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MOLECULAR ANALYSIS OF DUCHENNE MUSCULAR DYSTROPHY AND OTHER Xp MUTATIONS USING CLONED DNA SEQUENCES.

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I certify that this thesis does not contain material previously published or written by any other person except where referred to in the text. Results included in this thesis are my own work, unless otherwise stated, and have not been submitted for any other degree or diploma.

Elizabeth Gillard.

To my family: Margaret, Bernard, Bernard and Sue.

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LIST OF ABBREVIATIONS:

AHC	congenital adrenal hypoplasia
amp	ampicillin
ATP	adenosine tri-phosphate
BMD	Becker muscular dystrophy
bp	base pair
BRL	Bethseda research laboratories
BSA	bovine serum albumin
cDNA	complementary copy DNA
cM	centi-Morgan
CPDX	chrondrodysplasia punctata, X-linked dominant
CYBB	cytochrome b_{245} beta chain (chronic granulomatous disease gene)
D	dalton
dATP	2'deoxyadenosine 5'-triphosphate
dCTP	2'deoxycytodine 5'-triphosphate
dGTP	2'deoxyguanidine 5'-triphosphate
DMD	Duchenne muscular dystrophy
DMF	Dimethyl formamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dTTP	2'deoxythymidine 5'-triphosphate
EDMD	Emery-Dreifuss muscular dystrophy
EDTA	ethylene diamine tetra-acetic acid
e.g.	exempli gratia (for example)
et al.	et alia (and others)
FACS	fluorescence activated cell sorter
FVIII	Factor VIII locus
g	gram
GK	glycerol kinase (deficiency)
G6PD	glucose-6-phosphate dehydrogenase
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPRT	hypoxanthine-guanine phosphoribosyl transferase
i.e.	id est (that is)
K	thousand revolutions per minute

KAL	Kallmann's syndrome
k	kilo
kb	kilo-basepair
l	litre
LOD	logarithm of the odds
M	molar (concentration) or Morgan (distance)
m	milli
μ	micro (or "mu", a measure of the prior risk)
Mb	mega-basepair
MOPS	morpholinopropanesulfonic acid
Mr	relative molecular mass
mRNA	messenger ribonucleic acid
n	nano
NDP	Norrie's disease
OA	ocular albinism
OD	optical density
OTC	ornithine transcarbamylase
poly(A)	poly riboadenylic acid
PFGE	pulse field gel electrophoresis
pfu	plaque forming units
PGK	phosphoglycerate kinase (deficiency)
PIC	polymorphic information content
psi	pounds per square inch
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse A	ribonuclease A
rpm	revolutions per minute
RP3	retinitis pigmentosa type 3
RS	retinoschisis
SDW	sterile distilled water
SSC	standard saline citrate.
SDS	sodium dodecyl sulphate
SRO	smallest region of overlap
STS	steroid sulphatase
TDF	testes determining factor
TDH	"Tom, Dick and Harry"
Tris	tris (hydroxymethyl) aminoethane

VNTRs	variable number of tandem repeats
XLI	X-linked ichthyosis
XLIHA	X-linked ichthyosis, hypogonadotropic hypogonadotropism and anosmia.
Xce	X chromosome inactivation centre
XG	Locus for Xg blood group
XK	Kell blood group, McLeod syndrome
Xp	short arm of the X chromosome
Xq	long arm of the X-chromosome

SUMMARY

Duchenne muscular dystrophy (DMD) is the most common, lethal X-linked mutation in the United Kingdom, affecting one in every three thousand male livebirths. Affected males have progressive muscle weakness and die as a result of respiratory or cardiac involvement in their late teens or early twenties. Whilst the X-linked nature of DMD has been recognised for many years, the regional assignment of DMD to Xp21 is relatively recent. This was first proposed on the basis of X;autosome translocations and more recently has been supported by linkage analysis using polymorphic DNA probes from within and around the DMD locus.

X-linked ichthyosis (XLI) is the most common cause of early onset scaly skin (ichthyosis), affecting one in every five to six thousand male livebirths and is due to a deficiency of steroid sulphatase (STS). Although the X-linked nature of one form of ichthyosis had been recognised for many years, deficiency of STS in association with this condition was a relatively recent discovery. STS was assigned to Xp22 to Xpter on the basis of somatic cell hybrid mapping and to Xp22.3 to Xpter on the basis of a male with an XY translocation. The STS locus is of particular interest as it is one of the few known X-linked loci which escape inactivation in females.

The main aim of this project was to isolate DNA sequences from the short arm of the X-chromosome (Xp) which could be used to study either DMD or XLI.

In order to achieve this, an X-chromosome specific EcoRI library was constructed from flow-sorted chromosomes. In the initial screening of this library, one hundred and twenty plaques were selected for further study of which twenty eight had human inserts. Sixteen of these were single-copy or low-copy sequences with homology to the human X-chromosome, four were sequences with homology to the autosomes, six had homology to repeat sequences and a further recombinant had homology to multiple sequences in both man and mouse.

The X-specific inserts were further characterised by a somatic cell hybrid mapping panel and four were assigned to Xp;

GMGX9(DXS237) to Xp22.3-pter, GMGX10(DXS238) to Xp21-cen and GMGX11(DXS239) and GMGX12(DXS240) to Xp21-Xp22.3.

The ability of these four probes to detect restriction fragment length polymorphisms (RFLPs) was studied using panels of twelve to fourteen X-chromosomes digested with each of thirteen restriction enzymes. Only GMGX9(DXS237) detected an RFLP with HindIII. This had alleles of 4kb and 2.5kb + 1.5kb, which occurred at frequencies of 0.67 and 0.33 respectively, and a PIC value of 0.44.

These four probes were also tested in eleven individuals with DMD, BMD or contiguous gene syndromes including DMD, who all had deletions of pERT87(DXS164) and/or XJ(DXS206) (which are deleted in 6-10% of males with DMD/BMD). GMGX11(DXS239) was deleted in one individual with adrenal hypoplasia (AHC), glycerol kinase deficiency (GK) and DMD, whilst GMGX10(DXS238) was deleted in three individuals with large deletions.

These eleven individuals were also examined with twenty seven other Xp probes. Only two of these eleven deletions could not be resolved from the others by these probes and there was no smallest region of overlap. The deletion in one boy (pedigree number 5097) with BMD, apparently encompassed that of a second (5265) with DMD, thus excluding the notion of an unique BMD domain in Xp21.

Deletion studies in these eleven individuals and in somatic cell hybrids mapped GMGX10(DXS238) proximal to pERT84(DXS142), GMGX11(DXS239) between JBir(DXS270) and L1-4(DXS68) and GMGX12(DXS240) between C7(DXS28) or B24(DXS67) and GMGX9(DXS239).

GMGX10(DXS238), GMGX11(DXS239), GMGX12(DXS240), p20(DXS269), JBir(DXS270) and J66HI(DXS268) were used to screen one hundred and three boys with DMD or BMD, including nine of the eleven deletions described above. GMGX10(DXS238) and GMGX12(DXS240) detected no additional deletions. Thirty six additional deletions or altered fragment sizes were detected with GMGX11(DXS239), p20(DXS269), JBir(DXS270) and J66HI(DXS268). Thus 46% of boys in this group had deletions or altered restriction fragment lengths visualised with DNA probes. 18% were detected by GMGX11(DXS239) and 22% by p20(DXS269), with a slight degree of overlap.

GMGX11(DXS239) detected fifteen novel deletions (and three

altered band fragments) amongst EcoRI digests and fourteen novel deletions (and three altered band fragments) amongst HindIII digests. p20(DXS269) detected eight deletions, five partial deletions and ten individuals with novel fragment sizes (or novel fragment sizes associated with partial deletions) amongst HindIII digests. Notably four partial deletions and two complete deletions of p20(DXS269) were observed amongst the fifteen families with BMD. None of the mutations detected by GMGX11(DXS239) were associated with BMD.

Pulse field gel electrophoresis (PFGE) data and mapping with respect to translocation breakpoints in females with DMD has placed GMGX11(DXS239) between JBir(DXS270) and J66HI(DXS268). Both genomic and cDNA clones which detect deletions at high frequency have been assigned to this interval. Comparative mapping studies show that GMGX11(DXS239) is not conserved and is thus unlikely to be expressed. GMGX11(DXS239) is linked to J66HI(DXS268) and p20(DXS269) to JBir(DXS270) by PFGE although the two loci have not been compared directly.

The study of new mutations in DMD/BMD should help determine how deletions arise, although such studies are complicated by germ-line mosaicism which makes reliable definition of a new mutation difficult. The origin of the mutation was identified in three DMD families (5313, 3485 and 7866). In each family the mothers were heterozygous for probes deleted in their sons, suggesting that, in the absence of gonadal mosaicism, these were new mutations in a maternal gamete.

A deletion of probes L1-4(DXS68), B24(DXS67) and C7(DXS28) was detected in a male (5996) with AHC and mental retardation but neither GK deficiency nor DMD. His deletion was encompassed by that of a male with DMD, GK and AHC (whose deletion included probes from pERT84(DXS142) to C7(DXS28) / B24(DXS67)). A second individual with DMD, GK and AHC, was deleted from GMGX10(DXS238) to J66HI(DXS268) inclusive. Comparison of these three deletions mapped AHC distal to GK and both genes between J66H1(DXS268) and L1-4(DXS68). A further patient (5194) with AHC but not mental retardation was probed with the same probes but no deletion was detected.

The GMGX9(DXS237) HindIII RFLP was studied in normal three generation families (in which it was shown to segregate as a Mendelian trait) and in families segregating for X-linked ichthyosis (XLI). GMGX9(DXS237) was deleted in males from thirty seven out of forty-four (84%) unrelated families with XLI and could be used to predict carrier status in some families. Multipoint linkage analysis showed tight linkage of GMGX9(DXS237) to STS deficency.

Comparative mapping studies showed that GMGX9(DXS237) was not conserved and was thus unlikely to represent an exon of the STS gene. STS cDNA clones, p2a4 and STB14 were also studied in thirty one affected males and detected deletions in the same individuals with only one exception. The exceptional male was deleted for p2a4 but not the X-specific band of STB14. The X-specific band of GMGXY19 but not that of GMGXXY3 was also deleted in this individual.

The deletion in a male with XLI and Kallmann's syndrome (KAL) was apparently identical to those of patients with XLI only, whilst three patients with KAL only were not deleted for any probes tested. Two patients with XY translocations were additionally deleted for M1A(DXS31).

All but one of the deletions at the STS locus in this study were identical, in contrast to those at the DMD locus. Resolution of these deletions, however, was limited by the probes available and flow cytometry (FACS) suggested a range of deletion size up to 5.2Mb in affected males.

Deletions in XLI arising by reciprocal XY translocations were excluded by inheritance of Xg(a) in many affected males, and the mechanism by which deletions are generated at high frequency is unclear. Molecular studies on the origin of new mutations might define possible deletion mechanisms but as yet none have been described.

The present study emphasises the value of "reverse genetics" in the study of human disease. This procedure entails identifying gene-specific DNA sequences, by virtue of their chromosomal location. These are then used to isolate (or predict the structure of) the protein product. This approach is best exemplified by

current progress in the study of DMD, but "reverse genetics" has been successfully applied to other conditions and is expected to make an important contribution towards the cloning of the genes involved in more than four thousand two hundred mendelian phenotypes.

CHAPTER 1: INTRODUCTION

1:1 GENERAL INTRODUCTION.

The primary aim of this project was to perform a molecular analysis of two X-linked disorders; Duchenne muscular dystrophy (DMD) and X-linked ichthyosis (XLI). These two disorders therefore provide the main focus of the introduction (sections 1:3 and 1:4 respectively), following on from a general introduction.

This general section (section 1:1) introduces the concepts of X and Y linked inheritance, of X-inactivation and of mapping genes to the X-chromosome. Section 1:2 addresses the regional localisation of genes on the X-chromosome by X;autosome translocations, deletions, somatic cell hybridisation studies and linkage analyses.

1:1:1 Sex-linked inheritance and X-inactivation.

The X and Y chromosomes are particularly amenable to a molecular approach in the study of human development and inherited disorders in man, as both chromosomes lack homologues in males. Mutations of many genes on the X- and Y- chromosomes are thus expected to be evident in males, regardless of whether the traits are dominant or recessive. Phenotypic changes can thus be directly correlated with those changes evident at the molecular level.

The characteristic mode of inheritance of X-linked genes has resulted in recognition of the X-linked nature of many disorders. Both recessive and dominant X-linked traits have characteristic patterns of inheritance.

A recessive X-linked condition will affect hemizygous males, with a single X-chromosome, but not heterozygous females with two X-chromosomes (with only rare exceptions, as discussed below). "Carrier females" (who are asymptomatic) "carry" one X-chromosome with the defect and one X-chromosome without the defect. Half of the sons and half of the daughters of a carrier female receive each chromosome and thus half of the sons will be affected and half of the daughters will be carrier females. One consequence of this means of transmission is the characteristic "knight's move" pattern of affected males within a pedigree.

An X-linked dominant condition affects both males and females within a family. It can be distinguished from an autosomal dominant

condition according to the sex of affected children born to an affected male. An affected male with an X-linked dominant condition will transmit this to all of his daughters but to none of his sons (whereas a male with an autosomal dominant trait would transmit the trait to both sons and daughters equally). Females heterozygous for an X-linked dominant trait are as severely affected as hemizygous males and transmit the condition to half their children, regardless of sex.

Occasionally an X-linked dominant disorder may be lethal in males. In such circumstances only affected females will be observed. If the disorder is also incompatible with female fertility every individual observed will represent a new mutation.

X-linked recessive conditions may occur in females with Turner's syndrome (45,X karyotype). It may also occur in females homozygous for a defective allele (who usually have consanguinous parents) providing the condition does not affect male fertility (either directly or by early death). Females with chromosome aberrations involving the X-chromosome (e.g. X;autosome translocations which disrupt specific loci) may also be affected as a result of non-random inactivation of the "normal" X-chromosome (section 1:2:1).

Inactivation of one or other X-chromosome is one means of ensuring the same relative dosage of X-specific and autosomal genes in males and females. The X-inactivation hypothesis was first proposed by Lyon in 1961. X-inactivation, reviewed by Gartler & Riggs (1983), generally occurs at random in cells of the inner cell mass (which give rise to adult somatic cells) in female embryos. X-inactivation "spreads" from a single inactivation centre, Xce, situated on the proximal long arm of the X chromosome (Xq). The inactivated X is stably inherited by cells of clonal origin, yet the process is reversible for example in the female germ-line.

Carrier females may manifest an X-linked recessive disorder if the X-chromosome with the normal allele is inactivated in a high proportion of cells of the appropriate type as a result of non-random X-inactivation. The phenotypic severity in such individuals is dependent on the proportion of cells inactivated. More rarely carrier females may manifest the disorder as a result

of a second mutation in the normal allele.

Y-linked (holandric) inheritance should be characterised by limitation of a trait to males and transmission from father to son. No confirmed Y-linked diseases have been described although hairy ears was at one time suspected.

Difficulties can arise in distinguishing between a sex-linked disorder and an autosomal dominant condition whose expression is limited to males. These may be resolved for some X-linked conditions in which random X-inactivation (or "Lyonization") can be demonstrated in carrier females e.g. testicular feminisation (Meyer, Migeon & Migeon, 1975). Failure to observe random inactivation does not necessarily exclude X-linked inheritance however, as certain genes assigned to the X-chromosome escape X-inactivation e.g. steroid sulphatase (STS). This phenomenon will be addressed in section 1:4:5.

Genes within "the pairing segment" which is responsible for the initiation of pairing of the X and Y chromosomes in male meiosis, will have homologues on both Xp and Yp. Certain loci within the pairing segment e.g. 29Ci (DXYS14) undergo obligatory recombination in male meiosis and therefore mimic autosomal inheritance (Cooke, Brown & Rappold, 1985). These loci have been termed "pseudoautosomal". Other more proximal pseudoautosomal loci recombine at frequencies less than 50% (Rouyer et al., 1986) and are more obviously X-Y homologous.

The importance of pedigree analysis in assigning genes to the X-chromosome can be be appreciated by the high proportion of (confirmed) loci known to be X-linked (table 1), given that the X-chromosome represents only 5-6% of the haploid genome (Mayall et al., 1984; Harris, Boyd & Ferguson-Smith, 1986). Almost half of the loci currently mapped to the X-chromosome were assigned before 1966 (table 1), prior to the development of chromosome banding techniques which could distinguish the X-chromosome.

The colour-blindness gene on the X-chromosome was the first to be assigned to a specific chromosome in man (Wilson, 1911). Since then, more assignments have been made to the human X-chromosome than to any other chromosome in metazoa, apart from those those of Drosophila (McKusick, 1986). One hundred and forty independent

Mendelian phenotypes (expressed loci) have been assigned to the X-chromosome, whilst a further one hundred and sixty eight Mendelian phenotypes have tentatively been designated X-linked (McKusick, 1987).

1:2 REGIONAL LOCALISATION OF X-LINKED TRAITS.

Over sixty genes have been regionally localised on the X-chromosome (Read, 1987). Regional localisation can be acheived (i) by examination of breakpoints in translocations and/ or other chromosomal rearrangements in affected indviduals, (ii) by association of a disorder with other well-localised phenotypes as a "micro-deletion syndrome" or "contiguous gene syndrome", (iii) by somatic cell hybrid analysis (for defined gene products) or (iv) by linkage to a suitable marker. The precision of any regional localisation is dependent largely on the breakpoints and markers available. These methods are considered in sections 1:2:1-1:2:4.

1:2:1 X;autosome translocations.

Structural chromosome aberrations often provide the first means of mapping a disorder to a specific locus. These generally fall into one of the following categories: translocations, inversions, duplications or deletions.

In general, translocations and inversions only disrupt loci at their breakpoints. De novo translocations (or inversions) in association with a "new mutation" can thus provide information to localise an autosomal dominant or X-linked disorder to a specific chromosome band.

X-chromosome inactivation generally occurs at random in undifferentiated cells within the inner cell mass, as described earlier. In individuals with X-chromosome abnormalities, however, the inactivation pattern observed is usually that which would result in the most balanced genotype. Thus, for abnormalities involving the X-chromosome alone, (e.g. ring-chromosomes and isochromosomes), the abnormal X-chromosome is generally inactivated (Therman & Patau, 1974).

The best genetic balance results from inactivation of the normal X-chromosome in balanced X;autosome translocations and from

inactivation of the translocated X in unbalanced X;autosome translocations. Since the normal X-chromosome is inactivated in individuals with a balanced translocation, it follows that the expression of any X-linked genes disrupted by a balanced translocation will be adversely affected (section 1:3:3).

Clinically, a balanced X;autosome translocation is suspected when a female manifests an X-linked trait with the severity expected of an hemizygous male, and this provides an indication for chromosome analysis.

Mattei et al. (1982) summarised the data from one hundred and five X;autosome translocations. The normal X was consistently inactivated in 81% of individuals with balanced translocations, whilst the translocated X was consistently inactivated in 76% of individuals with unbalanced translocations. Most of the remaining individuals (15-18% in each category) had cells in which each X-chromosome was inactivated, 50% (or more) of which resulted in the most balanced genotype.

The translocated X-chromosome will be inactivated in individuals with an unbalanced translocation providing that it carries an inactivation centre. In such circumstances inactivation may spread to include autosomal sequences in some cells and result in partial monosomy for these loci. Somatic cell hybrids (section 1:2:4) were used to show inactivation of an autosomal gene (esterase D) by spreading of X-inactivation (Mohandas, Sparkes & Shapiro, 1982). Derivative X-chromosomes which form part of an unbalanced translocation and lack an inactivation centre can not be inactivated, and will result in dosage imbalance.

1:2:2 Deletions

Duplications and deletions both disrupt gene dosage. Deletions are generally more deleterious than duplications and are more frequently detected. They may occur in association with a variety of X-linked and autosomal diseases as either dominant or recessive mutations and as either microscopic (cytogenetically detectable) deletions or sub-microscopic deletions (detectable only by molecular methods).

Complex phenotypes (involving several disorders) may result

from micro-deletions ("microdeletion syndromes") or other chromosomal abnormalities resulting in loss of contiguous genes ("contiguous gene syndromes"). These often form an heterogeneous group in which the region(s) altered or deleted in all individuals define(s) essential sequences in the genome. These regions are referred to as the smallest region of overlap or "SRO". This principle will be applied to ordering genes around DMD in this project.

The largest well documented micro-deletion in which random X-inactivation still occurred, was that of KC, a female with mild mental retardation, who had partial ornithine transcarbamylase (OTC) deficiency and was heterozygous for chronic granulomatous disease. Her deletion was estimated to represent less than 10% of Xp by cytogenetic means (Francke, 1984). This deletion, together with other deletions of Xp21 will be discussed more fully below (section 1:3:6).

Clinically, a deletion is indicated when several X-linked disorders co-exist or when mental retardation occurs in association with an X-linked condition when this is not a usual feature.

The majority of sub-microscopic deletions on the X-chromosome, described in the literature, were derived from regions of the genome which are well characterised at the molecular level e.g. deletions of hemophilia A (Factor VIII), (Gitschier et al., 1985), hemophilia B (Factor IX), (Gianelli et al., 1983), OTC (Old et al., 1985) and Lesch-Nyhan disease (HPRT), (Yang et al., 1984). It is thus likely that sub-microscopic deletions will be described for other disorders as these are characterised more completely at the molecular level.

Males with interstitial deletions of their (unique) X chromosome are especially valuable for localisation of DNA markers, as they can be studied directly avoiding the necessity to construct somatic cell hybrids (section 1:2:3). Large deletions of the X-chromosome are less well tolerated in males than in females, however, so that deletions in males are likely to be more appropriate to refining the position of a probe than to its initial localisation.

	YEAR			
PHENOTYPE	1958	1966	1978	1987
Autosomal dominant	285	269 (+568)	736 (+753)	1390 (+1084)
Autosomal recessive	89	237 (+294)	521 (+596)	623 (+852)
X-linked	38	68 (+51)	107 (+98)	140 (+168)
Proportion of "confirmed" loci assigned to the X-Chromosome.	10%	13%	8.5%	7.0%

Table 1: Numbers of autosomal and X-linked assignments since 1958.

(After McKusick 1987. Figures in parenthesis represent loci which are not fully identifed or validated).

1:2:3 Somatic cell hybrid analysis.

Somatic cell hybrids are invaluable in separating the two derivative chromosomes of an X;autosome translocation. Such hybrids were instrumental in the assignment of PGK, G6PD and HPRT to the long arm of the X-chromosome (e.g. Shows & Brown, 1973,1974) and the construction of the earliest gene maps of the X-chromosome (Pearson et al., 1974).

The earliest such assignment was that of PGK to Xq (Grzeschik et al., 1972). This study failed to assign HPRT and G6PD to Xq due to a rearrangement in the hybrid used. The positions of HPRT and G6PD were resolved by 1975 (Brown et al., 1975).

A panel of somatic cell hybrids is an effecient way of localising X-linked (or autosomal) biochemical markers which are expressed in mouse cells. This technique can also be applied to the localisation of DNA sequences. Somatic cell hybrids have also been derived from females heterozygous for interstitial deletions of the X-chromosome (such as that of KC, described above) and provide a valuable contribution to mapping panels.

1:2:4 Linkage analysis.

In the absence of other indications, linkage of an X-linked disorder to a defined marker is often the first step towards its regional localisation. Suitable markers include (i) other disorders, (ii) expressed antigens e.g. Xg, (iii) enzymatic activities e.g. Glucose-6-phosphate dehydrogenase (G6PD) or (iv) restriction length polymorphisms ("RFLPs", described below).

Two loci are "linked" when they fail to show independent assortment at meiosis. Recombination between two loci is expressed as a recombination fraction ,theta, with values from 0-0.5 or 0-50%, and is related to the distance between them measured in centi-Morgans (0-50cM). This relationship is only linear over short distances (theta less than 0.25) due to double cross-overs. The maximum measurable distance between two loci by linkage analysis is comparable to that of two loci on separate chromosomes, which assort independently (i.e. greater than or equal to 50cM). The X-chromosome has been estimated at 200cM (Renwick & Schulze, 1964).

Linkage between two loci is measured in terms of the most

likely recombination fraction between them. The probability of linkage (as opposed to that of obtaining the same results by chance) at any given recombination fraction is expressed as a logarithm, "LOD" score (or logarithm of odds). Probabilities from separate investigations can be most readily combined in logarithmic form (by addition as opposed to multiplication). It is accepted that two loci are linked if the LOD score is greater than 3 and excluded if the LOD score is lower than -2 (Maynard-Smith, Penrose & Smith, 1961).

Linkage is normally expressed as a maximum LOD score at the appropriate recombination fraction with confidence limits at the appropriate values of theta at a LOD score of the maximum LOD score minus one.

Linkage of two loci on the X-chromosome can be implied from lower LOD scores than for autosomal conditions, as the X-linked nature of a disorder (and thus linkage to an X-specific marker) can be readily discerned from its mode of inheritance.

Prior to 1958, colour blindness, which affects only 6% of males, was the only useful marker for mapping loci on the X-chromosome. In 1958, Childs et al. demonstrated the X-linked nature of G6PD deficiency. Unfortunately, since G6PD deficiency was extremely rare amongst Northern Europeans, G6PD was not a very useful marker for linkage studies in Northern European families.

The Xg blood group described by Mann et al. (1962), in contrast, was heterozygous in 45% of Northern European females (data summarised by Sanger et al., 1971). Linkage of Xg was established to X-linked ichthyosis (XLI), ocular albinism (OA) and retinoschisis (RS), and excluded with G6PD, hemophilia A, hemophilia B, deutan and protan colour blindness and Duchenne muscular dystrophy. These findings are summarised in Race & Sanger (1975).

Restriction fragment length polymorphisms (RFLPs) reflect small differences between alleles at a restriction enzyme recognition site, which result in different fragment lengths on DNA digestion with the appropriate enzyme. These differences are detected by DNA sequences which share (or partly share) sequence homology to the restriction fragments in question and segregate as

mendelian traits. RFLPs can be considered as genetic markers and greatly enhance the reference points available for linkage analysis in the localisation of genetic disorders.

The first two X-linked RFLPs (RB6 and RC8) were reported at the Sixth International Human Gene Mapping workshop in 1981 (Skolnick & Francke, 1981). One hundred and twenty five X-linked RFLPs have now been described (Ninth International Human Gene Mapping workshop (Paris), (table 2)).

As at least one hundred and forty genes are presently assigned to the X chromosome, it follows that any DNA marker isolated on the X chromosome is likely to be closely linked to a number of disorders and to prove a valuable resource for the study of many conditions.

Linkage is difficult to establish at a distance of more than 20cM, by virtue of the number of analyses required to obtain a significant LOD score. Ideally, the closest marker should be no further than 10cM from the locus under study. Thus, for the entire human genome (of approximately 30M), one hundred and fifty evenly spaced loci would be required (Botstein et al., 1980). Theoretically over ten times this number of randomly spaced probes are required to acheive this (Lange & Boehnke, 1982). Even then, RFLPs would not be informative in all families studied.

If the X-chromosome is assumed to represent 2M then 100 randomly spaced RFLPs should ensure that no X-linked gene is further than 10cM from an RFLP. One hundred and twenty five X-linked RFLPs have now been described (table 2) and thus linkage studies are now theoretically possible for any locus on the X-chromosome.

For an X-linked condition, "Phase" (i.e. the determination of the combination of alleles derived from each chromosome) may be demonstrated by analysis of the maternal grandfather, mother and either an affected or an unaffected son. Alternatively it may be deduced using other relatives. This situation is considerably simpler than the situation for autosomal recessive conditions, which normally require results from an affected individual within the family. Thus diagnosis of the carrier state and prenatal or antenatal diagnosis (where appropriate) are often possible for

X-linked disorders even in the absence of a surviving affected individual. The reliability of such a diagnosis is dependent on the distance of the disorder from the closest informative marker(s). The most reliable results will be obtained from multiple closely linked markers flanking and/or within the gene.

Individuals are more likely to be multiply informative if high frequency RFLPs (with a rare allele frequency greater than 20%) are applied. Such individuals enable linkage and family studies to be performed more readily. One measure of the value of a probe is the polymorphic information content or "PIC" (Botstein et al., 1980). For X-specific loci the PIC value is considered formally equivalent to heterozygosity i.e. 2pq for di-allelic probes, where p and q are the allele frequencies (Willard et al., 1985). The closer the PIC value is to one the more useful a given marker will be.

Multi-allelic probes, for which a greater proportion of individuals will be heterozygous, are especially valuable. The first multi-allelic locus to be described was that of a randomly isolated probe detecting fifteen alleles (Wyman & White, 1980). Subsequently, many additional loci have been described e.g. 5' to the insulin gene (Bell, Selby & Rutter, 1982). These may occur as multiple independent restriction site alterations or as variable numbers of short tandem repeats ("minisatellites" or "VNTRs"), 11 to 60bp in length (Nakamura et al., 1987), in which the numerical variation may be revealed using any restriction endonuclease which lacks a restriction site(s) within the repeat unit.

Hypervariable probes will also prove invaluable in new applications e.g. mapping of human disease by linkage to specific bands in "DNA fingerprints" (multiple fragments detected by a consensus core repeat sequence), (Jeffreys, Wilson & Thein, 1985; Jeffreys et al., 1986).

		DNA SEGMENTS		
YEAR	LOCATION	GENES	ARBITARY DNA	TOTAL
1981	X-specific	NONE	10 (2)	10 (2)
(HGM6)	Total	16 (6)	35 (18)	51 (24)
1983	X-specific	7 (3)	75 (23)	82 (26)
(HGM7)	Total	104 (35)	215 (95)	319 (130)
1985	X-specific	12 (5)	202 (63)	214 (68)
(HGM8)	Total	249 (88)	559 (245)	808 (333)
1987	X-specific	32 (11)	273 (114)	305 (125)
(HGM9)	Total	610 (216)	2057 (977)	2667 (1193)

Table 2: Numbers of cloned genes, arbitary DNA segments and RFLPs reported for the X chromosome and total genome since 1981.

(After Willard et al (1985) and Pearson et al (1987). Numbers in parenthesis indicate the number of polymorphic DNA segments in each category).

1:3 DUCHENNE MUSCULAR DYSTROPHY (DMD).

This section focusses on Duchenne muscular dystrophy with reference to its phenotype, its X-linked nature and its regional assignment on the X-chromosome by means of both cytogenetic and linkage data. Contiguous gene syndromes which include DMD and a preliminary characterisation of the DMD locus using restriction fragment length polymorphisms are also presented.

1:3:1 Phenotype.

Duchenne muscular dystrophy (reviewed by Moser, 1984 and Emery, 1987) is a progressive myopathy which occurs at a frequency of 1 per 3000-4000 male live-births. DMD has an onset in the first five years of life and is characterised by pseudohypertrophy of the calves with wasting and weakness of the proximal muscles. Affected individuals become progressively weaker and are generally confined to a wheelchair by their twelfth birthday. In the terminal stages of DMD, muscle involvement is generalised and most boys with DMD will die as a result of respiratory or cardiac involvement in their teens or early twenties. Becker muscular dystrophy (BMD), which occurs in 1 in 30 000 male live-births is similar to DMD but has a milder clinical course.

Emery & Skinner (1976) reported that 97% of DMD patients were wheel-chair bound by age eleven and 94% died before attaining their sixteenth birthday. A child with DMD/BMD is thus usually defined as BMD if he is still ambulant at his sixteenth birthday. As both "mild Duchenne" and "severe Becker" mutations have been described, the division is somewhat arbitary and growing evidence from linkage analysis and deletion studies (see later) suggest that the two disorders are allelic.

DMD/BMD may occur in females (i) with Turner's syndrome (e.g. Walton, 1957), (ii) as a result of a second mutation in a carrier female (iii) as a result of non-random X-inactivation in carrier females (e.g. Emery, 1963) or (iv) in girls with X-autosome translocations disrupting the DMD gene (see below).

Inactivation of the normal X in the majority of cells in critical tissue(s) is thought to be responsible for the DMD phenotype in obligate carriers of Duchenne muscular dystrophy

(Dubowitz, 1982). Considerable heterogeneity in the severity of phenotype amongst manifesting carriers was attributed to the variation in the proportion of muscle cells in which the normal-X was inactivated (Moser & Emery, 1974).

Many muscle enzymes, notably creatine kinase, are elevated in individuals with DMD or BMD. These may also be elevated in individuals with other conditions but not to as great an extent as in males with BMD/DMD. Raised creatine kinase levels are evident in affected males before other symptoms become apparent. Many obligate carriers of DMD also have elevated levels of creatine kinase.

Prior to the application of RFLP analysis to the study of Duchenne muscular dystrophy (section 1:3:4), carrier analysis was dependent solely on the detection of elevated levels of creatine kinase in the heterozygous state and on pedigree analysis. Fetal sexing (and termination of all male pregnancies) was the only option available to those desiring prenatal diagnosis.

A third X-linked muscular dystrophy, Emery-Dreifuss muscular dystrophy (EDMD), (Emery & Dreifuss, 1966), with onset in childhood and slow progression, is characterised by wasting and weakness of muscles predominantly affecting peroneal muscles. It can be distinguished from DMD and BMD by contractures involving the neck, elbows and ankles which are recognisable at an early stage and by the absence of pseudohypertrophy which is indicative of DMD/BMD. EDMD is frequently associated with cardiac involvement and sudden death.

1:3:2 Mapping DMD to the X-chromosome.

Duchenne muscular dystrophy was first described in detail by Duchenne in 1868 and subsequently (in English) by Gowers in 1879. The majority of affected individuals were male and many pedigrees were clearly consistent with X-linked inheritance (e.g. Kostakov, 1934, cited in Gates 1946). This strongly suggested X-linkage but autosomal inheritance with limitation to males could not be excluded (section 1:1:1).

Emery (1964) reviewed three hundred and forty three families (including many previously described in the literature) with

sufficient clinical information to indicate a diagnosis of Duchenne muscular dystrophy. Affected females were described in only seven of two hundred and thirty one sporadic cases, three of fifty three familial cases (in which affected individuals occurred in more than one branch of the family) and five of fifty nine isolated affected sibships.

Further evidence for the X-linked nature of Duchenne muscular dystrophy was provided by brothers with DMD with the same mother but different fathers (e.g. Walton, 1955), females with Turner's syndrome (e.g. Walton, 1957; Ferrier, Bamater & Klein, 1965) and clinical manifestations in obligate carriers of DMD (Emery, 1963).

1:3:3 Evidence from X;autosome translocations for the regional localisation of DMD on the X-chromosome.

A female with a de novo X;1 translocation-inversion rearrangement and a myopathy with the severity and clinical characteristics of DMD (but with no family history of DMD), was a vital lead in the localisation of DMD on the X-chromosome (Lindenbaum et al., 1979).

Lindenbaum et al. (1979) proposed that the de novo translocation was likely to be responsible for DMD in this girl (unless the DMD locus was smaller than 3kb) and postulated two locations on Xp for the DMD locus (Xp11.06 and Xp21.07) based on the breakpoints in this patient. Distal Xp, broken at Xp21.07, was exchanged with distal 1p, broken at 1p34.00 whilst proximal Xp was simultaneously paracentrically inverted between Xp21.07 and Xp11.06.

Twenty females with DMD/BMD and X;autosome translocations have been described to date (reviewed in Boyd et al., 1986). The clinical course in females with X;autosome translocations is often less severe than in affected males although considerable variation in phenotypic severity is observed. This may reflect the heterogeneity found in males and depend on the exact position of the breakpoint or it could result from variation in the proportion of cells (up to 10%) in which the normal X is expressed and the translocation chromosome is inactivated (Worton, 1986).

Boyd & Buckle (1986) examined translocation breakpoints in

prometaphase chromosomes of nine females with X;autosome translocations. They were able to show cytogenetic heterogeneity of the breakpoints amongst these individuals, suggesting that the DMD gene was large, but could not correlate the differences observed with those evident at the phenotypic level. Six breakpoints were defined within Xp21.2, two within Xp21.1 and a third within either Xp21.2 or Xp21.3. These bands were estimated to represent 5 Mega-basepairs (Mbp), 2Mb and 4Mbp respectively, assuming the X-chromosome is equivalent to 150Mbp or 5% of the haploid genome.

1:3:4 Linkage analysis and DMD.

Linkage of DMD to both Xg (reviewed in Race & Sanger, 1975) and colour-blindness (Emery, 1966; Greig, 1977) was excluded. Localisation of DMD to Xp21 was supported by linkage to the DNA probes L1.28 and RC8 on Xp (Murray et al., 1982; Davies et al., 1983).

RC8 and L1.28 each lie about 15cM from DMD but on opposite sides (Davies et al., 1983) and were used in the first RFLP determination of carrier status (Wieacker et al., 1983a). Individuals doubly heterozygotes for both these probes are rare, and account for only 10% of the population, which severely limits their usefulness.

By 1985, however, seven additional Xp markers flanking DMD which were closer to DMD than either L1.28 (754 and OTC) or RC8 (pXUT23, 99.6, D2, B24 and C7) had been isolated. At least one of these probes was heterozygous in 95% of individuals and a diagnostic reliability of greater than 96% for greater than 80% of potential carriers was predicted, (Bakker et al., 1985). The positions of these two probes and many others described throughout this project are illustrated in the discussion (figure 14).

A maximum LOD score of 1.25 at theta = 0.25 gave a suggestion of linkage of BMD to colour-blindness on distal Xq (Skinner, Smith & Emery, 1974), which would have excluded the two phenotypes being allelic. BMD was subsequently linked to L1.28 (Kingston et al., 1983), suggesting a close association with, or allelism to, DMD. Linkage of EDMD has been shown to both the factor VIII gene and DX13 (DXS15) in distal Xq (Boswinkel et al., 1985; Hodgson et al.,

1986b; Thomas et al., 1986b; Yates et al., 1986).

1:3:5 Application of pERT 87 and XJ RFLPs to define the DMD locus.

The pERT87 locus (Kunkel et al., 1985a) was cloned from a male,"BB", with a microdeletion of Xp21 (Francke et al., 1985). The XJ locus (Ray et al., 1985) was cloned from a female with an X;21 translocation which disrupted both the DMD/BMD locus and the ribosomal RNA cluster on chromosome 21 (Verellin-Dumoulin et al., 1984). The methods applied to clone both these loci will be summarised in the discussion (section 4:1)

Probes derived from the pERT87 locus (DXS164, Kunkel et al., 1985a) and from the XJ locus (DXS206, Ray et al., 1985) were shown to detect deletions in 6-10% of boys with either DMD or BMD (Monaco et al., 1985; Kunkel et al., 1986). It was soon apparent that no probe which detected deletions was consistently missing in all individuals with deletions.

X;autosome translocations were also studied with probes from these two loci. Three breakpoints were described proximal to both pERT87 and XJ, two breakpoints distal to both pERT87 and XJ and one (from which the XJ locus was derived) to the distal edge of the XJ locus but proximal to pERT87 (Boyd et al., 1986). These results indicated heterogeneity at the DMD locus, consistent with the findings of high resolution chromosome analysis (Boyd & Buckle, 1986).

RFLPs at the pERT87 (Kunkel et al., 1985a) and XJ (Ray et al., 1985) loci recombined with the DMD phenotype at a frequency of 5% (Berteleson et al., 1986; Davies (report on Fifth Muscular dystrophy group workshop), 1986; Fischbeck et al., 1986; Thompson et al., 1986). These probes were heterozygous in greater than 90% of individuals, but their relative positions with respect to the DMD mutation was dependent on the families studied, (Kunkel et al., 1986).

It thus became apparent that it was advisable to use a combination of intragenic probes and probes flanking DMD, unless the precise site of the mutation in the family under study was known. The closest proximal marker is 754 (Hofker et al., 1985), approximately 6cM from DMD, whilst the closest distal markers are

C7 (de Martinville et al., 1985) and B24 (Aldridge et al., 1984), more than 10cM from DMD.

The high recombination frequency, together with the detection of deletions in 5-10% of boys with DMD/BMD, indicated that the pERT87 and XJ loci were close to "the DMD mutation" and probably intragenic. The DMD gene was thus required to be either a single gene (larger than any previously described gene), or a large gene complex, which could encompass all translocation breakpoints and both XJ and pERT87 (which both recombined at a frequency of 5% with DMD). Either hypothesis could explain the phenotypic heterogeneity found amongst boys with DMD/BMD if certain domains or certain genes could exert specific effects and it was thus apparent that additional DNA markers were required to help define the gene.

1:3:6 Complex phenotypes in Xp21.

A number of individuals with complex phenotypes (or "contiguous gene syndromes") including Duchenne muscular dystrophy (i.e. with several distinct phenotypes, one of which is DMD) have been described. Many are associated with micro-deletions of Xp21 and represent deletions of adjoining genes.

The microscopic deletion in KC, a girl with mild mental retardation, partial ornithine transcarbamylase (OTC) deficiency and heterozygous for chronic granulomatous disease (CYBB), was estimated (by cytogenetics) at less than 10% of Xp (Francke, 1984). Random X-inactivation was observed in KC which explains her partial OTC deficiency.

BB, a male with an interstitial deletion of Xp21, was affected with chronic granulomatous disease (CYBB), McLeod red blood cell phenotype (Kell antigen), (XK), Duchenne muscular dystrophy (DMD) and one form of retinitis pigmentosa (RP3), (Francke et al., 1985). His deletion confirmed that DMD mapped to Xp21 and was central to molecular studies of Xp21 (see section 4:1). As KC's deletion encompassed that of BB (Francke et al., 1985), it must also have included the loci for DMD, XK and RP3.

A similar deletion was reported in a male child with oroticaciduria, lacticaciduria and glyceroluria (consistent with OTC deficiency and glycerol kinase (GK) deficiency) and

histological alterations of his adrenal glands, consistent with a phenotype of congenital adrenal hypoplasia (AHC). A cytogenetic deletion was observed in his phenotypically normal mother, with presumptive breakpoints at Xp11.2 and Xp21 (Hammond et al., 1985). It can be assumed by comparison to the deletions in BB and KC that this deletion also included the loci for DMD and CYBB.

Many patients have been described with (infantile) GK deficiency in association with AHC, mental retardation and myopathy (DMD), (Renier et al., 1983). These were shown to have microscopic (Saito et al., 1986) and/ or molecular (Wieringa et al., 1985; Clarke et al., 1986; Dunger et al., 1986; Francke et al., 1987) deletions in Xp21. GK in association with AHC, without myopathy, has also been reported (Bartley, Miller & Hayford, 1982; Francke et al., 1987). One of these patients, YB, had a small microscopic deletion of Xp21 (Francke et al., 1987).

These deletions assign OTC, CYBB, XK, RP3, GK and AHC (and DMD) to Xp21. The clinical course of all these disorders are described by McKusick (1986). The chromosomal position of these genes and of others described throughout this project are illustrated in the discussion (figure 15).

Molecular techniques have permitted more detailed comparisons of micro-deletions and have enabled the relative order of many of these genes to be established (see discussion). Interstitial deletions of Xp21, particularly those in males, which avoid the need to construct somatic cell hybrids, are also of value in localising new probes.

1:4 STEROID SULPHATASE (STS).

This section introduces X-linked ichthyosis (XLI) associated with steroid sulphatase (STS) deficiency. It focusses on the aetiology of XLI and the regional mapping of STS to the X-chromosome by means of somatic cell hybridisation studies, chromosome aberrations and linkage analyses. It concludes with mechanisms of inactivation and evidence that STS escapes from inactivation.

1:4:1 Steroid sulphatase (STS) and STS deficiency.

Steroid sulphatase (STS), (reviewed by Shapiro, 1985) is a microsomal enzyme, present in all mammalian tissues, involved in the hydrolysis of 3- beta- hydroxy steroid sulphates (dehydroepiandrosterone sulphate, estrone sulphate and cholesterol sulphate) and necessary for the conversion of sulphated steroid precursors to estrogen during pregnancy in man.

Within microsomes, STS occurs as a polymer (Mr 533 000), (Noel et al., 1983). The largest form of the monomer found in fibroblasts is a glycoprotein (Mr 63 000), which is subject to post-translational processing by removal of N-linked oligosaccharide chains within two days of its synthesis. The mature form of the monomer (Mr 61 000) has a half-life of four days (Conary et al., 1986).

STS is deficient in individuals with X-linked ichthyosis and also deficient or severely reduced in individuals with multiple sulphatase deficiency, an autosomal recessive disorder in which arylsulphatases A, B and C are also deficient or reduced. Purified STS protein has itself been shown to have arylsulphatase C activity.

Complementation experiments indicated that these two disorders represent discrete defects, as indicated by their modes of inheritance (Ballabio et al., 1985). The primary defect in multiple sulphatase deficiency has not been determined, but may involve post-translational processing or a co-factor (Steckel, Hasilik & von Figura, 1985).

X-linked ichthyosis, associated with scaly skin, occurs in 1

in 5000-6000 male live-births. The rare female individuals with X-linked ichthyosis are either Turner's (45X) individuals (Solomon & Schoen, 1971) or homozygous for the deficiency e.g. as a result of consanguinity (Stern, 1973). STS escapes inactivation (see below) and thus affected females are not observed as a result of non-random inactivation.

Scaling in males with X-linked ichthyosis is believed to be due to an accumulation of cholesterol sulphate, a substrate of STS, which can induce similar scaling in mice when applied directly. Onset of ichthyosis usually occurs at about 3-6 months of age although it may be present from birth (Shapiro, 1985).

Deficiency of STS is also associated with lack of placental production of estriol in pregnancy and prolonged and delayed labour. These prenatal manifestations may often be the first to be appreciated.

The link between placental sulphatase deficiency and X-linked ichthyosis remained unnoticed until relatively recently (Jobsis et al., 1976; Koppe et al., 1978; Shapiro et al., 1978). Individuals with X-linked ichthyosis enjoy good health and fertility although some patients have been observed with testicular anomalies. Shapiro (1985) postulated that these patients may have deletions involving additional loci.

1:4:2 Regional localisation of STS to Xp22.3.

Cockayne (1933) recognised the X-linked nature of one form of ichthyosis but was unable to distinguish this clinically from the autosomal dominant form. This clinical distinction was first made by Kerr & Wells in 1965.

STS was localised in the region Xp22 to Xpter using a panel of human-Chinese hamster somatic cell hybrids (Mohandas et al., 1979) and to Xp22.3 on the basis of a boy with an XY translocation and X-linked ichthyosis, whose translocation breakpoint was at Xp22.3 on his X-chromosome (Tiepolo et al., 1980).

Males with XY translocations are effectively nullisomic for distal Xp and generally have X-linked ichthyosis and short stature. Most of these are not associated with additional disorders (see below), although chondrodysplasia punctata (CPDX) was not

excluded in several cases. Metaxotou et al. (1983) described a male with a familial XY translocation and ichthyosis, hypogonadism and mental retardation.

Male fertility, but not female fertility is lost as a result of XY translocations. Female XY translocation carriers demonstrate prefential transmission of the translocated X-chromosome to their progeny, as demonstrated by the 13:3 ratio of translocation bearing to non-translocation bearing siblings (Allderdice et al., 1983). There is no evidence, as yet, to suggest that such anomalous segregation is found in the progeny of obligate carriers of X-linked ichthyosis.

A female who carried such an XY translocation was central to the assignment of Xg to distal Xp (Xp22.3 to Xpter). Her mother was Xg(a-) and her father Xg(a+) whilst she was Xg(a-). Her failure to inherit her father's Xg(a) allele, allowed Xg to be assigned to the distal portion of the X chromosome lost in the translocation (Ferguson-Smith et al., 1981).

Deletions were cytogenetically detectable in Xp22.3 in four affected individuals, obligate carriers and some potential carriers from two kindreds segregating for X-linked ichthyosis and chondrodysplasia punctata (CPDX), all of whom had short stature (Curry et al., 1982, 1984). The deletion in UCLAB2, a hybrid derived from one of these individuals (Curry et al., 1984) was estimated at 5Mb (Mondello et al., 1987) and encompassed Xp22.3 including MIC2, STS and probe M1A (DXS31).

X-linked ichthyosis has also been described in association with hypogonadism, anosmia and neurological defects (Sunohara et al., 1986) and with Kallmann's syndrome (hypogonadotropic hypogonadism and anosmia) alone (Ballabio et al., 1986).

1:4:3 Linkage analysis and X-linked ichthyosis.

Early studies showed linkage of Xg to X-linked ichthyosis, the first of which was by Kerr,Wells & Sanger in 1964. This data is summarised in Race & Sanger (1975) and in Keats et al. (1979) and placed XLI 11cM and 15cM from Xg respectively.

STS levels in XX males (which arise from the interchange of Yp material, including the testis determining factor (TDF), with Xp

material, Ferguson-Smith, 1966) were typical of female levels with few exceptions, whilst the distribution of Xg phenotypes was male-like. This suggested that Xg, but not STS, had been lost in the interchange of Xp and Yp sequences, and thus that STS was proximal to Xg (Ropers et al., 1981).

Wieacker et al. (1983b) examined sixteen families for linkage with RC8 (which was known to have positive LOD scores with Xg). They found only one informative family which resulted in a maximum LOD score of 0.45 at 25cM. Ballabio et al. (1986) were able to show a suggestion of linkage to both Xg and Dic56 (DXS143) in a family segregating for X-linked ichthyosis, hypogonadotropic hypogonadism and anosmia (XLIHA). Maximum LOD scores of 1.44 for XLIHA-Xg and 1.55 for XLIHA-Dic56 were obtained respectively, at theta = 0 (since no recombinants were observed).

1:4:4 The maintenance and "spreading" of X-inactivation.

One of the most interesting features of STS is that it escapes X-inactivation. Evidence for this will be presented in section 1:4:5. Methylation is considered to be the most likely mechanism by which the pattern of X-inactivation is maintained, since methylation patterns are somatically heritable in mammalian cells and many genes have been reported which are underexpressed when methylated at their 5' ends. (Other possible mechanisms are reviewed in Gartler & Riggs, 1983).

This is supported by experiments with 5-azacytidine (which inhibits methyltransferase activity and hence promotes demethylation of DNA). 5-azacytidine treatment is most effective in late S phase when the inactive X chromosome is replicating. It requires two cell cycles before a phenotypic effect is observed (which presumably allows complete demethylation).

5-azacytidine can cause reactivation of both HPRT and PGK in mouse-human hybrids and results in a stable revertant phenotype in the absence of selection, and in spite of the lack of spreading of inactivation in tissue culture (Mohandas et al., 1981).

X-inactivation may spread to adjacent autosomal loci in human X;autosome translocations (Mohandas, Sparkes & Shapiro, 1981). Similar spreading of inactivation to autosomal sequences has been

observed for Cattanach's translocation in the mouse, in which part of chromosme 6 with several well-defined markers, has been inserted into the X-chromosome. Coat colours are more frequently inactivated at either edge of the inserted segment than at its centre, and are maintained more stably. It may be that proximity to inactivated X-linked sequences stabilises inactivation and that those autosomal sequences at the centre of the insert are furthest from this influence. This predicts that sequences (or structures) on the X-chromosome can in some way enhance X-inactivation or that sequences (or structures) on the autosomes can hinder inactivation (reviewed in Gartler & Riggs, 1983).

The STS gene may thus lack sequences which enhance inactivation or contain sequences which hinder it. Schorderet et al. (1987) have reported mouse cell revertants which demonstrate STS activity following treatment of two STS deficient cell-lines (which themselves arose from STS proficient lines) with 5-azacytidine. It would thus appear that, in these deficient cell-lines, STS deficiency was due to aberrant methylation and that inactivation can spread to regions of the X-chromosome which normally escape inactivation.

STS was shown to escape X-inactivation (Keitges et al., 1985; Keitges & Gartler, 1986) in the mouse, but to be pseudoautosomal with a functional Y-linked allele (Keitges et al., 1985, 1986).

The pattern of inheritance of X-linked ichthyosis precluded a functional Y-linked allele for STS in man. This begged the question as to how much homology (if any) there was to STS on the human Y-chromosome. This will be addressed further in the discussion.

1:4:5 Evidence that Xg, STS and MIC2X escape X-inactivation.

Xg, STS and MIC2X (which codes for a polypetide recognised by 12E7 antibody), in distal Xp, are all known to escape (or partially escape) X-inactivation. Only MIC2X has a functional Y-homologue (MIC2Y).

The first indication that Xg escaped inactivation came with the realisation that all cells from Xg(a)/ Xg individuals reacted with anti-Xg(a) antibody (Gorman et al., 1963). Similarly, both cell populations in females doubly heterozygous for X-linked

sideroblastic anemia and Xg, expressed Xg(a) equally (Weatherall et al., 1970). Furthermore Xg(a) was expressed from the inactive (maternal) X in individuals heterozygous for both HPRT and Xg, (Fialkow, 1970).

Escape of STS from inactivation was demonstrated by both fibroblast and somatic cell hybrid studies. Fibroblast clones derived from obligate carriers of STS deficiency, and from individuals doubly heterozygous for G6PD variants and STS deficiency, all demonstrated STS activity, (Shapiro et al., 1979). Mohandas et al. (1980) demonstrated that human STS was expressed in somatic cell hybrids which contained an X-chromosome in an otherwise inactive state.

Normal males, who possess one dose of STS, express STS at approximately half the level of normal females (with two doses). The ratio of expression in females to that in males is consistently less than two throughout a range of tissues e.g. fibroblasts and lymphocytes (Muller et al., 1980) and placenta (Bedin et al., 1981). This implied that STS may not completely escape X-inactivation. This contention was supported by somatic cell hybrid studies in which the STS activity of the inactive X appeared lower than that of the active X (Migeon et al., 1982).

The MIC2 locus was cloned by Darling et al. (1986). Evidence that MIC2X escapes X-inactivation was provided by the expression of 12E7 antigen in two independent human-rodent cell-lines with a single inactivated X-chromosome, in which three unrelated X-linked enzymes were not expressed (Goodfellow et al., 1984). However, 12E7 antigen was expressed less strongly than in control hybrids, with either a Y chromosome or an active X-chromosome. This indicated that either the inactive X-chromosome had been lost from a proportion of cells, or that 12E7 was expressed at a lower level from the inactived X-chromosome, as proposed for STS.

1:5 AIMS OF PRESENT RESEARCH.

The aim of this project was to perform a molecular analysis of Duchenne muscular dystrophy (DMD) and of X-linked ichthyosis (XLI) and of disorders found in association with either condition. It was thus necessary to isolate X-specific probes from within Xp21 (for DMD) or Xp22.3 (for XLI).

The aims, outlined below, involve the isolation of Xp-specific sequences, an assessment of their value in the study of DMD or XLI, and the applications of suitable probes. They can be summarised as follows:

1) To construct an X-chromosome specific library using flow-sorted X-chromosomes.

2) To screen the library for single copy human recombinants.

3) To use somatic cell hybrids to determine which of these sequences were X-linked and to regionally localise these sequences on the X-chromosome.

4) To submit sequences assigned to Xp to further analysis by
(i) searching for restriction fragment length polymorphisms (RFLPs)
(ii) screening for deletions in affected males.

5) To study the DMD locus by
(i) using appropriately placed probes which detected RFLPs to undertake family studies and perform a linkage analysis.
(ii) using probes which detected deletions in males with DMD to examine the frequency at which deletions occur.

6) To study the XLI locus by
(i) using appropriately placed probes which detected RFLPs to undertake family studies and perform a linkage analysis.
(ii) using appropriately placed probes to study the molecular pathology of X-linked ichthyosis.

7) To compare data obtained using these probes with that obtained using pre-existing probes, and to determine the extent of DMD deletions.

8) To investigate conservation between mammalian species of any Xp sequences of particular interest, which could serve to indicate whether or not they might be expressed.

CHAPTER 2: MATERIALS AND METHODS.

2:1 MATERIALS

2:1:1 General

Centrifuge tubes and other "disposable plastics" were supplied by Sarsdedt, with the exceptions of 50ml Falcon 2070 tubes, SW41 ultra-centrifuge tubes (Sorvall), universals and bijoux (Sterilin 128C and 129B), 1ml (blue) pipette tips (Treff), plastic pastettes (Alpha-Labs) and plastic petri dishes ("plates"), (Nunc 140mm and 100mm).

The centrifuges used were (i) Sorvall RC-5B (HB4, SM24 and HS-4 rotors), (ii) IEC DPR-6000, (iii) Sorvall ultra-centrifuge and (iv) IEC centra-4X microfuge.

Other equipment and consumables are described at appropriate points in the text. Details of the solutions used are presented in section 2:1:2.

2:1:2 Solutions

All solutions were prepared with Sterile distilled water in clean glass-ware and were sterilised in a pressure cooker at 15psi (pounds per square inch) for fifteen minutes unless otherwise stated. These are listed in the order of their first appearance in the methods section which follows.

SM /litre:		
	NaCl	5.8g
	$MgSO_4.7H_2O$	2g
	1M Tris.Cl (pH7.5)	50ml
	2% gelatin	5ml.

L (Luria-Bertani)Broth /litre:		
	Bacto-tryptone	10g
	Bacto-yeast extract	5g
	NaCl	10g

L-plates were made 1.5% in Difco Bacto-agar, whilst top agar for preparing phage was 0.7% in Bacto-agar (or 0.5% in Bacto-agar for scraping protocol).

TM: 50mM Tris.Cl (pH7.8)
10mM $MgSO_4$

Phenol/ Chloroform: 50% Phenol,
(Not sterilised) 50% Chloroform,
0.1% 8-hydroxyquinoline.
Phenol/chloroform was saturated and equilibrated to pH7.5 with TE buffer.

DNA isolation buffer: 150mM NaCl
(Chromosomal) 10mM Tris.Cl (pH8.0)
10mM EDTA

TE Buffer: 10mM Tris (pH7.5)
1mM EDTA (pH8.0)

10 x Ligase buffer: 0.5M Tris.Cl (pH7.4)
(DTT was filter 0.1M $MgCl_2$.
sterilised) 0.1M Dithriothreitol (DTT)
1mg/ml Bovine serum albumin (BSA)

Depurination solution: 0.2M HCl.

Denaturation Solution: 0.5M NaOH
1.5M NaCl.

Neutralisation Solution: 3M NaCl
0.5M Tris.Cl (pH 7.4).

20xSSC: 3M NaCl
300mM Na Citrate (pH7.4)

Prehybridisation Buffer: (Not sterilised)	50% Formamide (deionised) 5x Denhardts 5x SSC 50mM Sodium Phosphate (pH6.8) 380µg/ml sonicated, denatured salmon sperm DNA 0.2% SDS 10µg/ml Poly(A).
Hybridisation Buffer: (Not sterilised)	50% Formamide (deionised) 1x Denhardts 5x SSC 20 mM Sodium Phosphate pH 6.8 100µg/ml sonicated, denatured salmon sperm DNA, 20µg/ml Poly (A) 10% Dextran Sulphate.
100x Denhardts Solution:	2% Ficoll 2% Bovine Serum Albumin 2% Polyvinylpyrrolidone
Phosphate buffer/500ml:	NaH_2PO4 37g (anhydrous 28g) Na_2HPO4 44g (anhydrous 35g)
Dextran sulphate/ litre:	750ml water was boiled in a 2 litre beaker and 500g dextran sulphate added. This was stirred, heating as necessary until dissolved, and the volume adjusted to one litre.

poly A: 100mg was dissolved in 5ml sterile distilled water.

Deionised formamide: This was prepared immediately prior to use. 5g ion exchange resin (Bio-Rad Ag 501-X8 20-50 mesh) was added per 50ml, stirred with a magnetic stirrer at room temperature for one hour and filtered twice through Whatman Number one filter paper.

TES buffer: 0.3M Tris (pH9.0)
0.15M EDTA
1.5% SDS

"Oligo" reaction mix: solutions A:B:C in a 100:250:150 ratio:

Solution A: 1ml solution O
(stored at-20°C) 18µl 2-mercaptoethanol
5µl each of 100mM dATP,
dGTP and dTTP (in TE).
Solution O: 1.25M Tris.Cl (pH8.0),
(stored at 4°C) 0.125M $MgCl_2$

Solution B: 2M HEPES (pH6.6)
(stored at 4°C)

SolutionC: Hexadeoxyribonucleotides (Pharmacia
(stored at -20°C) PL number 2166) evenly suspended at
90 OD units per ml.

1x "oligo" stop mix: 20mM NaCl
20mM Tris.Cl pH7.5
2mM EDTA
1µM dCTP (normally omitted)
0.25% SDS

SOB: Bacto tryptone 2%
Bacto yeast extract 0.7%
NaCl 10mM
KCl 2.5mM

Filter sterilised 1M $MgCl_2$ and 1M $MgSO_4$ were each added to 10mM prior to use. SOB botttom agar (for plates) was prepared by the addition of Difco bacto-agar to 1.5%.

RF1 :

RbCl	100mM
$MnCl_2.4H_2O$	50mM
Potassium acetate (pH7.5)	30mM
$CaCl_2.2H_2O$	10mM
glycerol	15%w/v

The pH was adjusted to pH5.8 with 0.2M acetic acid. RF1 was sterilised by filtration (prior to the addition of sterile glycerol).

RF2:

MOPS (Morpholinopropanesulfonic acid)	10mM
RbCl	10mM
$CaCl_2.2H_2O$	75mM
glycerol	15%w/v

The pH was adjusted to pH6.8 with NaOH. RF2 was sterilised by filtration (prior to the addition of sterile glycerol).

Ampicillin: Ampicilin was dissolved in sterile distilled water at 25mg/ml and stored in 1ml aliquots at -20°C.

X-gal: 100mg X-gal was dissolved in 5ml Dimethyl formamide (DMF) and stored at -20°C.

STET:
50mM Tris.Cl (pH8.0)
50mM EDTA
8% sucrose
5% Triton X-100 (BDH Chemical Ltd.)

Bacterial lysis mix:
50mM Tris.Cl (pH7.5)
50mM EDTA
15% sucrose

Anticoagulant:
4.5% (w/v) EDTA,
0.7% (w/v) NaCl

Lysis Buffer: 0.32M Sucrose
10mM Tris (pH 7.5)
20mM $MgCl_2$
1% Triton X100

NaCl/ EDTA: 0.075M NaCl
(proteinase K buffer) 0.024M EDTA (pH 7.5)

Loading mix: 50% Glycerol
2% Ficoll
50mM EDTA
Pinch of Orange G.

50x EB/ litre: Tris base 242g
glacial acetic acid 57.1ml
0.5M EDTA (pH8.0) 100ml

Prehybridisation/ hybridisation solution:
50% formamide
3x Denhardt's
5xSSC
25mM sodium phosphate (pH6.8)
250µg/ml sonicated denatured salmon sperm DNA
1% SDS
20µg/ml poly A
10% dextran sulphate.

2:2 LAMBDA PHAGE METHODOLOGY.

2:2:1 Preparation of plating cells (Maniatis, Fritsch & Sambrook, 1982).

100ml cultures in L-broth (see section 2:5:1) of the appropriate plating cells were incubated overnight, with vigorous shaking, at 37°C. The cells were pelleted in sterile Falcon 2970 tubes by centrifugation (IEC DPR-6000) at 2.8K for ten minutes at 4°C and resuspended in 10-20mls SM buffer. The optical density at 600nm (OD_{600}) was read on a Perkin-Elmer 6000 spectrophotometer and the cell concentration adjusted to an OD_{600} of 2.0 (or approximately 1600 million cells/ ml).

2:2:2 Titration of Phage stocks (plate lysis) (Maniatis et al, 1982).

Serial dilutions (eg 10^{-3}, 10^{-5} and 10^{-7} dilutions) of the phage stock to be titred were prepared in SM buffer. 100µl of each dilution was added to 100µl of the appropriate plating cells and left to adsorb for twenty minutes at 37°C in a bijou (Sterilin). 3mls of 0.7% top agar were added to each bijou, the contents mixed gently and poured on L-plates. Control plates with (i) cells only and (ii) cells + SM buffer were also set up. The plates were inverted and incubated at 37°C overnight. The titre per ml was then calculated by multiplying the dilution factor by ten times the number of plaques obtained.

2:2:3 Optimisation of liquid lysis conditions.

A series of small scale liquid lysis preparations were performed in order to determine the optimum ratio of phage to cells. Ratios from 1:200 to 1:20 bacteriophage per cell are generally required to obtain lysis, dependent on the bacteriophage strain used. Ratios from 1:50 to 1:1000 were prepared. The appropriate amount of a 10^{-2} dilution of the bacteriophage stock was added to 100µl plating cells in a universal and the volume adjusted to 200µl with SM buffer. After twenty minutes at 37°C in order to allow adsorption to occur, 10ml L-Broth was added, and the universal taped in position in the shaking incubator overnight (or

until lysis occurred). The ratio(s) at which optimum lysis occurred (eg 1:500 for NM1149 with NB78) were then used for large scale preparations (section 2:2:4).

2:2:4 Large scale Lysis (Blattner et al,1977; Maniatis et al, 1982).

The optimum ratio, determined as described in section 2:2:3 was scaled up to 6.25mls cells (ie 10^{10} cells) and 2 x 10^{8} phage (for NM1149). Adsorption was performed at 37°C for twenty minutes. 500mls L-broth (prewarmed to 37°C) was added and the culture incubated at 37°C with vigorous shaking for six hours. Lysis was generally apparent. 10ml chloroform (Analar reagent grade) was added to each flask and incubation continued for a further thirty minutes.

2:2:5 Purification of bacteriophage lamda (Yamamoto et al, 1970; Vande Woude et al, 1979; Maniatis et al, 1982).

The culture was transferred to a second sterile flask and left to equilibrate at room temperature. DNaseI (Boehringer Mannheim) and RNaseA (Sigma) were each added to a final concentration of 1µg/ml and the culture incubated at room temperature for thirty minutes. Solid sodium chloride was added to a final concentration of 1M and dissolved by swirling. The culture was left for one hour on ice. The debris was pelleted by centrifugation at 2.8K for ten minutes at 4°C (Sorvall RC-5B, HS-4 rotor) and the supernatant transferred to a third sterile flask. Solid polyethene glycol (Sigma) was added to 10% (weight/ volume) and dissolved by slow stirring at room temperature. The bacteriophage suspension was then cooled on ice water overnight to enable the bacteriophage to precipitate.

Bacteriophage were recovered by centrifugation (Sorvall RC-5B, HS4 rotor) at 2.8K for ten minutes at 4°C. The supernatant was discarded and the pellet drained thoroughly. The phage pellet was resuspended in 2.5-5ml TM (50mM Tris 7.8, 10mM MgSO4) and extracted once with chloroform. The suspension was then loaded on top of a glycerol step gradient prepared in a Sorvall SW41 tube. The step comprised 3ml 40% glycerol in TM, followed by 4ml 5%

glycerol in TM. The volume was "made up" with TM. The step gradient was subjected to centrifugation at 35 000 rpm for one hour at 4°C (Sorvall OTP-65 ultracentrifuge, SW41 rotor). The supernatant was discarded and the bacteriophage pellet resuspended in 1ml TM.

2:2:6 Preparation of bacteriophage lambda DNA.

Pure DNaseI and boiled RNAse A were added to the bacteriophage suspension to 5µg/ml and 10µg/ml respectively. Incubation for thirty minutes at 37°C was performed. 0.5M EDTA (pH 8.0) was added to 20mM, 10mg/ml proteinase K (Gibco, Bethesda Research Laboratories (BRL)) to 50µg/ml and 10% SDS (Sigma) to 0.5%, prior to incubation at 65°C for one hour. The phage suspension was then combined with an equal volume of phenol in a 13ml Sorvall tube, mixed by gentle inversion and the phases separated by centrifugation (Sorvall RC-5B, HB4 rotor, 6K, five minutes, room temperature).

The aqueous phase was removed with a plastic pastette (Alpha Labs), extracted once with equilibrated phenol:chloroform (50 parts : 50 parts) and then once more with chloroform. Phage DNA was precipitated at-20°C overnight, by the addition of sodium acetate (pH7) to 0.2M and an equal volume of isopropanol. Phage DNA was pelleted by centrifugation (10K, 10minutes, 4°C, Sorvall RC-5B, HB4 or SM24 rotors), washed twice with 70% ethanol and resuspended in 500µl TE.

2:3 CONSTRUCTION OF X-CHROMOSOME LIBRARY (Fuscoe, Clark & van Dilla, 1986).

The X-chromosome specific library was derived from complete (EcoRI) digestion of flow sorted X-chromosomes. This avoided the need to subclone recombinants prior to their localisation on Xp.

The library was constructed in bacteriophage lambda NM1149 which accepts inserts from 0-11kb in size. The host bacteria NM514 permitted only those phage disrupted at the cI gene (ie at the EcoRI or HindIII insertion sites of the vector) to enter a lytic cycle and thus selected for recombinants. Non-recombinant phage were constrained to undergo a lysogenic cycle. NM514 was recombination proficient, however which is likely to have selected

against inserts containing inverted repeats.

2:3:1 Preparation of bacteriophage (vector) arms.

50µg phage DNA was digested with 30U EcoRI (BRL) using BRL core buffer for three hours at 37°C. 25µg were treated with 1U CIP (calf intestinal phosphatase, Boehringer Mannheim (22U/µl), diluted in core buffer) for thirty minutes at 37°C. The CIP was inactivated by heating to 70°C for five minutes and cooling on ice, twice.

Three phenol/chloroform extractions and a chloroform extraction were performed and the vector arms prepipitated by the addition of 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of 95% ethanol overnight at -20°C. The arms were pelleted by centrifugation (twenty minutes, 12 000 rpm, IEC Centra-4X microfuge, room temperature), washed with 70% ethanol, vacuum dried (HETO CD3 vacuum drier) and resuspended at 100µg/µl in TE.

2:3:2 Growth of cell-lines and sorting of X-chromosomes.

Lymphoblastoid cell lines were established and maintained by L. Snaddon or M. Clarke, esssentially as described by Neitzel (1986). Samples for flow karyotype analysis were prepared and analysed by A. Cooke, essentially as described in Harris et al (1985).

X-chromosomes were sorted by virtue of their relative fluoresence after staining with ethidium bromide. The X-chromosomes used in his study were derived from the lymphoblastoid cell line of MD, a patient with a 4;7 translocation. This translocation alters the relative fluorescence of one chromosome seven and removes it from the chromosome peak which contains two chromosome sevens and two X-chromosomes (and some chromosome 8) in normal female individuals, such that the relative proportion of X-chromosomes obtained in this peak is correspondingly increased.

2:3:3 Preparation of Chromosomal DNA (Fuscoe et al, 1986).

The sorted chromosomes were combined in a 1.5ml eppendorf tube (Sarsdedt). This was placed within a 13ml Sarsdedt tube and spun at 20K (SM24 rotor) for one hour at 4°C (Sorvall RC-5B centrifuge). The supernatant was removed and the chromosomal pellet resuspended

in 100µl DNA isolation buffer (150 mM NaCl, 10mM TrisCl pH 8.0, 10mM EDTA). 10% SDS was added to 0.5% and 10mg/ml proteinase K to 100µg/ml prior to overnight incubation at 37°C.

The sample was transferred to a 0.5ml eppendorf tube (Sarsdedt), an equal volume of phenol/chloroform added and the tube mixed by gentle inversion for ten minutes at room temperature. The phases were seperated by a two minute spin in a microfuge (IEC centra-4X) and the aqueous layer transferred to a fresh tube. The phenol/ chlorofom layer was extracted with 50µl TE (10mM TrisCl pH8.0, 1 mM EDTA pH8.0) in the same manner and the aqueous phases combined. A further phenol/ chloroform extraction was performed with 150µl phenol/ chloroform, the organic layer extracted with 50µl TE and the aqueous phases combined.

The aqueous phase was then extracted with 200µl chloroform and transferred to a mini-collodion bag (Sartorius). Dialysis against TE was performed for seven hours at room temperature with two changes of buffer. The DNA solution was then transferred to an 0.5ml eppendorf and stored at 4°C.

2:3:4 Digestion of chromosomal DNA (Fuscoe et al, 1986).

Chromosomes were digested with 50U EcoRI in a reaction volume of 200µl under the manufacturer's recommended conditions (BRL 1x core buffer) but without the addition of spermidine. Digestion was for four hours at 37°C. EcoRI was inactivated by placing the reaction at 65°C for fifteen minutes.

The digest was mixed with an equal volume of phenol/chloroform for ten minutes at room temperature and the phases seperated by a two minute spin in a microfuge. The aqueous phase was transferred to a 1.5ml eppendorf and the organic phase extracted with 50µl TE. The aqueous phases were combined and extracted with an equal volume of phenol/ chloroform. The aqueous phase was again transferred to a 1.5ml eppendorf and the organic phase which remained extracted with 50µl TE. The aqueous phases were combined and extracted once with an equal volume of chloroform.

The aqueous phase from this extraction was transferred to a mini-collodion bag and dialysed against sterile distilled water overnight at 4°C with two changes of buffer.

2:3:5 Co-precipitation and ligation of vector and insert (Fuscoe et al, 1986).

The X-chromosome digest was transferred to an 0.5ml eppendorf tube and combined with 2µg (100mg/ml) lambda phage EcoRI arms to give a total volume of 400µl. The sample was then placed in the freeze dryer for two hours to reduce the volume to 50-100µl. One-tenth volume of 3M sodium acetate (pH5.0) and two volumes of cold 95% ethanol were added and the sample mixed well. The sample was then subjected to three freeze/thaw cycles for thirty minutes each at -70°C. The DNA was then pelleted in the Centra-4X microfuge using a horizontal rotor (10 minutes, room temperature), washed once with 70% ethanol, vacuum dried for three minutes and resuspended in 10µl TE overnight at 4°C.

1µl 10x ligase buffer (0.5M Tris.Cl (pH 7.4), 0.1M $MgCl_2$, 0.1M dithiothreitol, 1mg/ml BSA), 2µl 5mM ATP (Sigma) and 1µl 1U/µl T4 ligase (Boehringer Mannheim) were added and the reaction incubated at 15°C overnight. A further 0.5µl each of 1U/µl T4 ligase, 10x ligase buffer and 5mM ATP were added to give a reaction volume of 16µl and the reaction incubated for a further three hours. 4µl of the ligated reaction was then packaged as described below.

2:3:6 Packaging of the X-chromosome library.

Packaging of DNA into bacteriophage particles (Becker & Gold, 1975) was done using Amersham packaging kits (Amersham International, Code 334), derived from two strains of mutant phage with complementary defects in phage assembly.

The packaging effeciency was tested by packaging 1µg wild type lambda. Serial dilutions of the packaged phage were made in SM buffer and the titre/ µg packaged phage determined as 1.8×10^8 pfu/µg, which compared well with the packaging effeciency of 2×10^8 plaque-forming units (pfu) per µg wild-type lambda DNA claimed by the manufacturers (Amersham batch analysis).

The phage extracts were thawed on ice. 1µg (4µl) of the ligated reaction was added to extract B and 15µl of extract A added immediately. The extracts were mixed gently with a yellow tip and the contents collected by a brief (ten second) spin in a microfuge. The reaction was then incubated at room temperature for two hours,

after which time 0.5mls SM buffer and 10µl chloroform were added. The packaged phage were then stored at 4°C as normal.

Aliquots of the λ -library were plated on both NB78 and NM514 to determine the proportion of recombinants in the library and the approximate titre. Phage were then plated out on NM514 at a density which allowed the optimum number of plaques to be screened at any given time. This number approached 2-300 for a small plate (87mm in diameter) and three times this number for a large plate (137 mm in diameter) and represented the maximum plaque density at which plaques were well resolved.

2:4 SCREENING OF THE X-CHROMOSOME LIBRARY.

Plaque lifts of the X-chromosome library were hybridised with ^{32}P labelled total human (female) DNA in order to select against recombinants which contained repeated sequences (and would thus give a strong signal with total human DNA). The single (or low-copy) clones which did not result in a signal were then selected for further study (see sections 2:4:1-2:4:7 and 2:5:1).

2:4:1 Plaque Transfers.

Plaque transfers (Benton & Davis, 1977) to Hybond-N membrane (Amersham International plc) were performed according to the manufacturers recommended conditions (Amersham International, 1985). Hybond-N membranes (RPN.87N (87mm) or RPN.132N (132mm)) were placed on the plates and orientated by corresponding ink markings on both the membrane and the plates. Membranes were removed after one minute and placed (plaque-side up) on a pad of Whatmann 3MM paper saturated with denaturation solution (0.5 M NaOH, 1.5M NaCl). After seven minutes they were transferred to a pad saturated with neutralisation solution (3M NaCl, 0.5M TrisCl pH7.4), left for three minutes and then transferred to a fresh pad saturated with neutralisation solution for a further three minutes. The membranes were finally washed in 2xSSC (0.3M NaCl, 30mM NaCitrate pH7.4), left to air dry, and the DNA bound to the filter by ultraviolet light treatment (plaque side down on a UV transilluminator (UVC incorporated)) for four minutes or latterly by baking for five hours at 80°C (Gallenkamp BS Oven 250).

2:4:2 Screening plaque transfers.

Plaque lifts were pre-wet in 1xSSC, 0.1% SDS and prehybridised for a minimum of two hours in prehybridisation solution (50% deionised Formamide, 5x Denhardts (0.1% ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrollidone), 5xSSC, 50mM Sodium phosphate pH6.8, 380µg/ml sonicated denatured salmon sperm DNA,0.2% SDS and 10µg/ml Poly (A)) at 42°C in a shaking water-bath (Tecam SB-16).

A nick-translated probe was prepared from total human DNA (see below). This was boiled for five minutes, cooled on ice and added to ten volumes (generally five millilitres) hybridisation buffer (50% deionised Formamide, 1x Denhardts (0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrollidone), 5xSSC, 20mM Sodium phosphate pH6.8, 100µg/ml sonicated denatured salmon sperm DNA, 20µg/ml Poly (A) and 10% dextran sulphate).

The prehybridisation buffer was replaced with hybridisation buffer containing the appropriate probe and left to hybridise overnight at 42°C.

Filters were washed at 1xSSC, 0.1%SDS for thirty minutes at room temperature, at 0.5xSSC, 0.1%SDS for a further thirty minutes at room temperature, blotted dry, placed between plastic sheets in an autoradiography cassette and covered with a sheet of Kodak XAR-5 (fast) film. The casette was equipped with Dupont lightning plus intensification screens and was placed at -70°C overnight. Films were developed using Kodak XAR developer and fixative and a Fuji RGII automatic film processor.

2:4:3 Selection of plaques.

Phage which failed to give a signal on hybridisation with nick-translated total human DNA were selected by comparing the autoradiograph with the original plate and picked using a sterile pasteur pipette (Bilbate) with a rubber bulb. Each agar plug was expelled into 1ml SM buffer, in a 1.5ml eppendorf tube. A drop of chloroform was added and the tube was left at room temperature for one hour to enable the phage (1-10 million) to diffuse out of the agar. The picked plaques stocks were then stored at 4°C as normal, (Maniatis et al, 1982).

2:4:4 Plaque purification and preparation of high titre stocks.

10^{-3} dilutions of picked phage stocks were made in SM buffer. 10µl of each dilution was added to 100µl of plating cells and allowed to adsorb at 37°C for twenty minutes in a bijou (Sterilin). 3mls of 0.7% top agar were addded. The contents of each bijou were mixed by gentle inversion and then poured on top of an L-plate. Once the top agar was set the plates were inverted and incubated overnight at 37°C. Well-separated plaques were obtained and these "purified plaques" were picked with a sterile pasteur pipette and expelled into SM buffer/ chloroform as described above.

10µl of each "purified plaque" stock was addded to 112µl of plating cells in a bijou. Adsorption was for twenty minutes at 37°C after which time 3mls of 0.7% top agar were added, the bijoux inverted gently and the cells and phage poured over pre-warmed L-plates. The plates were incubated at 37°C overnight but were not inverted as confluent lysis was desired. Phage were recovered from the top agar by one of two methods (Maniatis et al, 1982):

For the first method, 5mls SM buffer were added to each plate. Plates were left shaking gently at 4°C for four hours (Luckham R100 shaking platform). The SM buffer was transferred to a 13 ml sarsdedt tube and chloroform added to 1%. The tubes were spun at 8K (ten minutes, 4°C, SM24 rotor, Sorvall RC-5B) and the supernatant transferred to a fresh tube. The resultant high titre stock was made 0.05% in chloroform and stored at 4°C.

Alternatively, the top agar (0.5% for this protocol) was scraped into a 13ml sarsdedt tube tube using a sterile bent pasteur pipette. 5mls of SM buffer and 100µl chloroform (to 2%) were added and the contents mixed in the shaking incubator (New Brunswick G25) at 150-200rpm for thirty minutes. The tubes were spun at 8K (ten minutes, 4°C, SM24 rotor), the supernatant transferred to a fresh tube and chloroform added to 0.5%. The resultant high titre stock was stored at 4°C.

For applications in which only a small amount of high titre stock was required e.g. the inital screen of the library the first approach was used. The second method was easier to scale up and was used to produce a larger volume of high titre stock for storage.

2:4:5 Preparation of liquid lysates.

1µl of each high titre stock (approximately 3x 10^6 phage) was added to 112µl (10^{10}) of the appropriate plating bacteria and allowed to adsorb for twenty minutes at 37°C in a universal (Sterilin). 5mls of L-broth were added and samples incubated overnight in the shaking incubator (New Brunswick G25). Lysed cultures were transferred to 13ml Sarsdedt tubes, 100µl chloroform added to each, and incubation continued for a further fifteen minutes. The bacterial debris was pelleted (Sorvall RC-5B, SM24 rotor, 8K, twenty minutes, room temperature) and the supernatant transferred to a fresh tube. A further 20µl of chloroform was added (to 0.4%) and the liquid lysates stored at 4°C, (Leder, Tiemeier & Enquist, 1977; Maniatis et al, 1982).

2:4:6 Small scale phage DNA preparations (Cameron, Phillipson & Davies, 1977).

2mls of liquid lysate were transferred to a 13ml Sarsdedt tube. 10mg/ml DNase was added to 70µg/ml and the lysate incubated at 37°C for one hour. 0.8mls TES (0.3M Tris (pH9.0), 0.15M EDTA and 1.5% SDS) was added, samples placed at 70°C for 15 minutes and then cooled to room temperature.

0.8mls of 5M Potassium acetate (pH8.0) was added, the samples placed on ice for thirty minutes and spun at 9K for twenty minutes (Sorvall SM24 rotor at 4°C) with the brake off. 4mls of supernatant were transferred to a fresh tube, mixed with an equal volume of isopropanol and spun for a further twenty minutes (9K, 4°C).

The phage pellet was washed with 70% ethanol, dried, resuspended in 100µl TE and transferred to an 1.6 ml eppendorf tube. 10µl boiled RNaseA (25mg/ml) was added and incubation performed at 37°C for thirty minutes. 4µl each of 10% SDS and proteinase K (10mg/ml) were added, prior to incubation at 55°C for fifteen minutes.

A tenth volume of 3M sodium acetate (pH7.0) was added and phenol/chloroform extractions performed until the interface was clear. Two volumes of 100% ethanol were added to the aqueous phase at this point and samples placed at -20°C for ten minutes.

Phage DNA was pelleted by centrifugation (9K, twenty minutes ,

4°C), washed once with 70% ethanol, dried and resuspended in 32µl sterile distilled water, prior to digestion with the appropriate restriction enzyme.

2:4:7 Isolation of phage inserts.

EcoRI digestion of phage recombinants was performed in a total reaction volume of 40µl (containing a tenth volume of BRL "React 2" (or BRL "core") buffer, a twentieth volume of 0.1M spermidine (final concentration 5mM) and 1-2µl (10-20U) EcoRI) overnight at 37°C.

5µl of the digest was placed aside for sub-cloning at a future stage (refer to section 2:5:2). 5µl loading mix (50% Glycerol, 2% Ficoll, 50mM EDTA with a "pinch" of Orange G) was added to the remaining sample which was then loaded on a 1% LMP agarose (BRL) gel prepared in EB buffer. Gels were subjected to constant current electrophoresis at 250 mA, stained in a 5µg/ml solution of ethidium bromide in EB buffer, and visualised over ultra-violet light.

"Insert bands" were excised using a sterile scapel blade, transferred to 1.6ml sarsdedt tubes, weighed and a volume of sterile distilled water (SDW) equivalent to twice the weight of the insert added. The tube containing DNA/agarose/SDW was placed in boiling water for ten minutes and used directly for oligonucleotide labelling (see section 2:5:2) or stored at -20°C.

2:5 PREPARATION OF RADIOACTIVELY LABELLED PROBES.

Alpha-^{32}P dCTP (Amersham international, specific activity 3000 Ci/mmol (or 110TBq/mmol) labelled probes were prepared by (i) nick-translation of whole plasmid DNA or genomic DNA or (ii) random oligonucleotide primed labelling of phage or plasmid inserts.

2:5:1 Nick translation (Kelly et al, 1970; Maniatis et al, 1982).

Nick translation was performed using nick translation kits (Amersham international, N5000). Essentially, 1-2µg of DNA (made up to 17µl with SDW) was combined with 6µl of 5X Amersham reaction mix (100µM each in dATP, dGTP and dTTP), 5µl (approximately 50µCi) ^{32}P dCTP and 2µl of Amersham enzyme solution (which contained 0.5U/µl DNA polymerase I and 10pg/µl DNase I). The reaction was incubated

at 14-16°C for an hour and a quarter and then stopped by the addition of 5µl 0.5M EDTA. Labelled DNA was separated from unincorporated oligonucleotides by chromatography through a Sephadex G-50 column.

Amersham reaction mix and enzyme solution were in Tris/HCl and $MgCl_2$ at pH7.8 and pH7.5 respectively. The molarities were not specified but probably approximate to final concentrations of 50mM Tris.HCl and 10mM $MgSO_4$ as specified in Maniatis et al (1982).

2:5:2 Oligo-nucleotide primed ("oligo-") labelling (Feinberg & Vogelstein, 1983, 1984).

20µl of excised insert (diluted with SDW) was boiled for ten minutes and equilibrated at 37°C for fifteen minutes. This was added to 15µl SDW, 10 µl 10x "oligo" reaction mix and 2µl 10mg/ml bovine serum albumin (BSA, Sigma). 20-30µCi ^{32}P dCTP was added to each reaction together with 0.5-1µl (5-10U) Klenow fragment (Amersham).

The reactions were incubated at 20°C overnight and stopped by the addition of 50µl of 4x stop mix (1x stop mix: 20 mM NaCl, 20mM Tris-Cl pH7.5, 2mM EDTA, 1µM dCTP (normally omitted) and 0.25% SDS). Incorporated label was resolved from unincorporated nucleotides on a Sephadex G-50 column.

2:6 PLASMID TECHNIQUES.

2:6:1 Storage and culture of bacterial strains and plasmid recombinants.

Plasmids were firstly stored as a DNA stock (at 4°C). They were then transformed (see section 2:5:2) into an appropriate bacterial strain (generally pUC19), plated out and single colonies selected. Bacteria containing recombinant plasmids were stored by (i) "stabs" (ii) glycerol stocks and (iii) (for short-term storage only) by streaking out on to L(-amp) plates. Bacterial strains with no plasmid recombinants were also stored in this manner (eg NM514 and NB78, which were used in constructing the library) but without anti-biotic selection.

The specifications of plasmid DNA clones, which were used in

this study but were obtained from other sources (see acknowledgements), are summarised in table 3.

To create "stabs" part of the colony was picked with a sterile wire loop, stabbed into a bijou or 2ml starsdedt tube containing 1.5% L-agar and grown up (loosely capped) at 37°C overnight. These were tightly capped, wrapped in Nesco-film to prevent evaporation and stored at room temperature.

Glycerol stocks were established by selecting part of the colony with a sterile wire loop to innoculate 5ml L broth (+/- 50-100µg/ml ampicillin) in a sterile universal. The culture was grown overnight at 37°C in a shaking incubator with vigourous shaking (200-300 rpm). 1ml was removed to a sarsdedt 1.6ml sterile screw-cap tube, 0.6ml sterile glycerol added and the two mixed well, resulting in a bacterial stock just less than 40% in glycerol. This was stored at -20°C

Single colonies of interest were also streaked onto a fresh L(-amp) plate with a sterile wire loop to give decreasing concentrations of bacteria proceeding clockwise round the plate. After overnight incubation at 37°C well spaced single colonies were almost invariably observed in some area of the plate, thus ensuring ready access to single colonies. The plates were wrapped in Nesco-film and stored at 4°C for a maximum of two months.

Single colonies were (i) selected directly from the appropriate plate stored at 4°C, (ii) obtained by streaking out of a stab or glycerol stock or (iii) when only plasmid DNA was available, by transforming bacteria, plating out and selection of the appropriate recombinants (see section 2:6:2).

Single colonies were used to prepare overnight cultures by inoculating 5mls L-broth (which was 50-100µg/ml in ampicillin where appropriate) and incubating overnight at 37°C with vigorous shaking. Where large scale cultures were required the 5ml culture was grown up for several hours and then used to "seed" 100ml of pre-warmed L-broth. These overnight cultures provided the starting point for plating cells (section 2:2:1) and plasmid preparations (sections 2:6:4 and 2:6:5)

PROBE	DNA SEGMENT	VECTOR	SITE+SIZE OF INSERT	RFLP	CONSTANT BANDS	ALLELES SIZE	FREQ.	PIC	REFERENCE
29C1	DXYS14	pUC9	1.5 PstI	EcoRI		over 20 of 4-15kb		0.7	Cooke et al, 1985
pSG1	MIC2	pUC8	1.1 EcoRI	MspI	1.2,1.1	2.35 2.55			Goodfellow et al, 1986
p19b	MIC2	pUC8	1.0 EcoRI	TaqI		2.5 3.2			Goodfellow et al, 1986
M1a	DXS31	pBR322	2.1 EcoRI/ PstI	XmnI		3.4 3.0	0.19 0.81	0.31	Koenig et al, 1984
STB14	NA	pUC13	1.8 HindIII	NP					Ballabio, personal communication
p2a4	NA	pUC13	1.4 EcoRI	NP					Ballabio, personal communication
Dic56	DXS143	pUC8	1.4 HindIII/ BamHI	BclI		7.4 8.9	0.56 0.44	0.49	Middlesworth, Berteleson & Kunkel, 1985
RC8	DXS9	gtWES.B	6.1 EcoRI	TaqI	6.6	3.2 5.3 3.0	0.84 0.10 0.06	0.28	Wieacker et al, 1984
782	DXS85	pAT153	4.3 HindIII	EcoRI		14.0 7.0	0.60 0.40	0.48	Hofker et al, 1985
XUT23	DXS16	pUC8	2.1 BamHI	BglII		17.5 12.5	0.84 0.16	0.27	Bakker et al, 1985
D2	DXS43	pBR322	1.7 EcoRI	PvuII		6.0 6.6	0.71 0.29	0.41	Aldridge et al, 1984
99-6	DXS41	pBR322	1.6 BamHI	PstI		22.0 13.0	0.71 0.29	0.41	Aldridge et al, 1984
C7	DXS28	pBR322	2.5 EcoRl	EcoRV		7.5 8.0	0.85 0.15	0.26	Dorkins et al,1985

Table 3: Specifications of DNA probes.
(This table gives the DNA segment (DXS assignment), cloning vector, cloning site, RFLP (if any), allele sizes (in kilobases), allele frequencies (according to HGM9, or to the reference given), PIC value and the earliest reference, for each probe used, except for those isolated as part of this project. NA, not assigned; NP,not polymorphic; FREQ,frequency; ?,unknown; Bluesc.,bluescript. Continued overleaf).

PROBE	DNA SEGMENT	VECTOR	SITE+SIZE OF INSERT	RFLP	CONSTANT BANDS	ALLELES SIZE	ALLELES FREQ.	PIC	REFERENCE
B24	DXS67	pBR322	0.4 HindIII/ EcoRI	MspI		1.6	0.96	0.08	Aldridge et al, 1984
						1.4	0.04		
				BstNI		1.6	0.85	0.26	
						1.4	0.15		
						0.5	0.0		
L1-4	DXS68		0.75 HindIII	NP					Kunkel et al, 1985a
J66H1	DXS268	C2RB	1.7 HindIII/ EcoRI	PstI	2.8	1.5	0.60	0.54	Monaco et al, 1987
						1.4	0.30		
						1.35	0.10		
p20	DXS269	pKUN1	2.7 PstI	EcoRV	14	7.5	0.40	0.48	Wapenaar et al, HGM9
						7.0	0.60		
				MspI	1.5,1.0	6.0	0.40	0.48	
						3.5	0.60		
JBir	DXS270	pUC18	1.1 KpnI/ HindIII	BamHI	16	21	0.29	0.38	Monaco et al, 1987
						5	0.71		
pERT87-30	DXS164	pUC18	1.8 HindIII	BglII		8	0.37	0.47	Monaco et al, 1987
						30	0.63		
pERT87-15	DXS164	pUC18	1.5 HindIII	BamHI		9.4	0.38	0.47	Kunkel et al, 1986
						7.1/2.3	0.62		
				TaqI		3.3	0.33	0.44	
						3.1	0.67		
				XmnI		2.8	0.32	0.44	
						1.6/1.2	0.68		
pERT87-8	DXS164	pUC18	1.3 XbaI	BstXI		4.4	0.64	0.46	Kunkel et al, 1985a
						2.2	0.36		
						2.1	0.00		
				TaqI	1.0	1.1/2.7	0.74	0.38	
						3.8	0.26		
						1.9	0.0		

Table 3: Continued. Continued overleaf.

PROBE	DNA SEGMENT	VECTOR	SITE+SIZE OF INSERT	RFLP	CONSTANT BANDS	ALLELES SIZE	FREQ.	PIC	REFERENCE
pERT87-1	DXS164	pUC18	1.35 KpnI/SalI	XmnI		8.7	0.69	0.43	Kunkel et al, 1985a
						7.5	0.31		
				BstNI	0.7	3.1	0.65	0.46	
					2.45/0.65		0.35		
				MspI		4.05	0.35	0.46	
						1.8	0.65		
XJ10.1	DXS206	pUC13	0.6 HindIII	NP					Worton, Personal communication
XJ2.3	DXS206	pUC13	1.1 BamHI/EcoRI	TaqI		6.4	0.70	0.42	Thompson et al, 1986
						7.8	0.30		
XJ1.1	DXS206	pSP65	1.0 EcoRI or HindIII	TaqI		3.1	0.72	0.40	Ray et al, 1985
						3.8	0.28		
XJ1.2	DXS206	pUC19	0.6 XbaI/EcoRI	BclI		2.0	0.70	0.42	Thompson et al, 1986
						1.7	0.30		
HIP25	DXS230	?(4kb)	0.6 XbaI/EcoRI	NP					Smith et al, 1987
XJ5.1	DXS206	Bluesc.	0.6 XbaI/EcoRI	SphI		17.0	0.80	0.32	Worton et al, HGM9
						24.0	0.20		
JMD	NA		1.7 HindIII	NP					Monaco et al, 1987
J47	DXS272		1.8 HindIII	NP					Monaco et al, 1987
pERT84	DXS142		1.9 HindIII	NP					Kunkel et al, 1985
754-11	DXS84	pUC12	0.9 EcoRI/SalI	BglII		5.3	0.60	0.48	Hofker et al, 1986a
						6.3	0.40		
				EcoRI		2.4	0.84	0.27	
						4.2	0.16		
754-6	DXS84	pUC12	0.9 EcoRI/SalI	HindIII		1.7	0.60	0.48	Hofker et al, 1986a
						5.2	0.40		
754	DXS84	pAT153	2.2 HindIII	PstI		12.0	0.62	0.47	Hofker et al, 1985
						9.0	0.38		

Table 3: continued. Continued overleaf.

PROBE	DNA SEGMENT	VECTOR	SITE+SIZE OF INSERT	RFLP	CONSTANT BANDS	ALLELES SIZE	FREQ.	PIC	REFERENCE
0TC	OTC	pUC8	1.2 PstI	MspI	17.5,1.9	6.6	0.61	0.48	Rozen et al, 1985
					5.4,3.5	6.2	0.39		
					2.0	5.1	0.73	0.39	
						4.4	0.27		
L1.28	DXS7	pBR322	1.25 EcoRI	TaqI		12.0	0.68	0.44	Wieacker et al, 1984
						9.0	0.32		
58.1	DXS14	pBR322	1.2 HindIII	MspI		4.0	0.65	0.46	Aldridge et al, 1984
						2.5	0.35		
GMGXY3	NA	pUC19	0.6 EcoRI/ HindIII	NP					Mitchell, Personal communication
GMGXY19	NA	pUC19	1.5 HindIII	NP					Mitchell, Personal communication

Table 3: Continued.

2:6:2 Preparation of competent cells and bacterial transformation.

Frozen competent cells (JM83) were prepared by frozen storage protocol III of Hanahan (1985). A freshly streaked SOB plate was prepared from a single colony. Ten colonies, 2-3mm in diameter, were picked from this plate, dispersed in 1ml SOB by vortexing and used to inoculate 100mls SOB in a 1 litre Erlenmyer flask.

The culture was grown to a density of 4-7 x 10^7 cells/ml, split between two Falcon 2070 (or Sarsdedt 60.547) tubes and placed on ice for fifteen minutes. Cells were pelleted (2800rpm, fifteen minutes, 4°C, IEC DPR-6000), drained and resuspended in one-third volume (2x16mls) RF1 buffer and incubated on ice for fifteen minutes. Cells were pelleted once more by centrifugation (as above), resuspended in 2x 4mls RF2 buffer and left on ice for a further fifteen minutes. Competent cells were then aliqoted into chilled 1.6ml sarsdedt screw-cap tubes, "flash-frozen" in liquid nitrogen and stored at -70°C.

These were thawed at room temperature as required for transformation. Cells were divided into 100µl aliqouts in Sarsdedt 1.6ml screw-cap tubes and placed on ice. 100ng-1µg of DNA in a volume of up to 10µl was added and the tube swirled to mix the sample well. Samples were left on ice for twenty to forty five minutes prior to "heat-shock" at 42°C for thirty to forty five seconds. Samples were immediately returned to ice after heat-shock and plated onto L-plates (+/- ampicillin). Control plates were prepared using competent cells with no transforming DNA.

2:6:3 Sub-cloning of phage inserts.

pUC19 vector was prepared by digestion with EcoRI and calf intestinal phosphatase treatment (as described in section 2:2:1). 1µl (20ng) of plasmid vector was ligated with 2µl of the appropriate phage digest (section 2:3:8) for one hour at room temperature and then at 4°C overnight. The total reaction volume of 10µl comprised: 1µl plasmid vector, 2µl phage digest, 1µl 10x ligase buffer, 2µl 5mM ATP, 1U T4 DNA ligase and 3µl SDW.

The ligation reaction was used directly in transformation of JM83 cells without phenol/chloroform extractions to remove T4 DNA ligase.

2:6:4 Selection of transformants.

Transformed cells and controls were spread with a sterile glass rod onto L- plates (made 50-100µg/ml ampicillin for sub-cloning into pUC19, or for any ampicillin resistant plasmids cloned in either the pUC or pBR322 series of vectors).

X-gal selection, was used whenever possible (eg for "pUC" vectors using JM83 competent cells). 40µl of 20mg/ml X-Gal in dimethyl formamide was added to each L-amp plate prior to use. JM83 has a LacZ mutation and thus cannot produce beta-galactosidase necessary to metabolise X-gal (5-bromo- 4-chloro- 3-indoyl-beta-D-galactopyrosanide). The lacZ' gene at the polylinker (or multiple insert) site in pUC19 complements the defect and thus JM83 cells containing this plasmid metabolise X-gal. This reaction results in a blue colour. Recombinant plasmids (which interrupt the lacZ' gene) are "white" and can be distinguished from (blue) religated vector clones. Small scale DNA preparations were performed for each well-spaced single colony selected (see section 2:6:5).

2:6:5 Rapid (small scale) plasmid preparation by the boiling method (Holmes & Quigley, 1981).

5ml cultures, derived from 20µl glycerol stock or, preferably, from single colonies were grown overnight in L-broth (made 50-100µg/ml in ampicillin if appropriate), in sterile Universals (Sterilin) within a shaking incubator at 37°C.

1.5ml of overnight culture was transferred to an eppendorf tube. Cells were pelleted by centrifugation for five minutes in a microfuge (IEC centra-4X). (The remainder of the culture was made 40% in glcerol and stored at -20°C as a glycerol stock). The supernatant was discarded, the cells resuspended in 700µl ice-cold STET (50mM Tris 8.0, 50mM EDTA, 8% sucrose and 5% Triton X-100) and 50µl 10mg/ml lysozyme (in STET) added. The tubes were left on ice for fifteen minutes to enable the cells to lyse, placed in boiling water for forty five seconds, and returned to ice.

The bacterial debris was pelleted by centrifugation at maximum speed for thirty minutes and the supernatant transferred to a fresh tube. Sodium acetate was added to 0.3M and phenol/ chloroform extractions performed until the interface was clear. Plasmid DNA

was precipitated at -20°C for thirty minutes, by the addition of 0.6 volumes of isopropanol. DNA was pelleted by centrifugation, washed twice with 70% ethanol and air- or vacuum- dried. The pellet was resuspended in TE (for storage at 4°C) or more often in a small volume of sterile distilled water which formed the basis of the desired restriction digest.

Small scale preparations yielded plasmid DNA of a quality suitable for "oligo"-labelling (section 2:5:2), normally sufficient for twenty to thirty labelling reactions.

2:6:6 Large scale cleared lysate plasmid preparation (BRL protocol).

100ml cultures were grown overnight in L-broth at 37°C within a shaking incubator. These were generally "seeded" with 5ml cultures grown up from a single colony (or 20µl glycerol stock of the appropriate colony) during the day. Ampicillin was added to 50-100µg/ml if appropriate.

Cells were pelleted by centrifugation at 2500 rpm for fifteen minutes at room temperature. The supernatant was discarded. Cells were resuspended in 10mls bacterial lysis mix (50mM Tris 7.5, 50mM EDTA and 15% sucrose) and left at room temperature for fifteen minutes. 10mg of powdered lysozyme (Sigma) was added to 1mg/ml and the cells were then left for a further twenty minutes at room temperature.

0.5mls 10% SDS (to 0.5% SDS) was added and the tube inverted gently. 1ml 5M Potassium acetate (to 0.5M) was then added, the tube inverted gently once more and placed on ice for thirty minutes.

Bacterial chromosomal DNA was removed by centrifugation (10K, thirty minutes, 4°C, HB4 rotor) and the supernatant transferred to a fresh tube. Plasmid DNA (and RNA) were precipitated on ice for twenty minutes by the addition of two volumes of cold 95% ethanol and pelleted by centrifugation (10K, twenty minutes, 4°C, HB4 rotor).

The pellet was then resuspended in 4mls TE and solid ammonium acetate added to 2.5M (0.77g). This was left on ice for a further twenty minutes and then subjected to centifugation (10K, twenty minutes, 4°C, HB4 rotor). The supernatant was transferred to

a fresh tube and a further two volumes of 95% ethanol added. This was left on ice for a further twenty minutes and spun down (10K, twenty minutes, 4°C, HB4 rotor) to precipitate plasmid DNA.

The pellet was dried and resuspended in 2mls of 0.05M NaCl in TE (in 13ml Sarsdedt tubes). Boiled RNAse A was added to 25µg/ml and the tube incubated at 37°C for forty five minutes. 0.6 volumes of 2M NaCl in TE were added and phenol chloroform extractions performed until the interface was clear. Plasmid DNA was precipitated at -20°C for thirty minutes by the addition of a further two volumes of cold 95% ethanol.

Plasmid DNA was pelleted by centrifugation (10K, twenty minutes, 4°C, HB4 rotor), resuspended in TE and subjected to two further rounds of purification by ammonium acetate precipitation. (Each round of purification involved the addition of a half volume of 7.5M ammonium acetate and of two volumes of 95% ethanol. Plasmid was precipitated at -20°C for thirty minutes and then recovered by centrifugation). The final pellet was dried prior to resuspension in 200-500µl TE.

2:7 SOUTHERN ANALYSIS.

2:7:1 Preparation of genomic DNA from blood and cell lines (Kunkel et al, 1977).

Genomic DNA was prepared as described below from blood (10-20mls in NaCl anticoagulant) or from lymphoblastoid and somatic cell hybrid cell-lines. Fibroblast DNA was also prepared according to the same protocol with the quantities scaled down ten fold.

Blood samples were thawed (if frozen). 10mls blood was placed in each of two 50ml Falcon 2070 (or Sarsdedt 60.547) tubes. 40mls of lysis mix (0.32M Sucrose, 10mM Tris pH 7.5, 5mM $MgCl_2$, 1% Triton X-100) was added to each tube and the contents were mixed thoroughly. Cell pellets were resuspended directly in 50ml lysis mix and the tubes left on ice for ten minutes to ensure complete cell lysis.

Cell nuclei were pelleted in the IEC DPR-6000 centrifuge (2.8K, fifteen minutes, 4°C) and resuspended in 10mls NaCl/EDTA (0.075M NaCl, 0.024M EDTA). Any sample which had been split into

two, was recombined at this point. 0.75mls 10% SDS and 200µl 10mg/ml proteinase K were added, the tube inverted gently and incubated at 55°C for one to three hours.

The samples were allowed to cool to room temperature. A tenth volume of 3M sodium acetate (pH7.0) and an equal volume of phenol/chloroform (50% Phenol, 50% Chloroform, 0.1% 8-hydroxyquinoline equilibrated with TE) were added. The tubes were mixed gently by rotation (Stuart tube rotator TR-2) for fifteen minutes and the phases separated by centrifugation in the IEC centifuge (2.8K, fifteen minutes, room temperature).

The aqueous phase was removed with a plastic pastette and transferred to a fresh Falcon 2070 tube. Further phenol/chloroform extractions were performed until the interface was clear and the aqueous phase was once more transferred to a fresh tube. Two volumes of cold 100% ethanol were added to precipitate the DNA which could then be spooled around a sealed sterile pasteur pipette. The DNA was washed in a small quantity of 100% ethanol and allowed to air dry. DNA samples were rehydrated in 500µl-1ml TE. These were left at 4°C for a minimum of twenty four hours before use to ensure that they had dissolved adequately, mixed with a wide bore 1ml blue tip (Treff) and 10-20µl removed with which to measure the optical density at 260nm (Perkin Elmer 6000 spectrophotometer). For a 10µl dilution in 1ml TE, the DNA concentration in µgms/ml was obtained by multiplying the optical density by five thousand.

2:7:2 Restriction Digests, electrophoresis and Southern blots (Southern, 1975).

Restriction enzyme digests were performed using 7µg genomic or somatic cell hybrid DNA at 37°C overnight. The reaction consisted of DNA, one-tenth volume of the recommended 10x buffer (generally supplied), one-twentieth volume of 0.1M spermidine (Sigma), 10-20U restriction enzyme (BRL, New England Bio-labs, or Anglian Biotechnology) and sterile distilled water to an appropriate volume (normally 40µl). The samples were mixed well with a yellow pipette tip and spun briefly to collect the contents, prior to incubation.

Samples were spun briefly to collect the contents of the tube and one-sixth volume loading mix (50% Glycerol, 2% Ficoll, 50mM

EDTA and "a pinch" of Orange G.) added. The sample was loaded onto an 0.8% agarose (Sigma A6013) gel prepared in electrophoresis buffer (EB, ie in 40mM Tris-acetate, 2mM EDTA). Electrophoresis was performed in EB at 150-250mA constant current for three to four hours, or until the orange-G marker dye had reached the foot of the gel. Gels were stained in a dilute ethidium bromide solution (5µg/ml in EB) and photographed with a polaroid GU-5 land camera using polaroid 667 (black and white) film over the ultraviolet transilluminator.

Gels were soaked in acid (0.2M HCl) for twenty minutes, in denaturation solution (0.5M NaOH, 1.5M NaCl.) for 2x twenty minutes and in neutralisation solution (3M NaCl, 0.5M Tris.HCl pH 7.4) for a further 2x twenty minutes. DNA was transferred from the gel to the membrane (Amersham Hybond-N) in the presence of 10x SSC (1.5M NaCl, 0.15M sodium citrate) as described below.

A tank was filled with 10xSSC. A wick was prepared by laying a double thickness of Whatman 3MM paper over a glass plate placed across the tank such that either end of the 3MM paper was within the buffer. Once it was ensured that the wick was entirely wet and uniformly flat, the gel was placed on top of the wick. Hybond-N membrane (Amersham international plc), labelled and cut to size, was laid gently on top of the gel. Two sheets of 3MM paper (soaked in 2xSSC and cut to size) and two additional (dry) sheets of 3MM paper (also cut to size) were placed on top of the membrane. The remaining area of the wick was shrouded with plastic sheeting and a stack of paper hand-towels (Bowater-Scott), a glass plate and a small weight placed on top of the gel.

Transfer occurred overnight after which time the blotting apparatus was dismantled, the membrane rinsed briefly in 2xSSC to remove traces of agarose and baked at 80°C for five hours (or subjected to uv treatment for four minutes).

2:7:3 Hybridization of Southern Blots

Filters were prehybridised and hybridised as described in section 2:3:2) or prehybridised and hybridised in the same buffer, using 3-5ml buffer per 10cm x 13cm filter. This buffer was 50% formamide, 3xDenhardt's, 5xSSC, 25mM sodium phosphate pH6.8,

250µg/ml sonicated denatured salmon sperm DNA, 1% SDS, 20µg/ml poly A and 10% dextran sulphate. The denatured probe was added directly to the plastic bag containing the prehybridised filters. Hybridisation was overnight (sixteen hours) for probes labelled by nick translation or for two nights (forty hours) for probes labelled by oligonucleotide priming.

Excess probe was removed by washing the filter in 2xSSC, 0.1% SDS at room temperature for twenty minutes. Filters were washed sequentially in solutions of increasing stringency for twenty to thirty minutes each as required; 1xSSC, 0.1% SDS (room temperature), 1x SSC, 0.1% SDS (65°C), 0.5x SSC, 0.1% SDS (65°C), 0.1x SSC, 0.1% SDS (65°C) and 0.05x SSC, 0.1% SDS (65°C). The strength of the signal was assessed between washes with a "gm mini-monitor" (Type 5.10). Filters were blotted dry and placed between plastic sheets. The filters were then placed on a sheet of Kodak XAR-5 film in an autoradiographic cassette with "Dupont lightning plus" intensification screens and exposed overnight at -70°C (or from four hours to ten days as necessary). Films were developed as described in section 2:4:2.

2:8 PHOTOGRAPHY.

Autoradiographs were photographed using a Pentax 35mm camera (p30 SLR) with a SMC Pentax-A macro 50mm f/2.8 lens and Ilford PanF film. Films were developed for five and a half minutes in an 1:9 dilution of Ilford Ilfospeed developer, stopped in 3% acetic acid for thirty seconds, fixed in an 1:4 dilution of Hypam fixative (with 1/40th part Aculux hardener) for three and a half minutes minutes and rinsed with tap water and a few drops of photoflow (Kodak) for fifteen minutes. Negatives were enlarged on an Leitz (Wetzlar) enlarger and prints produced on Ilford number 3 paper. Prints were developed for one minute using an 1:9 dilution of Ilfospeeed developer at 20°C, rinsed in water, fixed for sixty seconds in an 1:9 dilution of Hypam fixative, rinsed in water again and dried using an Ilford Ilfospeed 1050 print drier.

2:9 SOMATIC CELL HYBRIDS.

AMIR2N was the gift of Dr P Goodfellow (Goodfellow et al, 1983), derived from the translocation in AM (1729), 46,Yt(X;Y)(p22.3;q11.2), (Ferguson-Smith et al, 1981). The breakpoint at Xp22.3 is proximal to the locus for STS.

Hybrids LNA9, EHA9, W2A9 and W5A9 were cloned by N. Morrison and L.Florentin in this department. LNA9 was derived from a patient LN, with an 46,XXp- karyotype, EHA9 was derived from EH, with a 46,XX,t(X;11)(p22;p15) karyotype, W2A9 was derived from the lymphoblastoid line of an individual with a 46,XX,t(X;8)(p21;q24) karyotype and W5A9 was derived from the lymphoblastoid cell line of individual with an 46,XX,t(1;X)(p32;p21) karyotype and a paracentric inversion betweeen Xp11 and Xp21, described by Lindenbaum et al (1979). The region of the X-chromosome retained in these hybrids is illustrated in figure 1.

HORL9X (Goodfellow et al, 1980) was a somatic cell hybrid containing only the X-chromosome whilst 3E7, isolated by M.Marcus & R. Voss from the hybrid described by Marcus et al (1976), contained only the Y-chromosome.

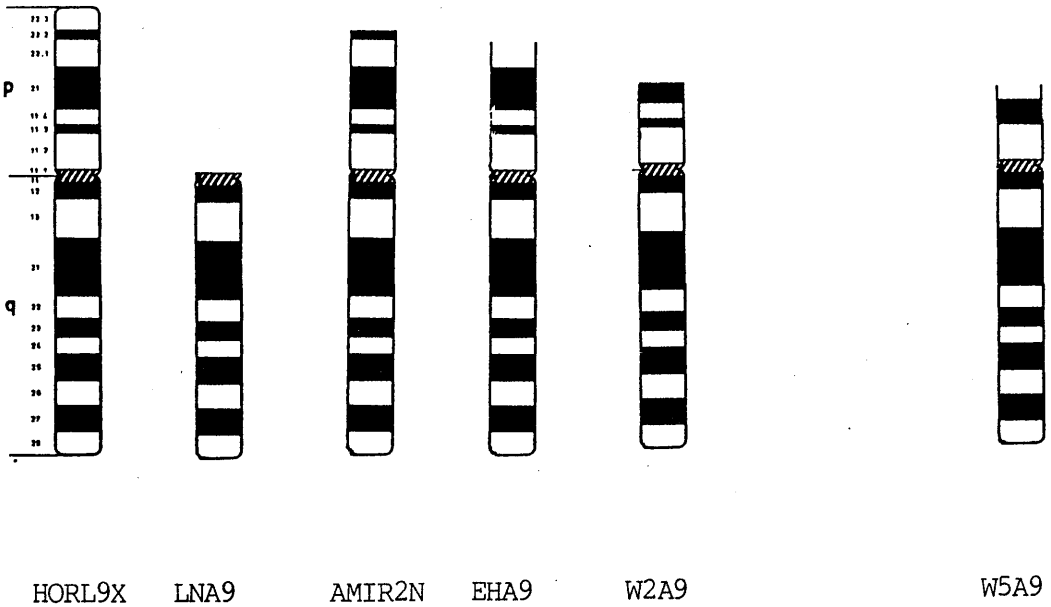


Figure 1: Diagrammatical representation of the extent of the X-chromosomal region in the somatic cell hybrids studied.

CHAPTER 3: RESULTS

3:1 CONSTRUCTION OF AN X-CHROMOSOME SPECIFIC LIBRARY IN LAMBDA-PHAGE NM1149.

An EcoRI library was prepared in lambda NM1149 from 1.13 x 10^6 sorted "X-chromosomes" (200-250ng DNA) as outlined in section 2:3. It was titred on both NB78 and NM514 and values of 116 000 and 68 100 plaque forming units (pfu) / total library (4ml) were obtained. As all bacteriophage will form plaques on NB78 and only recombinant phage form plaques on NM514, the difference in titre gives an indication of the proportion of non-recombinants. Thus, non-recombinants represent about one-third of this library.

The titre of the library fell prior to the second round of plating out, so that it no longer represented the entire X chromosome (which requires 50 000 recombinants). Thus, due to the reduced titre and the high proportion of non-recombinants, amplification of the X-library was undesirable and the library was plated out directly (on NM514 cells).

3:2 SCREENING OF THE LIBRARY.

Plaque transfers were performed and screened with ^{32}P labelled nick-translated total human (female) DNA in order to select for "single copy" inserts or inserts with low level repeats. Those recombinant plaques which failed to demonstrate hybridisation (and thus contained no repeat sequences or a low copy number of repeat sequences) were picked, 10^{-3} dilutions in SM buffer were prepared and putative recombinants were plated out again at low density. These techniques are described in section 2:4.

A second round of screening, which would have selected against some of the recombinants containing more highly repeated sequences, was not performed at this stage. This accounts for the moderate repeats apparent in some of the recombinants characterised (figure 2). A second round of screeening would also have increased the relative proportion of non-recombinants amongst the plaques selected.

One hundred and twenty single plaques were selected and DNA was prepared from liquid lysates as outlined in section 2:4. Fifty of these phage were shown to have inserts (figure 2) and were

labelled by the random hexa-nucleotide primed ("oligo-") method (section 2:5:2) .

3:3 REGIONAl MAPPING OF PROBES ON THE X-CHROMOSOME.

Each insert was hybridised to an X-chromosome EcoRl mini-panel, consisting of 7μg DNA from each somatic cell hybrid line (section 2:9) as follows:

1. female DNA
2. Horl9X X-only hybrid
3. LNA9 Xcen-Xqter
4. AMIR2N Xp22.3-Xqter
5. EHA9 Xp22.1-Xqter
6. W2A9 Xp21-Xqter
7. A9 mouse parent

Of fifty inserts, sixteen were X-specific (hybridised to the X-only hybrid), four were autosomal and six were highly repeated sequences. One was a moderate repeat with mouse homology. The remaining twenty two inserts demonstrated no, or non-specific, hybridisation and were presumably of phage or E.coli origin (figure 2).

Excluding data from EHA9 (see below), this panel assigned X-specific inserts to Xq or to one of three regions on Xp; (i) Xcen-Xp21, (ii) Xp21-22.3 or (iii) Xp22.3-Xpter. These assignments are summarised in table 4. Autoradiographs of these results (except for those obtained with X37 and X89) are shown in appendix 1. Probes X37, X38, X48 and X74 contained moderate repeats. Specifications of the X-specific inserts are shown in table 5.

Four Xp probes, including representatives of each of the three classes described earlier, were obtained. GMGX10 (X24) was assigned to Xp21-Xcen, GMGX11 (X66) and GMGX12 (X112) to Xp21-Xp22.3 and GMGX9 (X47) to Xp22.3-Xpter. These four inserts were subcloned into the EcoRI site of pUC19 (section 2:6:3).

The breakpoint in hybrid EHA9 was ascertained cytogenetically as Xp22.1 which was inconsistent with results with probe GMGX9 (X47). Data with GMGX9, (table 4 and appendix 1), indicated that

EHA9 had a more distal breakpoint than AMIR2N, at Xp22.3. Probes GMGX10, GMGX11 and GMGX12 were all present in EHA9. EHA9 thus has Xp sequences both proximal and distal to the breakpoint in W2A9. This anomaly might be explained by an interstitial deletion, a rearranged X-chromosome in EHA9 or a more distal breakpoint in EHA9 than AMIR2N. No conclusions were drawn from data with EHA9.

3:4 INITIAL CHARACTERISATION OF GMGX9-12.

Only the Xp-specific probes were characterised further. Probes are most useful when they detect RFLPs and/or deletions and thus these possiblities were explored for each of the four Xp probes (GMGX9, GMGX10, GMGX11 and GMGX12).

3:4:1 Search for RFLPs.

A search for high frequency restriction length polymorphisms (RFLPs) was performed for the four Xp probes (GMGX9, GMGX10, GMGX11 and GMGX12) using panels of two males and five females (or seven females) digested with each of the following restriction enzymes: AluI, BclI, BglII, DdeI, DraI, EcoRV, HaeII, HincII, HindIII, MboI, MspI, PstI, PvuII, RsaI, TaqI and XmnI.

No polymorphisms were detected with GMGX10, GMGX11 or GMGX12 with these sixteen enzymes. Only one polymorphism was detected with GMGX9 using HindIII. This is a two allele system which detects either a 4kb band or two bands of 2.5kb and 1.5kb in normal individuals, and is illustrated in figure 3.

Forty normal males and forty normal females were screened in order to calculate the allele and heterozygote frequencies for GMGX9. These were determined as 67% for the 4kb and 33% for the 2.5/1.5kb alleles respectively. The heterozygote frequency was 53% and the polymorphic information content (PIC) value was 0.44 (i.e. 2x 0.67 x 0.33).

Applications of this polymorphism will be discussed below (section 3:4:2 and sections 3:7:1-3).

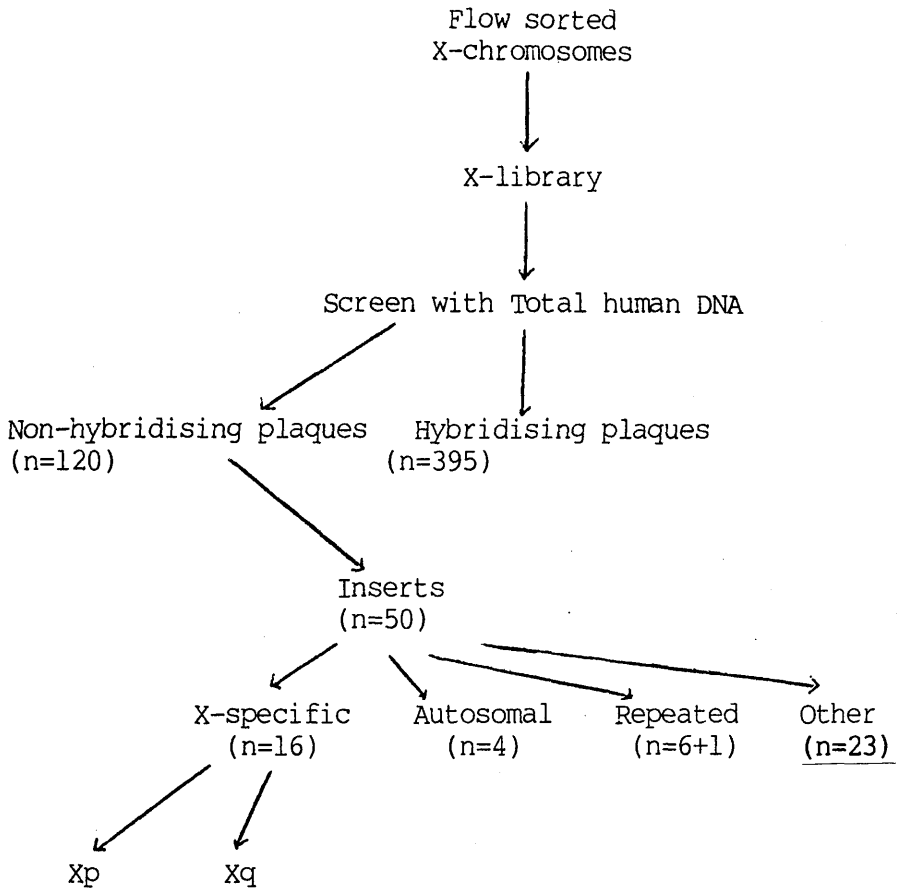
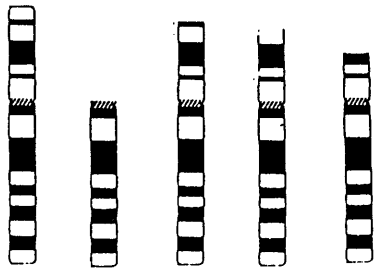


Figure 2: Characterisation of the X-chromosome library.



	FEMALE	HORL9X	LNA9	AMIR2N	EHA9	W2A9	A9
INSERT	I	2	3	4	5	6	7
X23/GMGX8	+	+	+	+	+	+	-
X24/GMGX10	+	+	-	+	+	+	-
X30/GMGX19	+	+	+	+	+	+	-
X37/GMGX22	+	+	+	+	+	+	-
X38/GMGX20	+	+	+	+	+	+	-
X47/GMGX9	+	+	-	-	+	-	-
X48/GMGX18	+	+	+	+	+	+	-
X53/GMGXY12	+	+	+	+	+	+	-
X60/GMGX16	+	+	+	+	+	+	-
X66/GMGX11	+	+	-	+	+	-	-
X74/GMGX13	+	+	+	+	+	+	-
X89/GMGX17	+	+	+	+	+	+	-
X92/GMGX14	+	+	+	+	+	+	-
X94/GMGX15	+	+	+	+	+	+	-
X110/GMGX23	+	+	+	+	+	+	-
X112/GMGX12	+	+	-	+	+	-	-

Table 4: Hybridisation of recombinants to the EcoRI somatic cell hybrid panel. These hybrids are described in materials and methods. Autoradiographs of these results are shown in appendix 1.

Name	Lab name	Vector	Insert	Location	DXS number
GMGX8	X23	NM1149	5kb	Xq	236
GMGX9	X47	pUC19	7kb	Xp22.3-pter	237
GMGX10	X24	pUC19	6kb	Xp21-cen	238
GMGX11	X66	pUC19	1.2kb	Xp21-p22	239
GMGX12	X112	pUC19	4.5kb	Xp21-p22	240
GMGX13	X74	NM1149	2.5kb	Xq	241
GMGX14	X92	NM1149	2.5kb	Xq	242
GMGX15	X94	NM1149	3.5kb	Xq	243
GMGX16	X60	NM1149	4kb	Xq	244
GMGX17	X89	NM1149	5kb	Xq	245
GMGX18	X48	NM1149	2.5kb	Xq	246
GMGX19	X30	NM1149	8kb	Xq	247
GMGX20	X38	NM1149	4kb	Xq	248
GMGX22	X37	NM1149	2.5kb	Xq	250
GMGX23	X110	NM1149	5kb	Xq	251
GMGXY12	X53	pUC19	1.6kb	Xq/Y	/

Table 5: Specifications of X-specific recombinants.

All are inserts are released by EcoRI digestion.

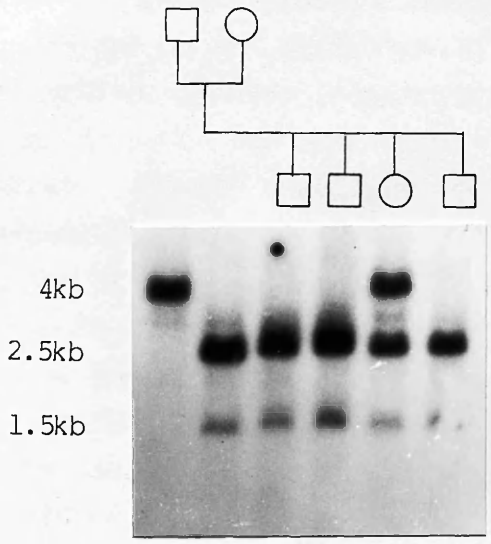


Figure 3: The GMGX9(HindIII) polymorphism, shown in a normal family.

3:4:2 The preliminary deletion screen with GMGX9, GMGX10, GMGX11 and GMGX12.

The panel which was used to screen for deletions at the DMD locus consisted of two individuals with complex phenotypes (BB and MJ, described below) and nine individuals with BMD or DMD with deletions of pERT87-8 and/or XJ1.1. These nine boys had been detected from amongst eighty seven boys with DMD or BMD prior to beginning this project (D. Wilcox, unpublished observations). The pedigrees of these nine individuals are illustrated in appendix 2A.

Scottish individuals are identified by both initials and pedigree numbers (indicated in parenthesis) in the text and by pedigree numbers in the figures and appendices. Other individuals are identified by their initials and/or place of origin eg "(Oxfo)"; Oxford, "(Card)"; Cardiff.

BB had chronic granulomatous disease, retinitis pigmentosa and the McCleod syndrome in association with DMD (Francke et al, 1985) whilst MJ (Oxfo) had DMD, congenital adrenal hypolplasia (AHC) and glycerol kinase deficiency (GK), (Davies et al, unpublished observations). AM (5996) and JR (5194) who both have AHC but not DMD or GK deficiency are also included in this panel. AM(5996) but not JR is mentally retarded. Results for AM(5996) and JR (5194) will be presented in section 3:5:6.

GMGX10 was deleted in BB and MJ (Oxfo) and also in SS (and his cousin JD, 5099), (table 6 and appendix 2). GMGX11 was only deleted in MJ (Oxfo) (table 6 and appendix 2B) and GMGX12 was not deleted at all (table 6).

BB and SS (and his cousin JD, 5099) had large deletions (visible on FACS analysis, Wilcox et al, 1986) as did MJ (Oxfo) (A. Cooke, unpublished observations) which suggested that both GMGX10 and GMGX11 might be further from "the DMD gene" than either pERT87 or XJ.

GMGX9 was not deleted in any of these individuals (appendix 2B) as predicted by its assignment to Xp22.3. It was, however, unexpectedly shown to detect deletions in individuals with X-linked ichthyosis. This became apparent whilst undertaking RFLP analysis in these families (sections 3:7:1-3) and greatly increased the value of GMGX9.

	C a r d	O x f o	5 0 9 9	5 0 9 9	3 4 8 5	5 1 1 6	5 1 1 3	4 0 9 7	5 3 1 1	0 1 2 0	5 2 6 5	5 0 9 7	5 9 9 6
	DH	MJ	BB	SS	JD	SJ	SB	NJ	SM	PE	AB	AK	JB	AM
Telomere														
GMGX12	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D2		+	+	+	+	+	+	+	+	+	+	+	+	+
99.6		+	+	+	+	+	+	+	+	+	+	+	+	+
B24	-	+	+	+	+	+	+	+	+	+	+	+	+	-
C7	-	+	+	+	+	+	+	+	+	+	+	+	+	-
L1-4	-	+	+	+	+	+	+	+	+	+	+	+	+	-
J66H1	-	-	+	+	+	+	+	+	+	+	+	+	+	+
GMGX11	-	-	+	+	+	+	+	+	+	+	+	+	+	+
p20		-	+	+	+	+	+	+	+	+	+	+	+	+
J-Bir		-	+	-	-	+	+	+	+	+	+	+	+	+
pERT87.30		-	-	-	-	+	-	-	-	-	+	+	+	+
pERT87.15		-	-	-	-	-	-	-	-	-	+	+	+	+
pERT87.8		-	-	-	-	-	-	-	-	-	+	+	+	+
pERT87.1		-	-	-	-	-	-	-	-	+	+	+	+	+
XJ10.1		-	-	-	-	-	-	+	+	+	+	-	-	+
XJ2.3		-	-	-	-	-	-	+	+	+	-	-	-	+
XJ1.1		-	-	-	-	-	-	+	+	+	-	-	-	+
XJ1.2		-	-	-	-	-	-	+	+	+	-	-	-	+
HIP25		-	-	-	-	-	-	+	+	+	-	-	-	+
XJ5.1		-	-	-	-	-	-	+	+	+	-	+	-	
JMD		-	-	-	-	-	-	+	+	+	-	+	+	
J47		+	-	-	-	+	+	+	+	+	-	+	+	
pERT84	-	-	-	-	-	+	+	+	+	+	+	+	+	+
754-11		-	-	-	-	+	+	+	+	+	+	+	+	+
754-6		-	-	-	-	+	+	+	+	+	+	+	+	+
754	-	-	-	-	-	+	+	+	+	+	+	+	+	+
GMGX10		-	-	-	-	+	+	+	+	+	+	+	+	+
OTC		+	+	+	+	+	+	+	+	+	+	+	+	+
L1.28		+	+	+	+	+	+	+	+	+	+	+	+	+
p58.1		+	+	+	+	+	+	+	+	+	+	+	+	+
Centromere														

Table 6: Results of hybridisation of Xp probes to the DMD deletion panel.

(+, hybridisation; -, deletion; blank, not tested. See appendices 2 and 3 for autoradiographs. The position of GMGX12 is approximate.)

3:4:3 A summary of initial findings.

In summary a library was constructed and sixteen X-specific recombinants were defined, four of which mapped to Xp.

GMGX9 was assigned to Xp22.3-pter and was the only probe to detect a polymorphism.

GMGX10 was mapped to the interval Xp21-cen and detected deletions in three unrelated individuals with DMD and/or complex syndromes including DMD.

GMGX11 and GMGX12 were assigned to Xp21-p22.3. GMGX11 was deleted in an individual with DMD, GK defeciency and AHC, whilst GMGX12 did not detect deletions.

3:5 FURTHER CHARACTERISATION OF GMGX10, GMGX11 AND DMD DELETIONS.

3:5:1 Extension of the preliminary deletion screen to include other probes.

Of the nine deleted boys with BMD/DMD described above, three were deleted for XJ1.1 alone, three for pERT87-8 alone and three for both probes corresponding to 7% (six out of eighty seven) boys at each locus. All eighty seven boys were screened with XJ1.2, early in the project but no new deletions were found.

These nine individuals, together with BB, MJ (Oxfo) and AM (5996) were studied with twenty seven probes (listed in table 6) which spanned Xp21 from Xp11 to Xp22 (figure 14). A composite of most of the autoradiographs obtained is presented in appendix 2C, whilst the results are tabulated in table 6.

These eleven deletions could be resolved into ten groups as follows (table 6) : MJ (Oxfo) was deleted from GMGX10 to J66H1, BB from GMGX10 to pERT87-30, SS (5099, and his cousin, JD) from GMGX10 to JBir, SB (5116) from JMD to pERT87-30, SJ (3485) from JMD to pERT87-15, NJ (5313) and SM (4907) from pERT87-1 to pERT87-30, PE (5311) from pERT87-8 to pERT87-30, AB (0120) from J47 to XJ10.1, AK (5265) from Hip25 to XJ2.3 and JB (the only Becker deletion, 5097) from XJ5.1 to XJ2.3 Only NJ (5313) and SM (4907) were not resolved by these probes.

A further individual, DH (Card) (from P.Harper), with a "complex phenotype" (DMD/ GK/ AHC and mental retardation) was included belatedly and has not been completely characterised. DH (Card) had an extensive deletion including probes pERT84 and C7/B24 (table 6 and figure 4).

An attempt was made to relate the extent of the deletion to the severity in phenotype but no clear correlation was apparent, for example the deletion in JB (5097), with BMD, encompasses that of AK (5265), with DMD. This argued against a BMD-specific domain.

Only JB (5097) was classified as BMD. SS (5099) was severely mentally retarded (and his cousin JD less so). JD (5099) was included in the analysis, although he was not an independent mutation, because of this discrepancy. The other eight boys were not mentally retarded and did not have glycerol kinase defeciency or congenital adrenal hypoplasia.

Anomalous hybridisation was observed with probe J47 in MJ (Oxfo) (table 6 and appendix 2), which suggests that his deletion is not contiguous or that a complex rearrangement or inversion has occured prior to the generation of his deletion. Other anomalies appear in the flow karyotype of his lymphoblastoid cell line (A. Cooke, personal communication) but it is nevertheless difficult to explain how this might have arisen.

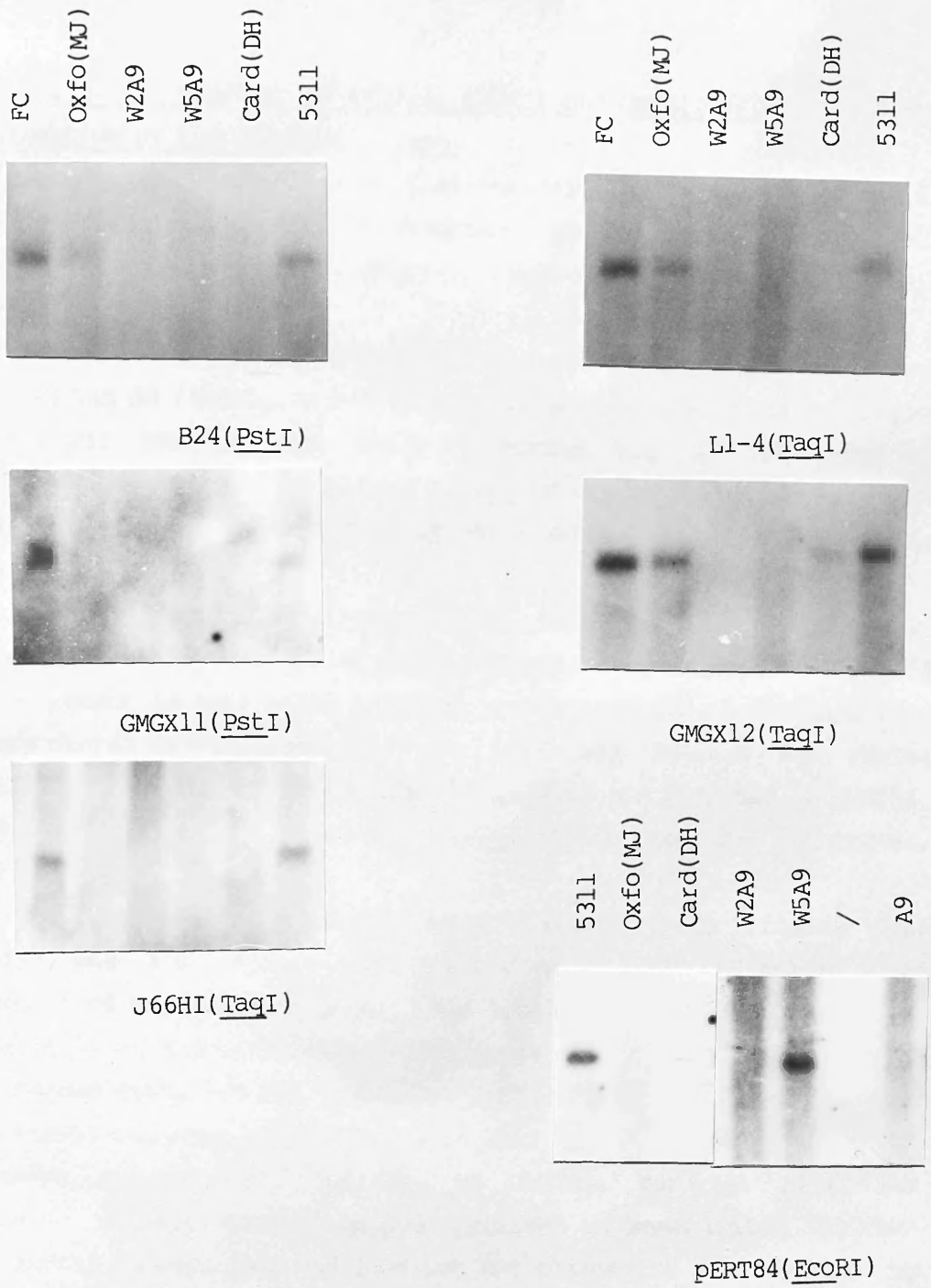


Figure 4: Results of hybridisation to Card(DH), Oxfo(MJ) and to somatic cell hybrids W2A9 and W5A9.

(FC, female control; 5311, male with DMD only; A9, mouse parent cell line. Details of Card(DH) and Oxfo(MJ) are given in the text whilst W2A9 and W5A9 are described in materials and methods).

3:5:2 Fine mapping of GMGX10, GMGX11 and GMGX12 arising from the extension of the deletion screen.

Based on the assumption that the deletions were contiguous, it was possible to deduce the approximate position of any new Xp21 probes isolated with respect to those already available (table 6 and figure 5).

GMGX10 was assigned to the region of overlap in the deletions of SS and JD (5099), BB and MJ (Oxfo), and excluded from the region of their overlap with other deletions e.g. SJ (3485). 754 and pERT84 both detected deletions only in these individuals. This localised GMGX10 between OTC and J47, (which flank 754 and pERT84), (table 6 and figure 5).

Two somatic cell hybrid lines, W2A9 and W5A9, (section 2:9) were used to further refine the localisation of GMGX10. Since 754 was present in both these hybrids, the breakpoints in W5A9 and W2A9 were distal to 754. W2A9 but not W5A9 was deleted for pERT84 (figure 4) and thus the breakpoint in W2A9 was proximal to pERT84. GMGX10 was present in W2A9 which localised it proximal to pERT84, (figure 5).

GMGX11 was deleted in MJ (Oxfo) (and DH (Card) (figure 4)). J66H1 was also deleted in these two individuals but not in other members of the deletion panel. This localisation was supported by the following observations. Firstly, as GMGX11 was absent in W2A9, it mapped distal to 754. Secondly, GMGX11 was not deleted in SS or JD (5099) and thus mapped distal to JBir (which is deleted in these cousins, see table 6). Thirdly, as GMGX11, but not L1-4, was deleted in MJ, GMGX11 mapped proximal to L1-4. GMGX11 was thus localised between JBir and L1-4 but not orientated with respect to J66H1 (figure 5). Further evidence pertinent to the localisation of GMGX11 (and of GMGX10) will be presented in the discussion (section 4:2:1).

GMGX12 was absent in W2A9, placing it distal to 754 and present in DH (Card), (figure 4), placing it distal to B24/C7. GMGX12 was also present in AMIR2N which placed it proximal to the Xp22.3 breakpoint i.e. proximal to GMGX9 and STS (figure 5).

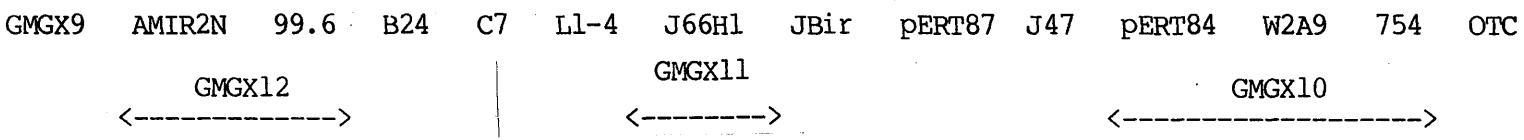


Figure 5: Preliminary localisation of GMGX10, GMGX11 and GMGX12 with respect to the deletion map and somatic cell hybrid mapping panel.

(The approximate positions of these DNA probes are shown in figure 14. Hybrid breakpoints are illustrated in figure 1)

3:5:3 Screening of all DMD/BMD boys for deletions with GMGX10, GMGX11 and GMGX12.

EcoRI and HindIII digests were prepared from eighty seven unrelated DMD males, fifteen unrelated BMD males and a further male, SR (2161), aged ten, with either BMD or DMD. These were screened with GMGX10, GMGX11 and GMGX12 in order to search for new deletions.

GMGX10 detected only those deletions which had already been described in SS and JD (5099), BB and MJ (Oxfo) (section 3:4:2). GMGX12 detected no deletions. GMGX11 detected sixteen deletions amongst the EcoRI digests, fifteen deletions amongst HindIII digests and altered fragment sizes in a further two patients, NR(7589) and PS (4033) (table 7). Thus GMGX11 detected abnormalities in eighteen of one hundred and three (18%) boys. Autoradiographs showing hybridisation of GMGX11 to HindIII filters of these boys are presented in appendix 3.

Differences were detected by GMGX11 between EcoRI and HindIII digests in four boys. Two of these, SR (5561) and MT (0107), revealed a larger than normal HindIII fragment but were deleted for the EcoRI fragment detected by GMGX11. The third, DM (3328), was deleted for the HindIII fragment but revealed a larger than normal EcoRI fragment whilst the fourth, PS (4033), had two EcoRI fragments smaller than the expected size but was not deleted for the HindIII fragment (table 7 and appendix 3).

These altered bands were not due to plasmid contamination, partial digestion or degradation as normal band sizes were detected on sequential hybridisation of these filters with other probes (section 3:5:4). They may therefore represent rare polymorphisms, partial deletions, point mutations altering the restriction site in question or endpoints associated with other chromosomal aberrations such as inversions or duplications.

The endpoints of the deletion (or rearrangement) are most precisely defined for NR(7589) and PS(4033) who are not completely deleted for either EcoRI or HindIII fragments but differentiate

between the two enzymes at the GMGXl1 locus.

3:5:4 Extension of the deletion analysis to include probes in the vicinity of GMGXl1.

JBir and p20 (proximal to GMGXl1) and J66Hl (distal to GMGXl1) were used to examine the extent of the deletions detected by GMGXl1 and to search for further mutations. These probes were tested on the EcoRI and HindIII filters used in the screen for deletions with GMGXl0, GMGXl1 and GMGXl2.

p20 was tested on the HindIII filters and detected eight deletions, five partial deletions and nine individuals with novel fragment sizes (including seven with two or more of the three HindIII fragments detected in the majority of individuals, see table 7).

p20 demonstrates an MspI polymorphism with constant bands of 1.5kb and 1.0kb and alleles of 6kb and 3kb (table 3). MspI digests were performed on all boys with deletions or alterations detected by GMGXl1, JBir or p20. Complete deletion of p20 (i.e. deletion of both constant bands and alleles) was observed in JB(5534), AS (2672), SMcK (7501), AW (7866), GN (4947), PM (3623), EH and JL (5190), DMcC (1596), JS (3896) and DC (4051)), whilst the allelic bands ("Al" (3kb) or "A2" (6kb)) but not the "constant bands" were deleted in three families, (SR (2161), RW, (3468) and DW (3995)), (table 7). A small extra band was observed in MT(0107) and JB(4629). Examples of these results are illustrated in appendix 4.

The number of "deletions" detected by p20 differed according to the restriction enzyme (MspI or HindIII) used (table 7 and appendix 4). At least nine of the individuals tested exhibited enzyme specific differences (WB (3405), AW (7866), SMcK (7501), GN (4947), JC (4221), JB (4629), MB (0187), BP (3552) and AP (5110)). Some individuals deleted for the MspI fragments of p20 but not HindIII fragments may not have been detected as MspI digests were only performed for those individuals already known to be deleted.

As p20 was derived from two sequences some 30-40kb apart, either fragment, both fragments or neither fragment might be altered or deleted in the vicinity of a restriction site dependent on the restriction enzyme used, thus accounting for the variation

observed.

It is worthy of note that six of the families which show an altered pattern of hybridisation to p20 have been classified as BMD: 4629, 5190, 3468, 3995, 4002 and 4051. SR (2161) may represent a seventh BMD family with an altered pattern of hybridisation to p20 but his DMD/BMD status is still in question. These six families represent at least 40% BMD families in this study. Three of these have the same HindIII fragments (table 7), which are also shared by SR (2161) and DP (4786) with DMD. Examples of autoradiographs obtained with p20 are presented in appendix 4.

JBir was hybridised to HindIII filters and J66H1 to EcoRI filters. JBir detected deletions in SS and JD (5099), as described earlier (table 6), MT (5657) and IM (4180). JBir also detected an altered fragment size in JL(1406). These results are also presented in appendix 4.

J66H1 detected a fragment of altered size in MT(5310) (appendix 4). Sixteen individuals were observed to have a fragment of similar size to that in MT(5310), in addition to the normal fragment. Ten of these individuals are deleted or have fragments of altered size for JBir, p20 or GMGX11 and are amongst those listed in table 7. Mutations were not detected with p20, GMGX11 or J66H1 amongst the other six affected males; JT (3568, BMD), AC (5113, DMD), RC (7500, DMD), ML (7502, DMD), SM (3997, DMD) and MP(7497, DMD). Autoradiographs showing hybridisation of J66HI to MT(5310), AC (5113) and RC (7500) can be found in appendix 4.

The significance of this finding with J66HI is not yet determined. Some of these individuals may have contiguous deletions with an endpoint detected by J66HI, but as some are additionally deleted for p20, but not GMGX11, (table 7), it seems more likely that the extra band represents a polymorphism rather than the endpoint of a mutation.

The pedigrees of individuals listed in table 7 are shown in appendix 5. Pedigree 5099 is shown in appendix 2.

Name	Ped.No	JBir(H)	p20(H)	p20(M)	GMGXl1(H)	GMGXl1(R)	J66(R)
JB	5534	D	+	-	-	-	+
JB	0198	D	+	123	12A2	-	+
WB	3405	D	+	13H	12A1	-	+
JH	5098	D	+	123	?	-	+L
SR	5561	D	+	123	12A2	H	+L
JS	5538	D	+	?	12A1	-	+
AS	2672	D	+	-	-	-	+
MT	0107	D	+	123	12A2L	H	+L
AW	7866	D	+	123	-	-	+
NR	7589	D	+	123	12A2	H	+L
NMcD	5540	D	+	123	12A2	-	+
FMcG	4045	D	+	123	12A2	-	+
SMcK	7501	D	+	I	-	-	+L
PS	4033	D	+	123	12A1	+	2x L +
GN	4947	D	+	L	-	-	+L
SM	5120	D	+	123	12A2	-	+
KM	5120	D	+	123	12A2	-	+
PM	3623	D	+	-	-	-	+
DM	3328	D	+	123	12A2	-	H +
MT	5310	D	+	123	12A1	+	+ L
MT	5657	D	-	123	12A2	+	+ +
IM	4180	D	-	123	12A2	+	+ +
SS	5099	D	-	123	12A2	+	+ +
JD	5099	D	-	123	12A2	+	+ +
JL	1406	D	H	123	12A1	+	+ +
MJ	Oxfo	D	-	-	-	-	- -
JC	4221	D	+	13H	12A2	+	+ +
JB	4629	B	+	123L	12A2L	+H	+ +L
MB	0187	D	+	12I	12A2	+	? +L
EH	5190	B	+	-	-	+	+ +
JL	5190	B	NT	-	-	NT	+ +
DMcC	1596	D	+	-	-	+	+ +
SR	2161	B/D	+	23	12	+	+ +
BP	3552	D	+	13H	12A1	+	+ +
AP	5110	D	+	13H	12A2	+	+ +
JS	3896	D	+	-	-	+	+ +
RW	3468	B	+	23	12	+	+ +
JH	3468	B	+	23	NT	+	NT +
RMcL	3468	B	+	23	NT	+	NT +
DW	3995	B	+	23	12	+	+ +
CS	3995	B	+	23	NT	+	+ +
DC	4051	B	+	-	-	+	NT +
SB	5972	D	+	123L	NT	+	NT +
CK	4002	B	+	23	NT	+	+ +
DP	4786	D	+	23	NT	+	+ +L

Table 7: Deletions detected by JBir, p20, GMGXl1 or J66HI.

(+, positive signal; -, deletion; NT, not tested; ?, unclear result.(H),HindIII; (M),MspI; (R),EcoRI. 123, normal restriction fragment pattern with p20 (HindIII); 12A1 or 12A2, normal pattern with p20 (MspI), 1 and 2 are constant bands, A1 represents the 3.5kb allele and A2 the 6kb allele. H, higher than normal fragment; L, lower than normal fragment; I, novel p20 HindIII fragment of intermediate size (between "2" and "3"). Sample autoradiographs are shown in appendices 3 and 4).

3:5:5 Contiguous gene syndromes involving DMD, ordering of loci in Xp21 and the definition of a deletion in an individual with AHC.

BB was affected with DMD and also with chronic granulomatous disease (CYBB), retinitis pigmentosa type 3 (RP3) and the McCleod syndrome (XK). BB was deleted from GMGX10 to pERT87.30. His deletion was very similar to that of SS (5099) which extended from GMGX10 to JBir inclusive (table 6). SS (5099) had DMD and mental retardation but none of the other disorders described in BB. SS's deletion extends more distally than BB's which suggests that CYBB, RP3 and XK are all proximal to DMD (figure 6).

DH (Card) and MJ (Oxfo) both suffered from DMD, GK and AHC. MJ(Oxfo) was deleted from GMGX10 to J66H1 whilst DH was deleted from 754 to B24/C7. SS (5099) had neither GK nor AHC and was deleted from GMGX10 to JBir. Direct comparison of the deletions in MJ (Oxfo) and SS (5099) assigns GK and AHC distal to JBir and proximal to L1-4 (which is present in MJ (Oxfo)) (table 6 and figure 6).

AM (5996) was affected with AHC and was also severely mentally retarded. Thus, although he did not suffer from GK deficiency or DMD, he was a "good candidate" for a micro-deletion. AM (5996) was deleted for C7 (appendix 2C) and also for B24 and L1-4 (appendix 2C and figure 7) but not for J66H1 or D2, or other Xp probes tested, (table 6 and figure 7).

As AM (5996) is not deleted for J66HI, his deletion places AHC distal to J66HI. Assuming that the deletions in MJ and AM (5996) are overlapping this deletion also suggests that AHC is distal to GK deficiency (figure 6).

The mother of AM (5996) , but not his grandmother, was mildly mentally retarded which suggested his mother (but not his grandmother) carried the deletion. His grandmother was found to be heterozygous for B24 confirming that she did not carry the deletion. (C7 was not informative and L1-4 is not polymorphic).

DNA studies were performed on flanking markers and AM (5996) was shown to have inherited proximal markers from his grandfather (754 and J66H1) and the distal marker GMGX9 from his grandmother. Other distal markers tested (99.6, D2, 782 and RC8) were

uninformative.

Although the GMGX9 result suggests that the deletion in AM (5996) might have occurred by recombination in a maternal gamete, GMGX9 (within Xp22.3) is not tightly linked to B24/C7 (within Xp21.2 -Xp21.3). It seems more probable that the deletion occurred in a grandpaternal gamete, which was passed to his daughter and then to AM. This would explain why AM's mother but not his grandmother was mildly mentally retarded.

JR (5194), with AHC, but not affected by DMD, GK or mental retardation, was tested for a sub-microscopic deletion in this region. JR (5194) was not deleted for L1-4, B24 or C7 (figure 8) although AHC segregated with pERT87.1 (Msp I) and B24 (MspI) in his family (figure 8).

3:5:6 Summary of deletion data.

Thirty-six new mutations were detected with JBir, GMGX11 and p20 (table 7) and nine deletions with pERT87 and XJ (table 6). DNA analysis therefore detected mutations in a minimum of 45% of one hundred and three boys with DMD or BMD. Only MJ and DH, who had complex phenotypes and extensive deletions, were deleted for GMGX11, p20 and J66HI in addition to probes from the pERT87 and XJ loci. The thirty three deletions detected by GMGX11 and p20 thus represent a discrete deletion-rich region.

18% deletions are detected by GMGX11 and 22% by p20. There is considerable heterogeneity amongst these thirty three deletions (table 7) and only three individuals (JB (5534), AS (2672) and PM (3623)) are consistently deleted for both GMGX11 and p20. Eight of the nine deletions at the pERT87 and XJ loci were also resolved in this study (table 6) which suggests that there is also considerable heterogeneity amongst deletions at these loci.

AM (5996) with AHC, but not GK or DMD, whose deletion includes L1-4, B24 and C7, allows the ordering of the genes for DMD, GK and AHC in Xp21.

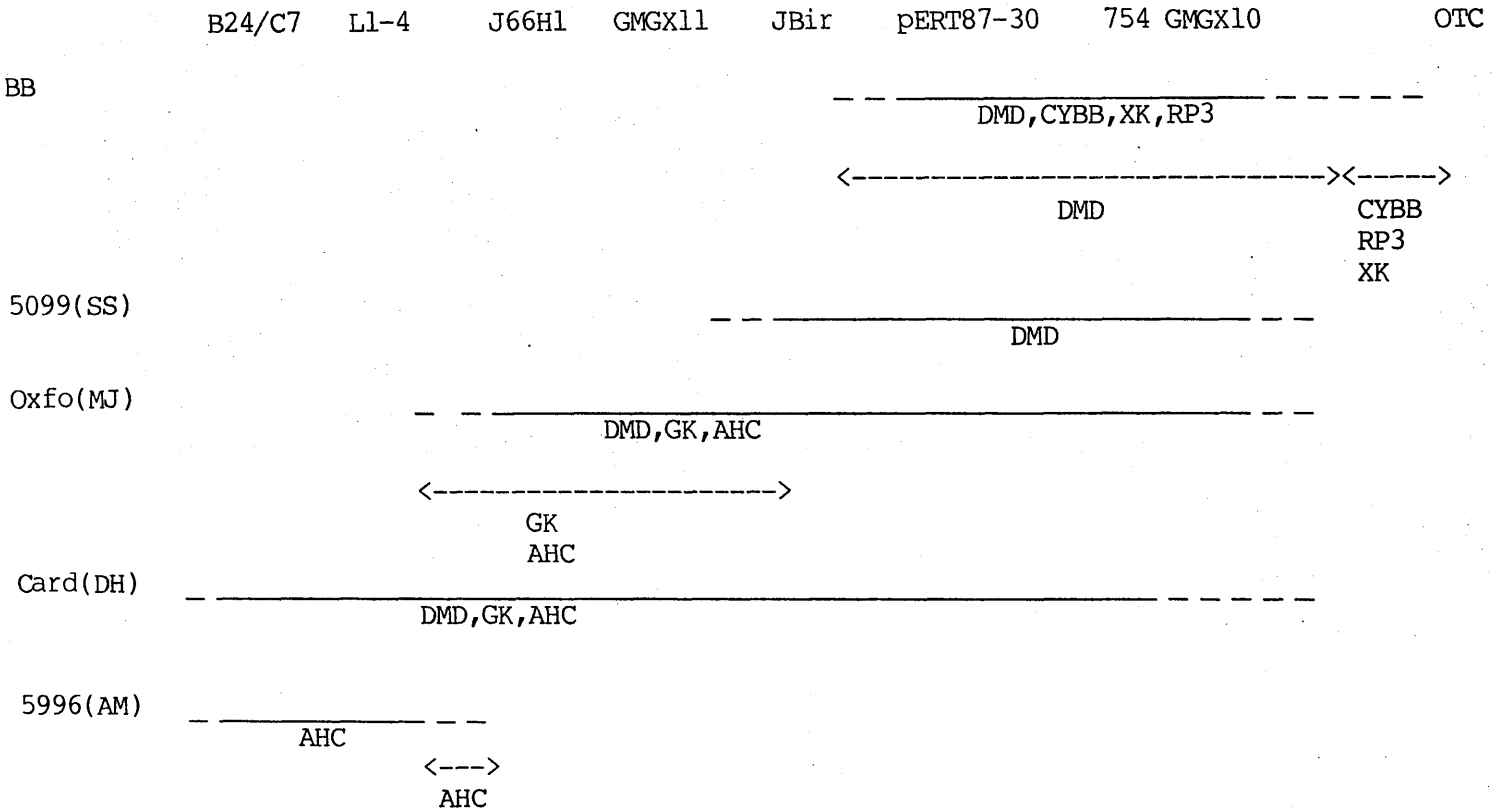


Figure 6: Gene localisations arising from the relative postions of microdeletions in Xp21.

(Not to scale. These individuals are described in the text. All five are mentally retarded. Solid lines represent deleted regions, whilst broken lines indicate the maximum limits of each deletion. Gene symbols are according to McKusick, 1987).

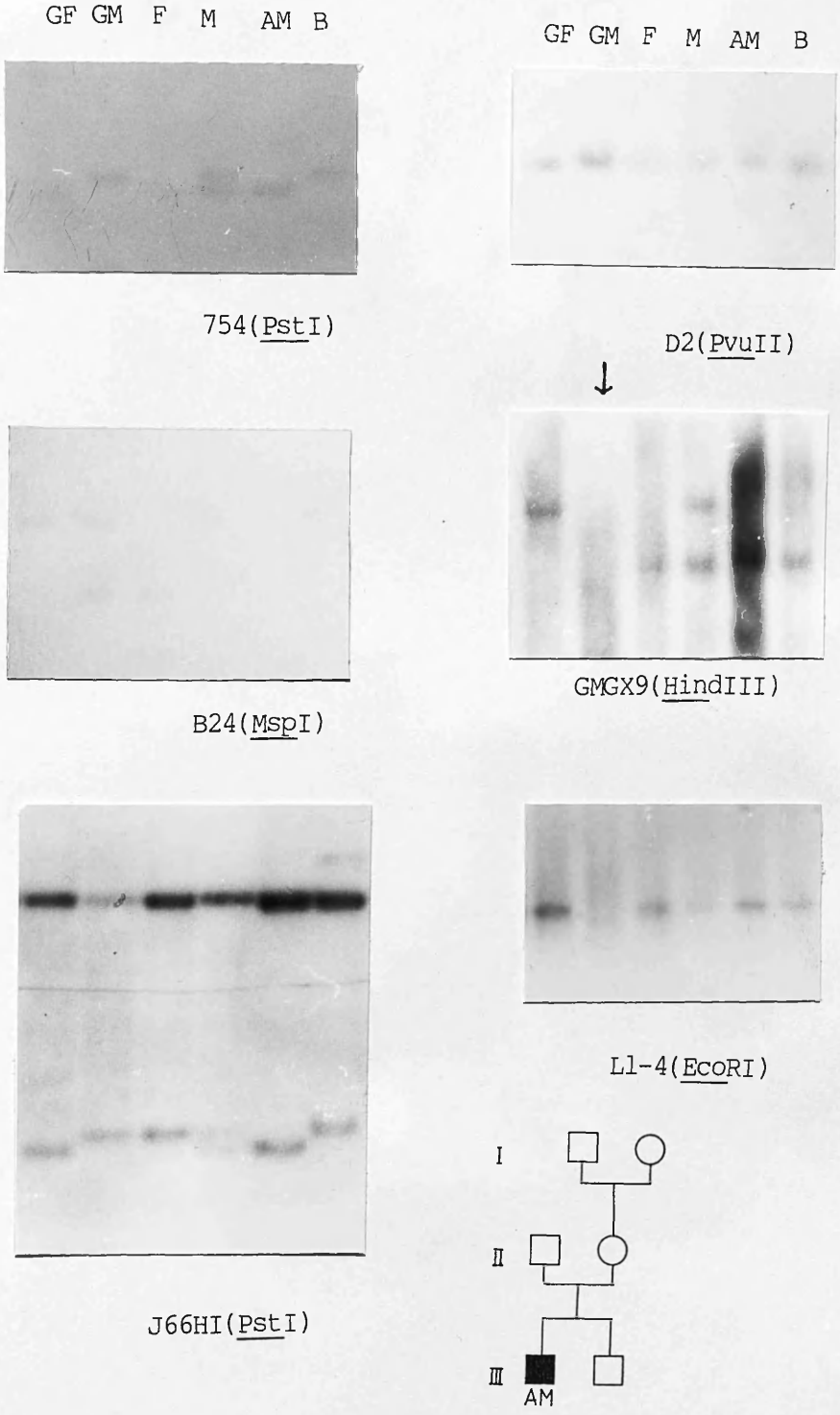


Figure 7: Results of deletion analysis of AM (5996) and his family.

(GF,grandfather (I_1); GM,grandmother (I_2); F,father (II_1); M,mother (II_2); AM,(III_1); B,brother (III_2). The grandmaternal track, marked with an arrow in the HindIII digest (probed with GMGX9) is degraded).

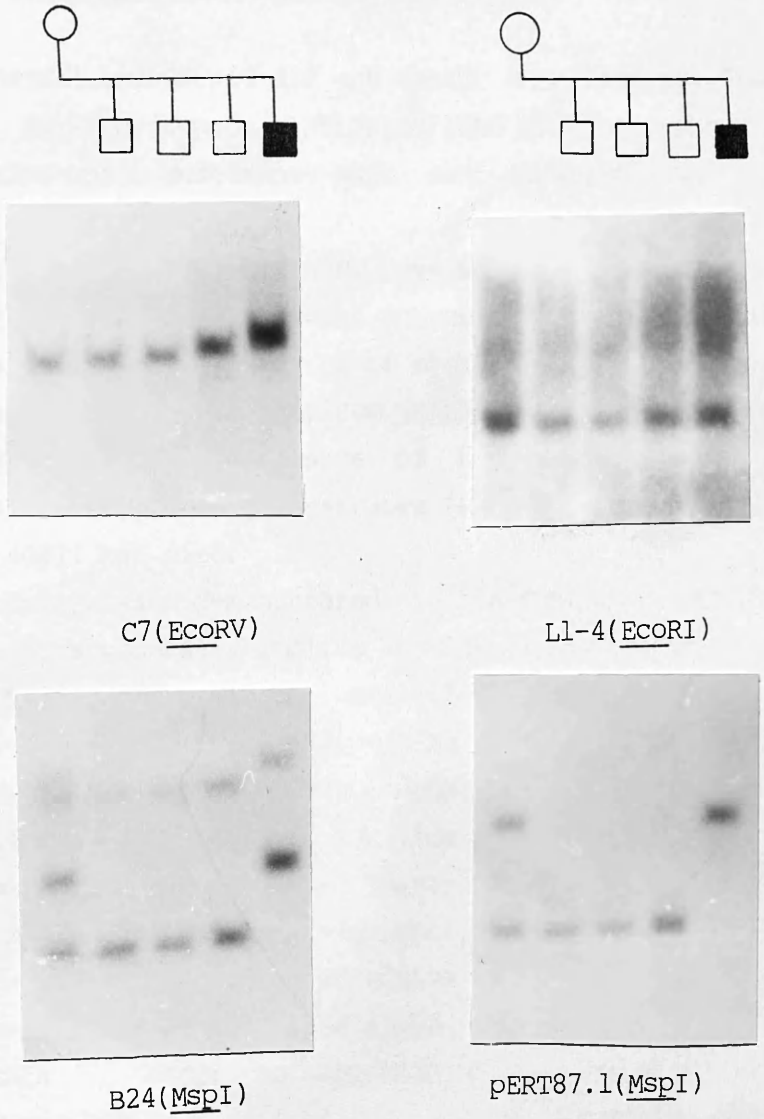


Figure 8: Results of deletion analysis of JR (5194) and his family.

3:6:1 RFLP analysis of deletions at the DMD locus.

GMGX10, GMGX11 and GMGX12 did not detect RFLPs and so linkage analysis was not performed. RFLPs defined by other probes were used to determine which deletions were new mutations and their origins.

The mothers of seven of the nine boys with deletions of pERT87 or XJ (table 6) were screened with polymorphic probes deleted in their sons, in order to determine their status with respect to the deletion. These results are summarised in table 8. The mother of SS (5099) was not studied as inheritance of his deletion from his grandmother had already been demonstrated (Wilcox et al, 1986). The mother of SM (4097) had died.

Heterozygosity was demonstrated in the mothers of NJ (5313) and SJ (3485). This generally implies absence of the deletion but may also indicate germ-line mosaicism. NJ's mother was heterozygous for pERT87-30 (BglII), pERT87-15 (BamHI), pERT87-8 (TaqI) and pERT87-1 (MspI). SJ's mother was heterozygous for pERT87-1 (MspI) and XJ5.1 (SphI). NJ (5313) and SJ (3485) are therefore likely to represent new mutations which most probably occurred in a maternal gamete, unless their mothers are germ-line mosaics. This will be considered further in the discussion.

The mothers of NJ (5313) and SJ (3485) were also analysed with flanking markers in order to determine which grandparent(s) the deleted chromosome had been derived from and to determine whether the deletion had arisen as a result of recombination. NJ failed to inherit his grandpaternal 99.6 (PstI), XJ2.3 (TaqI), XJ1.1 (TaqI), XJ1.2 (BclI), XJ5.1(SphI), 754 (PstI) and 754-11 (BglII) alleles and so appears to have inherited his grandmaternal X-chromosome without recombination. These results are presented in appendix 6.

SJ's grandfather was dead but it can be inferred that SJ has inherited his grandfather's 99.6 and 754-11 alleles, and thus his grandfather's X-chromosome, with no evidence of recombination. This suggests that his grandmother was not a carrier as had been assumed, on the basis that his grandmother's brother (individual II_5 in the pedigree reproduced in appendix 2) had been affected with DMD. The results for this family are presented in appendix 7.

The mothers of AB (0120), AK (5265), JB (5097), PE (5311) and SB (5116) were not heterozygous for any of the probes deleted in their sons (table 8) and may be homozygous or hemizygous at these loci. Grandparental samples, which might assist in defining maternal carrier status were only available for AB (0120).

The mother of AB (0120) exhibited the alternative XJ1.1, XJ1.2, XJ2.3 and XJ5.1 alleles from her own mother. Thus AB (0120) had inherited his deletion from both his mother and his grandmother. These results are presented in appendix 8.

The mothers of AK (5265), JB (5097), PE (5311) and SB (5116) are hemizygous or homozygous for three, four, three and eight loci respectively for which their sons are deleted (table 8).

If XJ1.2, XJ1.1 and XJ2.3 (which are in linkage disequilibrium, Thompson et al, 1986) and pERT87-1 and pERT87-8 (which are also in linkage disequilibrium, Lindlof et al, 1987) are each considered as one locus, MB, the mother of SB (5116) can be considered to be hemizygous or homozygous for five loci. These loci have heterozygote frequencies of 0.32-0.46, with a mean value of 0.42 (table 3). The theoretical probability that none of these five loci are polymorphic is approximately $(0.58)^5$ or 6-7%. This is in reasonable agreement with the findings of Bakker et al. (1986) who found that 97% of females were heterozygous for at least one pERT87 or XJ RFLP.

This estimate, combined with a prior risk of 4μ (Emery, 1976) that MB (5116) is a carrier and CK values which give odds of 1.6:1 that MB (5116) is a carrier, suggests that MB (5116) is a carrier (presumably deleted) with a probability of 0.99 and that SB (5116) does not represent a new mutation.

PE (5311) has an affected brother with Duchenne muscular dystrophy and mental retardation whilst JB (5097) has an uncle affected with Becker muscular dystrophy (ambulant until 14, died age thirty nine). These pedigrees are shown in appendix 2. It is thus unlikely that PE (5311) and JB (5097) represent new mutations and probable that the mothers of these boys do carry the deletion. However, as neither affected relative of these boys was available for study, it was not possible to confirm that DMD segregated with the deletion in these families.

The sister of PE(5311) exhibited different XJ5.1 and pERT87-1 alleles from her brother PE (5311). She was also heterozygous for pERT87-30 for which he was deleted. Thus she does not have the deletion. Similarly the sister of JB(5097) was not a deletion carrier as she was heterozygous for XJ1.2, for which JB (5097) was deleted. These results are presented in appendix 9.

As GMGX11 was not polymorphic, family members of boys from nine families deleted for GMGX11 (WB (3405), JH (5098), AS (2672), AW (7866), FMcG (4045), SMcK (7501), GN (4947), SM and KM (5120) and PM (3623) were investigated with two polymorphic markers which flanked GMGX11; J66H1 (PstI) and p20 (MspI).

None of the mothers of these boys were doubly heterozygous for p20 and J66HI. Five of these boys (AS (2672), AW (7866), SMcK (7501), GN (4947) and PM (3623)) were also deleted for p20 (MspI). None were deleted for J66HI(PstI), although J66HI detected an additional EcoRI band in both SMcK (7501) and GN (4947), (section 3:5:4). The mother of AW (7866) was heterozygous for p20 and thus he is likely to represent a new mutation. His sister is also heterozygous and thus does not carry the deletion. This result is also presented in appendix 9.

Time did not allow the extension of this analysis to include JBir and pERT87 proximally or B24/C7 distally or to analyse deletions subsequently revealed by p20.

3:6:2 Summary of RFLP analysis at the DMD locus.

In summary, RFLPs, detected by DNA probes other than GMGX10, GMGX11 and GMGX12, demonstrated that the mothers of three boys with deletions (NJ (5313), SJ (3485) and AW (7866)) were heterozygous for deleted probes and and were thus unlikely to be carriers for the deletion. The mother and grandmother of a fourth boy AB(0120) were shown to carry the deletion. The mother of a fifth boy SB (5116) was implicated as a deletion carrier by virtue of absence of heterozygosity at eight loci deleted in SB (5116).

M	PED.	S	<---------- PROBES---------->							
			XJ5.1	XJ1.2	XJ1.1	XJ2.3	87.1	87.8	87.15	87.30
MJ	3485	SJ	+	.	.	.	+	.	.	.
MB	5116	SB	.	.	.	.	.	.	.	.
MJ	5313	NJ	/	/	/	/	+	+	+	+
JE	5113	PE	/	/	/	/	/	.	.	.
MB	0120	AB	.	.	.	.	/	/	/	/
IK	5265	AK	/	.	.	.	/	/	/	/
JB	5097	JB	.	.	.	.	/	/	/	/

Table 8: Results of the screen for heterozygosity in the mothers of DMD boys with deletions.

(M, mother; PED, pedigree number; S, son; +, heterozygous; ., hemizygous or homozygous; /, not applicable (son not deleted). 87.1, pERT87.1; 87.8, pERT87.8; 87.15, pERT87.15; 87.30, pERT87.30. Some of these results for families 0120, 5313, 3485, 5097 and 5113 are presented in appendices 6-9).

3:7 FURTHER CHARACTERISATION OF GMGX9 POLYMORPHISM AND DELETIONS AT THE STS LOCUS.

3:7:1 RFLP analysis with GMGX9.

Linkage studies with GMGX9 were first performed in normal three generation families with three or more sons - "Tom, Dick and Harry" familes (TDH familes) - which each provided three or more informative meioses. Eight out of ten mothers in these families were heterozygous. These results are illustrated schematically in appendix 10, together with autoradiographs from three TDH families. The 4kb allele is represented by an "A" and the 2.5/1.5kb allele by a "B" in these schematic diagrams. These results show that GMGX9 segregates as a mendelian trait.

GMGX9 was then applied to the study of five families segregating for XLI (steroid sulphatase deficiency). Eight obligate carrier females from five families (two from family 3673, three from family 3496 and one each from families 4024, 1348 and 2994), were screened with GMGX9 in order to select for heterozygotes. No heterozygotes were found.

However, one of three obligate carrier sisters, (JP, 3496) had the alternative allele to her two sisters (MJ and HR, figure 9B). This indicated inheritance of a null allele from their father, as these sisters shared an affected father and each had affected sons. DNA samples were available from nine affected males in family 3496 and GMGX9 was found to be deleted in all nine (figure 9C).

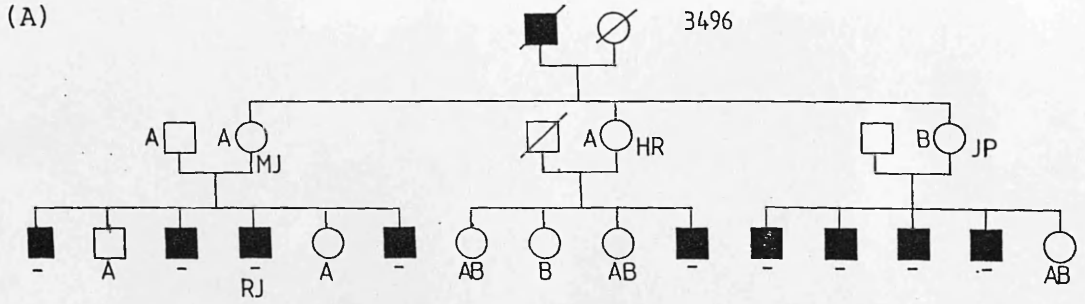
These five families were then studied in full. These results are also illustrated schematically in appendix 10. Affected individuals were consistently deleted in all but one family (4024), in which GMGX9 was also uninformative. Persistence of the deletion through three generations was demonstrated in family 3673 (figure 10) and implicated by inheritance patterns in families 1348, 2994 and 3496 (appendix 10).

GMMGX9 results for families 1348, 2994, 3496, 3673 and 4024, (described above), for a sixth family segregating for XLI (1034) and for the TDH familes (appendix 10) were combined with those with D2, 782, Dic56 and Mic2 (D.Goudie) and Xg (P.Tippett) for

multipoint linkage analysis (Yates et al, 1987b). The results of this linkage analysis are summarised in the discussion.

An Italian family (Ital) segregating for X-linked ichthyosis was studied and shown to segregate for a deletion of GMGX9. This result is shown in appendix 11. Prediction or exclusion of carrier status is possible using GMGX9 in families which segregate for a deletion, providing that the parents of the individual under investigation have different alleles.

It is possible to predict that individuals III_7 (Ital), and III_8 (3496) who failed to inherit maternal alleles are carriers, and that III_7 (3496), III_9 (3496) and III_{15} (3496), who are heterozygous for GMGX9, are not (appendix 11 and figure 9A). The accuracy of these predictions depends on the frequency of recombination between STS and GMGX9 and will be addressed in the discussion.



(B) GMGX9(HindIII)

Figure 9: Result of hybridisation of GMGX9 to family 3496.

(A: Pedigree of family 3496. "A",4kb allele ;"B",2.5/1.5kb allele; "-",deletion. (B): hybridisation of GMGX9 to MJ (II2), HR (II4) and JP (II6) respectively. (C): hybridisation of GMGX9 (upper panel) and pERT87.1 (lower panel) to the entire family). Continued over.

(C)

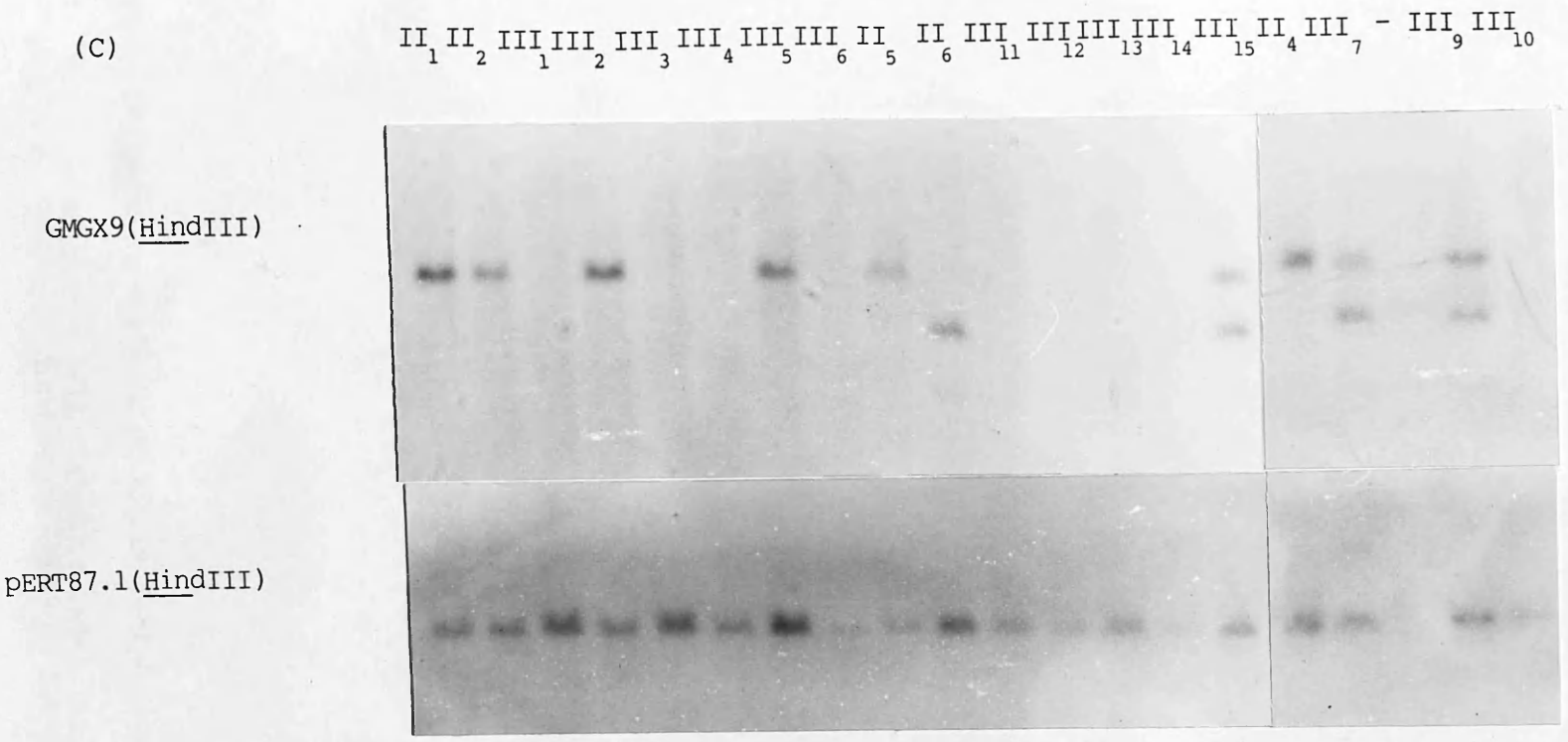
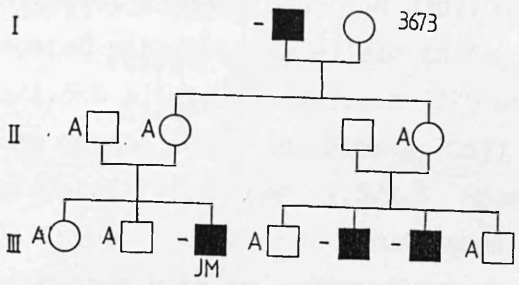
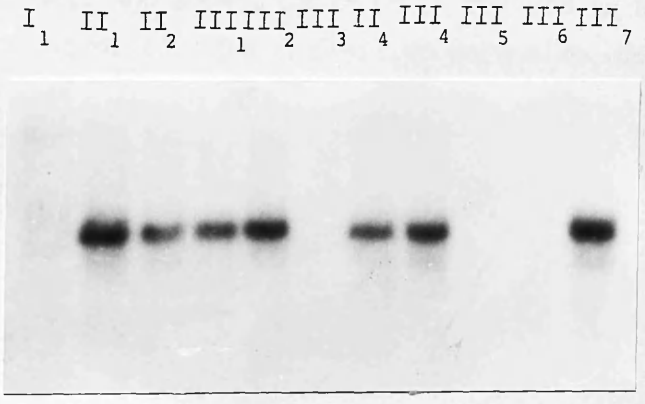


Figure 9 continued: Result of hybridisation of GMGX9 to family 3496.

(A: Pedigree of family 3496. "A",4kb allele; "B",2.5/1.5kb allele; "-",deletion. (B): hybridisation of GMGX9 to MJ (II2), HR (II4) and JP (II6) respectively. (C): hybridisation of GMGX9 (upper panel) and pERT87.1 (lower panel) to the entire family).





GMGX9 (HindIII)

Figure 10: Deletion analysis of family 3673 with GMGX9.

(A: Pedigree of family 3673. "A", 4kb allele; "B", 2.5/1.5kb allele; "-", deletion. (B): hybridisation of GMGX9 to the entire family).

3:7:2 Deletion analysis with GMGX9 and other distal Xp probes.

Males from twenty three independent "Glasgow" families segregating for STS deficiency, including the six families described above, were screened with GMGX9 (table 9). Only four of these families (4024, 7664, 3000 and 3307) were not deleted for GMGX9. XLI segregated with the 4kb allele in families 4024 and 7664 and with the 2.5/1.5kb allele in families 3000 and 3307.

Eight of nine German families (from A. Gal) were deleted for GMGX9. XLI segregated with the 2.5/1.5 kb allele in the ninth family (2/85 Br). These results are illustrated schematically in appendix 12, together with an autoradiograph showing results in families 1/85 (Ln), 2/85 (Br) and 3/85 (Ls). Segregation of the deletion with XLI in family 5/85 (Ge) was first inferred by the failure of II_2 to inherit the 2.5/1.5 kb allele from I_5 and was confirmed by A. Gal, who showed GMGX9 to be deleted in an affected male (III_2). Two out of four Dutch families were also deleted for GMGX9 (appendix 12).

Seven of eight affected Italian individuals (from A. Ballabio) were deleted for GMGX9 (figure 11). The eigth individual, Ba, has the 4kb allele of GMGX9 and will be discussed more fully below.

Thus, thirty-six of forty-two males with XLI (i.e. 86%) were deleted for GMGX9. These forty-two males were also investigated (by A. Ballabio) with p422, situated 3' to the STS gene. Thirty-seven of them were deleted for p422 (A. Ballabio, personal communication). These included the thirty-six males detected by GMGX9 and also Ba, who was not deleted for GMGX9. The cloning of STS will be presented in the discussion.

The twenty-three "Glasgow" families were examined with other distal Xp probes and markers e.g. Xg (P. Tippett), Dic56, 782, Mic2 (pl9b), MlA, and STBl4 to determine the extent of these deletions. (The location and details of these probes are presented in figure 14 and table 3). Dic 56, 782 and pl9b results in families 1034, 1348, 2994, 3496, 3673 and 4024 were the work of D. Goudie.

p2a4 and the X-specific bands of three Xp-Yq homologous probes, GMGXXY3, GMGXYl9 and STBl4 were deleted in the nineteen individuals deleted for GMGX9. (All GMGXXY3 and GMGXYl9 results

referred to throughout this thesis were obtained by M. Mitchell). All affected individuals hybridised to 782 and Dic56 and to the X-specific sequence of M1A. The majority of affected males tested (i.e. ten/ fifteen) were Xg(a) +ve which demonstrates the interstitial nature of these deletions. This data is summarised in table 9. Examples of the autoradiographs obtained with these probes are illustrated in appendix 13.

Ba, who was deleted for p422 but not GMGX9 was tested with additional probes in distal Xp. He was deleted for p2a4 (figure 11) and also for GMGXXY3, but not for STB14 or GMGXY19. Ba is included in table 9 for comparison. The other Italian patients were deleted for p2a4 (figure 11), GMGXXY3 and GMGXY19.

Thus, Ba had a deletion breakpoint close to or within the STS gene which divided probes into two groups; those within his deletion (p422, p2a4 and GMGXXY3) and those outwith his deletion (GMGX9, STB14 and GMGXY19). STSB14 is known to lie at the 5' end of STS but GMGX9 and GMGXY19 could lie to either side of the deletion. In common with all males with XLI, Ba was not deleted for the X-specific band of M1A.

Several distal Xp probes e.g. M1A, GMGXXY3, GMGXY19 and STB14 demonstrate homology to Yq whilst others e.g. p19b and 29Ci demonstrate homology to Yp. STS sub-clone p2a4 (and also p422, A. Ballabio, personal communication) failed to demonstrate Y homology at normal stringency. GMGX9 also failed to detect any homology to the Y-chromosome (figure 12), even when hybridisation was performed under conditions of low stringency (30% formamide,42°C).

PED.NO.	STS	782	DIC56	XXY3	X9	XY19	p2a4	MlA	Xg(a)	pl9b/PSGl
JM 3673	nil	+	+	-	-	-	-	+	+	+
SC 2994	nil	+	+	-	-	-	-	+	+	x
DG 1034	2.4%	+	+	-	-	-	-	+	+	x
JA 3710	nil	+	+	-	-	-	-	+	+	x
TR 4024	nil	+	+	+	+	+	+	+	+	+
TM 1348	nil	+	+	-	-	-	-	+	+	+
RJ 3496	nil	+	+	-	-	-	-	+	+	+
JD 0698	nil	+	+	-	-	-	-	+	+	x
SL 7568	NT	+	+	-	-	-	-	+	+	x
AB 4076	nil	+	+	-	-	-	-	+	-	x
DC 3000	nil	+	+	+	+	+	+	+	-	x
DC 7474	nil	+	+	-	-	-	-	+	NT	x
FM 7685	NT	+	+	-	-	-	-	+	NT	+
GB 7664	nil	NT	+	+	+	+	+	+	-	+
NJ 7750	nil	NT	+	-	-	-	-	+	-	+
EC 3307	nil	+	+	+	+	+	+	+	NT	+
HL 7605	nil	NT	+	-	-	-	-	+	NT	x
RF 7304	nil	NT	+	-	-	-	-	+	NT	x
YM 8056	nil	NT	+	-	-	-	-	+	NT	+
CK 5626	nil	NT	+	-	-	-	-	+	+	+
RM 4143	nil	NT	+	-	-	-	-	+	-	x
WR 7672	nil	NT	+	-	-	-	-	+	NT	x
RH 8009	nil	NT	+	-	-	-	-	+	NT	x
AM 1729	nil	+	+	-	-	-	-	-	-	-
JH 7079	nil	+	+	-	-	-	-	-	-	-
Ba ITALY		NT	NT	+	+	-	-	+	NT	NT

Table 9: Screening of Scottish XLI males with distal Xp probes.

(Affected males are identified by their initials and pedigree numbers. AM (1729), JH (7079) and Ba are included for comparison. +, hybridisation (heterozygote for pl9b/pSGl); -,deletion; NT, not tested; x, not informative; XY3,GMGXY3; X9;GMGX9; XY19,GMGXY19. PED.NO.,Pedigree number; STS, STS levels measured in hair-roots (Ferguson-Smith & Aitken, 1982), using ^{3}H-Dehydroepiandrosterone sulphate ($DHEASO_4$) as substrate with the exceptions of 3673, 2994 and 1034 (measured in fibroblasts) and 7474 (measured in white blood cells). The assay was a modification of that described by Shapiro et al (1977)).

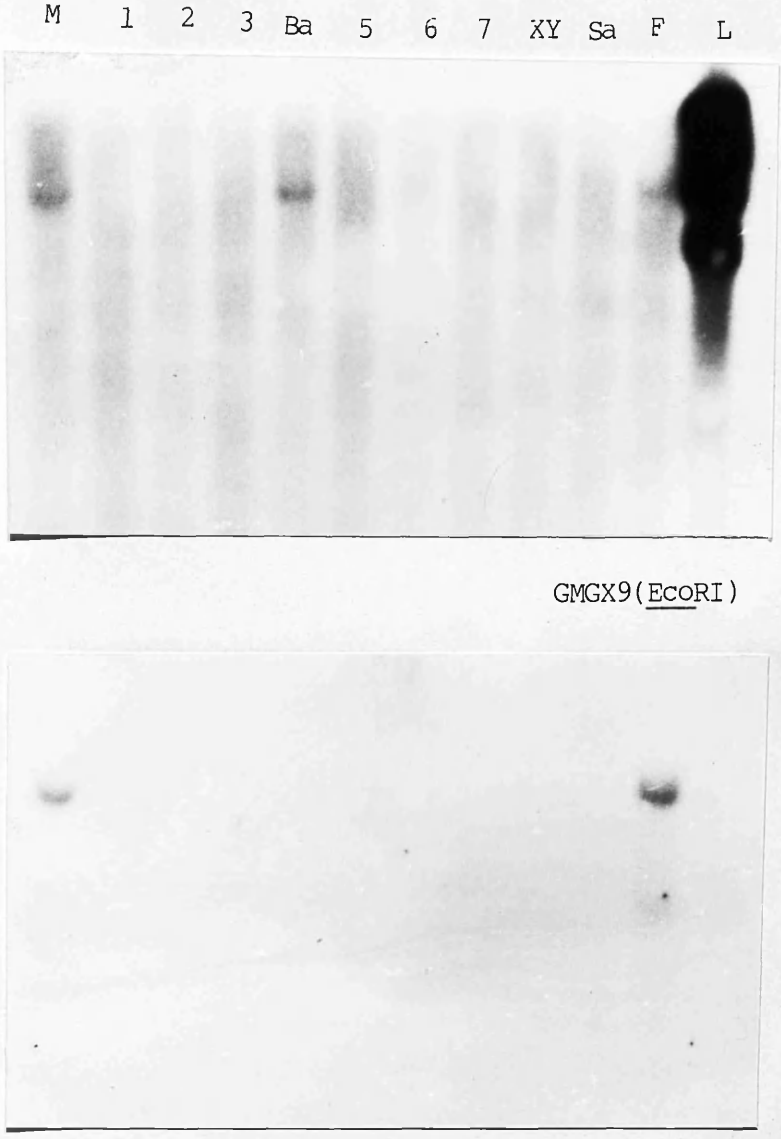


Figure 11: Deletion analysis of Italian males.

(M,male control; 1-7,males with XLI; Sa, male with XLI and KAL; XY,male with XY translocation; F,female control; L,lambda marker. Ba (male 4) and Sa are further described in the text. This blot was provided by A. Ballabio).

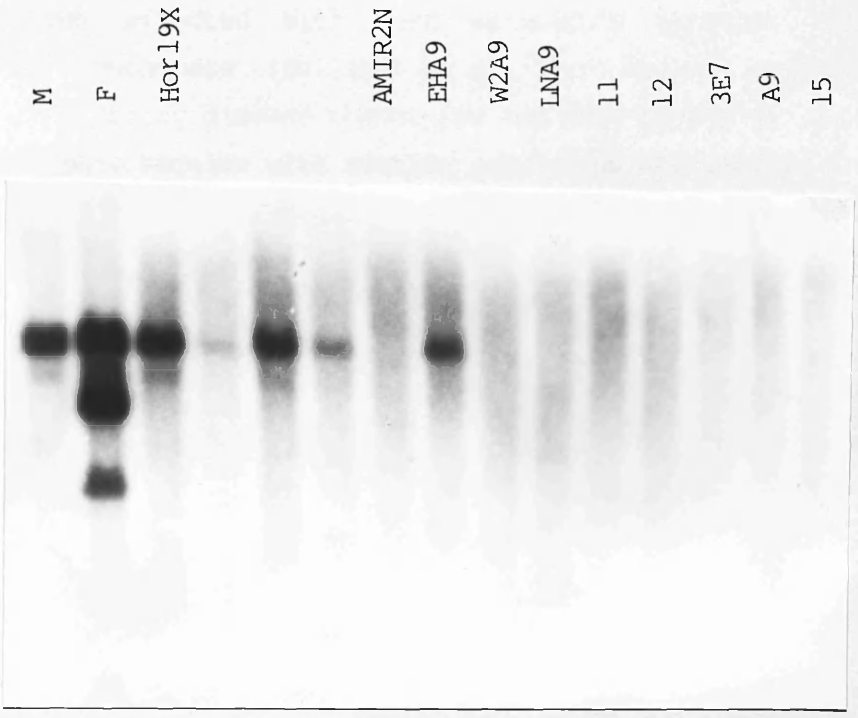


Figure 12: Hybridisation of GMGX9 to 3E7 (Y-chromosome specific hybrid).

(M,male control; F,female control. Hybrids are as described in materials and methods. Unlabelled tracks represent further hybrids isolated within this department;lanes 11 and 12 contain only Xq sequences, lane 15 is a further mouse cell line).

3:7:3 Analysis of complex phenotypes associated with XLI.

Sa was affected with both Kallmann's syndrome (KAL) and X-linked ichthyosis (Ballabio et al, 1986) and was potentially of value in ordering disease phenotypes and gene probes in distal Xp (compare this section with section 3:5:5). He was deleted for GMGX9 and p2a4 (figure 13), GMGXXY3 and p422 (Ballabio et al, 1987a) but not for Dic56 (Ballabio et al, 1986). Thus, his deletion was not distinguished from deletions associated with XLI alone.

Three individuals, RR (8050), SS(8012) and JG(8049) affected with KAL but not XLI were tested with 782, Dic56, GMGX9, p2a4, GMGXXY3, GMGXY19 and p19b, all of which failed to demonstrate a deletion (appendix 14). These individuals represent sporadic cases of Kallmann's syndrome, however, and it is possible that they are not associated with an abnormality in this region of the X-chromosome.

3:7:4 Deletion analysis with GMGX9 and other probes in individuals with XY translocations.

AM (1729) and JH (7079) are males with XLI as a consequence of XY translocations. AM (1729) is deleted for Xg. JH (7079) was Xg(a)-ve although his mother and grandparents were Xg(a)+ve. It is thus likely (but not proven) that JH (7079) is also deleted for Xg.

Both AM (1729) and JH (7079) are deleted for GMGX9 and p2a4 and for the X-specific bands of STB14, M1A and p19B. Some of these results are illustrated in appendix 15. The position of M1A relative to GMGX9 is not known. AM (1729) and JH (7079) are also deleted for the X-specific bands of GMGXXY3 and GMGXY19. Neither individual is deleted for 782 or Dic56.

3:7:5 A brief summary of STS deletion data.

In summary GMGX9 detects deletions in thirty-six of forty-two (86%) families segregating for STS deficiency. These deletions are all detected by other probes including STS cDNA clones and appear very similar at the molecular level. Only one of thirty-one patients studied in detail revealed a difference between GMGX9 and STS cDNA clones. Furthermore the deletion in an individual with

both KAL and XLI could not be resolved from that in deleted males with XLI. Two XY translocations also failed to separate GMGX9 and STS cDNA clones.

3:8 COMPARATIVE MAPPING

3:8:1 Comparative mapping with GMGX11.

It was of interest to determine whether GMGX11 was homologous to DMD cDNA clones which detected deletions at high frequency or represented an additional subset of microdeletions. An EcoRI blot was prepared with DNA from different mammals as follows: Lanes (1) human male, (2) human female, (3) male gorilla, (4) female gorilla, (5) male orang-utan (1), (6) male orang-utan (3), (7) male chimpanzee, (8) ram, (9) ewe, (10) dog, (11) bitch, (12) stallion, (13) mare and (14) bull, in order to establish whether this sequence was conserved between species.

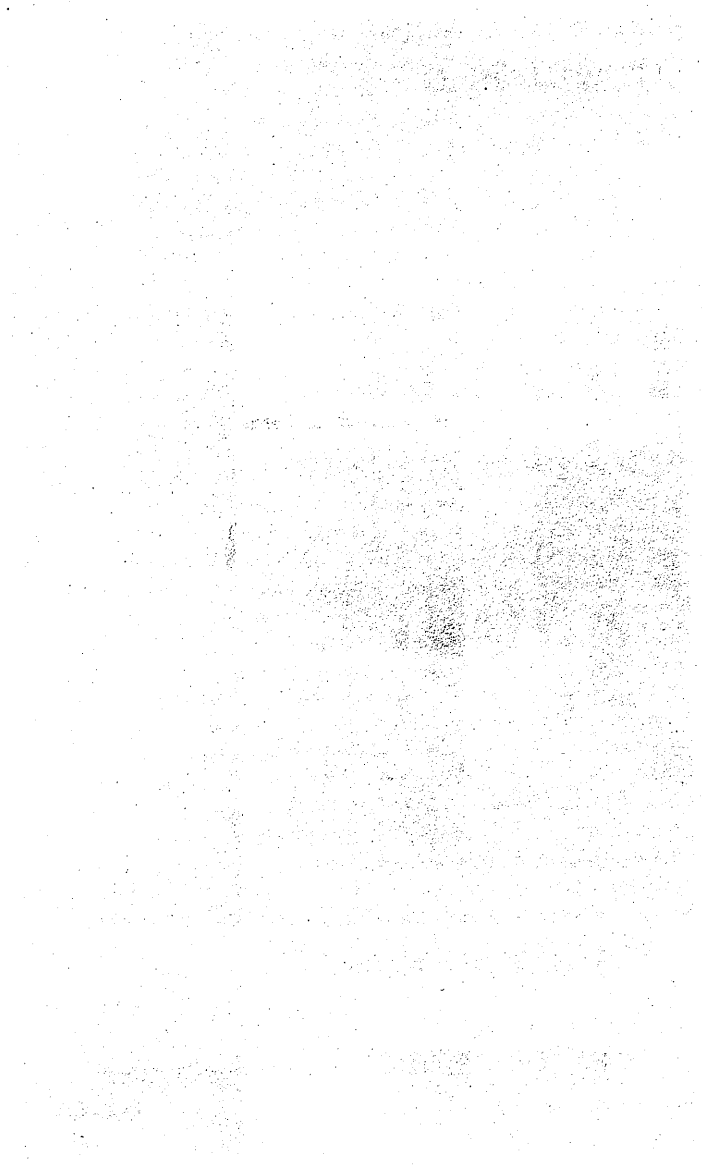
GMGX11 was conserved in primates (gorilla, orang-utang and chimpanzee) but not in other mammals (figure 13A). It thus seems improbable that GMGX11 is expressed.

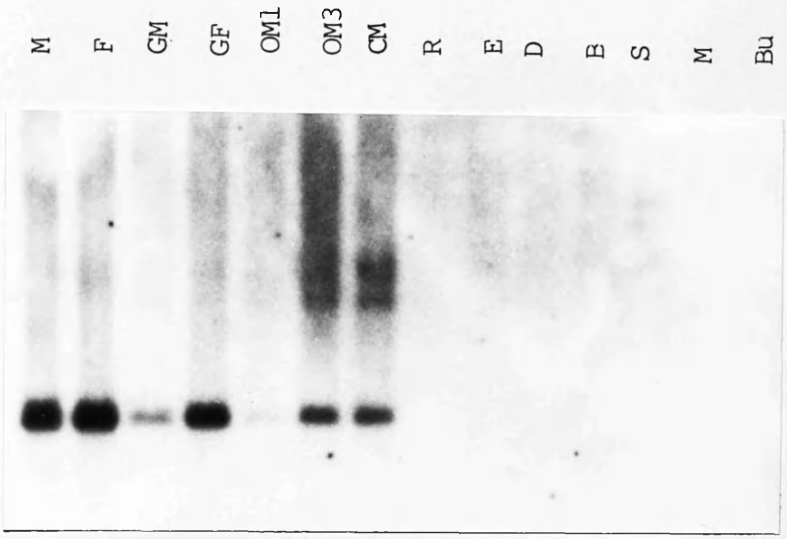
3:8:2 Comparative mapping with GMGX9.

The high proportion of deletions detected by GMGX9 in STS defecient individuals suggested that it might form part of the STS gene. It was thus of interest to determine if GMGX9 represented part of the coding sequence of STS. Two blots were prepared for comparative mapping studies using GMGX9. The first consisted of primate DNA digested with HindIII as follows: Lanes (1) human male, (2) human female, (3) male gorilla, (4) female gorilla, (5) male orang-utan 1, (6) male orang-utan 3, (7) male chimpanzee and (8) male with XLI (RH, 8009). The second consisted of DNA from other mammals digested with HindIII as follows: Lane (1) human male, (2) human female, (3) ram, (4) ewe, (5) dog, (6) bitch, (7) stallion, (8) mare, (9) bull and (10) cow.

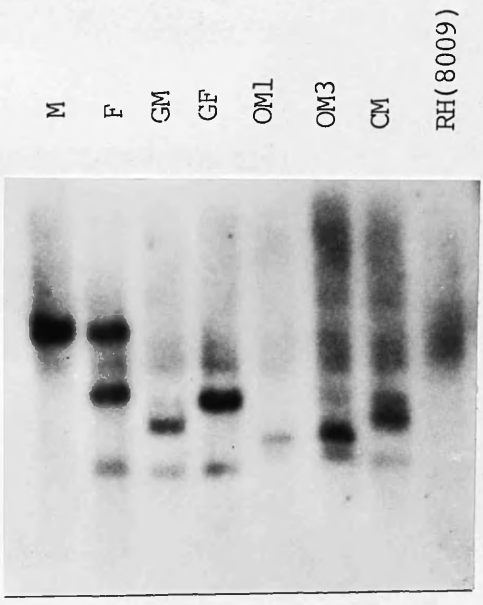
GMGX9 was conserved throughout primates (figure 13B) and also detects homology with bovine DNA (figure 13C). Weak homology to bovine DNA is common to many single copy sequences and does not imply that these sequences are expressed (Monaco et al., 1986).

Comparative mapping would thus suggest that GMGX9 is not expressed. GMGX9 also detects differences between male and female gorilla which might represent a polymorphism in these species.





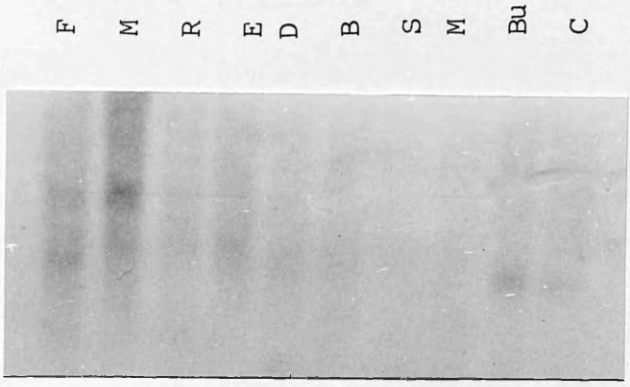
A) GMGX11(EcoRI)



B) GMGX9(HindIII)

Figure 13: Hybridisation of GMGX9 and GMGX11 to "comparative mapping" blots.

(M,male; F,female; GM,gorilla male; GF,gorilla female; OM1,orang-utan male 1 (faint track); OM3,orang-utan male 3; CM,chimpanzee male; R,ram; E,ewe; D,dog; B,bitch; S,stallion; M,mare; Bu,bull; C,cow; RH (8009);male with XLI. (A) EcoRI filter hybridised with GMGX11. (B) HindIII "primate" filter hybridised to GMGX9).



C) GMGX9(HindIII)

Figure 13 continued. (C) HindIII "farm" blot hybridised to GMGX9 (faint hybridisation).

CHAPTER 4: DISCUSSION

4:1 THE ISOLATION OF Xp SPECIFIC PROBES.

This section considers the likelihood of isolating DNA sequences which lie within Xp21 or Xp22.3 by "random" selection from an X-chromosome library, based on estimates of their size with respect to the X-chromosome as a whole. Similar estimates are given for the isolation of sequences within the DMD gene, within deletions in individuals with XLI and within the large Xp21 deletion described by Francke et al., (1985). Both random and targeted approaches to cloning are discussed with particular emphasis on X-chromosome libraries.

4:1:1 Predicted success of random cloning strategy.

This aim of this project was to isolate Xp DNA sequences from a chromosome specific library, in order to conduct an analysis of Xp, focussing on Xp21 and the analysis of DMD and on Xp22.3 and the analysis of STS deficiency, in particular.

The X-chromosome represents approximately 5% of the haploid genome or 150Mb, determined by both flow cytometry (Harris et al., 1986) and cytophotometric data (Mayall et al., 1984). Xp accounts for approximately one-third of the X-chromosome whilst Xp21 represents 20% of Xp (or 10Mbp). The size of Xp22.3 has been estimated at 5Mb (or 10% of Xp), (Mondello et al., 1987).

On the basis of these approximations, it may be predicted that, without targeted selection (see below), one-third of X-linked clones would map to Xp, one in fifteen X-linked clones would map to Xp21 and one in thirty X-linked clones to Xp22.3 (table 10).

Of sixteen X-linked probes isolated at random from this library, four were Xp specific: GMGX9 (DXS237), GMGX10 (DXS238), GMGX11 (DXS239) and GMGX12 (DXS240). GMGX10 (DXS238) and GMGX11 (DXS239) were mapped to Xp21 and GMGX9 (DXS239) to Xp22.3. GMGX12 (DXS240) mapped between GMGX11(DXS239) and GMGX9(DXS237), (table 10).

DNA sequences have now been cloned from three hundred and five loci on the X chromosome (table 2). There is considerable variation in size between these sequences from less than 1kb to several over 100kb. An (arbitary) average value of 10kb indicates that only 3Mb or one-fiftieth of the X-chromosome has been cloned.

Interval	"p"	"P"	Probes isolated from this interval
Xp	1/3	0.998	GMGX9, GMGX10, GMGX11, GMGX12
Xp21	1/15	0.67	GMGX10, GMGX11
Xp22.3	1/30	0.42	GMGX9
"BB" deletion	1/25	0.48	GMGX10
"DMD"	1/75	0.19	GMGX11
"STS."	1/1000 to 1/75	0.015 to 0.19	GMGX9

Table 10: Predicted success of a random approach in cloning DNA sequences within specific intervals of interest on Xp21.

(The probability, P, of isolating at least one probe in a given region is $1- [(1-p)^n]$), where p is the theoretical probability that any one probe will lie within that region (based on its size) and n is the number of X-specific probes isolated. This has been calculated for each category assuming n = sixteen, the number of X-linked probes isolated in this study. The "BB" deletion is described by Francke et al (1985)).

A minimal estimate of 2Mbp for the size of the Duchenne gene has recently been deduced by extrapolation of the mean intron:exon size ratio (Koenig et al., 1987). The smallest deletions visualised by flow cytometry (FACS) analysis at the STS locus are estimated at 2Mb (Cooke et al., in press) whilst the STS gene has been estimated to represent 140kb (Yen et al., 1987b). Cloning of these loci will be presented later.

These data predict that one in seventy-five probes assigned to the X-chromosome would lie within the DMD gene and between one in a thousand and one in seventy-five probes would be deleted in individuals with STS deficiency. Of sixteen X-linked probes derived from the library constructed in this project, GMGX11(DXS239) mapped within the DMD gene but outwith the deletion described by Francke et al. (1985), and GMGX9(DXS237) was deleted in 84% of individuals with steroid sulphatase deficiency, (including some with deletions not detected by FACS analysis), (table 10).

It can be seen that the probability of isolating one or more probes in each of the categories defined in table 10 (except that of STS deletions) was 0.19 or more, based on a group of sixteen probes isolated at random. Such calculations are only an approximation, but serve to demonstrate that attempts to isolate probes which mapped within Xp21, Xp22.3, the deletion described by Francke et al. (1985) or even the DMD gene were realistic. A more accurate determination of the minimum size of STS deletions, possibly by pulse field gel electrophoresis (PFGE), will be required to assess the probability of detecting deletions at this locus more accurately.

4:1:2 Random and targeted approaches to cloning.

Many single copy Xp DNA sequences presently in common use were derived from flow-sorted X-chromosome libraries by "random" selection for single copy sequences and were localised to Xp using somatic cell hybrids. One disadvantage of this approach, however, is that selection for single copy sequences results in an increased background of non-recombinants (self-ligated vector) and of non-human recombinants. Fuscoe (1987) used an oligonucleotide complementary to the insertion site of the vector to detect

non-recombinants. This had the added advantage of detecting all recombinants, regardless of their status with respect to repeated sequences.

Flow cytometry can resolve all human chromosomes except chromosomes 10, 11 and 12 in individuals with appropriate karyotype anomalies (Harris et al., 1985). Libraries are usually constructed from the complete digestion of one to two million chromosomes (Fuscoe et al., 1986), although Kunkel et al. (1985b) reported a library constructed from partial digestion of ten million X-chromosomes. Complete digestion of human DNA will result in some DNA fragments with sizes outwith the acceptance range of the chosen vector which cannot be represented in the library. Representation may be slightly improved by construction of complementary libraries using different restriction enzymes. Inserts containing inverted repeat sequences are also resistant to cloning in recombination proficient hosts (Wyman, Wolfe & Botstein, 1985; Donlon et al., 1986) and may be rearranged or lost entirely.

Fuscoe et al., (1986) predicted that only 65% of a given chromosome is clonable in lambda Charon 21A, due to constraints on insert size. Lambda Charon 21A has a comparable insert acceptance range (1-9kb) to lambda NM1149 (0-11kb). The range of these vectors is more representative of the fragment distribution in digested genomic DNA than that of lambda gtWES (5-14kb) and results in fewer non-X recombinants (Hofker et al., 1985).

Sixteen of fifty inserts isolated from the X-chromosome library constructed in lambda phage NM1149 were X-specific. This was comparable with the representation of X-specific recombinants in the lambda phage Charon 21A library described by Hofker et al. (1985), (table 11). The proportion of non-recombinants in the NM1149 library (almost two-thirds of the plaques selected) was much higher than in the lambda Charon 21A library, however, despite selection for recombinants disrupted at the insert site which should have reduced this, and was comparable with that in lambda gtWES (Hofker et al., 1985 and table 11),.

Other Xp probes have been derived by cosmid cloning e.g. cX5(DXS148), (Hofker et al., 1986b) or by "targeted approaches" based on knowledge of the location of a mutation. Cosmid clones are

generally constructed using somatic cell hybrids. Selection for human inserts depends on hybridisation to human repeated sequences and thus repeated sequences are present in all recombinants obtained. Cosmids facilitate the characterisation of large contiguous regions of the genome but are less appropriate in the direct isolation of single copy sequences, as sub-clones (free of repeated sequences) must first be isolated.

Two "targeted approaches", applied to cloning sequences from the DMD locus, are described below. A targeted approach is most valuable when the target is represented by only a few clones. Fuscoe et al. (1986) state that five chromosome equivalents are required for a 0.99 probability of including any given (clonable) sequence.

One targeted approach, using the "phenol enhanced reassociation" technique, relied on a large Xp21 deletion in a male with chronic granulomatous disease, Duchenne muscular dystrophy, McLeod Syndrome and retinitis pigmentosa type 3 (Francke et al., 1985). Digested DNA from a normal male was reassociated with an excess of sheared DNA derived from the affected boy, and thus the proportion of clonable fragments from within his deletion was enhanced. Four of eighty-one clones (eighteen of which were X-specific), including pERT84(DXS142) and pERT87(DXS164), were mapped within the deletion in this way (Kunkel et al., 1985a).

This deletion was estimated to represent 6000kb by flow cytometry (Wilcox et al., 1986), or three-fifths of Xp21. Thus, one in twenty-five randomly selected X-linked probes could be expected to map within this deletion. The targeted approach thus increased the proportion of clones within this deletion to a considerable extent. One of the sixteen X-linked clones isolated in this project, GMGX10(DXS238), mapped within this deletion (table 10).

A second targeted approach depended on a female with DMD and an X;autosome translocation, which disrupted both the ribosomal rNA (rRNA) gene cluster on chromosome 21 and the DMD gene (Verellen-Dumoulin et al., 1984). DNA clones from the rRNA locus (Wellauer and Dawid, 1979) were used to initiate "chromosome walks" (i.e. to continually select for overlapping sequences) in order to locate the junction fragment in somatic cell hybrids derived from

BACTERIOPHAGE VECTOR:	gtWES	Charon21A	NM1149
Insert sizes (kb):	5-14	1-9	0-11
Low copy clones:	134	86	120
"single copy clones":	79	73	/
Clones with inserts:	45	70	50
Human inserts:	26	69	27
Of which:			
Repetitive:	15	13	7
X-linked:	3	22	16
Autosomal:	2	26	4
Not tested:	6	8	/

Table 11: Comparison of the X-chromosome library with two others constructed in different vectors.

(After Hofker et al, (1985) The HindIII libraries in bacteriophage lambda gtWES and Charon21A are described in Hofker et al (1985). "Low copy clones" corespond to those selected after Benton-Davis type screening with total human DNA. "Single copy clones" correspond to those selected after a second round of screening by dot blot analysis. This was not performed for the NM1149 library).

each translocated chromosome (and free of additional acrocentric chromosomes with rRNA loci), (Worton et al., 1984). The first clone isolated in this manner was XJ1(DXS206), (Ray et al., 1985).

4:2 DUCHENNE MUSCULAR DYSTROPHY.

Restriction fragment length polymorphisms were not detected by GMGX10(DXS238), GMGX11(DXS239) or GMGX12(DXS240). The emphasis in this project was thus to study deletions at the DMD locus, rather than to perform linkage analyses. This section reviews the contribution of pulse field gel electrophoresis (PFGE) in determining the limits of the DMD gene. Cloning of the complete DMD cDNA and comparison of the genomic and cDNA maps are presented along with data which maps DMD, GK and AHC relative to each other. Deletion data is discussed in the light of other genomic and cDNA probes and correlated with that obtained by PFGE. Possible relationships between the size and localisation of deletions at the DMD locus and the severity of phenotype are presented. The problems of a causal relationship between deletions and DMD/BMD and of germ-line mosaicsm in deletion analysis are addressed. The origin of deletions and mechanisms by which deletions might arise are discussed. This section concludes with recent work which defines the DMD gene product, "dystrophin" and its implications for future work and therapy for DMD.

4:2:1 Physical mapping of the DMD gene.

Pulse field gel electrophoresis (PFGE) can resolve DNA fragments from 50kb to 2000kbp in size (Schwartz and Cantor, 1984). PFGE has complemented deletion analysis and linkage analysis in ordering Xp21 probes, and has defined maximum and minimum distances between many loci. Burmeister and Lehrach (1986), for example, predicted that XJ(DXS206) and pERT87(DXS164) could be no further than 300kb apart. Burghes et al. (1987) confirmed this by linking the two loci in a chromosome walk, resulting in a contiguous DXS206/DXS164 clone of 350kb.

PFGE has thus been of value in constructing a map of the DMD gene (Burmeister and Lehrach, 1986; van Ommen et al., 1986). The

map positions of loci which lie proximal or distal to DMD are less well defined. There is conflicting evidence to support either C7 or B24 as the closest distal polymorphic marker for DMD. A male with DMD, GK and AHC and a deletion of C7 and L1-4 but not B24 (van Ommen et al., 1986) suggests C7 is the closest distal marker whilst a recombination event places C7 distal to B24 (and further from DMD), (Norby and Scwhartz, 1987). PFGE data places C7 and B24 more than 2Mb from DMD and 500kb apart. PFGE fragments are shared by B24 and C7 and also by L1-4 and B24 but not by C7 and L1-4. This supports the order Xcen---L1-4---B24---C7---pter (Burmeister et al., unpublished observations).

Interestingly, distances on the pulse field map for DMD failed to correspond to those of the genetic (recombination) map. Although 754 is deleted in several individuals affected solely with DMD or with DMD and mental retardation (e.g. in family 5099, (Wilcox et al., 1986) or the family described by Greenberg et al. (1987)), the genetic distance between 754 and DMD is large (approximately 10cM). van Ommen et al. (1986) infer from PFGE data that the physical distance between 754 and DMD can be little more than 1-2 Mbp and thus that, in this region, 1% recombination is comparable to 100-200kbp instead of the usual 1000kb.

Wieacker et al. (1984) proposed that the genetic (recombination) and physical (cytogenetic) maps of Xp do not correspond and that genetically, the distal half of Xp may be twice as long as the proximal half (55-60cM v 25-30cM). Such disparity between physical and genetic maps is also observed in the pseudoautosomal region on the Y chromosome, (Rouyer et al., 1986), where it is attributed to an obligatory XY cross-over in a relatively small region.

GMGX11 was mapped between JBir and J66HI by PFGE, (Burmeister et al., unpublished observations), thus orientating GMGX11 with respect to J66HI. This localisation was confirmed using somatic cell hybrids with breakpoints between JBir and J66HI, derived from girls with X;autosome translocations (Boyd et al., unpublished observations). Wapenaar et al. (1987) assigned p20 to the same region, by PFGE.

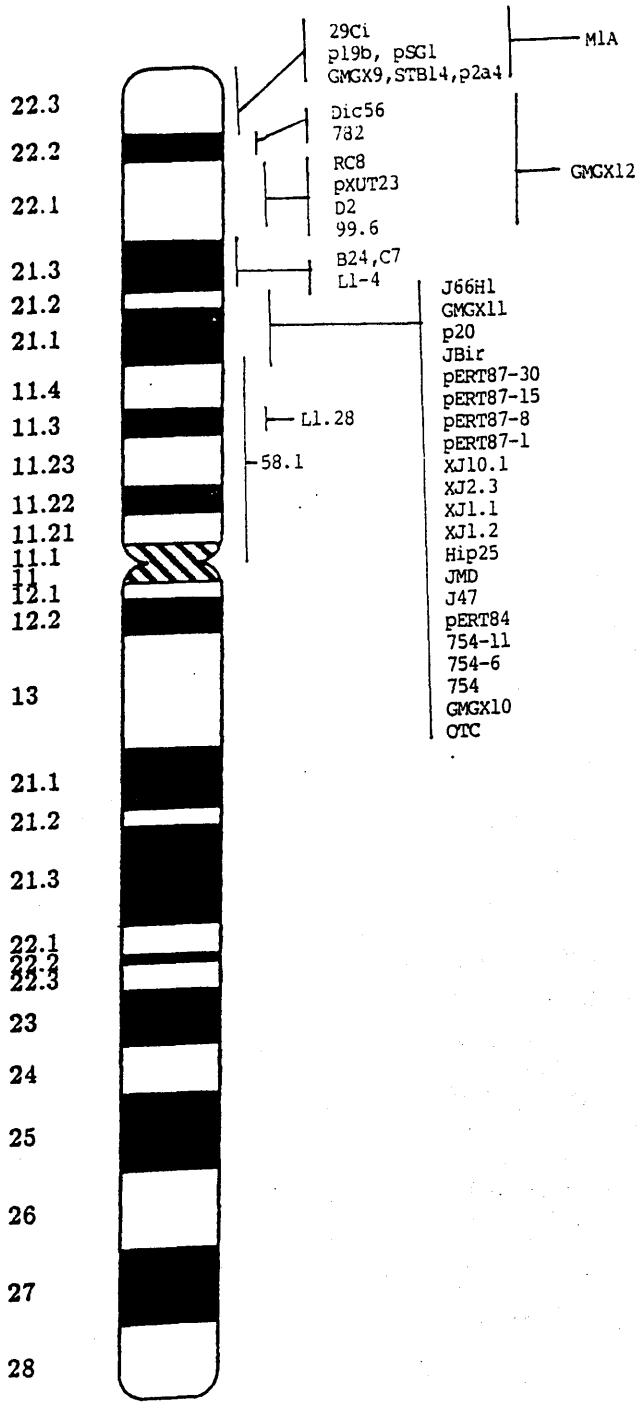


Figure 14: Chromosomal localisation of Xp specific probes. (Probe details are summarised in table 3. The relative positions of probes separated by a comma are not known.).

4:2:2 Cloning of the DMD cDNA and comparison to the genomic map.

Cloning of the DMD cDNA has also refined the map of the DMD gene. The first candidate cDNA clones from the pERT87 locus were isolated on the basis of conservation of exons of a key developmental protein between mammalian species (man, cebus monkey, cow and hamster) rather than by more conventional approaches, such as hybridisation to Northern blots or to a cDNA library, (Monaco et al., 1986). One candidate clone, pERT87-25, which hybridised to a 14kb transcript, was expressed in fetal muscle, but not in human myoblast cells or in He-La transformed cells.

cDNA clones from the XJ region were derived by direct screening of an adult muscle cDNA library, constructed in the expression vector lambda gt11, (Burghes et al., 1987), with subclones from within the XJ region. Cross species homology was again observed.

The 5' and 3' ends of the DMD gene were obtained by searching for overlapping clones in a second fetal muscle library, (Koenig et al., 1987). The resulting 14kb cDNA was divided into subclones of approximately 1kb and detected sixty-five X-specific HindIII fragments. As there were five HindIII sites within the 14kb cDNA itself, this indicated that the mRNA was transcribed from at least sixty exons.

Some fragments were ordered with respect to each other using deletions and translocation breakpoints, whilst others were ordered by comparison of the restriction patterns obtained to those of DNA sequences derived from chromosome walks around defined Xp21 loci.

All probes from pERT84 to J66HI inclusive (figure 14), were shown to be intragenic, with at least one third of the DMD gene lying distal to J66. This indicated that GMGX11 was intragenic. The first 6kb of DMD transcript were seen to correspond to a minimum of 800kb on the genomic map - from the first (most 5') exon within the pERT84(DXS142) locus to an exon within the JBir locus, distal to pERT87.

Since GMGX11 is only conserved amongst primates (and not amongst other mammals), it is unlikely to be a coding sequence and may be situated in one of the introns associated with cDNA subclones 7 or 8 (Koenig et al., 1987) and/or Cf23a (Forrest

et al., 1987a) or Cf56a (Forrest et al., 1987b). These cDNA clones detect a high frequency of deletions in the region between JBir and J66HI as observed for GMGX11. p20 may also be situated in one of these introns.

Relatively few reference points which might facilitate genomic mapping of the 3' end of the gene have been described. Extrapolation of the mean intron and exon sizes (35kb and 200bp respectively), however, predicts that the DMD locus extends over 2Mb and thus represents nearly 0.1% of the human genome (Koenig et al., 1987). This corresponds to the predictions of PFGE data (Burmeister et al., unpublished observations) and provides a partial explanation for the high frequency of recombination within the DMD gene itself, for heterogeneity in the site of the mutation and also for extreme variation in the severity of phenotype.

4:2:3 The ordering of Duchenne muscular dystrophy, glycerol kinase deficiency and congenital adrenal hypoplasia (AHC) and the characterisation of a deletion associated with AHC.

AM (5996) represents the first individual described with AHC and mental retardation (but without GK or DMD) with a micro-deletion of Xp21. His deletion could be visualised microscopically, by FACS analysis and by molecular studies (Yates et al., 1987a). JR (5194) with AHC but not mental retardation, was also studied by cytogenetic, FACS and molecular analysis but no deletion was detected.

The deletion in AM (5996) (which includes L1-4, B24 and C7), overlaps with that of DH (Card) with DMD, GK and AHC (section 3:5:5). The deletion in MJ (Oxfo) also affected with DMD, GK and AHC, included probes GMGX10 to J66HI (within the DMD locus) but not L1-4. These three deletions placed AHC between J66HI and L1-4. Furthermore as MJ (Oxfo), but not AM (5996), is additionally GK deficient, this suggested that AHC was at the distal end of the region of overlap. This supports the order DMD-GK-AHC-pter (figures 6 and 15).

Other molecular studies, concurrent with this study and reviewed by Francke et al. (1987), placed the AHC and GK genes between JBir (proximal to J66HI) and L1-4. Chelly et al. (1987b)

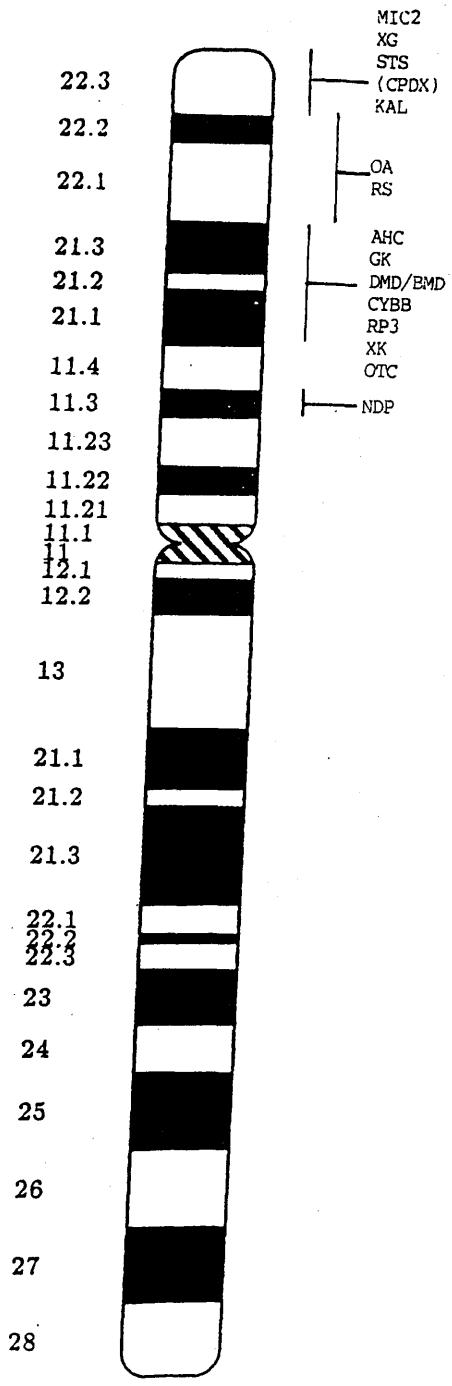


Figure 15: Regional localisation of genes on Xp. (Gene symbols are according to McKusick, 1987. Genes in parenthesis are provisional localisations. Only those genes which are mentioned in the text are included).

placed GK and AHC between J66HI and L1-4 in agreement with the above data.

As the deletion in KC (Francke, 1984) extends distally from OTC to exclude B24 and C7 (Francke, 1985), it must also include the genes for AHC and GK (figure 6). Thus KC must also be hemizygous for AHC and GK .

The distance between J66H1 (the most 3' probe known to lie within the DMD gene) and L1-4 represents some 2Mb or more as estimated by PFGE (Burmeister et al., unpublished observations). There are no points of reference in this interval which might allow GK and AHC to be localised more precisely. L1-4, which might provide an appropriate starting point for obtaining further probes, seems particularly resistant to chromosome walking and related cloning strategies (Kunkel et al., 1985b).

The deletion in AM (5996) was estimated by flow cytometry (FACS analysis) to represent some 2.3% of his X-chromosome or 3.4Mb (Yates et al., 1987a). Much of his deletion may lie outwith the gene itself, although L1-4 and C7 are at least 1000kb apart, (Burmeister et al., unpublished observations). The parents and grandparents of AM (5996) were also studied and his mother (who was mildly mentally retarded), but not his grandmother (of normal intelligence), was shown to be deleted by FACS analysis. This was supported by the finding that his grandmother was heterozygous for B24 (section 3:5:5).

Marlhens et al. (1987) have recently described an individual with a microscopic deletion of Xp21.2 with both GK and AHC who was not deleted for JBir, L1-4 or C7. This might be explained by the lack of suitable molecular markers in the 2Mb interval which represents the smallest region of overlap (SRO) of deleted individuals with AHC and GK deficiency.

Many boys with DMD have been assessed for the presence of glycerol in their urine as an indication of glycerol kinase deficiency. Clarke et al. (1986) and Chelly et al. (1987a) observed slightly elevated levels of glycerol in the urine of one of thirty, and five of three hundred and two, males with DMD, respectively. The increase was ten to one hundred fold less than that in males with both GK deficiency and AHC. Only one of the five individuals

described by Chelly et al. (1987a) was shown to be glycerol kinase deficient serologically. It thus appears that GK is more closely associated with AHC than with DMD.

The uniqueness of AM's phenotype suggests that his proximal breakpoint separates the genes for GK and AHC, and could represent a valuable starting point in cloning the two genes. Polymorphic probes within his deletion might also represent suitable flanking markers for the study of DMD, which would be closer and possibly more polymorphic than either B24 or C7. It would thus be worthwhile to search or select for X-specific clones deleted in AM (5996).

4:2:4 Deletion analysis (genomic probes)

In a preliminary screen, which was included in the collaborative study of Kunkel & co-authors (1986), deletions were detected in nine of ninety (10%) Scottish boys with either DMD (seventy eight boys) or BMD (twelve boys) by pERT87-8 and/or XJ1.1, (Wilcox et al., unpublished observations). These nine boys included one, JB (5097), with BMD.

That collaborative study involved twenty laboratories in the screening of one thousand three hundred and forty-six DMD and BMD males with probes from the DXS164 locus. Eighty-eight males (6.5%) were deleted for at least one of these probes, fifty-three of which were further investigated with additional pERT87 clones. Screening with pERT87-1, pERT87-8, pERT87-15 (and XJ1.1) was advocated, as a means to determine the majority of deletions, (Kunkel & co-authors, 1986).

Using this approach, Hart et al. (1986) detected fifteen deletions amongst one hundred and forty DMD families (10.7%) whilst Thomas et al. (1986a) detected nine deletions for at least one sub-clone in one hundred and seventeen unrelated individuals (8%) with DMD. Thomas et al. (1986a) also studied forty-eight BMD patients in whom they failed to detect a deletion.

One hundred and three Scottish DMD/BMD boys were screened for deletions with XJ1.2, GMGX10, GMGX11, GMGX12, p20, JBir and J66H1 in this study, and mutations were detected in a further thirty six (36%) individuals. No additional mutations were detected with XJ1.2, GMGX10 or GMGX12 whilst eighteen new mutations (18%) were

defined using GMGXl1 and twenty-two (22%) with p20. Seven individuals had mutations detected by both GMGXl1 and p20. J66HI detected a fragment of altered size in one individual whilst JBir detected a further three mutations.

The thirty-three mutations detected by GMGXl1 and p20 represent a new and distinct deletion-rich region from that of pERT87/XJ (figure 16). In this study, only MJ(Oxfo) and DH(Card), who have complex phenotypes, have deletions which encompass both pERT87/XJ and GMGXl1/p20.

GMGXl1 and p20 both detect restriction fragments of altered size which may represent partial deletions, point mutations producing rare polymorphisms or one endpoint of an inversion or duplication. These alternatives may also explain the altered fragments detected by JBir in JL (1406) and by J66HI in MT(5310), and could be investigated by cloning the junction fragments in these boys.

The detection of altered fragment sizes and/or deletions with GMGXl1 and p20 is partly dependent on the restriction enzyme involved. Only three individuals were deleted for HindIII and MspI fragments of p20 as well as the EcoRI and HindIII fragments of GMGXl1. As p20 was derived from two sequences some 30-40kb apart (van Ommen et al, personal communication), either fragment could be independently altered or deleted in the immediate vicinity of a deletion, dependent on the restriction enzyme used. GMGXl1, in contrast, consists of a unique fragment 1.2kb in length, which suggests that enzyme specific differences at this locus are small deletions or subtle changes in which at least one endpoint is well-defined.

J66Hl detected an additional EcoRI fragment, approximately 2kb in size (in addition to the "normal" EcoRI fragment of approximately 4kb) amongst sixteen males with DMD. The significance of this finding has not yet been determined, but could be investigated in several ways. Firstly, the families of those boys with this extra band should be studied, to determine if it segregates as a mendelian trait. Secondly EcoRI digests of normal males should be screened for the presence of this fragment. Finally, it might also be valuable to screen other restriction

enzyme digests of these sixteen males to determine if this represents a site specific mutation.

Den Dunnen et al. (1987) demonstrated deletions in 17% (eighteen of one hundred and four) DMD patients using probe JBir in addition to pERT87 and XJ1.1. Bakker et al. (1987b) reported that XJ, pERT87 and JBir were deleted in 10% individuals with DMD, and that p20 was deleted in an additional 18% individuals from the same group, in good agreement with the 22% observed in this study. J66H1, which was very effective at detecting deletions by pulse field analysis (as described below, section 4:2:5), detected only four deletions amongst four hundred and fifty individuals by Southern analysis, (Ginjaar et al., 1987).

Amongst those genomic probes which detect deletions by Southern analysis, p20 and GMGX11 detect the most deletions (figure 16). Some cDNA clones, however, are able to detect deletions in more than 50% affected individuals (Forrest et al., 1987b) and this has increased the proportion of affected individuals with deletions detectable by Southern analysis to 70% (Forrest et al., 1987b). Deletions of cDNA probes are described below (section 4:2:6).

4:2:5 Deletion analysis (PFGE)

Den Dunnen et al. (1987) were able to detect twenty deletions and a duplication amongst thirty-nine DMD patients by PFGE as compared to only seven deletions by Southern analysis. They used partial SfiI digests and the probes XJ1.1, pERT87 (sub-clone not specified), JBir and J66H1 (which span 2.2Mb using partial SfiI digests). Sixteen of these deletions (and one duplication) were detected with either JBir or J66H1. This predicted an especially deletion-rich region in the 950kb between JBir and J66H1.

The resolution of PFGE is greatest for large DNA fragments (outwith the "compression zone" of unresolvable fragments). Thus deletions as small as 30kb can be detected using probes as distant as 800 kb (van Ommen et al., 1986). GMGX11(DXS239) is about 300kb proximal to J66 and 400-500kb distal to JBir (Burmeister et al., unpublished observations). Thus deletions of more than 30kb at the DXS239 locus would be readily detected by PFGE analysis with either

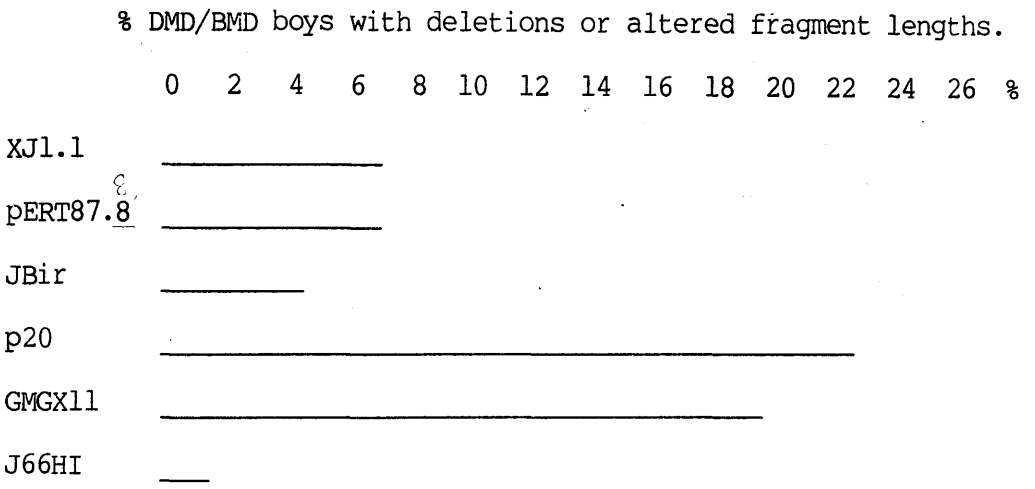


Figure 16: Schematic representation of mutations described in this study.

(pERT87-8 results were obtained by D.Wilcox. Figures are expressed as percentages of the total number of boys screened with each probe. Eighty seven individuals were studied for XJ1.1 and pERT87-8. One hundred and three individuals were studied with JBir, p20, GMGX11 and J66HI. Those boys with an extra EcoRI J66HI fragment in addition to one of normal size are not included).

J66 or JBir. Additicnal deletions might also be detected by PFGE with GMGXll or p20 if these probes are situated far enough (several hundred kilobases) from a deletion rich region.

p20, cDNA sub-clones 7 and 8 (Koenig et al., 1987), and Cf23a and Cf56a (Forrest et al., 1987a, 1987b) are also situated between JBir and J66 and deletions of these loci are likely to correspond to many of those detected by PFGE with JBir or J66HI. The extent to which deletions detected by these probes overlap and the distances between them have not been established.

4:2:6 Deletion analysis (cDNA probes).

Koenig et al. (1987) detected deletions in 50% (fifty three of one hundred and four) DMD patients using subclones of the 14kb cDNA, approximately lkb in size. They noted (i) a non-random distribution of deletions, (ii) a slight increase in the proportion of deletions associated with sub-clone lb (which roughly corresponds to the XJ/ pERT87 junction on the genomic map), (iii) a large increase in the frequency of (small) deletions associated with sub-clones 7 and 8 (which lie within the region defined by JBir and J66 on the genomic map) and (iv) an absence of deletions associated with five of the six distal subclones tested. Forty-two out of fifty-three (80%) deletions detected were deleted for sub-clone lb (fifteen deletions) or sub-clone 8 (twenty seven deletions).

Forrest et al. (1987a, 1987b) described two distal cDNA clones Cf23a and Cf56a, which detect deletions in DMD and BMD patients. Cf23a extends distal to JBir and detects deletions in 28% individuals with DMD and 51% those with BMD. Cf56a, which lies 3' to Cf23a, detects deletions in 51% individuals with DMD and in 52% those with BMD. The extent to which these cDNA clones overlap with cDNA clones 7 and 8 (Kunkel et al., 1987) is yet to be defined.

Deletions were detected in 70% DMD/BMD families by Southern analysis using Cf23a and Cf56a, in conjunction with two further cDNA sub-clones, CalA which is distal to pERT87-30, (Cross et al., 1987) and Cf27 (in the vicinity of XJ1.1 and HIP25). These subclones represent twenty-seven exons from the 5' and central regions of the DMD gene.

As cDNA deletions potentially define the critical domains of the DMD gene, cDNA clones which detect deletions at high frequency are likely to supercede random DNA clones in deletion screening.

4:2:7 Deletion/ phenotype correlations

There is no clear correlation between the size or location of deletions at the DMD/BMD locus and either the severity of phenotype or mental retardation. Three conclusions can be drawn from the data outlined below: (i) that no "Becker-specific" domain of the DMD gene exists, (ii) that the extent of the deletion is not a rigorous guide to the severity of phenotype and (iii) that deletions of certain probes e.g. GMGX11 are more frequently associated with a severe phenotype than others e.g. p20 or Cf23a.

Assessment of intelligence quotient (IQ) in eight hundred and ten boys with DMD (summarised in Emery, Skinner & Holloway, 1979) suggests that about a third of such individuals have an IQ less than seventy-five. Mental retardation in association with BMD is not unknown (Emery and Skinner, 1976) but is not a common feature.

SS (5099), with the largest deletion in this study, and to a lesser extent JD, (his cousin who has the same deletion), were severely mentally retarded with short stature. Not all mentally retarded boys have such large deletions, however. Greenberg et al. (1987) described a family with a similar microscopic deletion, including 754, pERT87 and XJ, in which affected males had "normal growth parameters" and were described as "slow-learners". Probes which might distinguish between these two deletions (e.g. GMGX10) could prove of especial value in examining mental retardation in boys with DMD.

In this study, JB (5097) with a severe Becker phenotype was deleted for DNA probes from the XJ/DXS206 locus. JB was quite severely affected (wheel-chair bound at thirteen, presently aged twenty). He had an affected uncle who was wheel-chair bound at fourteen and died aged thirty nine. His deletion encompassed that of AK (5265) with a Duchenne phenotype.

Liechti-Gallati et al. (1987) have recently reported an XJ/DXS206 deletion (including XJ1.1 and XJ2.3) in a mildly affected BMD patient. Other deletions which result in a BMD phenotype, have

been reported within the pERT87/DXS164 locus (Hodgson et al., 1986; Kunkel & co-authors, 1986; Hart et al.,1987). Cross et al. (1987) reported a patient with mild BMD whose deletion extends distally from pERT87-30 and includes J-Bir whilst Lindlof et al. (1987) recently reported a family with three BMD males deleted for XJ2.3 in addition to pERT87-1, pERT87-8 and pERT87-15 (but not pERT87-30).

Hart et al. (1987) described three BMD individuals with pERT87 deletions. One of these was only 6kb in size and was associated with a mild BMD phenotype. The other two deletions encompassed probes pERT87-1, pERT87-8 and pERT87-15. One was at the severe end of the BMD spectrum (wheel-chair bound at sixteen years and two months) whilst the other was milder in phenotype (able to walk until thirty-eight years).

Four out of fifteen BMD families in this study had a deletion breakpoint within p20, whilst a further two BMD families had deletions of p20. p20 also detected deletions in sixteen DMD families. Whilst one third of all alterations to the restriction fragment length pattern detected by p20 were associated with a BMD phenotype, all mutations detected by GMGX11, were associated with a Duchenne phenotype. This suggests that GMGX11, which lies 3' to p20, may lie close to an essential region of the DMD gene.

Individuals with BMD have also been reported with deletions of exons from cDNA clones CalA (distal to pERT87-30, Cross et al., 1987), Cf23a and Cf56a, which lies 3' to Cf23a. Cf23a detected more BMD deletions than Cf56a, including all those detected by Cf56a. Cf56a detected twice as many DMD deletions as Cf23a. Most of the deletions detectable by both probes had an endpoint between two particular exons of Cf23a, which also represented an endpoint for 30% of DMD deletions, (Forrest et al., 1987a, 1987b).

Cf23a thus appears to detect mutations in a deletion-prone region, of which several result in a less severe phenotype, as observed for p20. Disruption or loss of these loci thus appears less deleterious than disruption or loss of GMGX11. Separate patient groups were tested for deletions with p20 and Cf23a, so it is not yet known if these two groups are partially-, completely- or non- overlapping.

Cross et al. (1987) reported a deletion of a minimum of nine exons from the DXS164 locus (normally transcribed in adult DMD mRNA) in a sixty-one year old patient with an exceptionally mild BMD phenotype. The most extreme example of failure to correlate phenotype with deletion size was that of a normal male who was deleted for at least two exons of the DMD cDNA, (Koh et al., 1987). The severity of phenotype may be governed by whether a deletion causes an in-frame mutation, resulting in a shortened protein product, or an out-of-frame mutation which might completely disrupt transcription of the gene. This would predict that disruption of the GMGXll domain normally prevents formation of the DMD gene product and that disruption of the p20 and Cf23a loci could give rise to a defective gene product.

The forty-five mutations detected at the DMD/BMD locus in this study are highly heterogeneous, representing at least twenty-five distinct groups. It is apparent that it will be necessary to precisely define many of these at the DNA sequence level before accurate correlation with phenotype can be assessed. It may eventually be possible to predict the extent to which a well-characterised deletion will disrupt protein formation, and thus to predict the severity of phenotype from a defined mutation.

In rare instances, a muscular dystrophy which is clinically indistinguishable from DMD is observed. This appears to be inherited as an autosomal disorder (McKusick number 25310), (Somer et al., 1985). In such instances Xp21 RFLP haplotypes will fail to segregate with a muscular dystrophy phenotype.

4:2:8 Causal nature of DMD deletions

Although it has generally been accepted that a deletion in the pERT87 and/or XJ regions is causal to DMD/BMD, Koh et al. (1987) have recently reported a normal male with a deletion of DXS206 including XJ1.1 and XJ10.1 and at least two exons of the DMD gene. This boy's brother, with DMD, apparently has the same deletion. This dramatic difference in phenotype may be a consequence of subtle differences betwen the two deletions or alternatively to a compensatory factor present in the unaffected boy. Neither boy was deleted for XJ5.1 (or pERT87-42). Whatever the reasons for the

difference in phenotype, this deletion, in a normal individual, has serious implications for prenatal diagnosis based on deletion analysis. As there is no reason to suppose that such an anomaly need be restricted to this region, it would now seem appropriate to include unaffected male siblings of boys with DMD/BMD in any deletion screening.

Similarly, but less dramatically, two affected brothers have been reported with classical BMD whose great-great uncle appears to have the same deletion and yet is much more mildly affected (Forrest et al., 1987a).

4:2:9 Germline Mosaicism and DMD.

Recent reports (Lanman et al., 1987, Monaco et al., 1987 and Bakker et al., 1987a) have described females who are heterozygous for a given RFLP at the DMD locus, but have two or more offspring deleted for this same probe.

Transmission of deletions by unaffected males has also been reported e.g. transmission of a JBir deletion at the DMD locus to two daughters and to subsequent affected offspring (Darras and Francke, 1987) and of a pERT87-15 deletion to at least two daughters and subsequent affected offspring (Bech-Hansen et al., 1987).

Three hypotheses have been put forward to explain this phenomenom (i) germline mosaicism (ii) translocation of the "deleted sequences" to an autosome in the parent and (iii) a "pre-mutation" or predisposition to a deletion which gives rise to the same deletion in multiple independent events (unlikely). The first hypothesis is favoured but the second could be formally excluded by in situ hybridisation.

Romeo et al., (1986) have similarly identified a BMD pedigree in which a deletion recurs. Their data is most readily explained if the grandmother represents a germ-line mosaic.

Germ-line mosaicism is not unique to DMD/BMD or to X-linked disorders. It was invoked to explain three sisters with the autosomal dominant disorder achondroplasia, born to normal parents (Fryns et al., 1983) and also an individual heterozygous for a rearranged Factor VIII (Hemophilia A) gene who transmitted a 40kb

factor VIII deletion to one daughter and an affected son, the normal X-chromosome to two sons and two daughters and the rearranged X-chromosome to a further daughter (Gitschier, 1986).

In the light of these observations it is difficult to be convinced that a sporadic deleted individual truly represents a new mutation. It is thus possible that the mothers of SJ (3485) and NJ (5313) (who are heterozygous at two and four loci respectively for which their sons are deleted) are germline mosaics and could give rise to subsequent affected males. They should therefore be offered prenatal diagnosis.

4:2:10 The origin of DMD mutations.

The origin of the deleted chromosome has been demonstrated for two individuals, NJ (5313) and SJ (3485) for whom grandparental samples were available. The mutation in these two individuals occured in a maternal gamete, derived from grandmaternal chromosome in NJ (5313) and the grandpaternal chromosome in SJ (3485), with no apparent recombination. AW (7866) also represents a new mutation which arose in a maternal gamete.

Two further deleted individuals for whom grandparental samples were available, SS (5099, Wilcox et al. 1986) and AB (0120) did not represent new mutations. Their deletions were apparent in their maternal grandmothers.

New mutations are generally believed to account for one-third of individuals with DMD, based on the static frequency of the disorder in the population and a calculation using Haldane's formula (Haldane, 1935), which assumes that mutation rates are equal in male and female gametes and that male fertility in DMD is effectively nil. (Only one DMD male with a child has been reported to date, (Thompson, 1978)).

This is supported by segregation analysis performed on siblings of affected cases of DMD. Carrier analysis based on creatine kinase measurement has also suggested that about a third of boys with DMD represent new mutations (reviewed in Moser, 1984) although these results were unavoidably based on incomplete ascertainment. In contrast, a low proportion of new mutations (four non-carrier mothers/ twenty isolated cases) has been observed for

Lesch-Nyhan disease (Francke et al., 1976).

Witkowski et al. (1987) studied twenty seven three- generation families, six of which represented families with deletions. The deletion arose in the grandpaternal X-chromosome in two of these "deletion" families and in the grandmaternal X-chromosome in a third, giving rise to carrier females. The mutation occurred in a maternal gamete in the remaining three "deletion" families as for SJ(3485) and NJ(5313).

In this study the mothers of four boys were homozygous or hemizygous at all polymorphic loci shown to be deleted in their sons, and thus it was not possible to determine whether these individuals carried the deletion. Hemizygosity at a given locus is often difficult to prove by Southern analysis. This question could be best addressed by PFGE. Within the limits of resolution of the system, any probe which is close to, but not within, the deletion, may detect an anomalous fragment in addition to the fragment of normal size (which acts as an internal control). Kenwrick et al. (1987) described a similar phenomenon in a female DMD patient with an X;1 translocation using probes pERT84 and HIP25. PFGE might also allow the detection of a rearrangement, such as an inversion, which might result in a predisposition to deletions.

4:2:11 Possible mechanisms for the high frequency of deletions and duplications at the DMD locus.

Deletions seem to account for a higher proportion of mutations in certain disorders than in others at both the microscopic and molecular levels. Molecular (sub-microscopic) deletions represent 18% (five in twenty eight) mutations in Lesch-Nyan disease (Yang et al., 1984) and 7% (one in fifteen) mutations in OTC deficiency (Rozen et al., 1985).

To a certain extent, the high proportion of deletions detected at the DMD locus (sections 4:2:4-4:2:6) is a consequence of its considerable size. Koenig et al. (1987) state that the incidence of deletions is no greater for any 200kb region of the DMD gene than for other disorders e.g. the FVIIIc locus (Youssoufian et al., 1987) or the HPRT locus (Yang et al., 1984) although certain regions of the DMD gene are indisputedly more deletion-prone than

others (sections 4:2:4-4:2:6).

The mechanism by which deletions arise at the DMD locus is not known, although it has been proposed that deletion-rich regions might contain direct or indirect repeats and/or sequences with strong homology to others elsewhere within the gene. These may create a region susceptible to non-homologous pairing, thus increasing the likelihood of unequal crossing over at diplotene in meiosis (and resulting in both deletions and duplications). Alternatively direct and/or indirect repeats might allow stem and loop structures to form during replication, which could result in DNA loss by intra-chromosomal recombination.

Characterisation of the molecular structure of GMGX11, which is only 1.2kb in size, by DNA sequencing and/or detection of homologous sequences elsewhere in the gene (e.g. by screening an X-chromosome specific library at low stringency) may help to resolve these possibilities.

Duplications have been reported in association with DMD/BMD. Berteleson et al., (1986) described a male with a duplication encompassing all pERT-87 sub-clones. This duplication was determined by DNA dosage studies. Hu et al. (1987) identified duplications of one (or more) exon(s) in three patients (two with DMD and one with BMD) amongst one hundred and twenty individuals with either DMD or BMD. RFLP studies indicated that one tandem duplication had not arisen by unequal exchange between non-sister chromatids and may have resulted from sister chromatid exchange. Den Dunnen et al. (1987) used pulse field gel electrophoresis to demonstrate the presence of a duplication at the JBir locus in an affected individual and in his mother.

The breakpoint has been precisely defined in the t(X;21) female DMD patient described by Verellen-Dumoulin et al. (1984). No extensive homology was observed between the chromosomes from which the translocation arose, in the vicinity of the breakpoint (Bodrug et al., 1987).

Monaco et al. (1987) observed that six deletion endpoints in the DXS164 locus occured in single copy, repeated and long interspersed repeat ("LINE", Singer, 1982) sequence homology sequences, so that there would appear to be little sequence

specificity for deletion breakpoints.

It should be possible to gain insights into the means by which deletions arise at deletion-prone loci by comparison of the deletion junctions in affected individuals with the sequence in normal males. The many small deletions associated with GMGX11 and p20 should prove particularly amenable to such a molecular analysis.

4:2:12 The DMD gene product "dystrophin" and its implications for future research.

Isolation and cellular localisation of the DMD gene product, "dystrophin" have recently been described. Fusion proteins derived from two distinct mouse cardiac muscle cDNA clones were used to raise antibodies which defined the gene product "dystrophin". These mouse cDNA clones and others showed over 90% homology to the first 4.3kb of the human DMD cDNA at the level of both amino acid and DNA sequences (Koenig et al., 1987; Hoffman et al., 1987a).

Dystrophin is a 400kD protein representing 0.002% total protein in striated muscle, consistent with the low abundance of the DMD mRNA (0.01-0.001% total mRNA). Dystrophin is also present in cardiac muscle and at trace amounts in brain but was absent from muscle biopsies from two boys with DMD (Hoffman et al., 1987b).

The predicted structure of dystrophin was rich in alpha-helix, with significant homology to the actin binding site of alpha-actinin (Hammonds, 1987), suggesting a binding function for cell membranes or helical proteins. Recently, dystrophin has been shown to represent a minor constituent of triads, which are responsible for the release of calcium ions as a signal for myofibril contraction. DMD may thus result from aberrant calcium homeostasis, possibly due to the increased activity of calcium-dependent phospholipases (Hoffman et al., 1987c).

mdx, a muscular dystrophy mutation in the mouse which maps close to G6PD and HPRT, has a mild phenotype comparable to that of BMD in man. On the basis of its map position, it was initially proposed that mdx was homologous to EDMD, (Avner et al., 1987), which maps to Xq28-qter in man. However, cDNA clones from the DMD

locus mapped in the same interval of the mouse X-chromosome as mdx (Brockdorff et al. 1987a, 1987b; Heilig et al., 1987), whilst dystrophin was not expressed in two mdx mutations (Hoffman et al., 1987b). This argued that mdx is the murine equivalent of DMD rather than EDMD and that mdx would represent an appropriate model for the study of DMD, despite its milder phenotype.

It should now be possible to determine defects at the protein level and to confirm proposals that DMD results from a complete absence of dystrophin whilst BMD is a consequence of a defective protein product, using material from muscle biopsies.

The key to an appropriate therapy for DMD may lie in determining why the disorder is so much more severe in man than in mouse. Hoffman et al. (1987b) suggest that fibrosis of muscle fibres in DMD, which is not observed in mdx, may prevent the regeneration of muscle fibres which occurs in mdx and account for the more severe phenotype. If this is so, agents which inhibit fibrosis may ameliorate DMD in man.

4:3 STEROID SULPHATASE (STS) DEFICIENCY.

In this section, linkage analysis with GMGX9 and the cloning of STS are summarised. The frequency of deletions detected with GMGX9 and other genomic probes are compared with those of cDNA clones, and possible mechanisms for the high deletion frequency observed at this locus are discussed. Flow cytometry (FACS) data, the absence of heterogeneity amongst these deletions, XY translocation breakpoints and the escape of STS from X-inactivation are presented. Finally, clinical applications of GMGX9 and possible applications of GMGX9 to future work are discussed.

4:3:1 Linkage studies with GMGX9 and the relationship of GMGX9 to STS cDNA clones.

GMGX9 was mapped to Xp22.3 by somatic cell hybrid mapping and more precisely localised to the vicinity of STS by virtue of co-segregation of GMGX9 deletions with steroid sulphatase deficiency (Gillard et al., 1987).

GMGX9 detected a high frequency polymorphism (PIC 0.44) and was included in a multi-point linkage analysis with distal Xp

markers D2(DXS43), 782(DXS85), Dic56(DXS143) and Xg in both normal (TDH) families and families segregating for X-linked ichthyosis, (Yates et al., 1987b). A deletion of GMGX9 was considered as a third rare (null) allele for the purposes of this analysis. GMGX9(DXS237) was linked to STS with a maximum LOD score of 8.73 at 0 recombination (with confidence limits of 0-0.07).

In practice, since both GMGX9 and STS cDNA clones are deleted in at least 86% males with XLI, and two co-deleted loci will be linked at zero recombination regardless of the distance between them, the precise distance between STS and GMGX9 remains unknown. The maximum distance between STS and GMGX9 will correspond to the smallest deleted region these individuals have in common. This is below the limit of resolution of FACS analysis, as some deletions cannot be visualised by this technique, i.e. less than about 2Mb, which is normally assumed to represent 2% recombination.

Thus, the maximum error in assuming that GMGX9 and STS are tightly linked is small, providing that the distance between these loci does not differ substantially in deleted and non-deleted individuals.

The multi-point analysis gave the most likely order as D2-22cM-782-10cM-Dic56-5cM-(STS/GMGX9)-13cM-Xg-Xpter. This order was supported by the deletion of Xg, STS and GMGX9 (but not Dic56) in two males (1729 and 7079) with XY translocations.

STS cDNA clones were isolated independently by several groups using polyclonal antibodies (raised to purified STS protein) to screen placental cDNA libraries (constructed in lambda phage expresssion vectors), (Yen et al., 1987a; Ballabio et al., 1987a; Conary et al., 1987; Bonifas et al., 1987).

The 2.7kb STS cDNA clone of Yen et al. (1987a) failed to hybridise to RNA from fibroblasts of an affected individual and cross-reacted with STS antibodies when cloned into the mammalian expression vector pMSG. The cDNA clone of Ballabio et al. (1987a) was localised to Xp22.3 by in situ hybridisation. STS cDNA clones detected genomic deletions in eight out of ten (Yen et al., 1987a), eight out of eight (Ballabio et al., 1987a), two out of three (Conary et al., 1987) and fourteen out of fifteen (Bonifas et al., 1987) patients with XLI, respectively. These results confirmed that

these clones represented at least part of the STS gene. The 2.7kb mRNA transcript was sequenced and found to contain an open reading frame of 1542bp with a signal peptide of 22 amino acids and a coding region of 492 amino acids, (Yen et al., 1987a).

GMGX9 and the p422 and p2a4 STS cDNA clones (Ballabio et al., 1987a) demonstrated the same pattern of deletion in forty-four individuals with XLI with only one exception, yet comparative mapping suggested that GMGX9 was not expressed. GMGX9 might therefore represent an intron or a flanking marker.

4:3:2 Deletion analysis of males with XLI.

GMGX9 detected deletions in thirty-seven of forty-four (86%) unrelated individuals with XLI. Thirty-eight of these forty-four individuals were deleted for p422, including the thirty-seven deleted for GMGX9.

Thirty-one affected males, including the exceptional male,"Ba" were screened with GMGX9, STB14 (a genomic clone at the 5' end of the STS gene), p2a4 (an STS cDNA clone), GMGXXY3 and GMGXY19 (genomic probes independently isolated from X and Y chromosome libraries, (Mitchell et al., unpublished observations)). Four males were not deleted for any of these probes whilst twenty-six had deletions of (X-specific) bands of all five probes. Only one deletion, that of "Ba", could be resolved from the others, indicating that deletions at the STS locus represent a highly homogeneous group.

The STS gene was shown to span at least 140kb on the X-chromosome including at least ten exons, with introns from 100bp to over 20kb in size (Yen et al., 1987b). Twenty-one of a group of twenty-six males with XLI had entire gene (ten exon) deletions, whilst two had partial deletions. The first of these included all but the first exon whilst the second included at least 25kb and 4 exons, (Shapiro et al., 1987). Thus these individuals also represented a highly homogeneous group of deletions (table 12).

"Ba" was deleted for the X-specific bands of p2a4, p422 and GMGXY19 but not those of GMGX9, STB14 or GMGXXY3. These probes can be divided into two groups accordingly. STB14 is at the 5' end of this deletion but GMGX9 and GMGXXY3 may lie to either side of it.

This exceptional deletion demonstrates the value of deletion breakpoints.

It is difficult to determine the relative order of these probes, although they are all known to lie distal to Dic56. None of these probes, with the notable exception of GMGX9, detects an RFLP and thus ordering of these probes will depend on PFGE (in X-hybrids or females) or on screening of further deletions.

4:3:3 XY homology of probes which detected deletions in males with XLI.

Neither p2a4 nor GMGX9 demonstrate any homology to the Y chromosome, yet GMGXXY3 and GMGXY19, STB14 (Ballabio et al., unpublished observations) and the 2.7kb STS cDNA clone (Yen et al., 1987a) all have homologues on Yq. Overlapping Y-specific genomic clones, detected with STS cDNA clones, have shown that Y homology is limited to the 5' end of the STS gene, and consists of two EcoRI fragments of 20kb and 1.5kb and an intervening intron of 15kb (Yen et al., 1987a, 1987b). Thus homologous sequences to STS on the Y-chromosome represent a (non-functional) diverged gene or "pseudogene".

The Y-pseudogene was assigned to Yq11-Yqter using somatic cell hybrids (Yen et al., 1987a). Yq homologues have also been observed for other DNA probes in distal Xp e.g. M1A/DXS31 (Koenig et al., 1984), CRI-S-232 (Donnis-Keller et al., 1987) and GMGXXY3 and GMGXY19 (Mitchell, unpublished observations). Xg and MIC2X, however, which are analogous to STS in that they escape X-inactivation, have homologues on Yp.

Such observations suggest a peri-centric inversion on the Y chromosome, during its evolution from an ancestral sex-chromosome, which would result in an increased genetic distance between STS and the STS pseudo-gene and the transfer of the testes determining factor (TDF) close to the pairing region. It should be interesting to compare physical maps of the region around the X and Y homologues of STS and to assess the extent of their divergence.

PROBE AND DELETION STATUS

COUNTRY OF ORIGIN	GMGX9			p422			STB14			cDNA*		
	Complete	non-	partial-deletion	Complete	non-	partial-deletion	Complete	non-	partial-deletion	Complete	non-	partial-deletion
Scotland	19	4	0	19	4	0	19	4	0	NT	NT	NT
Italy	7	1	0	8	0	0	7	1	0	NT	NT	NT
Germany	8	1	0	8	1	0	NT	NT	NT	NT	NT	NT
Netherlands	2	2	0	2	2	0	NT	NT	NT	NT	NT	NT
USA (Y)	NT	NT	NT	NT	NT	NT	NT	NT	NT	21	3	2
Germany (C)	NT	NT	NT	NT	NT	NT	NT	NT	NT	2	1	0
USA (B)	NT	NT	NT	NT	NT	NT	NT	NT	NT	14	1	0

Table 12: Summary of deletion data for X- Linked ichthyosis.

(NT, Not tested; *cDNA clones isolated by:(Y); Yen et al, 1987a; (C); Conary et al, 1987; (B); Bonifas et al, 1987. Results with cDNA(Y) are presented in Shapiro et al, 1987, those with (C) and (B) in the references given. The results with p422 were obtained by A. Ballabio (unpublished observations)).

4:3:4 Deletion analysis of additional individuals with anomalies of distal Xp.

A number of individuals with anomalies of distal Xp were investigated in an attempt to define new breakpoints which could distinguish betweeen the probes deleted in males with XLI and facilitate their ordering on distal Xp. The first of these, "Sa", had both Kallmann's syndrome (KAL) and XLI. His deletion proved to be no different from those of other individuals with XLI alone, by either molecular or FACS analysis.

KAL is thought to be proximal to STS, as several male individuals with 46,Yt(X;Y)(p22.3;q11.2) translocations (effectively deleted for all of distal Xp) are affected with XLI but not KAL.

Three further patients with KAL but not XLI were analysed but were not deleted for any of the probes tested. This may be due to deletions or point mutations not detected by available probes in Xp22.3. Alternatively, KAL may not be associated with a mutation in this region of the X-chromosome in these individuals.

Perrin et al. (1976) described a family segregating for "X-linked ichthyosis, hypogonadotropic hypogonadism, mental retardation and anosmia" which failed to demonstrate linkage with Xg (maximum LOD score of -0.096 at theta = 0.40). This indicates a distinct mutation from that of the families described by Ballabio et al. (1986, 1987b) and an alternative localisation for KAL. STS levels were not assessed in the family described by Perrin et al. (1976), however, so the diagnosis of XLI was not confirmed.

Two males with XY translocations, AM (1729) and JH (7079) were also studied. These boys were deleted for the (X-specific) bands of all probes deleted in males with XLI (GMGX9, GMGXXY3, GMGXY19, STB14 and p2a4) and also for M1A. M1A may be either distal or proximal to the other five deleted probes.

These results suggest that there are definite hotspots in distal Xp which give rise to deletions and XY translocations associated with XLI and that these form two homogeneous groups or that there are as yet insufficient DNA probes to distinguish between individuals in each group. These possibilities might be resolved by cloning several translocation and deletion junctions.

The majority of XX males have levels of STS activity characteristic of normal female individuals. At least three XX males have been reported to possess only a single dose of STS activity, (Pierella et al., 1981; Wieacker et al., 1983c). This may have arisen as a result of the loss of one STS locus through X-Y interchange. Such XX males would be hemizygous for GMGX9 and might represent novel breakpoints for inclusion in a a deletion panel.

Petit et al. (1987) described an X-specific polymorphic sequence, 68b, which was tightly linked to the pseudoautosomal region. They observed the loss of the paternal 68b allele in two of six XX males and possibly of a third (on dosage). Loss of the paternal Xg allele was demonstrated in one of these two XX males.

GMGX9 and STS are estimated to be 13cM proximal to Xg (Yates et al., 1987b), which is itself X-specific and proximal to the pseudoautosomal region. Thus, loss of GMGX9 or STS in association with XY interchange might be predicted to occur more rarely than loss of either 68b or Xg.

HindIII digests of seventeen XX males were analysed with GMGX9. Six XX males were heterozygotes. The remining individuals were appparently homozygous (by DNA dosage) for the 4kb allele and were consistent with the inheritance of one allele from each parent, (Jamieson and Affara, unpublished observations).

4:3:5 FACS analysis of deletions

FACS analysis was performed for individuals from sixteen Scottish families, thirteen of which were deleted for GMGX9. Deletions were visualised in eight of thirteen families deleted for GMGX9 (Cooke et al., in press). The largest deletions detected in males with X-linked ichthyosis were those of AB (4076), MG(1034) and YM(9056) whose deletions correspond to 2.8% of the X chromosome i.e. 4Mbp or 8% of Xp. The deletion in AB (4076) could not be visualised microscopically using standard banding techniques. The smallest deletion was that of NJ (7750) which corresponded to 1.2-1.5% of his X-chromosome. The deletions in five affected males, deleted for GMGX9 were not visualised by FACS analysis which demonstrates that hitherto undetected heterogeneity exists amongst these deletions.

The eight individuals with FACS deletions are not clinically impaired in any way (other than by their ichthyosis) and in particular are not mentally retarded. This implies that there are relatively few genes encoding vital functions in the deleted segment. The ichthyotic males who demonstrate deletions on FACS analysis also have normal fertility (e.g. AB (4076) is a grandfather), in contrast to males with XY translocations, which suggests that XY pairing is not impeded in any way.

STS, GMGX9, GMGXXY3 and GMGXY19 must lie within a region of less than 2Mb (the smallest deletion detected by FACS analysis) as all these probes were deleted in JM (3673) whose deletion could not be detected in this way. The STS gene spans at least 140kb on the X-chromosome or just under one tenth of this distance (Yen et al., 1987b).

4:3:7 Comments on the high deletion frequency at the STS locus and future work with GMGX9.

The knowledge of carrier status is of particular value to obstetricians as steroid sulphatase deficiency results in low estrogen levels and delayed onset of labour in pregnancy. Carrier status may be assessed by an assay of steroid sulphatase in hair-roots (Aitken, Perry & Ferguson-Smith, 1981). Levels in carrier females are usually considerably reduced. It is possible to use GMGX9 to acccurately predict carrier status in certain families e.g those segregating for a deletion in which the parents have differing alleles, or those for which GMGX9 is not deleted in affected individuals but for which the phase is known. Such studies are not possible with cDNA clones at the STS locus (Yen et al., 1987a; Ballabio et al., 1987a; Conary et al., 1987; Bonifas et al. 1987) as no RFLPs have been described with these probes.

It was hoped to investigate the origin of new mutations using GMGX9 and additional DNA markers in distal Xp, in order to determine if deletions could arise in both male and female meioses. A new mutation arising from errors in female meiosis might, for example, be indicated by an isolated deleted individual whose mother was heterozygous for GMGX9. None of the affected individuals in this study represented new mutations, however, which precluded

such investigations.

Failure to determine new mutations at the STS locus may indicate that these are rare occurences and that the majority of individuals with XLI are due to familial rather than sporadic mutations. This could account for the observation that deletions at the STS locus form a fairly homogeneous group which are only rarely separable by molecular analysis e.g. one of thirty-eight individuals (this study) and two of twenty-three individuals (Shapiro et al., 1987, Table12). Alternatively deletion homogeneity could be explained by the existence of (a) preferential hotspot(s) for deletions.

FACS analysis suggests that further heterogeneity does exist, but is not detected by the available probes. The apparent lack of new mutations might thus be due to ascertainment bias, especially as multiple affected males within a family are a good indicator for the X-linked form of ichthyosis and families with multiply affected males are of greatest value for linkage analysis.

The extraordinarily high proportion of deletions detected amongst mutations at the STS locus, might be a direct result of the proximity of STS to the pairing segment and of illegitimate recombination between the X and Y chromosomes, although the mechanism by which this might occur remains unclear. Reciprocal translocation between the X and Y chromosomes can often be excluded due to inheritance of Xg(a), (which also demonstrates the interstitial nature of these deletions). Deletions might also occur as a result of unequal cross-over events in female meiosis although this process should also result in duplications at the GMGX9 locus (which have not yet been observed).

In order to investigate the mechanisms by which XY translocations and deletions occur at the STS locus it will be necessary to characterise the region around the existing probes in detail. PFGE electrophoresis may provide a suitable frame-work in which to construct a physical map of the STS locus or to size and to analyse deletions not detectable by FACS analysis, although additional DNA markers may first be required. It may thus prove worthwhile to expand the GMGX9 locus by chromosome walking or even by chromosome jumping (Collins and Weissman, 1984) to obtain probes

several hundred kb from GMGX9. Additional DNA markers might also permit more deletions to be resolved by Southern analysis.

A further DNA probe, CRI-S-232, recently described in distal Xp is also highly polymorphic (PIC value of 0.9), (Donnis-Keller et al., 1987) and may also prove useful in the analysis of STS mutations. This probe has a Yq homologue (which is not polymorphic), is linked to Dic56 and is about 15cM from several pseudoautosomal loci, (Knowlton et al., 1987).

A screen of "sporadic" STS deficient patients and their families might prove profitable in terms of defining the origin of mutations, whilst the screening of additional STS deficient individuals (and in particular those with complex phenotypes associated with XLI) or of patients with translocation breakpoints in distal Xp, should help to define additional breakpoints and to build up a deletion map.

There is evidence to suggest that the signal for resistance to X-inactivation is a function of the genes themselves (and not of their environment) in mice. For example, Sxr, which is active on the mouse Y chromosome, becomes subject to inactivation after transposition to the pairing region, whilst Sts is active within the pairing segment, (Keitges and Gartler, 1986). Yen et al. (1987b) report no significant difference in methylation comparing the 5' and 3' ends of the human STS gene on the active and inactive X-chromosomes, consistent with its escape from X-inactivation.

It should also prove possible to define the regions responsible for the escape of STS from X-inactivation by molecular means, providing that these control regions exert their action in cis. STS is not essential for cell proliferation and may thus prove an excellent model for investigating X-inactivation by means of site selected mutagenesis.

4:4 GENE CLONING AND REVERSE GENETICS

Of those genes assigned to Xp, only a small proportion have been cloned including OTC, MIC2, and more recently CYBB (the chronic granulomatous disease gene), DMD and STS. OTC was cloned independently by two groups (Horwich et al., 1984 and Davies et al.,1985) using the principle of interspecies homology, following the isolation of the rat gene from a rat liver cDNA library enriched for OTC mRNA (Horwich et al., 1983). MIC2 (Darling et al., 1986) and STS (Yen et al., 1987a; Ballabio et al., 1987a; Conary et al, 1987; Bonifas et al.,1987) were cloned by the "classical" approach which proceeds from the gene product to gene whilst CYBB (Royer-Pokora et al., 1986) and DMD (Monaco et al., 1986; Burghes et al., 1987) were cloned by "reverse genetics" which proceeds from the gene to the gene product (figure 17).

Glycerol kinase (GK), congenital adrenal hypoplasia (AHC) and Norries' disease (NDP) have all been associated with molecular deletions of Xp and their positions are thus well defined. GK and AHC have been assigned to a 2Mbp region between J66H1 and L1-4 and NDP to Xp11.3 close to L1-28 (Gal et al., 1985a, 1986; de la Chapelle et al., 1985). These three disorders are thus suitable candidates for the application of reverse genetics and should prove to be amongst the next Xp loci cloned.

Cloning of GK could be readily verified as glycerol kinase activity can be assayed in white blood cells. GK could also be cloned by classical methods. Isolated GK deficiency is not particularly deleterious, however,and cloning of this gene does not represent a clinical priority. AHC and NDP are far more serious conditions in which the primary defects are unknown. Cloning of these genes and of many others on Xp or elsewhere will thus only be possible by reverse genetics.

It is hoped that a complete map of Xp may eventually be constructed as part of a more ambitious plan to map the entire human genome with contiguous cosmid clones. These clones could prove an invaluable resource for reverse genetics and result in a rapid growth in the number of genes cloned, dependent solely on knowledge of their localisation. The precision of localisation should continue to improve as additional polymorphic loci are

defined and it may be that linkage to a single band of a DNA fingerprint (Jeffreys et al., 1986) will prove sufficient to enable many genes to be cloned by reverse genetics.

This project has centred on obtaining DNA sequences from specific intervals of the X-chromosome (Xp21 and Xp22.3) and on their application to the study of DMD and STS or to complex phenotypes associated with either disorder. The principles involved are not specific to the X-chromosome, however, and could be applied to cloning sequences from any specific chromosome or chromosomal interval and ultimately to cloning the genes for any human disorders mapped to a specific interval.

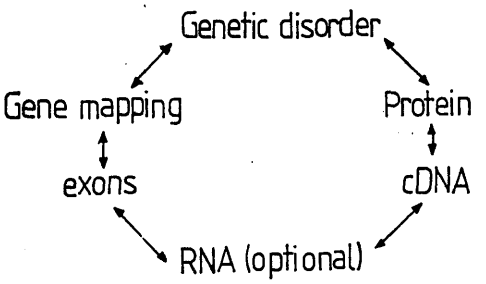


Figure 17: "Reverse genetics" and the classical approach to gene cloning.

(The classical approach proceedes clockwise from recognition of a disorder whilst a "reverse" approach proceedes anti-clockwise).

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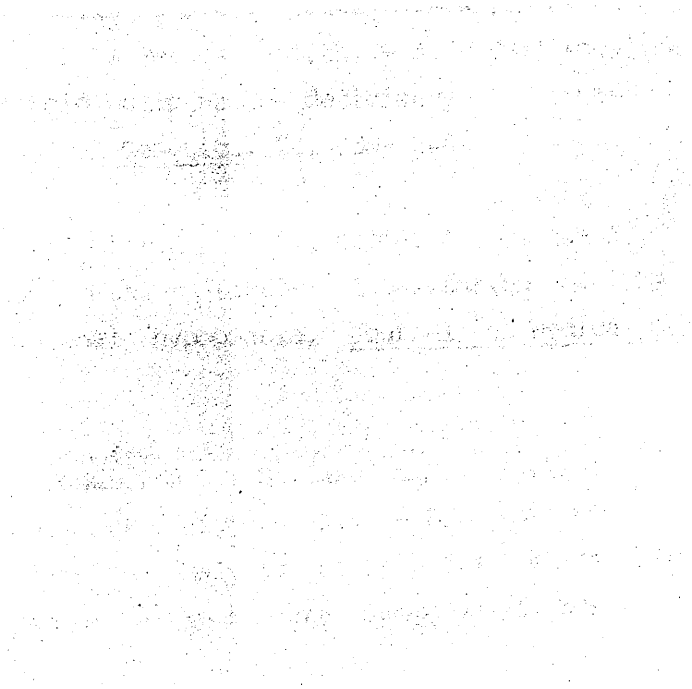
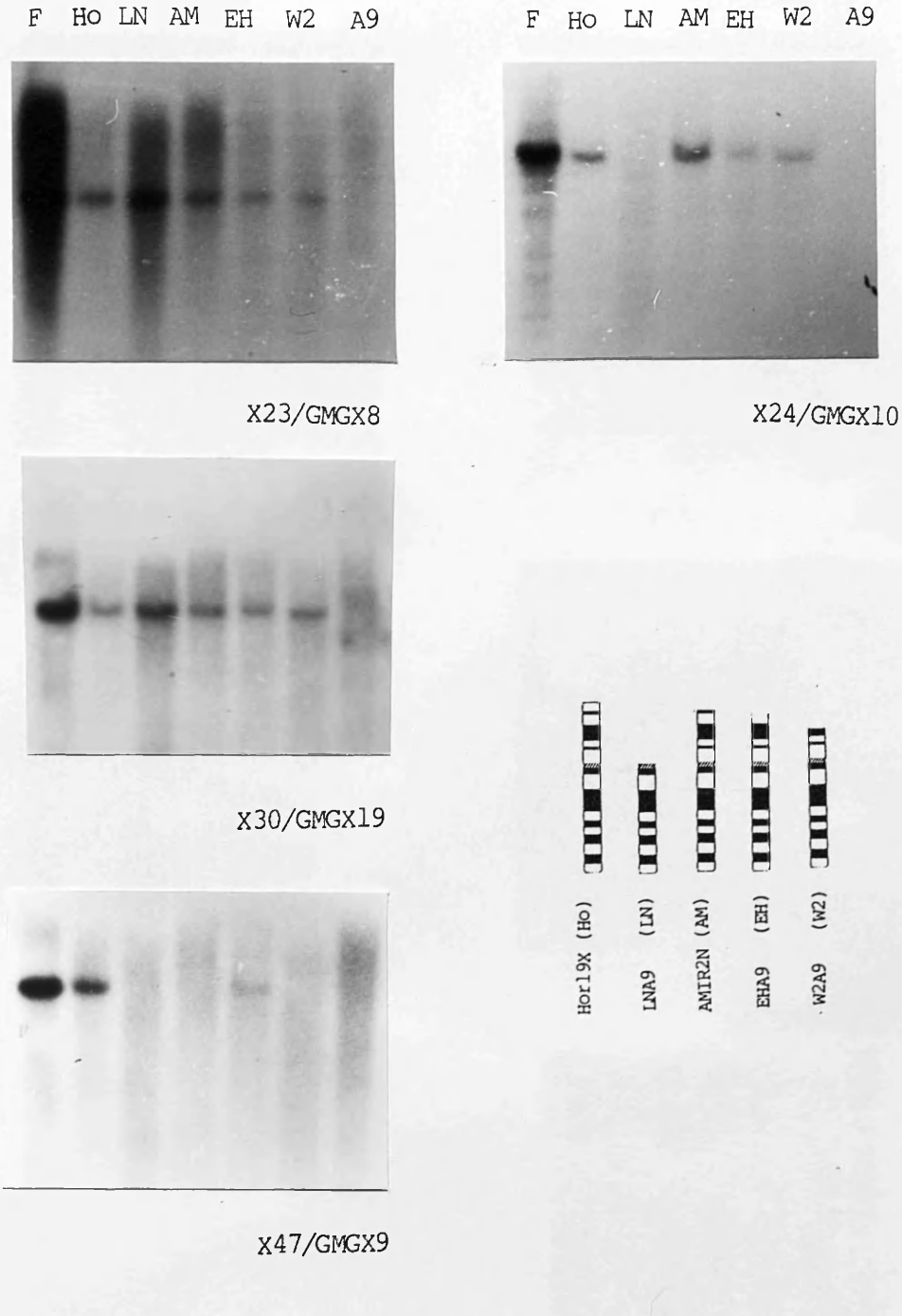


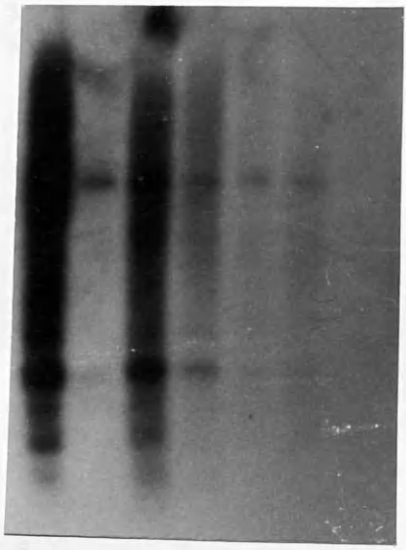
APPENDICES.



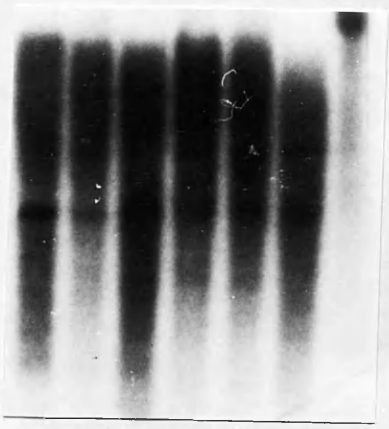
Appendix 1: Hybridisation of X-specific inserts to EcoRI somatic cell hybrid mapping panels (refer to table 4). Continued overleaf.

(F,female control. Hybrids are as described in materials and methods (and see inset). Probes are labelled with both the number of the picked plaque and the "GMGX" number assigned to it (see table 5)).

F Ho LN AM EH W2 A9

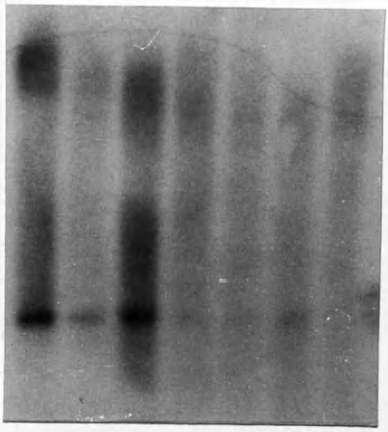


X38/GMGX20

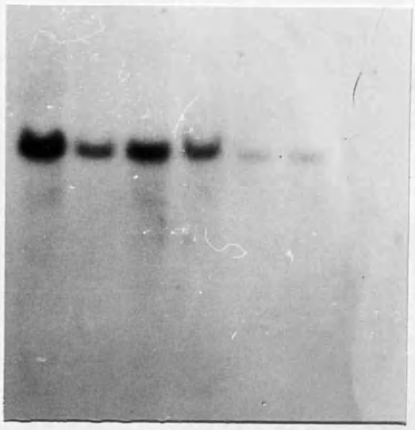


X48/GMGX18

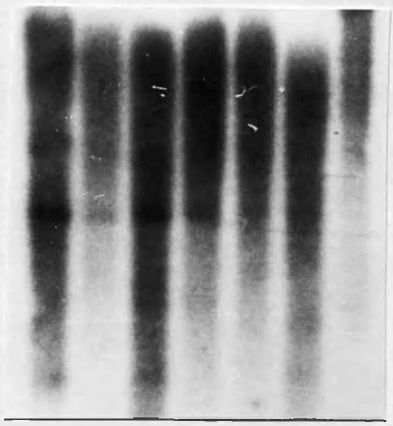
F Ho LN AM EH W2 A9



X53/GMGXY12



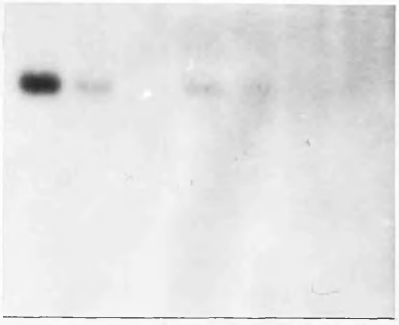
X60/GMGX16



X74/GMGX13

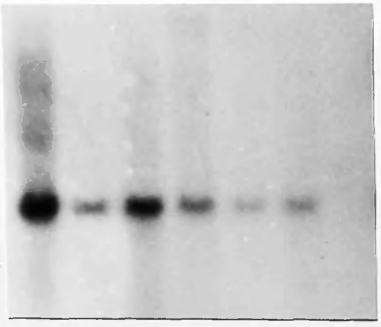
Appendix 1 (continued)

F Ho LN AM EH W2 A9

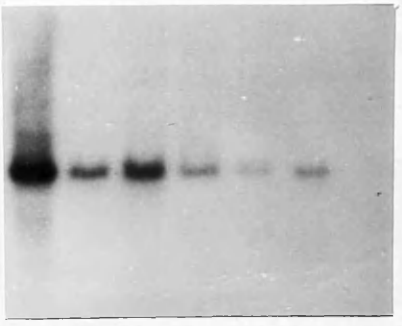


X66/GMGX11

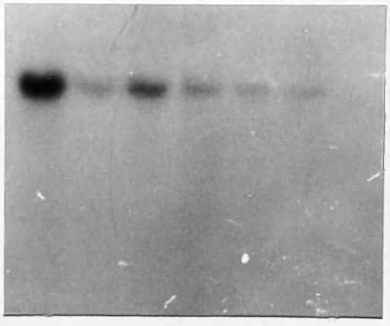
F Ho LN AM EH W2 A9



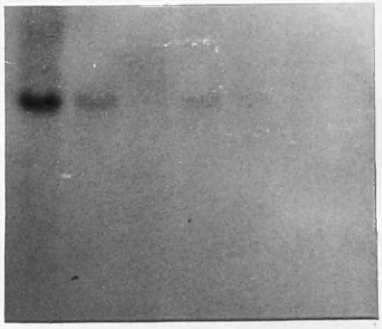
X92/GMGX14



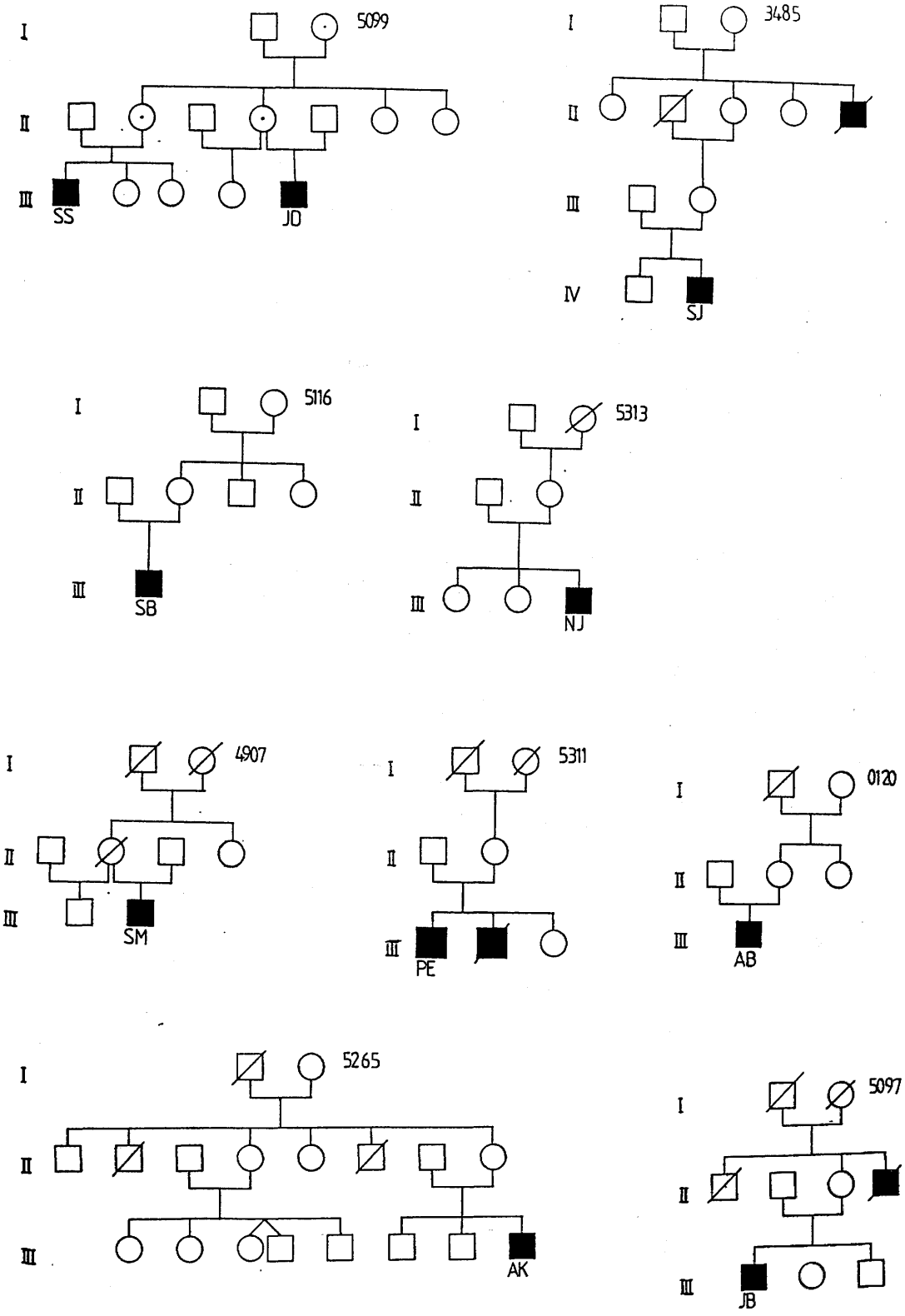
X94/GMGX15



X110/GMGX23

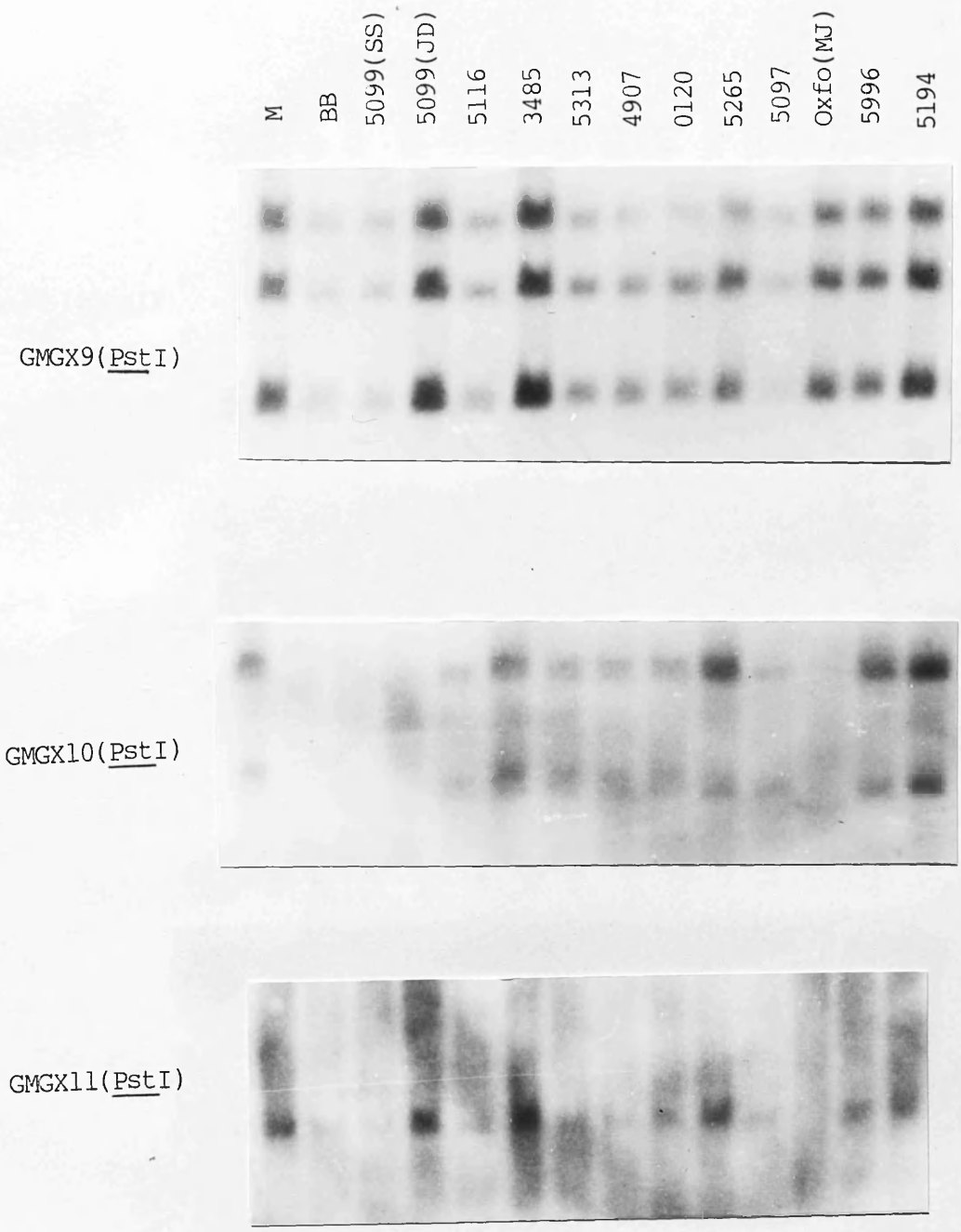


X112/GMGX12



Appendix 2: Deletion studies in DMD/ BMD families based on deletions of the pERT87 and XJ probes.

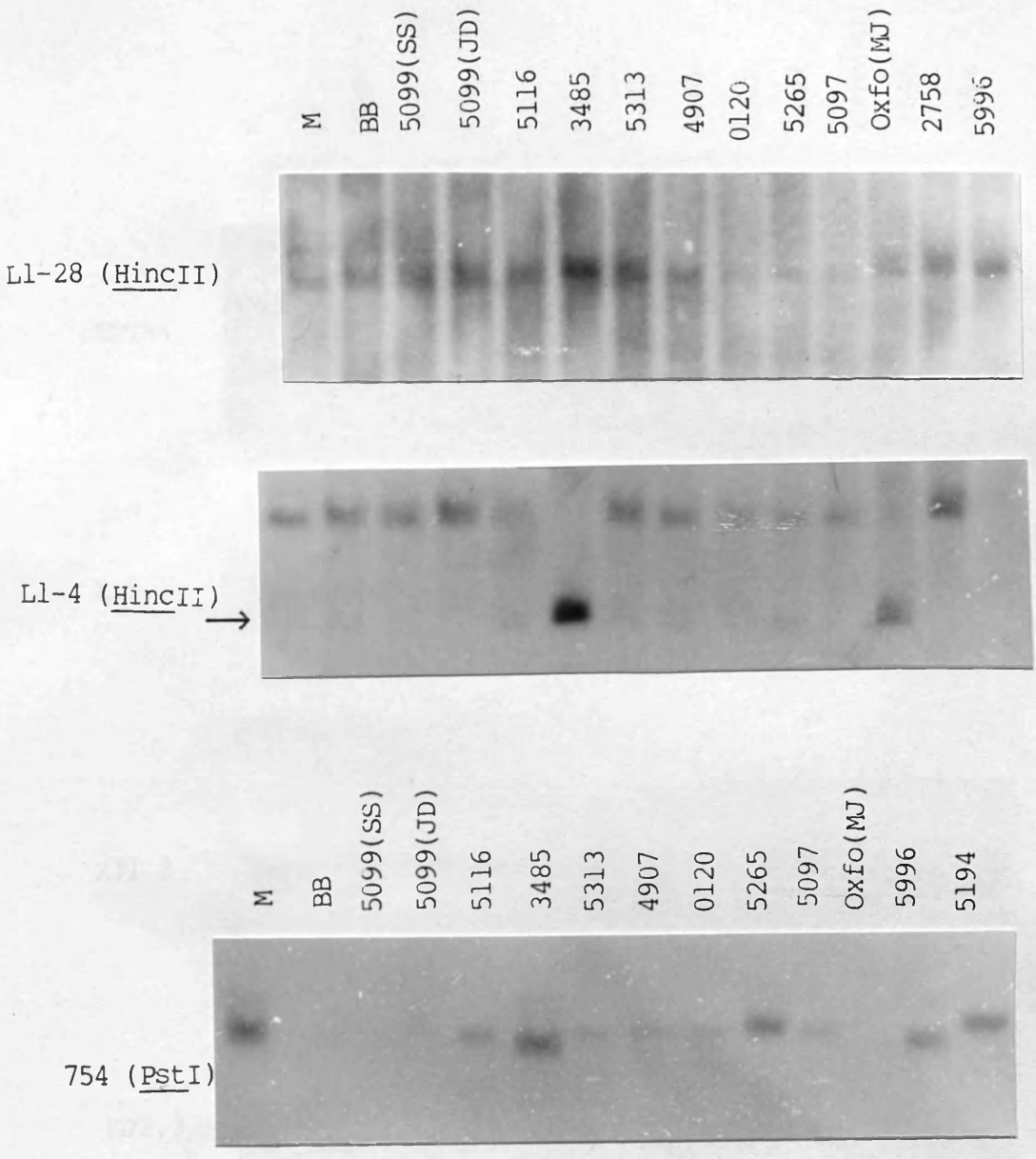
(A); Pedigrees of the nine boys with deletions detected by pERT87 and/ or XJ probes (refer to table 6).



Appendix 2: Deletion studies in DMD/ BMD families based on deletions of the pERT87 and XJ probes.

(B); Hybridisation of GMGX9, GMGX10 and GMGX11 to the deletion panel (refer to table 6).

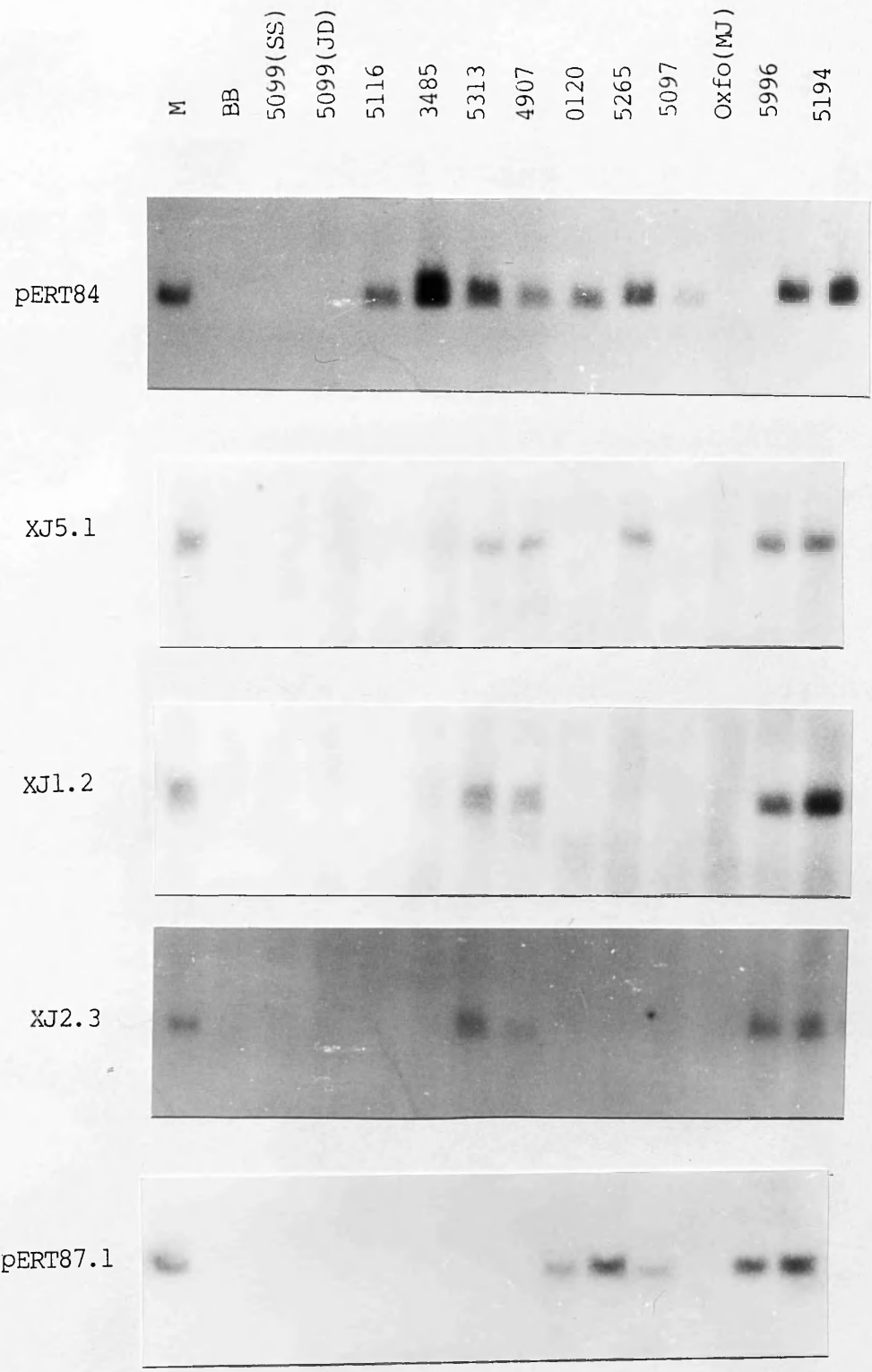
(M,male control. Other individuals are described in the text. These results are summarised in table 6).



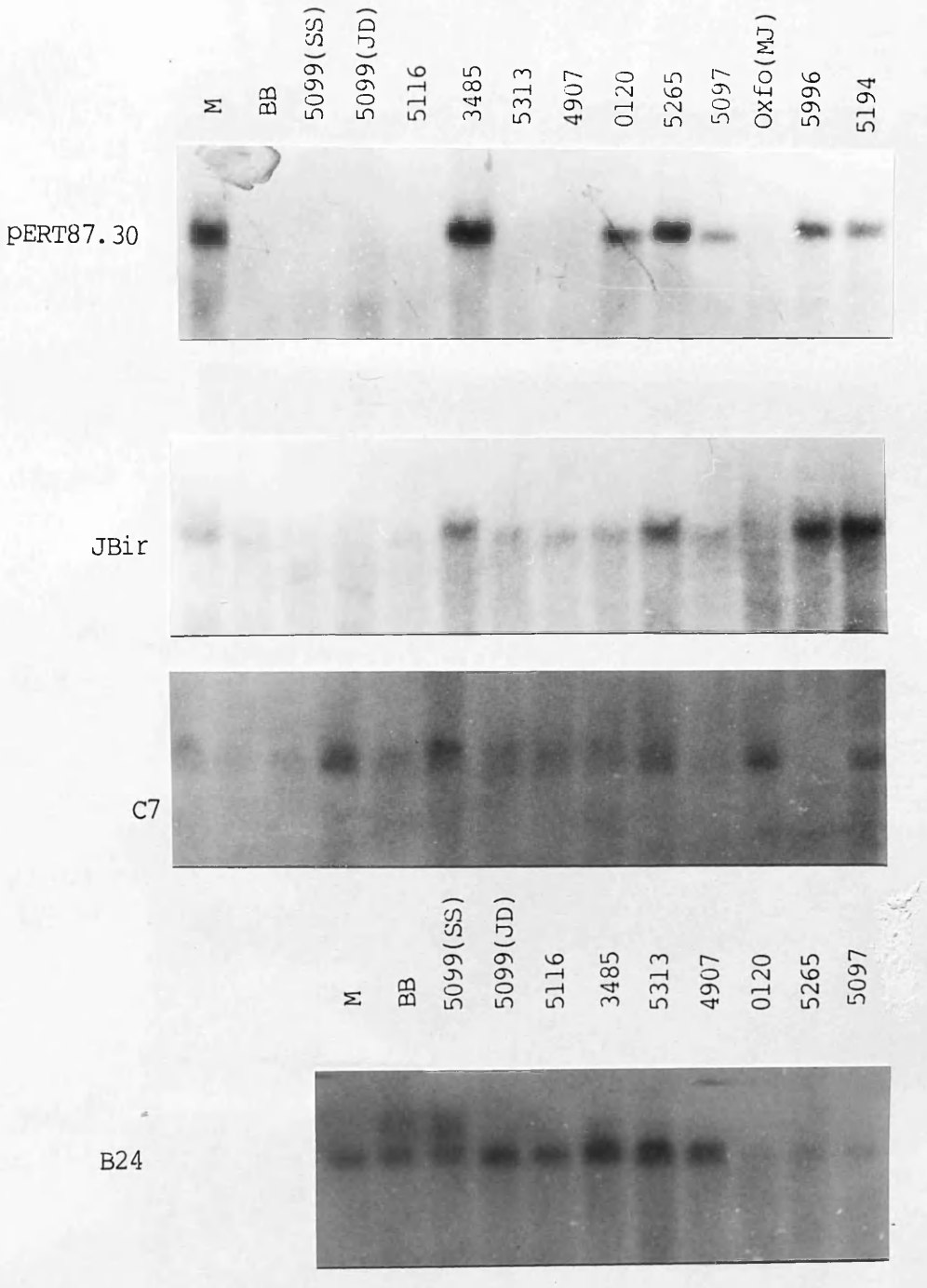
Appendix 2: Deletion studies in DMD families based on deletions of the pERT87 and XJ probes. (C); Hybridisation of additional Xp probes to the deletion panel (refer to table 6). Continued overleaf.

(M,male control. Other individuals are described in the text. These results are summarised in table 6. Filters were PstI digests unless otherwise specified (in parenthesis).

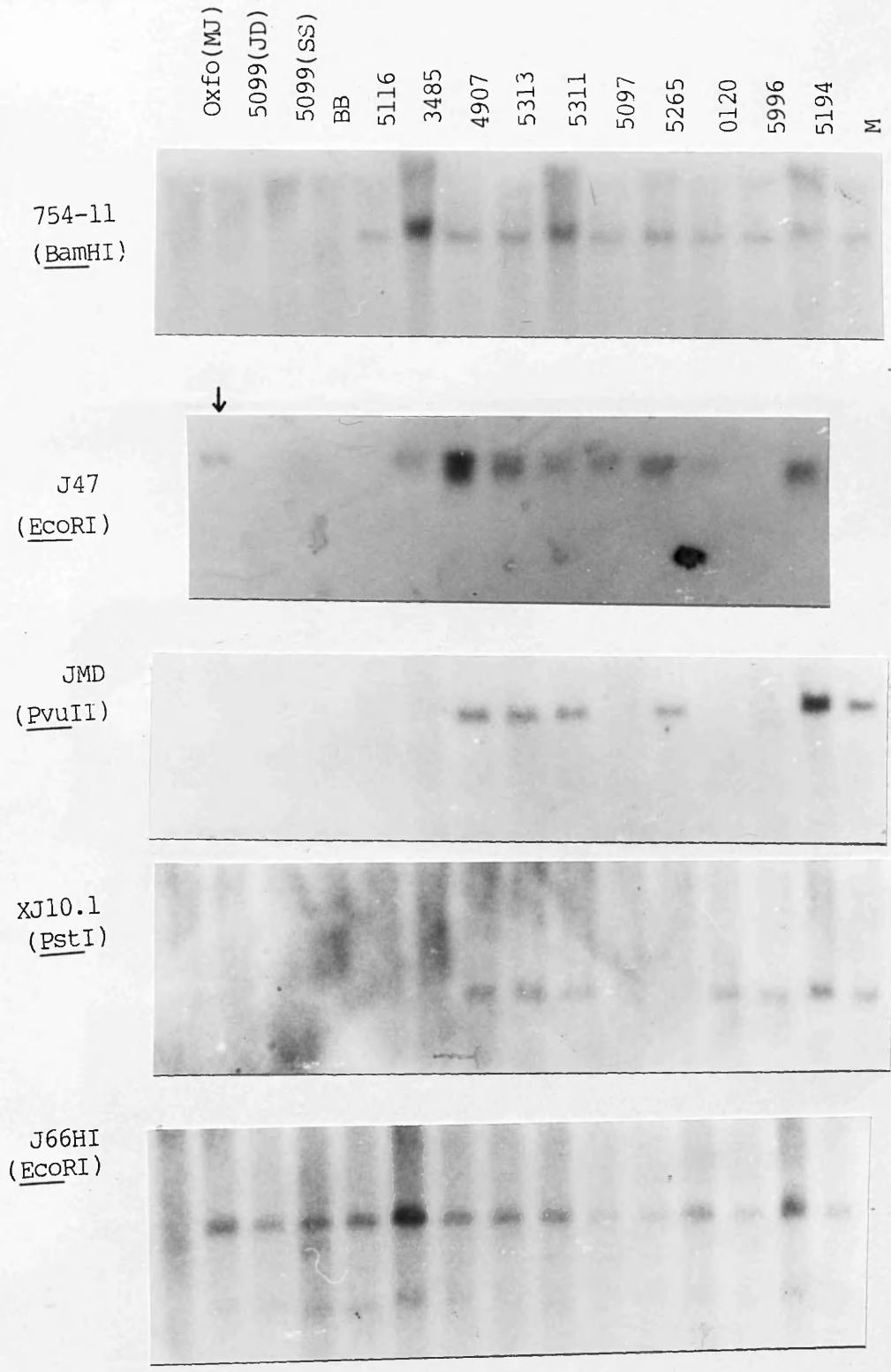
Notes: The anomalous fragment detected by J47 in MJ is marked with an arrow. Anomalous fragment sizes detected by L1-4 (Hinc II digests) in MJ and SJ (3485) may represent an RFLP, as a fragment of normal size was detected by L1-4 in a TaqI digest of MJ (refer to figure 6).



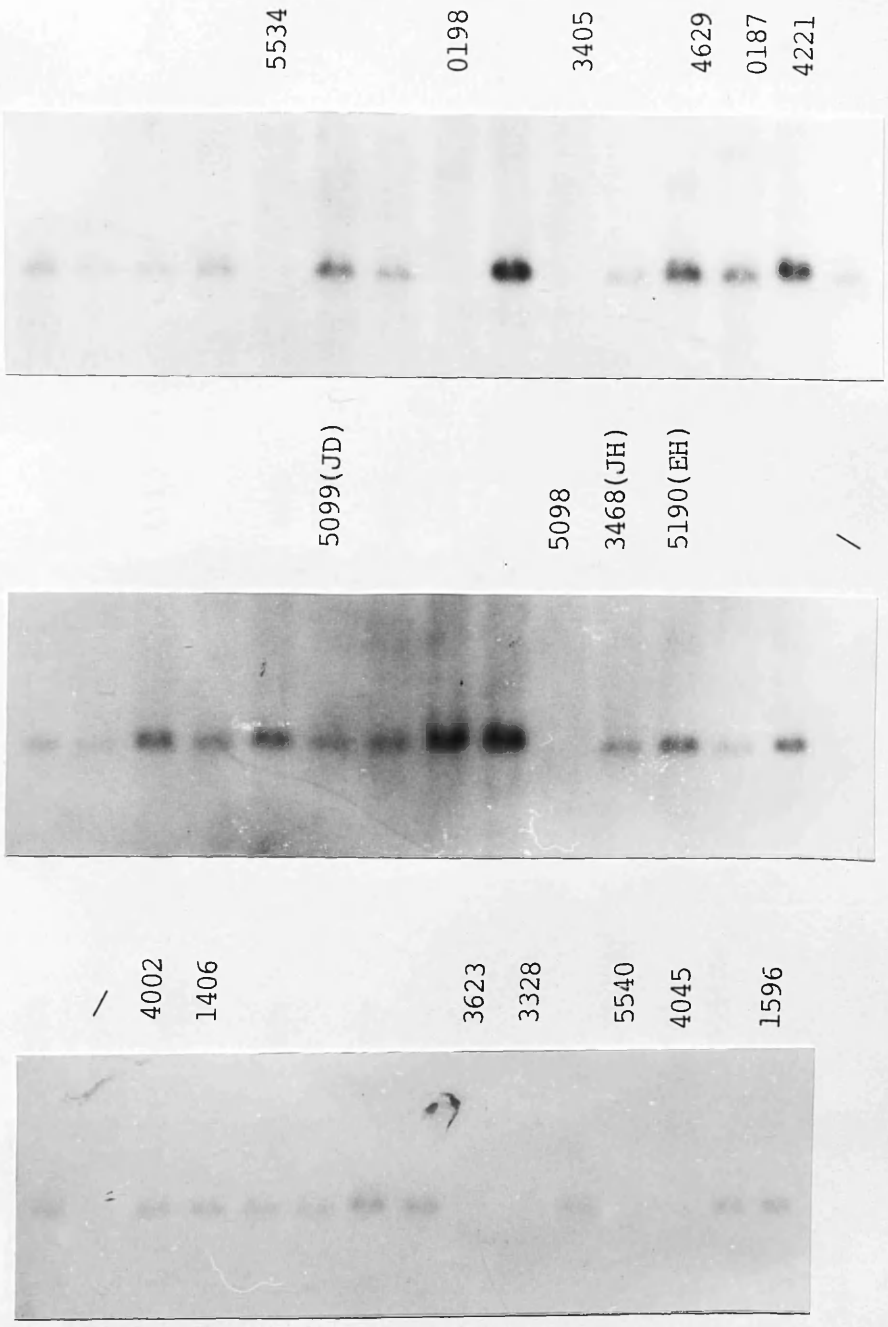
Appendix 2C continued



Appendix 2C continued



Appendix 2C continued

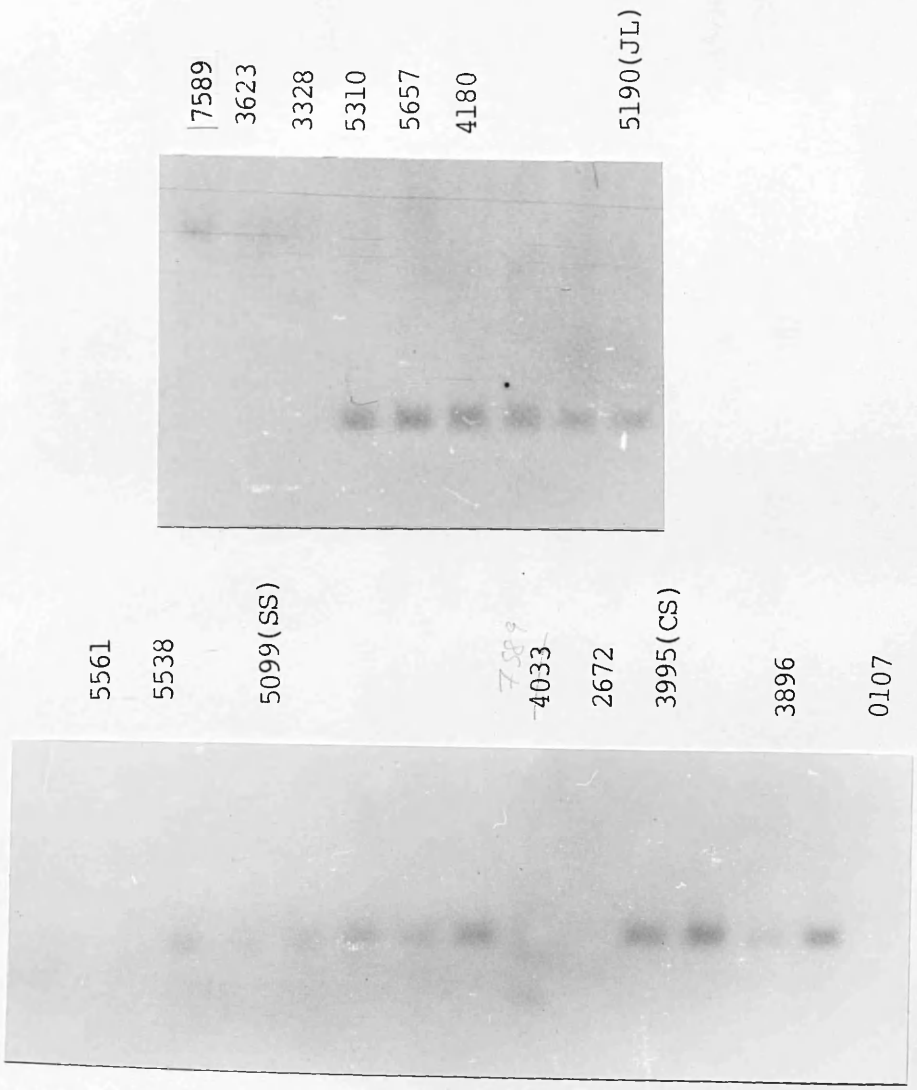


Appendix 3: Deletion studies in males with DMD/BMD with GMGXll.

(A); Hybridisation of GMGXll to HindIII digests of all boys. (Individuals listed in table 7 are identified by their pedigree numbers).

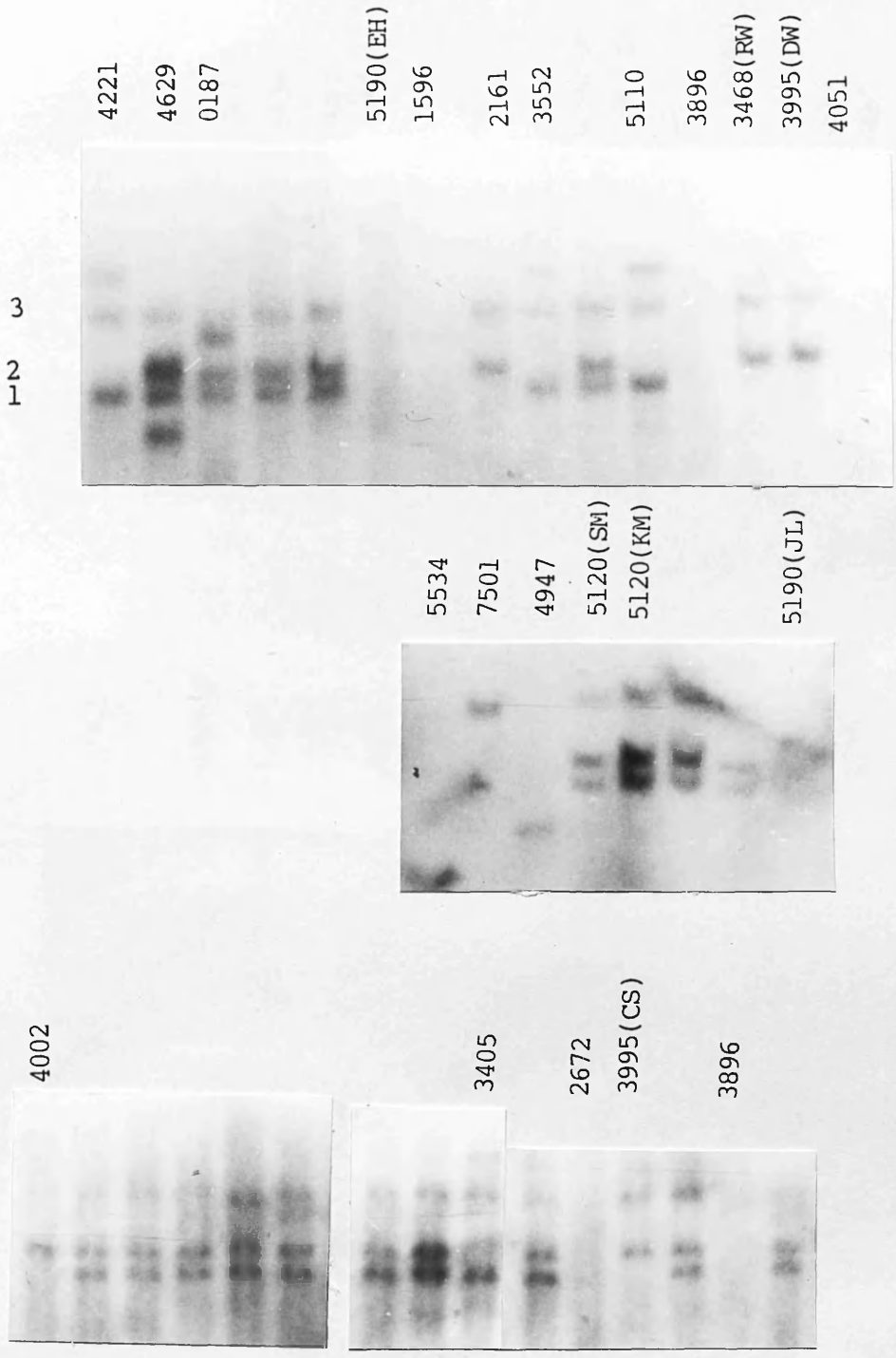
Appendix 3 continued.

Appendix 3 continued.



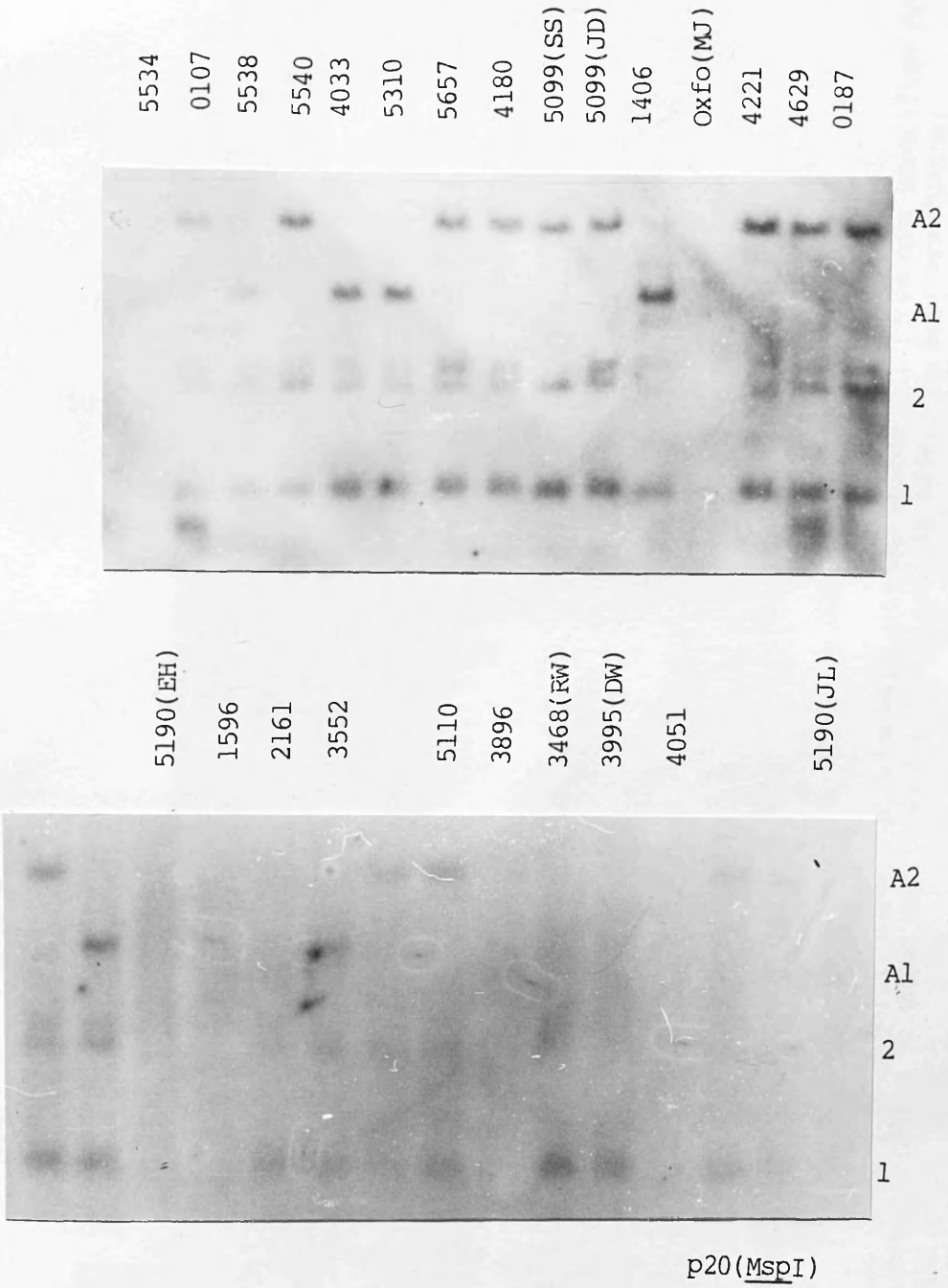
Appendix 3: Deletion studies in males with DMD/BMD with GMGX11.

(B); Hybridisation of GMGX11 to EcoRI digests of boys showing anomalous fragment sizes (refer to table 7). These are indicated by arrows (Individuals are identified by their pedigree numbers).

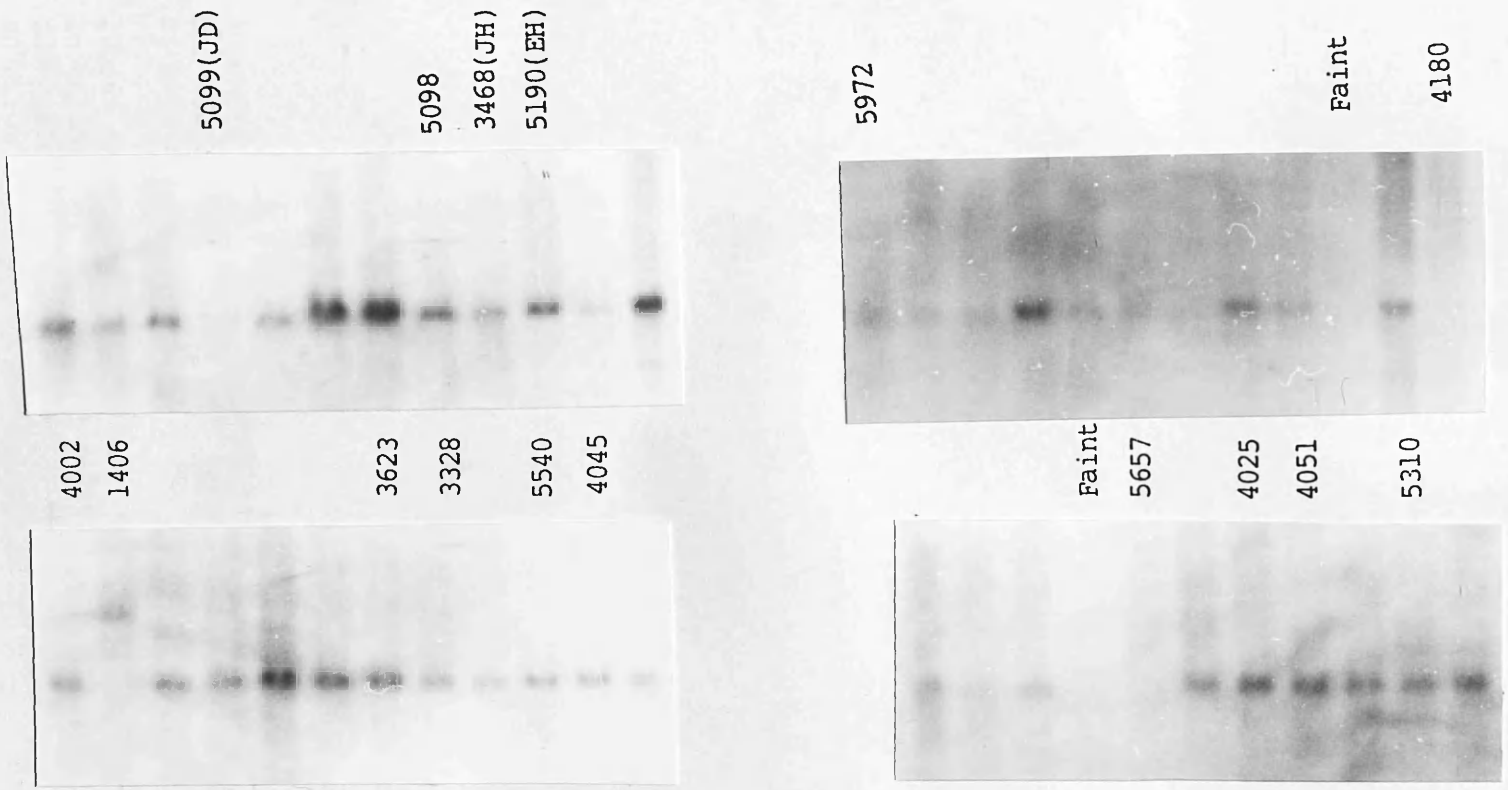


Appendix 4: Deletion studies in males with DMD/BMD using p20, JBir and J66HI.

(A); examples of hybridisation of p20 to HindIII digests of boys with DMD/BMD (see table 7). (Boys are identified by their pedigree numbers. For those families in which more than one individual is affected, boys are further identified by their initials).

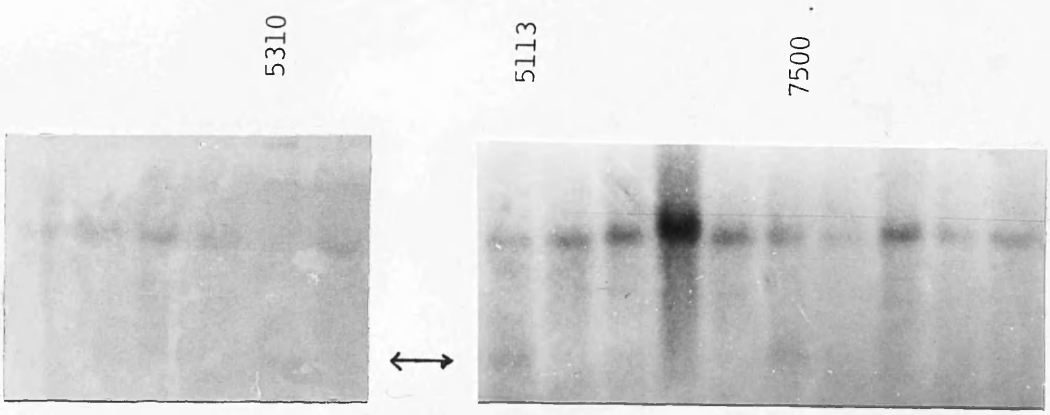


Appendix 4: Deletion studies in males with DMD/BMD using p20, JBir and J66HI. (B); examples of hybridisation of p20 to MspI digests of boys with DMD/BMD (see table 7).
(Boys are identified by their pedigree numbers. For those families in which more than one individual is affected, boys are further identified by their initials. Note: the "doublet" observed with constant band 2 (1.5kb) was not seen in other MspI digests probed with p20. This may be due to improved resolution of these particular gels (eg due to more prolonged electrophoresis) or to contamination in the batch of enzyme used for these digests. Track 1406 is partially digested).



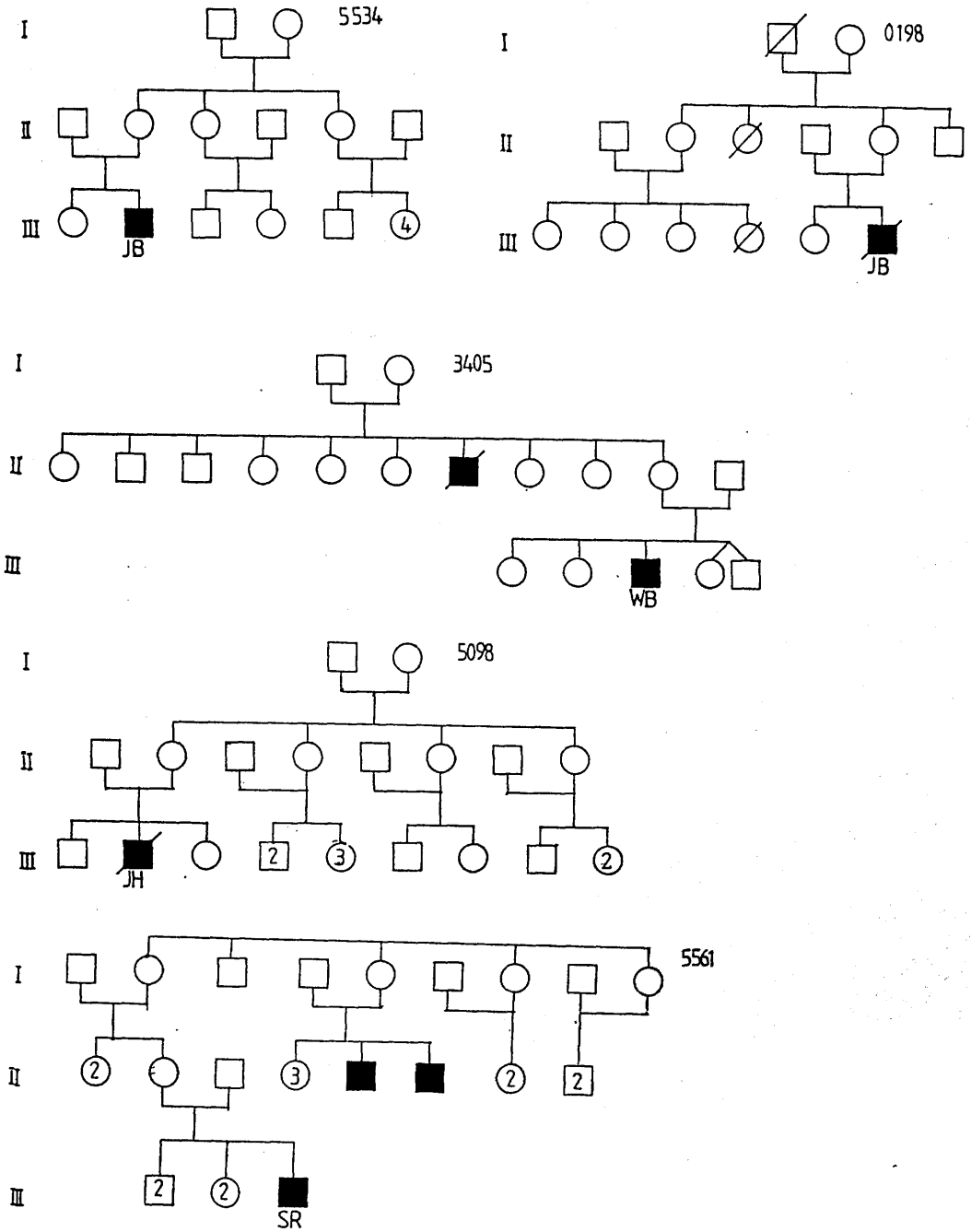
Appendix 4: Deletion studies in males with DMD/BMD using p20, JBir and J66HI.

(C); examples of hybridisation of JBir to HindIII digests of boys with DMD/BMD (table 7). (Boys are identified by their pedigree numbers. For those families in which more than one individual is affected, boys are further identified by their initials).

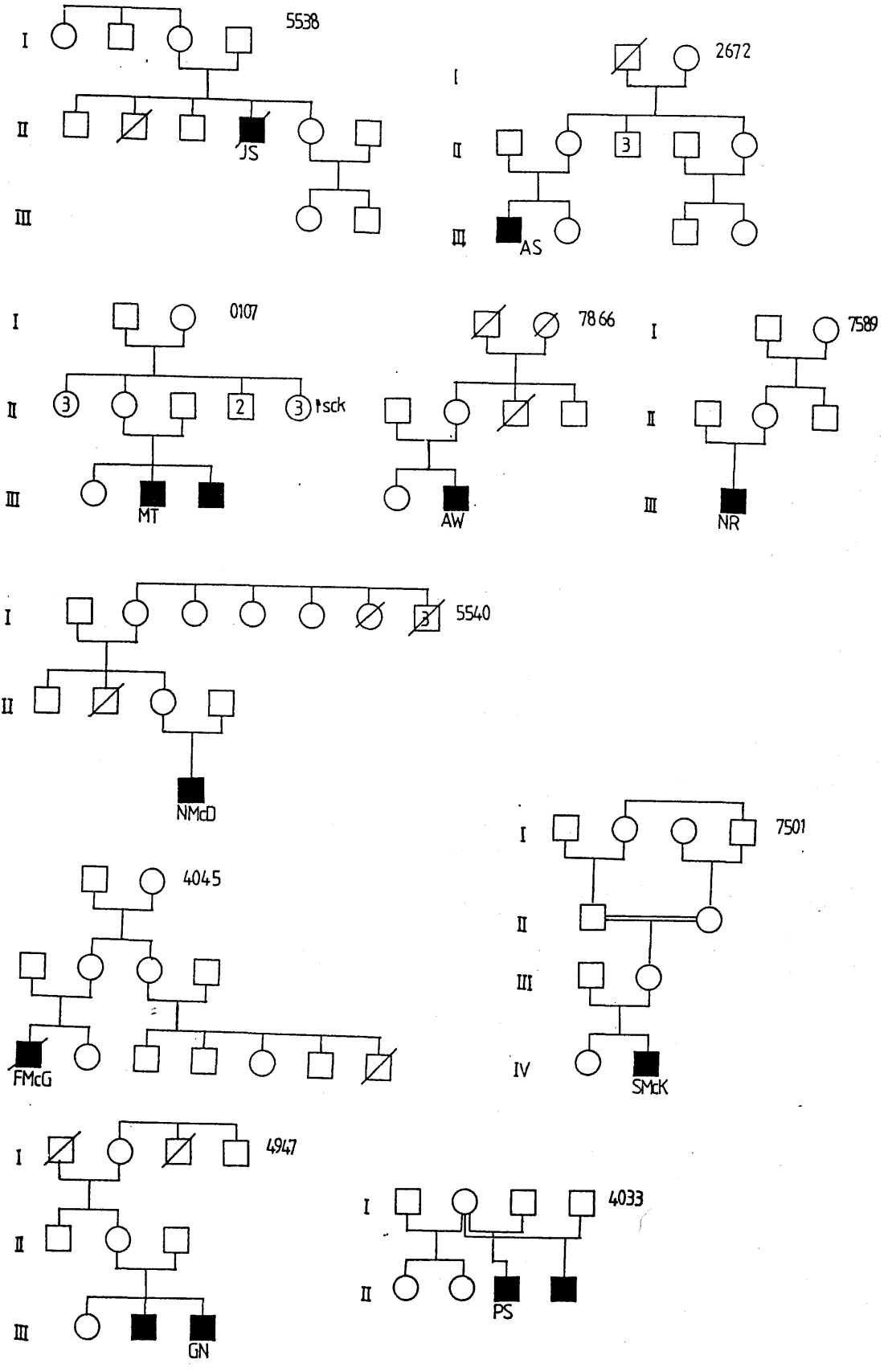


Appendix 4: Deletion studies in males with DMD/BMD using p20, JBir and J66HI. (D); examples of hybridisation of J66HI to EcoRI digests of boys with DMD/BMD (table 7).

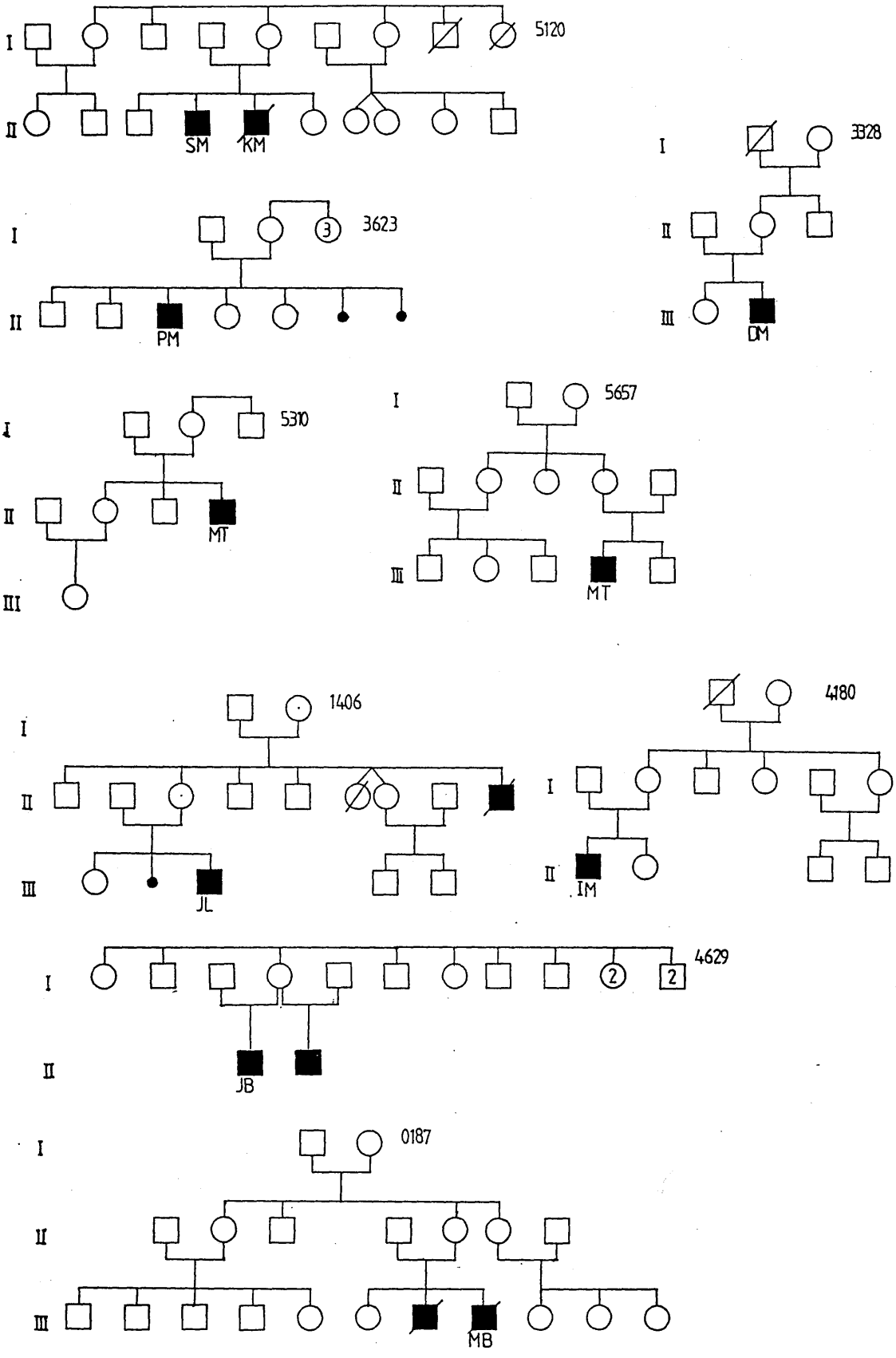
(Boys are identified by their pedigree numbers. For those families in which more than one individual is affected, boys are further identified by their initials. Anomalous fragments are indicated by an arrow).



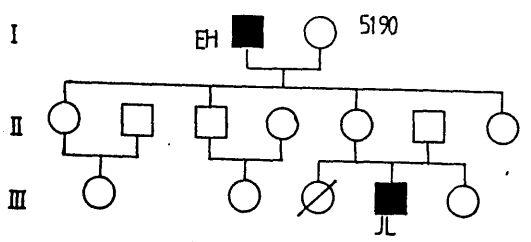
Appendix 5: Pedigrees of individuals with deletions or altered restriction fragment lengths detected by GMGX11, p20, JBir or J66HI. (Pedigrees are shown only for those individuals listed in table 7 and are presented in the same order as table 7. The pedigree for SS and JD (5099) is presented in appendix 2. The family history of MJ is not known). Continued overleaf.

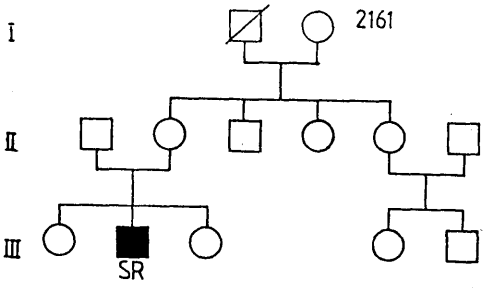


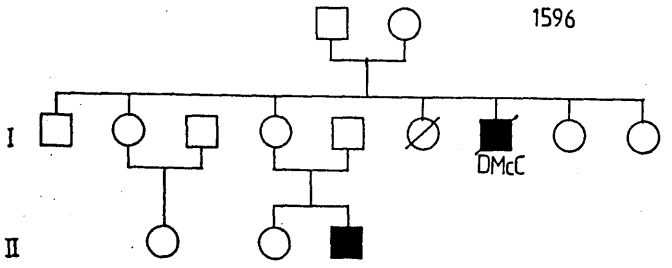
Appendix 5 continued. Continued overleaf.

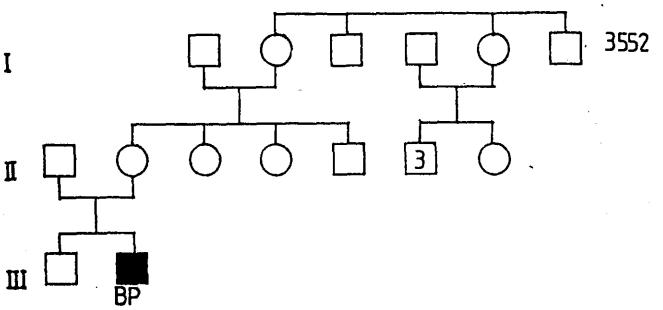


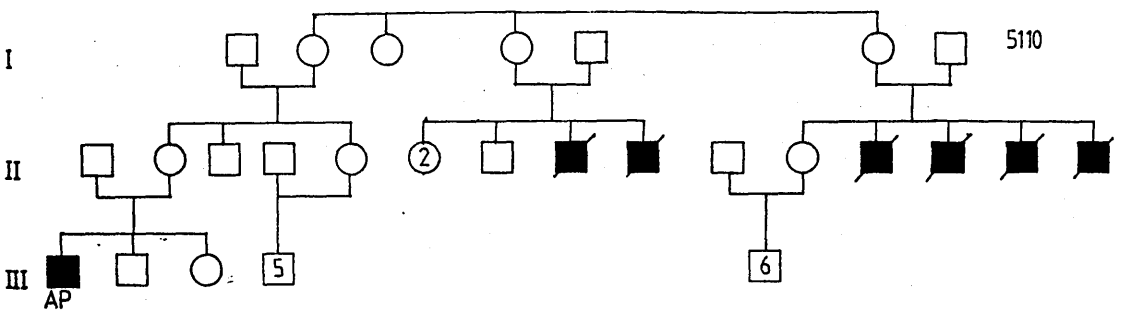
Appendix 5 continued. Continued overleaf.

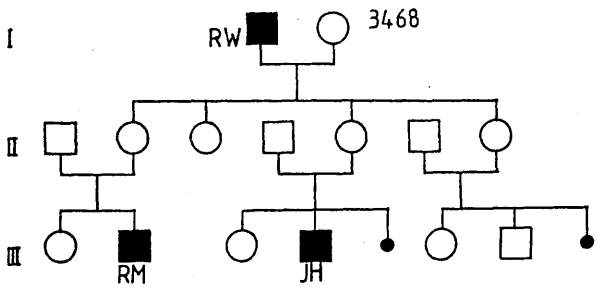


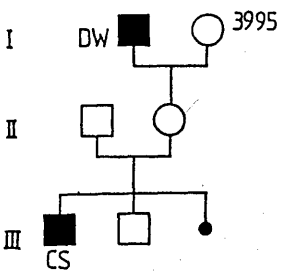




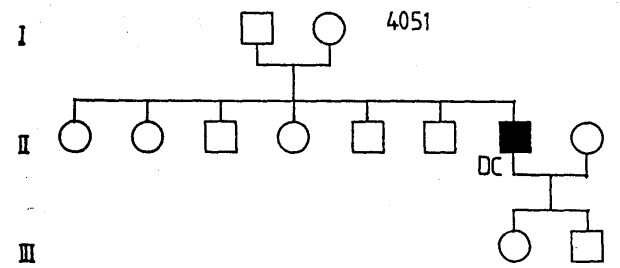


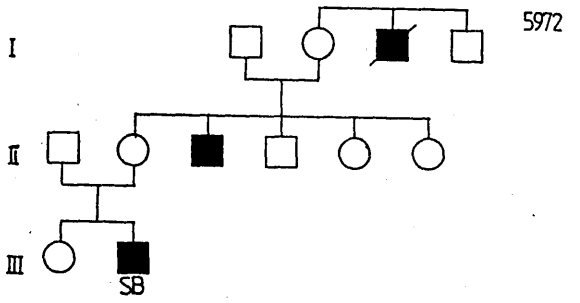


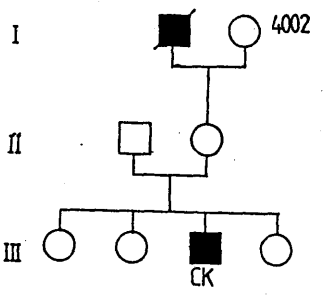


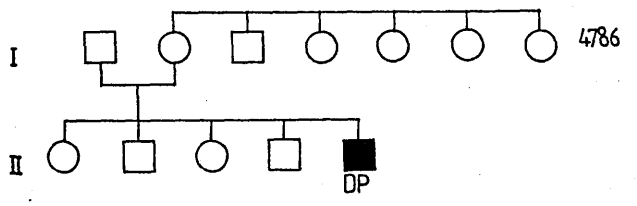


Appendix 5 continued.

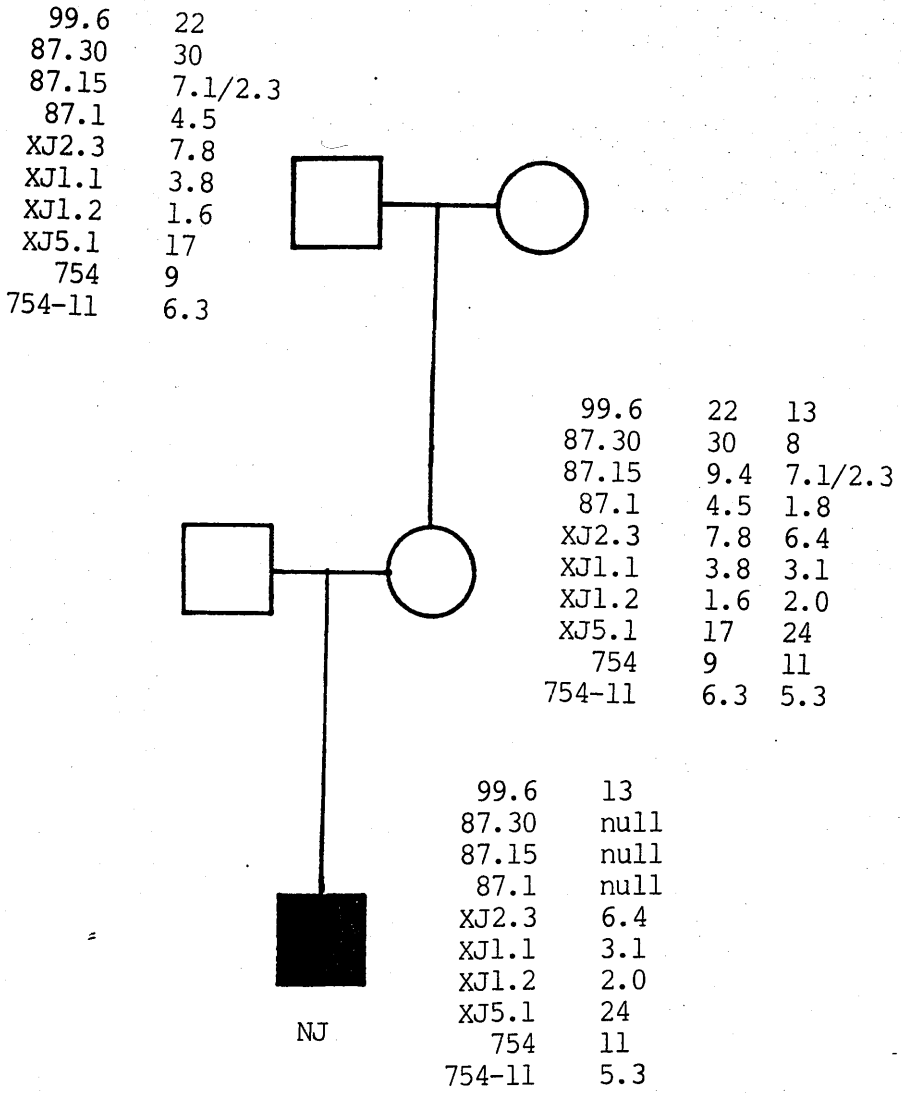








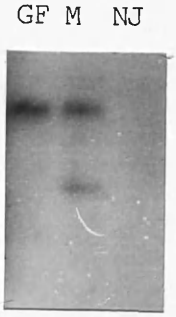
Appendix 5 continued.



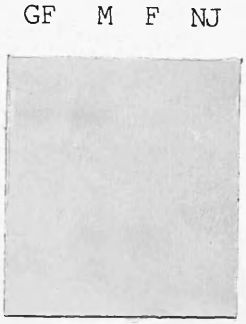
Appendix 6: RFLP analysis of the deletion in NJ (5313).

(A) Schematic representation of informative results. 87.1, pERT87.1; 87.15, pERT87.15; 87.30, pERT87.30. The following digests were used 99.6, PstI; pERT87.30, BglII; pERT87.15, BamHI; pERT87.1, MspI; XJ2.3, TaqI; XJ1.1, TaqI; XJ1.2, BclI; XJ5.1, SphI; 754, PstI; 754-11, BglII.

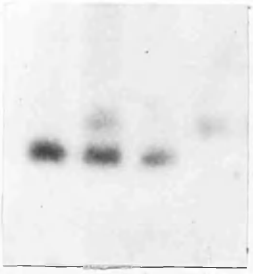
99.6(PstI)



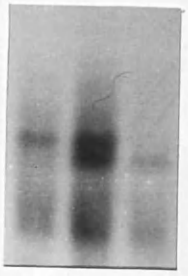
pERT87.30(BglII)



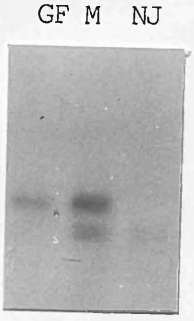
pERT87.1(MspI)



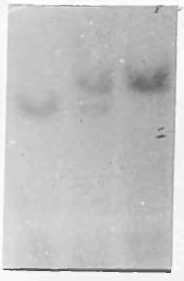
XJ1.2(BclI)



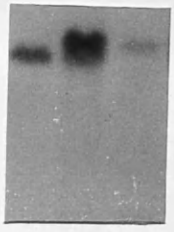
XJ2.3(TaqI)



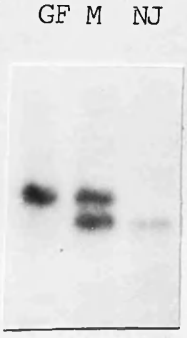
XJ1.1 (TaqI)



XJ5.1(SphI)



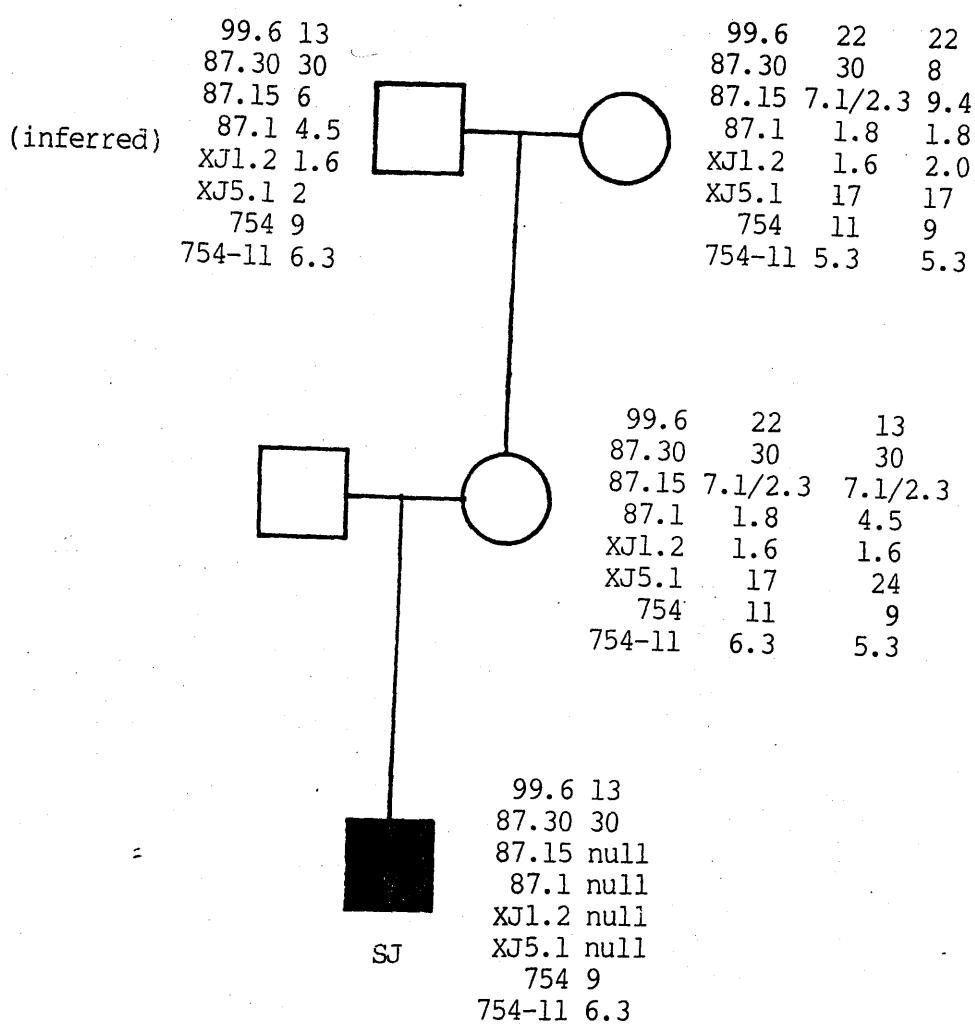
754(PstI)



754-11(BglII)

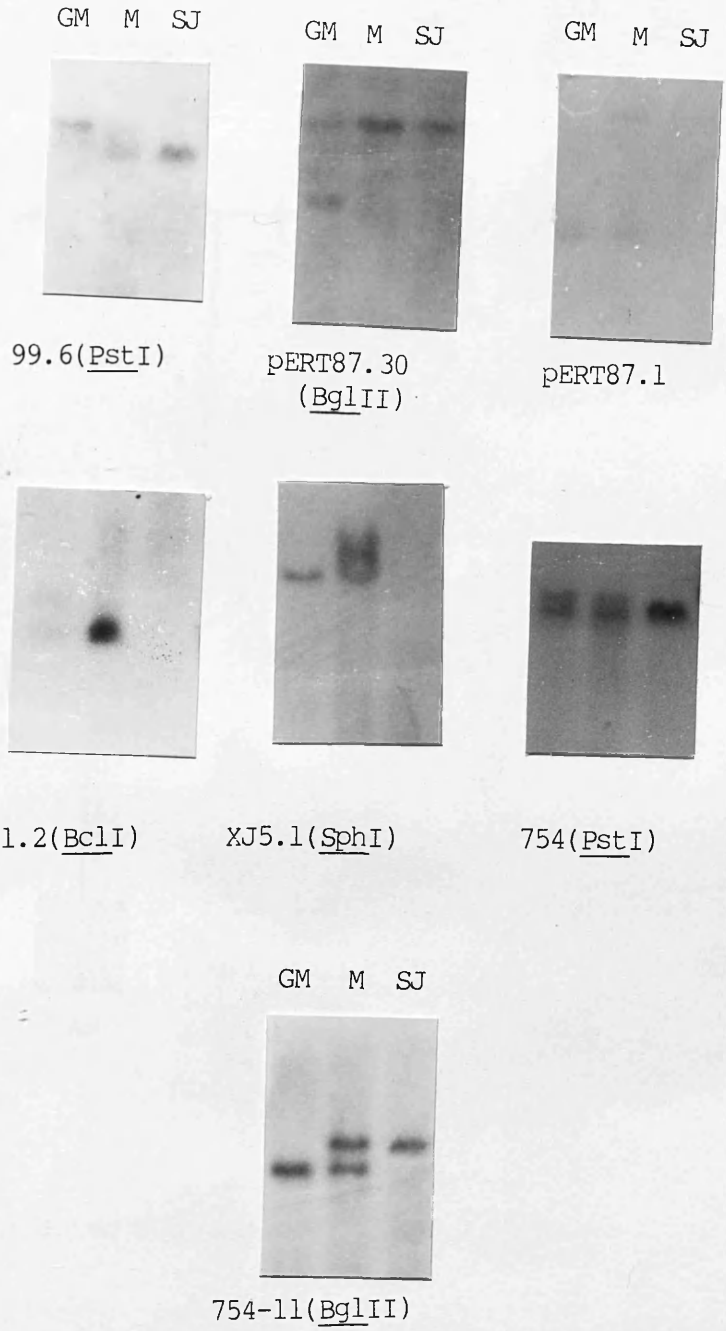
Appendix 6: RFLP analysis of the deletion in NJ (5313).

(B) Autoradiographs of results obtained. (pERT 87.15 results are not included. GF,grandfather; F,father; M,mother).

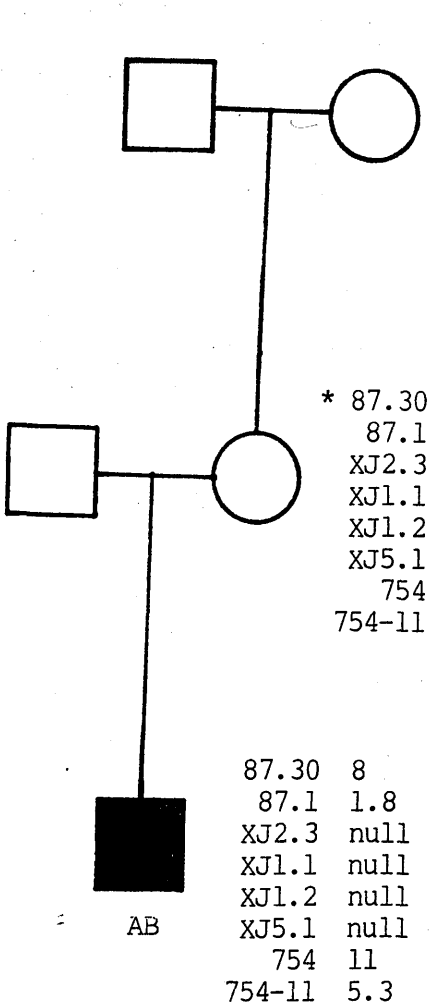


Appendix 7: RFLP analysis of the deletion in SJ (3485).

(A) Schematic representation of informative results. 87.1; pERT87.1; 87.15; pERT87.15; 87.30; pERT87.30. The following digests were used 99.6, PstI; pERT87.30; BglII; pERT87.15; BamHI; pERT87.1, MspI; XJ1.2, BclI; XJ5.1; SphI; 754; PstI; 754-11; BglII.



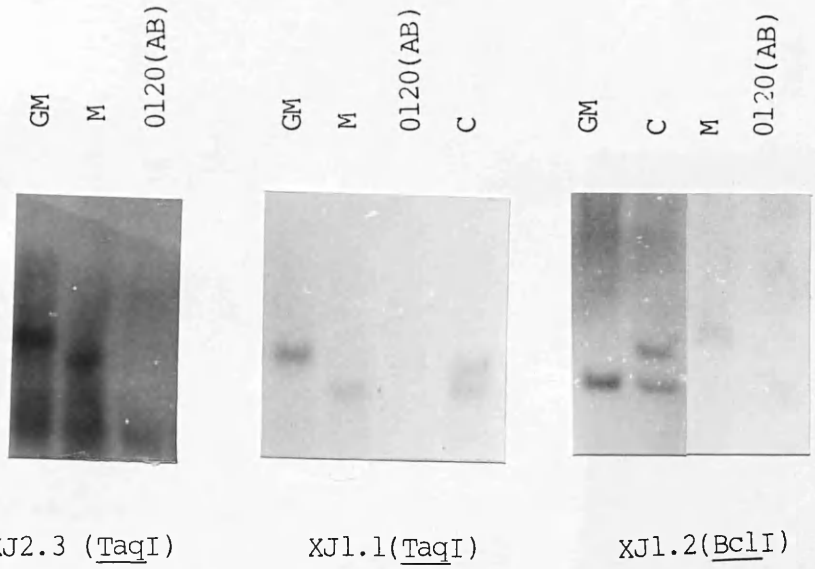
Appendix 7: RFLP analysis of the deletion in SJ (3485). (B) Autoradiographs of results obtained (pERT 87.15 results are not included. GM,grandmother; M,mother).



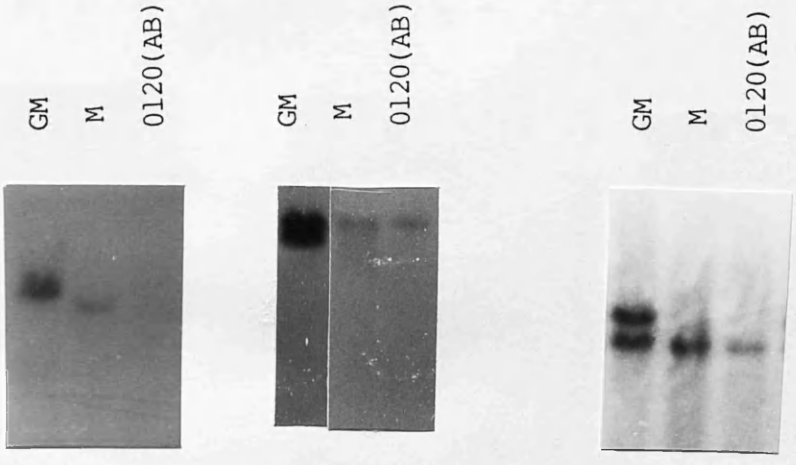
(* phase inferred from AB)

Appendix 8: RFLP analysis of the deletion in AB (0120).

(A) Schematic representation of informative results. 87.1, pERT87.1; 87.15, pERT87.15; 87.30, pERT87.30. The following digests were used pERT87.30, BglII; pERT87.1, MspI; XJ2.3, TaqI; XJ1.1, TaqI; XJ1.2, BclI; XJ5.1, SphI; 754, PstI; 754-11, BglII.



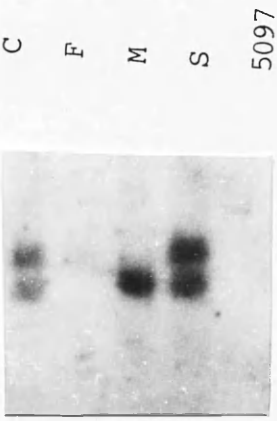
XJ2.3 (TaqI) XJ1.1(TaqI) XJ1.2(BclI)



XJ5.1(SphI) 754(PstI) 754-11(BglII)

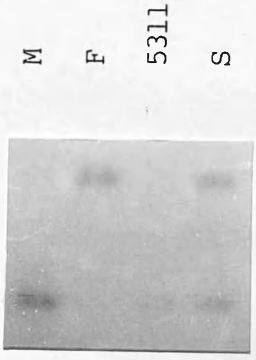
Appendix 8: RFLP analysis of the deletion in AB (0120).

(B) Autoradiographs of results obtained (pERT 87.1 results are not included. GM,grandmother; M,mother; C,control female).

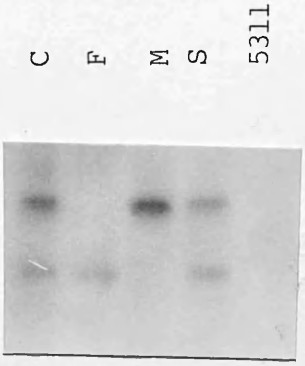


(A) XJ1.2(BclI)

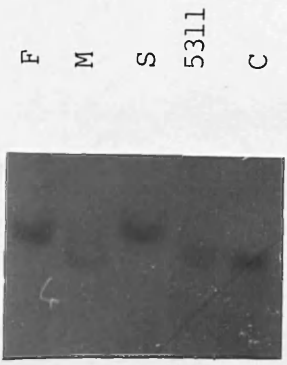
(B) p20(MspI)



(C) pERT87-1(MspI)



pERT87.30(BglII)



XJ5.1(SphI)

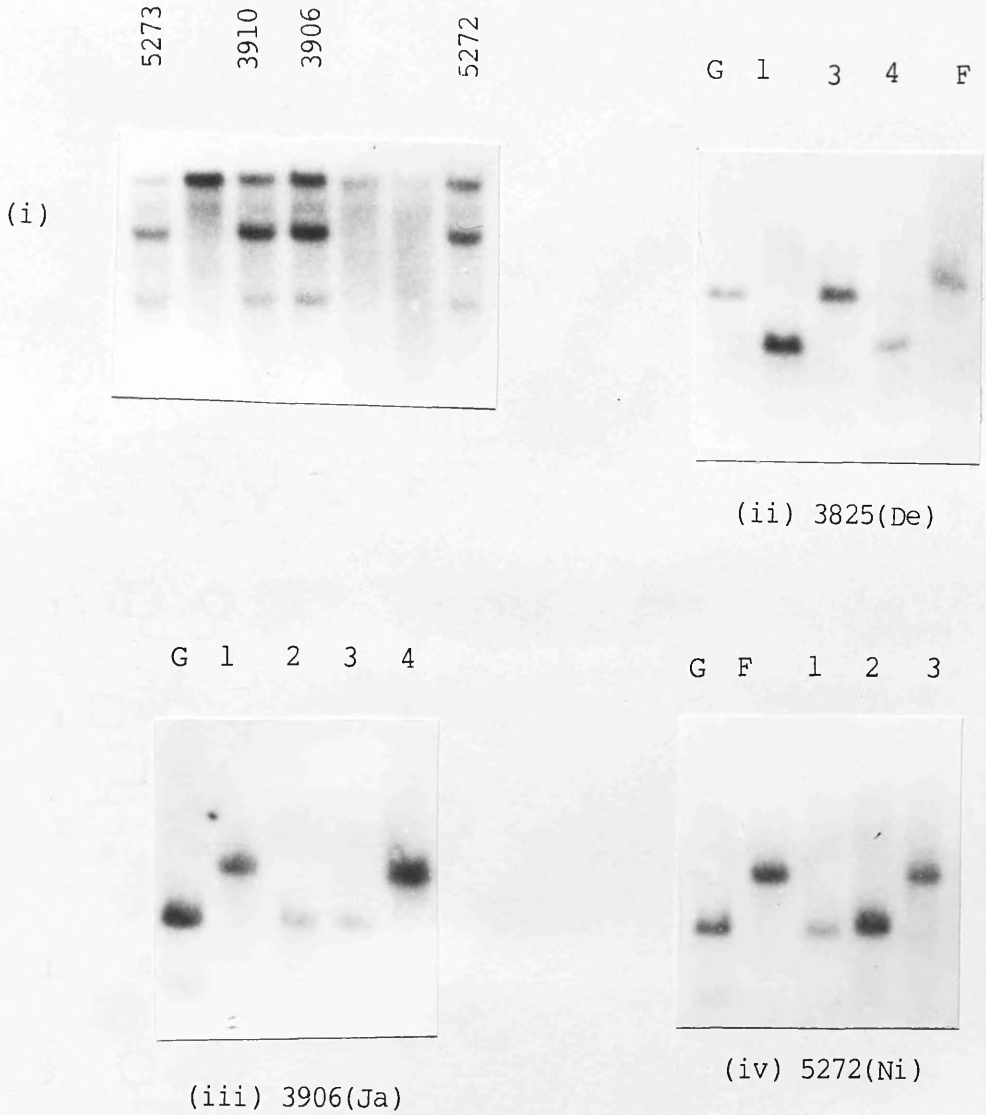
Appendix 9: RFLP results for the sisters of JB (5097), PE (5311) and AW (7866).

(A) XJ1.2 (BclI) results for the sister of JB (5097).

(B) p20 (MspI) results for the sister of AW (7866).

(C) pERT87.1 (MspI), pERT 87.30 (BglII) and XJ5.1 (SphI) results for the sister of PE (5311).

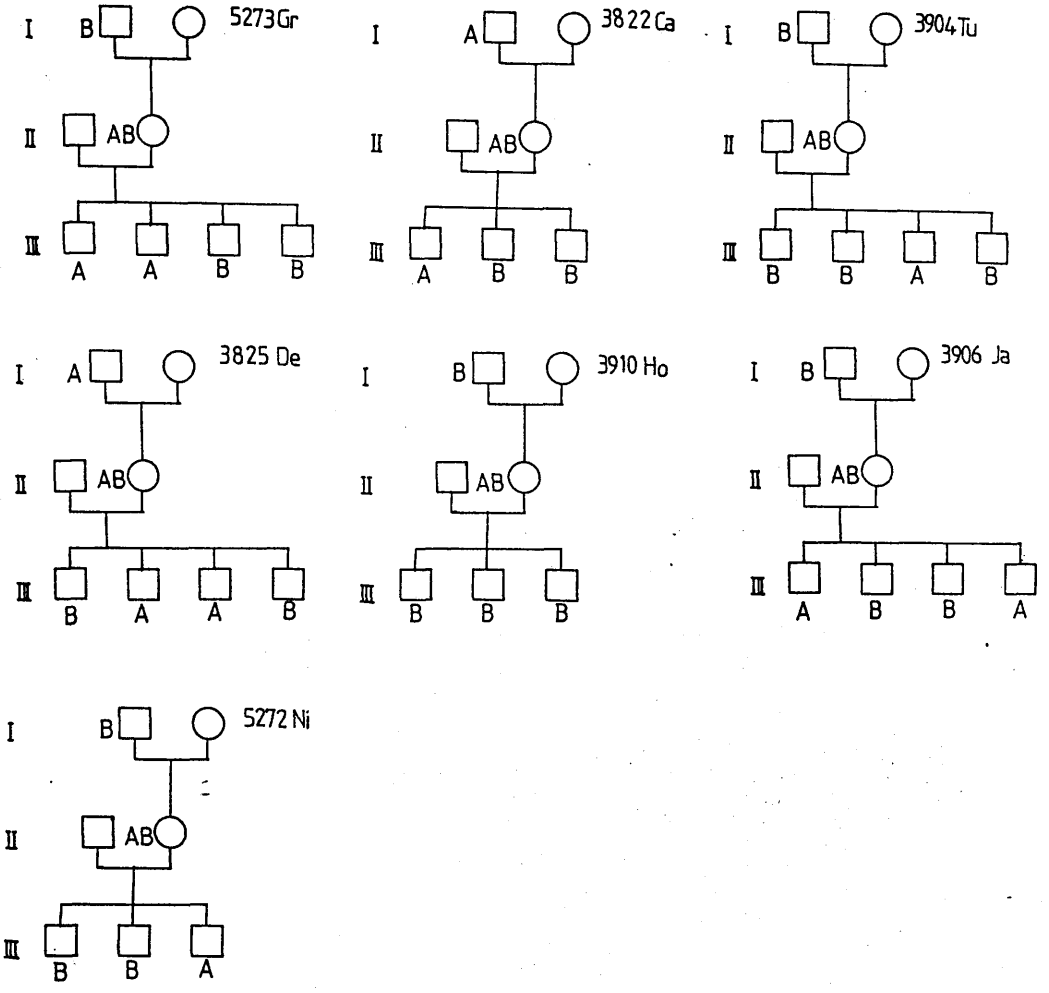
(C,female control; F,father; M,mother; S,sister).



Appendix 10: Results obtained with GMGX9 in TDH families and six Scottish pedigrees segregating for XLI.

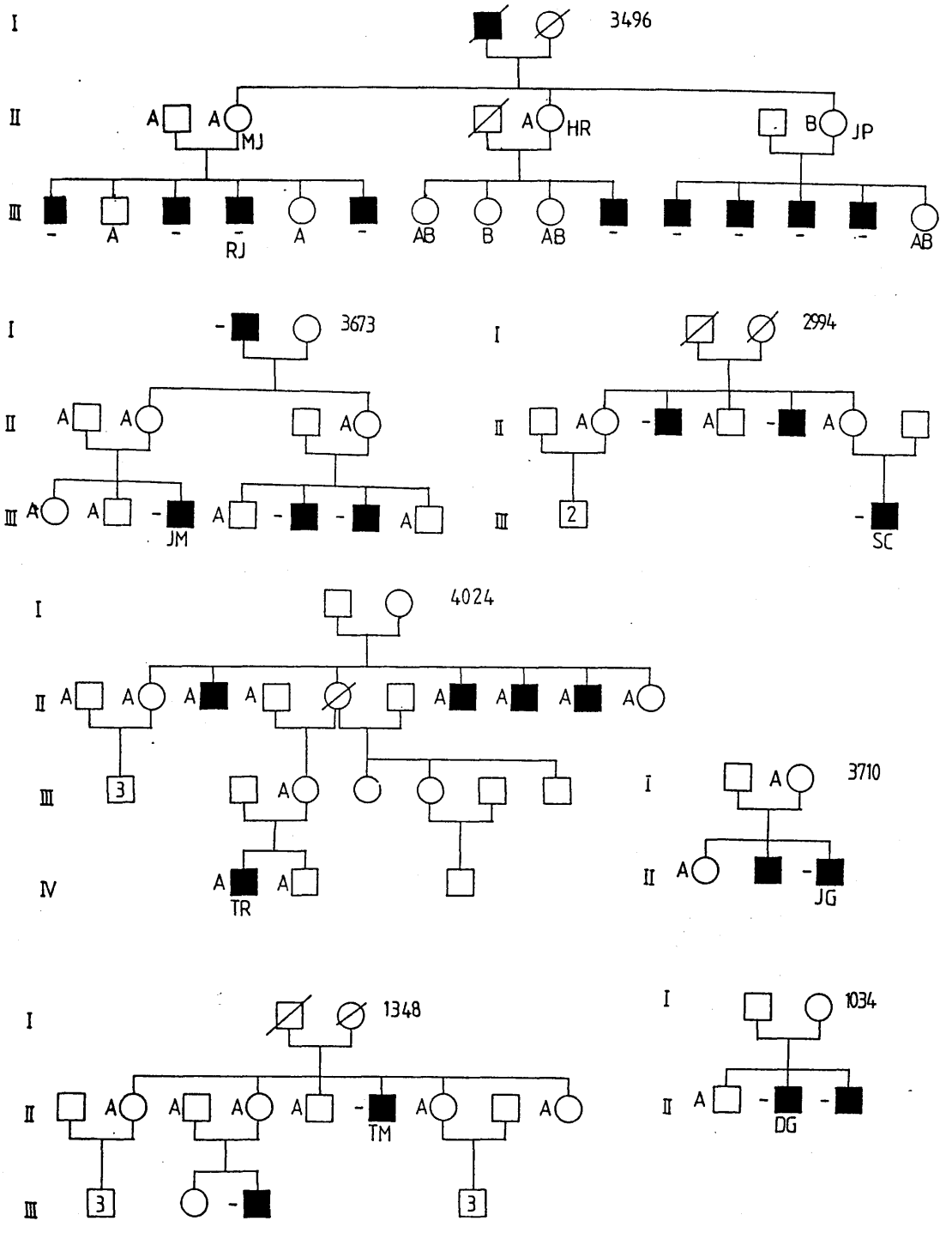
(A) (i) Hybridisation of GMGX9 to mothers from TDH families. (Heterozygous individuals are identified by their pedigree numbers).(ii) Hybridisation of GMGX9 to family 3825 (De). (iii) Hybridisation of GMGX9 to family 3906 (Ja). (iv) Hybridisation of GMGX9 to family 5272 (Ni).

(G,grandfather; F,father; 1-4, first to fourth sons respectively. The result for the second son in family 3825 (De) is not included).



Appendix 10: Results obtained with GMGX9 in TDH families and six Scottish pedigrees segregating for XLI.

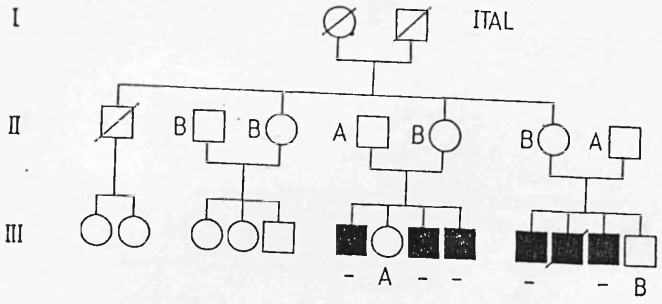
(B) Schematic illustration of results with GMGX9 in TDH families informative for GMGX9. ("A",4kb allele; "B",2.5/1.5kb allele).



Appendix 10: Results obtained with GMGX9 in TDH families and six Scottish pedigrees segregating for XLI.

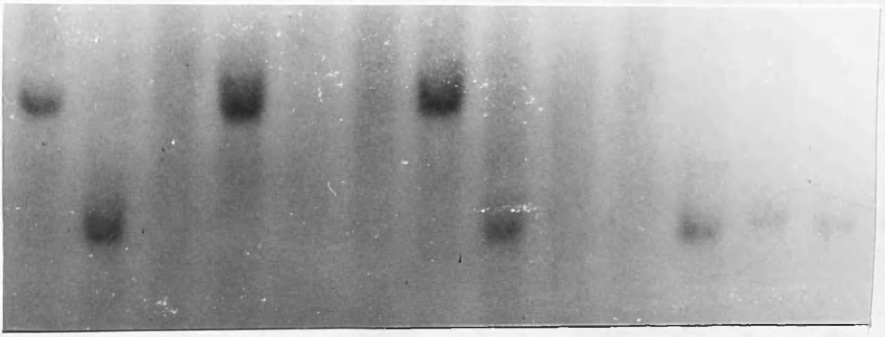
(C) Schematic illustration of results with GMGX9 in six of the Scottish families segregating for X-linked ichthyosis. ("A";4kb allele; "B",2.5/1.5kb allele; "-",deletion).

A



B

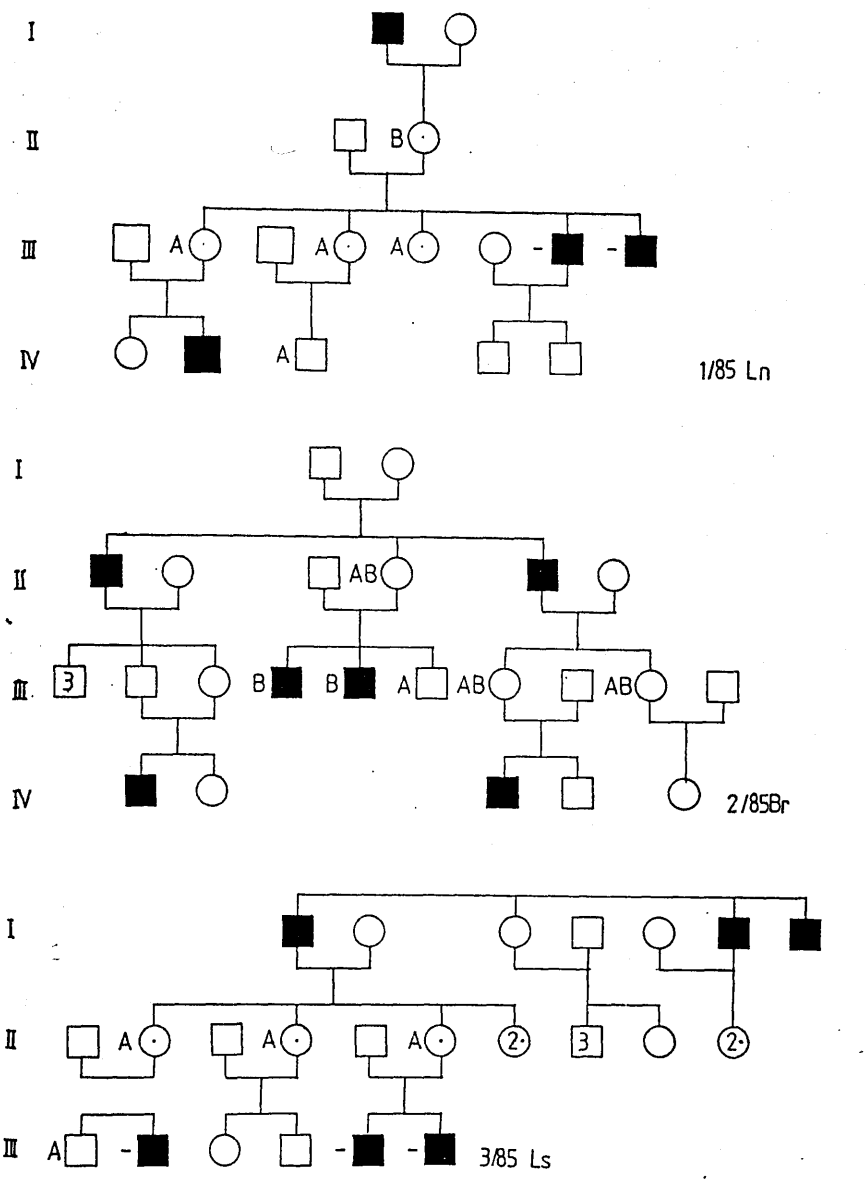
II_4 II_5 III_6 III_7 III_8 III_9 II_7 II_6 III_{10} III_{12} III_{13} II_2 II_3



Appendix 11: Hybridisation of GMGX9 to the Italian family (ITAL).

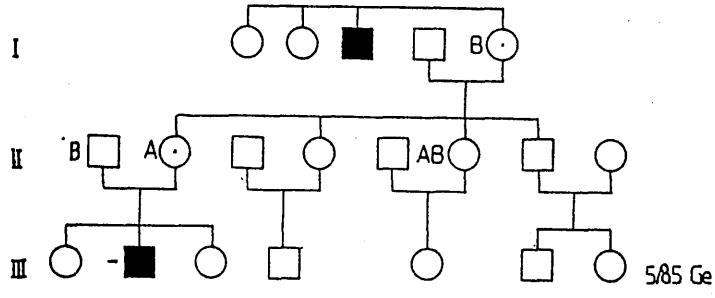
(A) Schematic representation of the results obtained. ("A",4kb allele; "B",2.5/1.5kb allele; "-",deletion).

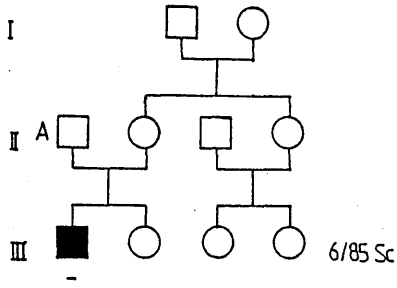
(B) Autoradiograph of hybridisation with GMGX9. (This blot was provided by A. Ballabio.)

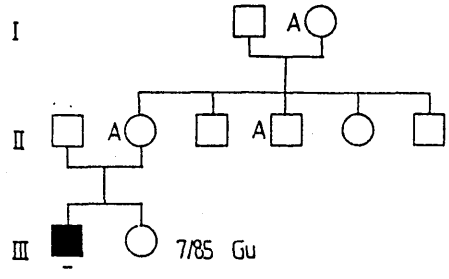


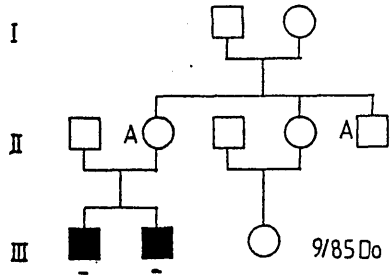
Appendix 12: Deletion analysis with GMGX9 in German and Dutch families segregating for XLI.

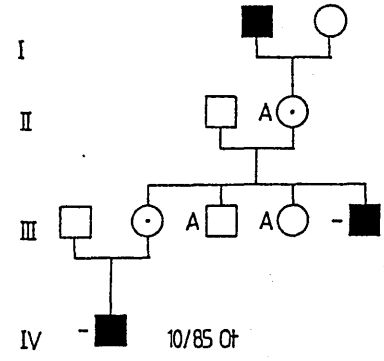
(A) Schematic representation of the segregation of GMGX9 in German families.("A",4kb allele; "B",2.5/1.5kb allele; "-",deletion). Continued overleaf.

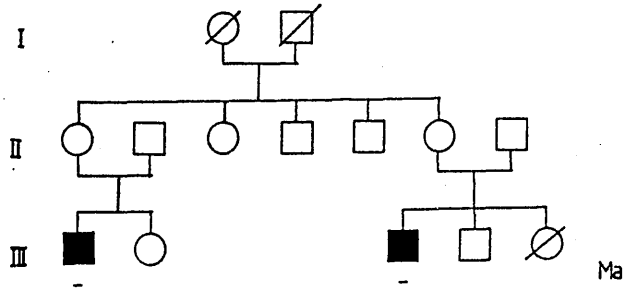




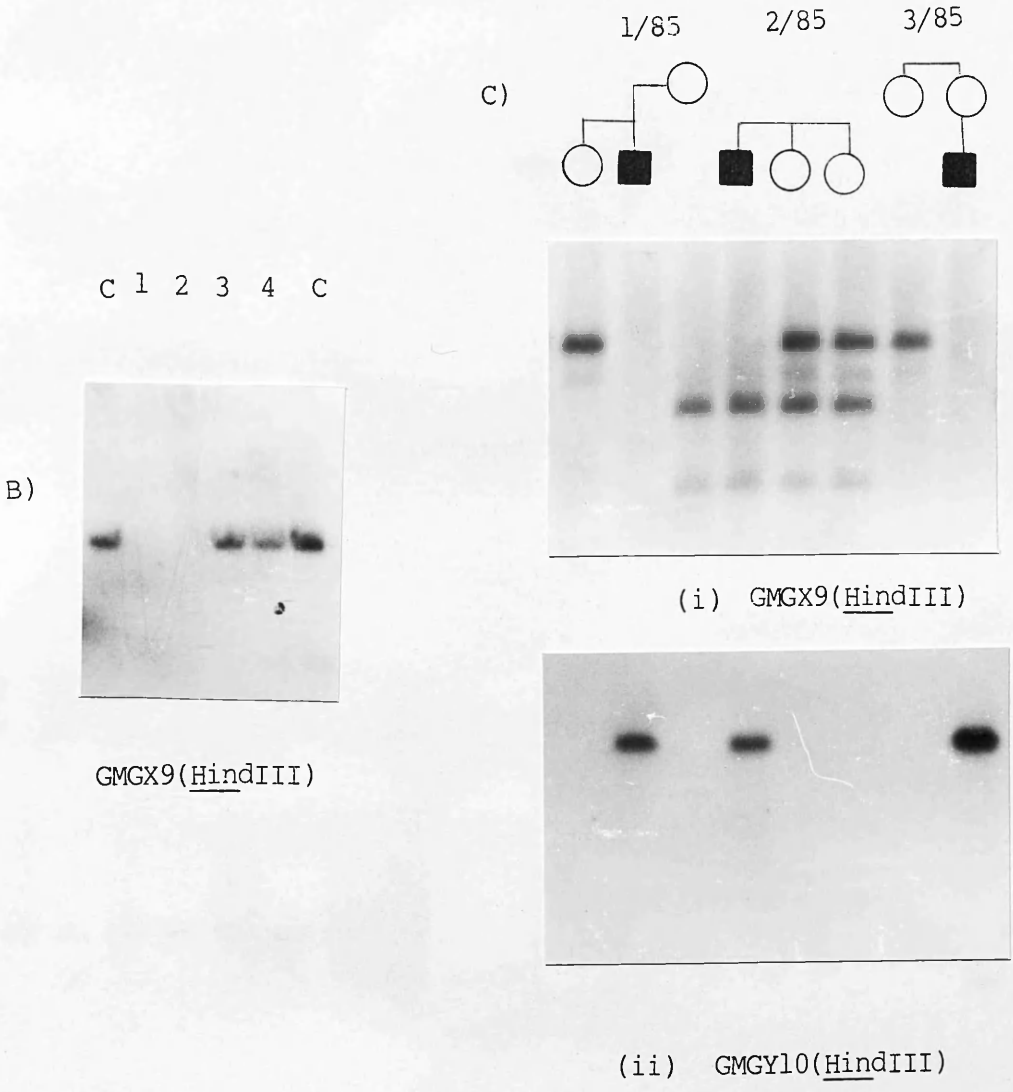








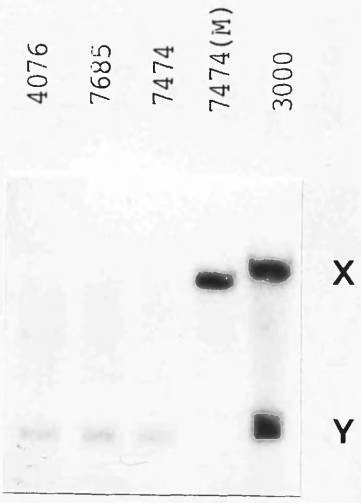
Appendix 12A continued.



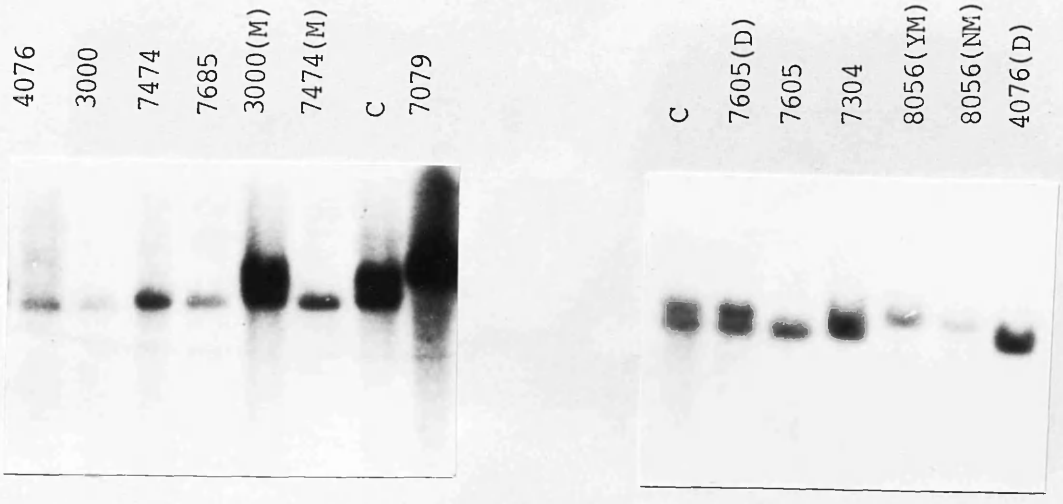
Appendix 12: Deletion analysis with GMGX9 in German and Dutch families segregating for XLI.

(B) Deletion analysis with GMGX9 in four Dutch males with XLI. (C; male control).

(C) Hybridisation with GMGX9 and GMGY10 to members of three German families. (i) Hybridisation with GMGX9. (ii) Hybridisation of the same filter with a Y-specific probe GMGY10 (Affara et al, 1986).



(i) STB14(HindIII)

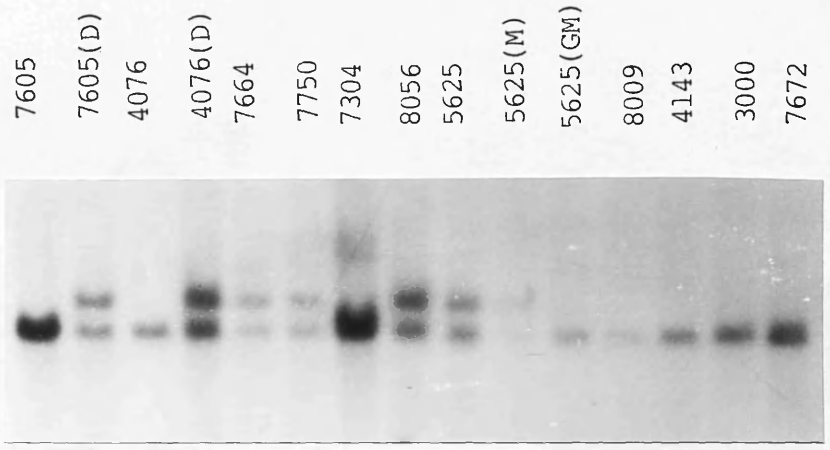


(ii) Dic56(BclI)

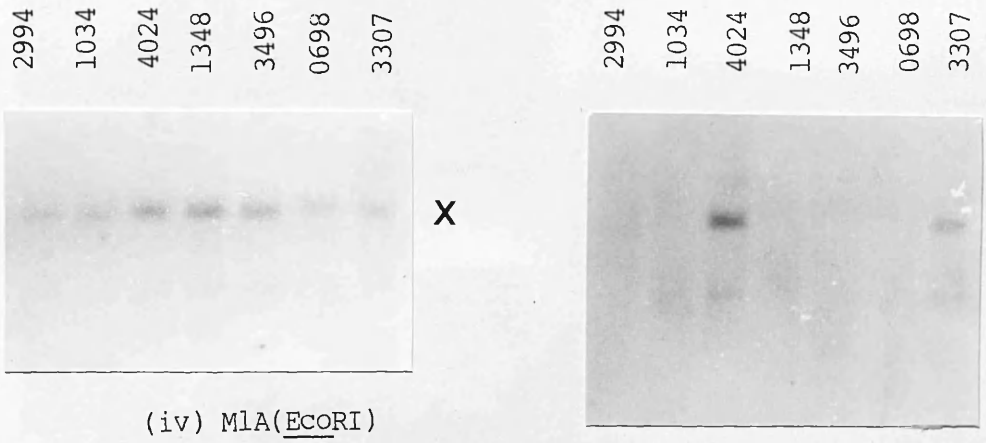
Appendix 13: Examples of hybridisations with distal Xp probes to males with XLI (refer to table 9). Continued overleaf.

(i) Hybridisation of STB14 to affected males and relatives from families 4076, 3000, 7474 and 7685. ((M),mother of affected male).

(ii) Hybridisation of Dic56 to the individuals shown in (i) above and also to a male with an XY translocation (7079) and individuals from families 7605, 7304, 8056 and 4076, which segregate for XLI. (C;female control; (M),mother of affected male; (D),daughter of affected male).



(iii) p19b(TaqI)



(iv) M1A(EcoRI)

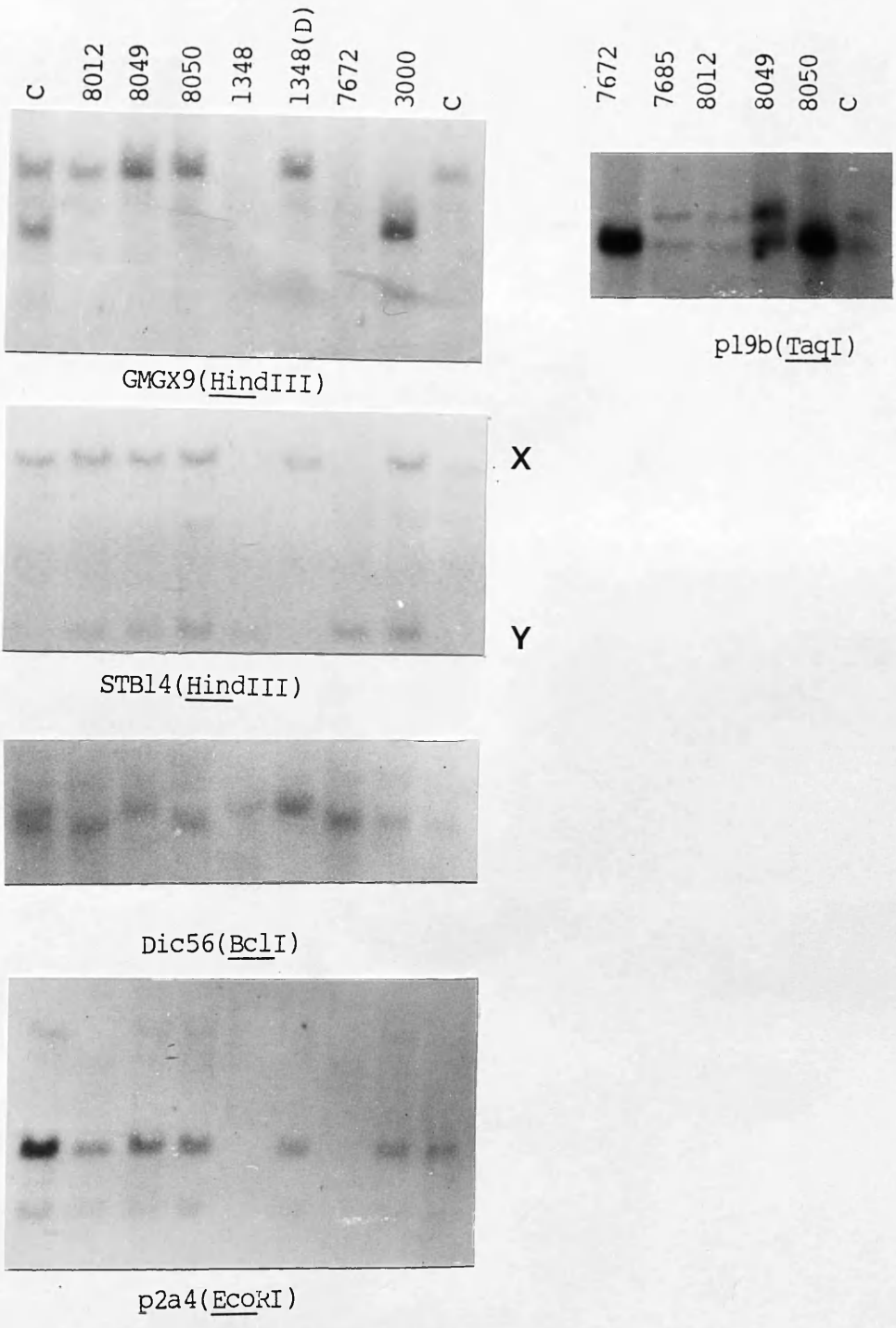
(v) p2a4(EcoRI)

Appendix 13 (continued): Examples of hybridisations with distal Xp probes to males with XLI (refer to table 9).

(iii) Hybridisation of p19b to affected males from families 7605, 4076, 7664, 7750, 7304, 8056, 5626, 8009, 4143, 3000. (C, female control; (M),mother of affected male; (GM),grandmother of affected male; (D),daughter of affected male)

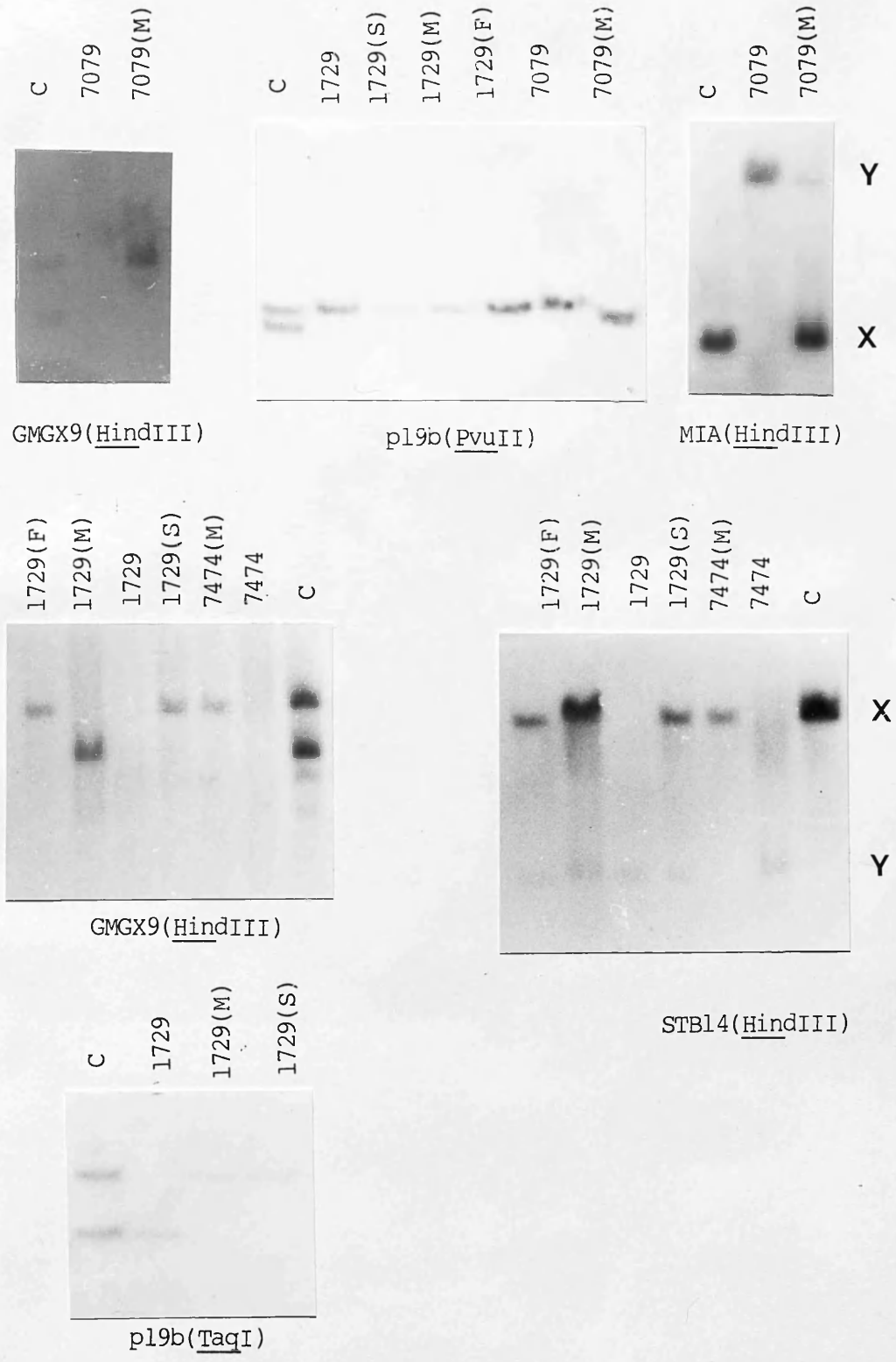
(iv) Hybridisation of M1A to affected males from families 2994, 1034, 4024, 1348, 3496, 0698 and 3307.

(v) Hybridisation of p2a4 to the affected males indicated in (iv) above).



Appendix 14: Deletion analysis with distal Xp probes in three males, SS (8012), JG (8049) and RR (8050), with Kallman's syndrome.

(C,female control; (D),daughter of affected male. Other individuals from families segregating for XLI are also included).



Appendix 15: Deletion analysis with distal Xp probes in two males, AM (1729) and JH (7079) with XY translocations.

(C,female control; (M),mother; (F),father; (S),sister. A male with XLI from family 7474 is also included).

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