a tightly linked DNA segment.Nature 316, 842-845.

Monk, M. and Harper, M.I. (1979) Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. Nature 281, 311-313.

Mueller, C.R., Davies, K.E. and Ropers, H.H. (1984) DNA sequence homologies on human X and Y chromosomes. Cyt. and Cell. Genet. 37, 545.

Müller, U., Lalande, M., Donlon, T. and Latt, S.A. (1986) Moderately repeated DNA sequences specific for the short arm of the human Y chromosome are present in XX males and reduced in copy number in an XY female. Nucl. Acids Res. 14, 1325-1340.

Müller-Hill, B., Crapo, L. and Gilbert, W. (1968) Mutants that make more lac repressor. Proc. Natl. Acad. Sci. USA 59, 1259-1264.

Murray, J.M., Davies, K.E., Harper, P.S., Meredith, L., Mueller, C.R. and Williamson, R. (1982) Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy. Nature 300, 69-71.

Murray, N.E., Brammar, W.J. and Murray, K. (1977) Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. 150, 53-61.

Nei, M. and Tajima, F. (1983) Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. Genetics 105, 207-217.

New England Biolabs Catalogue (1985-86) Site preferences of restriction endonucleases. pp. 111.

Norrander, J., Kempe, T. and Messing, J. (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26, 101-106.

Ohno, S. (1967) Sex chromosomes and sex-linked genes. Monographs in Endocrinology 1. Springer-Verlag.

Ohno, S. (1969) Evolution of sex chromosomes in mammals. Ann. Rev. Genet. 3, 495-524.

Page, D.C., de la Chapelle, A. and Weissenbach, J. (1985) Chromosome Y-specific DNA in related human XX males. Nature 315, 224-226.

Page, D.C., Harper, M.E., Love, J. and Botstein, D. (1984) Occurrence of a transposition from the X-chromosome long arm to the Y-chromosome short arm during human evolution. Nature 311, 119-123.

Polani, P.E. (1982) Pairing of X and Y chromosomes, non-inactivation of X-linked genes, and the maleness factor. Hum. Genet. 60, 207-211.

Porteous, D.J., Morten, J.E.N., Cranston, G., Fletcher, J.M., Mitchell, A., van Heyningen, V., Fantes, J.A., Boyd, P.A. and Hastie, N.D. (1986) Molecular and physical arrangements of human DNA in HRAS-selected, chromosome-mediated transfectants. Mol. and Cell. Biol. 6, 2223-2232.

Poustka, A. and Lehrach, H. (1986) Jumping libraries and linking libraries: the next generation of molecular tools in mammalian genetics. Trends in Genetics 2, 174-179.

Pritchard, C. and Goodfellow, P.N. (1986) Development of new methods in human gene mapping: selection for fragments of the human Y chromosome after chromosome-mediated gene transfer. EMBO J. 5, 979-985.

Race, R.R. and Sanger, R. (1975) The Xg Blood groups. In Blood Groups in Man 6th Ed. pp. 578-639. Blackwell.

Rackwitz, H-R., Zehetner, G., Frischauf, A-M. and Lehrach, H. (1984) Rapid restriction mapping of DNA cloned in lambda phage vectors. Gene 30, 195-200.

Ray, P.N., Belfall, B., Duff, C., Logan, C., Vanora, K., Thompson, M.W., Sylvester, J.E., Gorski, J.L., Schmickel, R.D. and Worton, R.G. (1985) Cloning of the break point of an X; 21 translocation associated with Duchenne muscular dystrophy. Nature 318, 672-675.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251.

Rogers, J.H. (1985) Origin and evolution of retroposons: Part 2. Int. Rev. Cytol. 93, 231-279.

Röhme, D., Fox, M., Herrmann, B., Frischauf, A-M., Edström, J-E., Mains, P., Silver, L.M. and Lehrach, H. (1984) Molecular clones of the mouse t complex derived from microdissected metaphase chromosomes. Cell 36, 783-788.

Rosenfeld, R.G., Luzzatti, L., Hintz, R.L., Miller, O.J., Koo, G.C. and Wachtel, S. (1979) Sexual and somatic determinants of the human Y chromosome: Studies in a 46, XYp^- phenotypic female. Am. J. Hum. Genet. 31, 458-468.

Rouyer, F., Simmler, M-C., Johnsson, C., Vergnaud, G., Cooke, H.J. and Weissenbach, J. (1986) A gradient of sex linkage in the pseudoautosomal region of the human sex chromosomes. Nature 319, 291-295.

Russell, L.B. and Chu., E.H.Y. (1961) An XXY male in the mouse. Proc. Natl. Acad. Sci. USA 47, 571-575.

Sandford, J.P., Chapman, V.M. and Rossant, J. (1985) DNA methylation in extraembryonic lineages of mammals. Trends in Genet. 1, 89-93.

Sanger, F. and Coulson, A.R. (1978) The use of thin acrylamide gels for DNA sequencing. FEBS Letters 87, 107-110.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Schwartz, D.C. and Cantor, C.R. (1984) Separation of yeast chromosome - sized DNAs by pulsed field gradient gel electrophoresis. Cell 37, 67-75.

Sealey, P. G. and Southern, E.M. (1982) Electrophoresis of DNA. In Gel Electrophoresis of nucleic acids: A practical approach. (Ed. Richwood, D. and Hames, B.D.). IRL

Sealey, P.G., Whittaker, P.A. and Southern, E.M. (1985) Removal of repeated sequences from hybridisation probes. Nucl. Acid Res. 13, 1905-1922.

Shapiro, L.J., Mohandas, T., Weiss, R. and Romeo, G. (1979) Non-inactivation of an X-chromosome locus in man. Science 204, 1224-1226.

Sibley, C.G. and Ahlquist, J.E. (1983) Curr. Ornithol. 1, 245.

Sibley, C.G. and Ahlquist, J.E. (1984) The phylogeny of the hominoid primates as indicated by DNA-DNA hybridisation. J. Mol. Evol. 20, 2-15.

Silvers, W.K., Gasser, D.L. and Eicher, E.M. (1982) H-Y antigen, serologically detectable male antigen and sex determination. Cell 28, 439-440.

Simmler, M-C., Rouyer, F., Vergnaud, G., Nyström-Lahti, M., Ngo, K.Y., de la Chapelle, A. and Weissenbach (1985) Pseudoautosomal DNA sequences in the pairing region of the human sex chromosomes. Nature 317, 692-697.

Simpson, J.L. (1982) Abnormal sexual differentiation in humans. Ann. Rev. Genet. 16, 193-224.

Singh, L. and Jones, K.W. (1982) Sex reversal in the Mouse (mus musculus) is caused by a recurrent non-reciprocal crossover involving the X and an aberrant Y chromosome. Cell 28, 205-216.

Singh, L., Purdom, I.F. and Jones, K.W. (1980) Sex chromosome associated satellite DNA: Evolution and conservation. Chromosoma 79, 137-157.

Sirota, L., Zlotogora, Y. Shabtai, F., Halbrecht, I. and Elian, E. (1981) 49, XYYYY. A case report. Clin. Genet. 19, 87-93.

Smith, G.P. (1976) Evolution of repeated DNA sequences by unequal crossover. Science 191, 528-538.

Solari, A.J. (1980) Synaptonemal complexes and associated structures in microspread human spermatocytes. Chromosoma 81, 315-337.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Stoneking, M., Bhatia, K. and Wilson, A.C. (1986) More on mitochondrial DNA and human evolution. Cold Spring Harbor Symp. Quant. Biol. In press.

Sumner, A.T., Robinson, J.A. and Evans, H.J. (1971) Distinguishing between X, Y and YY bearing human spermatozoa by fluorescence and DNA content. Nature New Biology 229, 231-233.

Therman, E., Sarto, G.E., Palmer, C.G., Kallio, H. and Denniston, C. (1979) Position of the human X inactivation centre on Xq. Hum. Genet. 50, 59-64.

Venolia, L., Gartler, S.M., Wassman, E.R., Yen, P., Mohandas, T. and Shapiro, L.J. (1982) Transformation with DNA from 5 azacytidine-reactivated X chromosomes. Proc. Natl. Acad. Sci. USA 79, 2352-2354.

Vergnaud, G., Page, D.C., Simmler, M-C., Brown, L., Rouyer, F., Noel, B., Botstein, D., de la Chapelle, A. and Weissenbach, J. (1986) A deletion map of the human Y chromosome based on DNA hybridisation. Am. J. Hum. Genet. 38, 109-124.

Vieira, J. and Messing, J.(1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-269.

Wachtel, S. (1983) H-Y antigen and the biology of sex determination. Grune and Snatton. New York.

Walder, R.Y., Langtim, C.J., Chatterjee, R. and Walder, J.A. (1983) Cloning of the MspI modification enzyme: The site of modification and its effects on cleavage by MspI and HpaII. J. Biol. Chem. 258, 1235-1241.

Weatherall, D.J. (1985) The new genetics and clinical practice. 2nd Ed. Oxford University Press.

Weiss, B., Jacquemin-Sablon, A., Live, T.R., Fareed, G.C. and Richardson, C.C. (1968) Enzymatic breakage and joining of deoxyribonucleic acid. J. Biol. Chem. 243, 4543-4555.

Welshons, W.J. and Russell, L.B. (1959) The Y chromosome as the bearer of male determining factors in the mouse. Proc. Natl. Acad. Sci. USA 45, 560-566.

Wetmur, J.G. (1975) Acceleration of DNA renaturation rates. Biopolymers 14, 2517-2524.

Wetmur, J.G. and Davidson, N. (1968) Kinetics of renaturation of DNA. J. Mol. Biol. 31, 349-370.

Whittaker, P.A. and Southern, E.M. (1986) Ultraviolet irradiation of DNA: a way of generating partial digests for rapid restriction site mapping. Gene 41, 129-134.

Wieacker, P., Davies, K.E., Cooke, H.J., Pearson, P.L., Williamson, R., Bhattacharya, S., Zimmer, J. and Ropers, H.H. (1984) Toward a complete linkage map of the human X chromosome: Regional assignment of 16 cloned single-copy DNA sequences employing a panel of somatic cell hybrids. Am. J. Human. Genet. 36, 265-276.

Wilimzig, M. (1985) LiCl-boiling method for plasmid mini-preps. Trends Genet. 1, 158.

Wolf, S.F., Jolly, D.J., Lunnen, K.D., Friedmann, T. and Migeon, B.R. (1984) Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome. Implications for X-chromosome inactivation. Proc. Natl. Acad. Sci. USA 81, 2806-2810.

Wolf, S.F. and Migeon, B.R. (1982) Studies of X chromosome DNA methylation in normal human cells. Nature 295, 667-671.

Wolf, U. (1981) The H-Y antigen system: Interaction of several genes. In Development and function of reproductive organs. (Ed. Byskav, A. and Peters, H.) pp. 260-263.

Wolfe, J., Erickson, R.P., Rigby, P.W.J. and Goodfellow, P.N. (184) Regional localisation of 3 Y-derived sequences on the human X and Y chromosomes. Ann. Hum. Genet. 48, 253-259.

Wolfe, J., Erickson, R.P., Rigby, P.W.J. and Goodfellow, P.N. (1984) Cosmid clones derived from both euchromatic and heterochromatic regions of the human Y chromosome. EMBO J. 3, 1977-2003.

Yamamoto, K.R., Alberts, B.M., Benzingher, R., Lawhorne, L. and Treiber, G. (1970) Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology 40, 734-744.