MICROCLONING AND MOLECULAR MAPPING OF THE MOUSE X CHROMOSOME

by

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Abstract

Microdissection and microcloning have been applied to generate banks of chromosome specific probes for molecular analysis of the mouse X chromosome.

For the purposes of microdissection the CD strain of wild mouse (Mus musculus) was used - in which the X chromosome is clearly distinguishable from the rest of the karyotype. X chromosome fragments were microdissected from unstained metaphase spreads and were collected in a nanolitre micro-drop. Following collection of chromosome fragments DNA was purified and after restriction enzyme digestion was cloned into a lambda vector, all using special microprocedures.

Two microdissections of the whole X chromosome generated banks of 2000 and 1000 microclones. In addition a region specific microdissection was carried out. The mouse X chromosome was divided into four equally sized regions, designated centromeric, proximal, distal and telomeric regions. The 'proximal' region was chosen for microdissection because it contains a number of interesting genetic loci. The regional microdissection resulted in a bank of 650 microclones.

Microclones from a whole X chromosome microdissection and from the regional microdissection have been analysed for size and repeat sequence content. Mean average microclone size from both banks was observed to be smaller than expected - probably due to acid fixation of chromosomes during preparation of metaphase spreads for microdissection.

A number of microclones, mainly from the regional microdissection, were further analysed for X chromosome specificity and restriction fragment length variation between Mus musculus DNA and Mus spretus DNA. Suitable microclones have been mapped on the mouse X chromosome by classical genetic analysis, utilising a mouse pedigree derived from a Mus musculus female, heterozygous for two semi-dominant X linked coat mutations, crossed to a wild type Mus spretus male. Resulting female progeny were 'backcrossed' to male Mus musculus mice, resulting in over 230 progeny for genetic analysis of microclone positions. X chromosome specific probes were mapped in relation to the segregating coat mutations, Harlequin and Tabby and to the other probes, thus distancing and ordering clones.

Six microclone probes displaying suitable restriction fragment length variation have been positioned on the mouse X chromosome and span the expected genetic area. Individual markers display unusual physical characteristics - two probes appear to detect localised repeat sequence islands. The set of X chromosome specific molecular markers provide a basis from which further analysis of the mouse X chromosome may be undertaken.

To my family.

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Standard Abbreviations

(S. I. Units and abbreviations explained in the text are not given here)

A_{600}	optical absorbance at 600 nm
ATP	adenosine triphosphate
bp	base pair
cM	centiMorgan
uCi	microCurie
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
d.p.m.	disintegrations per minute
g	acceleration due to gravity ($9.8\ ms^{-2}$)
kb	kilobase pair
min	minute
RNA	ribonucleic acid
p.f.u.	plaque forming units
UV	ultra-violet
U	units
v/v	volume per volume
w/v	weight per volume
θ	recombination fraction

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Introduction

The field of molecular genetics lies between the two separate disciplines implicit in its name: between the genetic analysis of chromosome segregation and the molecular study of chromosome structure; between the phenotypic manifestations of genes and the physical genomic sequence. The thread which runs between the boundaries and through the whole of molecular genetics, is the structure and content of the chromosome.

The chromosome is a physical entity, with a functional genetic nature. The analysis of the genetic aspect of the chromosome concerns the segregation behaviour and phenotypic manifestations of genes. The analysis of the physical aspect of the chromosome concerns the organisation of DNA within the chromosome. This organisation operates at many levels from the packing of DNA into the form of a chromosome, to the positioning of DNA sequences in a linear array along the length of the chromosome.

A basis for the delineation of the genetic and physical aspects of the chromosome is provided by the chromosome molecular map.

A map represents a system of recognition and orientation within a defined area. In the case of the chromosome molecular map the defined area is the length of the chromosome, and the system of recognition and orientation along this two dimensional 'data string' is provided by the positioning of molecular markers. The analysis of these

markers and their DNA restriction fragment length variants represents common ground for the genetic and physical aspects of the chromosome. The molecular marker has a genetic nature - its chromosomal DNA homologue will segregate and behave as a genetic locus, and is mapped as a purely genetic locus but at the same time this is a genetic manifestation of the molecular marker, a physical entity, being a DNA sequence derived from the chromosome by chemical means.

Mapping The Mammalian Chromosome

Compared to an organism such as Drosophila, comparatively little is known of the mammalian chromosome map. A variety of factors is responsible for this situation, for example: genetic analysis of the chromosome is dependent upon the phenotypic manifestation of genes, and their observed segregation through large numbers of meioses - only a minute proportion of genes compared to the total number of genes in the mammalian genome have been observed in phenotypic segregation studies. Mammals breed slowly and produce relatively few progeny through which to study meiotic recombination; the mammalian genome is considerably larger than that of Drosophila and mammals do not produce the polytene chromosomes which have coordinated the genetic and physical maps of the Drosophila genome (Brown, 1985).

With the advent of molecular genetics and the use of DNA sequences as molecular markers, it is possible to extensively map a mammalian chromosome by observing the

segregation of chromosome specific probes through a number of meioses. This mapping is totally independent of the few known phenotypic markers, and the probes may be derived from any region of the chromosome, regardless of sequence function.

The first successful use of DNA markers for mammalian chromosome mapping was by Deisseroth et al (Deisseroth et al, 1977) who used a cDNA probe from the α globin gene. The probe was hybridised in solution to somatic cell hybrid DNAs containing different human chromosomes, and Cot analysis assigned the genes to human chromosome 16.

Filter hybridisation using Southern blots (Southern, 1975) not only improved the sensitivity and specificity of detecting probe hybridisation to genomic sequences, but also enabled investigators to make use of genomic restriction fragment length polymorphisms. In 1980 Botstein et al (Botstein et al, 1980) proposed a strategy for the construction of a complete linkage map of a mammalian genome, the human genome, utilising molecular markers. These probes could be used to detect different parental genomic DNA digestion products from the restriction of their genomic homologues. The different parental DNA fragments could then be observed in Mendelian segregation via meiosis, to progeny. Probe segregation is mapped in the same way as gene segregation, by classical genetic mapping.

Mammalian Chromosome Mapping In Mouse

In constructing a molecular map of a mammalian chromosome, the mouse has many advantages over other organisms. It is a well established laboratory animal and compared to other mammals, average litter size (6 - 8 progeny), generation time and breeding rate (on average 1 litter per 6 weeks) result in large overall numbers of progeny from individual crosses. Some laboratory mouse strains are sufficently inbred (from brother-sister, parent-child matings) to ensure background genetic homogeneity in the controlled matings necessary for large scale molecular genetic analysis. However the concommitant use of outbred strains of wild mice in matings greatly facilitates finding parental restriction fragment length variation (Robert et al, 1985).

Compared to other mammals, an outstanding number of genes (approximately 600, Davisson and Roderick, 1986) have been positioned in the mouse genome and the additional molecular data will greatly augment the existing map. Francke (Francke, 1980) states that because the mouse is genetically the best characterised mammal besides man, it is a preferred species when approaching animal models of human genetic disorders.

The Mammalian X Chromosome

In mapping human chromosomes the ease of identifying X linked genes has put the X chromosome well ahead of all others. The unusual behaviour of this chromosome and, for example, the early use of X chromosome selection in somatic

cell hybrids has meant that in man gene assignments to the X chromosome have been made disproportionately to its representation in the genome. This is not so apparent in the mouse, where the use of large numbers of controlled matings between defined phenotypes has led to the assignment of genes throughout the genome. However in constructing a molecular map of a mammalian chromosome, as a basis for the further analysis of the genetic and physical nature of the chromosome, the mouse X chromosome is an excellent subject. It provides a model system for the behaviour of a 'generalised' mammalian chromosome, but also exhibits unique and extraordinary genetic and physical phenomena.

The X chromosome is one of two mammalian sex chromosomes. It segregates and undergoes normal meiosis in females, but in all somatic cells (after early foetal stages) one of each X chromosome pair is genetically inactive (Lyon, 1961) and physically condensed throughout interphase. This inactive X chromosome replicates late with respect to the other chromosomes (Lyon, 1972).

X chromosome inactivity may vary between species and between tissues, from being totally random (as in embryonic and adult tissues of a eutherian mammal such as the mouse, Lyon, 1961) to being programmed for inactivation of the paternally-derived X chromosome, (as in certain extra embryonic tissues of the mouse, Takagi and Saski, 1975, or in all tissues of marsupials, Cooper, 1971).

In males the X chromosome is hemizygous and for most of its length it does not undergo recombination in meiosis, when it

forms a heterologous pair with the Y chromosome.

In 1964 Ohno and co-workers (Ohno et al, 1964) noticed that the absolute size of the euchromatic region of the mammalian X chromosome was strikingly constant in a wide variety of species. Ohno suggested that the unique features of the mammalian X chromosome (for example, hemizygous gene dosage - see below) led to the evolutionary conservation of an ancestral X linked syntenic group (Ohno, 1967). Ohno's hypothesis was proposed, stating that any gene locus that is X linked in man should be X linked in all other mammalian species.

All data so far examined regarding X linkage of genes, supports Ohno's hypothesis. X linked gene loci are conserved between species, and this conservation spans vast stretches of time. For example, G6pd and Hprt which are X-linked in mouse and man (and in all other eutherian mammals so far studied) are X-linked in the red kangaroo (Donald and Hope, 1981) indicating conservation of X linkage for at least 130 million years - since the time of the marsupial-eutherian radiation.

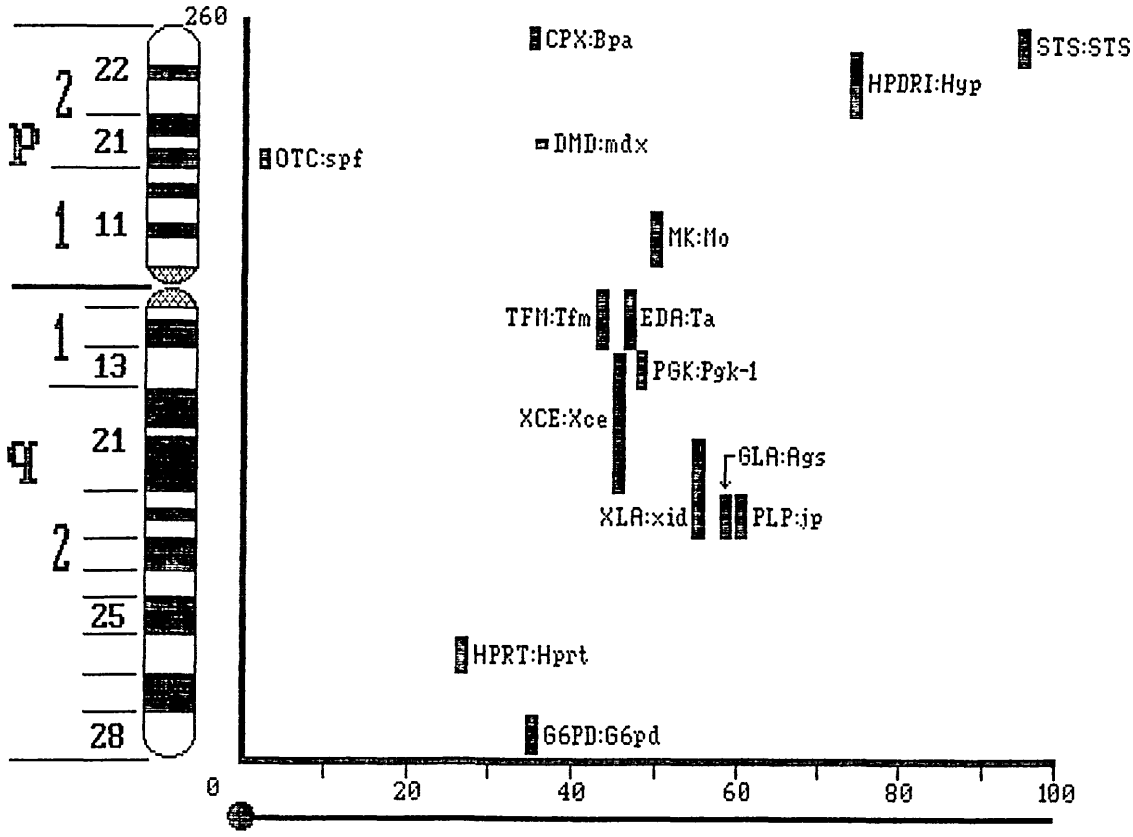
Whilst X-linkage appears to be totally conserved, locus order is only broadly conserved. Between man and mouse (for example) there have been chromosome rearrangements (see Fig. 1.1.).

Figure 1.1. Diagram of proposed homologies between mouse X chromosome and human X chromosome loci

Human Locus	Mapping Data	Mouse Locus	Mapping Data	Homology Data
STS	Craig and Tolley, 1986	STS	Craig and Tolley, 1986	Craig and Tolley, 1986
CPX	McKusick, 1986	Bpa	Davisson and Roderick, 1986	Buckle *et al*, 1984
HPDRI	McKusick, 1986	Hyp	Davisson and Roderick, 1986	Buckle *et al*, 1985
OTC	McKusick, 1986	spf	Davisson and Roderick, 1986	Buckle *et al*, 1985
DMD	McKusick, 1986	mdx	Davisson and Roderick, 1986	Bulfield *et al*, 1984
MK	McKusick, 1986	Mo	Davisson and Roderick, 1986	Buckle *et al*, 1985
TFM	McKusick, 1986	Tfm	Davisson and Roderick, 1986	Buckle *et al*, 1985
XCE	McKusick, 1986	Xce	Davisson and Roderick, 1986	Buckle *et al*, 1985
EDA	McKusick, 1986	Ta	Davisson and Roderick, 1986	McKusick 1984
PGK	McKusick, 1986	Pgk-1	Davisson and Roderick, 1986	Buckle *et al*, 1985
GLA	McKusick, 1986	Ags	Davisson and Roderick, 1986	Buckle *et al*, 1985
PLP	McKusick, 1986	jp	Davisson and Roderick, 1986	Dautigay *et al* 1986
XLA	Mensinck, *et al*, 1986	xid	Cohen *et al*, 1985	Lyon, (Pers. comm.)
HPRT	McKusick, 1986	Hprt	Davisson and Roderick, 1986	Buckle *et al*, 1985
G6PD	McKusick, 1986	G6pd	Davisson and Roderick, 1986	Buckle *et al*, 1985

Figure 1.1. Diagram of proposed homologies between mouse X chromosome and human X chromosome loci

The genetic mouse X chromosome map is shown along the X axis, with an approximate genetic length of 100 cM (Davisson and Roderick, 1986). The banded human X chromosome and mapped loci, is shown along the Y axis, with an approximate genetic length of 260 cM (Drayna et al, 1984); loci on the human map have been positioned by a variety of methods - see references.



The human loci symbols are shown first, then the proposed homologous mouse loci symbols.

Maintenance of X chromosome specificity may be largely due to gene dosage in certain X chromosome coding sequences which may be detrimental if transcribed in a double dose from two active chromosomes. Whilst at the level of chromosome linkage, synteny in the X chromosome is largely conserved between species, molecular structure at the level of the DNA sequence is not conserved between species. X chromosome DNA sequence divergence is comparable to that of autosomal sequences. For example, under conditions generally used in probe hybridisation to Southern blots, most randomly isolated (i.e. generally non-coding) human X chromosome DNA sequences have not shown any detectable homology to rodent sequences (Willard, 1983, for example see Bruns et al, 1982).

A dichotomy exists between the evolution of the X chromosome as a unique syntenic group and the evolution of X chromosome DNA sequences; this dichotomy illustrates the different levels of chromosome evolution and organisation. At one level of organisation, the molecular sequence level, the X chromosome is subject to the same evolutionary forces as autosomes, and its 'response' is similar to autosomes; almost no difference should be detected between them at this level. At the level of chromosome linkage, the X chromosome is also subject to the same forces as the autosomes, but its evolution has been different from the autosomes, and unique in the chromosome complement.

At the cytogenetic level there is limited X chromosome homology: mammalian X chromosomes can be metacentric (human)

or acrocentric (mouse), mostly euchromatic (human) or largely heterochromatic (hamster) (Pathak and Stock, 1974).

The Molecular Map As A Basis For Analysis Of The Genome

A molecular map provides a basis for beginning genetic and physical analyses of the mammalian chromosome. Each analysis of the chromosome presents a general problem of orientation within the vast genetic and physical distance spanned by a chromosome, and presents problems specific to the aim of the individual analysis. The general problem of orientation may be solved by the mammalian chromosome map. The particular problems of the individual analysis of chromosomal DNA sequences may also be resolved or aided because firstly, localising sequences of interest to a specific area is the beginning of the definition and characterisation of these sequences, and secondly because of the dual genetic and physical nature of the molecular map. Molecular markers provide access to the DNA because each marker is derived from the DNA, and represents and is available as, a cloned sequence.

Analysis Of Individual Loci

In the analysis of individual loci the use of the molecular map depends firstly on the genetic nature of the map to reveal a marker closely linked to a locus of interest, and secondly on the physical nature of the map to utilise the marker as a basis for physically defining and analysing the locus.

Throughout mammalian genetics the vast majority of known loci are genes which are defined by a mutation in gross phenotypic effect, for example genes which exert simple effects on, eye colour, or coat colour, or single loci which exert complex effects on a variety of tissues. In a large percentage of cases the molecular structure and function of the locus of interest is not known. A molecular definition of a locus is important for its integration into the biology of the whole organism, and in man the molecular definition has increasing clinical implications; firstly, in the prenatal diagnosis of genetic disease; secondly in the attempt to alleviate the symptoms of genetic disease; thirdly in gene therapy, the replacement or alteration of the host genotype in a proportion of cells.

One of the earliest examples of the use of molecular markers for the mapping of the mouse genome, was provided by Swan and colleagues in 1979 (Swan et al, 1979) who used cDNA molecular probes in conjunction with mouse-hamster somatic cell hybrids to give a chromosomal assignment to the immunoglobulin κ light chain genes.

In man, an example of the importance of the molecular definition of a locus was provided by Kan and Dozy in 1978 (Kan and Dozy, 1978), who performed the first prenatal diagnosis of a genetic disease utilising a closely linked probe. The probe mapped to a Hpa I site approximately 5 kb from the β globin gene, which in the pedigree examined was polymorphic between normal and sickle cell phenotypes. However for prenatal diagnosis, ideally there should be no

possibility of recombination between the probe and the mutant allele. This is the case first reported by Geever et al in 1981 (Geever et al, 1981) where a Dde 1 recognition site was altered as a result of the sickle cell mutation in the β globin gene.

The probes used by Swan et al, Kan and Dozy, and Geever et al were all derived from cDNA; in 1982 the first linkage between a random DNA marker and a known mammalian genetic locus was reported - Murray et al (Murray et al, 1982) established linkage between a human X chromosome specific single copy DNA sequence (polymorphic at a Taq 1 site) and the Duchenne muscular dystrophy locus.

Using genomic DNA probes and linkage studies to position genes to a chromosome is particularly important for loci in which the exact product cannot be determined - in either the normal or mutant form. This is particularly relevant to most human genetic diseases that have no distinguishable phenotype in cultured cells and cannot be localised by hybrid cell mapping (White et al, 1985) for example, Huntington's chorea (Gusella et al, 1983) or cystic fibrosis (Wainwright et al, 1985).

Molecular maps of the mammalian chromosome have a genetic form and a physical form. The genetic form is a linear array of segregating sequences. The physical form is a linear array of cloned sequences which give access to the DNA in the genome. The physical power of finding a probe which is closely linked to a locus of known phenotype, but

unknown gene product is demonstrated on the human X chromosome by Royer-Pokara et al (Royer-Pokara et al, 1986). They used a strategy based on molecular mapping data for the genetic and physical analysis of the locus responsible for X linked chronic granulomatous disease. Deletion data and linkage analysis utilising genomic X chromosome specific probes (Baehner et al, 1986) had mapped the gene locus to a specific region of the human X chromosome, and X chromosome specific probes derived from a chromosome walk in this region were analysed for mRNA transcripts. An mRNA transcript of the gene responsible for chronic granulomatous disease was discovered. Neither the protein encoded by this transcript nor the cDNA revealed any significant homology to known sequences.

This is the first example in mammals of the cloning of a gene that had been identified and characterised phenotypically but whose protein product was unknown. The use of molecular markers initially to establish linkage and to map the locus, and then to physically approach the gene was the basis of the cloning strategy.

Unique Analyses Of X Chromosome Genes

One type of analysis aimed at the cloning of genes and based on the mammalian chromosome map is largely confined to the X chromosome. The conservation of X chromosome loci between species, (specifically referred to in Ohno's hypothesis) means that molecular analysis of the mouse X chromosome has implications for the human X chromosome. In 1984 out of 29

loci positioned on the mouse X chromosome (Roderick and Davisson, 1984), 12 had documented homologues in man (Buckle et al, 1984).

For example, six X-linked muscular dystrophies have been described in man, (McKusick, 1984) and of these the Duchenne muscular dystrophy locus, the Becker muscular dystrophy locus and the Emery Dreifuss muscular dystrophy locus have been assigned to Xp21, Xp21 and Xq28 respectively. Duchenne muscular dystrophy is the most common of all (X linked and autosomal) muscular dystrophies, occuring with a frequency of up to a 1 birth per 5000 new born males (Murray et al, 1982). Becker muscular dystrophy may be allelic to Duchenne muscular dystrophy. All three mapped X linked muscular dystrophy loci have been assigned to specific regions of the human X chromosome by linkage analysis, but the gene product of all three is unknown.

In 1984 Bulfield et al, (Bulfield et al, 1984) described a spontaneous mutation arising in an inbred C57Bl/10 mouse colony that was an X chromosome linked muscular dystrophy (mdx). If Ohno's hypothesis is correct for this locus, then mdx is probably the homologue of one of the human X linked muscular dystrophies. On the basis of several common histopathological features, it has been suggested that mdx may be homologous to the Duchenne muscular dystrophy locus (DMD). These features include high serum levels of muscle enzymes, (for example, creatinine kinase, pyruvate kinase), degeneration of certain muscle fibres, proliferation of phagocytic cells in place of lost fibres and considerable

variation in muscle fibre size due to enlargement of some fibres and atrophy of others.

If a molecular probe derived from coding DNA was available for the mdx locus it is very likely that the probe would cross-hybridise at low stringency to human DNA. Mapping the probe to the human X chromosome would provide information about the comparative positions of genes on the human and mouse X chromosomes, and would also provide access to a human locus likely to be involved in one of the known muscular dystrophies. A molecular probe would facilitate cloning of the gene at the human dystrophy locus.

A gene specifically described on the mouse X chromosome is that at the Xce locus (Cattanach et al, 1971). This locus exerts an effect on X chromosome inactivation - different alleles alter the likelihood of inactivation of their chromosome (Rastan and Cattanach, 1983). Xce is defined by gross phenotypic effect, and has been mapped to a specific position on the mouse X chromosome. This locus is probably involved in chromosome inactivation, and may operate via chromosome condensation.

This important gene is uncharacterised, but the first step towards characterisation - mapping - has been made. Finding a closely linked molecular probe, for chromosome walking or jumping into the immediate region of the gene would be the next step.

One aspect of Xce locus function is recognition - recognition of the female XX and the male XY state,

recognition of the second Xce locus in a female cell, recognition in certain species and tissues of the paternally derived X chromosome. A mechanism of recognition between chromosomes, 'imprinting' of the chromosome, is implied from the functioning of the Xce locus and from data regarding the necessity of both the maternal and paternal contribution to a zygote to produce a fully viable organism (Anderegg and Markert, 1986).

The reason for imprinting and the mechanism of imprinting are absolutely unknown, but a molecular analysis of the Xce locus would probably help to explain this phenomenon.

Gene Expression

As well as identifying and characterising genes, molecular maps have been useful for examining the control of gene expression. For example, Menkes' disease, is an X linked disorder of copper metabolism with a homologue at the Mottled locus on the mouse X chromosome. The disease alters either metallothionein protein structure (Chan et al, 1978) or biosynthesis (Riordan and Jolicoeur-Paquet, 1982), and the metallothionein locus was therefore assumed to be X linked.

However Schmidt et al (Schmidt et al, 1984) hybridised cDNA metallothionein probes to various cell hybrids and mapped the human metallothionein sequences (including a processed pseudogene) to at least five autosomes; no metallothionein sequences were on the X chromosome, and in mouse metallothionein sequences are also autosomal. Therefore

metallothionein gene expression appears to be controlled in some way by an X linked transacting factor. Subsequently the tyrosine aminotransferase gene on mouse chromosome 8 was found to be affected by a transacting factor from the albino locus on mouse chromosome 7 (Muller *et al*, 1985).

These two cases have provided some of the earliest data on the control of eukaryotic gene expression *in trans*, and could have only been detected by the application of molecular mapping to specific genes.

Molecular markers derived from sequences which would not be present in cDNA libraries are necessary adjuncts to studying the control of gene expression in even well characterised genes; for example, most β thalassaemia mutations do not occur in coding sequences - there often appears to be a defect in transcriptional regulation or RNA processing (White, 1985); Lesh-Nyan disease results from *HPRT* deficiency, but it is known that this is seldom due to a lack of *HPRT* mRNA (Newmark, 1984).

Molecular markers may also provide information regarding gene regulation and position effects, for example, cytogenetic data has shown that for some cases of Burkitt Lymphoma in man, part of chromosome 8 is translocated to chromosome 14. Molecular mapping data has shown this chromosome translocation aligns c-*myc* sequences on chromosome 8, with V_H (immunoglobulin variable region of the heavy chain) sequences on chromosome 14. This may result in activation of c-*myc* sequences, ultimately resulting in Burkitt lymphoma (Dalla-Favera *et al*, 1982).

Molecular mapping has defined this chromosome translocation at a molecular level, and has indicated from information regarding gene position the nature of the aberrant gene control which probably results in oncogenesis.

In the above cases molecular probes have been used to study individual examples of control of gene expression; probes may also be used to look at broad mechanisms of control. For example, Cullen et al (Cullen et al, 1986) used selected restriction enzymes sensitive and insensitive to DNA methylation along with molecular markers mapped to, and derived from, gene sequences, to study methylation in relation to X chromosome inactivation and gene dosage. Using probe hybridisation to Southern blots of restricted male and female DNA samples they concluded that methylation probably does not have a role in the dosage compensation of tissue specific genes (unlike house keeping genes) but suggest that differences in DNA methylation related to X chromosome dosage compensation may be maintained regionally, and limited to areas with localised high concentration of CpG dinucleotides (see on).

The Physical Chromosome

The Organisation Of Gene Sequences

As well as providing information about the genic content of the chromosome, the physical nature of the mammalian chromosome may also be examined through molecular markers and the chromosome map. Molecular markers provide access to the different levels of chromosome organisation and very

little is known of the long range or short range organisation of the components of the mammalian chromosome. Classical genetic mapping based on gene phenotype has mapped too few genes, too loosely to be able to extrapolate information about, for example, the arrangement of genic and non-genic sequences. However, molecular analysis of the chromosome facilitates the delineation of chromosome organisation.

Examples of the use of molecular markers for this purpose were demonstrated by Bernardi and co-workers (Bernardi et al, 1985) and Brown and Bird (Brown and Bird, 1986).

Bernardi and colleagues separated unrestricted mouse nuclear DNA from warm blooded vertibrates, including mouse, by density gradient centrifugation. The DNA formed into four major components and several satellite and minor (such as ribosomal DNA) components. The major components generally included two light fractions and two (or three) heavy fractions. Using molecular probes Bernardi et al studied the distribution of genes, families of interspersed repeats and integrated viral sequences within the major components. Having observed this distribution a correlation was made between distribution, base composition and codon usage for the sequences.

Molecular probe hybridisation data revealed that the distribution of the three observed species is non-uniform within the genome. Gene sequences are preferentially distributed in the heavy DNA components (i.e. high GC

content). Families of interspersed repeat sequences, and integrated viral sequences tend to be located in the major components, but each repeat family or group of viruses is only located within one major component - for example mouse mammary tumor virus sequences were almost all found in one of the light components of the mouse genome.

In the heavy components Bernardi et al observed a higher GC content in exons, compared to their introns and surrounding heavy component environment, and a different codon usage between simliar genes in different components. For example the cardiac and skeletal mouse α actin genes are located in light and heavy components respectively. They differ by 8 % and 16 % in overall and third position GC content but the protein products show only a 1 % difference in amino acids. Bernardi et al conclude that the genome is compartmentalised into heavy and light 'isochores' of approximately 200 kb, and the codon usage of genes depends on the GC content of their isochores. 'Compositional compartmentalisation' by isochores appears to be an integral feature of chromosome structure.

Brown and Bird (Brown and Bird, 1986) used pulse field gradient gel electrophoresis to map cleavable sites for 'rare cutter' restriction enzymes. These enzymes generally recognise a 6 bp sequence composed entirely of CG dinucleotides, including 1 or more CpG dinucleotides which are rare in mammalian genomes. Brown and Bird predicted that cleavable sites for these methylation sensitive restriction enzymes would not be randomly distributed but

would be concentrated in stretches of genomic DNA called HTF islands (which span 0.5 - 2 kb).

HTF islands (Bird et al, 1985) were discovered from the cleavage of whole genome mouse DNA by Hpa II, which recognises CCGG (non methylated). About 1 % of the mouse genome is cleaved by Hpa II to give a discrete fraction and of this roughly 20 % is derived from ribosomal DNA. Genomic mapping using molecular probes derived from three non-ribosomal fragments showed that the fragments were derived from islands (Hpa II Tiny Fragment islands) within which Hpa II sites were highly concentrated.

The prediction by Brown and Bird was made because HTF islands are distinct from the bulk of whole genome DNA by being 65 % CG and unmethylated. The prediction was confirmed by the pulse field gel electrophoresis of rare cutter restricted genomes and observation of DNA fragment distribution, and by molecular probes derived from islands isolated at random from genomic DNA and from the dehydrofolate reductase gene which were utilised for the restriction mapping of HTF islands.

Molecular probes have shown these islands are associated with genes, mainly house keeping genes rather than genes expressed exclusively in one or a few tissues (Bird, 1986).

The work of Bernardi's group and Brown and Bird has utilised molecular probes from genes positioned on the chromosome molecular map to confirm that the distribution of gene sequences through the genome is non-random and is subject to an underlying organisation.

The Organisation Of Repeat Sequence DNA

Bernardi et al looked mainly at genic sequences, but also used molecular probes to report the non-random distribution of repeat sequences in different major isochores in mouse and other vertibrates.

Molecular probes derived from repeat sequences can be used to give a more specific positioning of repeat sequences within the genome. An example of the molecular investigation of a repeat sequence is provided by the Bkm sequence.

Bkm is a satellite DNA sequence which was first isolated from the female banded krait (Jones and Singh, 1981a), which is the heterogametic sex (ZW). Molecular probes derived from Bkm sequences were mapped by in situ hybridisation and were found to be largely concentrated on the W chromosome. Bkm hybridises to a wide variety of species, concentrating to varying degrees on the sex chromosomes. In Mus musculus Bkm sequences are male specific and have been mapped mainly to the Y chromosome (Jones and Singh, 1981b).

This faculty of Bkm sequences was used by Singh and Jones (Singh and Jones, 1982) to delineate the nature of the sex-reversal mutation, Sxr (Cattanach et al, 1971) in mouse. Sxr appeared to be an autosomal dominant trait, but considerable gene linkage analysis (Cattanach, Pers. comm.) failed to position the mutation. In 1982, Singh and Jones, having observed that Bkm sequences map mainly to a paracentric region of the mouse Y chromosome, hybridised Bkm molecular probes to chromosomes from normal males and

females, and sex-reversed females (XX ^{Sxr}) and Sxr carrier males (XY ^{Sxr}). The resulting data gave information about the Sxr mutation and the testis determining gene(s) in mouse.

Firstly, sex reversal of females was found to be due to a transfer of sequences from the telomere of the Y chromosome onto the telomere of the X chromosome. These sequences contained the testis determining gene(s) because they produced the male phenotype in an otherwise XX female karyotype. Secondly, up until that time the location of the testis determining gene(s) in mouse was unknown, but data from Bkm in normal XY male mice indicated it was located in the paracentric region of the Y chromosome. Thirdly, the above data and data from XY ^{Sxr} carrier males indicated that the Sxr mutation was due to a duplication of the testis determining region, the duplicate copy being present at the Y chromosome telomere.

Bkm sequences are one of many repeat sequence families. These families be high or low copy, localised or interspersed, transcribed or non-coding, but in few cases are their specific characteristics or their function, if any, known. Molecular probes can be used to map and investigate these otherwise inaccessible sequences, giving data on organisation, structure, function and evolution of what makes up the majority of the mammalian genome.

The Genetic-Physical Relationship

Molecular maps have been used to define the chromosome through the genetic mapping of markers along the chromosome (the molecular genetic map) and through the physical mapping of markers to the banded chromosome (the molecular physical map). These two forms of molecular map may be used to examine the relationship between the genetic and physical manifestations of the chromosome.

The genetic map of the chromosome is based on observing meiotic recombination events. If recombination occurs with an equal likelihood throughout the genome, the genetic map will simply parallel the physical map. However, if recombination events are preferentially located, there will be disparity between the genetic and physical maps.

Genetic maps show only a small fraction of genes in the genome, but enough genes have been mapped in the mouse genome to overcome errors due to sample size, when looking at gene distribution. The genes that have been mapped should be randomly selected and should be located throughout individual mouse chromosomes, (and should not be preferentially chosen from any physical region). If this is the case, then if crossing-over is randomly located, the genes should appear with a random and equal distribution throughout the chromosome. However, if crossing-over is not randomly located but occurs preferentially in certain locations, then genes will be widely spaced on the genetic map in regions of high recombination and genes will be closely linked in regions of little or no recombination.

Lyon (1976) studied the genetic map of the mouse genome and concluded that the distribution of genes on the mouse X chromosome (and chromosomes 2, 4, 5, 6, 7, 10, 11, 15 and 17) differed significantly from a Poisson distribution, and indicated a non-random distribution of genes (given the two initial parameters - the random selection of genes and the random location of crossover events).

This observation may be due to a number of factors - for example, true inhibition or facilitation of recombination at certain regions, or initiation of chiasmata at preferential points on a chromosome, with inhibition of recombination in surrounding regions as a consequence of the initial recombination event. However the assumption of a random distribution of genes is unproven and the second possibility exists, that gene clustering truly reflects the physical chromosome, recombination being a chance event with a random distribution.

In looking at the level of the whole genome, it has also been shown that there are differences in overall recombination values between the sexes. In general, recombination fractions from males are lower than those from females, but there are specific exceptions - such as in mouse chromosome 15 in the region of <u>caracul</u> gene (a region of tight clustering) where recombination values are higher in males than females (Wallace and Mallyon, 1972).

Molecular markers have been used in making observations across localised regions of a chromosome, (rather than the

whole chromosome). These probes (often derived from random DNA sequences) show that in defined areas of the chromosome meiotic recombination does not occur at random. There are are areas of recombination suppression and of abnormally high recombination (recombination hot spots).

An example of recombination suppression spanning up to one third of a chromosome is provided by the t-complex on mouse chromosome 17; the nature of the t-complex and its recombination suppression is being elucidated through the use of molecular probes.

When a t-complex haplotype is heterozygous with a wild type mouse chromosome 17, crossover suppression occurs between this region on the two chromosomes (Lyon et al, 1979). Recombination studies utilising molecular probes (derived from the microcloning of the physical region corresponding the the t-complex, from a wild type mouse chromosome (Rohme et al, 1984)) support the hypothesis that partial t-haplotypes suppress recombination in proportion to the extent of t-haplotype DNA that they contain (Fox et al, 1985). This may be due to reduced recombination or inviability of crossover products. Partial t-haplotypes are derived from rare wild type - t-complex recombination events.

t-complex crossover suppression is known to be a result of rearrangements between wild type and t-complex DNA (there is no recombination suppression in t-complex homozygotes), (Silver and Artzt, 1981). Shin et al (Shin et al, 1983) used molecular probes from the major histocompatibility

genes in the t-complex to show that these genes had taken part in a distal inversion compared to the wild type chromosome, which would account for non-homology of these sequences with the wild type chromosome. In 1986 Herrmann and colleagues used the markers derived from the microdissection of the t-complex to demonstrate the existence of a second inversion, within the proximal portion of the t-complex. The two described inversions are non-overlapping but encompass most of the DNA in the t-complex (Herrmann et al, 1986). The DNA rearrangement resulting from these two inversions appears to result in the massive reduction in recombination that occurs between the t-haplotype and the wild type chromosome.

Molecular evidence for a recombination hot spot is provided from the major histocompatibility complex (MHC) of mouse. Molecular maps that are available for large portions of the major histocompatibility complex are not congruent with the available classical genetic maps, which have been derived from recombination frequencies between marker loci (Uematsu et al, 1986). Meiotic recombination in the MHC is not random, but occurs with a high frequency at certain hot spots which vary dramatically in activity. For example, Uematsu et al, cloned (and sequenced) a hot spot from Mus musculus castaneous between the $A_{\beta 3}$ and $A_{\beta 2}$ genes. Five recombination break points were localised to this 3.5 kb stretch of DNA, and the frequency of recombination in this area is considerably greater than the average frequency per

unit length for the whole mouse genome. Meiotic recombination hot spots may possibly maintain polymorphism at MHC loci and be advantageous to the organism.

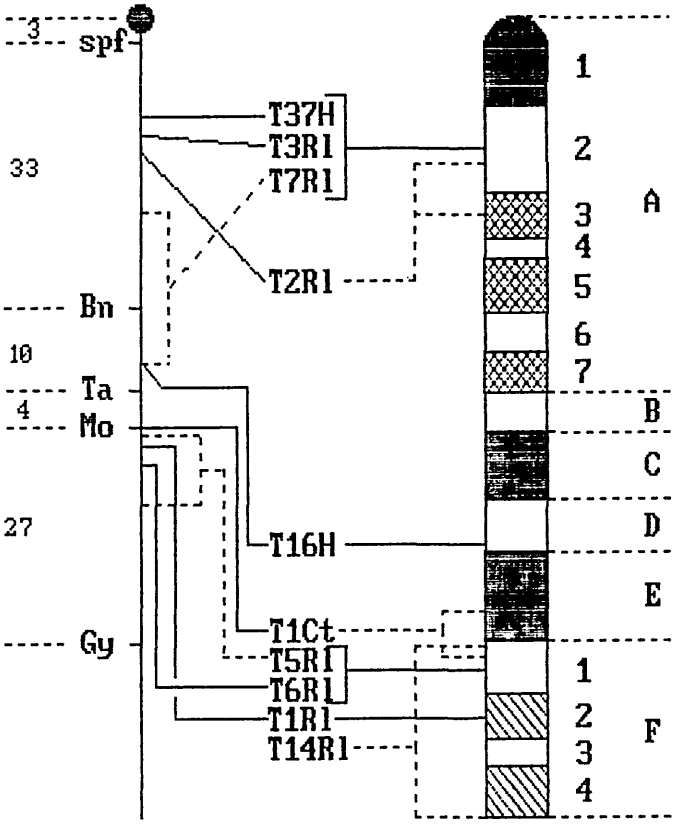
Molecular probes have been used to indicate the non-uniformity of meiotic recombination over small regions in the mouse genome, which will alter the genetic map in relation to the physical map of these regions.

Molecular probes may be used directly to analyse the genetic-physical relationship because of their genetic-physical nature. Until the use of molecular probes, little data were available on the physical position of genetic loci. These data were derived for example from the genetic mapping of rare translocation breakpoints which could also be positioned cytogenetically (Russell, 1983). 11 translocation break points have so far been mapped to the mouse X chromosome and these are not co-linear between the genetic and physical maps (Fig. 1.2.).

Molecular probes may be mapped genetically through linkage data, or physically for example by in situ hybridisation (Dautigay et al, 1986). The genetic and physical position of a specific probe may be compared. The use of pulse field gel electrophoresis (Brown and Bird, 1986) will also facilitate the physical mapping of probes over vast physical distances, corresponding to resolvable genetic distances of up to a cM.

Figure 1.2. Diagram of mouse X chromosome:autosome translocations (Searle and Beechey, 1981)

Schematic representation of the genetic and physical mouse X chromosome.

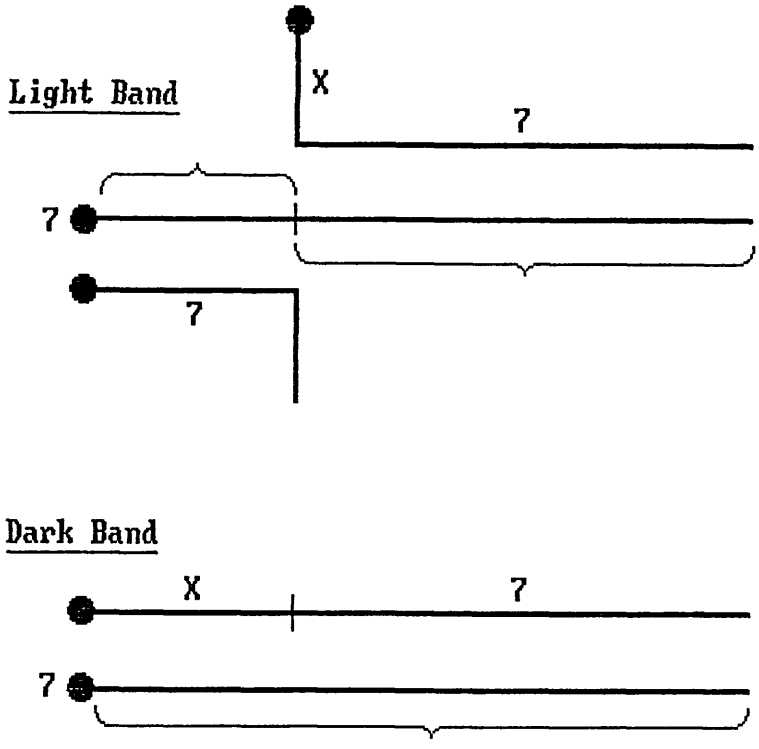


Direct physical and genetic analysis of molecular probes and the chromosome map have been used very successfully for an investigation of the X chromosome and its unique meiotic behaviour in males.

Rouyer et al (Rouyer et al, 1986) followed the genetic segregation through male meiosis to progeny, of three probes polymorphic between male parental X and Y chromosomes, known from physical data to map to the distal short arms of the human X and Y chromosomes. Segregation data revealed a gradient of linkage - recombination per unit of physical length appears to increase towards the telomere of Xp and Yp, culminating in a recombination fraction of 50 % at the tip of the telomere, in male meiosis. Telomeric sex-chromosome specific probes appear to be 'pseudoautosomal' and physical data (i), confirms that a probe is sex chromosome specific prior to linkage studies, (ii), indicates the physical position of the probes towards the telomere where genetic mapping indicates vast distances with recombination per unit of physical length increasing towards the telomere.

Physical data on the positioning of molecular markers provides an insight not only into the positions of abnormal recombination, but may also help to elucidate the exact correspondance between highly extended interphase chromosomes and highly condensed metaphase chromosomes. The packaging of DNA between chromosome types and between bands remains to be elucidated.

Figure 1.3. Diagram of synapsis between translocated and non-translocated chromosomes, according to Ashley (Pers. comm.) (G-banded chromosomes)



Where translocation breakpoints occur in light bands, homologous synapsis only occurs (bracketed regions), with no suppression of recombination

Where translocation breakpoints occur in dark bands, non-homologous synapsis occurs (bracketed region), with recombination suppression

There appears to be a possible association of chromosome banding with translocation breakpoints and recombination; this association should be analysed at the molecular level. Ashley (Pers. comm.) observes that in mouse X:autosome translocations, if the translocation breakpoint occurs in a light band, (giemsa staining) there is synapsis between homologous chromosome regions (see Fig. 1.3.) presumably with normal meiotic recombination. However, if the breakpoint lies in a dark band non-homologous chromosomes pair, and it is assumed that crossing-over is suppressed. Positioned molecular markers could be used to investigate the extent of recombination suppression.

Molecular Markers And Evolution

Molecular maps have significant contributions to make to the investigation of various aspects of the evolution of the genome.

At the level of the individual chromosome it has been observed by Bennett et al using mapped molecular markers (Bennett et al, 1986) that extremely large differences in recombination exist between the sexes of the marsupial Sminthopsis crassicaudata, with much closer linkage occuring in females. If the X chromosome of this animal undergoes little or no meiotic recombination in male or female meiosis, then it is being inherited as an essentially haploid chromosome. Molecular probes would analyse the result of this novel form of chromosome evolution.

At the level of mapped individual loci, genic and non-genic,

molecular maps can, for example, yield data on the fluidity of loci on the chromosome, or be used for delineating chromosome lineages.

In observing individual loci and the non-random distribution of crossing-over in the mouse genome, Lyon (Lyon, 1976) states that consigning recombination to certain regions of the chromosome may have evolutionary significance as this would favour the development of blocks of syntenic genes that are relatively undisturbed by crossing-over.

Comparative molecular mapping between species has been used to provide data of the evolution of the human Y chromosome. For example, Page and colleagues, (Page et al, 1982) mapped a molecular marker, DXYS1, to loci on the long arm of the human X chromosome and the short arm of the human Y chromosome. This data would appear to correlate with Ohno's suggestion that the sex chromosomes are derived from an ancestral pair. Therefore if the X and Y loci represent a region of ancient homology these sequences should be present on both X and Y chromosomes in all the great apes. However, it is possible these homologous sequences could be the result of a modern event, such as a transposition between sex chromosomes which has occured since, for example, man diverged from chimpanzees.

Page and co-workers examined DNA from chimpanzee, gorilla, orangutan and man (Page et al, 1984), and found the locus was only present on the X chromosomes of the great apes, therefore the X-Y homology at the human locus appears to be of recent origin.

There was less divergence between DXYS1 X and Y chromosome sequences than the divergence of the human genome from that of its closest relative, the chimpanzee - this is consistent with this suggestion of a recent transposition from X to Y chromosomes.

A similar observation of sequences of recent evolutionary origin has been made as a result of molecular mapping in the mouse, where up to 3 % of the Y chromosome is related to retroviral sequences not present on the X chromosome (Phillips et al, 1982).

Comparisons of the X chromosome genetic map of mouse and man (Fig. 1.1.) also show examples of large movements of DNA and relative divergence in chromosome organisation, despite the fact that in terms of linkage, the X chromosome is the most conserved of all mammalian chromosomes.

At the level of individual sequences, molecular maps give new insights into the derivation of chromosome component sequences. For example, one of the first uses of molecular probes in mouse was to map the mouse globin genes. The molecular hybridisation of the globin cDNA probes resulted in the detection of more genes than had been expected, and so the first mouse pseudogenes were described (Proudfoot, 1980). Phenotypic studies had given no indication of the existence of pseudogenes.

Vanin (Vanin et al, 1980) detected by molecular mapping a pseudogene related to the mouse α globin locus. Subsequent sequence analysis identified several additions and deletions

to the gene, from 1 - 12 bp in length, which prevent the production of a functional ∝globin polypepetide (due to in-phase termination codons). This gene also lacks the two introns normally found in the functional ∝globin gene.

The discovery of pseudogenes and gene families from the use of molecular markers and molecular maps, has led to different theories regarding genome evolution and how these sequences arose. For example, the intronless pseudogene arose from either: (i) precise excision of the intron DNA, which had been present in an ancestral gene; (ii) from an insertion of an ∝globin reverse transcript into the genomic DNA; (iii) from an ancestral duplication event from an intronless ancestral gene - the duplication failed to receive the promotor sequences so with no phenotypic selection pressure it was allowed to drift and diverge. However pseudogenes arose, in their current form they are 'no more than relics of evolution'. Proudfoot (Proudfoot, 1980) has estimated that one in four (mammalian) genes may be pseudogenes and that 'one quarter of our genes may be dead'.

Until the use of molecular probes, pseudogenes were an unknown aspect of chromosomal organisation and gene evolution. The detailed study of the DNA by these probes has generated a variety of hypotheses concerning the mechanisms of sequence evolution - such as gene duplication or the incorporation of reverse transcripts into the genome. The individual mechanisms have wider implications concerning the overall pattern of chromosome evolution involving, for

example molecular drive (Dover, 1982), or selfish DNA (Ford Doolittle and Sapienza, 1980, Orgel and Crick, 1980).

The Current State Of The Molecular Map

To be useful a map must fulfil certain criteria. It must have defined orientation points which are recognisable and distinct, and have direction and order. These orientation points must be sufficiently dense for an appropriate resolution of the features of the chromosome.

The mammalian molecular map fulfils the criteria but also has one outstanding feature, which transcends the genetic map of the chromosome - it provides access to the DNA because the orientation points are cloned DNA sequences.

A molecular map of the mouse X chromosome is the basis for an investigation of the genome.

Molecular chromosome maps have been responsible for insights into the structure, organisation, functioning and evolution of mammalian chromosomes. As a result the mechanisms underlying complex biological phenomena are being elucidated.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

All chemicals used were Analar grade supplied by British Drug Houses (BDH), Poole, U.K. with the exception of: ethanol - James Burroughs Ltd., London, U.K.; phenol - Rathburn Chemicals Ltd., Walkersburn, U.K.; dithiothreitol (DTT), ethidium bromide, sodium chloride, spermidine, trizma base (TRIS) - Sigma Chemical Co., St. Louis, Mo., U.S.A.

2.1.2. Tissue Culture Reagents

Heparin was supplied by Pains and Byrne Ltd., Greenford, U.K. One unit of heparin is defined as the quantity which will prevent 1 ml of citrate of sheep plasma from clotting for 1 hour after the addition of 0.2 ml of 1 % (w/v) calcium chloride solution. RPMI 1640 was from Flow Laboratories Ltd., Irvine, U.K. Heat inactivated foetal calf serum, glutamine, penicillin, streptomycin, and colcemid were from Gibco Ltd., Paisley, U.K. One unit of penicillin is the equivalent of 0.6 ug penicillin. Concanavalin A was provided by P. H. Glenister of M.R.C. Radiobiology Unit, Harwell. Tissue culture dishes were from Nunc, Roskilde, Denmark.

2.1.3. Microdissection Equipment

The microscope and the microforge were supplied by Micro Instruments (Oxford) Ltd., Oxford, U.K. The de Fonbrunne Micromanipulator was supplied by Bachofer Laboratorium, Reutlingen, F.R.G. Oil chambers were from Bischoff

Glastechnik, Bretten, F.R.G. Cover slips were from AB Termoglas, Göetenborg, Sweden. Spectroscopic grade paraffin oil (Merck no. 7161) was from E. Merck, Darmstadt, F.R.G.

2.1.4. Bacteriological Materials

Tryptone, yeast extract, and agar were supplied by Difco Labs., Detroit, Mi., U.S.A. Ampicillin was from Sigma. All plastic vessels including 90 mm petri dishes were from Sterilin Ltd., Teddington, U.K.

2.1.5. In vitro Packaging Kits

λ in vitro packaging kits were supplied by Amersham International, Amersham, U.K.

2.1.6. Bacterial Strains, Lambda Strains And Plasmid Vectors

Escherichia coli strains C600hfl150 and SM32 were for use with the vector λgt10. Plasmids were grown in E. coli strains WT217 and DH1. Genotypes are as follows:

C600hfl150 : F- hsdR- hsdM+ thi leuB6 lacY1 supE44 thr-1 tonA21 hflA150 (Young and Davis, 1983)

SM32 : F- gal SA500 his pyrD lon Δ100sulA strA (Mizusawa and Ward, 1982)

WT217 : F- hsdR recA56 leu lacx74 galU ara falK rps1 Tc (derived from MC1061, Casadaban and Cohen, 1980)

DH1 : F- hsdR17(rk-,mk-) recA1 thi-1 supE44 endA1 gyrA96 (Meselson and Yuan, 1968; Low, 1968; Maniatis et al, 1982)

Bacteriophage λgt10 (Huynh, 1985) has the genotype $\underline{Lsr}IL1^{o}$ $\underline{b527}$ $\underline{sr}IL3^{o}$ $\underline{imm434}$ ($\underline{sr}I434^{+}$) $\underline{sr}IL4^{o}$ $\underline{sr}IL5^{o}$. The λgt10 stock was prepared by J.S. Cavanna. Wild type λ DNA was supplied by Bethesda Research Laboratories (BRL), Cambridge, U.K. Microclones were subcloned into the plasmid vectors pSP64 (Melton et al,1984) (provided by G. Bates) and pGem-4 (Promega Biotec, 1985) (bought from Promega Biotec, Maddison, Wi., U.S.A.). Both plasmids are derived from pBR322, have an ampicillin resistance gene and a polylinker with a unique Eco R1 site.

2.1.7. Enzymes

For all restriction enzymes used one unit is defined as the amount of restriction enzyme which cuts 1 ug λ DNA to completion in one hour at 37°C under manufacturers' assay conditions.

Restriction enzymes: Eco R1 (200 U/ul) was supplied by Cambridge Biotechnology Labs., London, U.K. Eco R1 (25 U/ul) and all other restriction enzymes were from Anglian Biotechnology Ltd., Caterham, U.K.

Other enzymes: deoxyribonuclease 1 (DNAse), ribonuclease A (RNAse A) and proteinase K were from Sigma. T4 DNA ligase was from New England Biolabs, Beverly, Ma., U.S.A. One unit of T4 DNA ligase is defined as the amount of enzyme which gives 50 % ligation of Hind III digested fragments of λ DNA in 30 mins at 16°C in 20 ul at a 5' termini concentration of 0.12 mM (approximately 330 ug/ml). Klenow fragment (DNA polymerase I large fragment - 'Klenow') was from Anglian

Biotechnology. One unit of Klenow is defined as the amount of enzyme activity that incorporates 10 mM of total nucleotide into an acid precipitatable form in 30 mins at 37°C. Calf Intestinal Phosphatase was from Boehringer Mannheim, Lewes, U.K. One unit of calf intestinal phosphatase is defined as the amount of enzyme activity that hydrolyses 1 uM of 4-nitrophenyl-phosphate in 1 min at 37°C in 1 M diethanolamine buffer, 10mM 4- nitrophenyl-phosphate, 0.25 mM magnesium chloride, pH 9.8.

Enzyme grade bovine serum albumin (BSA) was supplied by BRL.

2.1.8. DNA Samples

ØX174 (RF fragment) DNA and wild type λ were from BRL. Cell line DNAs were sent by Dr. P. D'Eustachio and Dr. F. Ruddle.

2.1.9. Materials For Chromatography, Electrophoresis, Southern Blots And Hybridisations

Agarose and low melting point agarose were from BRL. Nylon blotting membrane, "Hybond", was from Amersham. Sephadex G-50 and ficoll 400 were from Pharmacia AB, Uppsala, Sweden. Polymer wool was from Interpet Ltd., Dorking, U.K. Bromophenol blue, bovine serum albumin (Fraction V), dextran sulphate and polyvinyl-pyrrolidone were from Sigma.

2.1.10. Materials For Radiolabelling Nucleic Acids

Radionucleotides were supplied by Amersham, all other nucleotides were from Pharmacia.

2.1.11. Photography And Autoradiography

Chromosomes were photographed with a Reichart polyvar micro-

scope with a 10 x eyepeice, 100 x objective and an automatic camera, all supplied by Reichart-Jung, Vienna, Austria. Polaroid PolaPan 4 x 5 Land Film was supplied by Polaroid Ltd., St. Albans, U.K. XAR-5 Film was from Eastman Kodak, Rochester, N.Y., U.S.A. Phenisol developer and Hypam fixer were from Ilford Ltd., Mobberley, U.K. The UV trans-illuminator was from UV Products, San Gabriel, Ca., U.S.A.

2.1.12. Mouse Stocks

CD strain mice were provided by Dr. H. Winking, Lubeck, F.R.G. *Mus spretus* were from Dr. R. Nash, London U.K. and Dr. G. Bulfield, Edinburgh, U.K. All other mice came from M.R.C. Genetics Unit, Harwell, U.K.

2.2. Methods

2.2.1. Tissue Culture And Preparation Of Metaphase Spreads

Tissue Culture - Lymphocyte Culture

With the minimum size breeding colony available (2 members) of small mice (average CD adult mouse weight is 15 - 20 g) orbital sinus bleeding (Breckon and Goy, 1979) was the most appropriate method of obtaining a supply of CD mouse lymphocytes. This method yielded at least the minimum 0.4 ml blood found to be necessary for an effective lymphocyte culture, with little risk to the animal.

A mouse was anaesthetised with diethyl ether and the skin surrounding the eye was pulled taut, causing the eye to bulge out. A capillary tube was gently inserted under the lower eyelid, towards the back of the orbit, dislodging the

eyeball and entering a blood filled sinus at the back of the eye; blood flowed freely into the tube. Capillary tubes were washed through with heparin before bleeding (1000 U/ml heparin). Heparin without bacteriocide was used because the additives affect mouse lymphocyte cultures (P.H. Glenister, Pers. comm.). Blood from one animal was collected in 5 ml wash medium (RPMI 1640 x 1, 50 U/ml heparin, 150 U/ml penicillin, 150 ug/ml streptomycin). After centrifugation (1000 x g, room temperature, 10 mins) cells were resuspended in 5 ml fresh wash medium and centrifuged again (same conditions). Cells were then resuspended in 5 ml complete medium (RPMI 1640 x 1, 50 U/ml heparin, 100 U/ml penicillin, 100 ug/ml streptomycin, 17 % (v/v) foetal calf serum (heat inactivated), 5 uM/ml glutamine, 8 ug/ml concanavalin A), the lid of the vessel was closed tight and the culture was incubated for 2 days at 37 °C.

Making Metaphase Spreads

Two day old lymphocyte cultures were incubated for 2 hours with colcemid (0.15 ug/ml) to arrest the cells in metaphase (by microtubule disruption). Lymphocytes were then centrifuged (100 x g, room temperature, 5 mins) and the pellet was resuspended in 2 ml hypotonic solution (0.075 M potassium chloride) at room temperature, in order to swell the cells. After 15 mins the cells were centrifuged (100 x g, room temperature, 5 mins) and the pellet was vigorously resuspended in 2 ml ice cold fixative (3 vols methanol:1 vol glacial acetic acid). The fixative was added drop by

drop to avoid cell clumping. The preparation was centrifuged (100 x g, 4°C, 5 mins), and cell fixation repeated twice before the final resuspension in 100 ul fresh fixative. Cells were dropped from a drawn out Pasteur pipette onto acid washed cover slips (see below), and the preparation was allowed to air dry.

2.2.2. Microdissection And Microcloning

Microinstruments

Microscope

The microscope had a long range working distance condenser lens, allied with a long working distance objective (x 32) used with phase contrast illumination; it was focused with the microscope tube, not the stage.

de Fonbrune Micromanipulator

The instruments used for the microtechniques were held and manipulated by a de Fonbrune micromanipulator. The receiver of the micromanipulator (placed opposite the trough of the oil chamber), was joined to a control stick by three thick walled rubber tubes containing air. Movements of the control stick within the three axes applied pressure to three orthogonal syringes, which by means of three rubber tubes acted on aneroid membranes at the receiver. The micromanipulator decreased the amplitude of movement of the hand so that the microinstrument moved distances appropriate to the microscope field. Reduction of movement was obtained (i) because of the area difference between the syringe cross sectional area and that of the the aneroid membranes; (ii)

because for the horizontal movements, the syringes are operated by levers which scale down the movements of the control stick - this provided the further facility of being able to change the lever ratio for horizontal movement. Vertical movement was controlled by a screw driven piston on the barrel of the control stick.

In addition to the above fine control, coarse control was possible by adjusting screws on the instrument holder of the receiver, for horizontal and vertical movement.

Glassware

Oil chambers (glass blocks 70 x 30 x 6 mm with a central trough of 24 x 30 x 3 mm) and narrow cover slips (6 x 32 x 0.17 mm) were acid washed in 0.15 M hydrochloric acid overnight, and rinsed in 10 changes of 100 % ethanol.

Cover slips used for microcloning (not metaphase spreads) were siliconised - aqueous drops attached to the siliconised surface under oil had a wetting angle of approximately 90°.

Siliconisation of glassware: glassware was left for 5 mins in 2 % dimethyl dichlorosilane in 1,1,1 trichloroethane, then air dried and rinsed in 1 mm ethylenediaminetetra acetic acid, sodium salt (EDTA).

Microneedles

Microneedles and micropipettes were pulled using a microforge which permitted the controlled melting of glass by a platinum-iridium filament, under a high power (x 200) monocular microscope.

Figure 2.1. Diagram of microneedle preparation

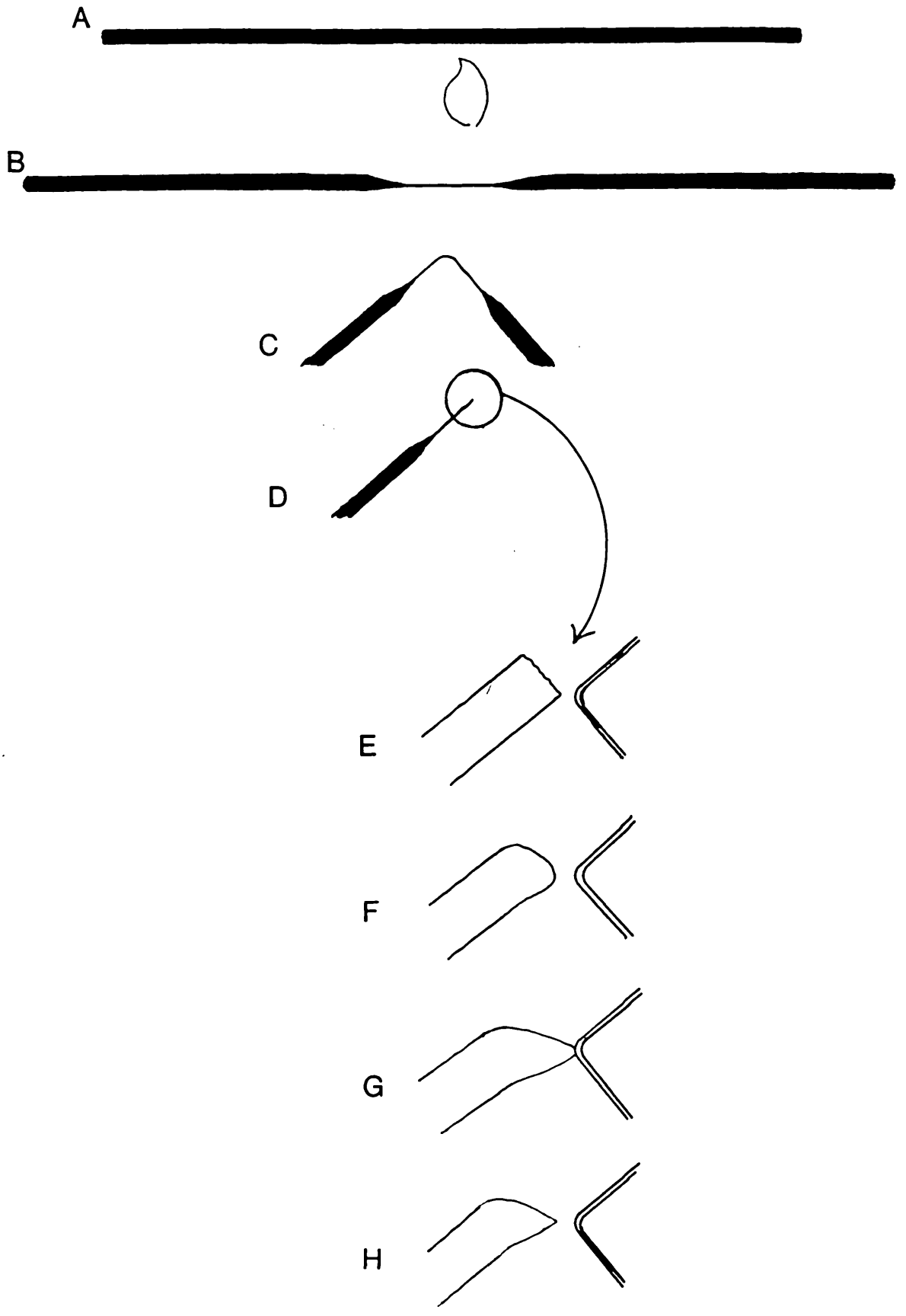


Figure 2.2. Diagram of micropipette preparation

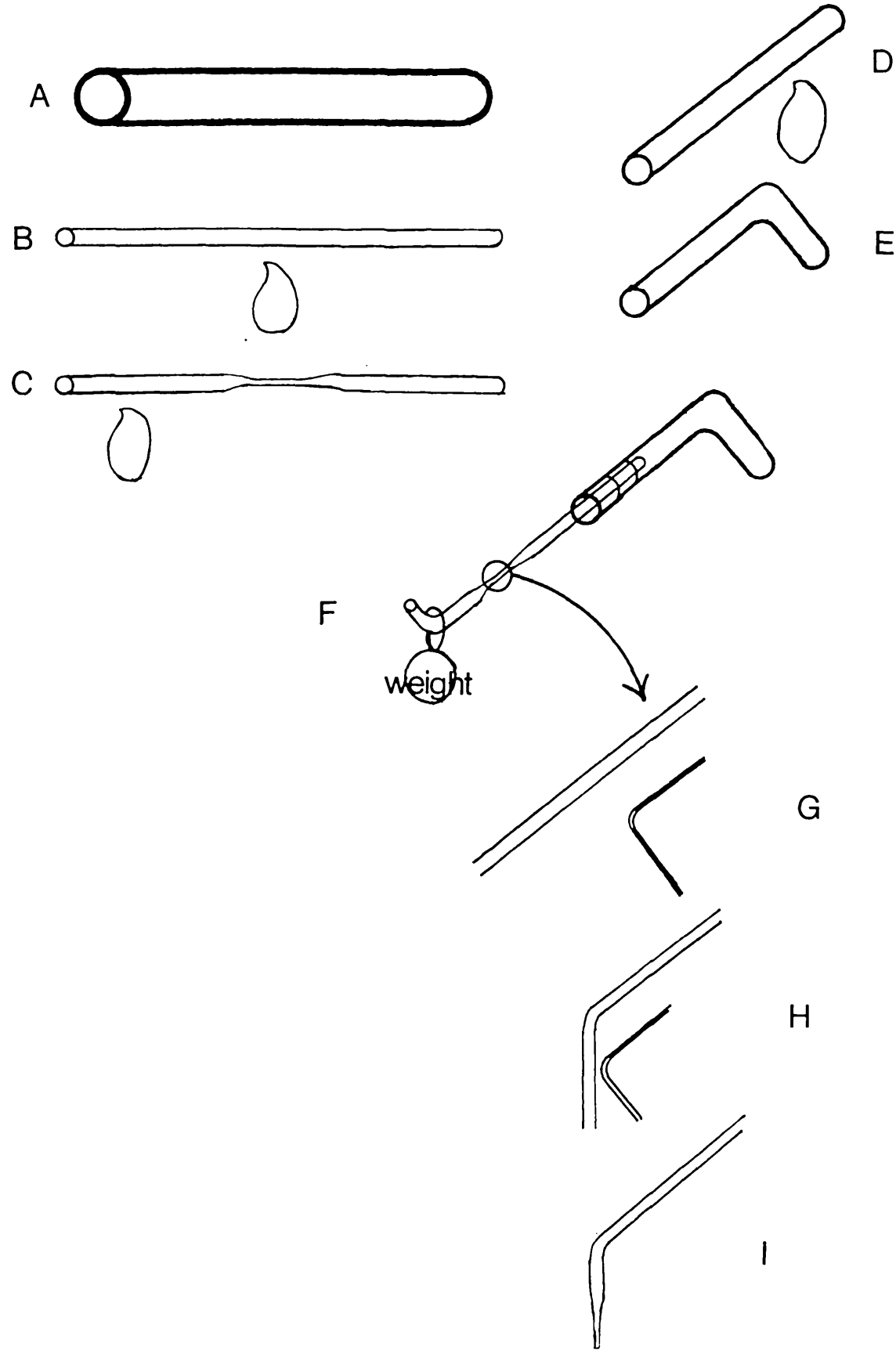


Figure 2.3:A Photograph of a micropipette positioned in the microforge

A 5 g weight is hanging from the micropipette hook.

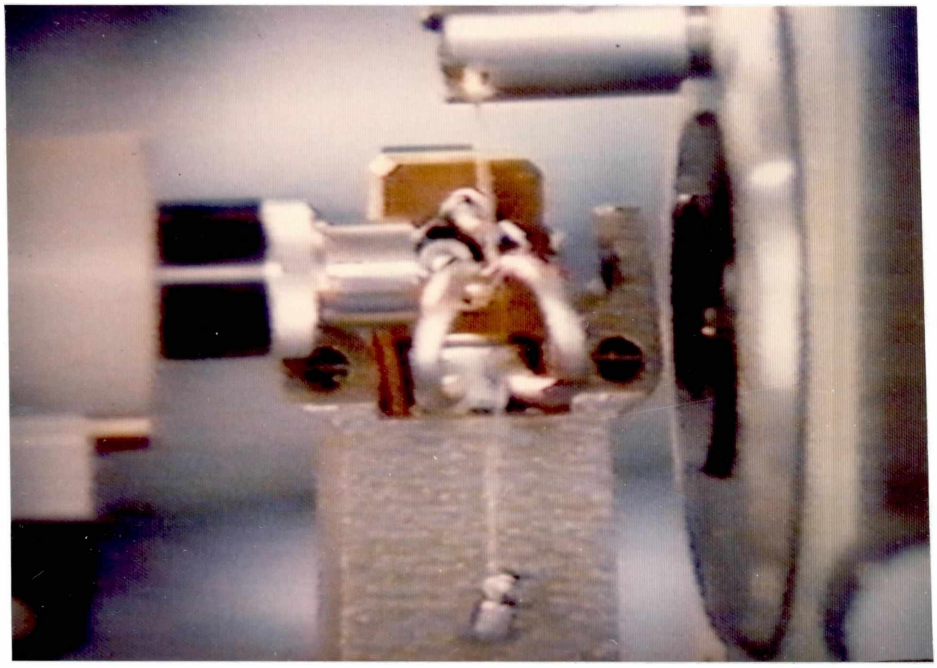
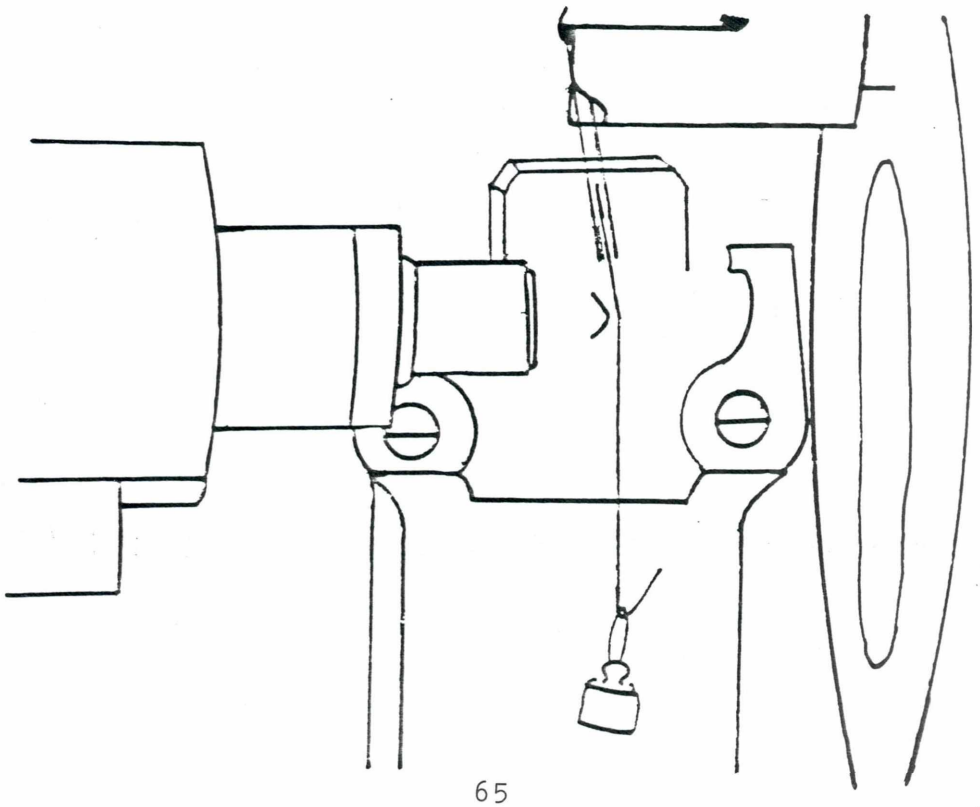


Figure 2.3:B Diagram of a micropipette positioned in the microforge



Microneedles were made from 20 cm x 2 mm diameter rods of soda glass which were pulled over over an alcohol flame (Fig.2.1:A) to give a thinning of about 0.5 mm diameter (Fig. 2.1:B), and were snapped at the thinning to form 2 needles (Fig. 2.1:C). A needle tip (Fig. 2.1:D) was placed adjacent to the microforge filament (Fig. 2.1:E). As the filament heated, the glass contracted (Fig. 2.1:F) and the needle tip (Fig. 2.1:G) bent towards the filament. The needle was then drawn away from the filament, forming a sharp tip with a high cone angle (Fig. 2.1:H). Finer needles (approximate diameter: 1 um) were forged for microdissection of chromosome regions, compared to those (approximate diameter: 2 um) used for whole chromosome microdissections. Microneedles were siliconised to prevent the collection drop attaching to them when entering the drop.

Micropipettes

Pyrex glass tubing 10 mm diameter with 1 mm thick walls (Fig. 2.2:A) was pulled over an acetylene flame to a diameter of approximately 1 mm, retaining the wall thickness:diameter ratio. These tubes were cut into 15 cm lengths and pulled in an alcohol flame 3 cm from one end, to give a thinning of approximately 0.5 mm diameter (Fig. 2.2:B). Using the alcohol flame this end of the tube was bent into a hook (Fig. 2.2:C).

A micropipette holder was formed by shaping (Fig. 2.2:D) 3 mm diameter soda glass tubing into a 90^{o} angle with arms of 9 cm and 4 cm (Fig. 2.2:E).

The long arm of a capillary was sealed air tight by paraffin wax, into the long arm of the holder, the thinned region projecting 3 cm. The micropipette was placed into the microforge at an angle of approximately 50° to the vertical, and a weight was attached to the hook (Figs. 2.2:F, 2.3.). Heating the filament adjacent to the pulled region allowed the capillary to bend to the vertical under the pull of the weight (Fig. 2.2:G). The micropipette was heated lower down (Fig. 2.2:H), forming a constriction which was pulled out and finally broke cleanly, under the force of the weight (Fig. 2.2:I). Micropipettes were then siliconised to inhibit bubble attachment and to overcome the surface tension which would decrease internal solution movement.

Micropipettes pulled with 2 g weights had fine elongated tips facilitating great control of liquid ejection and uptake, particularly important, for example, during phenol extraction. Micropipettes pulled with 5 g weights broke more quickly and so had wide mouths used during chloroform extraction - when a thin stream of chloroform would have knocked the microdrop from the cover slip and a wide low pressure wave front was necessary.

To pipette the required nanolitre volumes for microcloning the diameter of the microscope field (150 um) was estimated and bubble diameter corresponding to 1 nl (approximately 30 um) was calculated in relation to the microscope field diameter (1/5).

Preparation For Microcloning

Testing Of Restriction Enzyme

The restriction enzyme Eco R1, utilised for microcloning, was used in a very high concentration (from a stock of 200 U/ul) to minimise the possibility of inactivation and to ensure complete digestion of microdissected DNA. Aliquots of the microcloning enzyme were tested for (i) Eco R1* activity by the enzyme and (ii) endogenous exonuclease activity within the aliquot.

(i) Eco R1* Activity:

1 ug wild type λ DNA was digested with 200 U concentrated Eco R1. The number of U of enzyme per ug DNA in the final digestion mix was less than in the microcloning reaction (see on), but the sodium chloride and glycerol contents were similar. The enzyme was diluted with R buffer from a 10 x stock, (R buffer x 1: 100 mM sodium chloride, 10 mM TRIS pH 7.5, 10 mM magnesium chloride, 10 mM 2-mercaptoethanol) to a dilute stock Eco R1 concentration of approximately 50 U/ul in 12 % (v/v) glycerol and 2 x R buffer. Eco R1* activity is enhanced by high glycerol - low salt conditions and dilution with R buffer (a high salt buffer) lowered the percentage of glycerol (i.e. to 12 % (v/v)) and increased sodium chloride concentration to 200 mM (i.e. 2 x R buffer - 200 mM). 200 U of Eco R1 from the dilute stock were added to the λ DNA to give a digestion concentration of 100 mM sodium chloride and 6 % (v/v) glycerol.

Eco R1 functioning without Eco R1* activity restricts wild type λ into 6 fragments. After digestion for 2 hours at

37 °C the DNA was electrophoresed on a 0.8 % agarose gel and checked for the expected band pattern.

(ii) Endogenous Exonuclease Activity:

If the above gels displayed a smear, two fresh digests were set up with new buffer solutions and with wild type λ DNA; one digest was carried out with the concentrated Eco R1, and one with the 'normal' Eco R1. Results of these digests determined if DNA degradation was due to buffer or Eco R1 contamination by exonuclease.

Preparation Of Vector And Testing Of Ligase Enzyme

The cloning vector used was the insertion vector λgt10 which accepts inserts of up to 7.6 kb (Huynh, 1985) at a unique Eco R1 insertion site. Prior to microcloning the vector was checked for (i) complete digestion; (ii) religation potential; (iii) non-recombinant background (see below).

(i) Vector Digestion:

In preparation for microcloning λgt10 was digested with Eco R1, giving two fragments of 32.71 kb and 10.63 kb. In order to compare Eco R1 digested and undigested λgt10, an aliquot of the digest was then redigested with Bam H1. Bam H1 digests λgt10 into 6 fragments, including a 6.10 kb fragment containing the unique Eco R1 site. The double digested aliquot was electrophoresed with a marker track of Bam H1 digested λgt10 and the double digest was checked for the complete disappearance of the 6.10 kb Bam H1 fragment.

(ii) Religation Of Vector:

Another aliquot of Eco R1 digested λgt10 was religated to

check both degradation of vector arms, and functioning of T4 DNA ligase. A proportion of this aliquot was electrophoresed - one high molecular weight band of religated λgt10 with no other fragments indicated religation was successful.

(iii) Estimating Non-Recombinant Clear Plaque Background: In Vitro Packaging Of Vector

The remainder of the religated λgt10 was packaged in vitro. In vitro packaging kits utilise proteins from two different strains of λ which carry complementary mutations in the genes required for assembly of λ bacteriophage particles. The religated non-recombinant λgt10 DNA was mixed with the proteins necessary for packaging λ DNA into the protein capsid, according to the manufacturers' instructions, and after two hours at 20°C, the DNA had been packaged into infectious viable bacteriophage particles containing a linear monomer of λgt10.

λgt10 Plaque Morphology

The Eco R1 cut site, the λgt10 insertion site, is within the cI gene. Ligation of DNA fragments into the cI gene inhibits lysogeny; all recombinant λgt10 are cI^- and therefore lytic and produce clear plaques. However in non-recombinant λgt10 the normal plaque morphology is of turbid plaques being a mixture of lysogenic bacteriophage and a small proportion (of the order of 10^{-3}) of lytic bacteriophage. Thus recombinant bacteriophage can be distinguished because they produce clear, as opposed to turbid plaques.

Estimation Of Non-Recombinant Clear Plaque Background

In order to have effective selection of recombinant clones, the non-recombinant clear plaque background of Eco R1 digested and religated λgt10 had to be estimated.

The (i) packaged non-recombinant (digested and religated) λgt10 was plated out onto SM32 for this estimation.

Two other aliquots of λgt10 were also plated out onto SM32 - (ii) packaged undigested λgt10 and (iii) packaged digested (not religated) λgt10.

The number of clear plaques and turbid plaques from the three aliquots was counted. The packaged digested λgt10 (iii) gave no plaques and undigested λgt10 (ii) and digested and religated λgt10 (i) aliquots gave comparable non-recombinant clear plaque values of 1.6×10^{-3} and 1×10^{-3} respectively. Therefore the background was sufficiently low for effective selection of recombinant clones from the microdissection packaging reaction.

The digested λgt10 (iii) plating further indicated if Eco R1 digestion had been complete, and the digested and religated λgt10 (i) plating further indicated the success of the T4 DNA ligase.

2.2.3. Microdissection Of The Mouse X Chromosome

A cover slip with metaphase spreads was placed chromosome side down onto the oil chamber, adjacent to a collection drop cover slip. Paraffin oil was Pasteur pipetted into the chamber in order to stop microdrop evaporation (Fig. 2.4.). The oil did not affect reactions within the microdrop and

had been equilibrated over an aqueous layer of R buffer. Approximately 0.1 ul of SDS-proteinase K solution (0.5 mg/ml proteinase K, 10 mM TRIS pH 7.5, 10 mM sodium chloride, 0.1 % (w/v) sodium dodecylsulphate (SDS)) was pipetted from a drawn out sterile Pasteur pipette onto the supply cover slip. This was inverted (Fig. 2.5:A) and placed adjacent to the collection drop cover slip. Oil flowed around the supply drop and sealed it from the air. A micropipette was used to pipette SDS-proteinase K from the supply drop (Fig. 2.5:B) to form the 1 nl collection drop on the central cover slip (Figs. 2.5:C, 2.6., 2.7.).

Different micropipettes were used for each solution, these were connected by thick walled rubber tubing (which did not expand or contract with changes in air pressure) to a 2 way syringe for the uptake and ejection of liquids.

The oil chamber was placed on the microscope stage. A microneedle was positioned within the oil chamber (Fig. 2.8.) and was manipulated to scratch away (Fig. 2.5:D) whole X chromosomes or X chromosome fragments (Fig. 2.5:E) from the front (i.e. farthest from the operator) cover slip. After each microdissection the tip of the needle was placed in the collection drop (Figs. 2.9., 2.10.) and immediately a small mass could be seen to leave the tip of the needle (Fig. 2.5:F); these masses persisted throughout SDS-proteinase K action until phenol extraction, and may have been a proportion of protein which would not readily dissolve in the aqueous phase and so would not be easily attacked by SDS-proteinase K action.

Figure 2.4. Photograph of R buffer equilibrated paraffin oil being pipetted into an oil chamber

A pasteur pipette is being used to pipette oil into the oil chamber; two cover slips lie across the oil chamber and oil is beginning to flow between the cover slips and the oil chamber trough.

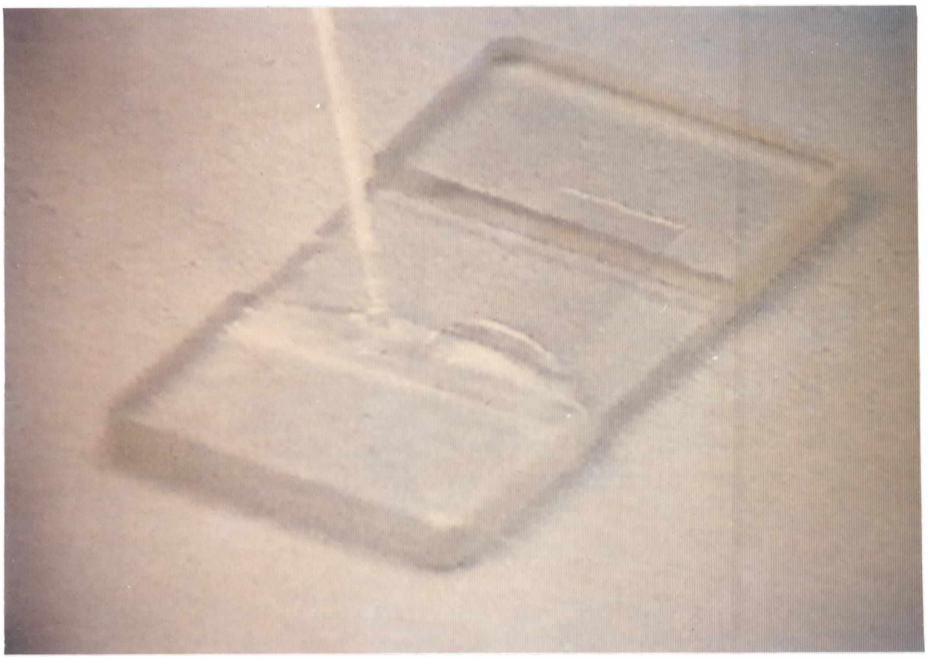


Figure 2.5. Diagram of microdissection and microcloning steps

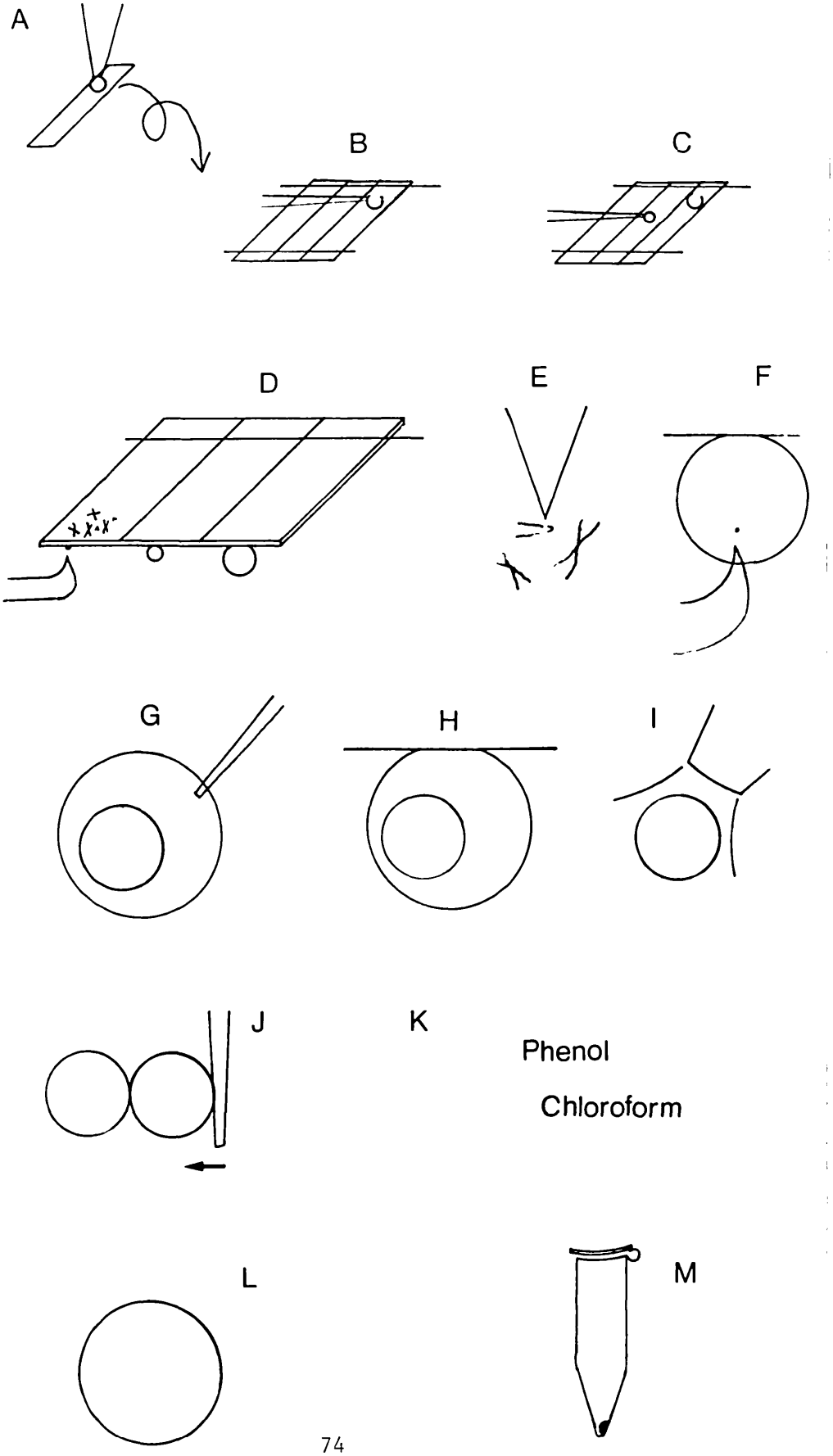


Figure 2.6. Photograph of a micropipette ejecting SDS-proteinase K to form a collection drop

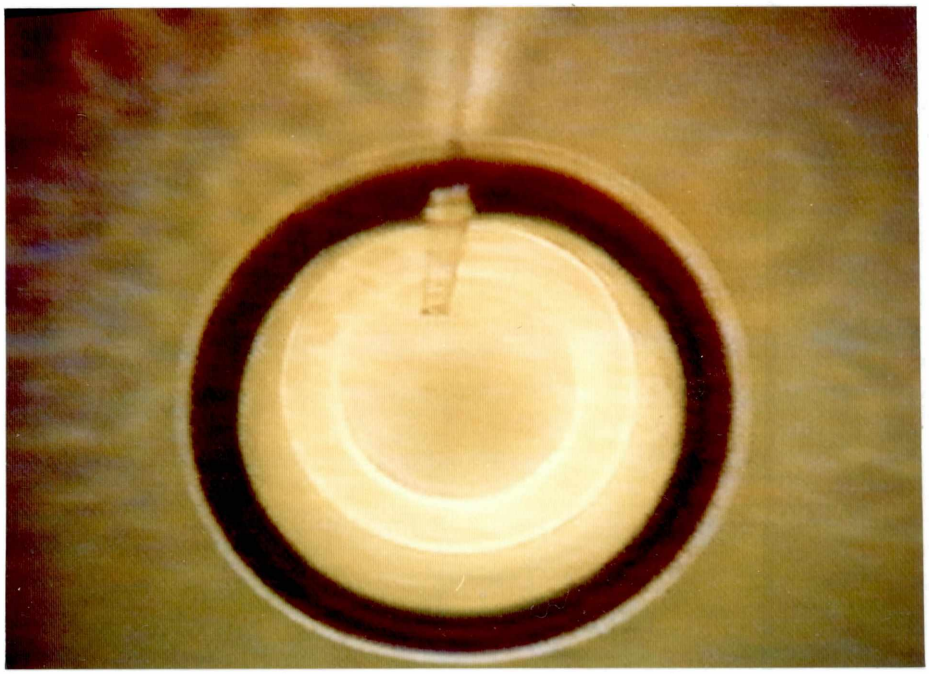


Figure 2.7. Photograph of side of an oil chamber

A microdrop is suspended in oil from a siliconised cover slip, the microdrop is circled in red.

Figure 2.8. Photograph of the microneedle being positioned within the oil chamber prior to microdissection

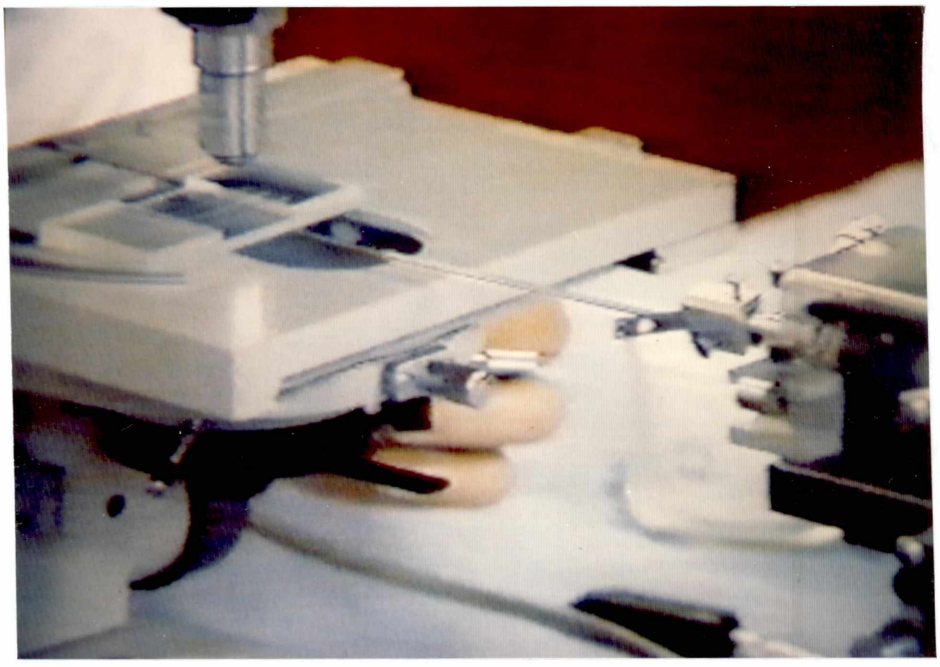


Figure 2.9. Photograph of a microneedle and microdrop

The microneedle is about to enter the microdrop

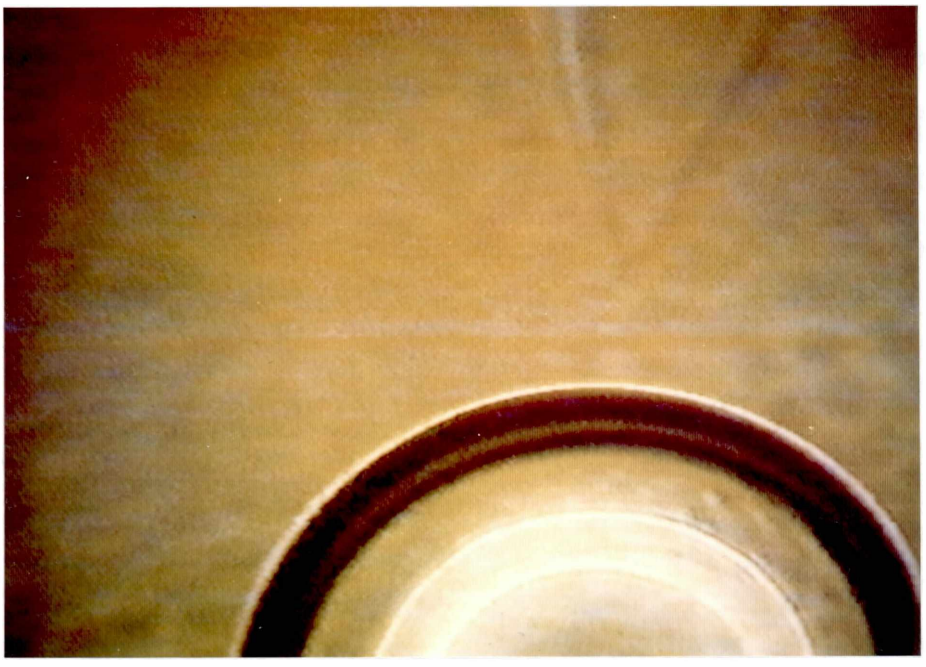
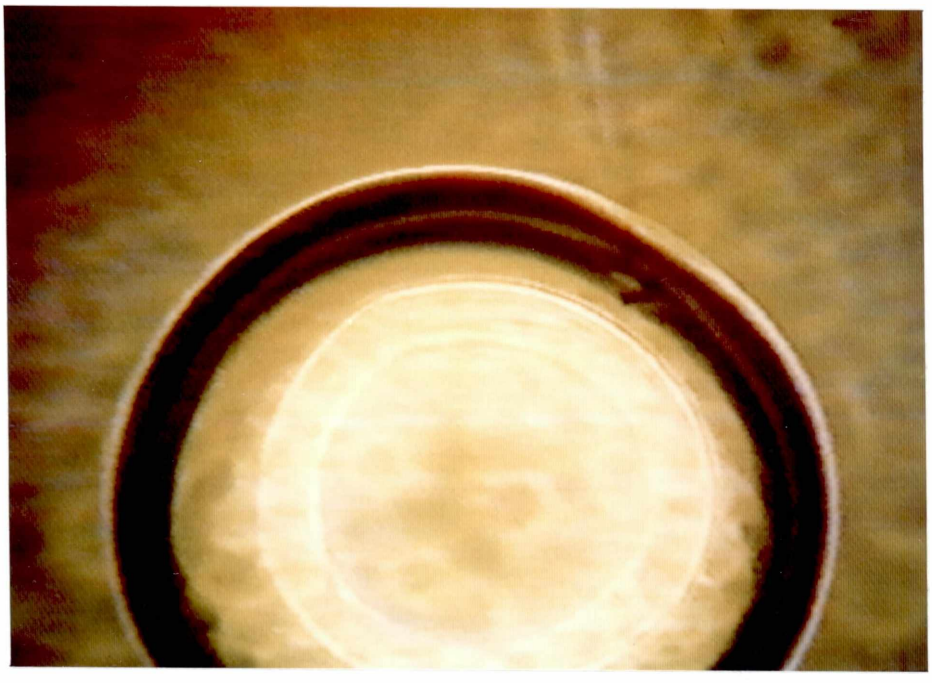


Figure 2.10. Photograph of a microneedle depositing microdissected chromosome fragments within the collection drop



2.2.4. Microcloning Of The Mouse X Chromosome Microextraction Of Microdissected DNA

When approximately 100 mouse X chromosomes or X chromosome fragments had been collected, a second nl drop of fresh SDS-proteinase K was fused to the collection drop (by pushing the two drops together with the micropipette). The oil chamber was removed from the microscope stage and a piece of R buffer moistened filter paper was slipped into the bottom of the trough to sustain the equilibration of the oil with the aqueous phase. The oil chamber was placed in a petri dish, which was in turn placed in a larger petri dish. Both dishes had a bottom lining of R buffer moistened filter paper, this 'double petri dish' arrangement created a humid chamber which was incubated for 90 mins at 37 °C.

After proteinase K digestion a supply drop of R buffer was placed in the oil chamber. One small supply drop of phenol was put on a cover slip behind the R buffer cover slip. The oil chamber was repositioned on the microscope stage and R buffer was micropipetted into the phenol drop to maintain aqueous saturation. The phenol, which had a blue tinge when viewed under a microscope, had been equilibrated with 10 mM TRIS pH 8.0 and then kept over R buffer. Phenol was used to denature and extract protein from the microdrop.

Phenol was pipetted from the R buffered supply drop until it surrounded the collection drop (Figs. 2.5:G, 2.11.), often detaching the collection drop from the cover slip (Fig. 2.5:H). After 3 - 4 mins the phenol was removed.

Figure 2.11. Photograph of a micropipette ejecting phenol

The phenol surrounds the collection drop

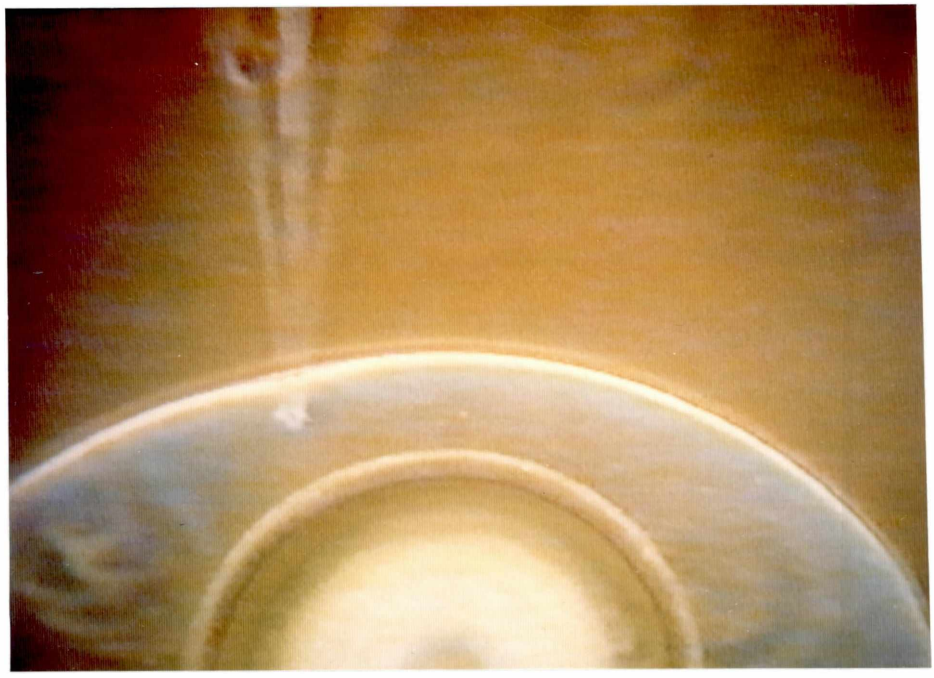
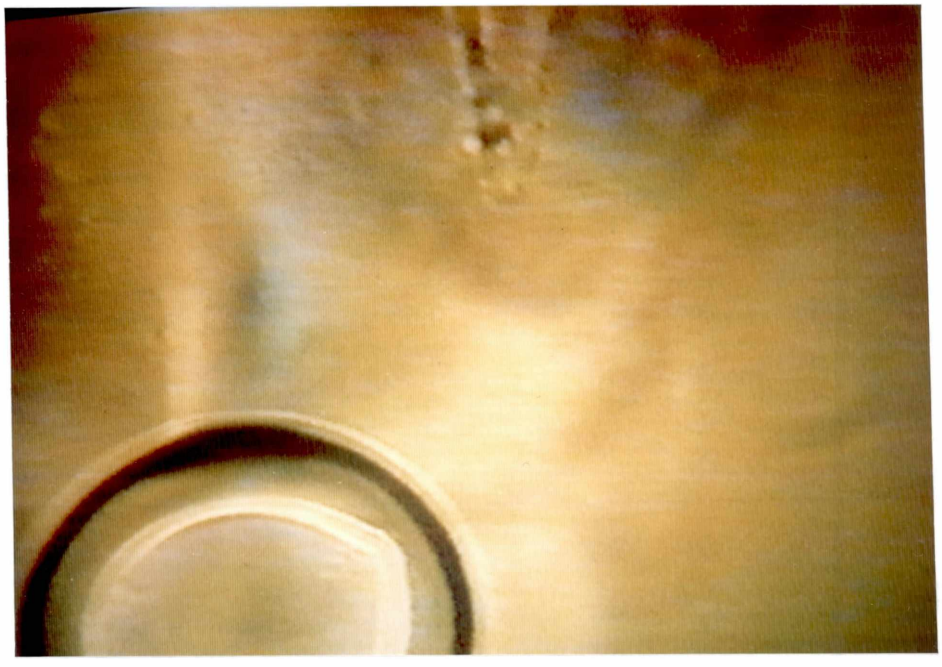


Figure 2.12. Photograph of a micropipette ejecting chloroform around a microdrop



Phenol extraction was repeated twice, and the phenol supply drop was removed from the oil chamber. As phenol dissolved in oil it was important to remove all traces of phenol from the vicinity of the collection drop so subsequent microcloning steps were not affected by phenol contamination; the phenol supply drop was also as small as possible for the same reason.

The collection drop cover slip was pushed onto a fresh oil filled chamber. A wide mouth micropipette was placed in a beaker of oil; oil was drawn into the capillary, chloroform was then drawn into the pipette from another beaker, followed by more oil. Chloroform could not be pipetted from a Pasteur pipette to form a supply drop because it instantly evaporated from cover slips, and in oil the negligible surface tension of a microdrop would allow the chloroform to dissolve immediately. Approximately 2 ul of chloroform was micropipetted over the collection drop to extract the phenol (Figs. 2.5:I, 2.12.). The cover slips were moved onto a fresh oil chamber and the chloroform extraction was repeated, followed by movement to two more fresh oil filled chambers to exclude any possibility of phenol contamination.

Microcloning Of Microdissected DNA

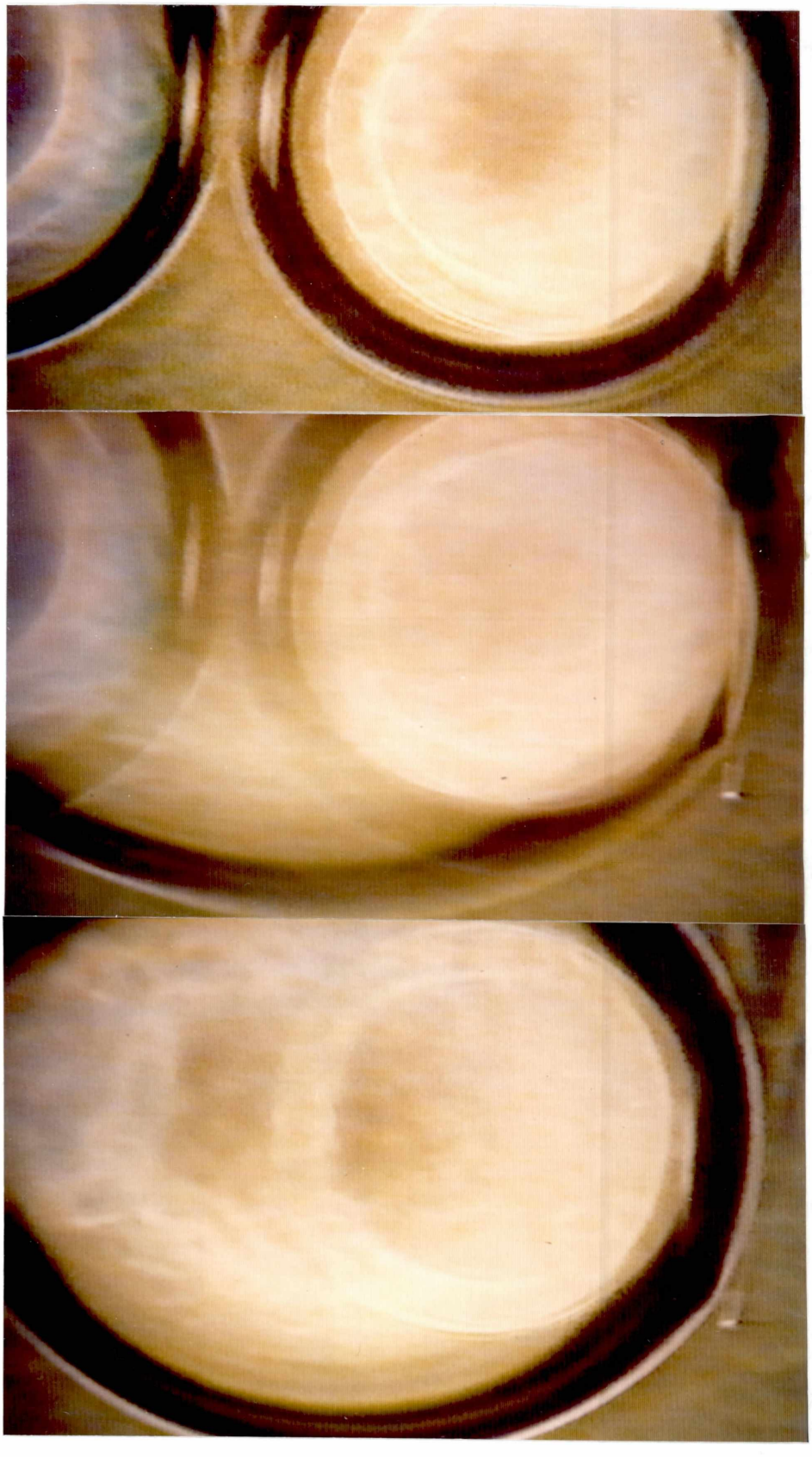
A nanolitre drop of the restriction enzyme Eco R1 was fused to the collection drop, from an Eco R1 supply drop (Fig. 2.5:J). The enzyme had been diluted in R buffer to a final Eco R1 concentration of approximately 50 U/ul (12 % (v/v) glycerol, 200 mM sodium chloride). Enzyme was diluted to

avoid Eco R1* activity (see above). One nl (0.05 U) added to the 2 nl collection drop resulted in an enzyme concentration of approximately 0.02 U/nl (4 % (v/v) glycerol, 70 mM sodium chloride) in 3 nl total volume. Following fusion of the collection drop and the Eco R1 drop, the collection cover slip was flanked by non-siliconised cover slips to maintain oil surface tension and avoid loss from hotter, less viscous oil running out of the sides of the oil chamber trough. The oil chamber was incubated for 90 mins at 37 ^{o}C in the double petri dish arrangement to allow complete DNA digestion, and was then incubated for 20 mins at 70 ^{o}C to inactivate the enzyme. Inactivation of the restriction enzyme was ensured by repeating the phenol and chloroform extractions (Fig. 2.5:K).

An equal volume of Eco R1 digested vector, λgt10, (3 nl) was added to the collection drop, in a fresh oil chamber (Fig. 2.13.). The vector concentration was 200 ug/ml with 4 mM ATP; 600 pg of vector were added to the Eco R1 digested mouse DNA, so vector DNA was in excess over the fragments to be cloned; (it is estimated (see Chapter 3) that the microdissection of 100 whole mouse X chromosomes results in the collection of approximately 34 pg DNA).

An equal volume (6 nl) of T4 DNA ligase was added to the microdrop. The concentration of the T4 DNA ligase was 4 x 10^{5} U/ml, and therefore 2.4 U were added to the ligation reaction. The microdrop (12 nl) was now clearly visible to the naked eye (Fig. 2.5:L),and the oil chamber was placed in the double petri dish arrangement at 4 ^{o}C overnight.

Figure 2.13. Photographs of a microdrop of vector DNA being fused to the collection drop



In Vitro Packaging Of Microcloned DNA

The efficiency of the in vitro packaging reaction is independent of the volume and concentration of λ DNA, so the microdrop was transferred via a wide mouthed micropipette from the oil chamber to an eppendorf tube containing 4 ul of TE buffer (10 mM TRIS pH 8.0, 1 mM EDTA) (Fig. 2.5:M).

The microcloned DNA was packaged in vitro into infectious recombinant bacteriophages. The packaging reaction was mixed with 10 ul chloroform and bacteriophage buffer up to 500 ul; it was stored at 4°C ready for plating out onto E. coli hosts.

Estimating Packaging Efficiency

SM32 is a permissive strain that will allow recombinant and non-recombinant λgt10 to grow. In order to estimate packaging efficiency one fiftieth (10 ul) of the packaging reaction was plated out onto 100 ul SM32 (A_{600} = 2). The resulting number of plaques was estimated and extrapolated to the number of plaques from the total packaging reaction.

2.2.5. Selecting Recombinant λgt10

The remaining packaging reaction was plated in 100 ul aliquots onto C600hfl150 (A_{600} = 2). C600hfl150 is a non-permissive strain which only allows growth of cI⁻ bacteriophage (i.e. resulting in clear plaques). The clear plaque titre within the microclone packaging mix was calculated and compared to the known clear plaque background for non-recombinant λgt10 (see above). This comparison confirmed if the microcloning had been successful, and clear

plaques visible on C600hfl150 plates were not due to background. Recombinants were selected by picking clear plaques from C600hfl150.

2.2.6. Preparing Microclone Stocks Of Recombinant λgt10

Packaged DNA was plated out onto C600hfl150 on L plates (15 g/l agarose in L broth; L broth: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride, 4 g/l maltose, 10 mM magnesium chloride). Low titre stocks were made by picking and ejecting a clear plaque into 1 ml bacteriophage buffer (10 mM TRIS pH 7.5, 10 mM magnesium sulphate) at 4°C. After leaving picked plaques overnight the bacteriophage eluant contained 10^7 - 10^8 pfu/ml.

High titre stocks were made from E. coli grown up overnight (37°C, shaking vigorously) in 10 ml L broth. 100 ul bacteria (A_{600} = 2) were mixed with 100 ul microclone low titre stock, and incubated at 37°C for 20 mins to allow bacteriophage adsorption. Infected bacteria were plated onto L plates and left overnight at 37°C. The resulting confluent clear plates were eluted with 5 ml bacteriophage buffer for 5 hours at 4°C. Eluant was centrifuged (100 x g, 4°C, 15 mins) to remove debris. Resulting high titre stocks contained 10^8 - 10^9 pfu/ml. Bacteriophage stocks were stored at 4°C, with 5 ul chloroform to lyse remaining bacteria.

2.2.7. Subcloning Of λgt10 Inserts Into Plasmid Vectors

Preparation Of Insert DNA

Eco R1 digested microclone DNA (see on) was ethanol precipitated overnight, and DNA was resuspended in water.

Ethanol precipitation: addition of sodium acetate to 0.3 M and 2 vols 100 % ethanol; followed by a variable period of precipitation at -20 °C, then a high speed centrifugation (15,000 x g, 4 °C, 20 mins); the pellet is washed with 70 % ethanol and vacuum dried.

Preparation Of Plasmid Vector DNA

5 ug of vector (pSP64 or pGem-4) were digested with Eco R1, and vector termini were phosphatased by a 30 min incubation with 1 U calf intestinal phosphatase at 37 °C. Enzymes were inactivated by two phenol extractions and one chloroform extraction. Phenol had been equilibrated with 1M TRIS pH 8.0 and then TE buffer (10 mM TRIS pH 8.0, 1 mM EDTA). Vector DNA was ethanol precipitated and stored at -20 °C.

Preparation Of Bacteria For Transformation

An E. coli colony (strains WT217 or DH1) from a streaked L plate was picked and incubated in 5 ml L broth at 37 °C, shaking vigorously for 2 hours (A_{600} = 0.3). Cells were then diluted 1:20 with 95 ml L broth and grown (for approximately 2 hours) under the same conditions until A_{600} = 0.48. Cells were chilled for 5 mins on ice, then centrifuged (650 x g, 4 °C, 5 mins) and resuspended in 20 ml filter sterilised Tfb I (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15 % (v/v) glycerol, to pH 5.8 with 0.2 M acetic acid) and left for 5 mins on ice. Cells were again centrifuged (650 x g, 4 °C, 5 mins) and resuspended on ice in 4 ml filter sterilised Tfb II (10 mM MOPS, 75 mM calcium

chloride, 10 mM rubidium chloride, 15 % (v/v) glycerol, to pH 6.5 with potassium hydroxide). After 15 mins 200 ul aliquots were snap frozen in dry ice and stored at -70 °C.

Ligation Of Insert DNA

The 10 ul ligation mix contained: 0.2 ug Eco R1 digested microclone DNA, 10 ng phosphatased plasmid vector DNA, 10 mM DTT, 2 mM ATP, ligase buffer (0.05 M TRIS pH 7.4, 0.01 M magnesium chloride, 1 mM spermidine, 0.1 mg/ml BSA) and 200 units T4 DNA ligase. This was left at 14 °C overnight with three controls i) a phosphatase control - no microclone DNA - to check for spontaneous vector religation; ii) a ligation control - no microclone DNA and restricted, not phosphatased vector; iii) a transformation efficiency control - 1 ng supercoiled vector in 10 ul water, only.

Transformation

Competent cells were thawed on ice, and left on ice for 10 mins. Ligation mix and controls were each added to a 200 ul cell aliquot, and this transformation mix was left on ice for 45 mins, heat shocked at 42 °C for 1.5 mins and returned to ice for 2 mins. 600 ul L broth at 37 °C were added to the cells, which were then gently shaken at 37 °C for 1 hour. 200 ul aliquots of transformed cells were plated on amp plates (8 g/l tryptone, 5 g/l sodium chloride, 5 g/l yeast extract, 15 g/l agar, 1 g/l glucose, 100 ug/ml ampicillin), and incubated at 37 °C overnight. Subsequent DNA preparation and analysis identified colonies with recombinant plasmids. Recombinant colonies were grown overnight at 37°C shaking

vigorously in 2 ml L broth with 100 ug/ml ampicillin and stored as a 50 % glycerol stock at -20°C.

2.2.8. Preparation Of DNA

Preparation Of λ DNA

100 ul *E. coli* at A_{600} = 2 were infected with 1 ul from a high titre stock, were incubated at 37°C for 20 mins, and were plated onto L plates containing 2 g/l glucose. After overnight incubation at 37°C plates were eluted with 5 ml bacteriophage buffer for 5 hours at 4°C. Eluant was centrifuged (1000 x *g*, 4°C, 15 mins) and supernatant was treated with RNAse A (to 20ug/ml) and DNAse (to 3ug/ml) at 37°C for 1 hour, then an equal volume of PEG solution (20 % (w/v) polyethyleneglycol, 2.5 M sodium chloride) was added. The sample was left overnight at 0°C. Centrifugation (14,500 x *g*, 4°C, 20 mins) collected precipitated bacteriophage particles and the pellet was resuspended in 1 ml bacteriophage buffer and incubated with 20 mM EDTA pH 8.0, 0.5 % (w/v) SDS at 65°C for 30 mins. Following two phenol extractions and one chloroform extraction DNA was ethanol precipitated, resuspended in 40 ul TE buffer and stored at -20°C.

Preparation Of Subclone DNA

5 ul from a glycerol stock was grown overnight in 10 ml L-broth with 100 ug/ml ampicillin at 37°C, vigorously shaking. Cultures were centrifuged (1000 x *g*, 4°C, room temperature) and resuspended in 200 ul solution I (50 mM glucose, 25 mM TRIS pH 8.0, 10 mM EDTA), followed by the addition of 400 ul

solution II (0.2 M sodium hydroxide, 1 % (w/v) SDS) then left on ice for 5 mins. 300 ul cold solution III (5 M potassium acetate pH 4.8) were added and preparations were left on ice for 5 mins and then centrifuged (1000 x g, room temperature, 1 min). Supernatant was transferred to a fresh tube with 450 ul propan-2-ol, left for 2 mins at room temperature then centrifuged (1000 x g, room temperature, 2 mins). The pellet was resuspended in 100 ul TE buffer, RNAse A was added to 10 ug/ul and the preparation incubated for 30 mins at 37°C. After one phenol and one chloroform extraction, DNA was ethanol precipitated and stored in 50 ul TE buffer at -20°C.

Preparation Of High Molecular Weight DNA

Mice were killed by breaking the neck; the liver and tail were removed and immediately frozen in liquid nitrogen prior to storage at -70°C. Liver samples were stored as a 'back-up' for DNA preparation. DNA was prepared from the tail (Grosschedl et al, 1984). Skin was removed, weighed and incubated overnight at 55°C in 1.0 ml solution 1 (0.1 M EDTA, 0.05 M TRIS pH 8.0, 0.5 % (w/v) SDS, 0.5 mg/ml proteinase K) on a rocking platform. After three phenol and two chloroform extractions DNA was ethanol precipitated, and resuspended in 200 ul TE buffer and stored at 4°C.
The DNA recovered was 2.5 - 4 ug per mg tail skin. Yields of over 300 ug per tail were achieved.

2.2.9. Restriction Enzyme Digestion

All DNA samples were digested with restriction enzymes

according to the manufacturers' recommended protocols.

2.2.10. Electrophoresis

Acrylamide Gel Electrophoresis

Acrylamide gels were made up from 6 % (w/v) acrylamide, TBE buffer (0.09 M TRIS, 0.09 M boric acid, 0.002 M EDTA pH 8.0), 55 ul NNN'N'-tetramethylethylene-diamine, 450 ul 10 % (w/v) ammonium persulphate and water to 50 ml. After sample loading with 10 % (v/v) bromophenol blue tracking dye (2 % (w/v) ficoll 400, 0.025 % (w/v) bromophenol blue) gels were electrophoresed (10 V/cm) for approximately 2 hours, vertically in TBE buffer, then fixed in 10 % (v/v) acetic acid for 10 mins, covered with cling film and autoradiographed.

Agarose Gel Electrophoresis

Agarose gels were made by dissolving the appropriate amount of agarose or low melting point agarose in boiling E buffer (0.04 M TRIS, 0.02 M sodium acetate, 0.5 mM EDTA pH 7.7) with 5 ug/ml ethidium bromide; hot agarose was allowed to set in a gel former with a suitable comb. Samples were loaded with 10 % (v/v) bromophenol blue tracking dye. Electrophoresis of DNA was carried out in 0.8 % - 1.0 % (w/v) agarose gels at 5 V/cm for 2 - 3 hours or 1 V/cm overnight; and in 1.8 % (w/v) l.m.p. agarose at 7 V/cm for 2-3 hours (at 4 ^{o}C). All gels were electrophoresed in E buffer with 5 ug/ml ethidium bromide. Ethidium bromide stained DNA was visualised with a 302 nm transilluminator and was photographed using a red filter, with polaroid film.

2.2.11. Extraction of DNA from Agarose Gels

The DNA fragment band of interest was excised from a l.m.p. agarose gel, weighed and melted at 100°C for 7 mins with 3 ml water per g gel. Samples were stored at -20°C (Feinberg and Vogelstein, 1984).

2.2.12. Transfer Of DNA Onto Nylon Membranes (Southern Blotting)

Agarose gels were denatured with 2 x 20 min washes of 0.5 M sodium hydroxide, 1.5 M sodium chloride, and then neutralised in 2 x 20 min washes of 0.5 M TRIS pH 5.5, 3 M sodium chloride. DNA was blotted overnight in 20 x SSC buffer (20 x SSC: 3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) onto Amersham Hybond nylon membrane, then washed in 3 x SSC and UV illuminated on both sides for 5 mins.

Prior to reprobing filters were washed in distilled water at 65°C for several hours.

2.2.13. Radiolabelling Of Nucleic Acids

Oligolabelling Of DNA

Oligolabelling buffer (OLB buffer) was made up from a mix of solutions A:B:C in a ratio of 100:250:150.

solution A: 1 ml solution O (solution O: 1.25 M TRIS pH 8.0, 0.125 M magnesium chloride), 18 ul 2-mercaptoethanol, 5 ul dATP, 5 ul dTTP, 5 ul dGTP (each triphosphate previously dissolved in TE buffer to a concentration of 0.1 M and stored at -20°C);

solution B: 2 M HEPES, adjusted to pH 6.6 with sodium hydroxide;

solution C: hexadeoxyribonucleotides evenly suspended in TE buffer at 90 O.D. units/ml.

10 ul OLB buffer, 2 ul 10mg/ml BSA, DNA (in TE buffer or l.m.p. agarose) up to 32.5 ul, 2 U Klenow, 20 uCi ^{32}P-dCTP were made up to 50 ul with water; this was left overnight at room temperature (Feinberg and Vogelstein, 1984).

Separation Of Unincorporated dCTP

Unincorporated nucleotides were removed by eluting the oligolabelling mix with 100 ul aliquots of 3 x SSC, 1 mM EDTA, through a 0.4 (diameter) x 4 cm sephadex G-50 column which had been plugged with polymer wool. Fractions were collected and the first excluded peak was pooled.

The number of counts per minute from ^{32}P β-particle emission was measured by Cerenkov counting.

End-labelling Of Samples For Acrylamide Gels

50 ng of Eco R1 digested microclone DNA was left for 20 mins at room temperature with 2 U Klenow and 10 uCi α-^{32}P-dATP in 100 mM sodium chloride, 50 mM TRIS pH 7.5, 10 mM magnesium chloride, 1 mM DTT. Samples were then mixed with 10 % (v/v) bromophenol blue tracking dye and loaded onto acrylamide gels.

2.2.14. Hybridisation Of Labelled DNA To Southern Blots

Filters were pre-hybridised in 10 x Denhardt's solution (10 x Denhardt's: 2 g/l ficoll 400, 2 g/l polyvinyl-pyrrolidone, 2 g/l BSA pentax fraction V), 6 x SSC, 0.1 % (w/v) sodium pyrophosphate, 0.5 % (w/v) SDS, at 65 ^{o}C overnight and then

hybridised overnight in fresh pre-hybridisation solution with 10 % (w/v) dextran sulphate, 10 mM EDTA and with a radiolabelled probe concentration of 10^6 d.p.m./ml. After hybridisation, filters were washed with several 30 min washes in 1 x SSC, 0.2 % (w/v) SDS at 65 °C.

2.2.15. Autoradiography

Filters and gels were exposed to Kodak XAR-5 film at -70 °C with intensifying screens for 1 - 14 days.

3. Results - Generation And Characterisation Of Microclone Banks

3.1. Cloning Strategies

A Chromosome Specific Genomic DNA Clone Bank

For the molecular mapping of the mouse X chromosome it was necessary to generate a large number of X chromosome specific genomic DNA probes. The resulting map could be used as a basis for the investigation of the genetic and physical nature of the chromosome, and the functional interactions of chromosomal DNA sequences. These investigations would include the analysis of individual loci which have been genetically mapped but are undefined in molecular terms, or for example the analysis of the physical make-up of the chromosome, such as repeat sequence organisation (see Chapter 1).

Molecular Marker Density

In order to make a molecular map of the mouse X chromosome it is necessary to produce a set of linked probes. This set of probes, the clone bank, need not be fully representative of all X chromosome sequences, but should contain sufficient probes to span the chromosome, at a density applicable to fine genetic and physical resolution of the chromosome. A map can orientate and provide 'landmarks' but is only useful when the scale of the map is appropriate.

Molecular probes may be positioned on the chromosome by either physical or genetic methods. Physical methods include the use of *in situ* hybridisation of chromosome

specific probes to banded metaphase chromosomes (for example, Dautigay, 1986) or hybridisation of probes to genomic DNA digests of cell hybrids in which (in this case) defined mouse X chromosome fragments would be present on a non-mouse chromosome background (for example see the localisation of human X chromosome specific probes by Riddell et al, 1986).

In situ hybridisation may resolve distances between probes down to the level of one chromosome band. In man, using banded metaphase chromosomes, this distance would be equivalent to approximately 3 cM (Ruddle, 1981). In man and mouse analysis of late prophase sub-banding has revealed more than twice the number of bands seen at metaphase, and chromosomal DNA segments down to 1200 kb (equivalent to approximately 1 - 2 cM - see on) may be visualised (Sawyer and Hozier, 1986).

Probes may be mapped by simply noting the presence or absence of their genomic DNA sequences within a cell hybrid, but resolution of probes depends on characterising the amount of X chromosome present by cytological techniques. Additionally, chromosomes in cell hybrids may be rearranged, and molecular probe order may be different from the wild type chromosome.

To create a fine genetic map with a resolution greater than that provided by physical methods, molecular probes were positioned on the chromosome by the application of classical genetic mapping (see on) in which map resolution depends on

the number of observed segregation events for each clone locus. To create a fine genetic map probes can be positioned with a resolution of less than 1 cM by observing segregation in relatively few mice progeny. One observed crossover event from 100 scored progeny is a value of 1 % recombination and is equivalent to approximately 1 cM distance between scored loci. If greater than 100 progeny are observed for clone segregation map resolution is increased to less than 1 cM (and a probe density of greater than 1 probe per cM may be resolved).

Optimal probe density for the analysis of the physical aspects of the chromosome depends on the application of physical techniques to molecular probes.

Physical distances of up to 100 kb may be covered by chromosome walking (for example, see Monaco et al, 1985), but a new technology, chromosome jumping (Poustka and Lehrach, 1986) is being developed for the large physical distances separating molecular markers from genes. In mouse 1 cM represents an average physical distance of 1000 - 2000 kb (Lewin, 1980); if a probe is mapped up to 0.5 cM (equivalent to approximately 500 kb - 1000 kb) distant from a locus of interest, it is intended that chromosome jumping will make this locus potentially accessible to molecular analysis. Therefore a probe density of greater than or equal to 2 probes per cM is appropriate for the physical investigation of X chromosome sequences.

To map probes to the mouse X chromosome with a resolution of 1 probe per 0.5 cM, appropriate to the physical nature of

the chromosome, 200 or more mice progeny would have to be scored; this also would result in a very fine genetic map. The mouse X chromosome spans approximately 100 cM, 10^8 bp, therefore to span the chromosome more than 200 clones must be produced.

Regional Specific Clone Banks

A clone bank to the whole mouse X chromosome has the potential to finely map any area and provide a closely linked marker to any gene on the chromosome. However it may be more useful to concentrate on a region (rather than the whole chromosome), and to span this area with the same marker density of 1 probe per 0.5 cM. It may be technically more difficult to create a regional clone bank, rather than a whole X chromosome bank; but a regional clone bank spanning an area of less than 50 cM would have a number of advantages over a whole X chromosome bank.

With a smaller absolute number of markers to be analysed, the creation of a fine genetic map would be faster; fewer probes would have to be analysed in order to find a closely linked probe to a particular gene within the region, so facilitating characterisation of loci which have been defined phenotypically but not in molecular terms.

Techniques For Creating Genomic DNA Clone Banks To The Whole Mouse X Chromosome And A Region Of The Mouse X Chromosome

A variety of techniques have become available for the molecular cloning of specific areas of the genome. Early protocols tended to employ clone banks to the whole genome,

followed by screening for clones to the chromosome or chromosome region of interest; these methods include the molecular cloning and screening of the whole genome (Page et al, 1982), and the screening of probes from somatic cell hybrid DNA, in which the chromosome of interest is present on a background of chromosomes (Kao, 1983) from a different taxonomic order.

In later methods selection of DNA from the area of interest is an inherent part of the cloning procedure; the most important of these methods involve the flow sorting of chromosomes (for example, Davis et al, 1981), deletion enrichment of a chromosome (Lamar and Palmer, 1984) or chromosome region (Kunkel et al, 1985) and the microdissection of chromosomes (Scalenghe et al, 1981; Rohme et al, 1984) prior to cloning.

Molecular Cloning Of The Whole Genome

One method for producing DNA probes to a specific region of the genome involved selecting probes from a whole genome library. Page et al screened unique sequence recombinant phage from a human genomic library, against panels of cell hybrids (Page et al, 1982) and selected a probe which hybridised to the human X and Y chromosomes.

This approach is extremely inefficient and too time consuming for isolating large numbers of probes to one chromosome or chromosome region. If applied to the mouse X chromosome, which represents about 6 % (Lewin, 1980) of the haploid physical genome, then given approximately equal

repeat sequence content and cloning potential of all chromosomes, only about 6 % of low copy sequences from a mouse genomic library or clone bank would be X chromosome specific i.e. an average of one X chromosome specific clone for every sixteen clones screened. Regional specificity, for example for clones located within one quarter of the mouse X chromosome, would require the screening of an average of sixty-four clones before finding one clone that was region specific.

Somatic Cell Hybrids

A huge refinement on the whole genome technique, involves the use of interspecific cell hybrids, combining somatic cell genetics with molecular genetics.

In a whole genome library of a cell line containing only the mouse X chromosome against a background of foreign chromosomes, all selected mouse sequences should be derived from the mouse X chromosome. Selection of mouse sequences would be performed by screening numbers of clones with mouse specific repetitive DNA (for example, as with human sequences - Gusella et al, 1980).

The advantage of this technique is the inherent selection provided by the chromosome content of the cell hybrid and the subsequent ease of screening large numbers of clones with species specific repeat sequence DNA. However, only a small proportion of the total numbers of recombinant clones produced will contain mouse DNA, and of these clones a small proportion will be missed because they do not contain

sufficient repeat sequence DNA. The final step of the procedure requires all positive mouse clones to be restricted into small fragments; repeat sequence containing fragments are selected out and low copy sequences are then subcloned.

The protocol has been very successful in producing chromosome specific probes (for example, see Gusella *et al*, 1980; Law *et al*, 1982) however, there are two major draw backs to the technique; firstly the production of cell lines and secondly the generation of genomic DNA probes to chromosome regions.

A permanent cell line containing mouse X chromosome material against a non-mouse background could be established by:

(i) fusing a non-mouse mammalian mutant cell line, lacking an active gene for the production of hypoxanthine phosphoribosyl transferase (*Hprt*) (see on), to normal mouse fibroblasts or lymphocytes. Non-hybrid mouse cells would then be selectively eliminated, (Kao, 1983);

(ii) chromosome mediated gene transfer (for example, McBride and Ozer, 1973; Klobutcher and Ruddle, 1979) which would involve the endocytic uptake of whole mouse chromosomes which are then degraded into subchromosomal fragments within *Hprt*$^-$ non-mouse cells;

(iii) DNA mediated gene transfer in which *Hprt*$^-$ non-mouse cells are transformed by purified mouse DNA, either by endocytosis or direct injection of DNA (for example, Tunnacliffe *et al*, 1983).

Hybrid cell lines may then be isolated in HAT medium

(Littlefield, 1964) which selects for the presence of an active Hprt locus, which is situated on the X chromosome i.e. all surviving hybrid cells would contain all or part of the mouse X chromosome.

Production of cell hybrids is slow and after forming the hybrid a period of 2 - 3 months must elapse, (during which chromosomes will be lost from the hybrids), before the cell line stabilises. After this lengthy period cell lines can be characterised by chromosome banding for chromosome content, or visible aberrations. The cell hybrid will contain mouse X chromosome sequences and possibly other mouse chromosomal DNA, necessitating an extra step in X chromosome specific clone production - screening the low copy mouse subclones for X chromosome specificity.

Transformed mammalian cells could be used to produce clones from a specific region of the mouse X chromosome but this is absolutely limited to the stochastic production of cell lines containing the region, and the availability of selectable markers within the region. It is possible to insert selectable markers (such as the dominant selectable gene Ecogpt (Tunnacliffe et al, 1983)) along the length of the mouse X chromosome; however the position of marker integration depends on chance events and cannot be controlled.

There is only minimal control over the size of the region which can be put on a non-mouse background for molecular cloning. Cell fusion generally results in maintenance of

whole (often rearranged) chromosomes (Ruddle, 1981), chromosome mediated gene transfer generally results in fragments which may be large enough to be detected by light microscopy (Ruddle, 1981; Murphy et al 1985) and DNA mediated gene transfer generally results in the submicroscopic integration of fragments usually less than 50 kb (Ruddle, 1981).

The use of somatic cell hybrids to generate X chromosome specific probes is realistically limited to the production of clones to the whole X chromosome or a region containing the Hprt locus.

Flow Sorting Of Chromosomes

In sorting chromosomes by flow cytometry, metaphase cells are lysed with detergent and chromosomes are stained with ethidium bromide and excited by a laser. A fluorescence activated cell sorter (FACS) measures the relative fluorescence of each chromosome and the fraction corresponding to the desired peak of fluorescence on a flow karyotype is collected (Young et al, 1981). DNA from recovered chromosomes is digested and cloned into suitable vectors.

Flow sorting depends on the ability to physically characterise, recognise and separate individual chromosomes. This method has been applied successfully to a number of chromosomes including the human X chromosome (Davies et al, 1981) and chromosomes 21 and 22 (Kraumlauf et al, 1982) however the availability of FACS is limited, and purity is a

continual problem with many of the chromosome libraries created after sorting, necessitating screening of all clones for chromosome specificity. For example, Amar et al (Amar et al, 1985) flow sorted the mouse X chromosome and estimate that only 25 - 30 % of unique sequences within the resulting clones are X chromosome specific. The human X chromosome library of Davies et al is thought to contain approximately 10 % chromosomes 7 and 8 (Kao, 1983).

Contamination of chromosome specific flow sorted libraries by other chromosome sequences may be due to factors such as insufficient resolution between the relative fluorescence peak of the desired chromosome and other chromosomes; or the sort 'window' from which the peak is selected being too wide.

It is not possible to utilise FACS to produce genomic DNA probes to a region of a chromosome unless a suitable cell line exists in which, for example, the mouse region alone is present on a non-mouse background (so therefore all mouse clones would be region specific) or is present as part of a translocated chromosome (so therefore probes would have to be screened for chromosome specificity).

Deletion Enrichment

Deletion enrichment is a method that can be applied when the chromosome, or chromosome region of interest is absent from a genome, such as the Y chromosome in XX cells or though a chromosome region deletion. The "deleted" genome is sonicated to produce random fragments of DNA, and the

equivalent "non-deleted" genome (ie. the complete genome, or a male XY genome) is digested with a restriction enzyme. If the two sets of genomic DNA fragments are denatured and then allowed to reanneal with an excess of deleted genome, the duplex DNA which is double stranded and has "sticky ends" will be mainly the sequences of interest and only these can be ligated back into a vector with complementary sticky ends.

Deletion enrichment was used in 1984 by Lamar and Palmer (Lamar and Palmer, 1984) to generate a clone bank highly enriched for mouse Y chromosome sequences, and in 1985 after modification of the protocol to include phenol enhanced DNA reassociation, Kunkel et al (Kunkel et al, 1985) successfully generated clones to a specific chromosome region by the use of a large fortuitous deletion of part of the Xp21 region of the human X chromosome.

It would be possible to use this method to generate clones to a region of the mouse X chromosome, depending upon available deletions, however this strategy could not be used to generate clones to the whole X chromosome.

Microdissection And Microcloning

Microdissection entails the physical removal of the chromosomes of interest (or a region of these chromosomes), from unstained metaphase spreads. The DNA collected for cloning is in pg quantities and as the ligation reaction is dependent upon DNA concentration, cloning steps (microcloning) take place in nl volumes of reagent. The

microdissection and microcloning of chromosomal DNA was first performed in 1981 by Scalenghe et al (Scalenghe et al, 1981), who isolated recombinant clones from microdissected fragments of the 3A - 3C region of the X chromosome of Drosphila; salivary gland giant polytene chromosomes were microdissected.

Subsequent development by Pirrotta et al (Pirrotta et al, 1983a) increased the resolution of microdissection and the yield of clones from microcloning, and in 1983 Pirrotta et al (Pirrotta et al, 1983b) microcloned the 3B1 - 3C2 region of the Drosophila polytene X chromosome.

Drosophila polytene chromosomes are giant chromosomes produced by approximately nine interphase replications without chromatid division, equalling approximately 2^9 (Lewin, 1980) copies of the unitene chromosome, therefore each individual microdissection will yield over a thousand times more DNA than microdissection of the same physical length of a Drosophila unitene chromosome. Using polytene chromosomes Pirrotta et al created a library of the 3C and part of the 3B region of the X chromosome in which all clonable restriction fragments were represented, having performed only 3 individual microdissections, (each spanning approximately 450 kb), from three polytene X chromosomes. The microdissection yielded 256 recombinant clones; the mean average size of 30 clones chosen at random was 3.8 kb, with a maximum insert of 10 kb and 6 of the inserts were less than 0.5 kb.

Subsequent microdissection of Drosophila chromosomes yielded

microclones to, for example, the Drosophila white locus (Pirrotta et al, 1983b) and the Drosophila Y chromosome (Hennig et al, 1983); then in 1984 the first microdissection of a mammalian chromosome was performed by Rohme et al (Rohme et al, 1984).

Microdissection Of Mammalian Chromosomes

Mammalian chromosomes are unitene and considerably smaller than Drosophila polytene chromosomes; they are microdissected in metaphase when the DNA is very compact. A 0.5 um diameter needle dissecting a Drosophila polytene chromosome would remove DNA from a small physical region - down to 200 kb (Pirrotta et al, 1983b) - due to the relatively elongated nature of the chromosomes. Using 0.5 um diameter needle on a mammalian metaphase chromosome may result in the dissection of up to 50 % of the entire chromosome. The minimum physical size of the region to be microdissected depends on needle diameter. The genetic size of the region depends on a number of factors (see Chapter 1), but is roughly proportional to the physical size.

A prerequisite of microdissection is that the chromosome of interest is easily distinguishable from the rest of the karyotype in an unstained metaphase spread. In mice a range of Robertsonian translocations allow the unequivocal identification of every mouse chromosome - either through metacentric fusion (Rohme et al, 1984) or chromosome exclusion (see below).

Rohme et al (Rohme et al, 1984) microdissected the proximal

half of mouse chromosome 17, including the physical region thought to contain the t-complex. The normal mouse karyotype consists of 40 acrocentric chromosomes, but Rohme et al were able to distinguish chromosome 17 by microdissecting from a karyotype containing a Robertsonian translocation. Chromosomes 8 and 17 were fused and formed the only metacentric in the karyotype, chromosome 17 forming the short arm of the metacentric.

270 proximal mouse chromosome 17 fragments were isolated and microcloned, yielding a clone bank of 212 bacteriophage; the average size of fragments which hybridised to mouse DNA (71 % of 41 non-repeat microclones of greater than 0.2 kb) was 1.15 kb. Only 1 out of 15 low copy microclones was not chromosome 17 specific when tested against a cell hybrid panel.

Microdissection and microcloning provide a fast and direct method for the isolation and production of a large number of genomic DNA clones to a whole mammalian chromosome or chromosome region. Any chromosome may be microdissected if it can be recognised and distinguished from the rest of the karyotype in an unstained metaphase spread; it is not necessary to construct and grow cell lines containing suitable chromosomes. The degree of contamination of the microclone bank by alien chromosome sequences is almost totally a function of the skill of the operator in microdissecting the chromosome of interest.

Regional Specific Clone Banks

As well as the above advantages microdissection and microcloning are the only widely applicable methods for the creation of clone banks to chromosome regions. All other available methods depend on fortuitous events, such as chromosome deletions.

3.2 Microdissection And Microcloning Of The Mouse X Chromosome

Microdissection and microcloning were the most applicable methods for generating a bank of genomic DNA probes to the whole mouse X chromosome and a region of the mouse X chromosome. These methods would speedily and efficiently generate pure banks of X chromosome and X chromosome region specific probes without the need to devise cell lines or use other stochastic or time consuming methods.

Two whole X chromosome collections and microclonings were performed, designated pmc 14 and pmc 15.

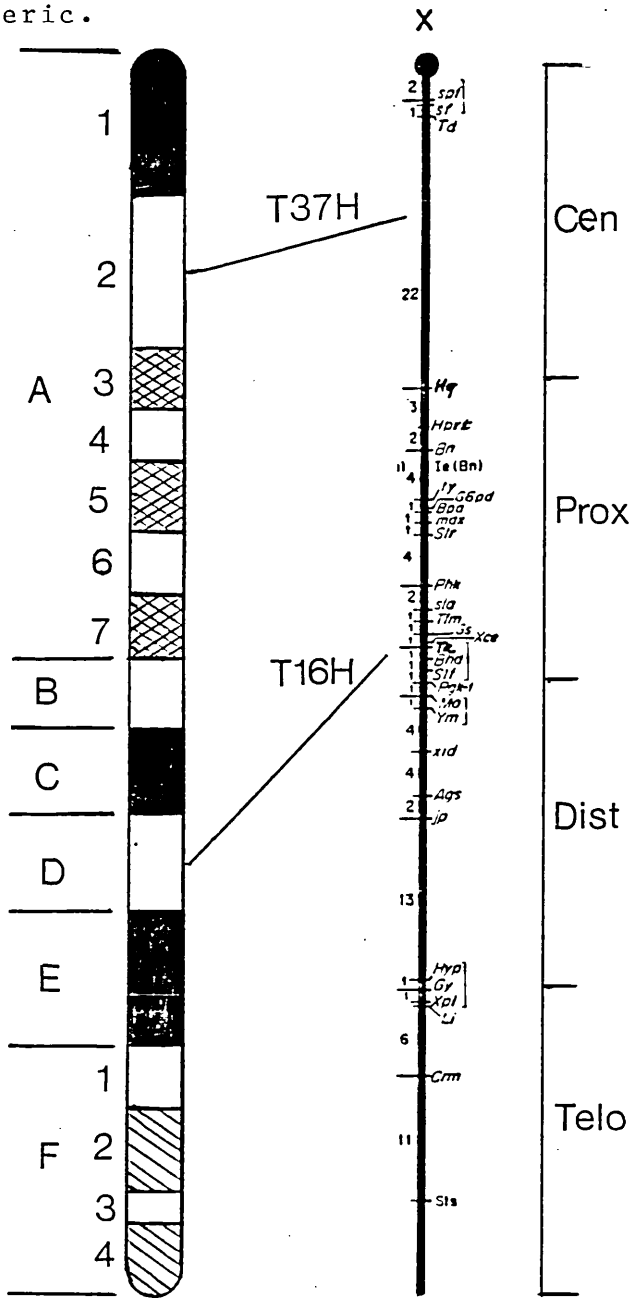
One regional mouse X chromosome collection and microcloning was performed, designated pmc 16.

The mouse X chromosome was divided into four physical regions which could be resolved by the dissecting needles forged for regional microdissections.

The 'microdissectable' regions were named centromeric, proximal, distal and telomeric regions. For pmc 16 the proximal region was microdissected, on the physical banded chromosome (Fig. 3.1.) this region roughly spans bands A3-B (giemsa banded chromosome).

Figure 3.1. Diagram of the four regions for regional microdissection on the mouse X chromosome

A giemsa banded chromosome (Searle and Beechey, 1981) is shown adjacent to the genetic map of the mouse X chromosome (Davisson and Roderick, 1986). The relationship between the physical map and the genetic map is unknown, however the physical and genetic locations of two translocation breakpoints, T37H and T16H (Searle and Beechey, 1981) are shown. Regions: Cen - centromeric; Prox - proximal; Dist - distal; Telo - telomeric.



There is no direct linear relationship between genetic and physical maps of the mouse X chromosome (see Chapter 1), however, X:autosome translocation data can very roughly position the two maps with respect to each other. Information from X:autosome translocations T37H and T16H (Russell, 1983) suggests the genetic region microdissected encompasses at least Hprt to Tfm.

The proximal region was chosen for microdissection because the approximate corresponding genetic region contains a large number of genetic loci, such as mdx which have not been defined or analysed in molecular terms. Some of these loci have clinical significance in man, (see Chapter 1). The area of the proximal region lying most towards the centromere may overlap a large area of very few genes. Either this dearth of genetic loci is a stochastic effect or possibly an unusually high amount of recombination is increasing the genetic distance (compared to physical distance) between loci in this area (see Chapter 1).

After microdissection DNA fragments were microcloned and the regional microclone bank was characterised for microclone size and gross repeat sequence content.

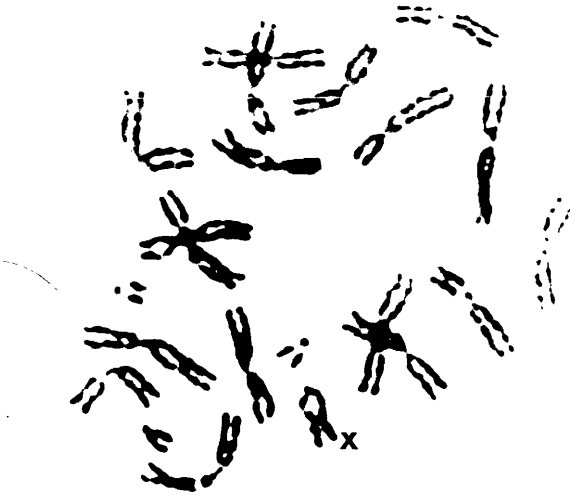
3.3. Microdissection Of The Mouse X Chromosome Metaphase Spreads From A Specific Mouse Karyotype

The process of microdissection is the physical dissection of the chromosome of interest (or chromosome region) from an unstained metaphase spread. This chromosome is readily distinguishable from the rest of the karyotype.

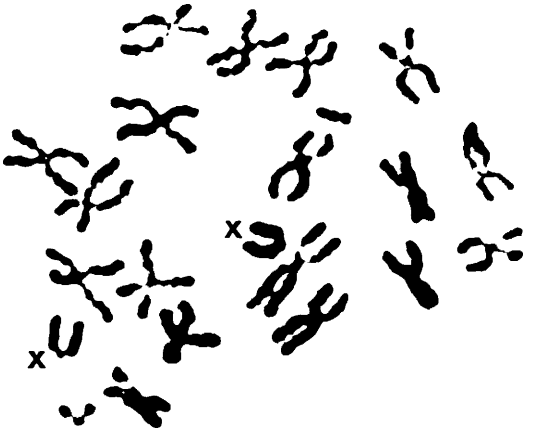
Figure 3.2. Photographs of metaphase spreads of the CD strain of mouse

Chromosomes were stained with 1 % giemsa stain and photographed under bright field conditions. All photographs of mouse chromosomes (Figs. 3.2., 3.3., 3.4.) were taken with a Reichart Polyvar microscope.

The X chromosome is marked.



MALE

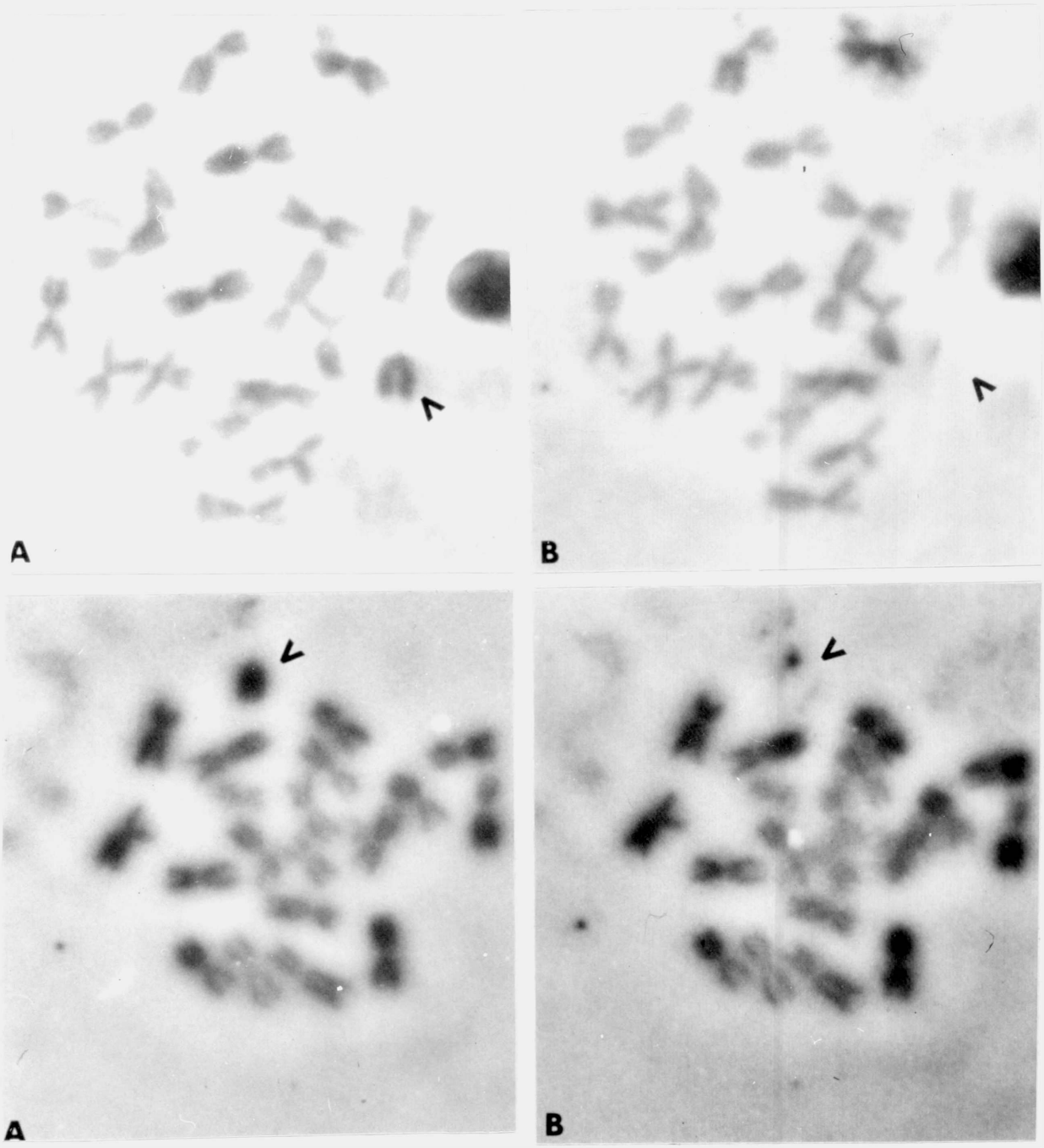


FEMALE

Figure 3.3. Photographs of unstained metaphase spreads of the CD strain of mouse, before and after microdissection of the whole X chromosome

Before microdissection (A); after microdissection (B). All unstained metaphase chromosomes were photographed under phase contrast conditions.

The X chromosome is marked.



Metaphase spreads were made from lymphocyte cultures of the CD strain of wild mouse (Mus musculus) in which all of the chromosomes apart from the 19, Y and X chromosomes, are fused as metacentrics in Robertsonian translocations (Capanna et al, 1975). Chromosomes 19, Y and X remain acrocentric, but the X is readily distinguishable for microdissection because it is approximately 2.3 times larger than the 19 or Y chromosomes (Fig. 3.2.).

Whole X Chromosome Microdissections

At each microdissection an entire X chromosome was scraped from the cover slip and deposited in the collection drop; the whole metaphase mouse X chromosome covered a surface area of approximately 5 um x 1.5 um (Fig. 3.3.). After collecting 100 X chromosomes, microcloning proceeded. Up to 100 X chromosomes could be dissected from an individual cover slip.

The mass of mouse DNA within the microdrop was equivalent to approximately 34 pg DNA.

This figure is derived on the basis that the total mouse haploid genome is 1500 cM and the mouse X chromosome is (in cM) 6.18 % of the total haploid genome (Roderick and Davisson, 1981); therefore the mouse X chromosome =

$$1500 \times 6.18\ \% = 92.7\ \text{cM}.$$

If 50 cM is equivalent to 9.0×10^7 bp in mouse (Lewin, 1980), then the amount of DNA in 92.7 cM =

$$(92.7 / 50) \times 9.0 \times 10^7$$

$$= 1.669 \times 10^8 \text{ bp}.$$

This is approximately equivalent to 0.17 pg (Lewin, 1980). Thus 100 chromosomes are approximately equivalent to 17 pg DNA. However each chromosome was microdissected during a diploid phase of mitosis therefore the amount of DNA microdissected was approximately 34 pg.

Regional Mouse X Chromosome Microdissection

100 proximal region mouse X chromosome fragments were microdissected and pooled for microcloning (Fig. 3.4.). The amount of DNA collected can be estimated as approximately 9 pg DNA (as above).

Microcloning Of The Mouse X Chromosome

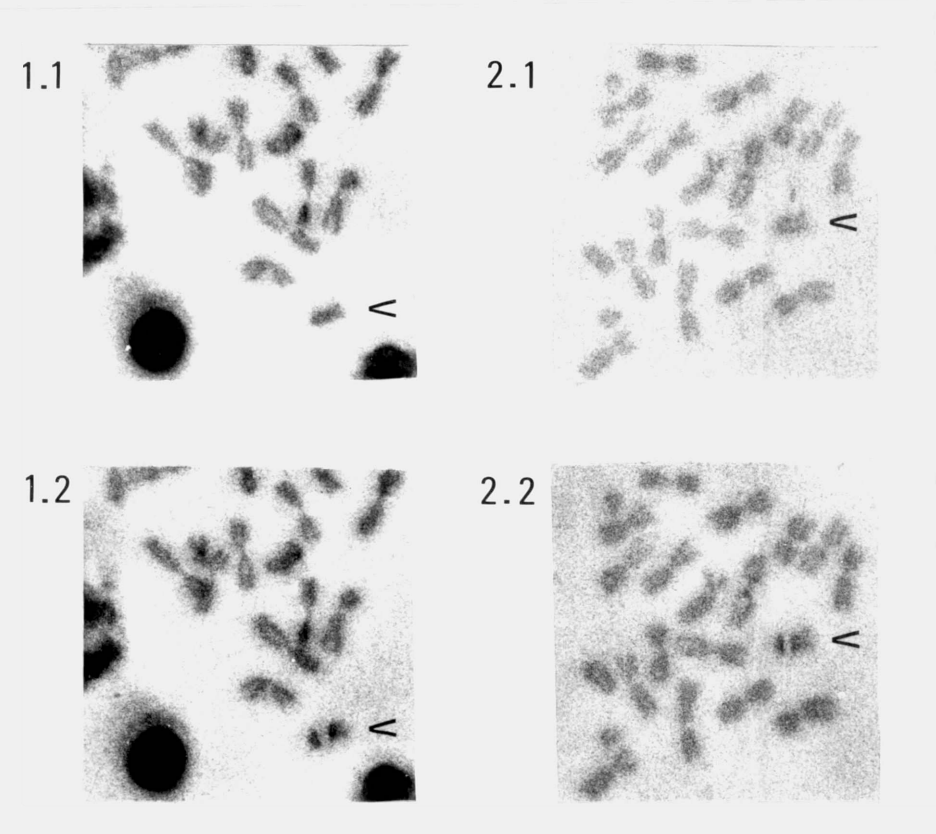
Microdissected X chromosomes or chromosome fragments were collected in a 1 nl proteinase K - SDS microdrop. Drop size was important because the ligation reaction is dependent upon the concentration of reactants, therefore with such a small amount of microdissected DNA it was necessary to microclone in nanolitre volumes.

After pooling of fragments, proteinase K digestion released the chromosomal DNA, and protein was removed by phenol extraction, followed by chloroform treatment to remove contaminating phenol. Resulting naked DNA was digested (to completion) with the restriction enzyme Eco R1. The mouse genome contains few recognition sites within X chromosome centromeric satellite sequences for Eco R1 (Brown and Dover, 1980). The microcloning strategy utilised Eco R1 restriction of microdissected genomic DNA to exclude these sequences from the microclones.

Figure 3.4. Photographs of unstained metaphase spreads of the CD strain of mouse, before and after microdissection of the proximal region of the X chromosome

Before microdissection (1.1, 2.1); after microdissection (1.2, 2.2).

The X chromosome is marked.



After further phenol and chloroform extractions DNA fragments were ligated into the Eco R1 insertion site of bacteriophage λgt10. Finally as the λ packaging reaction is independent of reactant concentration, the protocol was scaled up into microlitre quantities by micropipetting the collection drop from the oil chamber into 4 ul TE buffer in an eppendorf tube.

Parallel digestions and ligations on a microlitre scale were routinely performed with the microcloning enzymes. Analysis of results confirmed if enzymes and vector were suitable for the microcloning protocol.

Packaging and Plating of Microclone DNA

Microclones were packaged in vitro and plated out onto E. coli hosts SM32 and C600hf1150, to determine the total number of bacteriophage obtained from the packaging reaction, packaging efficiency and the total number of clear plaques (i.e. from recombinant and non-recombinant bacteriophage). Background levels of non-recombinant clear plaques were determined by plating non-recombinant λgt10 on SM32. (See Chapter 2).

3.4. Initial Analysis Of Microclone Banks

Table 3.1 shows data resulting from the two whole X chromosome microdissections (pmc 14, pmc 15), and the proximal region microdissection (pmc 16). The whole X chromosome is equivalent to 100 cM, and the regional X chromosome microdissection of one quarter of the physical chromosome was assumed to span a length of 25 cM, for this table.

Table 3.1

Initial Analysis of Microclone Banks

	pmc 14	pmc 15	pmc 16
Type of Microdissection	Whole	Whole	Regional
No. of Chromosomes (fragments) collected	100	100	100
Amount of Mouse X DNA Microcloned	34 pg	34 pg	9 pg
Restriction Enzyme	Eco R1	Eco R1	Eco R1
Vector	λgt10	λgt10	λgt10
Amount of Vector in Microcloning Reaction	600 pg	600 pg	600 pg
Packaging Efficiency (pfu/ug)	1.67×10^{8}	0.83×10^{8}	1.50×10^{8}
Total No. Plaques Recovered	100,000	50,000	90,000
Clear Plaque Background	10^{-3}	10^{-3}	10^{-3}
Total No. Clear Plaques Recovered (on C600hfl150)	2000	1000	650
Expected No. Non-Recombinant Clear Plaques	100	50	90
Expected No. Recombinant Clear Plaques	1900	950	560
Expected No. Recombinant Clear Plaques per pg Microdissected DNA	112	56	163
Expected Percentage Recombinant Clear Plaques out of the Total No. of Clear Plaques	95 %	95 %	86 %
Clear Plaque Microclone Levels above Background	20 times	20 times	7 times
Ratio of Clear Plaques: Turbid Plaques	1:50	1:50	1:138

(Table 3.1 continued)

Expected Mean Average Recombinant Microclone Density along the Mouse X Chromosome, Probes per cM (see above)	19	10	22

3.5 Size And Repeat Sequence Content - Characterisation Of One Whole X Chromosome And The Proximal Region X Chromosome Microclone Banks

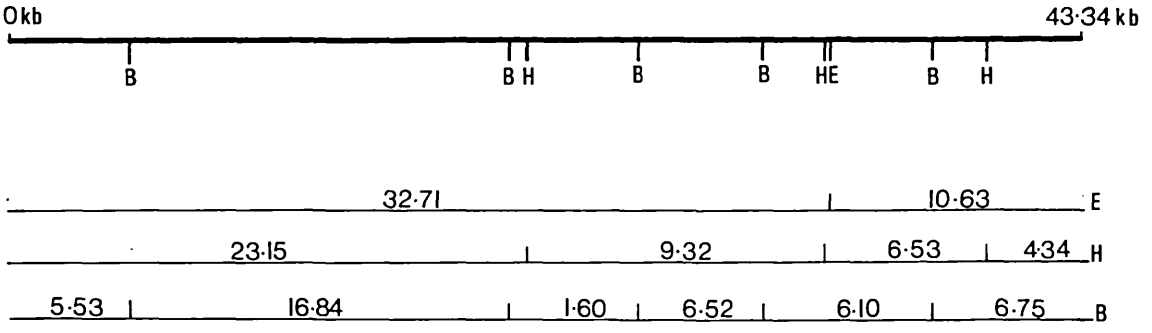
pmc 14 and pmc 16 were investigated for microclone size and repeat sequence content, pmc 15 was not further investigated.

Microclone Sizing

DNA from individual microclones was prepared, and digested with a restriction enzyme; insert size was estimated by electrophoresis and comparison of insert band migration to those of known electrophoretic markers, wild type λ digested with Hind III and ØX174 digested with Hae III.

Initially microclones were digested with Bam H1; Bam H1 digestion of non recombinant λgt10 results in 6 fragments, of 16.84 kb, 6.75 kb, 6.52 kb, 6.10 kb, 5.53 kb, 1.60 kb (Fig. 3.5.). The 6.1 kb fragment contains the single Eco R1 insertion site and so this fragment will migrate less far in recombinant compared to non-recombinant λgt10.

Figure 3.5. Diagram of restriction map of λgt10



Restriction Enzyme Cut Sites:

E-Eco RI

H-Hind III

B-Bam HI

The order of the cut sites for the above restriction enzymes, and their relative positions are correct, however the distances between cut sites are not shown on a comparative scale.

All pmc 14 microclones were sized by Bam H1 analysis (see Table 3.2) (Fig. 3.6.). However subsequently it was thought that Hind III digests of microclones were easier to interpret (see Table 3.3) (Fig. 3.7.). The recombinant Bam H1 band migrated up to the doublet (of the Bam H1 6.52 kb and 6.75 kb bands) making recombinant Bam H1 bands with inserts of 0.3 kb or greater difficult to resolve with the doublet. Hind III digestion of non-recombinant λgt10 DNA gives 4 fragments of approximately 23.15 kb, 9.32 kb, 6.53 kb and 4.34 kb. The λgt10 Eco R1 insertion site lies within the 6.53 kb fragment.

The majority of pmc 16 microclones were analysed solely from Hind III digests, however 30 microclones were analysed by cleavage with Eco R1, end-labelling and electrophoresis on acrylamide gels, followed by autoradiography of the gels (see Table 3.6) (Fig. 3.8.). These preparations consisted of a random group (13 microclones) and a group of microclones (17 microclones) preselected from Hind III digestion results, most being likely to contain longer inserts from the pmc 16 microclone bank. Gel electrophoresis on 6 % acrylamide gels with known markers, gave extremely accurate results of microclone size from 50 - 600 bp, a power of resolution that is difficult to attain on agarose gels. For approximately 50 % of microclones analysed by Hind III digestion on agarose gels and Eco R1 digestion on acrylamide gels, Hind III size estimates were overestimates by greater than 100 bp (see Table 3.6).

Figure 3.6. Photograph of Bam H1 digested pmc 14 microclones after electrophoresis in a 1 % agarose gel

Gels were run with wild type λ/Hind III markers and DNA was stained with 5 ug/ml ethidium bromide. Some samples show an extra 12.28 kb band, thought to be due to reannealing of λcos sites after digestion.

Wild type λ digested with Hind III gives 8 fragments of 23.13 kb, 9.42 kb, 6.68 kb, 4.36 kb, 2.32 kb, 2.03 kb, 0.56 kb and 0.13 kb.

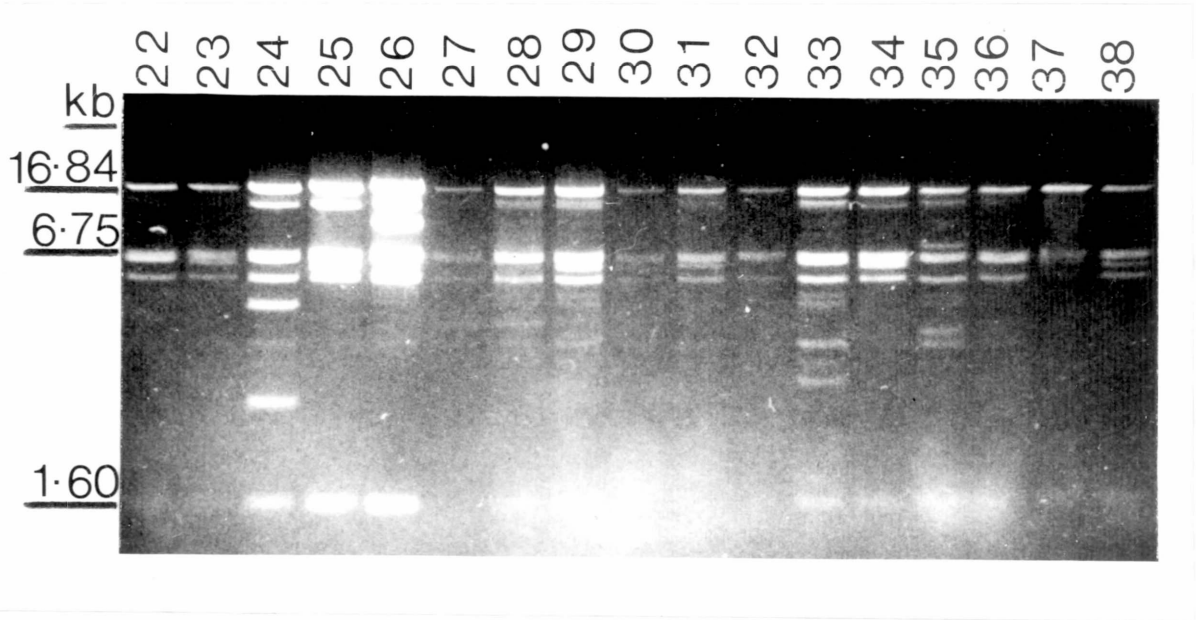


Figure 3.7. Photograph of Hind III digested pmc 16 microclones after electrophoresis in a 1 % agarose gel

Gels were run with wild type λ/Hind III markers (see back). DNA was stained with 5 ug/ml ethidium bromide (see Figs. 3.10. and 3.11.).

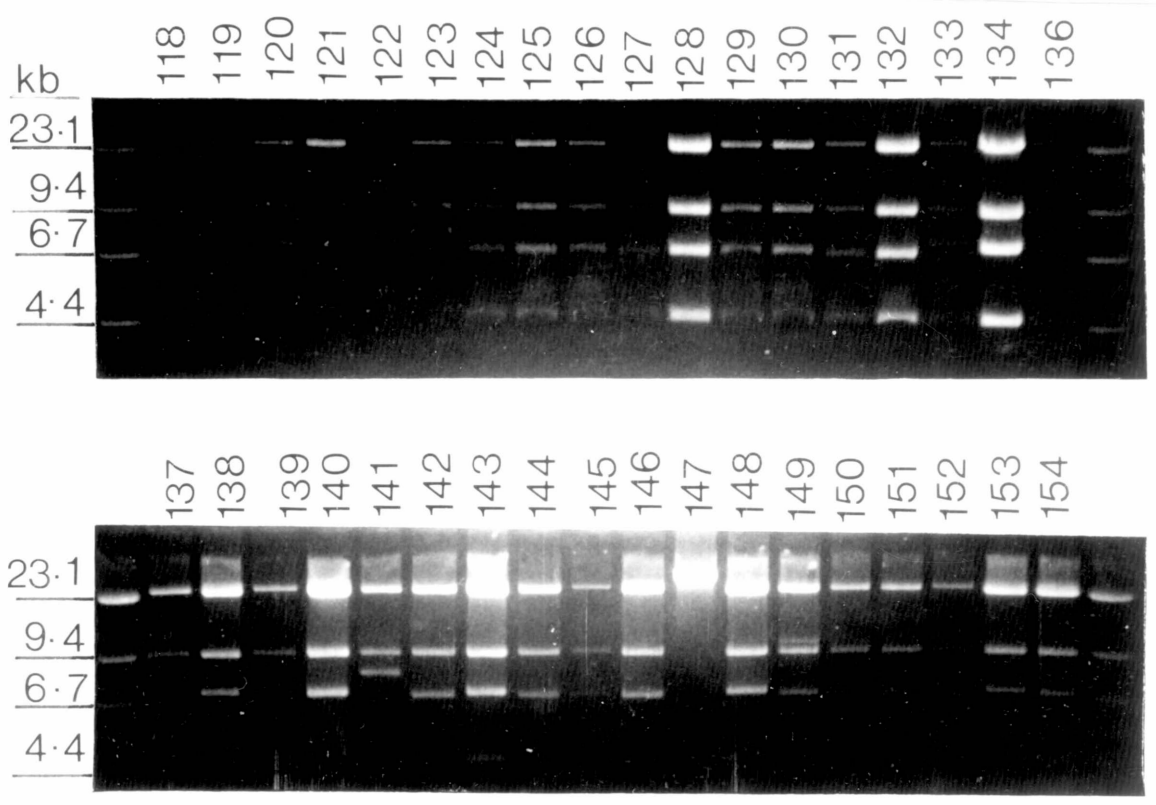


Figure 3.8. Autoradiograph of Eco R1 digested pmc 16 microclones after electrophoresis in a 6 % acrylamide gel

Gels were run with ØX174/Hae III markers. ØX174 digested with Hae III gives 11 fragments of 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 271 bp, 234 bp, 183 bp, 118 bp, and 72 bp.

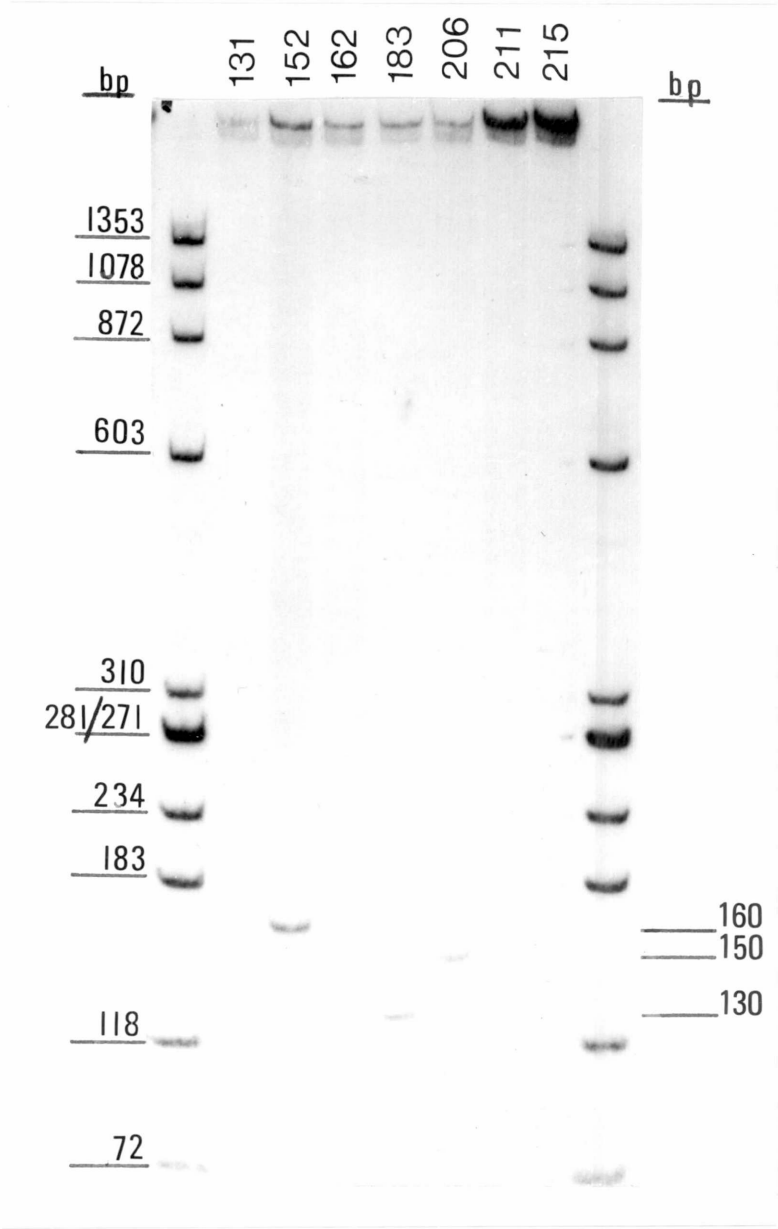
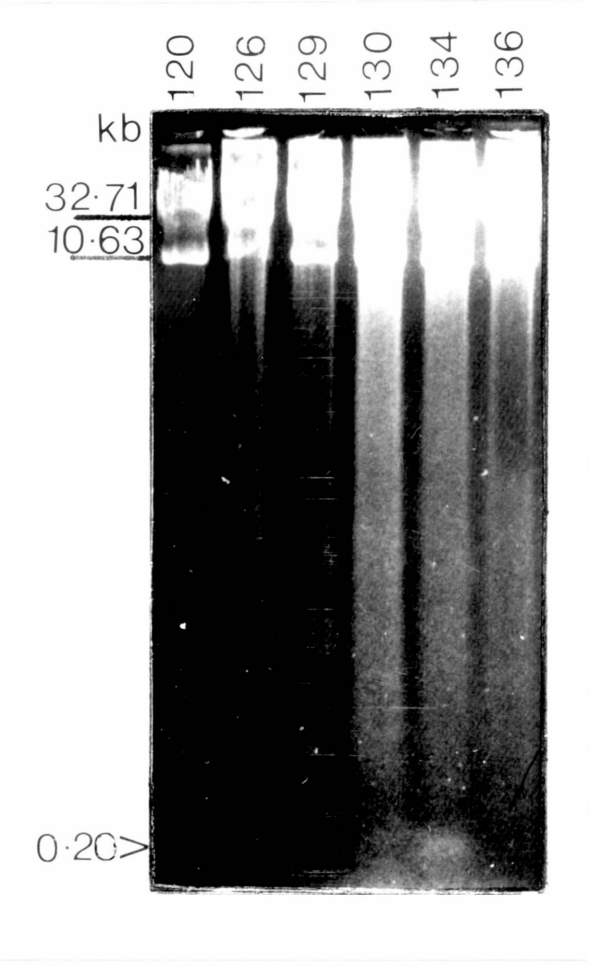


Figure 3.9. Photograph of Eco R1 digested pmc 16 microclones after electrophoresis in a 1.4 % agarose gel

Gels were run with ØX174/Hae III markers (see back) and DNA was stained with 5 ug/ml ethidium bromide. The 0.2 kb Eco R1 fragment of microclone pmc 16: 134 is visible.



A small number of pmc 16 microclone inserts (6 microclones) were analysed for size by Eco R1 digestion and electrophoresis on agarose gels (Fig. 3.9.). Eco R1 digestion of non-recombinant λgt10 results in 2 fragments of 32.71 kb and 10.63 kb.

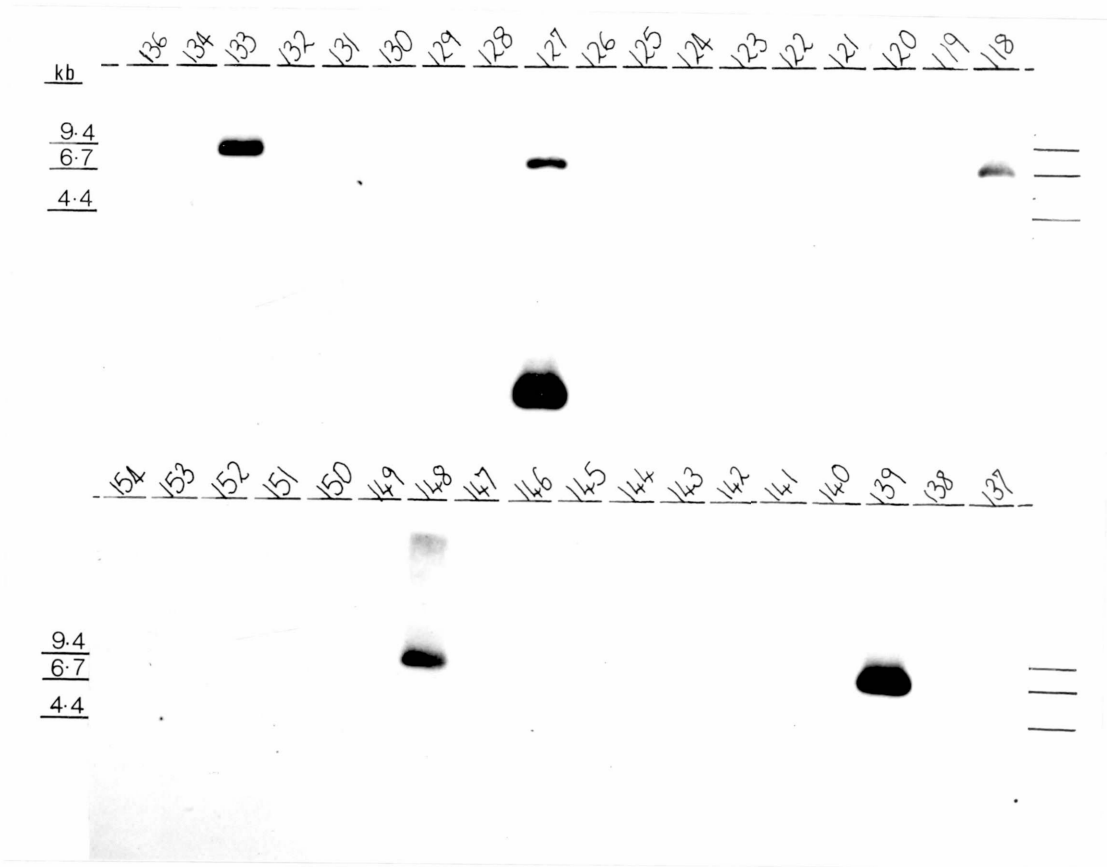
Repeat Sequence Analysis Of Microclone Banks

Microclones which had been analysed for size on acrylamide gels only (13 microclones) were reprepared, Hind III digested (to facilitate electrophoresis) and electrophoresed on agarose gels. DNA from these gels and all other Bam H1 and Hind III digest gels of microclone DNA was transferred by Southern blotting onto nylon membranes. Filters were probed with 1 ug oligolabelled total mouse DNA. Bands visible after autoradiography were from insert DNA containing repeat sequences (see Tables 3.2, 3.3), (Fig. 3.10.).

As the small size of the microclone banks became apparent, an advantage of Bam H1 and Hind III digests over Eco R1 digests appeared: large recombinant fragments (> 6 kb) were transferred with ease onto nylon membranes by Southern blotting, whereas the small Eco R1 insert only fragments (< 0.2 kb) would not transfer so well.

Figure 3.10. Autoradiograph resulting from hybridisation of 1 ug labelled total mouse DNA to a Southern blot of Hind III digested pmc 16 microclones

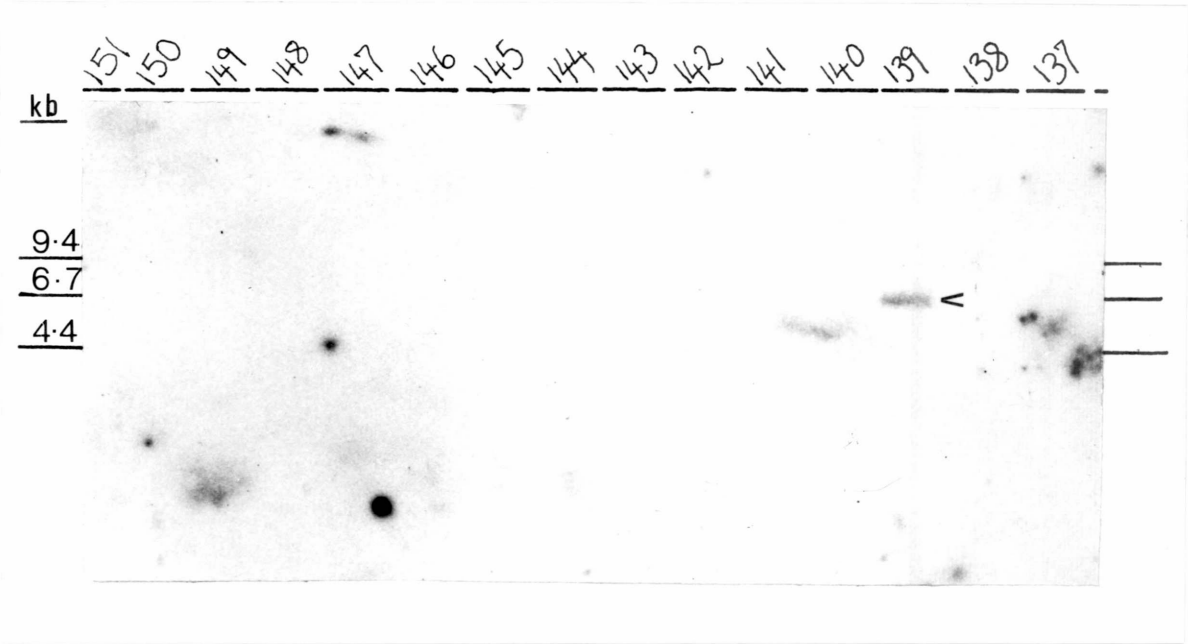
(See Figs. 3.7. and 3.11.)



Microclone pmc 16:127 contains an internal Hind III recognition site within the Eco R1 insert, and therefore gives two insert fragments after digestion with Hind III.

Figure 3.11. Autoradiograph resulting from hybridisation of 1 ug labelled total human DNA to a Southern blot of Hind III digested pmc 16 microclones

(See Figs. 3.7. and 3.10.)



One filter of Hind III digested microclones from pmc 16 was also probed with oligolabelled total human DNA. One microclone, 139, showed a band when the filter was autoradiographed (Fig. 3.11.). This clone had already been shown to contain mouse repeat sequences and the band resulting from the total human DNA probe indicated the presence of mouse DNA which hybridised to human repeat sequences. The microcloning strategy excluded the possibility of cloning satellite DNA therefore these repeat sequences were probably middle repetitive DNA.

Tables 3.2 and 3.3 :

All size estimates are given in kb.

'small' under 'Size Estimate' indicates a microclone size of 0.1 kb or less.

'E' under 'Size Estimate' indicates the estimate of microclone size from Eco R1 digestion followed by electrophoresis on agarose gels.

'High' under 'Copy Number' indicates that a microclone contained a repeat sequence under the autoradiograph criterion (see back).

Table 3.2

Size and Repeat Sequence Content of pmc 14 Microclones

All pmc 14 size estimates from Bam H1 analysis, in kb

Clone	Size Estimate	Copy Number	Clone	Size Estimate	Copy Number
22	0.4		61	small	high
23	0.2		62	small	
24	> 8	high	63	0.4	
25	small		64	0.4	
26	> 4	high	65	small	
27	0.4		66	0.6	
28	0.4		67	0.2	
29	small		68	0.2	high
31	small		69	small	
32	0.4	high	110	0.4	
33	> 4		124	0.4	
34	small		126	0.3	
35	> 7	high	127	0.3	
36	0.4		129	0.4	
38	small		130	0.4	
40	0.5	high	132	0.4	
41	2.0	high	138	0.4	high
42	small		142	0.3	
43	0.4		143	0.3	
44	0.4	high	146	0.4	
45	0.4		148	0.5	high
46	0.2		152	0.4	
47	0.2		156	0.5	high
48	4.0		159	0.2	
49	small		160	small	high
50	small		161	0.4	high
51	0.2		162	small	high
52	0.2		163	small	
53	2.0	high	164	> 4	high
54	0.2		165	small	
55	0.4		166	0.4	high
57	1.0		167	0.4	high
58	small		168	0.4	high
59	small		169	0.6	
60	small				

Table 3.3

Size and Repeat Sequence Content of pmc 16 Microclones

All pmc 16 size estimates from Hind III analysis in kb

Clone	Size Estimate	Copy Number	Clone	Size Estimate	Copy Number
1	small		117	0.4	
2	1.5 (E 2.2)		118	0.3	high
3	small		119	small	
4	small		120	0.4	
5	0.2		121	small	
7	small		122	small	
10	0.5		123	1.0 (E 0.8)	
11	small		124	small	
12	small		125	small	
13	small		126	small	
14	0.2		127	small	high
29	small		128	small	
30	small		129	small	
31	small		130	small	
32	small		131	0.3	
33	small		132	0.2	
34	small		133	0.5	high
35	small		134	small (E 0.2)	
36	small		135	rearranged	
37	small		136	small	
38	small		137	small	
39	0.2		138	0.2	
40	small		139	0.2	high
41	small		140	small	
42	small		141	1.0 (E 1.2)	
43	0.4		142	small	
44	0.8		143	small	
45	small		144	small	
47	0.3		145	small	
53	0.2		146	small	
54	0.2		147	small	
56	0.2		148	0.2	high
61	small	high	149	small	
62	0.4	high	150	small	
63	0.2		151	small	
69	0.2 (E 0.2)		152	0.2	
106	small		153	0.2	
107	0.2		154	small	
108	0.7		158	0.2	
110	2.9 (E 4.3)		159	4.0	
111	small		160	small	
112	0.2		161	0.3	
113	small		165	0.3	high
114	0.2		167	0.3	high
115	small		170	small	

Table 3.3 (continued)

Clone	Size Estimate	Copy Number
171	1.7	high
172	0.2	
174	small	
176	small	
179	small	
181	0.2	
182	small	
183	0.3	
184	0.3	
187	small	
188	small	
189	0.3	high
191	0.3	
193	small	
201	0.2	
206	small	
208	0.2	
209	small	
210	small	
211	small	
212	rearranged	
213	3.0	
214	small	
215	small	
216	small	
217	small	

Table 3.4

pmc 14 - Whole X Chromosome Dissection

(see Table 3.2). All size estimates from Bam H1 analysis

Total Number of Microclones Analysed	69
Mean Average Microclone Size: derived from 50 clones 0.2 kb or larger	1.0 kb
Modal Average Microclone Size: derived from 50 clones 0.2 kb or larger	0.4 kb
Range of Microclone Size: derived from 69 clones	0 - 8 kb
Number of 'small' Microclones	19
Percentage of 'small' Microclones	28 %
Number of Microclones Containing Repeat Sequences	20
Percentage of Microclones Containing Repeat Sequences: derived from 69 clones	29 %
Mean Average Size of Microclones Containing Repeats: derived from 17 repeat clones 0.2 kb or larger	1.9 kb
Mean Average Size of Microclones Not Containing Repeats: derived from 33 clones 0.2 kb or larger	0.6 kb

Table 3.5

pmc 16 Proximal X Chromosome Dissection

(see Table 3.3). All size estimates from Hind III analysis

Total Number of Microclones Analysed	116
Mean Average Microclone Size: derived from 47 clones 0.2 kb or larger	0.6 kb
Modal Average Microclone Size: derived from 47 clones 0.2 kb or larger	0.2 kb
Range of Microclone Size: derived from 116 clones	0 - 4 kb
Number of 'small' Microclones	67
Percentage of 'small' Microclones	58 %
Number of Microclones Containing Repeat Sequences	11
Percentage of Microclones Containing Repeat Sequences: derived from 116 clones	10 %
Mean Average Size of Microclones Containing Repeats: derived from 9 clones 0.2 kb or larger	0.5 kb
Mean Average Size of Microclones Not Containing Repeats: derived from 38 clones 0.2 kb or larger	0.6 kb

Table 3.6

Acrylamide Sizing of pmc 16 Microclones

(None of these microclones gave an autoradiograph signal when probed with total mouse DNA to detect repeat sequences).

Microclone	Size Estimate (bp)	Hind III Size Estimate (kb)	
Microclones previously sized by Hind III analysis:			
5	70	0.2	*
14	140	0.2	
35	70	small	
39	120	0.4	*
56	220	0.2	
117	120	0.4	*
120	190	0.4	*
122	60	small	
124	40	small	
152	160	0.2	
183	130	0.3	*
184	160	0.3	*
191	130	0.3	*
201	70	0.2	*
206	150	small	
208	180	0.2	
216	85	small	
Microclones not previously sized:			
69	190		
91	120		
109	500		
162	<50		
169	150		
198	150		
207	125		
211	<50		
219	650		
222	140		
223	70		
225	180		
227	250		

* - denotes difference in Hind III size estimate compared to Eco R1 size estimate of greater than 100 bp.

<50 - denotes that no band was observed on an acrylamide gel - although plaque morphology was recombinant - either the microclone was non-recombinant, or the insert was smaller than 50 bp, the detectable limit on the acrylamide gels.

Overall Results of Microclone Size Data

Tables 3.4 and 3.5 show a mean average microclone size difference (inserts 0.2 kb or larger) for pmc 14 and pmc 16 of 0.4 kb and a modal average microclone size difference (microclone inserts 0.2 kb or larger) of 0.2 kb. Size estimation of pmc 14 microclones was by Bam H1 digestion, size estimation of pmc 16 microclones was by Hind III digestion. The differences between pmc 14 and pmc 16 could reflect the inaccuracy of size estimation, but the greater maximum size of pmc 14 may indicate a true difference. Neither Bam H1 nor Hind III size analysis is as accurate as Eco R1 digestion and electrophoresis on acrylamide gels, and sizings may have been wrongly estimated by up to 100 bp, a substantial amount with the smaller microclones. However, sizings clearly show the similar small size of both microclone banks.

Both microclone banks contain a substantial percentage of 'small' microclones, and this figure is greater in pmc 16. Some of these microclones in which insert size could not be detected (by Hind III analysis) did hybridise to total mouse DNA, and therefore contained mouse genomic sequences.

Size estimation data also indicated that two pmc 16 clones were rearranged during microcloning.

Overall Repeat Sequence Results

The above data from the 46 pmc 14 microclones shows that microclones containing repeat sequences tend to be larger than non-repeat sequence containing microclones. The data for pmc 16 shows a very similar mean average size for non-

repeat sequence containing microclones.

Analysis of data from over 200 pmc 16 microclones - which includes those described here, and in Fisher et al, 1985 - showed that microclones containing repeat sequences tended to be larger than microclones without repeat sequences. If repeat sequences are randomly distributed throughout the X chromosome then the greater insert size, the greater the chance that the microclone will contain a repeat sequence - and the greater percentage of the total number of clones will contain a repeat sequence. However some of the largest clones (>2 kb) gave no autoradiograph signal after hybridising to labelled total mouse DNA. These microclones may not contain genomic mouse DNA (see Chapter 4).

The difference in the percentage of repeat sequence containing microclones may be due to the smaller sample size of pmc 14 and the effect of chance, but probably reflects a larger average insert size for pmc 14. In both clone banks the level of repeat sequence containing clones is low.

Non-Recombinant Microclones

Given that the background level of non-recombinant clear plaques is approximately 10^{-3} (see Chapter 2), by calculation approximately 95 % of pmc 14 and 86 % of pmc 16 microclone banks should be true recombinant microclones. An exact estimate of the percentage of recombinants in the microclone banks is impossible because of the number of very small inserts which may be less than 50 bp and undetectable on acrylamide gels.

3.6 Discussion Of Results

Microdissection And Microcloning

100 chromosomes or chromosome fragments were microdissected from unstained CD *Mus musculus* metaphase spreads, for each microcloning. This took an average of 10 hours of microdissection per collection. Theoretically smaller numbers of chromosomes could have been microdissected and microcloned; however low numbers of recombinant vector in the packaging reaction result in low numbers of clear plaques on the bacterial plates and so it is not possible to tell if the few observed clear plaques represent true recombinants or simply background non-recombinant vector. To be sure that a microcloning reaction has been successful the observed number of clear plaques must be well above the expected background level of non-recombinant clear plaques, and the largest number of plaques possible should be analysed.

Chromosome fragments were microcloned in nanolitre volumes, because of the sensitivity of the ligation reaction to DNA concentration. Working in microlitre volumes would have entailed the addition of a vast excess of vector DNA, to compensate for the low concentration of mouse DNA and this would lead to extremely high backgound levels of non-recombinant λ 'false positive' clear plaques.

The vector was added in excess, but it is not possible to give molar ratios for vector:insert, because the number of mouse DNA fragments within the microdrop is unknown.

The microclone DNA was packaged into λgt10, with a packaging

efficiency of approximately 10^8 pfu/ug. This magnitude of packaging efficiency is necessary to produce large number of recombinant bacteriophage from the microcloning reaction. A large number of resulting plaques must be analysed in order to be sure that the number of clear plaques is unequivocally well above non-recombinant background levels. The efficiency of the packaging reaction *per se* does not indicate if the microcloning has been successful - this is only evident from the levels of clear plaques observed above the expected background levels. pmc 14 and pmc 16 had comparable packaging efficiencies, but pmc 16 produced 4 times less clear plaques than pmc 14 - probably this was simply due to a 4 times decrease in the amount of mouse DNA that was collected.

Microclone Sizing

Microclones were initially sized by *Bam H1* and *Hind III* digestion. A group of microclones were analysed by *Eco R1* digestion on acrylamide gels, the resolution and accurate sizing make this a more suitable method of analysis than use of agarose gels for small (<0.3 kb) inserts.

Data from pmc 14 and pmc 16 is comparable to that of two other microclonings of mammalian chromosomes, both of which have utilised complete *Eco R1* digestion. Rohme *et al* (Rohme *et al*, 1984) showed a mean average microclone size of 1 kb and data from microcloning the short arm of human chromosome 2 (Bates *et al*, 1986) showed a mean average microclone size of approximately 0.4 kb.

Resolution of pmc 14 and pmc 16 microclone size by Bam H1 and Hind III enzymes was not very powerful, but these results show that average microclone size is considerably smaller than anticipated from the observed results of Eco R1 digestion of the mouse genome (Botchan et al, 1974). The mean average size of fragment from a complete Eco R1 digestion of mouse DNA has been empirically determined to be 2.4 kb i.e. 1.6 kb less than the mean average of 4 kb, calculated on the basis of random nucleotide distribution - this difference is thought to result from the relatively rich A-T content of the mammalian genome.

One possible explanation for small average insert size after microcloning is Eco R1* activity, but the Eco R1 aliquots used for microcloning were tested before use for Eco R1* activity and in the majority of recombinant λgt10 ('recombinant' in terms of plaque morphology and restriction fragment length analysis) the insert is recoverable by an Eco R1 digestion; therefore small insert size is not due to Eco R1* activity.

Small microclone size may be due to acid depurination followed by base hydrolysis of the chromosomal DNA during the fixation stage of metaphase spreading (Fisher et al, 1985). The chromosome fixative is pH 2.2 and total time of chromosome fixation was up to 1 hour. Brown (Brown, 1986) has analysed the Eco R1 size estimate data of microclones from pmc 16 and states that this data conforms to a distribution of insert sizes that can be mathematically described by two parameters - i) the frequency (per bp) of

Eco R1 sites within the DNA; - ii) the frequency (per bp) of a second event affecting the DNA, thought to be hydrolytic attack. Brown concludes for pmc 16 that a hydrolytic attack could have occured with a mean average of once every 100 bp. DNA fragments that have been subjected to hydrolytic attack probably cannot be cloned. Smaller Eco R1 fragments are less likely to suffer hydrolytic attack and so are more likely to be cloned, therefore the size averages of the microclone banks would be smaller than expected. Brown states (Pers. comm.) that for pmc 16, the the number of 'clonable' fragments may have been reduced by a factor of 0.03 - theoretically without hydrolytic attack approximately 22,000 clonable fragments (650/0.03) should have resulted from pmc 16, i.e. 2,300 fragments per pg microdissected DNA.

Such an expectation accords well with data derived from the cloning of non-fixed metaphase chromosomes i.e. the cloning of flow sorted metaphase chromosomes. Amar et al (Amar et al, 1985) estimate a recovery of approximately 5,600 clones per pg of DNA derived from flow sorting the X chromosome of CD mice. The average insert size of clones was 3 kb.

The time of fixation of the chromosomes in the microcloning of a region of mouse chromosome 17 (Rohme et al, 1984) is not stated, but for the microdissection of the Drosophila X chromosome regions from part of 3B to most of 3C (Pirrotta et al, 1983b) the maximum time of fixation in 45 % acetic acid was 1.5 mins and resulting mean average microclone size was 3.8 kb (microcloning utilised Eco R1 restriction).

Microclones Containing Repeat Sequence DNA

Ultimately microclones were to be positioned on the mouse X chromosome by classical genetic mapping (see Chapter 4), this method is not suitable for use with molecular probes containing highly repeat sequences. Use of the restriction enzyme Eco R1 provided an initial selection procedure against mouse satellite DNA sequences being microcloned. Hybridising total mouse probes to Southern blots of microclone DNA provided a second screen against repeat sequence containing microclones. However when a microclone insert fragment gave a signal with a total mouse probe, this may have been because the fragment was small but highly repeated, or long and less highly repeated, for example. Apart from indicating that a microclone contains a repeat, this data cannot further characterise the repeat in terms of for example, copy number, size, or number of homologous sequences.

Both microclone banks have a low percentage of repeat sequence containing microclones compared to the flow sorted Eco R1 library of the mouse X chromosome (Amar et al, 1985). Amar et al established by plaque hybridisation and probing individual clones with labelled total mouse DNA, that approximately 50 % of the total bacteriophage in the library contained mouse repeat sequences, (over 80 % of the total number of bacteriophage in the library contained inserts). The greater percentage of repeat sequence containing microclones is a reflection of the larger average insert size of the library.

Microcloning Strategy

Two microdissections of the whole mouse X chromosome and one microdissection of the proximal region of the mouse X chromosome were performed, resulting in three microclone banks. Two of these banks, one from the whole X chromosome and one from the proximal region of the mouse X chromosome, were analysed for microclone size and repeat sequence content. This analysis suggested that the microdissection and microcloning strategy for the generation of X chromosome and X chromosome region specific probe banks had been successful. A large number of clones had been generated and both clone banks had been shown to contain a large number of inserts (i.e. inserts greater than 0.2 kb), in excess of the number needed for a probe density of greater than 2 probes per cM.

Insert sizes were considerably smaller than expected from a complete Eco R1 digest of mouse genomic DNA but compared well with the results of two other microclonings of mammalian chromosomes. The great majority of clones did not hybridise to total mouse DNA, as small insert size diminishes the chance of a recombinant clone containing a repeat sequence. This characteristic of microclone banks is an advantage for the subsequent mapping of the clones, when high copy repeat sequence containing clones could not be mapped by classical genetic mapping (Chapter 4), without subcloning low copy sequences.

Both microclone banks should contain sufficient mouse X chromosome specific clones for the fine genetic mapping of the mouse X chromosome. In order to select microclones for mapping it was necessary to characterise individual clones.

4. Results - Analysis And Mapping Of Individual Microclones

4.1. Introduction

Banks of microclones have been generated from the microdissection of the whole mouse X chromosome and a region of the mouse X chromosome. Two clone banks were characterised for microclone size and repeat sequence content.

Over 20 microclones, almost exclusively from the regional microdissection, were individually characterised. Suitable clones were mapped to the mouse X chromosome, creating molecular locations definable in genetic terms of distance and order. Clones were mapped by applying classical genetic mapping to molecular probes; this approach to mapping is discussed.

Classical Genetic Mapping

Classical genetic mapping orders and distances loci into one dimensional arrays, by surveying the number of meiotic recombinations that occur between pairs of loci, out of a total number of observed meiotic events. Generally the chance of a recombination event occuring in meiosis between two loci increases with increasing distance between the loci (however regions of unusually high recombination and conversely, recombination suppression, are known - see Chapter 1). Recombinant events between two loci observed in progeny are taken as a percentage of the total numbers of meiotic results observable, this percentage is equivalent to

the distance between the loci and this distance is measured in map units called centiMorgans (cM).

Classical genetic mapping has two absolute requirements - and a number of limitations in consequence. The first requirement is for genetic variation - for two distinct alleles at the same locus. The need for two separate, scorable alleles from each of the two loci involved in a pairwise analysis arises from the need to follow the meiotic behaviour of parental chromsomes. One parent in the test cross must be heterozygous at both loci, and the phase of the alleles must be known. The segregation of one allele at one locus can be then compared to the segregation of another allele at a second locus, and linkage (and therefore distance) may be established from a large number of observed meiotic results.

The second requirement of classical genetic mapping is that each locus involved must have a recognisable phenotype for scoring the progeny.

Limitations arising from this system are varied :

- most importantly, classical genetic mapping is entirely dependent on phenotype. Whether coat colour, or protein isoelectric focussing point is the phenotype, this is always the result of a transcribed and translated DNA sequence. Therefore approximately 90 % of the genome (estimated non-transcribed sequence content (Lewin, 1980)) cannot be mapped by classical genetic means.
- loci can only be mapped if there is more than one allele.

Certain genes may not have more than one known phenotype, and therefore cannot be mapped;

- genetic mapping is the result of observations of meiotic exchange, and there are differences in the amount of recombination per unit of physical length - see Chapter 1;
- the two variants of a gene in an interspecific hybrid must occupy corresponding positions on their homologous chromosomes;
- a locus of unknown position must be mapped with two known loci to discover locus order, as well as separating distances;
- lastly, and with reference to the physical - genetic relationship of the chromosome (see Chapter 1), it is not possible to ascertain the physical position of a genetic locus, by classical genetic mapping.

Molecular Techniques

With the introduction of molecular biology, the DNA of a chromosome became accessible for direct analysis, and mapping techniques have developed to try and overcome the limitations of classical genetic mapping. These techniques centre around the use of DNA probes, such as those generated by microdissection, which are used as markers, or reference points, on the chromosome map.

Microdissected markers to the mouse X chromosome were mapped in genetic distances (cM) by applying molecular techniques to classical genetic mapping.

Mapping Microclone Probes

The principles of the genetic mapping of molecular probes are the same as those of classical genetic mapping, but there is no requirement for phenotypic expression, so removing the main limitation of classical genetic analysis from the study of the genome. Molecular probes must still be mapped in relation to two loci and the requirement for allelic variation in both loci of one parent is still necessary, but with molecular probes the allelism is that of restriction fragment length differences.

All sequences in the genome which undergo recombination, may be mapped providing a restriction fragment length polymorphism exists and in the case of repeat sequences, the sequences are localised and not interspersed.

Mapping Strategy - Mouse Crosses

A variety of crosses could have been set up for the mapping of the microclone probes - such as crosses between different inbred lines, different recombinant inbred lines, or outbred mice. However an interspecific cross would result in a greater chance of finding parental probe restriction fragment length variation because of the greater parental divergence. Therefore the mapping cross was set up between a *Mus musculus* female and a *Mus spretus* male. These species are known to interbreed under laboratory conditions (Bonhomme *et al*, 1978) and this interspecific cross was used for the first time in 1985 (Robert *et al*, 1985) to follow

the segregation of different restriction fragment length variants derived from myosin alkali light chain and actin genes.

Two million years of divergence (Brown, Pers. comm.) lie between *M. musculus* and *M. spretus* but this is not thought sufficient to alter locus position on the different X chromosomes. Of the mouse mapping data derived from these crosses so far, there is no reported example in which *M. musculus* and *M. spretus* loci occupy different positions within the genome (for example see Robert *et al* 1985, Amar *et al* 1986, Dautigay *et al*, 1986). Also no gross chromosomal reorganisations have been observed between pachytene *M. musculus* and *M. spretus* chromosomes, and genes known to be either linked or unlinked in *M. musculus* are equally linked or unlinked in *M. spretus* and *M. musculus*/*M. spretus* interspecific crosses (Amar *et al*, 1986). It is known for example, that the recombination frequency observed between the two X-linked genes *Pgk-1* (phosphoglycerate kinase-1) and *Ags* (alpha-galactosidase A) is comparable in *M. musculus*/*M. spretus* interspecific crosses to *M. musculus* intraspecific crosses (Chapman *et al*, 1983).

Mapping Strategy - Restriction Enzyme

A second strategy for increasing the likelihood of parental restriction fragment length variation was employed during the restriction of mouse DNA samples. DNAs can be digested with a restriction enzyme that contains the highly mutable CpG doublet within its recognition site. CpG dimers are

relatively rare in the genome (Skolnick and White, 1982) and are often methylated at the C position, a site implicated as a hotspot for C to T transition mutations. Therefore digesting DNA samples with a restriction enzyme that recognises this dimer (such as Taq 1) increases the chance of finding different parental recognition sites, and hence different parental restriction fragment length variants.

4.2. Results - Mouse Crosses

Cross C1

A cross (C1), was set up between Mus spretus and Mus musculus. For successful production of fertile hybrid progeny Bonhomme and colleagues observed that the female parent should be M. musculus and the male parent M. spretus (Bonhomme et al, 1978). Cross C1 was set up between a male M. spretus and an outbred M. musculus female. The female mouse carried 3 semidominant X linked coat mutations: Harlequin, Tabby, Lined.

Harlequin, (Hq): Hemizygous males and homozygous females are almost completely bald, and smaller than normal. They are reported to be fully viable and fertile. Heterozygous females have bald patches of less than 50 % of the total suface area, as a result the coat appears to have very broad transverse stripes. Heterozygous females are reported to be fully viable and fertile (Green, 1981).

Tabby, (Ta): Hemizygous males and homozygous females have no guard hairs ~~or zigzags~~ in the coat. They have a bald patch behind each ear and a bald tail with kinks towards the tip.

The number of vibrissae is reduced and eyelid aperture is small. The males are reported to breed satisfactorily but homozygous females are often sterile. Heterozygous females have very fine transverse stripes of normal and dark coloured hair, and reduced numbers of vibrissae. Heterozygous females are reported to be fully fertile (Green, 1981).

Lined, (Li): Hemizygous males die in utero, (therefore the effect of Li in homozygous females cannot been observed). Heterozygous females appear to be fully viable although there may be a slight shortage of these females compared to normal female litter mates (0.75:1). The mutation is difficult to detect in heterozygous females - they have very fine transverse stripes, which often can be seen only on parts of the dorsal area of the body. Other data has shown Li may result in non-random X chromosome inactivation - the chromosome carrying Li being preferentially inactivated in at least 90 % of cells (Cattanach and Crocker, 1984).

Harlequin and Tabby were chosen as the 'known genetic loci' for mapping analysis, because they were thought to be encompassed by the region microdissected (Fig. 3.1.). Hq and Ta are easily distinguishable and fast to score.

By chance the only available female for the C1 cross carried the mutation Lined on one X chromosome.

The mutations (Hq, Ta, Li) were present in the female Mus musculus in the following phase:

$$\frac{Hq \quad + \quad Li}{+ \quad Ta \quad +}$$

The male Mus spretus was wild type for all coat mutations.

Cross C1:

$$\frac{Hq \quad + \quad Li}{+ \quad Ta \quad +} \times \frac{+ \quad + \quad +}{Y}$$

Mus musculus (♀) Mus spretus (♂)

Cross C2

Cross C1 resulted in four female progeny, a, b, c, and d. Each mouse inherited one Mus spretus X chromosome, wild type for coat mutations, and one Mus musculus X chromosome. Therefore the females would be heterozygous for any inherited coat mutation.

The phenotypes of Harlequin and Tabby are clearly distinguishable, individually, or when present in the same mouse; the Lined phenotype could be only distinguished by analysing the male:female ratio of C2 progeny. After coat scoring and sexing of the progeny of these four females their genotypes were established as:

Mice a and d: $\frac{Hq \quad + \quad +}{+ \quad + \quad +}$ $\frac{(M.\ musculus\ X)}{(M.\ spretus\ X)}$

Mice b and c: $\frac{+ \quad Ta \quad +}{+ \quad + \quad +}$ $\frac{(M.\ musculus\ X)}{(M.\ spretus\ X)}$

Two females were heterozygous for Harlequin, two females were heterozygous for Tabby. Subsequent progeny data (see below) indicated that Lined had not been inherited by any of the four females. Hq and Li are separated by 50 cM; in the two Hq heterozygotes meiotic recombination had occured during female gamete formation, resulting in separation of Hq and Li from the same chromosome.

To follow segregation of Hq and Ta the four female C1 progeny were backcrossed, to M. musculus males. Only female C1 progeny would be backcrossed, so that X chromosome recombination during C1 female progeny meiosis could be analysed, (also, following Haldane's law, males resulting from the C1 M. musculus/M. spretus cross would be sterile (Bonhomme et al, 1978)).

The four females, a(Hq), b(Ta), c(Ta), d(Hq), were backcrossed to M. musculus males from the 129 inbred line (Cross C2). These males were wild type for the coat mutations.

Cross C2:

$$\frac{Hq}{+} \times \frac{+}{Y} \qquad\qquad \frac{Ta}{+} \times \frac{+}{Y}$$

musculus X spretus X (a,d Hq ♀)	musculus X Y (129 ♂)	musculus X spretus X (b,c Ta ♀)	musculus X Y (129 ♂)

Cross	C2a(Hq)	C2d(Hq)	C2b(Ta)	C2c(Ta)
Females	a(Hq)	d(Hq)	b(Ta)	c(Ta)
Males	129.6f	129.6g	129.1g	129.1h

129 males, 129.6f and 129.6g were litter mates; 129.1g and 129.1h were also litter mates, from a different cross.

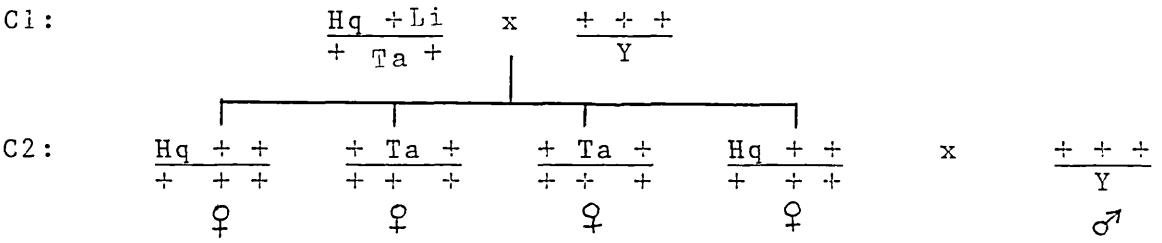
C2 Progeny

The reproductive capacity of female mice heterozygous for Harlequin or Tabby is not reported to be affected by their mutations, however analysis of progeny data (see Table 4.1) suggests that reproductive potential in Ta heterozygous females may be diminished - but this is a very small sample

from which to draw conclusions. Parental mice in crosses C2a(Hq), C2c(Ta) and C2d(Hq) were culled at the same time, whilst still breeding and with the potential to produce more litters. Cross C2b(Ta) was culled earlier as no litters had been produced for several weeks.

Progeny were scored for (i) sex and (ii) coat mutations; 232 progeny were produced overall.

Crosses C1 and C2



(Cross: C2a(Hq) C2b(Ta) C2c(Ta) C2d(Hq)

Resulting C2 progeny phenotypes and genotypes:

Crosses C2a(Hq) and C2d(Hq)

$\frac{Hq}{+}$	$\frac{Hq}{Y}$	$\frac{+}{+}$	$\frac{+}{Y}$
Harlequin female	Harlequin male	wild type female	wild type male

Crosses C2b(Ta) and C2c(Ta)

$\frac{Ta}{+}$	$\frac{Ta}{Y}$	$\frac{+}{+}$	$\frac{+}{Y}$
Tabby female	Tabby male	wild type female	wild type male

The C2 crosses resulted in over 230 progeny segregating for Harlequin and Tabby.

Table 4.1

C2 Progeny

Cross	C2a(Hq)	C2d(Hq)	Hq total	C2b(Ta)	C2c(Ta)	Ta total
Female Parent	a(Hq)	d(Hq)		b(Ta)	c(Ta)	
Male Parent	129.6f	129.1h		129.6g	129.1g	
Segregating Marker	Hq	Hq		Ta	Ta	
No. of Litters	12	10	32	5	10	15
Mean Average in Litters	7	8	8	7	4	6
No. of Mutant Males	13	21	34	8	11	19
No. of Wild Type Males	25	17	42	8	12	20
No. of Mutant Females	18	13	31	6	7	13
No. of Wild Type Females	25	24	49	11	13	24
Total No. of Males	38	38	76	16	23	39
Total No. of Females	43	37	80	17	20	37
Total Number of Mutants	31	34	65	14	18	32
Total Number Wild Types	50	41	91	19	25	44
Total Number of Progeny	81	75	156	33	43	76

Overall Total Number of C2 Progeny 232

Collection and Preparation of C2 Progeny

Having scored C2 progeny for coat mutations and sex, at 6 weeks old or later, progeny were killed and tail and liver tissue was immediately frozen in liquid nitrogen, then stored until required for DNA preparation. High molecular weight DNA was prepared then digested with the restriction enzyme Taq 1 and electrophoresed on agarose gels. After electrophoresis and photography of the gels, DNA was transfered to a nylon membrane by 'Southern blotting' (see Chapter 2). These membranes were stored until required for microclone mapping.

4.3. Results - Selection Of Microclones For Mapping

To facilitate using microclones as radiolabelled probes, inserts were subcloned into plasmid vectors. Microclone probes were then characterised and selected according to the requirements of molecular genetic mapping for positioning on the mouse X chromosome. Markers were suitable for mapping if they were low or single copy mouse X chromosome specific probes, and showed restriction fragment length variation between parental X chromsomes (Mus musculus and Mus spretus X chromsomes).

4.3.1. Subcloning Microclones Into Plasmid Vectors

To investigate individual microclones, insert DNA had to be radiolabelled for use as a molecular probe against Southern blots of mouse DNA. The length of λgt10 is 43.34 kb. If an entire recombinant λgt10 with an insert of 0.3 kb was oligolabelled, the insert DNA would represent 0.69 % of the

total incorporated ^{32}P-dCTP. Therefore labelling the whole recombinant λ molecule would be a very inefficient way of labelling the insert. To increase efficiency, recombinant λgt10 was digested with Hind III, electrophoresed in l.m.p. agarose and the insert band was excised from the gel for radiolabelling. This fragment consists of the 6.5 kb λ band plus insert. With an insert of 0.3 kb, still only 4.6 % of the incorporated ^{32}P-dCTP from a Hind III band labelling would be in insert DNA sequences. Hind III band labelling produced insert probes with a low specific activity, although, for example, microclone pmc 16: 153 was radiolabelled in this way (see on).

Radiolabelling the Eco R1 insert fragment would result in 100 % of labelled DNA being insert DNA. Using the 'Eco R1 fragment' was preferable therefore, to all other digest fragments. With large microclone inserts, for example pmc 16: 2, it was possible to excise the insert fragment from Eco R1 digests of recombinant λ on l.m.p. agarose. With microclones of 0.4 kb or less - the majority of pmc 16 microclones - it was extremely difficult to load and electrophorese sufficient recombinant λ DNA onto a l.m.p. agarose gel to see faint low molecular weight bands.

The most appropriate way to handle such small inserts was to subclone into plasmid vectors.

Plasmid Vectors

The plasmid vectors used, pSP64 and pGem-4 are 3.0 kb and 3.15 kb respectively, with a unique Eco R1 site of

insertion. Microclones of 0.3 kb or less were subcloned into these plasmid vectors, for characterisation against mouse genomic DNA (Fig. 4.1.).

Plasmid DNA is faster to prepare than λ DNA, and can more easily be prepared in greater quantities. With larger inserts it would have been feasible to radiolabel the whole recombinant plasmid and still obtain a high specific activity for insert sequences. However using whole recombinant plasmid as the probe tended to give higher backgrounds on autoradiography, than the insert alone.

Large microclones (pmc 14: 36, pmc 16: 141, 219) were also subcloned to make use of the advantages of plasmids.

Figure 4.1. Photograph of Eco R1 digested plasmid subclones after electrophoresis in a 1.8 % agarose gel DNA was stained with 5 ug/ml ethidium bromide.

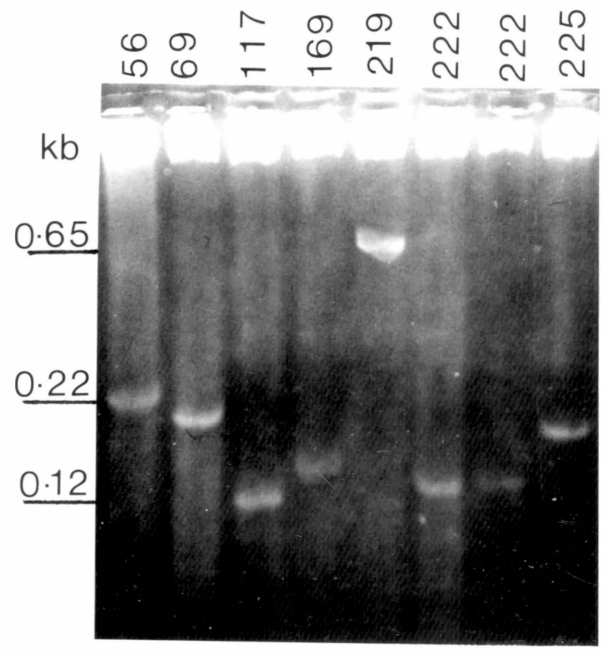


Table 4.2

Radiolabelling and Subcloning of Microclones

Clone	Size (kb)		Initial Characterisation	Vector	Bacterial Host
pmc 14:					
36	H	0.4	λ Hind III insert	pGem-4	DH1
pmc: 16:					
2	E	2.2	λ Eco R1 insert	-	-
14	A	0.14	Plasmid insert	pGem-4	DH1
56	A	0.22	Plasmid insert	pSP64	WT217
69	A	0.19	Plasmid insert	pGem-4	DH1
91	A	0.12	Plasmid insert	pGem-4	DH1
110	E	4.3	λ Eco R1 insert	-	-
117	A	0.12	Plasmid insert	pGem-4	DH1
120	A	0.19	Plasmid insert	pGem-4	DH1
123	E	0.8	λ Eco R1 insert	-	-
134	E	0.2	λ Eco R1 insert	-	-
138	H	0.2	λ Hind III insert	-	-
141	E	1.2	λ Eco R1 insert	pSP64	WT217
153	H	0.2	λ Hind III insert	-	-
169	A	0.15	Plasmid insert	pGem-4	DH1
184	A	0.16	Plasmid insert	pSP64	WT217
198	A	0.15	Plasmid insert	pGem-4	DH1
206	A	0.15	Plasmid insert	pGem-4	DH1
208	A	0.18	Plasmid insert	pGem-4	DH1
219	A	0.65	Plasmid insert	pSP64	DH1
222	A	0.14	Plasmid insert	pGem-4	DH1
225	A	0.18	Plasmid insert	pGem-4	DH1

Under Size, 'E' denotes that the size given is from Eco R1 analysis on agarose gels, 'H' denotes that the size given is from Hind III analysis on agarose gels and 'A' denotes that the size given is from Eco R1 analysis on acrylamide gels.

Under Initial Characterisation 'Plasmid insert' denotes that the plasmid Eco R1 insert fragment was used as the probe - not the whole plasmid.

4.3.2. Characterisation Of Microclone Probes

To select probes suitable for mapping, subclones were individually characterised for various criteria (such as copy number, or X chromosome specificity - see below) by hybridisation to genomic screening filters. All DNA on these filters was digested with the restriction enzyme Taq 1, as were the progeny filters (see below). This enzyme recognises the site TCGA, which contains a highly mutable CpG dimer. Taq 1 is an enzyme of choice when screening a population for restriction fragment length polymorphisms to specific probes.

The analysis was almost exclusively of the proximal microcloning, pmc 16.

Mouse DNA: Certain microclone probes repeatedly did not hybridise to filters containing mouse genomic DNA. The insert DNA in these probes was therefore probably not of mouse origin.

Copy Number: Low copy number probes were more suitable for the mapping protocol, and therefore all microclone probes were screened against Taq 1 digests of genomic Mus DNA, to observe band pattern.

Mus Spretus - Mus Musculus Restriction Fragment Length Variation: Microclone probes were screened against filter strips carrying Taq 1 digests of outbred Mus musculus DNA and Mus spretus DNA to ensure that there was sufficient variation between the two probe patterns to follow segregation of the microclone loci through a M. musculus - M. spretus hybrid family. Certain microclones produced the

same probe pattern on Taq 1 digests of M. musculus and M. spretus DNA.

X Chromosome Specificity: A limited number of probes were hybridised to panels checking for X chromosome specificity. These filters carried Taq 1 digests of 5 genomic DNAs :

i C57BL/10 mouse inbred line (Mus musculus)

ii SWR mouse inbred line (Mus musculus)

iii E36 Chinese hamster cell line (parent cell line for MAE 28 and MAE 32) (D'Eustachio et al, 1981, Old et al, 1962)

iv MAE 32 hybrid cell line containing mouse chromosomes X and 16 (derived from a BALB/c murine fibrosarcoma cell line) on a Chinese hamster background (D'Eustachio et al, 1981, Old et al, 1962)

v MAE 28 hybrid cell line containing mouse chromosomes X and 12 (derived from a BALB/c murine fibrosarcoma cell line) on a Chinese hamster background (D'Eustachio et al, 1981, Old et al, 1962)

Bands detected by microclone probes in tracks i, ii, (mouse DNAs), iv and v (cell hybrid DNAs), and not in track iii (parent chinese hamster cell line) represent mouse X chromosome specific fragments.

One microclone (pmc 14: 36) was hybridised to a cell hybrid panel containing a range of cell hybrids with different mouse chromosomes against a chinese hamster background. X chromosome specific fragments gave a unique autoradiograph pattern.

Ideally all probes should have been hybridised to panels checking for X chromosome specificity, however the hybrid cells lines were not freely available, and use of the filters was unfortunately limited. Therefore X chromosome specificity was also checked by hybridising a suitable probe to a progeny filter (see below) and comparing segregation of the probe with segregation of the coat mutation of the progeny. An X specific probe would give a recombination fraction, θ, of less than 0.5 in linkage analysis with either Tabby or Harlequin, unless it was derived from a very distant or pseudoautosomal region of the chromosome, (see on). In addition, all (non-pseudoautosomal) X linked genes would for example, produce no males displaying both the M. musculus and M. spretus pattern.

4.3.3. Mapping X Chromosome Specific Microclones

After putting microclone probes against genomic screening filters, suitable microclones were mapped along the mouse X chromosome by hybridisation to progeny filters. Individual progeny DNAs were then scored for the recorded coat marker mutations (Harlequin or Tabby) and microclone restriction fragment length variation (Mus musculus (M) or Mus spretus (S) pattern) to determine if progeny were 'parental' types or 'recombinant' types (i.e. 'recombinant' if a crossover had occured between the microclone locus and the coat mutation locus during female parental meiosis). For each C2 cross, eight types of progeny were possible, four parental types and four recombinant types.

(i) Coat Mutation Locus

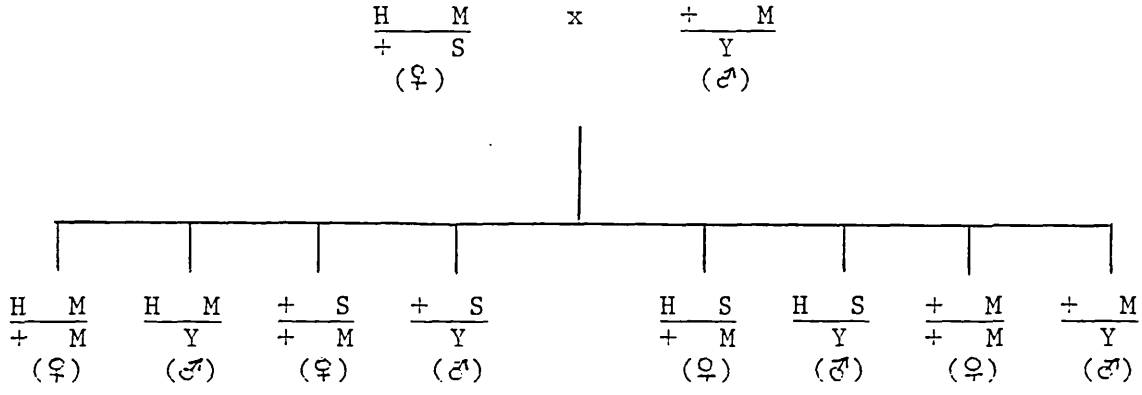
H - Presence of the coat mutation (Hq or Ta)
+ - Wild type at the coat mutation locus

(ii) Microclone Locus

M - M. musculus restriction fragment length variation
S - M. spretus restriction fragment length variation

C2 crosses were set up between M. musculus - M. spretus hybrid female mice and M. musculus males.

C2 cross:



N.B. It is not possible to generate SM males from the above cross, if the probe is X linked and in homologous positions on the M. musculus and M. spretus X chromosomes.

Calculation Of The Recombination Fraction, θ

The recombination fraction, θ, between a microclone probe locus and one of the known, positioned loci, (Tabby or Harlequin) is equivalent over small distances (see below) to the proportion of recombinant progeny in the set of progeny; i.e. θ =

$$\frac{\text{Number of Recombinants}}{\text{Number of Progeny}}$$

The value of θ is equivalent to the distance in cM between the two loci being scored.

However over large distances the occurance of double crossovers causes θ to be less than the true distance in cM.

The standard error (s.e.) of the recombination fraction is calculated from the following formula:

θ = recombination fraction

n = total number of progeny

$$\text{s.e.} = \sqrt{\theta \frac{(1-\theta)}{n}}$$

Determination Of Probe Order Along The Chromosome

To be positioned along the chromosome, probes must be mapped in relation to two known loci, mapping in relation to one known locus will not indicate if the probe is proximal or distal to that locus. All microclones were positioned relative to both Tabby and Harlequin, and then relative to other microclones.

Multipoint Data

When data from recombination fractions alone is insufficient to give an exact order between closely linked probes, 'multipoint' data may facilitate delineating probe order. Recombination fractions between individual probes may be calculated and probes positioned with respect to other microclones.

However the most decisive data comes from the placing of probes in the order which requires the lowest number of recombination events.

For example, if the positioning of the closely linked probes 2 and 3 is equivocal from Hq and Ta recombination fractions, but the scoring data for these two loci and two flanking loci, 1 and 4 is known in an individual mouse to be:

1 2 3 4

S M S M

then the most likely order for the four loci is the order dependent upon the fewest number of recombination events i.e.:

1 3 2 4

S S M M one recombination event between loci 3 and 2.

4.4. Results - Individual Microclone Data

4.4.1. Data Summary

The following table summarises data given in this section for each microclone probe. The microclones analysed were:

pmc 14: 36 (1 microclone)

pmc 16: 2, 14, 56, 69, 91, 110, 117, 120, 123, 134, 138, 141, 153, 169, 184, 198, 206, 208, 219, 222, 225. (21 microclones)

Table 4.4 - 'low' under Copy Number denotes a probe hybridising to sufficiently few fragments, to be mapped; 'low' does not refer to absolute numbers of sequences in the genome.

Table 4.3

Individual Microclone Data

Clone	Mouse DNA Probe	Copy Number	Spretus/ Musculus Polymorphic	X Chromosome Specific	Mapped
see section	4.4.2.	4.4.3.	4.4.4.	4.4.5., 4.4.6.	4.4.7.
non-mouse inserts					
pmc 16:					
2	no	-	-	-	-
110	no	-	-	-	-
123	no	-	-	-	-
high copy number inserts					
pmc 16:					
69	yes	high	-	-	-
134	yes	high	-	-	-
138	yes	high	-	-	-
153	yes	high	-	-	-
169	yes	high	-	-	-
198	yes	high	-	-	-
non-polymorphic inserts					
pmc 16:					
206	yes	low	no	-	-
208	yes	low	no	-	-
not mapped					
pmc 16:					
14	yes	low	yes	-	-
91	yes	low	yes	-	-
117	yes	low	yes	-	-
suspected autosomal inserts					
pmc 16:					
56	yes	low	yes	no	-
184	yes	low	yes	no	-
mapped X-specific microclones					
pmc 14:					
36	yes	low	yes	yes	yes
pmc: 16:					
120	yes	low	yes	yes	yes
141	yes	low	yes	yes	yes
219	yes	low	yes	yes	yes
222	yes	low	yes	yes	yes
225	yes	low	yes	yes	yes

4.4.2. Non-Mouse DNA Microclone Inserts

pmc 16:	2	2.2 kb
	110	4.3 kb
	123	0.8 kb

The above microclones were each hybridised three times to an X-specificity testing panel, which contained mouse genomic DNA digested with *Taq* 1. On no occasion was a signal detected after autoradiography of the filters. It is possible that these sequences may have been specific to the CD mouse genome (which was not present on the filters), but it seems unlikely that this should be true for all three sequences. A common factor between the probes is their unusually large sizes compared to the other probes in the bank. It was reported from the previous mammalian chromosome microdissection that these long inserts are anomalous, and may consist of bacterial DNA from microscope slides (Rohme *et al*, 1984).

4.4.3. High Copy Number Microclone Inserts

pmc 16:	69	0.19 kb	(see Figs. 4.2:A
	134	0.2 kb	4.2:B
	138	0.2 kb	4.2:C
	153	0.2 kb	4.2:D
	169	0.15 kb	4.2:E
	198	0.15 kb	4.2:F)

Microclones 134, 138, 153, were hybridised to X-specificity testing panels, microclones 69, 169, 198 were hybridised to *Mus musculus* - *Mus spretus* strips. 69, 138, 153 and 169 displayed a very large number of discrete bands.
69 and 169 showed extensive band variation between *M. musculus* and *M. spretus*.

Figure 4.2. Autoradiographs resulting from hybridisation of repeat sequence microclone probes to different genomic DNAs

Taq 1 strips

4.2:A - 69
4.2:E - 169
4.2:F - 198

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Band sizes are indicated in kb - in all cases sized fragments are in the 'M' lane.

X specificity testing panels

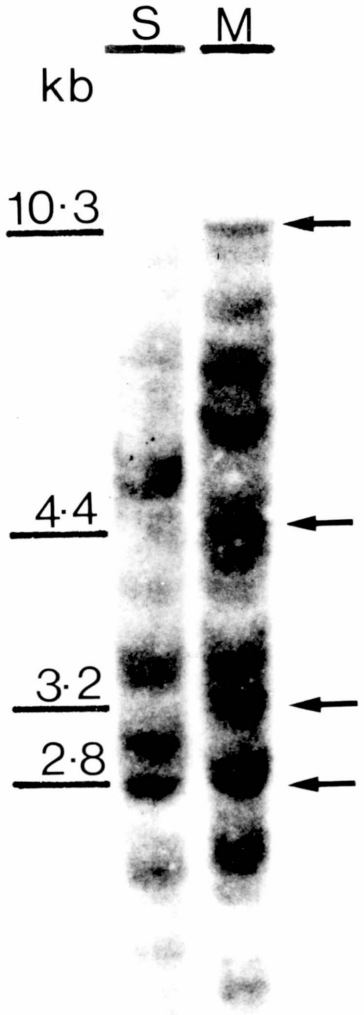
4.2:B - 134
4.2:C - 138
4.2:D - 153

'C57' lane - Taq 1 genomic digest of C57B1/10 M. musculus inbred line DNA
'SWR' lane - Taq 1 genomic digest of SWR M. musculus inbred line DNA
'E36' lane - Taq 1 genomic digest of chinese hamster parent cell line
'M32' lane - Taq 1 genomic digest of MAE 32 cell hybrid DNA
'M28' lane - Taq 1 genomic digest of MAE 28 cell hybrid DNA

Band sizes are indicated in kb - for microclone 134 sizes are for a λ marker lane (λ digested with Hind III), for microclones 138 and 153, sized fragments are in the 'SWR'lane.

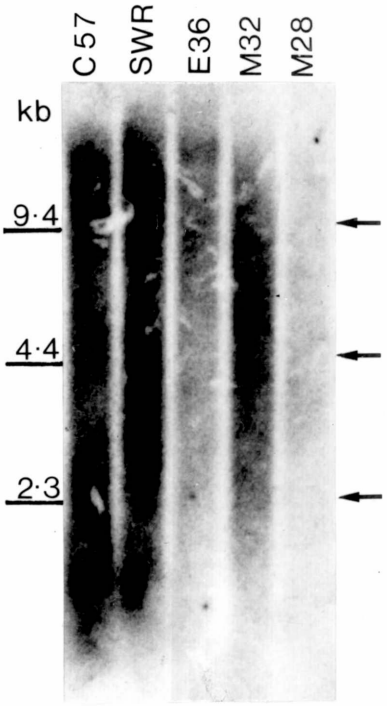
Figure 4.2.

A



69

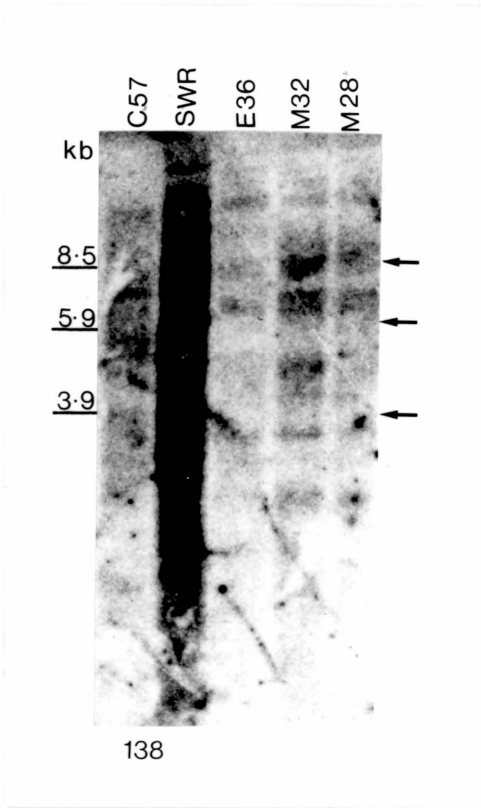
B



134

Figure 4.2. continued

C



D

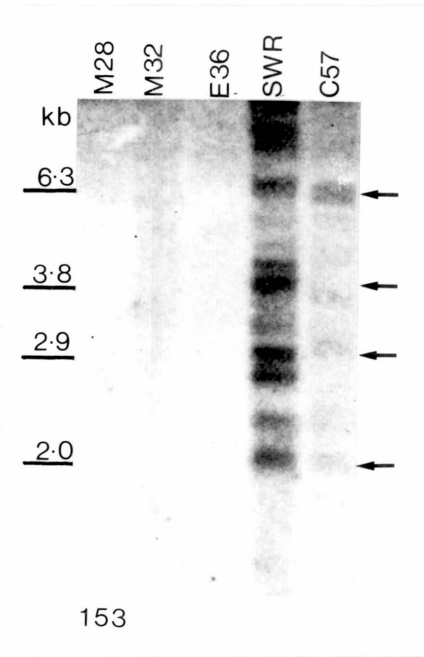
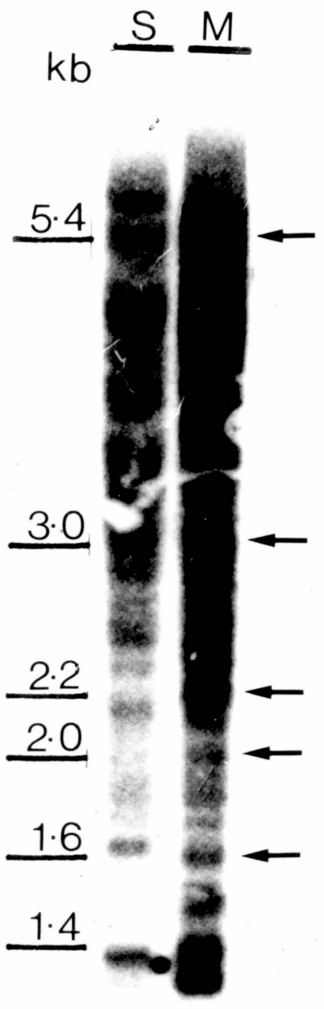
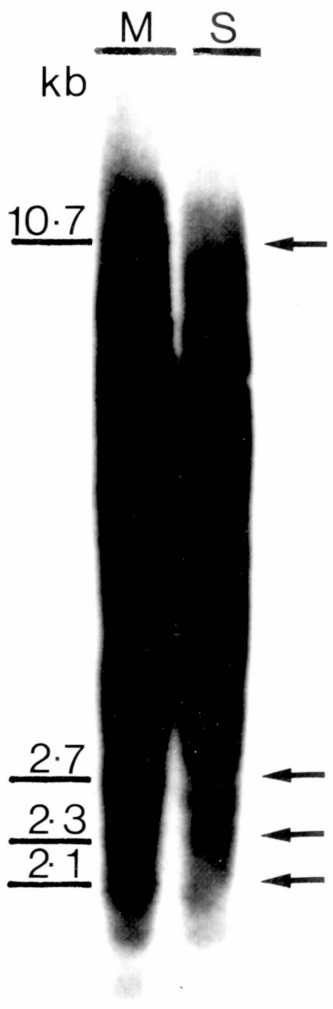


Figure 4.2. continued

E



F



138 gave a smear signal overlaid by specific bands on M. musculus genomic DNA and a number of discrete bands on the cell hybrid DNAs and the chinese hamster parent cell line DNA. 138 therefore represents a sequence with many copies in the mouse genome, the majority of which appear to be autosomal. This sequence had homologies in the chinese hamster genome.

Very few 153 bands hybridise to the cell hybrids, indicating that the majority of 153 sequences are autosomal.

134 and 198 displayed a smear throughout the genomic DNA, but variant M. musculus and M. spretus bands were 'superimposed' on top of the 198 smear.

All these high copy repeat containing probes escaped detection by screening with total mouse DNA (see Chapter 3), but due to their complexity could not be used for mapping.

4.4.4. Microclone Probes Without Mus musculus - Mus spretus Restriction Fragment Length Variation

pmc 16:	206	0.15	kb	(see Figs.	4.3:A
	208	0.18	kb		4.3:B)

Microclones 206 and 208 were hybridised to Mus musculus - Mus spretus Taq I strips and produced identical (206) or similar (208) patterns on each DNA sample. Both probes hybridised to M. musculus DNA and M. spretus DNA with similar intensities between the species.

Microclone 208 does show variation between the two Mus species, but when this probe is hybridised to progeny filters it is extremely difficult to distinguish the two

patterns. The most intense bands (2.9 kb and 4.8 kb) are of similar sizes in both species. The interspecific Taq 1 strips were loaded with approximately 15 ug DNA per track, the progeny filters were loaded with 5 - 10 ug DNA per track and variant bands were easier to distinguish on the strips. 208 was hybridised to six different progeny filters (each of 20 - 25 progeny) and in no case could a definite assessment be made about the banding patterns in individual tracks. A major restriction fragment length variation must be present between the species in order to map microclone probes.

4.4.5. Unmapped Microclones

pmc 16:	14	0.14 kb	(Fig. 4.4.)
	91	0.12 kb	(not shown)
	117	0.12 kb	(not shown)

Microclones 14, 91 and 117 clearly showed variation between Mus musculus and Mus spretus (for example, see Fig. 4.4.), however the clones were extremely difficult to use, probably because of their small sizes. Autoradiographs had to be left for two weeks in order to see a signal, by which time the non-specific background was very high. It is possible these probes hybridise less well to the M. musculus DNA because of sequence variation between CD mice and the M. musculus stock used in the mapping crosses. However this is unlikely to be the case for all three probes; also the probes would be expected to hybridise even less well to M. spretus DNA, but they all give a signal of similar intensity with both Mus DNAs.

Figure 4.3. Autoradiographs resulting from hybridisation of non-variant microclone probes to Mus musculus and Mus spretus strips

4.3:A - 206
4.3:B - 208

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Band sizes are indicated in kb - in both cases sized fragments are in the 'M' lane.

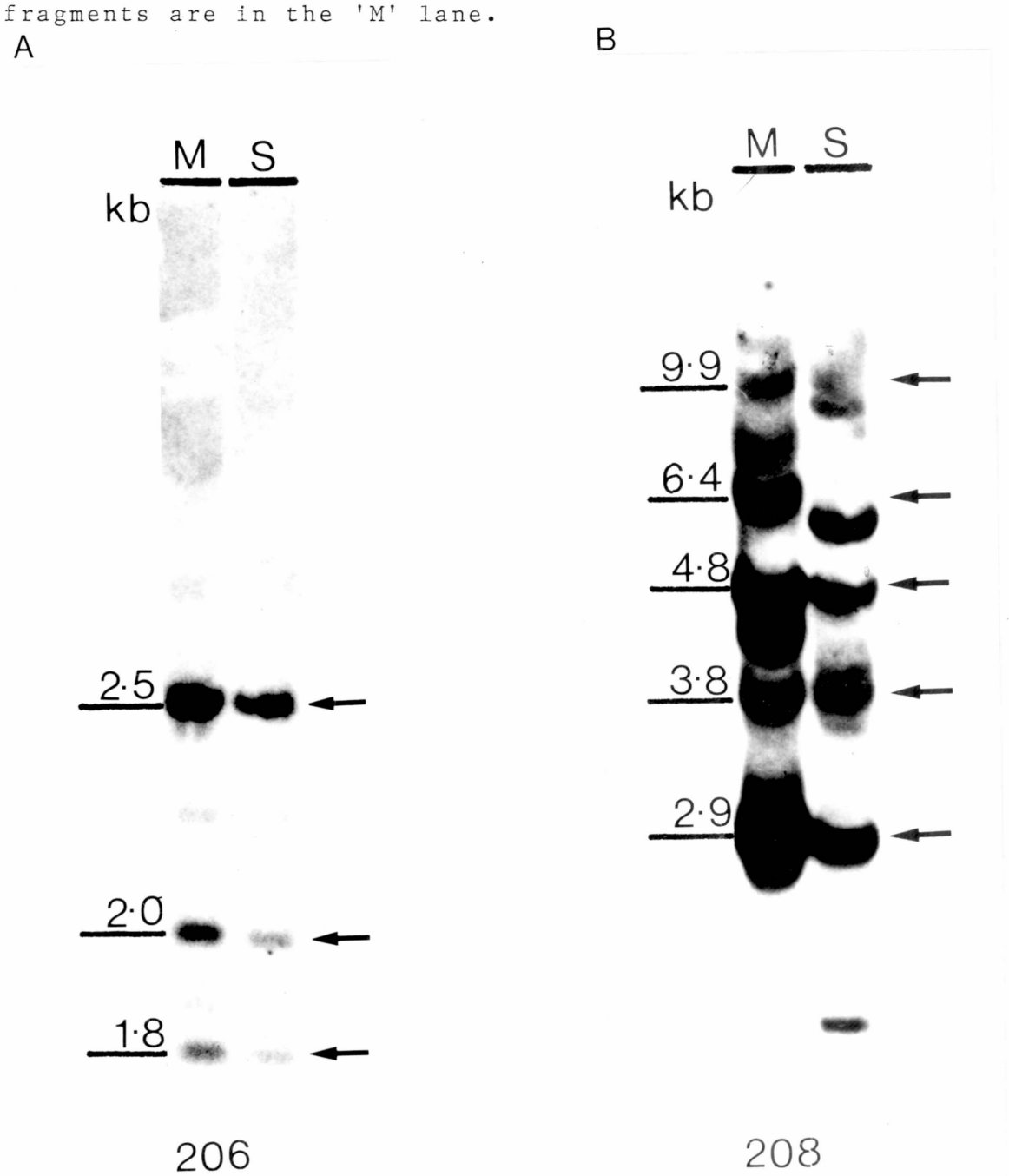
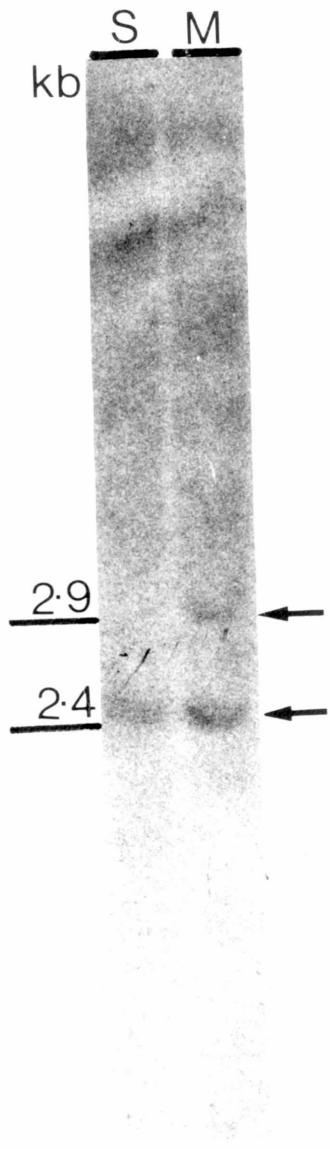


Figure 4.4. Autoradiograph resulting from hybridisation of an unmapped microclone probe, pmc 16: 14 to a Mus musculus and Mus spretus strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Band sizes are indicated in kb - sized fragments are in the 'M' lane.



4.4.6. Non X Specific Microclones

pmc 16: 56 0.22 kb
184 0.16 kb

Microclones 56 and 184 were hybridised to *Mus musculus* - *Mus spretus* *Taq* 1 strips. Microclone 56 hybridised to a *M. musculus* fragment of 2.8 kb and a *M. spretus* fragment of 2.5 kb. Microclone 184 hybridised to a *M. musculus* fragment of 4.3 kb and a *M. spretus* fragment of 2.3 kb.

Both probes were then hybridised to a progeny filter to check for X-specificity by following pattern segregation with that of the known coat mutation on the filter. In both cases the recombination fraction, θ', between the probes and the coat mutation was approximately 0.5, for over 40 C1 progeny; SM males were detected. These probes are probably autosomal sequences but could be sequences derived from a pseudoautosomal region of the mouse X chromosome - see on. Whilst this is extremely unlikely it is not possible to distinguish absolutely between these two cases with progeny filters. Cell hybrids would distinguish the results but were not available for testing with these probes.

4.4.7. Microclones Mapped to the Mouse X Chromosome

pmc 14:	36	0.4 kb
pmc 16:	120	0.19 kb
	141	1.2 kb
	219	0.65 kb
	222	0.14 kb
	225	0.18 kb

For each probe hybridisations were performed to establish:

(i) Mus musculus - Mus spretus banding pattern;

(ii) Parental banding pattern;

(iii) Mouse X chromosome specificity;

(iv) Map position;

Genomic copy number was estimated for 141.

Microclone pmc 14: 36

(i) Mus musculus - Mus spretus Banding Pattern

pmc 14: 36 is a microclone probe which hybridised to Taq 1 strips (of Taq 1 digested M. musculus DNA and M. spretus DNA) to give 3 intense bands on M. musculus DNA (of 5.2 kb, 3.4 kb and 2.8 kb), 2 of which (5.2 kb and 2.8 kb) had much less intense M. spretus equivalents (Fig. 4.5:A). The third band of 3.4 kb had no M. spretus equivalent. Autoradiograph signals came up extremely quickly compared to other microclone probes and although this probe may be greater than twice the length of other microclone probes, the signal intensity may indicate that pmc 14: 36 is repeated in the genome.

(ii) Parental Banding Patterns

pmc 14: 36 was hybridised to a panel of Taq 1 digests of the eight C2 parental mice (see Fig. 4.5:B).

Figure 4.5. Autoradiographs resulting from hybridisation of pmc 14: 36 to Mus genomic digests

Sizes are indicated in kb

A: Taq 1 strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Sized fragments are in the 'M' lane.

B: Parental DNAs - Taq 1 digests

'129.1h'- male C2 parent, exhibiting 'M' banding pattern
'a(Hq)' - female C2 parent, exhibiting 'SM' banding pattern

C: C2 Progeny DNAs - Taq 1 digests

'A7d' - 'A10a' - progeny DNAs, resulting from cross C2a(Hq). Progeny DNAs have been scored for the segregation of the 3.4 kb M. musculus X chromosome specific band (marked with an 'X').

On longer autoradiograph exposures the M. spretus 2.8 kb and 5.2 kb bands are visible.

A9e, A9d and A9h are showing an extra band, between the 3.4 and 2.8 kb fragments. These DNAs were digested in the same batch and this extra band, which is not displayed on any other autoradiographs, is possibly the result of a partial digest. See Fig. 4.6

Figure 4.5.

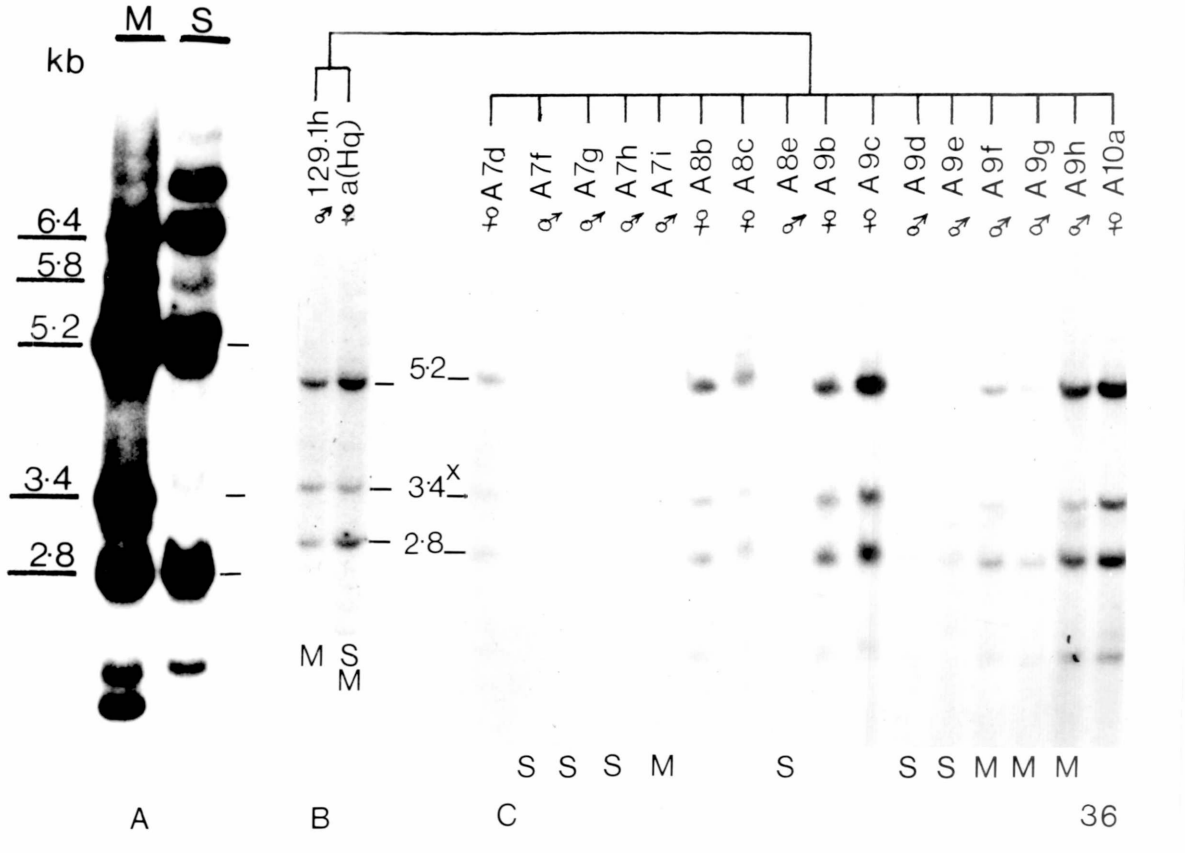

M S
kb
6·4
5·8
5·2
3·4
2·8
♂129.1h
♀a(Hq)
♀A7d
♂A7f
♂A7g
♂A7h
♂A7i
♀A8b
♀A8c
♂A8e
♀A9b
♀A9c
♂A9d
♂A9e
♂A9f
♂A9g
♂A9h
♀A10a
5·2
3·4X
2·8
M S
M
S S S M S S S M M M
A
B
C
36

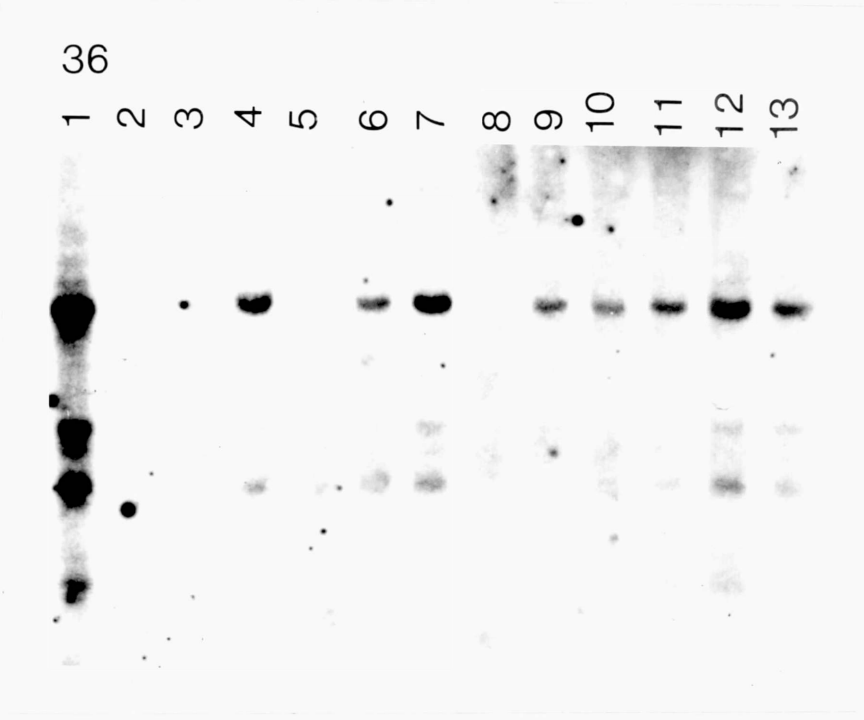
Figure 4.6. Autoradiograph resulting from hybridisation of pmc 14: 36 to a cell hybrid panel

Lane No.	TaqI Genomic Digest	Mouse Chromosomes Present																			Mouse X Chromosome
1	M. musculus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X	Yes
2	*E36																					No
3	*cM34	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X	Yes
4	*ma106												12								X	Yes
5	*EcmYe														14£	15						No
6	*TnCE126/7		2			5	6£		8£		10		12	13	14	15	16	17	18	19	X	Yes
7	*TnCE126/1					5	6£		8£				12	13	14		16	17		19	X	Yes
8	*YB31A73		2					7					12			15	16			19		No
9	*MACH2AC2	1	2	3	4			7	8	9	10		12	13		15	16	17	18	19	X	Yes
10	*mFE2/1/2		2	3	4		6	7	8	9	10		12	13	14	15		17	18	19	X	Yes
11	*mFE2/1/7	1	2	3			6	7	8	9			12	13		15		17		19	X	Yes
12	*mFE2/1/1	1	2	3	4		6	7	8	9	10		12	13		15		17	18	19	X	Yes
13	M. musculus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X	Yes

* Denotes DNAs provided by Dr. F. Ruddle
£ Denotes rearranged chromosome

E36 is a chinese hamster cell line
cM34 is a mouse sarcoma cell line

Figure 4.6. Autoradiograph resulting from hybridisation of pmc 14: 36 to a cell hybrid panel



Lane Number		TaqI Genomic Digest	Presence Mouse X Chromosome	Presence of 5.2, 3.4 and 2.8 kb bands
1		M. musculus	Yes	Yes
2	*	E36	No	No
3	*	cM34	Yes	Yes
4	*	mal06	Yes	Yes
5	*	EcmYe	No	No
6	*	TnCE126/7	Yes	Yes
7	*	TnCE126/1	Yes	Yes
8	*	YB31A73	No	No
9	*	MACH2AC2	Yes	Yes
10	*	mFE2/1/2	Yes	Yes
11	*	mFE2/1/7	Yes	Yes
12	*	mFE2/1/1	Yes	Yes
13		M. musculus	Yes	Yes

* denotes DNAs provided by Dr. F. Ruddle

The faint bands in lane 3 are clearly observable on the original autoradiograph.

Filters were scored for the presence (M) or absence (S) of the 3.4 kb band. The SM banding pattern and the M (or MM) banding patterns are indistinguishable (due to the presence of the 3.4 kb M. musculus band). Therefore the pattern of the four C2 parental females - which must be SM - was indistinguishable from the pattern displayed by the male C2 parental mice, which would be a M. musculus (M) pattern. The identical patterns of the C2 parental males and females did indicate there was no difference in banding pattern for pmc 14: 36 between the 129 inbred line and the C1 parental outbred M. musculus X chromosome DNA.

(iii) Mouse X Chromosome Specificity

pmc 14: 36 was hybridised to a panel of cell hybrids with different combinations of mouse chromosomes against a chinese hamster background (Fig. 4.6.). This panel was able to distinguish mouse X chromosome specific bands. All the bands of 36 are X chromosome specific. However the 5.2 kb and 2.8 kb bands cannot be mapped along the X chromosome because the M and S forms are invariant and it is impossible to score recombination between these two bands (or these bands and the 3.4 kb band).

(iv) Map Position

As it is not possible to distinguish between the female C2 progeny which are M. musculus homozygotes (MM) or M. musculus - M. spretus hybrids (SM) at the pmc 14: 36 locus, only male mice could be scored, according to whether they have an M or S pattern i.e. the presence or absence of the 3.4 kb fragment. Therefore progeny filters from Hq and Ta

crosses were scored for segregation of the S or M pattern in males (see Fig. 4.5:C) and the 3.4 kb fragment was mapped to the mouse X chromosome.

The minor less intense bands were not sufficiently distinct on progeny filters to score.

Cross: Harlequin
Total Number of Progeny Observed: 26
Total Number of Recombinants Observed: 2
Recombination Fraction: 0.077 +/- 0.052
Map units from Harlequin: 8 +/- 5 cM

Cross: Tabby
Total Number of Progeny Observed: 28
Total Number of Recombinants Observed: 7
Recombination Fraction: 0.250 +/- 0.082
Map units from Tabby: 25 +/- 8 cM

36 was positioned on the mouse X chromosome from the above recombination fractions, and from multipoint data with other mapped clones (see below), taking into account the minimum possible number of recombination events.

Approximate Map Position:

36

Hq Ta - = 2 cM

Microclone pmc 16: 120

(i) Mus musculus - Mus spretus Banding Pattern

pmc 16: 120 hybridised to Taq 1 strips to give a M. musculus band of 3.4 kb (M) and a M. spretus band of 4.0 kb (S), (see Fig. 4.7:A). The S band is marginally less intense than the M band. Autoradiograph signals were always faint on all filters, although 120 was a comparable size to other mapped microclone probes which gave very intense bands. 120 is probably a single copy unique sequence probe.

Figure 4.7. Autoradiographs resulting from hybridisation of pmc 16: 120 to Mus genomic digests

Sizes are indicated in kb

A: Taq 1 strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Sized fragments are in the 'M' lane.

B: Parental DNAs - Taq 1 digests

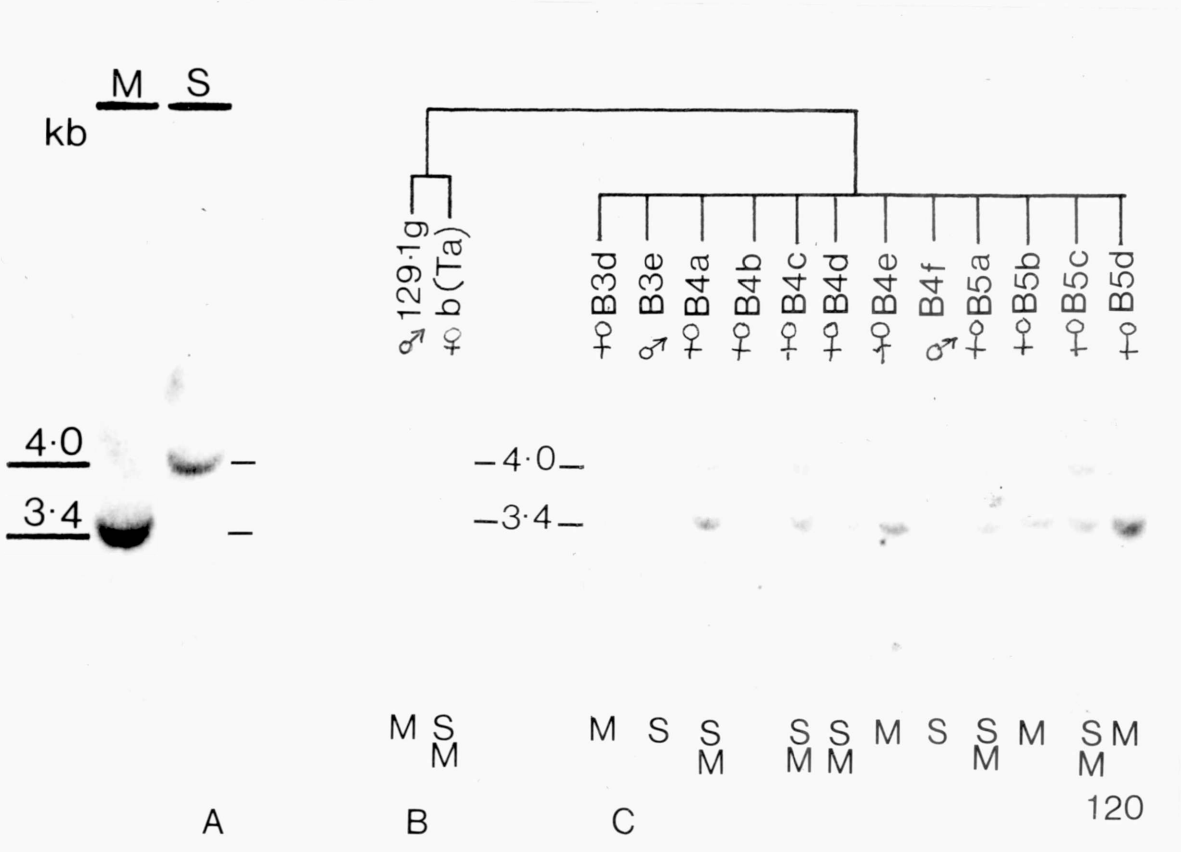
'129.1g'- male C2 parent, exhibiting 'M' banding pattern
'b(Ta)' - female C2 parent, exhibiting 'SM' banding pattern

C: C2 Progeny DNAs - Taq 1 digests

'B3d' - 'B5d' - progeny DNAs, resulting from cross C2b(Ta). Progeny DNAs have been scored for the segregation of the 3.4 kb M. musculus X chromosome specific band and the 4.0 kb M. spretus X chromosome specific band.

Too little DNA was present in lane B4b to be scored.

Figure 4.7.


M S
kb
4·0
3·4
♂129·1g
♀b(Ta)
♀B3d
♂B3e
♀B4a
♀B4b
♀B4c
♀B4d
♀B4e
♂B4f
♀B5a
♀B5b
♀B5c
♀B5d
–4·0–
–3·4–
M S M
M S S M S M S M M S S M M S M M
120
A
B
C

(ii) Parental Banding Pattern

120 was hybridised to the eight Taq 1 digested C2 parental DNAs (see Fig. 4.7:B), and the 3.4 kb M variant was clearly visible on all tracks. The fainter 4.0 kb S band was also visible, in the female tracks only. The identical M. musculus bands indicated no restriction fragment length variation between the two M. musculus chromosomes present in the C2 cross.

(iii) Mouse X Chromosome Specificity

pmc 16: 120 was hybridised to a progeny filter to check for X specificity. No SM males were observed and linkage of the 120 locus to the Ta locus indicated 120 was an X chromosome specific probe.

(iv) Map Position

Male and female C2 progeny from Hq and Ta crosses were scored for segregation of the 120 S and M variants (see Fig. 4.7:C).

Cross: Harlequin
Total Number of Progeny Observed: 24
Total Number of Recombinants Observed: 5
Recombination Fraction: 0.208 +/- 0.083
Map Units from Harlequin: 21 +/- 8

Cross: Tabby
Total Number of Progeny Observed: 48
Total Number of Recombinants Observed: 2
Recombination Fraction: 0.042 +/- 0.029
Map Units from Tabby: 4 +/- 3 cM

120 was positioned on the X chromosome by utilising the above Hq, Ta recombination fraction data, and multipoint data with other mapped clones, postulating the minimum number of crossovers. However, it has not been possible to

position 120 as being either proximal or distal to _Ta_.

Approximate Map Position:

```
                         120
---------------------------------------------
          Hq            Ta                      - = 2 cM
```

Microclone pmc 16: 141

_(i)_ _Mus musculus - Mus spretus Banding Pattern_

pmc 16: 141 hybridised to _Taq 1_ digests of _M. musculus_ DNA gives a complex series of bands (M) - 4 major intense bands (8.8 kb, 5.3 kb, 3.7 kb, and 2.6 kb) with a number of minor light bands. When hybridised to a _M. spretus_ DNA sample the 141 pattern is a faint doublet (7.1 and 6.9 kb) (S) (Fig. 4.8:A). The 141 _M. musculus_ pattern is more intense than any of the other microclones, and although this probe is considerably larger than the other probes, the intensity and the number of bands suggests this sequence may be repeated many times within the genome. The difference between the _M. musculus_ and _M. spretus_ banding patterns may be due to either a lack of homology between the _M. musculus_ sequence probe and the _M. spretus_ genomic sequence, or simply the presence of fewer 141 copies within the _M. spretus_ genome. Brown (Pers. comm.) has resolved this question by hybridising 141 to _M. spretus_ DNA at a considerably reduced stringency (3 x SSC, 50 ^{o}C); no further _M. spretus_ bands were observed or any increase in relative hybridisation, therefore there appear to be fewer 141 sequences within the _M. spretus_ genome compared to the _M. musculus_ genome.

Figure 4.8. Autoradiographs resulting from hybridisation of pmc 16: 141 to Mus genomic digests

Sizes are indicated in kb

A: Taq 1 strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Sized fragments are in the 'M' lane.

B: Parental DNAs - Taq 1 digests

'129.6f'- male C2 parent, exhibiting 'M' banding pattern
'd(Hq)' - female C2 parent, exhibiting 'SM' banding pattern

C: C2 Progeny DNAs - Taq 1 digests

'D1e' - 'D3e' - progeny DNAs, resulting from cross D2b(Hq). Progeny DNAs have been scored for the segregation of the set of M. musculus X chromosome specific bands and the M. spretus X chromosome specific doublet.

Lanes D2i and D3c contained sufficient DNA to be unequivocally scored on the original autoradiograph; lanes D2c and D3a did not.

In certain lanes (for example, male parent lane, 129.6f) the appearance of the 7.0 kb M. musculus band is faint, however it is clearly visible on the original autoradiographs.

Figure 4.8.

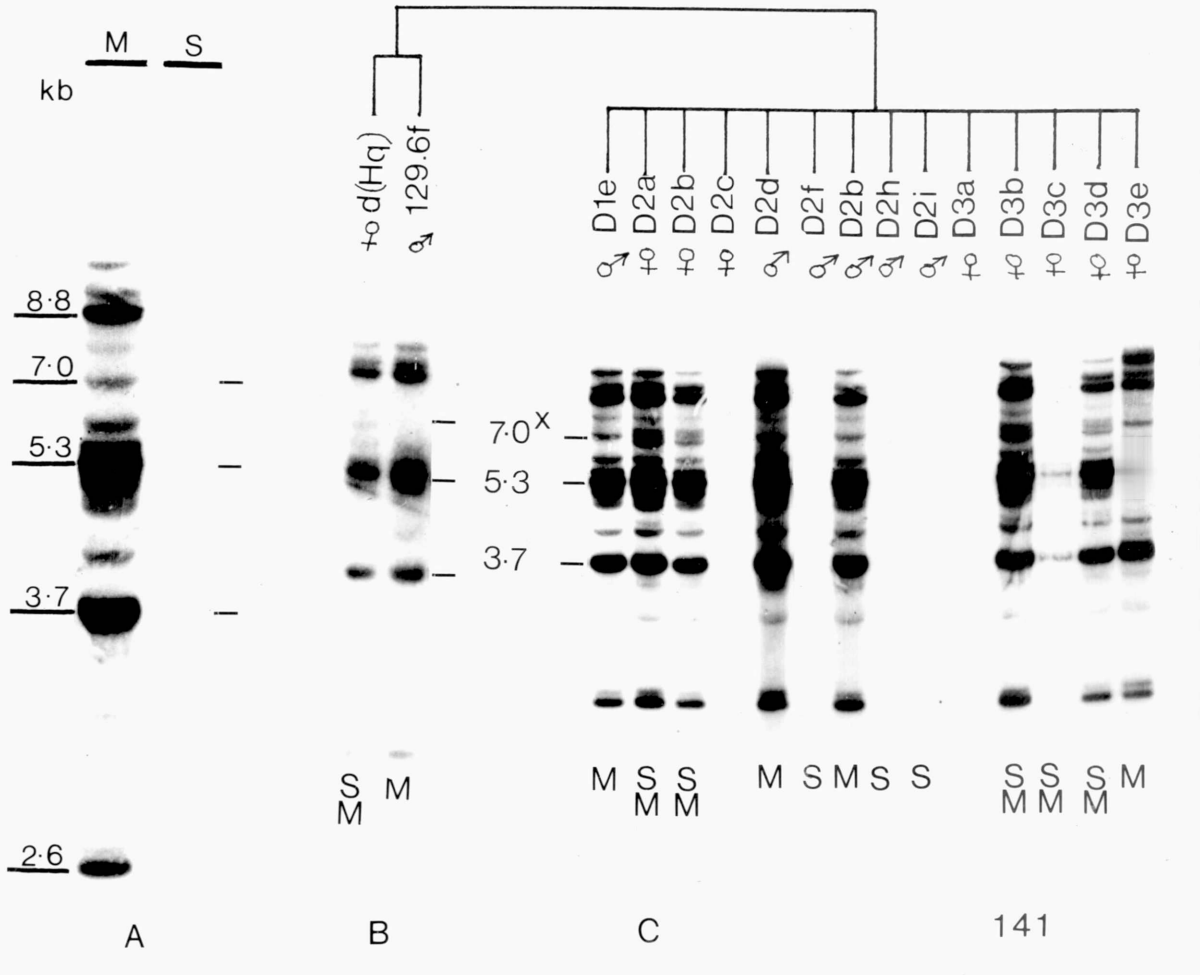
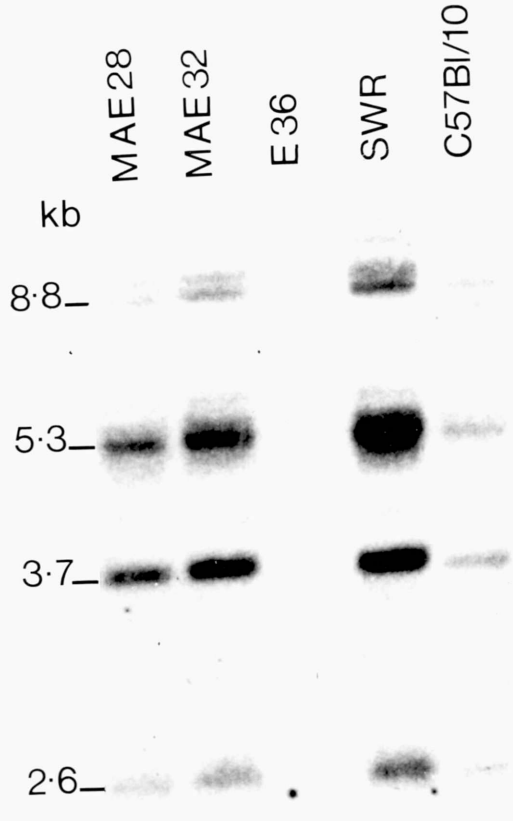

M
S
kb
8·8
7·0
5·3
3·7
2·6
A
♀ d(Hq)
♂ 129.6f
B
7·0 X
5·3
3·7
S M
M
C
♂ D1e
♀ D2a
♀ D2b
♀ D2c
♂ D2d
♂ D2f
♂ D2b
♂ D2h
♂ D2i
♀ D3a
♀ D3b
♀ D3c
♀ D3d
♀ D3e
M S M S M S S M S M S M
141

Figure 4.9. Autoradiographs resulting from hybridisation of pmc 16: 141 to X specificity testing panel

All sizes are in kb

MAE28 lane - Taq 1 genomic digest of MAE 28 cell hybrid DNA
MAE32 lane - Taq 1 genomic digest of MAE 32 cell hybrid DNA
E36 lane - Taq 1 genomic digest of chinese hamster parent cell line
SWR lane - Taq 1 genomic digest of SWR M. musculus inbred line DNA
C57BL/10 lane - Taq 1 genomic digest of C57B1/10 M. musculus inbred line DNA



(ii) Parental Banding Pattern

When hybridised to the Taq 1 C2 parental DNA panel (Fig. 4.8:B), the four female mice clearly displayed the SM pattern - the four intense M. musculus bands and the M. spretus doublet. The four males clearly displayed the M pattern; no polymorphism in any of the major or minor bands was observed between the two M. musculus X chromosomes on the parental filter (or progeny filters).

(iii) Mouse X Chromosome Specificity

pmc 16: 141 was hybridised to an X specificity testing panel (see Fig. 4.9.), all visible bands, major and minor were X chromosome specific, and later mapping analysis - scoring both male and female progeny - confirmed this result. No 141 homologous sequences were detected in the chinese hamster genome, (at a stringency of 1 x SSC, 65 ^{o}C). 141 was also hybridised three times to different Taq 1 digests of human DNA at a stringency of 6 x SSC at 50 ^{o}C and again no homologous sequences were detected.

(iv) Map Position

Male and female C2 progeny DNAs were scored for the segregation of the S and M patterns (Fig 4.8:C).

Cross: Harlequin
Total Number of Progeny Observed: 142
Total Number of Recombinants Observed: 13
Recombination Fraction: 0.092 +/- 0.024
Map Units from Harlequin: 9 +/- 2 cM

Cross: Tabby
Total Number of Progeny Observed: 63
Total Number of Recombinants Observed: 19
Recombination Fraction: 0.302 +/- 0.058
Map Units from Tabby: 30 +/- 6 cM

141 was positioned on the mouse X chromosome from the above data and using multipoint linkage analysis with the other mapped clones, taking into account the minimum possible number of crossover events.

Approximate Map Position:

$$\frac{141}{Hq \qquad\qquad Ta} - = 2\ cM$$

No recombination between fragments was observed in any of the 105 C2 progeny male DNAs i.e. all male progeny displayed either an S or M pattern. Therefore recombination between the fragments occurs with a frequency of less than 1 recombination per 100 meioses, and fragments are localised within a region spanning approximately 1 cM on the M. musculus and M. spretus X chromosomes. All 141 sequences appear to segregate as a single Mendelian locus.

(v) Copy Number

141 was hybridised to a panel of Eco Rl digests of C2 progeny DNA (Fig. 4.10.). The M. musculus variant pattern was of four intense bands of 7.0 kb, 3.5 kb, 1.5 kb and 1.2 kb and other less intense minor bands; the lower 1.2 kb band was most intense. The M. spretus Eco Rl variant pattern consisted of two faint bands in an upper doublet of 7.6 kb and 6.5 kb, and a less faint 1.2 kb band. Therefore 141 was definitely repeated within the M. musculus and M. spretus genomes.

The copy number of 141 within the M. musculus genome was determined by the electrophoresis and blotting of Eco Rl

digests of a known amount of male M. musculus DNA, and 141 subclone DNA. Varying amounts of Eco R1 digested 141 subclone DNA were loaded, equivalent to the amount of 141 sequence (in pg) in approximately 5 ug mouse DNA if 141 was present in 4 copies, 20 copies, 40 copies and so on. 141 Eco R1 fragment DNA was then oligolabelled and hybridised back to the filter (Fig. 4.11.). The autoradiograph was analysed by densitometry, giving curve integral values for each of the visible bands. A graph was drawn of curve integral against known 141 copy number. The graph displayed a linear relationship between these two parameters, at lower values. At higher copy numbers the relationship was non-linear, presumably because of the saturation of the autoradiograph film (not shown).

The male genomic M. musculus DNA displayed the four intense 141 bands and for each band a curve integral value was determined by densitometry. 141 copy numbers were determined for each of the four individual bands from the graph, see Fig. 4.11.

The haploid genomic M. musculus copy number of 141 was found to be approximately 32 copies. This value roughly agrees with the value independently derived by Brown (Brown, Pers. comm.) of 50 copies per haploid M. musculus genome, as determined by dot blots.

Figure 4.10. Autoradiograph resulting from hybridisation of pmc 16:141 to Eco R1 digested Mus DNAs

'M' lane - M. musculus 141 Eco R1 band pattern
'S' lane - M. spretus 141 Eco R1 band pattern

All sizes are in kb, sized fragments are in the 'M' lane.

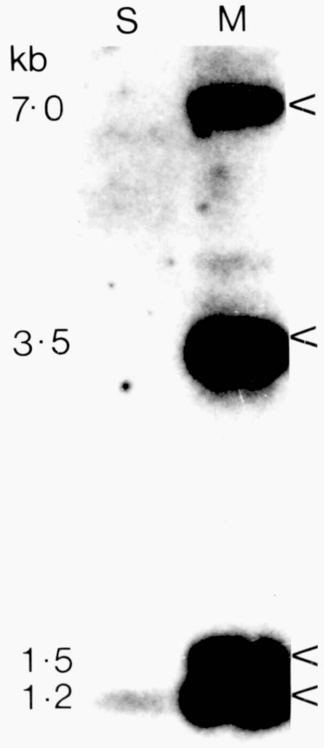


Figure 4.11:A Autoradiograph resulting from hybridisation of pmc 16:141 to 'copy number' filter

Lane	Digest	141 Copy Number
1	M. musculus (male)	2
2	141	4
3	141	20
4	141	40
5	141	(100)
6	141	(160)
7	141	(200)

Figure 4.11:B Graph of curve integral value against 141 copy number

Lane	DNA Sample	Curve Integral	141 Copy Number
2	141	4372	4
3	141	11600	20
4	141	17846	40

Extrapolated from graph:

1	M. musculus		
	7.0 kb band	3974	3
	3.5 kb band	5460	6
	1.5 kb band	4680	4
	1.2 kb band	10428	19
		Total	32

141 is present in the haploid M. musculus genome in approximately 32 copies.

Figure 4.11:A Autoradiograph resulting from hybridisation of pmc 16:141 to 'copy number' filter

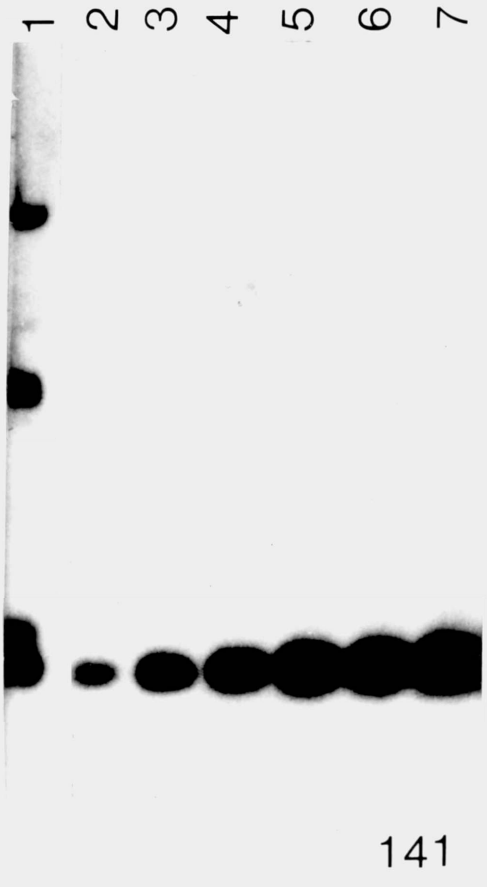
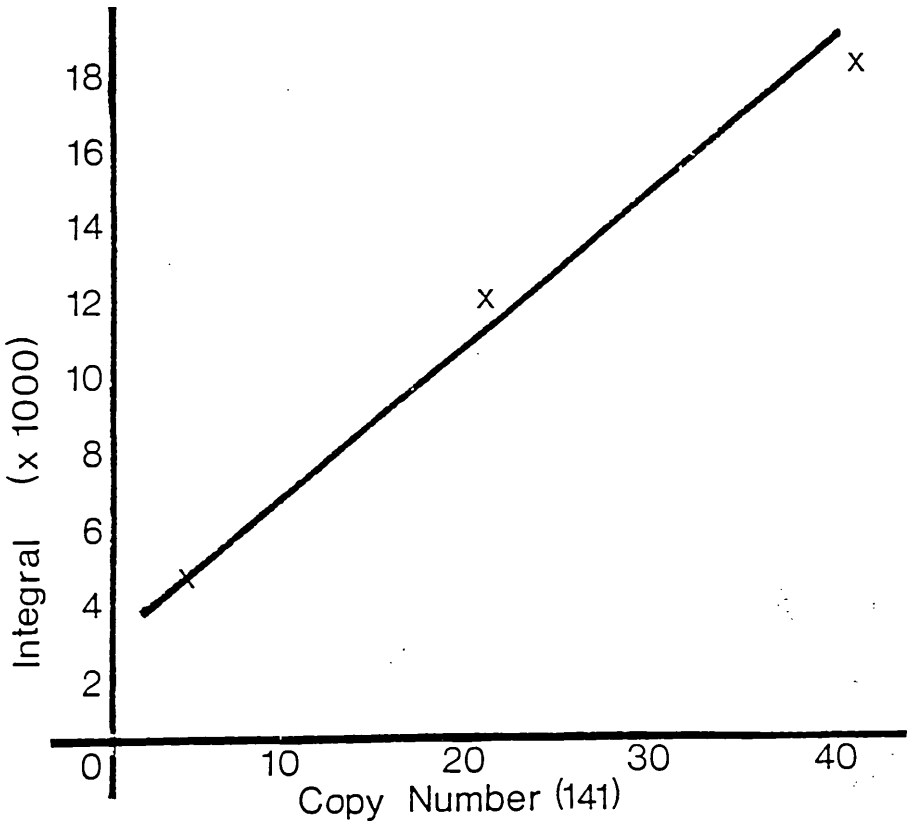


Figure 4.11:B Graph of curve integral value against 141 copy number



Microclone pmc 16: 219

(i) Mus musculus - Mus spretus Banding Pattern

When hybridised to M. musculus - M. spretus Taq 1 strips, pmc 16: 219 gives a 2.4 kb M band, and a 2.1 kb S band (see Fig. 4.12:A), the S band being slightly less intense than the M band. 219 also hybridised to a 5.1 kb fragment in both species.

219 was a relatively large probe, but autoradiograph signals were consistently slow to appear; possibly the 2.1 kb and 2.4 kb bands are single copy sequences and the faint 5.1 kb bands represent fragments with weak homology to 219, rather than fragments with less copies in the genome than the 2.1 kb and 2.4 kb fragments.

(ii) Parental Banding Pattern

On the C2 parental filters the four female DNAs displayed the expected SM patterns, and the four males displayed the M pattern only. No polymorphism was observed for the 2.4 kb band between the outbred and 129 inbred line M. musculus X chromosomes (Fig. 4.12:B).

(iii) Mouse X Chromosome Specificity

219 was hybridised to a C2 progeny filter to check for X chromosome specificity. Male and female mice were scored and linkage data indicated 2.1 kb and 2.4 kb fragments were X chromosome specific. No SM males were observed.

Two further faint bands of 9.0 kb and 6.5 kb were observed on progeny filters. It was not possible to score segregation of these bands because they were too faint, therefore their chromosomal localisation is unknown.

Figure 4.12. Autoradiographs resulting from hybridisation of pmc 16: 219 to Mus genomic digests

Sizes are indicated in kb

A: Taq 1 strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Sized fragments are in the 'M' lane.

B: Parental DNAs - Taq 1 digests

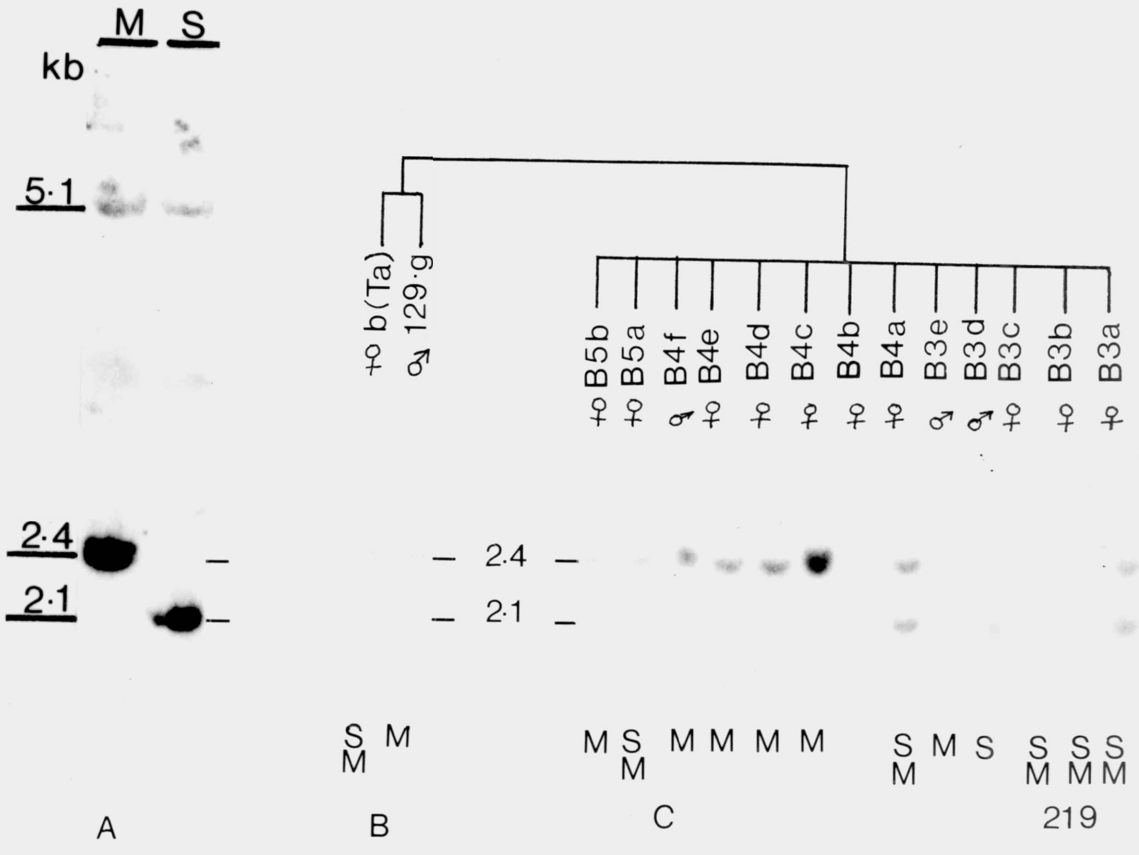
'129.1g'- male C2 parent, exhibiting 'M' banding pattern
'b(Ta)' - female C2 parent, exhibiting 'SM' banding pattern

C: C2 Progeny DNAs - Taq 1 digests

'B5b' - 'B3a' - progeny DNAs, resulting from cross B2b(Ta). Progeny DNAs have been scored for the segregation of the 2.4 kb M. musculus X chromosome specific band and the 2.1 kb M. spretus X chromosome specific band.

Lane B4b contained insufficient DNA to be scored.

Figure 4.12.


M S
kb
5·1
2·4
2·1
♀ b(Ta)
♂ 129·g
♀B5b
♀B5a
♀B4f
♂B4e
♀B4d
♀B4c
♀B4b
♀B4a
♂B3e
♂B3d
♀B3c
♀B3b
♀B3a
2·4
2·1
S M M
M S M M M M M
S M M S
S M S M S M
A
B
C
219

(iv) Map Position

Male and female progeny DNAs were scored for the segregation of the 2.4 kb (M) and 2.1 kb (S) bands (Fig. 4.12:C).

Cross: Harlequin
Total Number of Progeny Observed: 50
Total Number of Recombinants Observed: 1
Recombination Fraction: 0.020 +/- 0.020
Map Units from Harlequin: 2 +/- 2 cM

Cross: Tabby
Total Number of Progeny Observed: 46
Total Number of Recombinants Observed: 15
Recombination Fraction: 0.326 +/- 0.069
Map Units from Tabby: 33 +/- 7 cM

219 was positioned from the above data, multipoint data and analysis of the lowest number of possible crossovers.

Approximate Map Position:

```
          219
-------------------------------------------------
          Hq          Ta                    - = 2 cM
```

Microclone pmc 16: 222

(i) Mus musculus - Mus spretus Banding Pattern

pmc 16: 222 was hybridised to Taq 1 digests of M. musculus DNA to give one very intense band of 2.8 kb and a number of less intense bands (Fig. 4.13:A). The 2.8 kb band had a weak M. spretus counterpart, but none of the other M bands had S bands of the same size. M autoradiograph signals were strong, suggesting a difference in intensity due to increased numbers of 2.8 kb fragments in the M. musculus genome (compared to the M. spretus genome). Alternatively there may also be differences in homology between this and other bands.

Figure 4.13. Autoradiographs resulting from hybridisation of pmc 16: 222 to Mus genomic digests

Sizes are indicated in kb

A: Taq 1 strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Sized fragments are in the 'M' lane.

B: Parental DNAs - Taq 1 digests

'129.1h'- male C2 parent, exhibiting 'M' banding pattern
'a(Hq)' - female C2 parent, exhibiting 'SM' banding pattern

C: C2 Progeny DNAs - Taq 1 digests

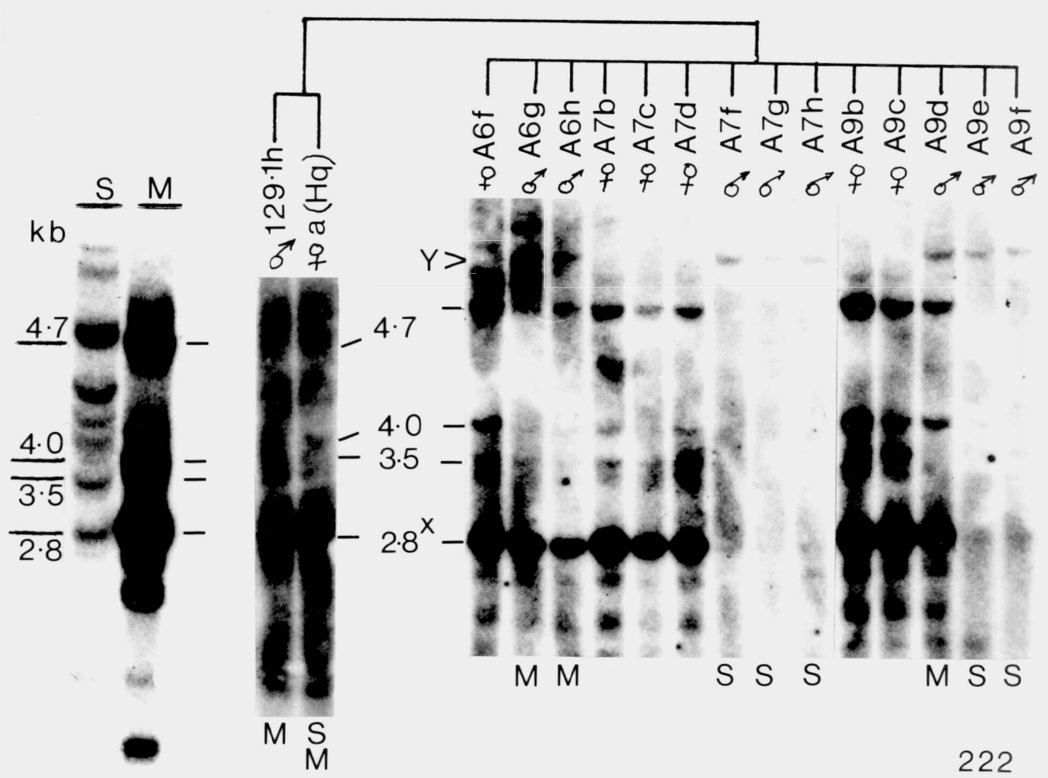
'A6f' - 'A9f' - progeny DNAs, resulting from cross A2b(Hq). Progeny DNAs have been scored for the segregation of the 2.8 kb M. musculus X chromosome specific band, marked 'X'.

The 4.0 kb band and the 4.7 kb band are both M. musculus specific and are segregating with the 2.8 kb band, and no exceptions have been found to this observation. The hybridisation of the 4.0 kb band (for example, see progeny lane A7c) is rather variable on this filter.

The 3.5 kb band (M. musculus specific) appears to be more loosely linked to the 2.8 kb band. Out of the total number of mice scored for segregation of 222, one recombination event was observed between the 3.5 kb band, and the 4.7kb, 4.0 kb and 2.8 kb bands - see mouse A9d.

The 5.4 kb band is segregating only with the M. musculus Y chromosome; this band is marked 'Y' on the photograph.

Figure 4.13.


S M
kb
4·7
4·0
3·5
2·8
♂129·1h
♀a(Hq)
4·7
4·0
3·5
2·8X
Y
♀A6f
♂A6g
♂A6h
♀A7b
♀A7c
♀A7d
♂A7f
♂A7g
♂A7h
♀A9b
♀A9c
♂A9d
♂A9e
♂A9f
M S M
M M S S S M S S
222
A
B
C

(ii) Parental Banding Pattern

222 was hybridised to a C2 parental DNA filter (Fig. 4.13:B). The 2.8 kb band was observed in all parental mice - indicating no observable polymorphism between the two M. musculus X chromosomes present on the filter. The other less intense M and S bands were difficult to observe.

(iii) Mouse X Chromosome Specificity

On a C2 progeny filter the segregation of 222 could only be scored in males, as MM females were indistinguishable from SM females, therefore males were scored for the presence (M) or 'absence' (S) of the 2.8 kb band. The 2.8 kb band was linked to the Harlequin locus; therefore the 2.8 kb band is X chromosome specific. Although the hybridisation of the 4.0 kb band was variable in some mice, this band and the 4.7 kb band were observed in C2 male progeny to segregate only with the 2.8 kb M. musculus fragment. Therefore the 4.7 kb and 4.0 kb bands are X chromosome specific and are linked to the 2.8 kb fragment. The 3.5 kb band segregated in a similar manner, but a recombination event was observed in mouse A9d between this band and the 4.7 kb, 4.0 kb and 2.8 kb bands, indicating a more distant X linkage. The 4.0 kb and 3.5 kb bands were never observed when the M. spretus X chromosome was present alone, and even on long exposures (1 week) the M. spretus equivalents of the M. musculus 4.7 kb and 2.8 kb bands were very faint, and clearly not of the 'M' type.

As no recombination has been observed between the 4.7 kb or 4.0 kb bands themselves or the 2.8 kb fragment throughout

47 meioses, all these bands are localised to a region spanning less than or equal to 2 cM.

A 5.4 kb band was observed to be segregating with male progeny only, and therefore represents a M. musculus Y chromosome specific locus with homology to 222. This band was observed on all progeny filters and was not detected on the original M. musculus - M. spretus Taq 1 strip, because these were made with female mouse DNA.

(iv) Map Position

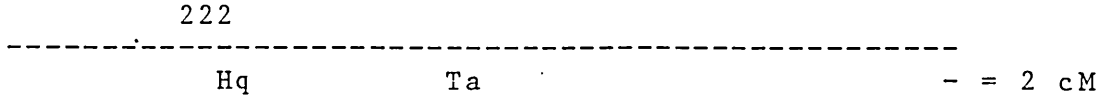
Male progeny only were scored for coat mutation and the presence (M) or absence (S) of the 2.8 kb band (Fig. 4.13:C).

Cross: Harlequin
Total Number of Progeny Observed: 26
Total Number of Recombinants Observed: 1
Recombination Fraction: 0.038 +/- 0.038
Map Units from Harlequin: 4 +/- 4cM

Cross: Tabby
Total Number of Progeny Observed: 22
Total Number of Recombinants Observed: 8
Recombination Fraction: 0.364 +/- 0.103
Map Units from Tabby: 36 +/- 10 cM

222 was positioned on the mouse X chromosome from the above data and use of multipoint data from other mapped microclones, taking into account the lowest possible number of recombinations.

Approximate Map Position:



Microclone pmc 16: 225

(i) Mus musculus - Mus spretus Banding Pattern

pmc 16: 225 hybridises to M. musculus Taq 1 digested DNA to give one very intense band of 4.6 kb, and at lease 4 fainter bands. 225 hybridises to a M. spretus fragment, slightly larger than the 4.6 kb fragment and less intense; it also hybridises to other faint M. spretus bands. (Fig. 4.14:A). 225 very quickly gave an intense signal for the 4.6 kb band, suggesting this band may be repeated in the genome a number of times, and the faint bands may represent sequences present in fewer copies, or with less homology to 225.

(ii) Parental Banding Pattern

When 225 was hybridised to the C2 parental filter the resulting autoradiograph displayed the M. musculus banding pattern for all parental mice (Fig. 4.14:B). No M. musculus polymorphism was observed. The M. spretus pattern was too faint to be visible in female mice.

(iii) Mouse X Chromosome Specificity

The difference in intensity between the M 4.6 kb band and its nearest M. spretus equivalent was sufficient to use as a scorable variation. Therefore progeny were scored for M - presence of the 4.6 kb band, or S - 'absence' of the M. spretus band. In longer exposures the larger M. musculus bands were clearly visible, as were the M. spretus bands - adding confirmatory data to the scoring of the 4.6 kb band by presence or 'absence'. However only male progeny were scored because of the difficulty of distinguishing MM and SM females.

Figure 4.14. Autoradiographs resulting from hybridisation of pmc 16: 225 to Mus genomic digests

Sizes are indicated in kb

A: Taq 1 strip

'M' lane - Taq 1 genomic digest of M. musculus DNA
'S' lane - Taq 1 genomic digest of M. spretus DNA

Sized fragments are in the 'M' lane.

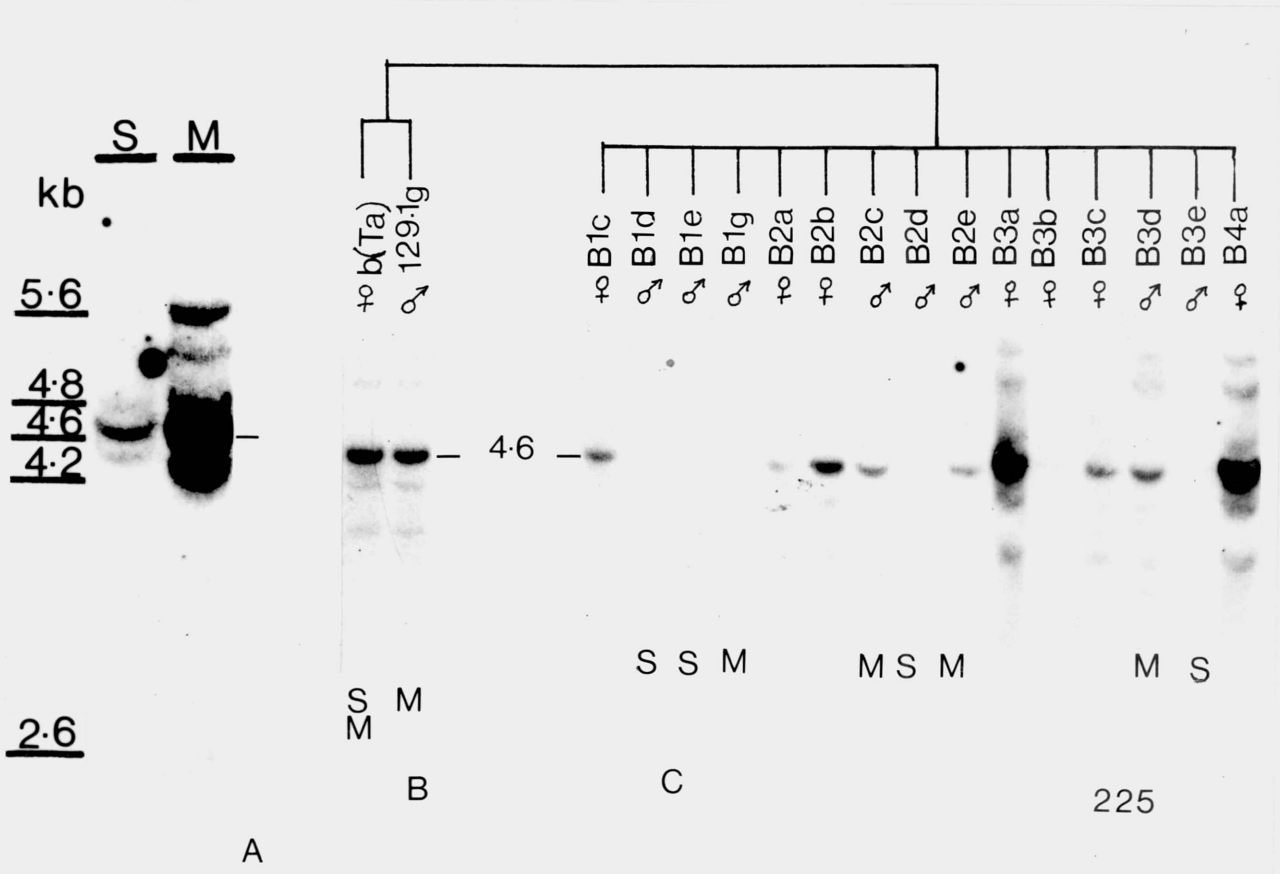
B: Parental DNAs - Taq 1 digests

'129.1g'- male C2 parent, exhibiting 'M' banding pattern
'b(Ta)' - female C2 parent, exhibiting 'SM' banding pattern

C: C2 Progeny DNAs - Taq 1 digests

'B1c' - 'B4a' - progeny DNAs, resulting from cross B2b(Ta). Male progeny DNAs have been scored for the segregation of the 4.6 kb M. musculus X chromosome specific band, marked 'X'.

Figure 4.14.


S M
kb
5·6
4·8
4·6
4·2
2·6
A
♀ b(Ta)
♂ 129·1g
♀ B1c
♂ B1d
♂ B1e
♂ B1g
♀ B2a
♀ B2b
♂ B2c
♂ B2d
♂ B2e
♀ B3a
♀ B3b
♀ B3c
♂ B3d
♂ B3e
♀ B4a
4·6
S M
M
B
S S M
C
M S M
M S
225

When hybridised to a C2 progeny filter, the 4.6 kb band was shown by linkage to be X chromosome specific. The other faint M. musculus bands of 5.6 kb, 4.8 kb, 4.2 kb and 2.6 kb all segregated with the 4.6 kb fragment. No recombination was observed in 42 males, suggesting all these sequences are localised to a region of the X chromosome of approximately 2 cM.

(iv) Map Position

Males only were scored for presence (M) or absence(S) of the 4.6 kb band (Fig. 4.14:C).

Cross: Harlequin
Total Number of Progeny Observed: 26
Total Number of Recombinants Observed: 12
Recombination Fraction: 0.462 +/- 0.098
Map Units from Harlequin: 46 +/- 10 cM

Cross: Tabby
Total Number of Progeny Observed: 19
Total Number of Recombinants Observed: 4
Recombination Fraction: 0.211 +/- 0.094
Map Units from Tabby: 21 +/- 9

225 was positioned from the above data and from multipoint data with other clones, taking into account the minimum possible number of crossovers.

Approximate Map Position:

$$\text{Hq} \quad \overset{225}{\text{---------}} \quad \text{Ta} \quad - = 2 \text{ cM}$$

Table 4.4

X Specific Microclone Mapping Data

Distances (in cM) between known loci and microclones.

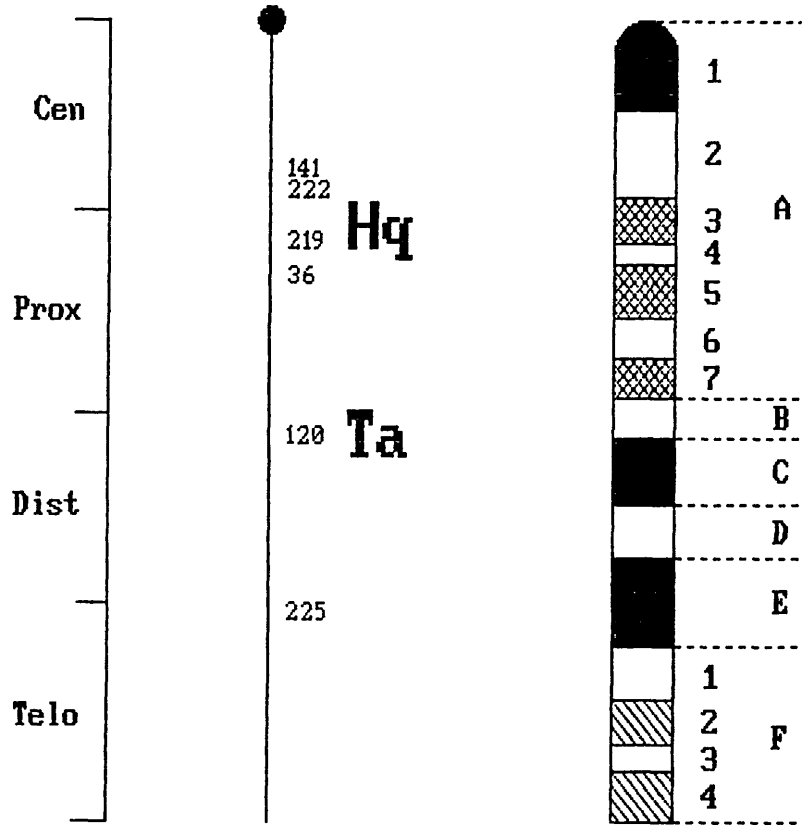
Standard errors in brackets beneath.

Microclone	141	222	Hq	219	36	120	Ta	225
141	-	2 (2)	9 (2)	13 (4)	9 (4)	32 (6)	30 (6)	53 (7)
222	2 (2)	-	4 (4)	7 (4)	11 (5)	39 (9)	36 (10)	53 (7)
Hq	9 (2)	4 (4)	-	2 (2)	8 (5)	21 (8)	21	46 (10)
219	13 (4)	7 (4)	2 (2)	-	5 (3)	30 (6)	33 (7)	48 (8)
36	9 (4)	11 (5)	8 (5)	5 (3)	-	30 (9)	25 (8)	43 (8)
120	32 (6)	39 (9)	21 (8)	30 (6)	30 (9)	-	4 (3)	19 (8)
Ta	30 (6)	36 (10)	21	33 (7)	25 (8)	4 (3)	-	21 (9)
225	53 (7)	53 (7)	46 (10)	48 (8)	43 (8)	19 (8)	21 (9)	-

See Fig 4.15 in which the 6 microclone probes are positioned on the mouse X chromosome.

Multipoint linkage recombination data and analysis of the crossover events displayed in mouse C2. progeny aided precise positioning of microclones on the mouse X chromosome.

Figure 4.15. Diagram of the mouse X chromosome and mapped X chromosome specific microclones



The microclones are shown in their approximate positions in relation to Hq and Ta on the genetic map of the mouse X chromosome. A diagram of a giemsa banded chromosome is adjacent (Searle and Beechey, 1981).

Table 4.5

Individual Mouse Mapping Data

Parental Cross	Mouse Progeny	Sex	Pheno -type	Scored Microclone Variant 141	222	219	36	120	225
C2: a(Hq)	A 1a	F	Hq	MM					
	A 1b	F	Hq	MM		MM			
	A 1c	M	+	S					
	A 1d	M	Hq	M	M	M	M	M	M
	A 2a	F	Hq	MM					
	A 2b	F	Hq	MM					
	A 2c	M	+	S	S	S	S		S
	A 2d	M	+	S	S	S	S		S
	A 3a	F	Hq	MM					
	A 3b	F	Hq	MM		MM			
	A 3c	F	Hq	MM		MM		MM	
	A 3d	M	Hq	M	M	M	M		S
	A 3e	M	Hq	M	M	M	S		S
	A 3f	M	+	S	S	S	S		S
	A 3g	M	+	S	S	S	S		S
	A 3h	M	+	S	S	S	S		S
	A 4a	F	+	MM		SM		SM	
	A 4b	F	+	SM					
	A 4c	F	+	MM		SM			
	A 4d	F	+	MM		SM			
	A 4e	M	Hq	M	M	M	M		S
	A 4f	M	+	S	S	S	S		M
	A 4g	M	+	S	S		S		M
	A 5a	F	+	MM		SM			
	A 5b	F	+	MM		SM			
	A 5c	F	+	MM		SM			
	A 5d	F	+	MM		SM			
	A 5e	M	+	S	S	S	S		S
	A 5f	M	+	S	S	S	S		S
	A 5g	M	Hq	M	M	M	M		M
	A 6a	F	Hq	MM		MM		MM	
	A 6b	F	Hq	MM		MM		MM	
	A 6c	F	Hq	MM		MM		MM	
	A 6d	F	Hq	MM		MM		MM	
	A 6e	F	Hq	MM		MM		MM	
	A 6f	F	Hq	MM		MM		MM	
	A 6g	M	Hq	M	M	M	M	S	S
	A 6h	M	Hq	M	M	M	M	M	M
	A 7b	F	Hq	MM		MM		MM	
	A 7c	F	+	SM		SM		MM	
	A 7d	F	Hq	MM		MM		MM	
	A 7f	M	+	S	S	S	S	M	M
	A 7g	M	+	S	S	S	S		S
	A 7h	M	+	S	S	S	S		M
	A 7i	M	Hq	M	M	M	M		S

Parental Cross	Mouse Progeny	Sex	Pheno -type	Scored Microclone Variant 141	222	219	36	120	225
	A 8a	F	Hq	SM					
	A 8b	F	Hq	MM		SM		MM	
	A 8c	F	+	SM		SM			
	A 8d	M	+	S					
	A 8e	M	+	S	S	S	S		S
	A 8f	M	+	S					
	A 8g	M	+	S					
	A 9b	F	Hq	MM		MM		MM	
	A 9c	F	+	SM		SM		MM	
	A 9d	M	+	M	M	S	S	S	S
	A 9e	M	+	S	S	S	S	S	S
	A 9f	M	+	S	S	S	M	M	M
	A 9g	M	Hq	M	M	M	M		S
	A 9h	M	Hq	M	M	M	M	M	S
	A 10a	F	Hq	MM		MM		MM	
	A 10b	F	+	SM		SM		SM	
	A 10c	F	Hq	MM					
	A 10d	M	+	S					
	A 10e	F	Hq	MM					
	A 10f	M	+	S					
	A 10g	M	Hq	M					
	A 11a	F	Hq	MM					
	A 11b	F	Hq	MM					
	A 11c	F	+	MM					
	A 11d	F	+	SM					
	A 11e	M	Hq	M					
	A 11f	M	+	S					
	A 11g	M	+	S					
	A 12b	F	+	MM					
	A 12c	F	Hq	MM					
	A 12d	M	+	M					
C2: B(Ta)	B 1a	F	+	MM					
	B 1b	F	Ta	MM		MM		MM	
	B 1c	F	+	SM		SM		SM	
	B 1d	M	+	S	S	S	S	S	S
	B 1e	M	+	M	M	M	M	S	S
	B 1g	M	Ta	S	S	S		M	M
	B 2a	F	+	MM		MM		SM	
	B 2b	F	Ta	MM		MM		MM	
	B 2c	M	+	S	S	S	S	S	M
	B 2d	M	Ta	M	M	M	M	M	S
	B 2e	M	Ta	S	S	S	S	M	M
	B 3a	F	+	MM		SM		SM	
	B 3b	F	+	SM		SM		SM	
	B 3c	F	Ta	SM		SM		MM	

Parental Cross	Mouse Progeny		Sex	Pheno -type	Scored Microclone Variant 141	222	219	36	120	225
	B	3d	M	Ta	S	S	S	S	M	M
	B	3e	M	+	M	M	M	M	S	S
	B	4a	F	+	SM		SM		SM	
	B	4c	F	+			MM		SM	
	B	4d	F	+	MM		MM		SM	
	B	4e	F	Ta	MM		MM		MM	
	B	4f	M	+	S	S	M	M	S	
	B	5a	F	+	SM		SM		SM	
	B	5b	F	Ta	MM		MM		MM	
	B	5c	F	+	MM		MM		SM	
	B	5d	F	Ta	MM		MM		MM	
	B	5e	M	+	S	S	S	S	S	S
	B	5f	M	+	S	S	S	S	S	S
	B	5g	F	+	SM		SM		SM	
	B	5h	M	Ta	M	M	M	M	M	M
	B	5i	M	Ta	M	S				
C2: C(Ta)	C	1a	F	+	MM				MM	
	C	1b	M	+	S	S				
	C	2a	F	Ta	SM					
	C	2c	M	+	M	M	S	S	S	S
	C	2d	M	+	S	S	S	S	S	S
	C	3a	F	+	SM		SM		SM	
	C	3b	F	+	SM		SM		SM	
	C	3c	F	Ta	MM		MM		MM	
	C	4a	F	Ta			SM		MM	
	C	4b	F	+			SM		SM	
	C	4c	F	+			SM		SM	
	C	4e	M	+	M	M	M	M	S	S
	C	4f	M	+	S					
	C	4g	M	+	S	S	S	S	S	M
	C	4i	M	Ta	M	M	M	M	M	S
	C	5a	F	+	SM				MM	
	C	5c	M	+	S	S	S	S	S	S
	C	5d	M	+	S	S	S	S	S	S
	C	5e	M	Ta	M	M	M	M	M	M
	C	6a	F	Ta	SM		SM		MM	
	C	6b	F	Ta	SM		SM		MM	
	C	6c	F	Ta	MM		MM		MM	
	C	6d	F	Ta	MM		MM		MM	
	C	6e	M	Ta	M			M		
	C	6f	M	Ta	M			M		
	C	7a	F	+	SM					
	C	7b	F	+	SM					
	C	7c	M	+	S			S		
	C	7d	M	+	M					

Parental Cross	Mouse Progeny	Sex	Pheno -type	Scored Microclone Variant 141	222	219	36	120	225
	C 7e	M	Ta	M			M		
	C 9a	F	+	SM					
	C 10a	F	Ta	MM					
	C 10c	M	Ta	M			M		
	C 10d	M	Ta	M			M		
	C 10e	M	+	S			S		
	C 10f	M	Ta	S			S		
	C 10g	M	+	S			S		
C2: D(Hq)	D 1b	F	+	SM					
	D 1c	F	+	SM					
	D 1e	M	Hq	M					
	D 2a	F	+	SM					
	D 2b	F	+	SM					
	D 2c	F	+	SM					
	D 2d	M	Hq	M					
	D 2e	M	+	S					
	D 2f	M	+	S					
	D 2g	M	Hq	M					
	D 2h	M	+	S					
	D 2i	M	+	S					
	D 3a	F	+	SM					
	D 3b	F	+	SM					
	D 3c	F	+	SM					
	D 3d	F	+	SM					
	D 3e	F	Hq	MM					
	D 3g	M	Hq	M					
	D 3h	M	Hq	M					
	D 4a	F	Hq	MM					
	D 4b	F	Hq	MM					
	D 4c	F	Hq	MM					
	D 4d	F	Hq	MM					
	D 4e	F	Hq	MM					
	D 4f	F	+	SM					
	D 4h	F	+	SM					
	D 4i	M	+	S					
	D 4j	M	+	S					
	D 4k	M	Hq	M					
	D 5a	F	Hq	MM					
	D 5b	F	Hq	MM					
	D 5c	M	+	S					
	D 5d	M	+	S					
	D 5e	M	Hq	M					
	D 6a	F	+	SM					
	D 6b	M	Hq	M					
	D 6c	M	Hq	M					

Parental Cross	Mouse Progeny	Sex	Pheno -type	Scored Microclone Variant 141	222	219	36	120	225
	D 6d	M	+	S					
	D 6e	M	+	S					
	D 6f	M	+	S					
	D 7b	F	+	SM					
	D 7c	M	+	S					
	D 7d	M	Hq	M					
	D 7e	M	+	S					
	D 7f	M	Hq	M					
	D 7g	M	Hq	M					
	D 7h	M	+	S					
	D 7i	M	Hq	M					
	D 8a	F	+	MM					
	D 8b	F	+	SM					
	D 8c	F	+	SM					
	D 8d	M	+	S					
	D 8e	M	Hq	M					
	D 8f	M	Hq	M					
	D 9a	F	+	SM					
	D 9d	F	+	SM					
	D 9e	M	+	S					
	D 9g	M	Hq	M					
	D 9h	M	Hq	M					
	D 9i	M	Hq	M					
	D 10a	F	Hq	SM					
	D 10b	F	Hq	MM					
	D 10c	F	+	SM					
	D 10d	F	+	SM					
	D 10e	F	+	SM					
	D 10f	F	+	SM					

	141	222	219	36	120	225
Total number scored Hq (crosses C2a(Hq),C2d(Hq))	142	26	50	26	24	26
Total number scored Ta (crosses C2b(Ta),C2c(Ta))	63	22	46	28	48	19
Total number scored males	105	48	45	54	28	45
Total number scored females	100	0	51	0	44	0
Total number scored progeny	205	48	96	54	72	45

Scored Mouse Progeny Results

The above table (Table 4.6) shows the results of mapping 6 X chromosome specific microclones to various C2 progeny mice. From these results 45 male mice were scored for 5 or 6 of the X specific microclones. Of these mice, 1 was a double recombinant (B 4f); 23 presented one crossover and 21 maintained either a M. musculus or a M. spretus chromosome between the scored loci (5 M. musculus; 16 M. spretus). One female double recombinant (A 8b) was observed, (females were only scored for 3 loci (120, 141 and 219)).

4.5 Discussion

Twenty-two microclones were individually analysed for a variety of criteria, and 11 of these microclones were selected for potential mapping to the mouse X chromosome. 6 microclones were ultimately mapped along the mouse X chromosome, 1 from pmc 14 and 5 from pmc 16.

The 22 microclones were not chosen at random, but were preselected (mainly for large insert size), so it is not possible to estimate the percentage of X specific sequences within the microclone banks. However, of the 7 pmc 16 microclones which fulfilled all the criteria for mapping (low copy number, interspecies variation, etc.) 5 clones (71 %) were X chromosome specific and 2 (29 %) appeared to be autosomal sequences.

Of the low copy microclones, only 2 out of 13 (15 %) were not sufficiently variant to be useful when hybridised to *Taq 1* digests of *M. musculus* and *M. spretus* DNA. This is an extremely high level of variation and demonstrates the power and advantage of using interspecific DNA restriction fragment length variation rather than allelic gene systems for chromosome mapping.

Individual Microclone Characterisation

Three microclones did not hybridise to genomic mouse DNA, these were the larger microclones from pmc 16. A set of large microclones containing non-mouse inserts had been reported from previous microdissections (Rohme *et al*, 1984). These inserts are probably derived from bacterial DNA

present on the cover slips used during the microdissection and microcloning.

Six microclones chosen for further analysis contained sequences too highly repeated to be mapped. The initial screening with total mouse DNA had not detected these microclones although microclones such as 198 appear to be relatively highly repeated. Probe size *per se* is presumably not responsible for the lack of detection by total mouse DNA, because total mouse DNA hybridised to some probes with very small inserts. Therefore the insensitivity of detection by this method may be due to a combination of insert size and repetitivity.

Two probes were non-polymorphic between *M. musculus* and *M. spretus* DNA. Avner (Pers. comm.) found that X chromosome specific probes which were non-variant between *M. musculus* and *M. spretus* DNA tended to be from coding regions. It was not possible to elucidate the chromosome specificity of pmc 16: 206 or 208 (cell hybrid panels were not available) or map position (progeny filters would have been uninformative for non-variant probes). Both microclones may have shown suitable interspecific variation with another restriction enzyme.

Three of the microclones were low copy and variant between *M. musculus* and *M. spretus*, but were not mapped because of the difficulty of detecting hybridisation. The microclone bank is small on average, and has the advantage of diminishing the number of microclones that contain repeat sequences, however it has the disadvantage of the difficulty

of using small probes. These probes could be analysed by, for example, ligating a number of inserts of individual microclones into one vector, so creating a larger probe, and aiding probe network formation.

Two of the probes gave an autosomal pattern of inheritance when hybridised to progeny filters. It is most likely that these probes are truly autosomal, but the possibility that they are derived from a pseudoautosomal region of the mouse X chromosome cannot be excluded. In human male meiosis (Rouyer et al, 1986) the telomere of the X chromosome has a gradient of recombination culminating in recombination frequencies of 50 % at the tip of the telomere. Data from the Sxr mutation in mice (Singh and Jones, 1982) (in which a copy of the testis determining region - normally positioned in the proximal half of the Y chromosome - is present at the telomere of the X or Y chromosome) indicates that an obligatory crossover also occurs at the tip of the telomere in male mouse meiosis. Other data from the segregation of the steriod sulphatase gene in mouse supports this pattern of segregation (Keitges, et al, 1985).

However, Rouyer and colleagues (Rouyer et al, 1986) report reduced recombination in human female meiosis compared to male meiosis at the telomere of the X chromosome, but little data is available for mouse. If there is a region of the X chromosome which displays pseudoautosomal inheritance in female mouse meiosis, then it is possible that these probes could be derived from this area, but this is very unlikely as the region would be very small (physically), and far away

physically (and genetically) from the region of microdissection.

Six of the probes that were individually analysed fulfilled all the criteria for mapping to the mouse X chromosome, and were positioned on this chromosome.

The Set Of Mapped X Chromosome Specific Microclones

Of the six probes mapped to the mouse X chromosome only two, pmc 16: 120 and pmc 16: 219 appear to be present in approximately one or two copies and these are not the two smallest probes. Four of the mapped microclones hybridise to a number of fragments and therefore appear to represent low copy repeat sequences:

pmc 14: 36 is a totally X chromosome specific clone which appears to hybridise to a number of fragments. Only one fragment could be localised on the X chromosome by classical genetic mapping, the other fragments could be interspersed throughout the chromosome, or localised to the same region. Brown (Pers. comm.) estimates the copy number of this probe to be 100 - 150 copies per haploid M. musculus genome.

Three of the microclones derived from pmc 16, 141, 225, and 222 were localised non-unique sequences:

pmc 16: 141 hybridises to approximately 50 sequences in the haploid M. musculus genome (at 1 x SSC, 65 ^{o}C) and fewer than 10 sequences in the haploid M. spretus genome (Brown, Pers. comm.). These sequences are present in the genome in a region spanning 1 cM and are entirely localised to this region.

All pmc 16:225 genomic sequences also appear to segregate as one X linked Mendelian locus, and 225 seems to be derived from a localised euchromatic region of the mouse X chromosome. This sequence is present in approximately 20 copies per M. musculus haploid genome and 5 copies per M. spretus haploid genome (at 1 x SSC, 65°C) (Brown, Pers. comm.).

pmc 16: 222 hybridises to a number of fragments. The most intense band and the other minor bands are X chromosome specific and most of the 222 fragments appear to segregate as a single unit. 222 also hybridises to a M. musculus Y chromosome specific band.

A large amount of data has been generated in man regarding sequences with homologies on the X and Y chromosomes (for example, see Page et al, 1982). In mouse very little data is available regarding X-Y homologies. For example, Nallaseth and Dewey (Nallaseth and Dewey, 1986) report sequences present in 100 - 200 copies on the mouse Y chromosome and as single copies in female DNA, but there is no direct data on X-Y homologies. The fact that this band is inherited as totally Y specific confirms that the 222 Y chromosome homologous sequence is not present at the telomeric pseudoautosomal region of the mouse Y chromosome.

Repetitivity Of Mapped Microclones

The above four out of the set of six X chromosome specific microclones hybridised to a large number of fragments indicating these sequences were not unique in the genome.

This level of repetitivity from a total of 6 random probes of 0.14 - 1.2 kb may be due to the small sample size or may reflect the true nature of the mouse X chromosome. There is no known reason why the microdissection or microcloning procedure should select for non-unique sequences or why non-unique sequences should have a greater chance of appearing in the microclone bank at a level above that of the mouse X chromosome level. Therefore these probes may mirror the chromosomal situation. The small average size of the microclones enabled clones such as 225 to be investigated whereas in a normal clone bank such a small clone would not have been of interest.

Two out of the six mapped microclones identified two genetically distinct sets of localised sequences on the mouse X chromosome.

The number of localised repeats out of the six mapped microclones, suggests these may be relatively frequent within the genome. As above, there is no reason why these sequences should be selected for by the microcloning procedure.

Mus musculus - Mus spretus Probe Hybridisation

In all cases the microclone probes hybridised with an equal or lesser intensity to M. spretus DNA compared to M. musculus DNA, this is either a reflection of the difference in homology between the M. spretus DNA sequences and the M. musculus microclone sequences, or of the difference in copy number between the species. In the case of 141 it is known

that the variation in intensity is due to a difference in copy number between the M. musculus and M. spretus genomes.

Mapping Strategy

The lack of any Mus musculus polymorphism highlighted the use of the interspecific cross in parental restriction fragment length variation. This, and the Taq 1 digestion of progeny DNAs were successful strategies for finding parental differences, the major requirement of molecular genetic mapping. Sufficient progeny resulted from the C2 cross for quite fine genetic mapping and Harlequin and Tabby provided coat mutation loci which were fast to score through large numbers of mice.

The repeat nature of the clones demonstrated the flexibility of the mapping strategy which was not confined to unique sequence probes, providing one band was variant between M. musculus and M. spretus and the repeat sequences were localised and segregated as a single Mendelian locus.

In mapping autosomes which are diploid in somatic cells, parental variation must consist of bands of different sizes because the heterozygote must be distinguishable from the homozygote. The X chromosome has the advantage of being haploid in males. Therefore in the cross used, probe segregation could be mapped (if necessary) entirely in males and parental variation, may take the form of presence or absence of a band, because a male will never be heterozygous at a totally (non-pseudoautosomal) X chromosome specific locus. This unique facility allowed three X chromosome

specific loci, (defined by pmc 14:36, pmc 16:222 and 225) to be mapped.

The mapping strategy complemented the use of the microcloning strategy, and having prepared and digested the progeny DNAs, large numbers of clones could be mapped against one set of filters, providing an efficient method for positioning clone banks.

The Genetic And Physical Positions Of Mapped Microclones

The genetic region between 141 and 225 spans the physical region thought to have been microdissected. By chance the microclone from the whole X chromosome microdissection, pmc 14: 36, maps within the proximal region.

Microdissection And The Molecular Map Of The Mouse X Chromosome

Microdissection produced banks of clones to the mouse X chromosome; less than 40 genetic loci have been mapped along the entire 100 cM (100,000 kb) length of this chromosome (Roderick and Davisson, 1986). All these loci exert a phenotypic effect and the majority have been mapped either through rare and fortuitous genic allelism or though selecting X linked mutations arising from mutation experiments involving vast numbers of mice. The number of molecular probes now available could increase the number of markers to the mouse X chromosome dramatically.

5. Discussion

5.1 The Microclone Molecular Map As A Basis For Analyses Of The Mouse X Chromosome

As stated in the introduction to this study, a molecular map provides a basis for beginning the genetic and physical analyses of the mammalian chromosome. In the creation of a molecular map to the mouse X chromosome data has consequentially been generated that is pertinent to various aspects of the genetic and physical analyses of this chromosome.

Genetic And Physical Data

The six microclones that were mapped along the mouse X chromosome generated two types of data: genetic data on the positioning of the clones, and physical data on the characterisation of the clones.

The six microclones define six new molecular orientation points on the mouse X chromosome genetic map. The clones were mapped through observation of recombination events between known loci and microclone loci in female meiosis. The set of microclones derived from the proximal region microdissection spanned the expected genetic region; fortuitously the microclone derived from the whole X chromosome microdissection mapped to a locus within this region.

Of the set of mapped probes, 120 and 219 are probably single copy, and the remaining four clones probably hybridise to low copy repeat sequences. Of these, two appear to be

localised repeat sequences, and one hybridises to a Y chromosome specific fragment as well as X-linked loci.

Repeat Sequence Islands - Physical Organisation Of The Chromosome

The most unexpected information derived from the six X chromosome specific microclones concerns the physical organisation of the mouse X chromosome, with respect to a novel type of repeat sequence DNA, the repeat sequence island.

Brown has defined a repeat sequence island as a finely localised region of the genome identified by a repeat sequence specific for that region, that is not a simple tandem array, but may be finely interspersed with other sequences. Repeat sequence islands are a phenomenon of the euchromatic areas of a mammalian chromosome. The fine localisation - due to the confinement of its component sequences to a limited area of a few cM (or less) of DNA - is the most striking and unusual feature of a repeat sequence island.

Microclones 141 and 225 are representitives of genomic sequences which make up repeat sequence islands, situated in two distinct areas of the euchromatic region of the mouse X chromosome.

The genetic data here shows these sequences are finely localised (present in an area spanning approximately 1 cM (141) to approximately 2 cM (225) of the chromosome i.e. approximately 1000 to 2000 kb respectively). Work in this

study and by Brown (Brown, Pers. comm.) has shown these sequences are repeated and data on their organisation has lead Brown to conclude that they are long complex repeats which are arrayed in a non-tandem fashion.

There have been reports of X chromosome specific repeat fragments in man, but these sequences appear to be tandemly arrayed and probably chromosome specific variants of satellite sequences. For example, Yang and co-workers (Yang *et al*, 1982) working with human DNA, reported a 2 kb *Bam Hl* repeat sequence which was X chromosome specific. These sequences were shown by physical mapping (by *in situ* hybridisation or the use of somatic cell hybrids) to map to locations at the centromere (Yang *et al* 1982) of the human X chromosome. The sequences are tandemly arranged (Willard and Smith, 1982). The 2 kb *Bam Hl* repeat sequence appears to be a satellite DNA variant (Waye and Willard, 1985) related to the human alphoid centromeric repeat DNA which has sites of hybridisation at several autosomal centromeres (Yang *et al*, 1982)).

The sequences described by the above groups have characteristics which are totally distinct from the sequences making up the 141 and 225 repeat sequence islands. The 2.0 kb *Bam Hl* repeats are localised in the heterochromatic regions of the chromosome and they are present in approximately 5000 copies per haploid genome (Waye and Willard, 1985), this is two orders of magnitude greater than 141 or 225 copy number. The sequences are arrayed in tandem and appear to be simply localised variants

of an extensive satellite DNA family - they are now known to be the fundamental amplified unit of ∝satellite DNA on the human X chromosome (Waye and Willard, 1985).

In 1985 Disteche and co-workers (Disteche et al, 1985) communicated the isolation of two repetitive DNA fragments located near the centromere of the mouse X chromosome. These sequences were repeated 20 - 50 times in the genome and like 141 and 225 were not selected out as repeat sequence containing probes by the hybridisation of a total mouse DNA probe to the individual clones.

One fragment was localised by in situ hybridisation to the distal edge of a centromeric block of heterochromatin on the X chromosome and on chromosome 11, and was thought to be arranged in tandem.

The second fragment, '68-36', was mapped by in situ hybridisation and was stated to be present only in band A3 of the X chromosome. 68-36 was present in approximately 50 copies in the haploid genome. Although the arrangement of 68-36 sequences was not rigorously determined, the authors suggested it may be tandemly arranged.

Whether 68-36 is localised in the same way as 141 or 225 could not be determined - in situ hybridisation cannot resolve chromosomal distances sufficiently to indicate the very tight localisations characteristic of repeat sequence islands. No detailed analysis of 68-36 sequence organisation was made. Whether 68-36 hybridises to a repeat sequence island is not clear, but it may represent another island on the mouse X chromosome.

Repeat Sequence Island Evolution

Clones 141 and 225 are representatives of repeat sequences of comparatively low copy number, and a chromosomal localisation that has been preserved across at least two million years of evolution and divergence, between M. musculus and M. spretus (although there are fewer copies of either sequence in M. spretus).

No satisfactory explanation is known for the mechanism of proliferation (or evolution) of repeat sequence islands. Any proposed theory must account for the interspersion of the sequences and their fine localisation.

Mechanisms of sequence proliferation are known, for example, slippage replication occurs relatively often in the genome (Tautz et al, 1986) but this mechanism results in the proliferation of small simple sequences, of a few bp, rather than the hundreds of bp which make up 141 or 225. A mechanism such as unequal crossing-over does produce localised arrays of repeated sequences, but these are repeated in tandem, in an organisation exemplified by the 2 kb Bam Hl repeat of the human X chromosome. Unequal recombination would not result in the interspersion of 141 and 225 repeat sequences observed by Brown. A mechanism such as transposition would result in the proliferation of interspersed sequences but this would also result in their distribution at least across the mouse X chromosome, if not dispersed throughout the genome.

141 and 225 are present in different copy numbers in M. musculus and M. spretus. The two Mus species evidently

had a common ancestral sequence for 141 and 225, but either both sequences have proliferated successfuly in M. musculus or sequences have been lost from the M. spretus genome. Sequences may have been lost from M. spretus by, for example, unequal crossing-over, (but conversely, this would not account for their proliferation).

The specific problems of the evolutionary lineages of 141 and 225 are unresolved, but they lead to a major question concerning the mammalian chromosome - if two, of a set of six random DNA probes spanning 50 cM are localised repeat sequence islands, how common are these sequences in the whole mammalian chromosome and in the genome?

Long Range Organisation Of The Mammalian Chromosome

Repeat sequence islands as defined by 141 and 225 may be very common in the genome and may contribute to a long range modular nature of the chromosome.

Over a short range of a few kb different sequence types, for example genic sequences and middle repetitive sequences seem to be finely interspersed, and the chromosome appears randomly heterogeneous, with a few exceptional regions, such as those defined by 141 and 225, or the heterochromatic regions of the chromosome.

Repeat sequence islands may make up regions spanning hundreds of kb, and they distinguish these regions from the surrounding chromosomal DNA. At this long range of organisation a modular nature of the chromosome is becoming apparent from for example, the work of Bernardi et al who

refer to the 'compositional compartmentalisation' of the genome, and who define this compartmentalisation of the genome in terms of isochores: isochores are distinguished by density gradient centrifugation, and span approximately 200 kb stretches (Bernardi et al, 1985 and see Chapter 1).

At the moment very little is known of the long range organisation of the mammalian chromosome, but whatever the origins and possible role(s) of the different forms of repetitive DNA (for example, see Heller et al, 1984), microclones 141 and 225 are a new addition to the known register of repeat sequences; unique in terms of the combination of copy number, arrangement and localisation and possibly evolution.

The Genetic-Physical Relationship Of The Chromosome And Recombination

The mapping of the microclone random DNA probes may illuminate the organisation and meiotic recombination behaviour of the mouse X chromosome.

The positions of probes 141, 219 and 222 (proximal microclones) corresponds well with the genetic region thought to have been microdissected, but 120 and 225 are rather distal - possibly they are derived from an inaccurate microdissection. Including 225 the microclones span a region of 50 cM, excluding 225 they span a region of 32 cM. These results indicate that on the gross scale of the whole chromosome the genetic position of loci roughly approximates their physical position. However, molecular probes have the

power to greatly refine the analysis of the genetic-physical relationship across the chromosome, and they can be used to observe recombination and possibly extend Lyon's study of 1976. On a finer scale there are likely to be anomalies between the genetic and physical maps.

Lyon, (Lyon, 1976) stated that given the random selection of genes, and if (and only if) recombination occurs with an equal likelihood along the mouse X chromosome, then the genes so far mapped appear to be clustered into regions of relatively higher gene concentration.

The appearance of clustering could be due to two sets of conditions: firstly (as Lyon states) that recombination is random along the length of the mouse X chromosome, and genes are physically clustered; secondly that recombination does not occur with an equal likelihood along the mouse X chromosome, but occurs in areas of high and low concentration, and genes are physically randomly and evenly dispersed.

If the first case is true, then randomly derived DNA probes will be randomly and evenly spaced along the length of the chromosome (and microdissected region). The even distribution of the clones will be a reflection of the equal chance of recombination per unit length occuring along the chromosome.

If the second model represents the true situation and recombination is not random, although the physical distribution of genes is random, then DNA probes mapped by

classical genetic mapping will be clustered in exactly the same sites as the mapped genes.

The six microclone probes mapped in this study are fairly well spaced along the X chromsome and no obvious clustering of loci is indicated from the microclone molecular map. This may be due to the small sample size of the clones, but extensive positioning of further microclones may help to delineate the true situation.

Ultimately a detailed investigation of the fine genetic - physical relationship of a region spanning a few cM may be possible by, firstly determining the frequency of crossover between two closely linked markers from probe segregation data through a maximum number of meioses, then secondly by finding the physical distance between probes (on the same mouse X chromosome as used for segregation data) by linking the probes through pulse field gel electrophoresis. For example on the human X chromosome, two probes mapping to either side of the Duchenne muscular dystrophy locus (C7 and 754, Davies et al, 1985) are 20 cM apart genetically, but have been shown by pulse field gel electrophoresis studies to be only 3000 kb apart, physically (Davies, Pers. comm.) i.e. indicating an area between the two probes of increased recombination compared to the rest of the human X chromosome.

The region between Harlequin and Tabby on the mouse X chromosome contains more known genes than any other 20 cM stretch of the chromosome. Microclone probes to this region will help in analysing whether this region is physically

much longer than the genetic data suggests. Possibly both recombination events and gene loci are non-randomly distributed along the mouse X chromosome.

X Chromosome Recombination

In analysing why and how translocations arise, and phenomena associated with recombination, microclone 141 maps to an area of interest in the vast uncharted region proximal to Harlequin.

The region of the mouse X chromosome between Harlequin and Tattered makes up 22 % of the entire mouse X chromosome genetic distance and is entirely devoid of known genes. Possibly such a large genetic distance may be due to increased recombination over this area, rather than true physical length. However until recently there has been no means of analysing the genetic-physical relationship of the area.

Microclone 141 provides only the second molecular 'handle' for the analysis of the area of the mouse X chromosome proximal to Harlequin (the first being clone 66 described by Amar et al, 1985). More microclones may be useful for dissecting the 22 cM length into smaller units, each limited by a molecular marker. Ultimately recombination and physical distance between molecular markers, and within the units, could be compared through molecular genetic maps and pulse field gel electrophoresis. If there is an overall increase in recombination in this area, then it is necessary to explain why and how recombination is

concentrated here. If recombination should be proportional to physical distance, then it is necessary to explain why no genes have been mapped to this region.

Access To Genes Of Interest On The Mouse X Chromosome

As stated above, microclone 141 provides access to a region of interest on the mouse X chromosome. Individual microclones may also provide access to genes of interest on the mouse X chromosome.

Thirty-two genes have been localised on the mouse X chromosome (Davisson and Roderick, 1986), fifteen of which lie within the region spanned by Harlequin and Tabby. Of these, in only four cases, Hprt, G6pd, Phk and Tfm, is the protein product of the gene known.

Genetic segregation data has positioned the microclones and these may provide useful start points in travelling along the chromosome (for example, by chromosome jumping, Poustka and Lehrach, 1986) to specific genes, ultimately for their analysis at the molecular level.

Furthermore, Kao (Kao, 1983) estimates that there is an average of 20 genes per cM in the mouse genome. Even if this estimate is 50 % too high, evidently a large number of genes remain to be discovered.

Some of the genes in the 'proximal' area, for example Tabby are of known clinical interest to man (Mckusick, 1985) and if Ohno's hypothesis is borne out then all the genes on the mouse X chromosome will be relevant to the biology of man.

In microdissecting the proximal region of the mouse X

chromosome it was hoped that resulting mouse X chromosome microclone probes might be useful in the analysis of a human locus, specifically the site of the Duchenne muscular dystrophy locus (DMD), the proposed human homologue to the mdx locus in mouse. This depends firstly, on finding a closely linked probe to mdx and secondly , on the homology of DMD and mdx.

Of the set of mapped microclones reported here, 36 is approximately 3 cM from mdx. This distance is too far away to reach mdx, using current technology, but further analysis of the microclones may yield potentially useful probes.

The problem of mdx:DMD homology cannot be solved by comparative mapping, but can be solved by examining the loci at the molecular level.

Relative map positions are known for a number of mouse - human homologous loci, and (see Fig. 1.1.) these clearly show movements of chromosomal material on the human X chromosome relative to the mouse X chromosome. In man these loci are in the order:

Telomere - sts - CPX - HPDRI- OTC - DMD - MS - Centromere - Tfm - Xce - EDA - Pgk - GLA - PLP - XLA - HPRT - G6PD - Telomere

and in mouse are in the order:

Centromere - spf - Hprt - G6pd - Bpa - mdx - Tfm - Xce - Ta - Pgk - Mo - xid - Ags - jp - Hyp - sts - Telomere

mdx could therefore correspond to DMD, or possibly to Emery-Dreifuss muscular dystrophy, which maps to the same band in

man as G6PD, (although it is not known if it is proximal or distal to G6PD).

A mouse probe that was linked at a few cM to mdx could not be used to distinguish absolutely between the two possibilities because of the extent of rearrangement between mouse and man. The homology can only be established by observing a probe derived from the mdx locus (or DMD) hybridising to the DMD (or mdx) locus.

The question of homology is important because although the gene for DMD is probably already cloned in humans (Monaco et al, 1986), if mdx is the mouse equivalent of DMD then the mouse mutant provides a valuable resource for analysis of the gene and gene product, for example in utilising the mouse model in the physiological analysis of the role of the gene product - and the effect of the mdx/DMD mutation, when human tissue culture may yield no information.

If mdx and DMD are not homologous, and the mdx locus corresponds to the Emery Dreifuss muscular dystrophy locus in man, analysis of this mutation would provide further information on a locus which affects various aspects of human biology, including mental development.

Mouse X Chromosome Evolution

The Dichotomy Of The Gene And The Molecular Sequence

The use of molecular probes may provide greater insight into the extent of chromosomal rearrangements between man and mouse, and possibly aid clinical studies in confirming the homologies of mouse - human loci.

Whether or not mdx is homologous to DMD or Emery Dreifuss, or even an uncharacterised muscular dystrophy from an unknown locus, the gene at the mdx locus presumably has a human counterpart somewhere on the human X chromosome, if Ohno's hypothesis is correct for this locus (and the use of molecular probes will confirm this).

However as stated in Chapter 1, Ohno's hypothesis is not postulated at the level of the DNA sequence, only at the level of the gene. The genes of the mammalian X chromosome, and the molecular sequences of the mammalian X chromosome evolve differently. All X-linked genes so far studied have been X linked in every tested mammal. The attempts to hybridise 141 to human DNA have shown that X linked DNA sequence conservation is not upheld in the way it is at the gene level. 141 represents a molecular sequence that is X linked in mouse, and present in a number of copies, in two tested mouse species. However no 141 sequences are present in human DNA, or in the genome of the chinese hamster. How 141 sequences evolved is unknown (see above) but they have either arisen since the separation of the mouse - chinese hamster ancestor, or (probably less likely) have been lost from non-mouse genomes.

The X And Y Chromosomes

141 has provided data on the evolution of X chromosome DNA sequences; the mapping and characterisation of 222 may provide further data at the chromosomal level on the evolution of the X and Y chromosomes.

In 1914 Muller (Muller, 1914) proposed a theory of Y chromosome evolution in which he stated that initially the X and Y chromosomes contained the same gene complement. This idea has been generally accepted and was corroborated by Ohno in 1967 (Ohno, 1967) who stated that the vertebrate sex chromosomes evolved from a common ancestral pair of homologues. The theory of common origin suggests that homologous sequences or possibly genes will be present between the two chromosomes.

Molecular probes have provided tools for the analysis of X and Y chromosome sequence homology - Page and co-workers (Page et al, 1982) reported the first X-Y homologous sequences (in man) and Goodfellow and colleagues (Goodfellow et al, 1983) reported the first X-Y shared gene (in man).

Molecular analysis of the human X and Y chromosomes has clearly shown that the presence of many sequences on the human Y chromosome may be due to relatively recent events, involving exchanges of sequences between the sex chromosomes (Cooke et al, 1984, Page et al, 1984).

However whilst data at the molecular level is yielding considerable information on the evolution of the human X and Y chromosomes there is almost no data available in mouse. Microclone 222 is one of the first DNA sequences to be described which hybridises to homologous loci on the Mus musculus X and Y chromosomes.

The position of several of the 222 homologous sequences has been genetically localised to one region of the mouse X

chromosome. The homologous Y sequences would have be to be mapped by physical methods such as *in situ* hybridisation. However the mapping data so far excludes the telomeric pseudoautosomal region of the mouse Y chromosome.

222 could represent a sequence that has been maintained on the X and Y chromosomes, since the time of their divergence, or a sequence that has transposed from one chromosome to another. One way to test an hypothesis of origin would be to hybridise 222 to male or female DNA from other *Mus* species or more distantly related animals as in the case of Page *et al*, (Page *et al*, 1984) - a recent movement of sequences from one chromosome to another would be displayed as either X or Y chromosome linkage without interchromosome homology in other species. There is a possibility that the mechanism which caused 222 to proliferate on the X chromosome should work on the Y chromosome. Therefore 222 could be hybridised to male *M. musculus* DNA at a lower stringency. If it detects multiple copies of 222 homologous sequences, then this sequence may have been present on the Y chromosome for some time. If it detects only one fragment then the existence of 222 homologues on the Y chromosome may only be from recent times.

5.4 General Conclusion

As with any area that is to be characterised and mapped, the chromosome may be defined in different ways. This study has utilised molecular probes, derived physically from the chromosome and positioned by exploiting the genetic behaviour of the chromosome.

These probes provide a simple molecular genetic map, but all maps are interrelated through their information content and all types of maps of the chromosome are relevant to each other. The molecular genetic map can provide information for more complex maps which define and characterise the chromosome architecture in terms of, for example chromosome regions, such as those made up of repeat sequence islands.

By whatever criteria the chromosome is mapped and defined, genetic or physical, it has a third axis of definition, because of the temporal dimension of its constitution. In looking, for example, at the homologous molecular loci of two different Mus species, the temporal aspect of the chromosome becomes apparent in the evolution of ancestral homologous sequences.

The landscape of the genome changes, and at different rates in different places - and definitions and reference points are necessary. One of the most important aims of genetics is to disseminate the rules regarding this change, and the effect on the biology of the organism.

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ADDENDUM

page 19

line 23 : should read,
"A map represents a system of recognition and orientation within defined boundaries. In the case of the chromosome molecular map the length bounded is that of the chromosome, and the system of recognition and orientation..."

page 30

line 23 : should read,
"Molecular maps of the mammalian chromosome have a genetic aspect and a physical aspect. The genetic aspect is of a linear array of segregating sequences. The physical aspect is of a linear array of cloned sequences..."

page 40

line 18 : should read,
"In Mus musculus Bkm sequences exhibit a largely male specific pattern, and have been mapped mainly to the Y chromosome (but they are also present on the X chromosome and autosomes)."

page 150

line 26 : should read,
"Ta and Li are separated by 30 cM (Davisson and Roderick, 1986)"

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Microdissection and microcloning of the mouse X chromosome

(*mdx*/genetic mapping)

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ABSTRACT **A wild mouse (CD) karyotype in which all the chromosomes bar the X, 19, and Y, are fused as metacentrics has been used for the microdissection and microcloning of a specific mouse X chromosome region. Dissection of a proximal region of the X chromosome encompassing the genetic loci *Hprt* to *Tfm* and including *mdx* has yielded 650 clones. A number of the recovered clones containing sizable inserts have been confirmed as X chromosome specific. This X chromosome bank of clones provides a start point for the isolation of the *mdx* locus. It is now clear that microdissection and microcloning can be applied to all mouse chromosomes, including the X chromosome, yielding premapped banks of clones that will greatly aid in the isolation and characterization of important genetic loci.**

The mouse X chromosome has become a paradigm for the study of a variety of biological phenomena of molecular interest: X chromosome inactivation (1, 2), sex determination (3–6), and the comparative evolution of mammalian chromosomes (7, 8). Yet, very little is known of the detailed molecular structure or long-range organization of this or other mammalian chromosomes. In addition, the mouse X chromosome contains a number of genetic loci of exceptional interest; e.g., some, such as *mdx* [X-linked muscular dystrophy (9)], putative homologues to human X-linked genes of medical interest, [e.g., Duchenne muscular dystrophy (10)]. To begin the molecular mapping of the mouse X chromosome and isolation of genetic loci of primary interest, we have used microdissection and microcloning (11, 12) to isolate DNA sequences from a specific X chromosome region. Genomic clones recovered from the microdissection and microcloning of a proximal region of the mouse X chromosome originate from the mouse X chromosome and provide a bank of clones highly specific to this chromosome.

METHODS

Preparation of Metaphase Spreads. Lymphocytes were collected from male and female CD mice by suborbital bleeding (16). Lymphocyte division was stimulated by concanavalin A treatment for 2–3 days and, subsequently, lymphocytes were arrested in metaphase by Colcemid. After treatment with KCl for 12 min, cells were fixed with methanol/acetic acid (3:1) and spread by air-drying. Metaphase spreads were stained with 1% Giemsa for photography.

Microdissection and Microcloning. Unstained metaphase spreads of the CD strain were prepared on coverslips according to the methods described above. Coverslips were inverted over an oil chamber and chromosomes were dissected by using glass microneedles with the aid of a de Fonbrunne micromanipulator as described (11, 12). All manipulations were observed under a phase-contrast microscope with a long working distance. Specially fine microneedles were drawn on a de Fonbrunne microforge and were used for the microdissection of the proximal region of X chromosomes well separated in the metaphase plate. After dissection, DNA was prepared and cloned essentially according to published micromethods (11, 12) but with some modifications. Briefly, fragments were pooled in a drop of proteinase K/0.5% $NaDodSO_4$ residing on a separate coverslip in the oil chamber. After dissection was completed, the oil chamber was incubated at 37°C for 2 hr. After incubation, the nanoliter drop was extracted several times with phenol. After chloroform extraction to remove residual phenol the nanoliter drop was transferred to a fresh oil chamber and a nanoliter drop of restriction enzyme (*Eco*RI, 50 units/μl) was added and digestion proceeded by incubation at 37°C for 2 hr. Phenol extraction to inactivate enzyme and chloroform treatment were repeated with subsequent transferance of the microdrop to a fresh oil chamber. A nanoliter microdrop ($\approx$400 pg) of *Eco*RI-digested vector, λgt10, was added, followed by a nanoliter microdrop (0.4 unit) of ligase. Ligation proceeded overnight at 4°C. After ligation, the microdrop was taken up in several microliters of buffer for *in vitro* packaging. Recombinants were selected by plating on an *Escherichia coli* C600*hflA*$^-$ host (14, 15). Individual plaques were picked and plate stocks were prepared by propagation on SM32 (16) using standard procedures.

Analysis of Microclones. A plate lysate method was used to prepare DNA from individual clones. Insert size of clones was determined by *Eco*RI or *Hin*dIII digestion and agarose gel electrophoresis. Two *Hin*dIII sites flank the RI insertion site of λgt10, and *Hin*dIII digestion provides a rapid and easy method for indirectly determining insert sizes of smaller clones without using large amounts of prepared DNA by observing the change in migration of a λgt10 *Hin*dIII 6.7-kilobase (kb) band. Gels were blotted onto nitrocellulose and hybridized to nick-translated total mouse DNA (10^8 dpm/μg) to identify clones containing repeat sequences (17). Prehybridization and hybridization conditions are as described below.

X Chromosome Localization of Microclones. Mouse inbred line DNAs and hybrid cell DNAs were digested to completion with *Taq* I and were fractionated on 0.8% agarose gels and transferred to Zetapore membranes. Filters were initially hybridized overnight in 3× NaCl/Cit/10× Denhardt's solution at 65°C followed by a second hybridization for 6 hr in 3× NaCl/Cit/10× Denhardt's solution/denatured salmon sperm DNA (100 μg/ml)/poly(A) (10 μg/ml)/0.1% $NaDodSO_4$ at 65°C (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). Microclone inserts were released from recombinants by *Eco*RI digestion and were separated on 1.4% low melting point agarose gels. Insert bands were cut out and water was added to 3 ml per g of gel and the gel was dissolved by heating in a boiling water bath for 7 min. Insert DNA was labeled by oligo-priming without

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Abbreviation: kb, kilobase(s).

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further extraction according to a published method (18). Hybridization was carried out for 16 hr in 3× NaCl/Cit/1× Denhardt's solution/10% dextran sulfate/denatured salmon sperm DNA (100 μg/ml)/poly(A) (10 μg/ml)/0.1% $NaDodSO_4$ at 65°C and a probe concentration of 1–5 × 10^6 dpm/ml. After hybridization, filters were washed several times with 1× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C and exposed on Kodak XAR-5 film with intensification for 1–7 days.

RESULTS

Microdissection of the Mouse X Chromosome. Microdissection and microcloning involves the physical dissection of chromosomes from unstained metaphase spreads and their cloning by using specialized microprocedures. To unequivocally identify the mouse X chromosome within an unstained metaphase spread, we have used a wild strain of mouse (*Mus musculus*), CD (19, 20), in which all chromosomes apart from the 19, X, and Y, are fused as metacentrics (Fig. 1). The 19, X, and Y chromosomes are the only acrocentrics and are readily distinguishable; the X chromosome is clearly distinguishable from the 19 and Y on account of its much greater size (size ratio, 2.3:1). Arbitrarily, for the purposes of dissection, we have divided the mouse X chromosome into four equally sized regions: centromeric, proximal, distal, and telomeric (Fig. 2). As there is no direct correspondence between the genetic and physical maps of the mouse X chromosome, the exact genetic region dissected in any case is unclear. In Fig. 2, a genetic map is shown and its relationship to the physical map is indicated in order to demonstrate those genetic regions that are likely to be included in any regional dissection. As there is necessarily some variation in the region dissected, it is expected that some sequences from the centromeric and distal regions will be collected. Thus, the genetic region encompassed by a proximal dissection lies within the limits of the genetic loci *Hprt* and *Tfm* and includes the *mdx* locus (9).

Microcloning of Dissected X Chromosome Fragments. Dissected proximal X chromosome fragments (Fig. 2) from CD karyotypes were pooled in a proteinase K/$NaDodSO_4$ nanoliter microdrop to release DNA. After phenol extraction, DNA was digested with *Eco*RI, phenol extracted again, and then ligated into the insertion vector λgt10, all using specialized microprocedures. After *in vitro* packaging, phage were plated on an *E. coli hflA* mutant host to selectively recover recombinant clones (14).

Analysis of X Chromosome Microclones. From 100 dissected proximal fragments (≈2 pg of DNA), a total of 650 cI^- phage clones were recovered (Table 1). Plating of a small proportion of the packaged microligation on a nonselective strain [SM 32(22)] indicated that, in total, ≈90,000 phage (recombinant and nonrecombinant) were recovered from the microligation and subsequent *in vitro* packaging of microdissected proximal X chromosome DNA. Control ligations of the *Eco*RI-digested λgt10 vector alone demonstrated that only 1 in 10^3 phage recovered successfully grow on an *E. coli hflA* mutant host, indicating that a high proportion (>85%) of the 650 recovered cI^- clones are true recombinants. A large number of the microclones were miniprepped and the insert size was estimated by restriction enzyme digestion. Some microclones (28 of 72) had very small (<0.1 kb) or undetectable inserts. Insert size of the remaining clones varied from 0.2 to 4 kb, with an average insert size of 0.4 kb. The miniprepped and digested clones were blotted and hybridized to total mouse genomic DNA to eliminate those clones containing repeat sequence DNA; 13% (9 of 72) of the clones hybridized strongly, including one microclone with a very small insert (<0.1 kb). The average insert size of sizable clones containing repeat sequences was 0.6 kb. The small average insert size has probably contributed to the relatively low number of clones containing significant repeat sequences; those clones containing repeat sequences are of higher average insert size. The overall small insert size of recovered genomic clones may be due to preferential acid hydrolysis of large *Eco*RI fragments during fixation of metaphase spreads.

To verify that the recovered clones do indeed originate from the X chromosome, we have hybridized a number of low copy microclones carrying sizable inserts to hybrid cell lines—MAE-28 and MAE-32 (21)—containing the mouse X chromosome. MAE-28 contains Chinese hamster chromosomes plus mouse chromosomes X and 12, while MAE-32 contains Chinese hamster chromosomes plus mouse chromosomes X and 16. E-36, the parent cell line, contains only Chinese hamster chromosomes. DNAs from the inbred mouse lines, C57BL/10 and SWR, and from MAE-28, MAE-32, and E-36 were digested with *Taq* I, electrophoresed on agarose gels, blotted, and hybridized to a variety of X chromosome microclones chosen at random (Fig. 3). Five clones were tested and all were shown to be X chromosome specific. Hybridizing fragments observed in mouse DNAs, C57BL/10 and SWR, are also observed in MAE-28 and

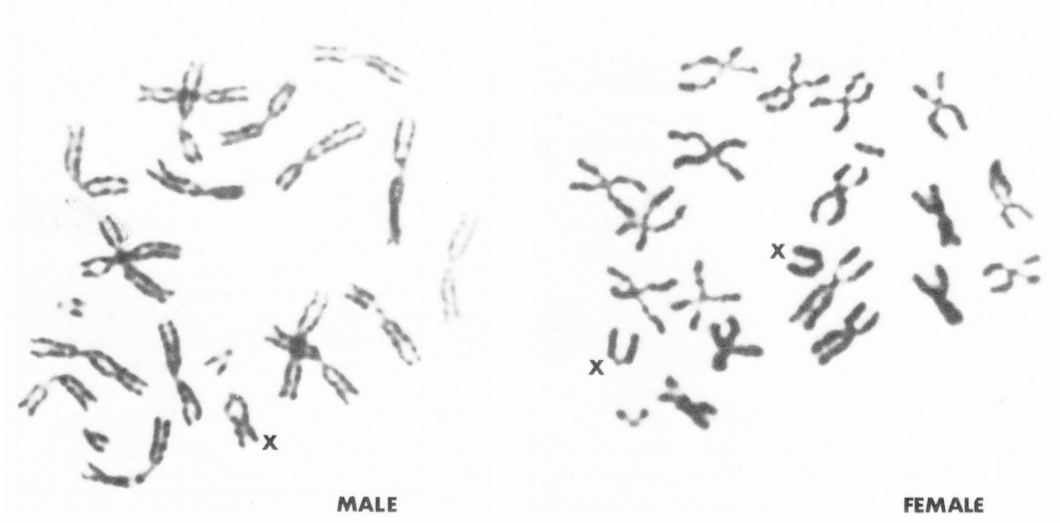

FIG. 1. Chromosomes of the CD strain of mouse. Metaphase spreads from lymphocyte cells of male and female individuals of the wild CD strain (19, 20) of *Mus musculus*. The X chromosome is marked.

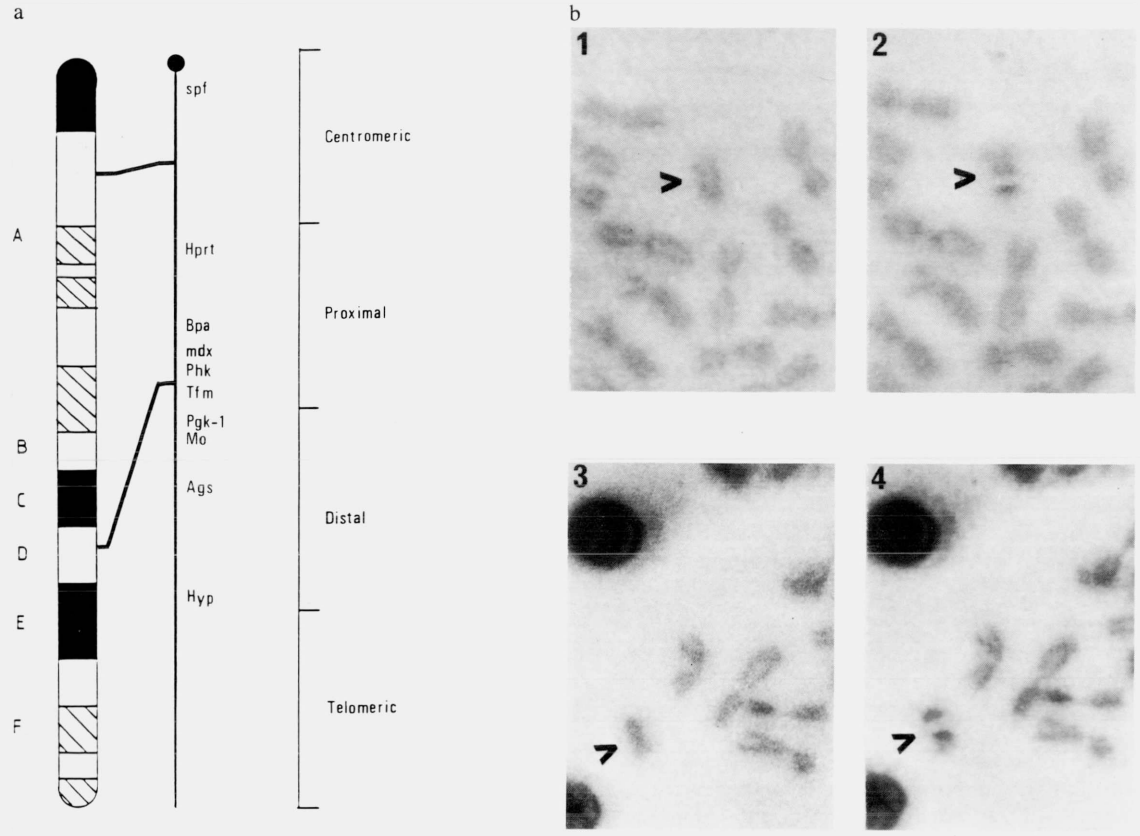

FIG. 2. Microdissection and microcloning of the mouse X chromosome. (*a*) Diagram to illustrate the four physical regions of the mouse X chromosome arbitrarily defined for microdissection. Note the genetic and physical maps of the X chromosome do not exactly correspond; the concordance between the physical and genetic maps where known (7, 20) is indicated. The genetic region dissected cannot be precisely determined and is only shown here to give an indication of those genetic markers that might be included (see text). (*b*) Unstained metaphase spreads of the CD strain observed by phase contrast before (*1* and *3*) and after (*2* and *4*) microdissection of the proximal region of the X chromosome.

MAE-32 but not in E-36, confirming the location of the microclones on the X chromosome. In the case of two clones, 10 and 44, weakly hybridizing fragments common to E-36, MAE-28, and MAE-32 are seen, representing weakly homologous sequences in the Chinese hamster genome. In addition, the larger of the two X chromosome fragments detected by clone 44 shows a *Taq* I restriction fragment length polymorphism between the SWR and C57BL/10 inbred strains. Clone 44 also shows some background hybridization to all lanes, indicating that it still carries some repeat sequence DNA not detected in the initial screening. In clone 43, apart from the strongly hybridizing X chromosome fragment common to C57BL/10, SWR, MAE-28, and MAE-32 DNAs, a variety of weakly hybridizing fragments of variable intensity are observed in mouse genomic DNAs but not in MAE-28 and MAE-32 DNAs even after long exposure. These fragments may represent weakly homologous sequences to the X chromosome clone 43 with an autosomal location.

Table 1. Microdissection and microcloning of the proximal region of the mouse X chromosome

No. of chromosome fragments dissected	100
No. of clones recovered	650
Size range of inserts (72 clones analyzed)	<0.1–4 kb
Average insert size (44 clones)	0.4 kb
% clones containing repeat sequences	13%
Average insert size of clones containing repeat sequences	0.6 kb

Average insert size was determined by using only microclones with sizable inserts, >0.1 kb (44 of 72 clones analyzed). Percentage clones containing repeat sequences was calculated on the basis of the total number of clones analyzed of which 9 hybridized strongly to total mouse DNA (see text). Average insert size of clones containing repeat sequences was also determined by using only clones with sizable inserts, >0.1 kb (8 out of the 9 strongly hybridizing clones).

DISCUSSION

By using Robertsonian translocations to identify individual autosomes in unstained metaphase spreads, regional microdissection can provide banks of clones from individual autosomes (22). But, the X chromosome does not participate in Robertsonian fusions (20). However, by a process of exclusion, involving the majority of autosomes in Robertsonian fusions and leaving the X chromosome free, we have demonstrated here the microdissection and microcloning of an individual region of the mouse X chromosome. Thus, it is now possible to microdissect and microclone all mouse chromosomes.

Genomic clones originating from dissected proximal X chromosome fragments are X chromosome specific and provide a bank of clones premapped to the mouse X chromosome. While there must be some variation in the region dissected, the technique clearly provides a method for the rapid isolation of clones from a discrete physical region of the genome. Genetic experiments will enable us to define exactly the genetic limits of the microclones obtained, and at the same time, to define those clones tightly linked to interesting genetic loci.

Some 550 microclones, of which 50% may contain usable genomic inserts, have been obtained from the microdissec-

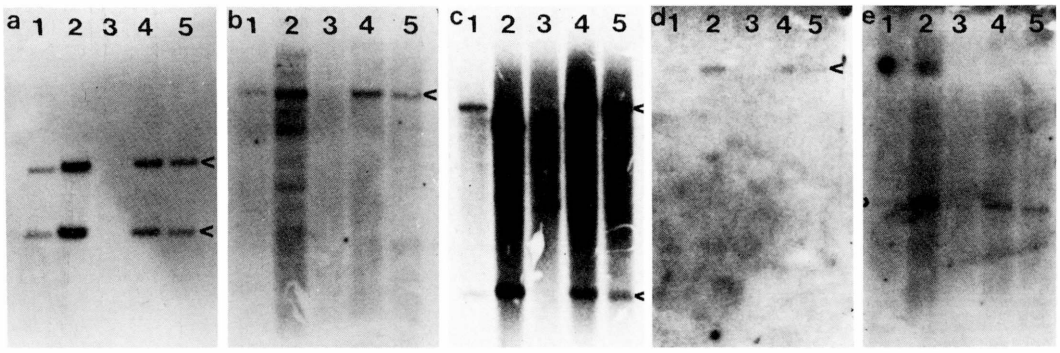

FIG. 3. Localization of microclones to the mouse X chromosome. Hybridization of proximal X chromosome microclones to *Taq* I-digested DNA from C57BL/10 (lanes 1) and SWR (lanes 2) mouse inbred lines; E-36 (lanes 3) (parent Chinese hamster cell line); MAE-32 cell line (21) (lanes 4) (containing Chinese hamster chromosomes and the mouse chromosomes X and 16) and the MAE-28 cell line (21) (lanes 5) (containing Chinese hamster chromosomes and the mouse chromosomes X and 12). Note that in all cases, lane 2 (SWR DNA) was loaded with greater amounts of DNA, accounting for the increased signal in these lanes. X chromosome-specific bands are indicated by arrows. (*a*) Clone 10 (insert size, 0.5 kb); (*b*) clone 43 (0.4 kb); (*c*) clone 44 (0.8 kb); (*d*) clone 54 (0.2 kb); (*e*) clone 101 (0.2 kb).

tion of a region of the mouse X chromosome, ≈25 cM long. Given a random distribution of proximal microclones along the chromosome region dissected, the clones may be able to differentiate very small genetic distances (≈0.1 cM). The proximal bank of X chromosome microclones might therefore be expected to contain DNA sequences tightly linked to important genetic loci, such as *mdx*, that will lead to their eventual isolation and characterization. The provision of highly specific banks of microdissected clones from all mouse chromosomes should rapidly facilitate the future mapping of the entire mouse genome.

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