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MOLECULAR BASIS OF FRAGILE X SYNDROME

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DECLARATION

The work described in this thesis was performed by the candidate except where acknowledged. No material in this thesis has been presented for any other degree or diploma except for publication No. 1 in Appendix II which formed part of the PhD thesis of Dr. G. K. Suthers, University of Adelaide, 1990. The author consents to the thesis being made available for photocopying and loan if accepted for the award of PhD degree.

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SUMMARY

Fragile X syndrome is the most common cause of mental retardation and is one of the most genetically complicated disorders in human beings. Segregation analysis revealed many unique aspects of the genetics of this syndrome but left them unanswered. Molecular cloning of the mutation is an essential step towards resolving the mystery.

Positional cloning of the fragile X (FRAXA) was applied since nothing was known about the gene products. The availability of DNA probes detecting loci near FRAXA and the newly developed yeast artificial chromosome (YAC) cloning system provided the bases for positional cloning of the fragile X. Four DNA probes were used to screen for YAC clones from an Xq YAC library constructed from a human/rodent somatic hybrid containing human Xq24-28 expressing the fragile X. All nine positive YAC clones isolated were studied either by the candidate (Chapter 2) or by coworkers. YAC XTY-26 was identified to be an important clone because it was shown by in situ hybridization to span the fragile X and it was also found to contain DNA probes which flanked FRAXA (Chapter 4).

Systematic cloning of FRAXA was carried out as a cooperative project (Chapter 5). A lambda library of XTY-26 was constructed and was screened for clones containing human DNA sequences. A lambda contig was constructed to cover the region between two closest markers which flanked the fragile X. The fragile site was defined to a 15 kb region by in situ hybridization, using lambda clones in this contig, to the metaphases expressing the fragile X. Subclone 5, a lambda clone mapping within the 15 kb region, identified the breakpoints of two somatic cell hybrids, which were constructed to have breaks at the fragile site, to be within a common 5 kb EcoRI fragment. Subclone 5 also identified a variable region in affected males in which the normal 5 kb EcoRI fragment was replaced by a larger band(s) of varying size in different patients. The variable region was further mapped to a 1 kb PstI fragment. A DNA probe, pfxa3, isolated from the 1 kb PstI fragment, was shown to be powerful in identifying the

fragile X genotype. In fragile X syndrome families, individuals carrying the fragile X mutation have the pfxa3 band of different sizes, indicating that the variable region is unstable during meiosis (Chapter 5).

Southern analysis of over 400 DNA samples of 49 fragile X families with probe pfxa3 revealed many novel genetic characteristics of the unstable element and provided an explanation for many puzzling aspects of fragile X syndrome (Chapter 6). Firstly, the size of the unstable element was found to be correlated with mental status. Secondly, the unstable element was observed to increase in size from generation to generation when the mutation was transmitted by a female, but not when transmitted by a male. The size of the unstable element in carrier mothers correlated with the size of the unstable element in their offspring. These observations provided an explanation for the Sherman Paradox, a phenomenon of increasing penetrance in the successive generations. Thirdly, a correlation was identified between the size of the unstable element and the methylation status of the SacII site at the fragile X associated CpG island. Fourthly, multiple pfxa3 hybridization bands were observed in lymphocyte DNA in a proportion of affected individuals, and pfxa3 bands of different sizes were observed in DNAs of different cell lines from the same individual. This indicated somatic variation of the unstable region. Finally, all fragile X syndrome patients including apparently isolated cases were identified to be familial.

CHAPTER 1

LITERATURE REVIEW

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1.1. SUMMARY

Fragile X syndrome is a distinct form of X-linked mental retardation associated with a cytogenetically inducible fragile site at Xq27.3. With a prevalence of 0.6/1000 in males and 0.4/1000 in females, it is the most common cause of familial mental retardation. Males with fragile X syndrome usually have moderate to severe mental retardation, express the fragile site at Xq27.3, and often have subtle phenotypic abnormalities, such as macroorchidism, long face and protruding jaw and ears. A proportion of females with the mutation have mental impairment of varying degrees along with the fragile site expression. The fragile X site associated with the syndrome can be detected in almost all affected males and females, only in some female carriers, but seldom in normal male carriers (transmitting males) even under the most appropriate culture conditions. Segregation analysis revealed a very unusual inheritance pattern of the fragile X syndrome. This includes the existence of transmitting males, a high frequency of mental impairment in female heterozygotes, penetrance determined by the sex and phenotype of the carrier parents, and a lack of new mutation in affected individuals. Complicated by these unique genetics, carrier detection and prenatal diagnosis based on the cytogenetic detection of the fragile site was not completely reliable, and genetic counselling was difficult. Many hypotheses were proposed to explain some aspects of the unique genetics of the disorder, but none of them was able to provide an explanation for all aspects of the syndrome. In recent years, many DNA markers have been isolated and mapped around the fragile X region. Linkage analysis with these DNA probes not only confirmed the unusual inheritance pattern, but also provided a tool for carrier detection, prenatal diagnosis and genetic counselling. More importantly, the linkage map of the fragile X region paved the way towards the molecular cloning of the fragile X.

1.2. INTRODUCTION

Fragile X syndrome is the most common cause of familial mental retardation. It has been attracting the attention of many professionals in medical specialty areas because of its high prevalence as well as its unique or enigmatic inheritance and expression patterns. Research into the fragile X syndrome spreads to various areas, including symptomatology, dermatoglyphics, neurology, neuropsychology, epidemiology, cytogenetics, molecular biology, genetic counselling, pharmacotherapy, behavioral problems and many more.

The literature review in this chapter will cover the major issues in terms of understanding the fragile X syndrome with emphasis on its molecular biological aspects. Included will be: 1) how the fragile X syndrome was recognized as the most common cause of familial mental retardation; 2) cytogenetics of the fragile X; 3) the unique segregation pattern of the syndrome; 4) hypotheses proposed to explain the enigma of the syndrome; 5) linkage analysis of the fragile X locus (FRAXA); 6) positional cloning and the strategy of this project.

1.3. RECOGNITION OF THE FRAGILE X SYNDROME

In mentally retarded populations, a male excess of 25% had long been recorded, but genetic elements had never been considered as the cause of the male excess. Not until the early seventies, was it proposed by Lehrke (1974) for the first time that X-linked genes might account for at least some of the male excess. This hypothesis was supported by observations that in some families mental retardation segregated in an X-linked manner (Martin and Bell, 1943; Allan et al., 1944; Dunn et al., 1963; Renpenning et al., 1962).

The association between X-linked mental retardation and a marker X chromosome was first reported by Lubs (1969) who observed a marker X chromosome in all four mentally retarded males and in one of the two obligate carrier mothers in a family with X-linked mental retardation. This finding was made before chromosome banding techniques were established. The marker chromosome was presumed to be the X chromosome by its morphology and more importantly by its apparent association with an X-linked disorder. The morphology of this marker X chromosome

was described as a secondary constriction near the end of the long arm giving the appearance of large satellites (Lubs, 1969).

However, Lubs' observation had been neglected for about seven years until similar findings were reported by other researchers (Giraud et al., 1976; Harvey et al., 1977). In the study of the relationship between chromosome structural variations and clinical abnormalities, Giraud et al. (1976) observed a constriction on the long arm of an X chromosome, similar to that observed by Lubs, in five unrelated mentally retarded boys and one girl. Of the five boys, one had family history of X-linked mental retardation and another had a mother who also expressed the constriction at Xq27. Similarly, Harvey et al. (1977) reported four families with X-linked mental retardation associated with a secondary constriction at the distal end of Xq27. The affected boys reported in both papers were found to have very subtle phenotypic abnormalities. With chromosome banding techniques, the secondary constriction (referred to as fragile site) on the X chromosome was located at Xq27 (Giraud et al., 1976). From these two reports, the association between the X-linked mental retardation and the marker X chromosome was established.

The reason why it took so long to confirm Lubs' observation was uncovered by Sutherland, who discovered that expression of the fragile site required special tissue culture medium conditions (Sutherland, 1977a). The fragile site at Xq27 associated with mental retardation, known as the fragile X, as well as a number of autosomal fragile sites were only expressed in cells after growth in tissue culture medium TC199, but not after growth in other commercial culture media such as RPMI1640, Ham's F10, Eagle's (basal) and CMRL1969 (Sutherland, 1977a). However, around that time many laboratories had switched from the old fashioned culture medium TC199 to other newly developed culture media. Soon after, a further discovery was made by Sutherland (1979a) that the effectiveness of TC199 in inducing fragile site expression was because of its relative lack of folic acid and thymidine. In other words, expression of the fragile site could be inhibited by folic acid and thymidine.

Since the culture medium requirement for fragile site expression was discovered, the fragile X has been consistently observed by many researchers in mentally retarded males and females,

especially in those with family history of mental retardation (Sutherland, 1977b; Turner et al., 1978; Sutherland, 1979b; Jacobs et al., 1980; Turner et al., 1980a; 1980b; Webb et al., 1981b). In the early papers the boys with mental retardation were reported to have very subtle physical abnormalities (Harvey et al., 1977). Careful examination of those affected individuals revealed some characteristic clinical features of the disorder, such as macroorchidism and facial abnormalities (Turner et al., 1978; Turner et al., 1980a). Furthermore, the fragile X associated mental retardation (fragile X syndrome) was estimated to contribute to 1/3 of the X-linked mental retardation (Turner et al., 1978). By the early 80's, it was generally accepted that fragile X syndrome was a specific form of X-linked mental retardation characterized by the presence of a fragile site at Xq27, macroorchidism and facial abnormalities, and that it was the major cause of X-linked mental retardation (Gerald, 1980). The family with X-linked mental retardation, originally reported by Martin and Bell (1943), was reinvestigated to demonstrate the fragile X. Of the seven affected males, five were found to show the fragile X site (Richards et al.,1981). Therefore, fragile X syndrome is also called Martin-Bell syndrome.

1.4. THE FRAGILE SITE AT Xq27

Fragile X syndrome differs from all other monogenic diseases studied, in that its abnormal phenotype is associated with the presence of an inducible cytogenetic marker, the fragile site at Xq27. This fragile site at Xq27 is the only fragile site that is associated with an abnormal phenotype (Sutherland, 1979b). In 1979, Sutherland (1979a) defined a fragile site as: (1) a non-staining gap of variable width which usually involves both chromatids, (2) the site is always at exactly the same point on the chromosome in cells examined from any individual patient or kindred, (3) the site is inherited in a Mendelian codominant fashion, and (4) fragility must be evident by the production (under appropriate in vitro conditions) of acentric fragments, deleted chromosomes, triradial figures, and the like. Because the secondary constriction on the X chromosome fulfilled the above criteria, it was then called the fragile X site. FRAXA, the gene

symbol for the fragile X locus, is used to differentiate it from other fragile sites on the X chromosome.

1.4.1. Factors Affecting Fragile Site Expression

The fragile X was classified as a folate sensitive fragile site since its expression was first noticed to be suppressed by folic acid (Sutherland, 1979a). Other factors were soon found to affect expression of the fragile X. The presence of folate antagonists such as methotrexate was shown to induce the expression of the fragile site (Sutherland, 1979a; Sutherland and Hecht, 1985; Hagerman and Silverman, 1991). The inhibiting effect of folic acid, but not that of thymidine, can be negated by addition of 5-fluorodeoxyuridine (FUdR), a potent inhibitor of thymidylate synthetase, to the culture medium (Glover, 1981; Tommerup et al., 1981a, 1981b; Vandamme et al., 1988). The final effect of these factors seems to limit the dTMP pool and, thus, the dTTP pool available for DNA synthesis. Later, it was found that the folate sensitive fragile sites could also be induced by a high level of thymidine, but not by high levels of 5'-bromo-2'-deoxyuridine (BrdU) (Sutherland et al., 1985). It appeared that not only a deficiency in dTMP but also a deficiency in dCTP available for DNA synthesis resulted in fragile X expression. Based on the role of the inhibitors and inducers of the fragile sites on DNA synthesis, the DNA at the folate sensitive fragile sites was proposed to be a section of repetitive polypurine/polypyrimidine rich DNA such as polyd(AG)/polyd (TC) (Sutherland et al., 1985).

1.4.2. Location of the Fragile X

Giraud et al. (1976) were the first to map the fragile X at Xq27 by a chromosome banding technique. With high resolution chromosome banding, the fragile X was further localized at Xq27.3 close to the Xq27-28 interface (Brookwell and Turner, 1983; Krawczun et al., 1985).

1.4.3. The Fragile X under Scanning Electron Microscope (SEM)

Under SEM, two types of fragile X morphology were observed by Harrison et al. (1983). One type of fragile X demonstrated almost complete breakage at the fragile site, producing an isochromatid gap with two fragments detached. The other type showed incomplete breakage at the fragile site, manifesting a chromatid gap at the fragile site with only one fragment completely detached. The fibres traversing the fragile site resembled those seen between the satellites and centromere in D group chromosomes (Harrison et al., 1983). Examination of metaphases in the SEM revealed that the fragile X was consistently located at the lower Xq27 sub-band, Xq27.3. This was consistent with the location of the fragile X under light microscopy (Brookwell and Turner, 1983; Krawczun et al., 1985).

1.4.4. The Fragile X Expression in Lymphocytes

The initial studies of fragile site expression were on cultured lymphocytes. Until now, detection of fragile X in lymphocytes has been the major means of diagnosis of fragile X syndrome. However, the fragile X is never seen in all lymphocytes, a phenomenon still not fully understood. The proportion of lymphocytes expressing the fragile X is larger in male fragile X syndrome patients than in female patients (Sherman et al., 1984; Sutherland and Hecht, 1985). Although fragile X syndrome males always express the fragile X, in fragile X families about 20% of males carrying the fragile X mutation showed neither the fragile site in their lymphocytes nor mental retardation, they are known as transmitting males (Sherman et al., 1984, 1985). Segregation studies showed that only 56% of females with the fragile X mutation could be identified by either having mental retardation of varying degrees, or by the presence of the fragile site at Xq27.3 (Sherman et al., 1984). Generally, affected females usually express the fragile X, but only a few female carriers (< 4%) express it (Turner and Jacobs 1984; Sherman et al., 1984; Sutherland and Hecht, 1985).

1.4.5. Prenatal Diagnosis of the Fragile X

Presence of the fragile X in cultured fibroblasts was considered a prerequisite for prenatal diagnosis of fragile X syndrome. However, the culture medium (TC199) initially used to induce fragile X in lymphocytes did not induce the fragile X in fibroblasts (Sutherland, 1977b; Sutherland, 1979a).

The fragile X was later successfully induced in fibroblasts by adding FUdR or methotrexate to culture medium (Tommerup et al., 1981a; Glover, 1981; Fonatsch, 1981; Mattei et al., 1981a; Steinbach et al., 1983). Since then, prenatal diagnosis of fragile X chromosome has become possible. The first case of prenatal diagnosis was performed by Jenkins et al. (1981). By adding FUdR to the culture medium, they identified the fragile X in 20% of amniocytes from an at risk male fetus. By 1985, at least 147 cases had been studied world-wide for prenatal diagnosis of the fragile X syndrome (Turner et al., 1986a). At the second International Workshop on the fragile X and X-linked mental retardation in 1985, the cytogenetic experience of prenatal detection of the fragile X chromosome in amniocytes was well summarized in three papers (Tommerup et al., 1986; Shapiro et al., 1986; Jenkins et al., 1986). These authors confirmed their prenatal diagnoses by follow-up of cases either in tissues from abortuses or in cord blood after birth and further evaluated the reliability of the tests. Overall, they concluded that there was no problem with the diagnosis when a high percentage of fragile X expression was observed. However, interpretation of the results became difficult in cases with low fragile X expression (around 1-2%). Both false positive and false negative results were reported (Turner et al., 1986a). These authors recommended that for prenatal diagnosis of the fragile X chromosome, several different tissue culture methods should be used and at least 150 cells should be scored; for at risk fetuses without cytogenetic fragile X expression, the complementary RFLP analysis should be applied to determine carrier status. Later, adding an excess of thymidine to cultures was found to be a more reliable way to induce the fragile X in fibroblasts (Sutherland and Baker, 1986).

1.4.6. The Fragile X in Somatic Cell Hybrids

Inconsistency of fragile site expression within pedigrees, among affected sibships, and even between different cell types within a single individual (Sutherland, 1979b) was well documented but poorly understood. Could such a variation be caused by autosomal loci, or other loci on the X chromosome, or was it purely an environmental effect? To answer these questions, a better controlled system was required. For this purpose, several laboratories isolated the fragile X chromosome in a rodent background, which permitted fragile X expression. This approach made it possible to study the fragile X from different patients on a homogeneous and controlled genetic background in order to determine whether genetic or environmental elements were important for the fragile site expression.

The first successful attempt was reported by Bryant et al. (1982a, 1982b). They established two human X/mouse somatic cell hybrids, one from a fibroblast and the other from a lymphoblastoid cell line from two affected males. Expression of the fragile X was induced in the hybrids by adding FUdR. Subsequently, a number of human-rodent somatic cell hybrids were established, with the fragile X chromosomes derived from affected males (Nussbaum et al.,1983; Warren and Davidson 1984; Ledbetter et al., 1986b; Lin et al., 1987), from female carriers (Ledbetter et al., 1986a, 1986b; Wohrle and Steinbach, 1991), or from transmitting males (Ledbetter et al., 1986a; 1986b; 1986c), or, with the normal X chromosomes from normal control males (Warren and Davidson 1984; Ledbetter et al., 1986c). Expression of the fragile X was induced by FUdR (Nussbaum et al., 1983; Warren and Davidson 1984; Ledbetter et al., 1986a; 1986c; Lin et al., 1987), methotrexate (Nussbaum et al., 1983), excess thymidine (Ledbetter et al., 1986c) or FUdR plus caffeine (Ledbetter et al., 1986a, 1986b). It was realized later that at least some of the "fragile X" sites (Ledbetter et al., 1986c) were in fact FRAXD, a common fragile site at Xq27.2 (Sutherland and Baker, 1990), distinct from FRAXA.

Nussbaum et al. (1986a) generated a reduced hybrid by fusing the parental chinese hamster cells with a lethally irradiated somatic cell hybrid containing a single human fragile X chromosome. This hybrid, X3000-11, contained only a proportion of the human X chromosome

(Xq24-qter) translocated onto a hamster chromosome and expressed the fragile site under FUdR induction. Furthermore, Warren et al. (1987) observed that under culture conditions of thymidine stress the fragile X chromosome in somatic hybrids was frequently involved in chromosome rearrangement. Using the two genes HPRT and G6PD which were located on either side of the fragile X for selection, they identified hybrids containing either Xpter-Xq27 or Xq27-Xqter of a fragile X chromosome translocated to a rodent chromosome arm (Warren et al., 1987, 1990). Such hybrids were further tested with DNA markers known to be located on either side of the fragile X (DXS51, F9, DXS105, DXS98, DXS52, DXS15 and F8). In each hybrid, the position of the translocation breakpoint on the X chromosome was found to be at or very close to the fragile site (Warren et al., 1987; 1990). In two of these hybrids, fragile X site expression was observed at the translocation junction, but at significantly lower frequencies than that seen in the intact fragile X of the parental hybrid (Warren et al., 1987). These translocation hybrids, referred to as fragile X hybrid or fragile X translocation hybrid later in the thesis, were of use in mapping DNA markers very close to the fragile site and also provided a reagent for cloning of the fragile site (see Chapter 5).

Several conclusions could be drawn from the study of fragile X expression in somatic hybrids (for review see Nussbaum and Ledbetter, 1986). Firstly, fragile X site expression was likely to be an intrinsic property of the site itself. The existence of human autosomal loci was not a prerequisite for fragile X expression since expression of the fragile site was induced in hybrids containing the fragile X chromosome as the only human DNA source (Nussbaum et al., 1983; Warren and Davidson 1984; Ledbetter et al., 1986a). The fact that the fragile X could be expressed in a hybrid containing Xq24-qter as the only human DNA (Nussbaum et al., 1986a) suggested that the autosomes and the rest part of the X chromosome were not important for the site expression. Therefore, fragile site expression was likely to be a property of the DNA sequences at the site under special culture conditions. Secondly, the fragile site was indeed fragile and tended to break under certain culture conditions (Warren et al., 1987). Moreover, the DNA sequence at the fragile site was likely to be a repeated sequence since the translocation

junctions of some fragile X hybrids showed fragility but at a lower frequency, thus retaining part of the repetitive sequence (Warren et al., 1987).

1.5. THE UNIQUE INHERITANCE PATTERN

When Lubs et al. (1969) first described a marker X chromosome in a family with X-linked mental retardation (XLMR), the inheritance of mental retardation was shown to be consistent with an X-linked recessive inheritance pattern. However, departures from classical X-linked recessive inheritance were later observed in families with fragile X syndrome. Segregation analysis of 206 fragile X families revealed many more unique aspects of this disorder (Sherman et al., 1984, 1985) and marked it as one of the most genetically complicated disorders in humans.

1.5.1. Existence of Transmitting Males

In an X-linked disorder, penetrance in males is expected to be 100%. But this is not the case in fragile X syndrome. Webb et al. (1981) reported a fragile X family in which a mentally retarded boy had inherited the fragile X from his maternal grandfather, who showed 26% fragile X expression but no mental retardation (Webb et al., 1981).

By linkage analysis with the human factor 9 (F9) RFLP, Camerino et al. (1983) was for the first time able to confirm the observation of transmission through a male in a large family. The grandfather who transmitted the fragile X chromosome to his seven daughters had neither the cytogenetic marker nor the clinical features of the disease (known as transmitting male). However, five of his daughters had seven mentally impaired offspring (Camerino et al., 1983). Subsequently, transmitting males were shown to be very common in fragile X syndrome pedigrees either by pedigree analysis (Nielson et al., 1981; Jacobs et al., 1983; Froster-Iskenius et al., 1984; Arinami et al., 1986) or by linkage analysis (Webb et al., 1986; Voelckel et al., 1988). It was then suggested that the grandpaternal relatives of a fragile X syndrome patient should also be tested for carrier status.

Segregation studies of 206 fragile X pedigrees revealed that the proportion of mentally retarded males (affected males) in fragile X families was 20% less than expected (Sherman et al., 1984, 1985). In other words, 20% of males carrying the mutation did not express it either cytogenetically or phenotypically. Therefore, in male, the penetrance of mental retardation was estimated to be 80%.

1.5.2. Penetrance in Female Heterozygotes

For an X-linked dominant mutation, the penetrance in female is expected to be very high, whereas for an X-linked recessive mutation the penetrance in female is very low. In fragile X syndrome, however, about one third of heterozygous females were reported to show mental impairment of varying degrees (Brown et al., 1978; Howard-Peebles, 1979; Turner et al., 1980b; Webb et al., 1982). Segregation analysis of a large sample of fragile X syndrome families revealed that penetrance in heterozygous females was about 35% (Turner and Jacobs, 1984; Sherman et al., 1985), which is much greater than that seen in typical X-linked recessive disorders, such as haemophilia or Duchenne muscular dystrophy. Furthermore, female heterozygotes for the fragile X mutation showed a mental handicap varying from borderline to severe (Turner, et al., 1980b; Webb et al., 1982; Turner and Jacobs, 1984; Sutherland and Hecht 1985). If those with learning disabilities were counted, the penetrance in females would be even higher. An X-linked dominant model with incomplete penetrance was proposed to account for the high penetrance in both sexes seen in fragile X syndrome (Mulley and Sutherland, 1987).

1.5.3. Parental Dependent Penetrance

1.5.3.1. Penetrance Determined by Sex of Carrier Parent

A female can obtain a fragile X mutation either from her mother or her father. The penetrance will differ depending on the sex of the carrier parent. Lubs et al. (1984a,1984b) studied a large four generation family segregating fragile X syndrome with transmission through a male (generation I). None of the nine obligate carrier daughters (generation II) of the male were

mentally retarded, and all of these daughters had very low or no fragile site expression. In contrast, in generation III and IV, one third of the females carrying the mutation were mentally retarded. To further study the parental sex dependent penetrance, Lubs et al. (1984a) reviewed other reported pedigrees. In nine pedigrees, no penetrance was found in daughters of transmitting males (0/49), while it was frequent (13/38) in the children and grandchildren of carrier women in these families. Segregation analysis of a large number of families confirmed the parental dependent penetrance of mental impairment in fragile X syndrome (Sherman et al., 1984, 1985). When the fragile X was transmitted from a carrier father, the penetrance in carrier daughters was close to zero. In another words, the daughters of transmitting males were seldom mentally impaired. In contrast, when the fragile X was inherited from a mother, the penetrance in carrier daughters was high.

1.5.3.2. Penetrance Determined by Phenotype of Carrier Mother

If the fragile X was transmitted from a mother, the penetrance of mental retardation would be different in the offspring, depending on whether the mother was mentally affected. If a mother was intellectually normal, the penetrance was 76% in her sons and 32% in her daughters. However, if a mother was mentally impaired, the penetrance increased to 100% in her sons and 55% in her daughters (Sherman et al., 1985).

1.5.4. Generation Dependent Penetrance (the Sherman Paradox)

Sherman et al. (1984; 1985) compared penetrance in the offspring of the mothers of transmitting males and that in the offspring of the daughters of transmitting males. Both the mothers and the daughters of transmitting males were mentally normal and without cytogenetic fragile site expression. They were assumed to have identical genotypes and thus should have had a similar ratio of affected sons. But this was not the case. The penetrance in the sons of the mothers of transmitting males was 18% compared with 74% in the sons of the daughters of

transmitting males (Sherman et al., 1985). This phenomenon, increased penetrance in succeeding generations, was termed the "Sherman paradox" (Opitz, 1986).

1.5.5. Lack of New Mutation in Affected Males versus High Mutation Rate

For X-linked recessive lethal genes, about one third of affected males were expected to result from new mutations (Haldone, 1941). In the case of fragile X syndrome (presumed to be an X-linked dominant disorder with incomplete penetrance both in males and females), the proportion of new mutants in affected males were estimated to be zero by segregation analysis. This implied that there were no new mutants in affected males (Sherman et al., 1984, 1985). In other words, all affected males inherited the fragile X mutation from their mothers, and all mothers of affected males were carriers. This conclusion was based purely on calculation, since no technique available then could tell whether an affected male was a new mutant. Therefore, assuming the classical one-step mutation model, and the new mutation only happening in sperm, the mutation rate was calculated to be 7.2×10^{-4}/gamete/generation (Sherman et al.,1984), by far the highest in human inherited disorders. Since some of the isolated cases (affected individuals with no affected relatives) were likely to be sporadic cases (new mutants), determining the proportion of isolated cases among affected individuals would give an estimation of the proportion of possible mutants (Jacobs et al., 1986). The results from the study of isolated cases was very similar to that from previous segregation analysis in that sporadic cases of the fragile X syndrome were rare, if they occurred at all, among retarded males and females (Jacobs et al., 1986). However, the proportion of sporadic cases among affected males was estimated to be 24% by segregation analysis of new family data (Sherman et al., 1988), which was significantly different from the estimation of 0 in the original study (Sherman et al.,1984). Neither cytogenetic testing nor linkage analysis could tell which rate was correct. Since the high prevalence of fragile X syndrome was obvious, a question arose about where the new mutation occurred.

1.6. HYPOTHESES

Various hypotheses have been put forward to explain the unusual genetics of the fragile X syndrome, such as high prevalence, existence of transmitting males, high penetrance and variable expression in female heterozygotes, parental and generation dependent penetrance as well as the associated fragile site.

1.6.1. Possible Explanation for High Prevalence

The prevalence of the fragile X syndrome was estimated to be 0.4-0.8/1000 in males and 0.2-0.6/1000 in females (Hagerman and Silverman, 1991), and thus the disorder was the second most common cause of mental retardation after Down syndrome. Based on the high prevalence data, the mutation rate was calculated to be 7.2×10^{-4} /gamete/generation if assuming the mutation only happened in sperm, or 2.4×10^{-4} /gamete/generation if mutation occurred with equal frequency in sperm and egg (Sherman et al., 1984; 1985). These data implied that the high prevalence of the fragile X syndrome resulted from a balance between very high mutation rate in conjunction with a selective disadvantage of retarded individuals. This unusually high mutation rate was questioned by other authors. If mutation occurred at such a high rate in sperm, it would be expected that 52% of carrier mothers of probands were new mutants (for calculation, see Vogel et al., 1985). However, in a segregation study of 112 informative families, it was found that the proportion of new mutants among mothers of probands was much lower than 52% (Vogel et al., 1985).

Alternatively, the high prevalence could be explained by a moderately high mutation rate in combination with the selective advantage of the normal carriers (Vogel, 1984; 1985). By assuming a moderately increased fertility in normal female carriers and in transmitting males, the mutation rate would be between 1.10×10^{-4} and 2.05×10^{-5}/gamete/generation (Vogel, 1984).

1.6.2. Two-Step Mutation Model

In fragile X pedigrees, daughters of transmitting males were rarely mentally retarded and showed a low level or no fragile X site expression. In contrast, the sons of these daughters were at a high risk of being affected. To explain this observation, a two-step-model for development of the fragile X syndrome was proposed independently by a number of authors (Sherman et al., 1985; Pembrey et al., 1985; Jacobs et al., 1986). These authors suggested that the fragile X mutation had two distinct forms: premutation, which has no phenotypic effect, and full mutation, which causes the fragile X syndrome phenotype. The conversion from a premutation to a full mutation would only occur in an egg. The transmitting males and carrier females would carry the premutation and all affected ones, the full mutation. Based on this hypothesis, the mutation rate from normal to premutation would be 1.67×10^{-4} (Winter, 1987), much lower than that proposed by Sherman et al. (1984).

Some authors further speculated on the nature of the premutation and the mechanism of the conversion from a premutation to a full mutation. Pembrey et al. (1985) suggested that the premutation could be an inherited sub-microscopic chromosome rearrangement in the fragile X region. It could generate a genetic imbalance through a recombination event in meiosis, and be converted to a full mutation when transmitted by a female (Pembrey et al., 1985). On the other hand, based on the functions of fragile site inducers and inhibitors, Sutherland et al. (1985) proposed that DNA in a folate sensitive fragile site (including the fragile X) was a repeating structure of polyd(AG)/polyd(TC). Nussbaum et al. (1986b) further suggested that the repetitive sequence could exist as a normal sequence in human X chromosomes; and during female oogenesis, this repetitive sequence could be amplified through cross-over between two X chromosomes to produce an initial lesion, or a premutation of the fragile X. Individuals carrying such a premutation would be phenotypically normal. In carrier female, however, when the X chromosome with a premutation went through meiosis, recombination in this region would result in an even longer stretch of repetitive DNA, a "full mutation".

To test the recombination mechanism for the transition from premutation to full mutation, Winter and Pembrey (1986) analysed the linkage data from loci around the fragile X locus (DXS51, F9 and DXS52) in daughters and grandsons of transmitting males. The daughters of transmitting males were assumed to carry the premutation and should produce affected sons by a recombination event in the Xq27 region if the hypothesis was correct. However, in the meioses giving rise to affected sons, recombination between flanking DNA markers was found to be infrequent. This observation was explained by double recombination or gene conversion in the fragile X region (Winter and Pembrey, 1986). Whether this explanation was appropriate was questioned by Brown et al. (1987a) who suggested that the analysis of Winter and Pembrey (1986) could be more easily interpreted if cross-over in the fragile X region was not necessary to generate a "full mutation". Indeed, linkage analysis using flanking DNA markers in many other laboratories clearly demonstrated that recombination between the two X chromosomes in a carrier mother was not a prerequisite for producing a fragile X syndrome offspring (Oberlé et al., 1986; Goonewardena et al., 1986; Mulley et al., 1987).

1.6.3. X-Inactivation Model in Fragile X Heterozygotes

This model was proposed to explain the varied degree of mental retardation in fragile X female heterozygotes. In females, one of the two X chromosomes is undergoing X inactivation in each cell. Whether the paternal X or the maternal X chromosome is inactivated in a given cell is a random event in normal females. As to female heterozygotes, the manifestation of mental retardation would depend on the proportion of cells in which the fragile X chromosome was active. If the fragile X chromosome was inactive in a large proportion of her cells, the female would be relatively normal. However, if the fragile X was active in most of her cells, the female would be affected. Therefore, mentally retarded females would be expected to show an excess of active (early replication) fragile X chromosomes, while the opposite would be expected for the normal carrier females. In other words, the percentage of cells with an active fragile X chromosome in a carrier female would be negatively correlated with her mental capacity. With

cytogenetic analysis, one can readily tell which X chromosome is active (early replicated) and which is inactive (late replicated) in a metaphase spread.

Although the replication pattern of the fragile X chromosome was reported in a few early papers (Lubs, 1969; Martin et al., 1980), it was Jacobs et al. (1980) who, for the first time, hypothesized that the mental status of a heterozygous female could correlate with the proportion of her cells with the active fragile X chromosome.

Froster-Iskenious et al. (1982) studied the fragile X chromosome replication pattern in a Klinefelter's syndrome patient who had a fragile X chromosome. In this patient, there was an excess of cells with active fragile X chromosomes, which was proposed to be responsible for the mental retardation in the patient. Replication status of the fragile X chromosome was determined in three heterozygous females by Howell and McDermott (1982). An excess of early replicating fragile X chromosomes was seen in a severely mentally retarded woman but not in two other women with normal intelligence. This observation supported the relationship between the replication status of the fragile X chromosome and mental capacity in a heterozygous female. The relationship was later confirmed by some authors (Uchida and Joyce 1982; Uchida et al., 1983; Paul et al., 1984; Arinami et al., 1987), but not by others (Fryns et al.,1985).

However, study of a large sample of heterozygous females generated contradictory results. Nielsen et al. (1983) studied the replication pattern in 63 obligate and potential female carriers of the fragile X syndrome. These authors divided the subjects into three groups: one group of mentally retarded females with a high percentage of fragile site expression; another group of normal intelligence carriers also with a high percentage of fragile site expression; and third group of normal intelligence carriers with a low percentage of fragile site expression. The pooled data showed that an excess of active fragile X chromosomes was evident in the first two groups with high percentage of fragile site expression, whereas there was an excess of inactive fragile X chromosomes in the third group with a low percentage of fragile site expression. Based on this result, they suggested that the level of fragile X expression should be taken into account when performing replication studies.

Obviously, more information was needed to clarify this confusion. Tuckerman et al. (1985) studied the replication status of the fragile X chromosome in a pair of monozygotic twin sisters with marked differences in intelligence. In both twin sisters, 7% of their lymphocytes were found to express the fragile X. However, in the sister with normal intelligence, 30% of her cells carried an active fragile X chromosome, whereas in the dull sister, 85% of her cells carried an active fragile X chromosome (Tuckerman et al., 1985). Furthermore, Rocchi et al. (1990) studied a group of phase-known double heterozygotes for the fragile X mutation and the G6PD variant for the fragile X chromosome inactivation. The proportion of somatic cells with an active fragile X chromosome was assessed via the G6PD phenotype observed in the red blood cells and in the cultured fibroblast cells. These authors pointed out that there was a significant negative correlation between the mental capacity and the proportion of fibroblasts with an active fragile X chromosome in female carriers tested. But, no significant correlation was obtained when the mental capacity and the data from red cells were compared (Rocchi et al., 1990). It seemed that fibroblasts were more closely related to brain cells than were red blood cells. In another study (Schmidt et al., 1991), probe M27ß (DXS255) was used in Southern blot analysis to assess the X inactivation pattern in fibroblast DNA from four fragile X heterozygotes. The methylation status in this locus (DXS255) was known to be different between the active and the inactive X chromosome in females (Schmidt et al., 1991), and was correlated with the expression of an X-linked gene (Brown et al., 1990). Among the four unrelated mentally retarded fragile X syndrome females, two had a random inactivation pattern, and the other two had a nonrandom X inactivation pattern. They concluded that there was no correlation between mental capacity and nonrandom fragile X chromosome inactivation in fragile X syndrome females tested.

In summary, the inactivation pattern of the fragile X chromosome could be determined in three ways: 1) cytogenetic analysis, adopted by most authors, relied on differentiation between the early and the late replicating X chromosome after incorporation of tritiated thymidine or BrdU. Whether a fragile X chromosome is inactive (late replicating) can be readily identified under a microscope. 2) a biochemical assay could be used (Rocchi et al., 1990). Informative

females for this study were those phase known double-heterozygotes for fragile X and for another gene which is located close to the fragile X. This gene, such as G6PD, is known to undergo X-inactivation and its known gene product is easily examined biochemically. The proportion of cells with an active fragile X chromosome could be determined through the phenotype of the known gene. 3) Southern analysis with probe M27ß (Schmidt et al., 1991) could determine the differences in methylation status between the active and inactive X chromosomes. Thus this method could be used to assess the X-inactivation pattern in a female. However, if X-inactivation is nonrandom in a fragile X female heterozygotes, this method can not tell whether the excess active (or inactive) X chromosome is the fragile X chromosome. The advantages and disadvantages of each method were discussed in the relevant paper(s). As far as the tissue type was concerned, peripheral lymphocytes were used in most of the X-inactivation studies. However, it was suggested that inactivation status of fibroblasts had a better correlation with mental status in a carrier female than that of blood cells (Rocchi et al., 1990).

Whether the X-inactivation pattern is correlated with mental capacity in fragile X heterozygous females is still inconclusive. Despite the technical variations between different researchers, and assuming that the mental capacity of a fragile X female is truly determined by the proportion of brain cells with an active fragile X chromosome, how the data obtained from red blood cells, lymphocytes or fibroblasts could reflect the situation in brain is an interesting, but yet unresolved issue. Nevertheless, the fact that the proportion of lymphocytes with an inactive fragile X chromosome increases with the age of a female carrier is probably due to the selection advantage for those cells carrying an inactive fragile X chromosome (Rousseau et al., 1991c; also see section 6.5.3.).

1.6.4. X-Inactivation Imprinting Model

Laird (1987) proposed a mechanism for the inheritance and expression of the fragile X syndrome, the X-inactivation imprinting model. According to this model, the fragile X mutation itself had no phenotypic effect but blocked locally the reactivation of an inactive fragile X

chromosome in a carrier female. If the mutation was located on an inactive X chromosome in a female carrier, the local fragile X region would remain inactivated in her offspring (imprinted), and her offspring would be affected. On the other hand, if the mutation located on an active X chromosome in a female carrier, the fragile X region would remain active (unimprinted) in her offspring, and her offspring would be a carrier female or a transmitting male.

This hypothesis did explain some unusual aspects of the fragile X syndrome, such as that the mutation had to be passed through a female in order to be expressed. The transmitting males and normal carrier females were supposed to carry an unimprinted fragile X mutation, the affected ones the imprinted fragile X mutation. The predicted penetrances for males and females from carrier mothers were 76% and 38% respectively, very close to the observed values (Sherman et al., 1985). Laird et al. (1990) further extended this model to explain the different penetrance among male sibships of transmitting males and among grandsons of transmitting males, namely the Sherman Paradox (Optiz, 1986). The estimated penetrance of 20% and 80% were very similar to the reported values of 18% and 74% (Sherman et al., 1984; 1985).

Since the X-inactivation imprinting model assumed that DNA sequences at the fragile X region were late replicated because of inappropriate methylation (Laird et al., 1987), the genes at or near this region were likely to be transcriptionally inactivated. Khalifa et al. (1990) tested the imprinting hypothesis by examining the methylation status of the CpG islands at the 5' end of four genes (HPRT, G6PD, P3 and GdX) and four anonymous loci (DXS98, DXS304, DXS52 and DXS15) which flank the fragile X. In eight fragile X-syndrome males and normal controls, there was no difference in the methylation status in all markers tested between normal and fragile X syndrome males. In fact, methylation was never observed in these loci in fragile X syndrome males (Khalifa et al., 1990). However, since the genes and loci tested by Khalifa et al. were located at least 5 centimorgans (cM) away from the fragile X locus (FRAXA), their results did not refute the imprinting hypothesis.

In another study, the activity of the hypoxanthine phosphoribosyltransferase (HPRT) gene, which was located at Xq26 proximal to FRAXA, was studied by measuring HPRT enzyme

activity and the level of HPRT-RNA in fibroblast cell cultures (Steen et al., 1991). There was no significant difference found in these tests between fragile X and normal cell cultures. Again, these data neither supported nor refuted the hypothesis because the HPRT locus is located millions of base pairs away from the fragile X locus.

To test if delayed DNA replication was involved in the fragile X region, Yu et al. (1990) compared the late replication pattern at Xq27 between normal males and fragile X syndrome males, by using BrdU incorporation R-banding. All of the affected males were found to have high frequency of late incorporation at Xq27, compared with a low frequency of late incorporation at the same region in normal males. One transmitting male and one carrier female had a similar frequency of late incorporation at the Xq27 region as the normal males had (Yu et al., 1990). These data supported Laird's hypothesis that late DNA synthesis is likely to occur at Xq27 of a fragile X chromosome but not at this position on a normal X chromosome.

1.6.5. Others

1.6.5.1. Maternal Effect

This hypothesis was proposed to explain why penetrance was higher when the mutation was transmitted from a mother but not when transmitted from a father. van Dyke and Weiss (1986) suggested a maternal effect in fragile X. The maternal effect might be mediated by the uterus or placenta by different inactivation patterns in those tissues between pregnancies. If the mother was a carrier, each of her embryos could be exposed to very different environments due to the different inactivation pattern of the fragile X chromosome in the uterine tissue at implantation sites of the uterus. Therefore, the carrier children born of a carrier mother would have a wide range of phenotypic expression. If the father was a carrier, but the mother was not, a carrier child would be phenotypically normal because of no maternal effect (van Dyke and Weiss, 1986).

1.6.5.2. Transposable Genetic Element

This hypothesis was proposed to explain the existence of transmitting males as well as the other unique aspects of the fragile X syndrome (Friedman and Howard-Peebles, 1986). They proposed that the fragile X mutation was due to a transposable genetic element, which had chromosomal and extrachromosomal activity. Hoegerman and Rary (1986) further suggested that the fragile X mutation might result from the insertion of transposable elements (TEs) within or adjacent to a normal gene at Xq27.3. Normal transmitting males were interpreted as transmitters of the factors necessary for TE insertion at the fragile site at Xq27.3. However, as these authors realized, father to son transmission, predicted by their hypothesis, had not been reported (Hoegerman and Rary, 1986).

1.6.5.3. Autosomal Modifier and Autosomal Suppressor Model

Segregation studies by Sherman et al. (1984; 1985) revealed that 1) transmitting males and their daughters seldom had mental retardation; 2) the grandchildren of transmitting males had a high penetrance of fragile X syndrome. To explain this observation, Steinbach (1986) raised an autosomal modifier model, assuming that mental retardation in fragile X syndrome was determined by several genes; a major gene at Xq27 and at least one autosomal modifier gene. The X-linked major gene was undergoing X-inactivation in female carriers. The effect of this major gene was modified by at least one non-X-linked gene. Most predictions with this model were compatible with segregation data (Sherman et al., 1984; 1985). However, the estimated penetrance in sibships of a transmitting male (58.6%) was much higher than that observed in segregation study (8%).

Israel (1987) adjusted the autosomal modifier model by assuming an autosomal suppressor gene (S) acting differently in males and females. The proposed suppressor acted as a recessive gene in males, while it acted as a dominant gene in females. Assuming the frequency of SS in the general population being 20%, this model could explain most of the observations by Sherman et al. (1984; 1985) with a few exceptions such as the Sherman Paradox.

1.7. LINKAGE ANALYSIS

Clinically, identification of carrier females and prenatal diagnosis based on the expression of the fragile site at Xq27.3 was associated with technical difficulty and penetrance problem. The only means by which to confirm transmitting males and normal carrier females with high confidence was the linkage analysis. Therefore, linkage study of the fragile X was of great use not only for the eventual cloning of both the fragile X site and the gene responsible for the disorder but also for diagnostic purposes.

1.7.1. Isolation of DNA Markers for Linkage Mapping of FRAXA

Genetic linkage between the genes for glucose 6-phosphate dehydrogenase (G6PD) and protan colour blindness and the fragile X locus (FRAXA) was first demonstrated by Filippi et al. (1983) in six families segregating both fragile X and G6PD or protan colour blindness. G6PD gene was shown by in situ hybridization to be distal but close to the fragile site (Szabo et al., 1984).

The first identified RFLP linked to the fragile X syndrome was a TaqI polymorphism of the coagulation factor IX gene (F9) reported by Camerino et al. (1983). Using the F9 gene probe to a large fragile X syndrome family, these authors were able to determine the carrier status of seven sisters and to confirm the transmission of the fragile X by a transmitting male, the father of these seven sisters. The significance of this study was that RFLP analysis was shown to be a powerful diagnostic tool, independent from cytogenetic detection, to identify transmitting males and normal carrier females (Camerino et al., 1983). Since Camerino et al. (1983) identified no recombinant among 17 informative meiosis in two families, the genetic distance between F9 and the FRAXA was estimated to be less than 12 cM at a 90% confidence level. However, other authors later reported very loose linkage between these two loci, such as 20 - 25 cM (Choo et al., 1984; Davies et al., 1985) and 33 - 35 cM (Buchanan et al.,1987; Goonewardena et al., 1986).

Encouraged by the RFLP study outlined above, many researchers made efforts to isolate informative DNA markers closer to FRAXA for linkage analysis. Drayna et al (1984) isolated a series of single-copy DNA probes from a flow sorted X chromosome specific DNA library

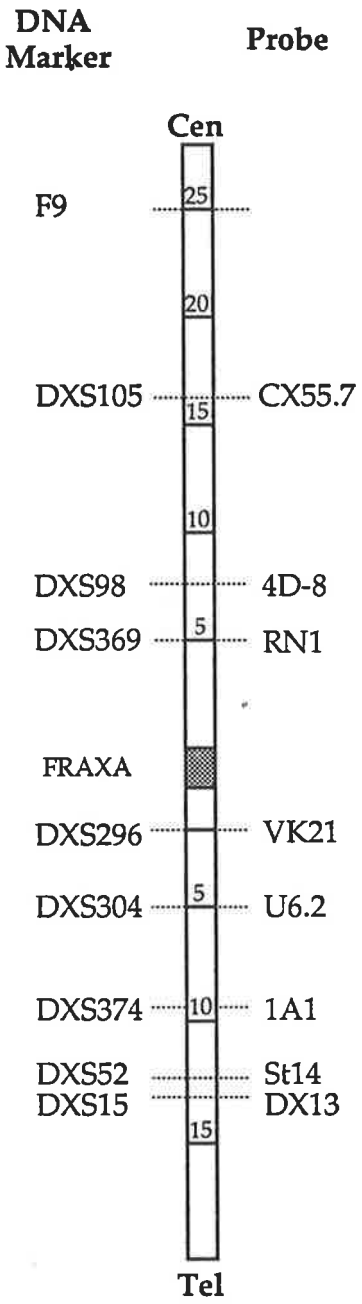


Fig. 1 - 1. Linkage map of the FRAXA. DNA markers (D number) on the left hand side, name of probes on the right. The genetic distance (in centimorgan) from FRAXA (hatched region) is indicated with number. Cen: centromere; Tel: telomere.

Probe 4D-8 (DXS98) was isolated from a genomic library made from X chromosomes isolated from a 49 XXXXY human lymphoblast cell line by fluorescence-activated sorting (Boggs and Nussbaum 1984). This probe detected a MspI RFLP with average heterozygosity of 30%. Linkage analysis revealed no detectable linkage between DXS98 (4D-8) and the HPRT locus (Boggs and Nussbaum 1984). Further study showed that DXS98 (4D-8) was located between the F9 locus and FRAXA with a genetic distance of 5 cM proximal to FRAXA (Brown et al., 1987b), thus it was the closest available DNA marker. To increase its heterozygosity (30%), the DXS98 locus was expanded by screening a lambda library with probe 4D-8. A positive clone (lambda 4d8-3) was isolated and it detected three additional RFLPs. The combined heterozygosity for the four RFLPs at DXS98 locus increased to 48% (Schnur et al., 1989). DXS98 was further mapped between DXS105 (cX55.7) and FRAXA (Patterson et al., 1988), 7 cM proximal to the FRAXA (Brown et al., 1988a). (see also Fig. 1 - 1).

The order of these DNA probes around FRAXA was determined as follows: DXS51(52A)-F9-DXS105(cX55.7)-DXS98(4D-8)-FRAXA-DXS52(St14), DXS15(DX13) (Fig. 1 - 1). This order was consistant in linkage studies of a large number of fragile X families (Buchanan et al., 1987; Veenema et al., 1987; Mulley et al., 1987, 1988; Patterson et al.,1988; Brown et al., 1987b, 1988a, 1988b). The order was also consistent between fragile X and normal families (Oberlé et al., 1987; Thibodeau et al., 1988) and was in good agreement with the physical map in this region (reviewed by Davies, 1986; Patterson et al., 1987). The closest proximal marker, DXS98(4D-8), was 7 cM, whereas the closest distal marker, DXS52(St14), was 10-12 cM from the fragile X locus.

1989 was a fruitful year for isolating DNA markers close to the fragile X locus. Early in 1989, probe 1A1 (DXS374) was isolated from a cosmid library made from human-rodent hybrid DNA containing a human X chromosome as its only human component. The probe detected a PstI RFLP with allele frequencies of 58% and 42%. The RFLP detected by 1A1 was located between FRAXA and DXS52(St14), with a distance of 3 cM proximal to DXS52 (Patterson et al., 1989).

Soon after, three more DNA markers were identified within 5 cM of the fragile X locus (reviewed by Brown, 1990). DNA probe, U6.2 (DXS304), isolated from a human-rodent hybrid

library, detected six RFLPs with enzymes TaqI, PstI, MspI, BclI, BglI, and *Stu*I. Linkage disequilibrium was observed for all six RFLPs, and a 30% heterozygosity was estimated for DXS304 (Dahl et al., 1989a). No recombination between DXS304 and FRAXA was observed in 29 informative meioses (Dahl et al.,1989a). In another large fragile X pedigree, recombination was not detected between DXS304 and FRAXA in 20 informative meioses, although recombination had been detected before between FRAXA and other available DNA markers in the pedigree (Lucotte, 1990). The genetic distance between DXS304 and FRAXA was estimated to be less than 5 cM (Vincent et al., 1989). The DXS304 locus was shown to be distal to FRAXA but closer than previously available markers (Vincent et al., 1989). The order of the DNA markers around FRAXA was established: F9-DXS105-DXS98-FRAXA-DXS304-DXS52 (Dahl et al., 1989b). Subsequently, two new RFLPs (BanI and BstEII) were identified at the DXS304 locus by chromosome walking (Rousseau et al., 1990). The combined heterozygosity at the DXS304 locus increased to 63%. Thus, locus DXS304 became a very useful marker for carrier detection and prenatal diagnosis owing to its tight linkage to FRAXA and its reasonably high heterozygosity.

Probe RN1 (DXS369) was isolated from a human-rodent hybrid library by screening with an Alu sequence (Oostra et al., 1990). This library was made from a hybrid cell line containing a small terminal portion of the long arm of the human X chromosome which retained markers distal to the fragile X such as F8 and DXS52 (St14) but not the proximal markers such as DXS98 (4D-8). Probe RN1 detected a two allele XmnI polymorphism with allele frequency of 0.41 and 0.59 (Hupkes et al., 1989). DXS369 (RN1) was localized between DXS105 (cX55.7) and FRAXA, about 5 cM proximal to FRAXA by linkage analysis in conjunction with the other probes around the FRAXA (Oostra et al., 1990). (also see Fig. 1 - 1).

Hyland et al. (1989) constructed a phage library from a mouse/human hybrid containing a der(16)t(X;16)(q26:q24) as the only human chromosome and subsequently isolated a series of anonymous human DNA probes from this library. Since the hybrid contained only the region Xq26-qter, the X-derived clones were ensured to be in this particular region. Overall 19 clones were mapped physically to Xq26-qter by using somatic hybrid cell panels with the translocation

or deletion breakpoints at Xq26-28 (Hyland et al., 1989; Suthers et al., 1990). Some of them were mapped genetically by linkage analysis in normal and fragile X families (Suthers et al., 1989; Suthers 1991a, 1991c). Of these clones, DXS296 (VK21) detected RFLPs with TaqI, MspI and BclI (Suthers et al., 1989; Yu et al.,1989). It was shown to be distal to FRAXA with a genetic distance of 1 - 2 cM by linkage analysis (Fig. 1 - 1). Thus DXS296 was the closest marker to FRAXA yet reported (Suthers et al., 1989). This result was consistent with the location of FRAXA being 2.2 cM proximal to DXS296 (Suthers, 1991).

In view of the linkage studies of FRAXA, some critical steps are summarized as following: Firstly, in order to isolate X chromosome specific DNA fragments efficiently for linkage analysis, an X chromosome-specific library is a prerequisite. A human genomic DNA library of this kind can be constructed from 1) flow sorted X chromosome DNA (Davies et al., 1981), or, 2) DNA from human-rodent hybrid cell lines containing only the human X chromosome (Patterson et al., 1989) or even a portion of the X chromosome (Hyland et al., 1989). Secondly, when a human-rodent hybrid library is used, human DNA sequences can be isolated from the background rodent DNA by hybridization with either total human DNA (Hyland et al., 1989) or Alu sequences (Oostra et al., 1990). In the case of using a flow sorted X chromosome DNA library, DNA probes were assigned to a specific chromosome region by hybridization with a set of somatic hybrids with breakpoints along the X chromosome or by their linkage with the loci of known location (Drayna et al., 1984). Thirdly, low heterozygosity of a DNA marker can be improved by either testing more restriction enzymes (Rekila et al.,1988) or by isolating other probe(s) in the vicinity by screening the original or a different DNA library with the initial probe (Schnur et al., 1989; Heilig et al., 1988). The latter has been proved to be a good means of increasing the heterozygosity of a DNA marker.

1.7.2. Is There Linkage Heterogeneity Around FRAXA?

An interesting issue encountered in the linkage analysis of FRAXA was the linkage heterogeneity. When DNA marker F9 was used for linkage analysis in families segregating

fragile X syndrome, the recombination rate between F9 and FRAXA was found to be high in some families, but not in others (Brown et al., 1985). Those families with low recombination rates were found to contain transmitting males, whereas those families with high recombination rates contained no transmitting males. Therefore, Brown et al. (1985) concluded that fragile X families could be divided into two classes: those with transmitting males and also with low recombination rate between F9 and FRAXA, and those without transmitting males and with high recombination rate. This phenomenon, named "linkage heterogeneity", was confirmed by the same group in an extended study with two more DNA markers (DXS51 and DXS52) and 8 additional fragile X families (Brown et al., 1986). In addition, the recombination rate in the DXA51(52A) and F9 interval was found to be strikingly different between fragile X families and normal families. It then seemed that genetic heterogeneity regarding recombination existed not only within fragile X families, but also between fragile X and normal families. Further evidence for genetic heterogeneity was obtained by linkage study with probes 52A, F9, DX13 and St14 (Brown et al., 1987c, 1988b). In combination with the previous data, these authors concluded that the presence of transmitting males in a pedigree was not an indicator of heterogeneity; heterogeneity between F9 and FRAXA did exist and was correlated with the frequency of the fragile site expression as well as with mental capacity (Brown et al., 1987c).

One possible model of genetic heterogeneity of recombination fractions was genetic heterogeneity of the locus. According to this model, linkage heterogeneity meant that two separate loci in the region between DXS51(52A) and DXS52 (St14) might code for fragile X mutation and that either of which could produce fragile X syndrome. To test the existence of these two loci, Clayton et al. (1988) undertook a multipoint linkage analysis in 57 fragile X families. However, the data did not suggest that there were two such loci within the interval between markers DXS51 (52A) and DXS52 (St14). Therefore, if linkage heterogeneity existed between F9 and the FRAXA locus, it must have other explanations.

Since DXS105(cX55.7) was mapped between F9 and FRAXA, it was feasible to test whether the linkage heterogeneity was in the interval between F9 and DXS105 (cX55.7) or between

DXS105 (cX55.7) and FRAXA. Veenema et al. (1987) used six DNA probes for linkage analysis in 67 members of a large family segregating fragile X syndrome. Among 25 descendants of a transmitting male, at least five recombinants were observed: four being between F9 and DXS105 (cX55.7) and one between DXS105 (cX55.7) and FRAXA. These data did not fit with the notion that a close linkage between fragile X and F9 existed in families with transmitting males. Again, recombinations between F9 and FRAXA were mainly within the interval from F9 to DXS105 (Veenema et al., 1987). Oberlé et al. (1987) performed linkage analysis with seven DNA probes in nine fragile X and 16 normal families. Their data suggested that the genetic distance between F9 and FRAXA did not differ between fragile X and normal families, thus contradicting the observation by Brown et al. (1986). Although the data from Buchanan et al. (1987) apparently supported the observation of Brown et al. (1986), it was pointed out that it was not appropriate to predivide fragile X families on the basis of whether transmitting males were present.

A collaborative linkage study provided an opportunity to examine the linkage heterogeneity around FRAXA in a large number of fragile X families (Suthers et al., 1991b). In this study, the authors tested five polymorphic loci (DXS369, DXS297, DXS296, IDS and DXS304) within 4 cM of the fragile X locus as well as four more distal or proximal loci (F9, DXS105, DXS98 and DXS52) in 112 fragile X families and 40 normal families. Recombination frequency between any two loci was obtained from two-point linkage analysis. The data failed to provide any evidence for linkage heterogeneity around the FRAXA either between fragile X and normal families or within fragile X families. The most plausible explanation for the discrepant conclusions of linkage heterogeneity around the FRAXA was misclassification of some individuals in some fragile X pedigrees because of the existence of a common fragile site (FRAXD) (Ledbetter and Ledbetter, 1988; Sutherland and Baker, 1990) just proximal to FRAXA (Suthers et al., 1991b). Therefore, the genetic map around FRAXA could be used for genetic counselling and risk estimation with confidence.

1.7.3. Diagnosis of Fragile X Syndrome by Linkage Analysis

About 20% of males and 44% of females carrying the mutation showed neither mental retardation nor fragile site expression (Sherman et al., 1984, 1985). Linkage analysis with DNA markers around FRAXA was the only means of diagnosis for those carriers before the mutation was cloned (Sutherland and Mulley, 1990). Linkage analysis can also be used in prenatal diagnosis of the fragile X genotype.

Linkage analysis in fragile X families allowed tracking of the mutation from parents to offspring by following the transmission of polymorphic DNA markers flanking the fragile X mutation. If no cross-over occurred between the two flanking markers, the linkage phase in a family could be determined by testing the key members of the family. In order to generate reliable information for carrier detection and prenatal diagnosis, DNA markers for analysis are required to be both highly polymorphic and very close to the fragile X locus, and flanking markers are recommended (Sutherland and Mulley, 1990). Strategies for linkage analysis in fragile X families were proposed to obtain the closest informative flanking markers with a minimum amount of laboratory effort. That included choosing appropriate enzyme(s) and DNA probe(s) for Southern analysis (Sutherland and Mulley, 1990; Suthers et al., 1991a). By using linkage analysis, many laboratories had success in identifying transmitting males and carrier females and in prenatal diagnosis (Goonewardena et al., 1986; Oberlé et al., 1985b, 1986; Tommerup et al., 1985; Veenema et al., 1987). However, there were also potential problems. First, many factors limited the utility of linkage analysis in certain fragile X families, such as the DNA sample of a key family member being unavailable or recombination occurring between flanking markers. Second, double recombination between the two flanking markers would give a wrong result, the chance of which depended on the distance between the two flanking markers (Sutherland and Mulley, 1990). Third, in prenatal diagnosis, linkage analysis could provide information on whether the fetus was carrying the fragile X genotype but not whether the fetus would be affected.

Overall, linkage analysis resulted in a detailed genetic map around FRAXA (Fig. 1 - 1). This map as well as the physical map in this region not only facilitated the carrier detection and prenatal diagnosis of the disorder but also paved the way towards molecular cloning of the FRAXA (Chapter 4 and Chapter 5).

1.8. POSITIONAL GENE CLONING

For a gene having known gene product, identification of the gene is straightforward. A good example was the characterization of the human factor VIII (F8) gene (William et al., 1984). In brief, cloning of the F8 gene was initiated with a 36-base oligonucleotide probe, which was synthesized according to the peptide sequences of one section of human F8. The oligonucleotide probe was used to screen a human genomic library in order to target the F8 gene. Then the isolated human genomic fragment, contained in a lambda clone, was extended in both directions by isolation of overlapping lambda clones until the entire gene was covered (William et al., 1984). This approach was named direct or functional gene cloning.

For genes without known products, gene isolation can be much more difficult. Since no information on the gene product is available to allow direct cloning of the gene, the remaining alternative has been to identify the gene on the basis of its known chromosomal map position, so called positional cloning or reverse genetics.

To isolate a gene by positional cloning, there are some prerequisites. First of all, genetic linkage analysis is essential to assign the gene to a chromosome and to refine the regional localization by the creation of a detailed genetic map around that gene. Then, a large amount of the surrounding DNA is cloned and physically mapped in order to further pinpoint the candidate gene sequences. Finally, the DNA lesion is pinpointed. This step has been greatly accelerated by the identification of gross chromosomal rearrangements with breakpoint(s) interrupting the relevant gene. One good example is the identification of the neurofibromatosis gene (Wallace et al., 1990; Cawthon et al., 1990). Two patients with neurofibromatosis were identified as having translocations involving chromosomes 1 and 17 in one patient, 17 and 22 in the other. The common

breakpoint on chromosome 17 was at 17q11.2 with the breakpoints in these two patients being only 60 kb apart determined by pulsed field gel electrophoresis. Subsequent cloning efforts were focused on the region between the two breakpoints. A gene, isolated in this region and interrupted by both breakpoints, was subsequently shown to be the neurofibromatosis gene (Wallace et al.,1990; Cawthon et al., 1990).

In addition, efficiency for positional cloning has been greatly improved by the introduction of the yeast artificial chromosome (YAC) cloning system (Burke et al., 1987). The YAC cloning system has the capacity of cloning several hundred kilobase pairs of DNA, compared with the traditional cosmid vectors carrying a maximum 50 kb of DNA. In the case of identification of the cystic fibrosis gene, ten genomic libraries had been constructed and 49 recombinant phage and cosmid clones were isolated and characterized to cover a contiguous chromosomal region of 280 kb (Rommens et al., 1989). Whereas, only one YAC clone containing a 270 kb human DNA insert covered the major region of the neurofibromatosis I gene (Wallace et al., 1990).

1.9 STRATEGY OF THIS PROJECT

This project was based on the detailed physical and genetic maps of DNA markers around the fragile X (see Fig. 1 - 1). The general aim was to characterize the fragile X at the molecular level. Since there was no information on the gene product to allow direct cloning of the fragile X, positional cloning was the alternative chosen to approach the associated gene and the DNA lesion(s). To achieve this goal, the following strategies were undertaken: 1) approaching and covering the fragile X by means of chromosome walking; 2) defining the fragile X region; 3) pinpointing the DNA lesion in fragile X syndrome. These are detailed as following:

1) Chromosome walking to cover the fragile X region. The YAC cloning system was used for chromosome walking because of its powerful capacity of cloning DNA fragments of up to several hundred kilobase pairs. An Xq YAC library which was constructed from a human/rodent hybrid X3000.11, (containing Xq24-qter as the only human DNA) provided an important source of YAC clones at the fragile X region. Four loci which mapped very close to the fragile X were chosen as

starting points for chromosome walking in the hope that bi-directional walking from multiple points would cover the fragile X region more efficiently. Four probes (VK21, VK23, VK16 and VK18) which detect the four loci, were used to screen the Xq YAC library and thus isolate YAC clones containing human DNA fragments in the vicinity of the fragile X region. Then both ends of the human DNA fragment in each YAC clone were isolated and used to screen the Xq YAC library for overlapping YAC clones. In this way, the walking was to continue until a YAC clone bridging the fragile site was identified.

2) Defining the fragile X region. Since the fragile X syndrome is well known to be associated with a fragile site at Xq27.3, the location of its locus on the X chromosome was thus determined. With this cytogenetic indicator, human DNA fragments cloned in each YAC can be readily mapped by in situ hybridization in relation to the fragile site. After identification of the YAC which bridged the fragile X, a lambda library of this YAC needed to be constructed in order to establish a lambda clone contig in the critical region. The fragile X region was further localized by using lambda clones from the contig for in situ hybridization. Moreover, since the fragile X translocation hybrids were made to have the breakpoints very close to or at the fragile X site, hybridisation of DNA fragments (very close to the fragile X) to the hybrid DNA samples provided independent evidence for defining the fragile X region.

3) Identification of the DNA lesion in fragile X syndrome. To identify whether a large scale DNA rearrangement was involved in the fragile X mutation, DNA samples containing a fragile X chromosome or a normal X chromosome were compared by PFGE with the probe(s) very close to the fragile X region. If no DNA differences between normal and affected individuals was identified, a small scale comparison was undertaken. This could be achieved by probing normal Southern blots containing DNA samples of fragile X and normal individuals with the probes very close to the fragile X region. The lambda clones in the contig provided contiguous probes to search for the mutation until the difference was identified.

CHAPTER 2

MATERIALS AND METHODS

2.1. INTRODUCTION

This project used molecular genetic technology to investigate the DNA lesions of the fragile X and the associated syndrome. Most of the methods used in this project were well established and used routinely in the laboratory of molecular genetics at Adelaide Children's Hospital (Adelaide, Australia). These methods include DNA isolation, cloning human DNA sequences into plasmid, genomic DNA analysis by Southern hybridization and pulsed field gel electrophoresis. They will be mentioned briefly in this chapter, with indications of some aspects of each technology. Only materials and methods used in more than one result chapter will be presented in this chapter, otherwise, they will be described in the corresponding chapter. This chapter will put considerable emphasis on the YAC cloning system, since the YAC analysis technologies described in this chapter were either introduced or developed by the candidate unless specified. Various aspects of the YAC cloning system will be described in detail to give a clearer picture of the cloning system and the related technologies.

2.2. DNA ISOLATION

2.2.1. Large Scale Isolation of Plasmid DNA (modification of Maniatis et al., 1982)

Ten ml Luria Bertani (LB) medium containing ampicillin (50 µg/ml) was inoculated with a single bacterial colony (MV1190). The culture was incubated at 37°C for 5 - 7 hours with vigorous shaking, and then transferred to 100 ml LB containing ampicillin (50 µg/ml). After overnight incubation at 37°C with vigorous shaking, the culture was transferred to two 50 ml falconer tubes. The tubes were left on ice for 15 minutes and then spun at 3000 rpm for 15 minutes in a Jouan CR3000 centrifuge at 4°C. The supernatant was discarded and the cell pellet was gently resuspended in 300 µl TE and glucose (50 mM Tris-HCl, 20 mM EDTA and 50 mM glucose) containing 60 µl of 80 mg/ml lysozyme (Boehringer). The cell suspension was left at room temperature for 4 minutes and on ice for 1 minute. Then 1.2 ml of 0.2 M NaOH/1% SDS was added to the cell suspension, gently mixed and left on ice for another 5 minutes. Nine hundred µl of ice cold 3 M potassium acetate (pH 4.3) was added to the suspension and mixed by

inverting. Then the mixture was spun in a BECKMAN J2-21M/E centrifuge in a JA20 rotor at 15K for 15 minutes and the supernatant was transferred to a fresh tube and spun again at the same speed. Then, the supernatant was well mixed with 5.5 ml of ethanol, and stood at room temperature for 5 minutes. The solution was spun in the BECKMAN centrifuge at 15K for 5 minutes, and the supernatant was discarded. The DNA pellet was washed twice in 2 ml of 70% ethanol, air-dried and then resuspended in 200 µl TE (10 mM Tris-HCl/1 mM EDTA, pH 8.0). To eliminate RNA in the DNA preparation, 10 µl of 1 mg/ml RNase (Boehringer) was added to the DNA solution and incubated for 15 minutes at 37°C. To eliminate proteins in the DNA preparation, 100 µl of 3 x proteinase K buffer (10 mM Tris HCl/10 mM NaCl/10 mM EDTA), 10 µl of 10% SDS and 2 µl of 10 mg/ml proteinase K (MERCK) were added to the DNA solution and incubated at 37°C for 1 hour. For DNA extraction, equal volume (to DNA solution) of phenol (previously distilled, then saturated with 10 mM Tris HCl) was added and gently mixed with the DNA solution by inverting for 5 minutes. The mixture was then spun in an eppendorf centrifuge at 10,000 rpm for 10 minutes, and the top, aqueous, phase was transferred to a fresh tube, gently mixed with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1). Centrifugation was repeated once more. Then the top, aqueous, phase was transferred to another fresh tube and mixed with an equal volume of chloroform: isoamyl alcohol (24:1) by inverting for 5 minutes. After centrifugation, the top, aqueous, phase was transferred to a fresh tube and mixed well with 1/3 volume of 7.5 M ammonium acetate. Then centrifugation was repeated once more. The supernatant was then mixed with 2 volumes of ethanol and left at -20°C overnight for DNA precipitation. After centrifugation, the DNA pellet was washed twice with cold 70% ethanol, desiccated, and dissolved in 200 µl of TE.

2.2.2. Small Scale Isolation of Plasmid DNA (modification of Birnboim and Doly, 1979)

A single bacterial colony was inoculated to 1.5 ml of LB medium containing ampicillin (50 µg/ml) in a 10 ml tube. The culture was incubated at 37°C overnight with vigorous shaking. The culture was transferred to an eppendorf tube and spun in an eppendorf centrifuge at 10,000 rpm

for 2 minutes. After discarding the supernatant, the cell pellet was well resuspended in 100 µl of cold fresh TES medium [5 ml of TES medium contained 4.1 ml of 15% Sucrose, 0.5 ml of 100 mM EDTA, 0.125 ml of 1 M Tris (pH 8.0) and 0.25 ml of 100 mg/ml lysozyme (Boehringer)]. The cell suspension was left at room temperature for 5 minutes before 200 µl of 0.2 N NaOH/1% sodium dodecyl sulfate (SDS) was added and mixed well. The mixture was left on ice for 5 minutes and then 150 µl of cold 3 M sodium acetate (pH 4.6) was added. After 5 minutes on ice, the mixture was spun in an eppendorf centrifuge for 4 minutes. After transferring to a fresh eppendorf tube, the supernatant was spun for a further 6 minutes and then the supernatant was transferred to a fresh eppendorf tube. DNA was precipitated with 2 volumes of ethanol. Then the DNA pellet was washed twice with 70% ethanol, air-dried and resuspended in 30 µl of TE.

2.2.3. Isolation of Peripheral Lymphocyte DNA (modification of Wyman and White, 1980)

Blood samples were collected in 10 ml tubes containing EDTA and were allowed to cool to room temperature before being stored at -70°C. For isolating lymphocyte DNA, the frozen blood sample was thawed and transferred to a falconer tube. Cell lysis buffer (0.32 M sucrose/10 mM TrisHCl/5 mM $MgCl_2$/1% Triton X-100) was added to the tube till 30 ml mark was reached. After mixing, the tube was left on ice for 30 minutes. The cell suspension was spun in the Jouan Centrifuge (4°C) at 3,500 rpm for 15 minutes. Supernatant was aspirated down to 5 ml then cell lysis buffer was added again to 30 ml mark. Centrifugation was repeated once more. The supernatant was carefully removed, 3.25 ml of Proteinase K buffer, 0.5 ml of 10% SDS and 0.2 ml of Proteinase K (10 mg/ml) were added and well mixed with the cell pellet. The tube containing cell suspension was sealed with parafilm, placed on a rotating wheel (10 rpm) and incubated overnight at 37°C. DNA extraction was performed twice with an equal volume of phenol and twice with an equal volume of chloroform : isoamyl alcohol (24:1). For DNA precipitation, 1/10 volume of 3 M sodium acetate (pH 4.6) and 2 volumes of cold ethanol was added to the tube and inverted several times until DNA precipitated. DNA was transferred to an eppendorf tube and washed twice with 70% ethanol. After desiccation, the DNA was

dissolved in 0.1 ml of TE (10 mM Tris HCl/0.1 mM EDTA). Gloves were used throughout the procedure and phenol and chloroform were handled in a fume hood.

2.2.4. Recovery of DNA from Agarose Gel

2.2.4.1. GeneClean

The following protocol was obtained from the BIO Inc product handbook (1989), the reagents used in this protocol were provided in the form of GeneClean II Kit. Agarose containing the DNA band was excised from the ethidium bromide (EtBr) stained agarose gel. The wet weight of each gel slice was recorded to estimate its volume (1 mg = 1 µl). Three volumes (to the volume of gel slice) of NaI stock solution was added to the gel slice in an eppendorf tube. The tube was placed in a 50°C water bath for 5 minutes and the contents were mixed by inverting every minute until the agarose was completely melted. Five µl of well-mixed "glassmilk" (silica matrix) suspension was added to the solution, mixed, and placed on ice for 5 minutes to allow DNA binding to the silica matrix. The silica matrix with the bound DNA was pelleted by spinning in an eppendorf centrifuge at 10,000 rpm for 5 seconds. The silica matrix was washed 3 times with 10 - 50 volumes of NaCl/ethanol/water. Then the DNA was eluted from the silica matrix with TE at 50°C for 2 - 3 minutes.

2.2.4.2. Electroelution (modification of Maniatis et al., 1982)

DNA was recovered from the low melting point (LMP) agarose gel or the agarose gel (Pharmacia) by electroelution after ethidium bromide (EtBr) staining and visualization with UV illumination. The dialysis tubing (Promega) used for electroelution was prepared by boiling for 10 minutes in one liter of 2% sodium bicarbonate/1 mM EDTA. After rinsing thoroughly in distilled water, the dialysis tubing was boiled for 10 minutes in distilled water, allowed to cool and stored in at 4°C. The tubing was washed inside and out with distilled water before use.

A slice of LMP agarose gel containing the DNA band was excised and placed into pretreated dialysis tubing containing 0.5 x TBE (1 x TBE: 89 mM Tris base, 89 mM boric acid and

10 mM EDTA). DNA was electrophoresed out of the gel slice at 100V for 2 - 3 hours in 0.5 x TBE. The current direction was reversed for 2 minutes and the buffer containing the DNA was recovered from the dialysis tubing. DNA was recovered by ethanol precipitation.

2.2.4.3. Phenol/chloroform Extraction (experience of Dr. E. Kremer, ACH)

DNA in LMP agarose gels could also be recovered by phenol/chloroform extraction. The gel slice was melted in an equal volume of water at 65°C. DNA extraction was performed sequentially with an equal volume of prewarmed (45°C) phenol, phenol/chloroform (1:1 v/v) and chloroform. Two volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 4.6) were added to the aqueous phase to precipitate DNA.

GeneClean was used most of the time to recover DNA fragments larger than 500 bp. However, if the DNA fragments were smaller than 500 bp, electroelution or phenol/chloroform extraction was used to recover the DNA fragments from an agarose gel. The DNA fragments recovered were further used as DNA probes or subcloned into plasmids.

2.2.5. Isolation of Genomic DNA from Yeast Cells (see section 2.6.4.)

2.3. SUBCLONING OF HUMAN DNA SEQUENCES INTO PLASMID

This protocol is a combination of the protocol in Maniatis et al. (1982) and the experience of Dr. M. Pritchard (ACH, Adelaide).

2.3.1. Preparation of Plasmid Vector DNA and Human DNA Inserts

Five hundred ng of plasmid vector pUC19 (Bresatec) was digested with an enzyme which cleaves the polylinker of pUC19 in a total volume of 20 µl at required temperature (usually 37°C) for 1 hour. Digestion was tested by running 1 µl of digested and undigested pUC19 DNA

samples side by side on a minigel, which was stained with EtBr and visualized under UV light. A single band of 2.7 kb for the digested sample indicated complete enzyme digestion.

Human DNA (cloned in YAC or in lambda) was digested with the same restriction enzyme that cleaved the plasmid vector. The digested DNA sample was checked on a minigel for complete digestion, then was extracted once with an equal volume of chloroform followed by ethanol precipitation.

2.3.2. Dephosphorylation of Plasmid Vector DNA

In order to prevent self-ligation of plasmid vector digested with a single restriction enzyme, the 5' terminal phosphate group was removed with alkaline phosphatase. The vector DNA was digested to completion with an appropriate restriction enzyme in a total volume of 20 µl. Then 2 µl (1/10 volume) of 10 mM EDTA (pH 8.0) and 1 µl of 2.8 u/µl Calf intestinal alkaline phosphatase (CIAP) (Boehringer) were added into the digests. The reaction was carried out at 37°C for 30 minutes, then 1 µl of CIAP (2.8 u/µl) was added and incubation was continued at 37°C for another 30 minutes. Then 5 µl of 5% SDS was added and the mixture was heated to 65°C for 10 minutes. After extraction with phenol/chloroform, DNA was precipitated with 2 volumes of ethanol. The DNA pellet was rinsed with two changes of 70% ethanol at room temperature to remove all traces of SDS, desiccated and dissolved in 100 µl of TE.

To test the efficiency of dephosphorylation, 1 µl of dephosphorylated vector DNA was ligated and transformed into *E. coli* strain MV1190. If the 5' terminal phosphate group was removed, the vector could not recircularize, therefore, only a few colonies were seen on the plate because linear form DNA is very inefficient in transformation. The vector was then ready for use.

2.3.3. Ligation Reactions

Ligation reactions were carried out with a vector : insert molar ratio of approximately 1 : 4 to maximize intermolecular ligation rather than intramolecular ligation. Usually, for 100 ng of linearised and phosphatase treated plasmid vector, 2 µl of 10 x ligation buffer [0.5 M tris HCl (pH 7.4), 0.1 M $MgCl_2$, 0.1 M dithiothreitol (DTT), 10 mM spermidine, 10 mM ATP and 1 mg/ml Bovine serum albumin (BSA)], 1 - 2 units of T4 DNA ligase (BIO-RAD) and insert DNA (~200 ng) were added and the reaction mixture (in a total volume of 20 µl) was incubated at 12 - 16°C overnight.

Usually a ligation control was set up simultaneously with 1 ng of PstI digested vector DNA for cohesive end ligation or 1 ng of SmaI digested vector DNA for blunt end ligation. After transformation, the number of colonies on the control plate indicated the efficiency of the ligation reaction. Alternatively, the efficiency of ligation could be checked by religation of the HindIII digested lambda DNA under the same conditions as the sample DNA. The religated and unreligated lambda DNA samples were separated on an agarose minigel. The disappearance of low molecular weight bands and increasing intensity of the large molecular weight bands indicated efficiency of the ligation reaction.

2.3.4. Introduction of Recombinant DNA into *E. coli* (modification of Chung et al., 1989)

E. coli strain MV1190 cells were made competent with a method modified from Chung et al. (1989). Stationary phase MV1190 cells from an overnight culture were diluted 1:100 (v/v) into 20 ml LB. The cells were grown at 37°C with constant shaking for 1.5 - 2 hours. The cells were pelleted by centrifugation in the Jouan centrifuge at 3,000 rpm for 10 minutes, then the cell pellet was resuspended in 2 ml (1/10 of the original volume) of ice cold fresh TEB [10 ml TEB contains 1 g polyethylene glycol (PEG) 3600 (Sigma), 500 µl Dimethyl sulfoxide (DMSO) (AJAX Chemicals, Univar) and 9 ml LB]. The cells were ready for use after leaving on ice for 10 minutes.

For transformation reactions, 1 µl or 5 µl of ligation reaction mixture was added to 100 µl of competent MV1190 cells and left on ice for 10 minutes. Then 30 µl of 0.1 M isopropylthio-ß-D-galactoside (IPTG) (BRL) and 30 µl of 0.2% 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) (BRL) were mixed with the cells and the mixture was spread onto agar plates (86 mm) containing 50 µg/ml ampicillin. The plates were inverted and incubated at 37°C for 16 hours.

2.4. ENZYME DIGESTION, GEL ELECTROPHORESIS AND SOUTHERN ANALYSIS

2.4.1. Restriction Endonuclease Reactions (product handbook of Biolabs)

Eight µg of human genomic DNA was cleaved overnight with 10 - 20 units of restriction endonuclease (Biolabs) in the compatible restriction buffer (Biolabs) in a total volume of 50 µl. The efficiency of digestion was monitored by separation of 5 µl digested DNA sample on an agarose minigel. The minigel was stained with EtBr and visualized under UV light. Complete digestion of genomic DNA was indicated by a smear in the lane, whereas, a thick high molecular weight band in the lane implied incomplete digestion of the DNA sample.

To ensure the enzyme activity was not affected by glycerol, the volume of restriction enzyme(s) should not exceed 1/10 of the final volume of reaction mix, especially when 2 or 3 different enzymes are used simultaneously.

2.4.2. Gel Electrophoresis (Maniatis et al., 1982)

DNA in the size range 0.5-15 kb was resolved by electrophoresis at 20V for 16 hours in 0.8 - 1.2% agarose gels. For human genomic DNA, 0.8% agarose gel was used for EcoRI digests, 1.0% gel for PstI digests and 1.2% gel for Sau3AI digests. Gels were cast and run in 0.5 or 1 x TBE. DNA samples were prepared by adding 0.1 volume of 10 x gel-loading buffer [0.1 M TrisHCl (pH 8.0), 0.2 M EDTA (pH 8.0), 2% Sarcosyl (w/v), 20% Ficoll 400 (w/v), 0.1% Bromophenol blue (w/v), 0.1% Xylene cyanol (w/v)].

Analytical agarose minigels containing 0.5 µg/ml ethidium bromide were electrophoresed at 100V for 1 hour.

2.4.3. Molecular Weight Markers

HindIII digested lambda DNA (Biolabs), EcoRI digested Spp1 phage (Bresatec) or DRIgest (Amersham) were used as molecular weight markers in southern blot analysis.

2.4.4. *Transfer of DNA from Agarose Gel to Nitrocellulose Filter (Maniatis et al., 1982)

After electrophoresis, DNA fragments in an agarose gel were denatured, transferred onto a nylon nitrocellulose filter (Hybond N+, Amersham) by the method modified from Southern (1975). The relative positions of the DNA fragments in the gel were preserved during their transfer to the filter.

The agarose gel was soaked in an ethidium bromide solution (0.5 µg/ml) for 20 minutes to stain the DNA, then rinsed in water to remove ethidium bromide traces on the surface of the gel. DNA was detected by fluorescence on a UV transluminator and photographed using Polaroid type 677 film (ASA 3000). Usually photos were taken with a ruler alongside the gel so that the positions of the molecular size markers could be read directly from the photographic image. After photography, the gel was soaked sequentially, with gentle shaking, in the following solutions :

1) 0.2 M HCl for 15 minutes to reduce the average molecular size of DNA (especially for HMW DNA on pulsed field gel).

2) 0.5 M NaOH/2.5 M NaCl for 30 minutes to denature double-stranded DNA.

3) 2.5 M Tris-HCl (pH 7.4)/1.5 M NaCl for 10-30 minutes to neutralize the gel.

Filters were prepared by soaking in deionised water and then in 10 x SSC (1 x SSC: 150 mM sodium chloride/15 mM sodium citrate) for 5-10 minutes. DNA in the agarose gel was transferred to the prepared filter in 10 x SSC by capillary action for 5 - 16 hours. DNA was

subsequently fixed to the filter by treating in 0.5 M NaOH for one minute, and then in 0.2 M Tris/2 x SSC (pH 7.5) for 1 minute. The filter was allow to dry at room temperature.

In some instances, vacuum blotting was used to accelerate the transfer. The LKB 2016 Vacugene Vacuum Blotting System uses a low pressure vacuum to transfer nucleic acids from a gel to a membrane within a short time (as short as 30 minutes). The system was operated according to the manufacturer's instructions.

2.4.5. ^{32}P-labelling of DNA Fragments

Double-stranded DNA fragments were ^{32}P-labelled using the multiprime DNA labelling system (Amersham) to incorporate ^{32}P-dCTP (Amersham) according to the protocol in the Amersham product handbook. Usually 20-30 ng of DNA was diluted with deionised water to a volume of 28 µl. The DNA solution was boiled for about 10 minutes to denature DNA. After cooling on ice, DNA was mixed with 10 µl of nucleotide/buffer solution, 5 µl of BSA/primer and 2 µl of enzyme (Klenow DNA polymerase). Then, 5 µl of ^{32}P-d CTP was added to the mixture behind a radiation protection screen. The reaction mixture (total volume of 50 µl) was incubated at 37°C for 1 - 1.5 hours or at room temperature overnight.

Labelled DNA probe could be used immediately or left at -20°C for later use. In some instances, unincorporated ^{32}P-dCTP was removed from labelled DNA by running the sample through a Sephadex G-50 (Pharmacia) column. The first peak detected by the radiation monitor was collected since it contained the ^{32}P-labelled-DNA. (Columns were run behind a radiation protection screen).

2.4.6. Hybridization of Southern Filter

^{32}P-labeled DNA or RNA were hybridized to DNA filters. Autoradiography was used to locate the position of any DNA complementary to the radioactive probe. This technique can be used to detect specific sequences in both genomic and cloned DNA.

The DNA filters were placed into a plastic bag, into which 20 ml of hybridization mix [1 M Na_2HPO_4/ NaH_2PO_4(pH 7.0), 7% SDS] was added. The filters were wetted evenly and the bag was sealed with as few bubbles as possible. The bag was placed flat in a plastic box in a 65°C water bath for prehybridization for at least 15 minutes with constant shaking. The ^{32}P-labelled DNA probe was boiled for 10 minutes to denature the DNA and left on ice for 5 minutes to prevent reannealing, just before it was put into the plastic hybridization bag. Hybridization was performed at 65°C for 6 - 16 hours. The filters were removed from the bag and washed sequentially in 2 x SSC, 0.5% SDS at 65°C for 30 minutes, and 0.1 x SSC, 0.1% SDS. The temperature and time for the second washing were adjusted according to the strength of the signal on the filter(s) detected by a radiation monitor. After washing, the filters were exposed to X0mat XK-1 film (Kodak) at -80°C in the presence of two intensifying screens.

To test whether two or more probes hybridized to the same fragment, the same filter was always hybridized sequentially with multiple probes. The first probe was removed from a filter by immersing the filter in boiling 0.5% SDS and allowing the solution to cool to room temperature. Then the filter was hybridized with the next probe.

2.5. PULSED FIELD GEL ELECTROPHORESIS (handbook of Pharmacia LKB 20.5)

Having the capacity of separating large DNA molecules up to several megabase pairs, pulsed field gel electrophoresis (PFGE) is very useful in analysis of the human DNA fragments cloned in yeast artificial chromosomes (YACs). In this project, Pulsaphor TM system (Pharmacia LKB 20.5) was utilized to construct restriction maps of human inserts in YACs.

In brief, the gel was prepared by casting 150 ml of 1% agarose in 0.5 x TBE directly into the gel frame (15 cm x 15 cm) on the gel support tray. For each sample, 50 - 100 µl of agarose beads containing high molecular weight (HMW) DNA (see section 2.6.4.) was loaded into the well using cut-off tips. PFGE was performed in 0.5 x TBE at 15°C for an appropriate time with a selected switch interval. The operation and application of the complete Pulsaphor system was according to the manufacturer's instructions.

2.5.1. Switching Interval

The most critical determinant of resolution is the switching interval (pulse time). Once the size of the human insert in a YAC was known, a variety of switching intervals were tested to separate the standard PFGE molecular markers (see section 2.5.3.). The pulse which gave good resolution at the size range of analysis, was then used to separate YAC DNA digests along with PFGE markers on either side of a gel. The switching intervals used for analysis of the two YACs in this project are described in Chapters 3 and 4.

2.5.2. Loading Agarose Beads (see section 2.6.4.) into the Wells

The agarose beads were loaded into the wells of pulsed field gel in two ways: while the gel was submerged in electrophoresis buffer (wet loading) or before the gel was put into the buffer (dry loading). When wet loading was used, the agarose bead digests were centrifuged and the liquid was removed as much as possible. Then 1/10 volume of 10 x gel-loading buffer was mixed with the agarose beads. The samples were loaded into the wells using cut-off tips. In dry loading, agarose beads were loaded into the wells without gel-loading buffer and the top of each well was sealed with 2 - 3 drops of low melting point agarose gel before the gel was put into electrophoresis buffer. Both methods worked equally well. Wet loading was used most of the time in this project. However, dry loading was later used instead because of its simplicity.

2.5.3. Molecular Weight Markers

Lambda DNA-PFGE (Amersham) and Lambda HindIII (Biolabs) were used as molecular weight markers independently or in combination in PFGE.

2.6. YEAST ARTIFICIAL CHROMOSOME (YAC) CLONING SYSTEM

YAC vectors have the capability of cloning DNA fragments of up to several hundred kilobase pairs in size and allow their propagation as linear artificial chromosomes in yeast

cells (Burke et al., 1987). This cloning system greatly improves genome mapping efficiency and accelerates the process of chromosome walking and gene cloning. In this project, a YAC cloning system was used to localize the fragile X locus. This approach has led to the characterization of the fragile X genotype (Chapters 4, 5; see also Yu et al.,1991).

2.6.1. YAC Vectors

Fig. 2 - 1 is a simplified map of a YAC vector (pYAC) (Burke et al., 1987). This vector is constructed from both yeast DNA sequences (hatched box) and the entire DNA sequences of plasmid pBR322 (solid line). It incorporates all necessary functions into a plasmid that can replicate in *E. coli*. As shown in the diagram, functional DNA sequences including centromere (CEN4), telomeres (TEL), autonomous-replication sequences (ARS1), selectable markers (TRP1, URA3) and SUP4 gene are derived from yeast DNA, whilst ampicillin-resistance gene (Amp) and origin of DNA replication (Ori) are from plasmid pBR322. The yeast autonomous-replication sequences (ARS1), centromere sequence (CEN4) and the telomere-repeat sequence (TEL) confer the DNA replication and mitotic/meiotic stability on the yeast artificial chromosome during propagation in yeast. Therefore, a YAC can replicate in the same manner as its host's chromosomes. The selectable marker URA3 is for positive selection of transformants in ura3 hosts. The ampicilin resistance gene (Amp) and the origin of DNA replication (Ori) are essential for growth and amplification of the YAC vector (pYAC) in *E. coli*. The cloning site in each vector is different, such as SmaI in pYAC2, SnaBI in pYAC3, EcoRI in pYAC4 and NotI in pYAC5, although all of the cloning sites are in SUP4 gene sequences. When extraneous DNA is cloned into the cloning site, SUP4, an ochre-suppressing allele of a tyrosine transfer RNA gene, is interrupted and thus produces red colonies instead of white ones. Since pYAC contains the ampicillin-resistance gene (Amp), *E. coli* cells containing any of the pYAC vector can grow in LB medium plus ampicillin.

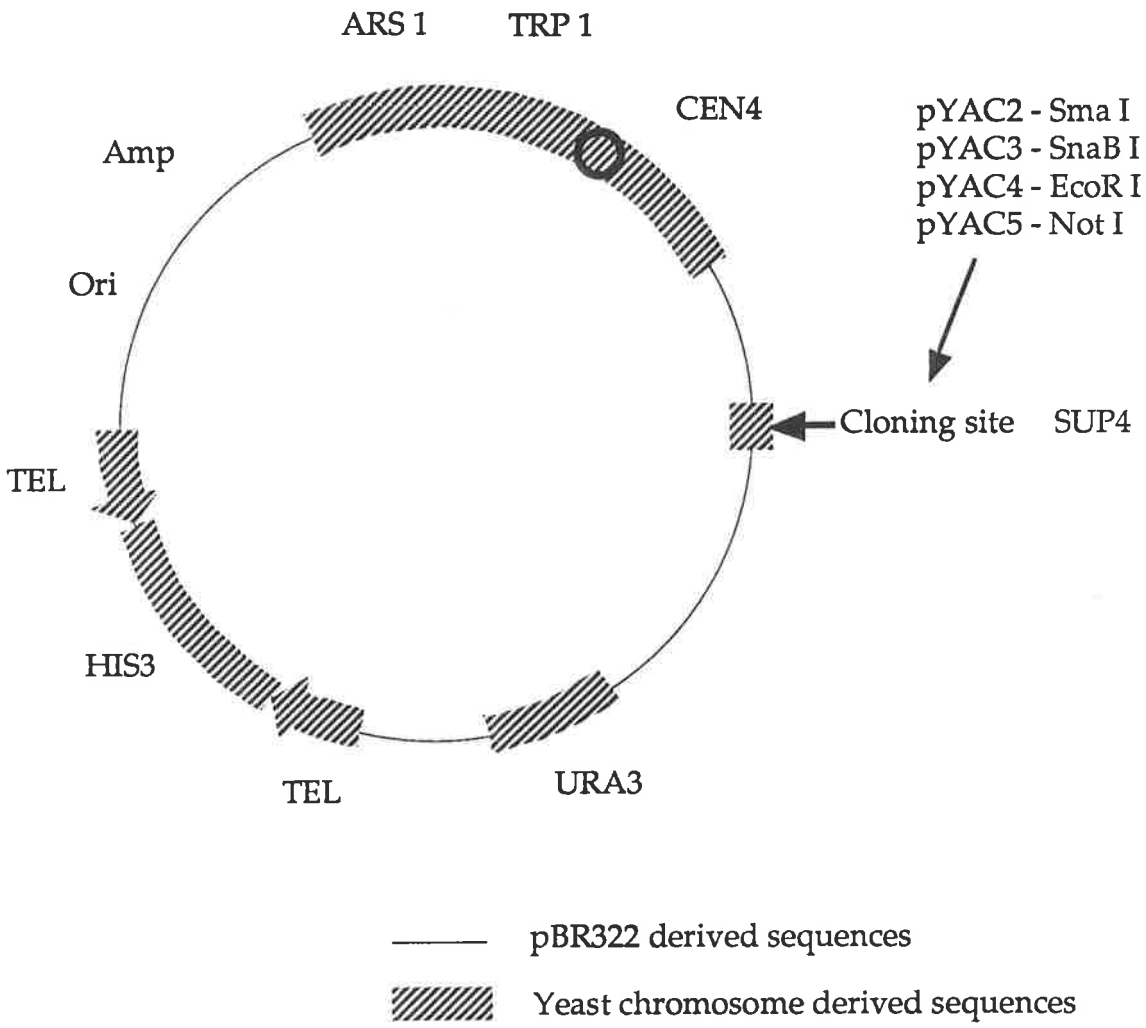


Fig. 2 - 1. The map of pYAC vector (adopted from Burke et al., 1987). The pYAC vector, approximately 11.3 kb in size, is derived from yeast DNA sequences (hatched box) and the entire pBR322 DNA sequences (thin line).

2.6.2. YAC Cloning Strategy

YAC libraries used in the present study were constructed by Dr. D. Schlessinger and his colleagues at the Washington University School of Medicine. The strategies for cloning large human DNA fragments into YAC vectors have been reported (Burke et al., 1987; Abidi et al., 1990). Fig. 2 - 2 illustrates the YAC cloning procedure with pYAC 2. Generally, the pYAC2 vector is prepared by double digestion with BamHI and SmaI (cloning site). As a result, three fragments are generated (see Fig. 2 - 2): the left arm (including the centromere), the right arm and a discard fragment (the BamHI fragment containing His3). The vector arms are dephosphorylated to prevent self-ligation. Human DNA inserts, obtained by partial digestion of HMW human DNA with a restriction enzyme that generates a blunt end (compatible with SmaI site), are ligated to the YAC vector arms. The ligation products are transformed into yeast spheroplasts. The transformants containing an extra linear form YAC are selected for complementation of a ura3 marker in the host by the URA3 gene in the vector. Then the transformants are screened for complementation of a host trp1 marker to ensure the YAC contains both arms of the vector. Finally, they are tested for loss of expression of SUP4 gene, which is interrupted by insertion of exogenous DNA at the SmaI cloning site (Burke et al., 1987). The expected structure of a YAC, shown on the bottom of Fig. 2 - 2, is that of a human DNA insert located between the right and left YAC vector arms.

2.6.3. YAC Clones Isolated with Human DNA Markers Close to FRAXA

DNA probes detecting markers close to the fragile X were used to screen the YAC libraries (see section 2.6.9). A number of yeast clones were isolated as a result of the screening by Dr. D. Schlessinger (St. Louis, USA) on a collaborative basis. Table 2 - 1 lists the names of the YAC clones isolated by various probes and the corresponding investigators responsible for their molecular characterization. Only the detailed studies of two YACs, XY-539 and XTY-26, are included in this thesis.

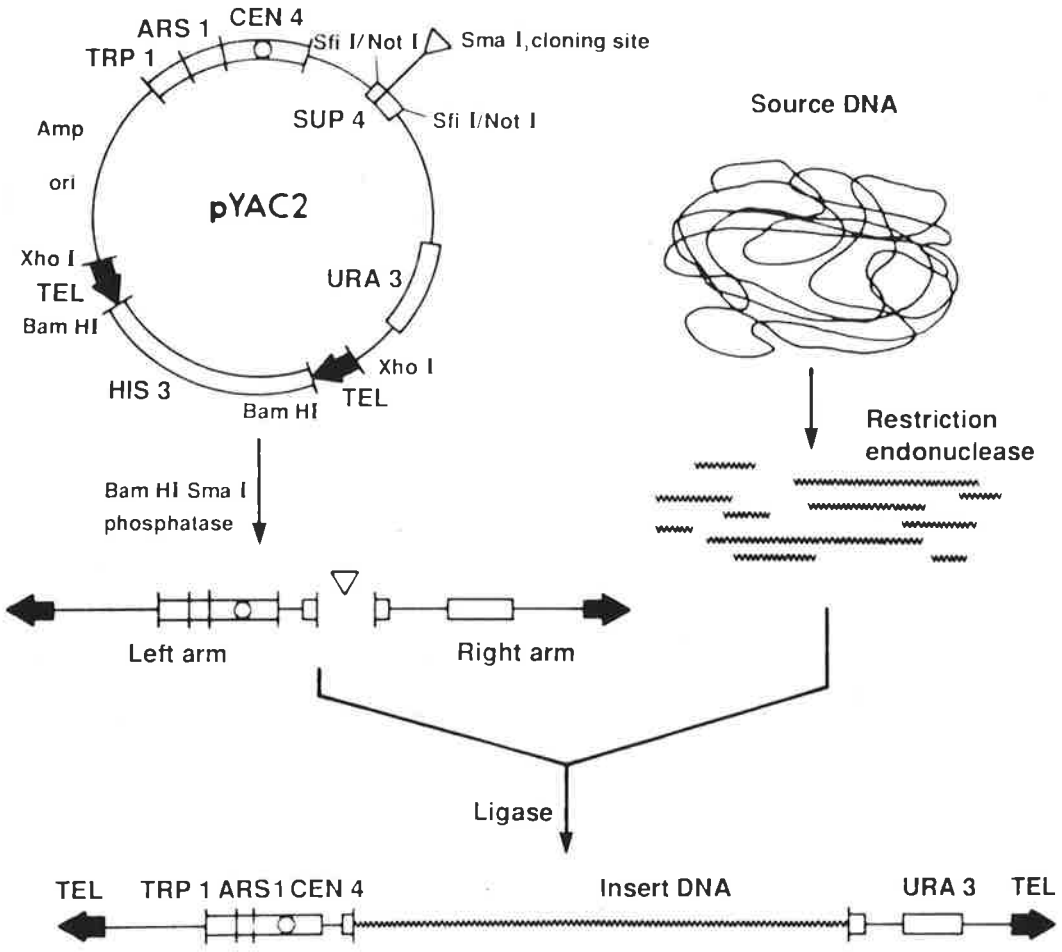


Fig. 2 - 2. Yeast artificial chromosome (YAC) cloning system (Burke et al., 1987). YAC vector pYAC2 is chosen to demonstrate YAC cloning procedure. The pYAC2 is digested with endonuclease BamHI and SmaI (cloning site). Three fragments are generated, the left arm (containing the centromere), the right arm and the discard BamHI fragment containing HIS3. The HMW human DNA is digested with the restriction endonuclease to generate large fragments with blunt ends (compatible with SmaI site). Then the two vector arms were ligated to either end of human DNA inserts.

Table 2 - 1. YACs Isolated by DNA Markers Close to FRAXA

DNA marker/Probe	YAC	YAC size (kb)	Investigator
DXS296/VK 21	XY-539	210	S. Yu
DXS293/VK16B3	XTY-26	280	S. Yu, E. Kremer
DXS295/VK18	XY-412	160	M. Pritchard
	XY-512	360	
	XY-861	340	
DXS297/VK23 B	XY202	80	E. Kremer
	XY436	900	
	XY497	460	
	XY686	280	
DXS296/XY-539-R*	A14D5	120	V. Kalatzis
	D49G8	470	

* Right end clone of XY-539 (see Fig. 3 - 9)

2.6.4. Isolation of HMW DNA from Yeast Cells (Overhauser and Radic, 1989)

Two hundred ml of AHC medium (1 liter AHC medium contains 6.7 mg of yeast nitrogen base, 10 mg of casein hydrolysate, 20 mg of adenine and 20 mg of glucose) in a one liter flask was inoculated with a yeast clone containing a YAC and grown at 30°C with constant shaking until stationary phase was reached (usually 3 - 4 days). After harvest, the yeast cells were embedded in agarose beads for further DNA preparation and restriction digestion to prevent shearing of the DNA (Overhauser and Radic, 1989).

In general, the method involves embedding yeast cells in agarose beads, preparing spheroplasts with zymolyase, lysing the spheroplasts with sarcosyl and eliminating proteins with proteinase K.

Yeast cells were pelleted at 3000 rpm for 10 minutes in the Jouan centrifuge at 4°C and the supernatant was removed. The yeast cell pellet was resuspended in 10 ml SE (75 mM NaCl/ 25 mM EDTA pH 8.0). After centrifugation, the cells were washed twice more with SE before being resuspended in 4 ml SE. The cell suspension and paraffin oil (AJAX Chemicals, labchem) were kept separately in a 45°C water bath. One percent low melting point agarose in SE was boiled and was put into the 45°C water bath. Five ml of 1% low melting point (LMP) agarose (45°C) was added to the cell suspension (45°C) in a 50 ml falconer tube and mixed well. Twenty ml of paraffin oil (45°C) was added to the cell suspension in agarose, then the mixture was swirled vigorously to form an emulsion. A 200 ml beaker containing 100 ml of ice cold SE and a magnetic stir bar was placed in an ice containing box on a stir plate set to medium speed. The emulsion was poured quickly into the ice cold SE. Stirring was continued for 5 to 10 minutes before the emulsion was transferred to several 50 ml falconer tubes. Centrifugation was performed at 3,500 rpm for 15 minutes in the Jouan centrifuge, then the mineral oil at the top of each tube was removed. After dispersion by repeated pipetting with a plastic pipette, the agarose beads were pelleted by centrifugation at the same speed. The supernatant was removed and the tube was wiped with a tissue to remove any excess mineral oil. Twenty ml of 1% SDS/25 mM EDTA was added to the beads and repeated pipetting was applied to break up any clumps. The

agarose beads were resuspended in 10 ml of SE containing 0.5 ml of 2-mercaptoethanol (Sigma) and 5 mg of zymolyase (Sigma) and incubated at 37°C for 2 hours. After centrifugation, the beads were resuspended in 20 ml of 1% (w/v) sarkosyl, 25 mM EDTA (pH 8.0) and 50 µg/ml proteinase K at 50°C overnight. After pelleting by centrifugation, the agarose beads were resuspended in 20 ml of TE containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) (Caution: PMSF is highly toxic and appropriate precautions are necessary). After having been washed at least twice in TE, the agarose beads were ready for use and stored at 4°C.

For restriction digestion, 50 - 100 µl of agarose beads from each sample were equilibrated with 1 ml of restriction buffer (1 x) for 10 minutes in an eppendorf tube followed by washing twice more with the buffer. Restriction endonuclease was added to the beads and the reaction was carried out at an appropriate temperature for about 16 hours.

2.6.5. End Probes for YAC Mapping (YAC-L, YAC-R and pUC19)

The YAC vectors were designed in such a way that both end probes were easily derived from pBR322. These end probes had no cross hybridization with either yeast or human genomic DNA sequences. They were particularly important in construction of a restriction map of a human insert in a YAC. When constructing the YAC vector (Fig. 2-1), yeast DNA SUP4 (containing the cloning site) was ligated into the BamHI site of pBR322 DNA (Fig. 2-3, position 375). The pBR322 DNA sequences from the BamHI site (position 375) to the PvuII site (position 2066) containing Amp and Ori form part of the centromere arm (left arm) of a YAC vector, the rest of the pBR322 sequences form part of the non-centromere arm (right arm). Therefore, any pBR322 DNA sequences in the left arm or the right arm of the YAC vector can be used as an end probe to detect the corresponding end of a human DNA insert in a YAC. In this project, pBR322 DNA was digested with three restriction enzymes (BamHI, PstI and NruI) to generate three DNA fragments. As shown in Fig. 2 - 3, YAC-L, a 1 kb BamHI-PstI fragment, detected the left arm of a YAC; while YAC-R, a 600 bp BamHI-NruI fragment detected the right arm. Plasmid vector pUC19 contains a PvuII-EcoRI fragment of pBR322 (from position

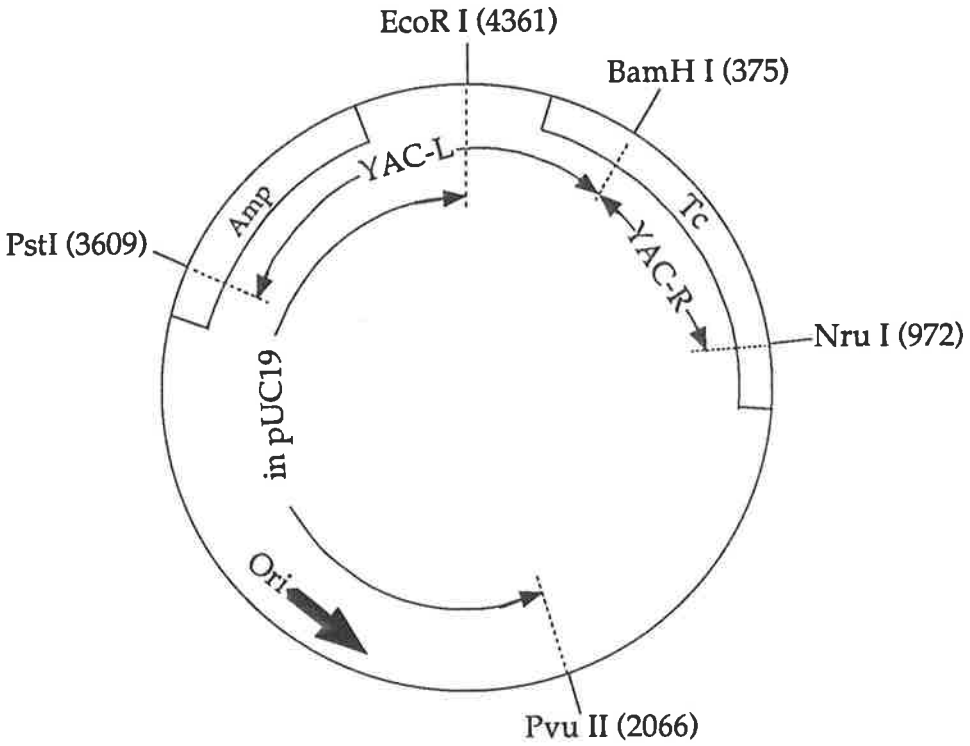


Fig. 2 - 3. Restriction map of pBR322 with indications of the YAC end probes. The entire pBR322 sequence is contained in pYAC vector, with the BamHI-PvuII fragment containing Amp and Ori in the left arm of a YAC vector and the rest of the pBR322 sequence in the right arm. For construction of the YAC restriction map, the BamHI-PstI fragment (YAC-L) or pUC19 containing the EcoRI-PvuII fragment was used as the end probe to identify the left arm of a YAC, whereas the BamHI-NruI fragment (YAC-R) was used to identify the right arm of a YAC.

2066 to 4361 containing Amp and Ori) (Fig. 2-3), so that it can be used as a end probe instead of YAC-L to identify the centromere arm of a YAC.

2.6.6. Cloning the Ends of a Human Fragment in YAC

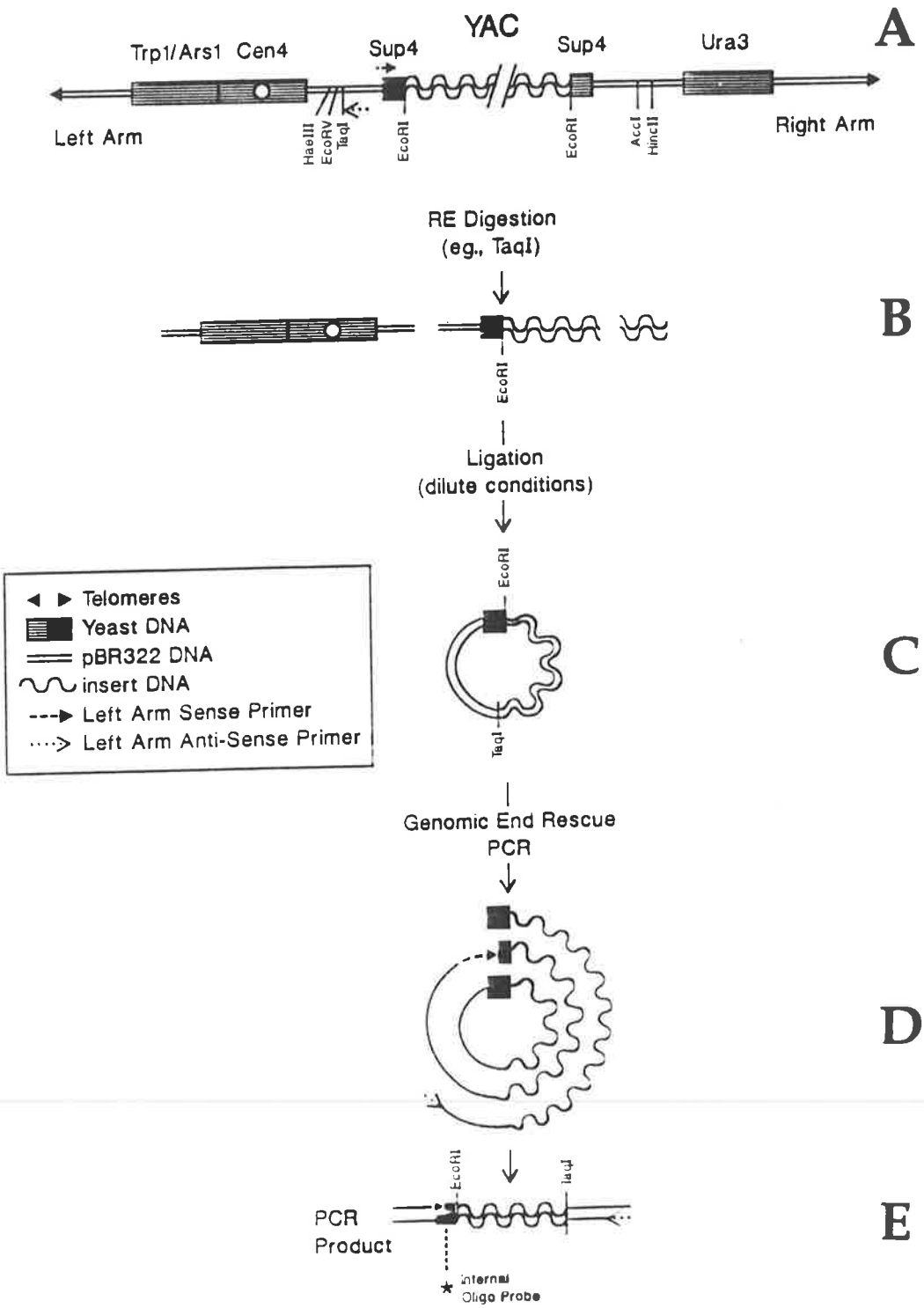
Subcloning both ends of the human insert in XY-539 was essential to orient the human fragment and to commence further chromosome walking towards the fragile X. Several approaches were reported to achieve this goal.

The plasmid rescue method, first described by Burke et al. (1987), was used to isolate human DNA sequences adjacent to the centromere arm (left arm). Since there was one XhoI site on each arm of the YAC vector (Fig. 2 - 2, map of pYAC2), when YAC DNA was digested with XhoI, two XhoI fragments at the vector/insert junction were generated. Both XhoI fragments had one XhoI site on the YAC vector adjacent to the TEL sequence and another XhoI site in source DNA. The XhoI digests were ligated under conditions that favor formation of monomer circles. The ligation products were then transformed into *E. coli* with ampicillin selection. Between the two XhoI fragments at the vector/insect junction, only one contained the pBR322 origin of Ori and Amp which was essential for plasmid replication and selection (Fig. 2-2). Therefore, only those cells carrying the XhoI fragment with Ori and Amp could form colonies, which were expected to contain human DNA sequences adjacent to the centromere arm of a YAC vector.

Inverse PCR method (Silverman et al., 1989) was another approach to isolating the ends of human fragment in a YAC (Fig. 2-4). With this method, YAC DNA was cleaved with a restriction enzyme, usually a four basepair cutter, to generate small fragments at the vector-insert junction. Then the restriction fragments were circularized (Fig. 2-4). Two primers were designed from the known vector sequences with opposite directions so that DNA synthesis would proceed across the cloning site (Silverman et al., 1989). With religated DNA as template, only the vector-insert junction fragment could give a PCR product.

Fig. 2 - 4. Demonstration of a left arm end rescue of genomic inserts in a YAC by inverted PCR (Silverman et al., 1989). A) The structure of a YAC with the human DNA sequence inserted into SUP4 gene between the left and the right arms of the YAC vector.

B) Generation of the vector-insert junction fragment. Enzyme TaqI has one known site close to the cloning site on the left arm of the YAC vector. C) Religation of the vector-insert junction fragment. D) PCR amplification of the vector-insect junction region with both primers from the pYAC vector sequences but with opposite directions. E) The PCR product. Human DNA sequences next to the left arm of the YAC vector is thus amplified.


A
YAC
Trp1/Ars1
Cen4
Sup4
Sup4
Ura3
Left Arm
Right Arm
HaeIII
EcoRV
TaqI
EcoRI
EcoRI
AccI
HincII
B
RE Digestion
(eg., TaqI)
EcoRI
Ligation
(dilute conditions)
C
Telomeres
Yeast DNA
pBR322 DNA
insert DNA
Left Arm Sense Primer
Left Arm Anti-Sense Primer
EcoRI
TaqI
Genomic End Rescue
PCR
D
E
EcoRI
TaqI
PCR
Product
Internal
Oligo Probe

2.6.7. YAC Mapping Strategy

To generate a restriction map of a human fragment in a YAC, complete digestion, partial digestion and double digestion with restriction endonucleases were performed. Since the human fragment in YAC is usually several hundred kilo base pairs in size, it is necessary to choose the enzymes that can generate large DNA fragments. The recognition sequences of these enzymes, such as NotI and NruI, are usually six to eight nucleotides in length and contain CpG. The CpG DNA sequence is infrequent or under-represented in the human genome, therefore, these enzymes cut very rarely in the human genome and generate large restriction fragments. These enzymes are therefore called "rare cutters" or "infrequent cutters", and are very useful in long range mapping.

Complete enzyme digestion is essential for YAC mapping. In general, high molecular weight YAC DNA is digested to completion with various "rare cutters" (Chapters 3 and 4). The restriction fragments are separated by PFGE, blotted onto membranes, and hybridized successively with ^{32}P labelled end probes, YAC-R, YAC-L or pUC19, as well as human DNA probes. The size of a restriction fragment detected by one end probe indicates the distance from the restriction site to the corresponding telomere, so the restriction map can be built up from both ends. Examples will be given in Chapter 3 and Chapter 4.

Complete digestion can generate a complete map only for the enzymes which have one or two cleavage sites in a human insert. If an enzyme has more than two cleavage sites in a human insert, partial digestion is essential to generate a complete map of all restriction sites. Partial digestion can be generated by either adding less enzyme or shortening digestion time. Shorter digestion time was found to give inconsistent results. However, lower enzyme concentration was found to generate reproducible and reliable results. To generate partial digestion of YAC DNA, enzyme concentration was initially tested from 0.001 unit to 10 units per 100 µl of agarose beads. At the right concentration, partial digestion was reproducible as long as the DNA sample was from the same agarose bead preparation. The restriction fragments were then separated by PFGE, blotted onto membranes, and hybridized successively with ^{32}P labelled YAC-L and

YAC-R. An ideal partial digestion pattern gave multiple bands although with different intensity. With a particular enzyme, the number of partial digest bands indicated the number of restriction sites; the sizes of the various bands suggested the distances from each restriction site to the probe end. The hybridization patterns with both end probes provided information not only for construction, but also for confirmation of a YAC map.

Double digestion was used to confirm the restriction map constructed by complete and partial digestions. In this instance, internal DNA probes as well as end probes were used. For double digestion, if both enzymes share the restriction buffer and the digestion temperature, they were added simultaneously. If both enzymes require different concentration of salt, the enzyme requiring lower salt was added first for an appropriate time (> 5 hours), and then the other enzyme plus additional salt were added. For enzymes demanding different temperatures, digestion was performed at the low temperature first.

2.6.8. Isolation of Unique Human DNA Sequences from YAC.

Methods for isolation of human-specific sequences from a YAC have been reported. As mentioned previously, plasmid rescue and inverse PCR were designed to isolate one or both ends of a human fragment in YAC (Burke et al., 1987; Silverman et al., 1989). Alu to Alu PCR was also applied to isolation of human DNA sequences from YACs (Nelson et al., 1989). However, the PCR products obtained with the above methods usually contain repetitive DNA sequences, which might give confusing results in further analysis. To obtain unique human DNA sequences, a subclone method was developed and proved to be practicable. Fig. 2 - 5 demonstrates the subcloning procedure step by step. YAC clone XY-539 DNA in agarose beads was applied to PFGE in order to separate the YAC from yeast chromosomes. To identify the gel section which contained the XY-539, a small proportion of the gel (2 - 3 lanes) was excised and used for Southern blot with VK21, a DNA probe that detected the YAC originally. Once the position of XY539 was determined, the corresponding region on the rest of the gel was excised. DNA in the gel slice was cleaved with PstI before being purified by GeneClean (see section

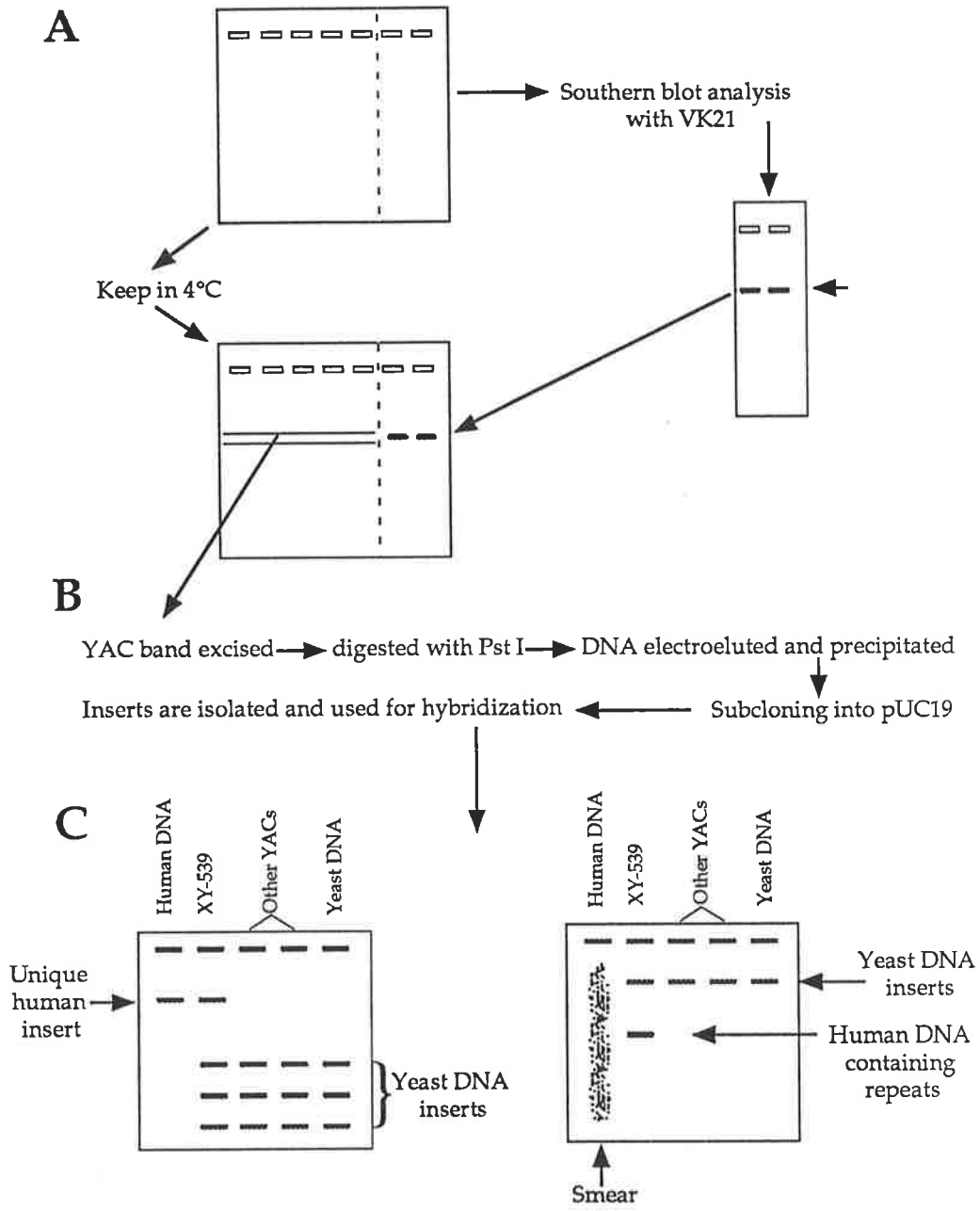


Fig. 2 - 5. Isolation of human DNA sequences by subcloning of YAC XY-539. A) the position of YAC XY-539 on a pulsed field gel is determined by hybridization with VK21(DXS296). B) YAC XY-539 DNA is isolated and subcloned into pUC19. C) The recombinants are randomly selected and the inserts of different sizes are hybridized simultaneously to the filter containing human DNA, DNA from YAC XY-539 and other YACs. Human DNA sequences are identified by hybridization only to human and XY-539 DNA.

2.2.4.1.). Then the DNA restriction fragments were ligated into the relevant sites of pUC19 and the ligation products were transformed into *E. coli* strain MV1190 (see section 2.3.4.). Recombinants (colourless colonies) were randomly selected and grown on a small scale. The inserts in those clones were carefully sized on a minigel and the band containing the inserts were excised. In order to identify unique human DNA sequences, each human insert could be tested individually to see if any one hybridized to a single band of human genomic DNA. In practice, however, this testing is too time consuming. To identify unique human DNA sequences more efficiently, a strategy was designed as follows: many inserts of different sizes were hybridized to one filter containing digested DNA samples from human, XY-539 and other YACs, and yeast. It was important that the DNA samples on the filter were digested with the same enzyme that was used to subclone the YAC DNA, so a unique human DNA fragment of YAC XY-539 could be recognized by its common size with a positive signal in the human track. Yeast DNA probes would give bands with common size in different YACs and yeast DNA, but no band in human DNA samples. Human DNA probes with repetitive DNA sequences gave smears in human tracks, a strong band in XY-539 DNA, and no band in other YAC DNA. In contrast, a unique human DNA probe detected bands of common size in human and XY-539 DNA, but not in DNA from other YACs. Furthermore, the size of the unique human DNA fragment(s) was indicated by the size of the hybridization band(s) seen at the human DNA track, so a unique human sequence can be identified from up to ten probes used simultaneously in single hybridization. Examples will be given in Chapter 3.

2.6.9. YAC Libraries

The two YAC clones analysed in this project were isolated from two different YAC libraries which were constructed by Dr. D. Schlessinger and his colleagues at Washington University, School of Medicine. Both YAC libraries were constructed from DNA of hybrid X3000.11 (Nussbaum, 1986a), which contained Xq24-Xq28 expressing the fragile site at Xq27.3. Since the human DNA in the hybrid X3000.11 was derived from a patient with Fragile X

syndrome, X3000.11 was thought particularly appropriate for characterization of the DNA sequences responsible for the fragile site and the associated syndrome.

2.6.9.1. Xq YAC Library

The library was constructed by ligation of partial EcoRI digested X3000.11 DNA with the pYAC4 vector. The vector had been digested with EcoRI (cloning site) and BamHI (Fig. 2 - 1). All the YAC clones containing human DNA inserts were selected on the basis of hybridization to total human DNA. The details of library construction are shown diagrammatically in Fig. 2 - 2. and have been published elsewhere (Burke et al., 1987). YAC XY-539 was isolated from this library by hybridization with VK21.

2.6.9.2. Telomeric YAC Library

This YAC library was also made from hybrid X3000.11 DNA by telomeric complementation. The YAC vector pTYAC1 could be digested with BamHI and either EcoRI or ClaI, to accommodate insert DNA digested with either EcoRI or TaqI (Riethman et al., 1989; Kremer et al., 1991b). X3000.11 DNA was partially digested with TaqI and ligated into the ClaI site of YAC vector pTYAC1 (Kremer et al., 1991b). As shown in Fig. 2 - 6, the YAC constructed in this way should have one telomere from the YAC vector and the other from the source DNA. However, circularized products are also expected. Circular YAC XTY-26 was the only positive clone in this library when probed with VK16B3.

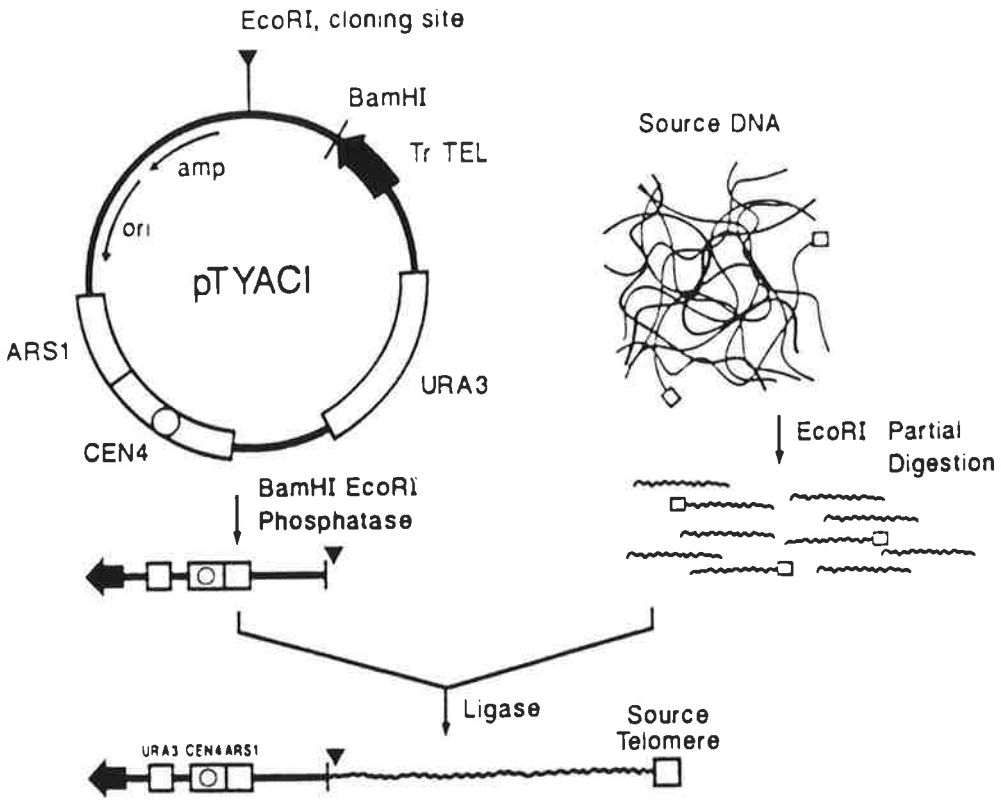


Fig. 2 - 6. Telomeric YAC cloning system (Riethman et al., 1989). The YAC vector pTYAC1 is digested with EcoRI (cloning site) and BamHI and treated with phosphatase. Only one arm is generated which contains a centromere and a telomere. Source DNA is partially digested with EcoRI and ligated to the vector arm. This YAC vector only provides one telomere for a YAC, the other telomere of the YAC is derived from the source DNA.

CHAPTER 3

STUDY OF A 210 kb DNA REGION AT DXS269 DISTAL TO FRAXA

3.1. SUMMARY

YAC XY-539 was isolated from an Xq YAC library by hybridization to VK21. It contained a 210kb human DNA fragment. A detailed rare cutting restriction enzyme map of the 210kb human insert in XY-539 was generated by application of pulsed field gel electrophoresis (PFGE). XY-539 was shown to produce restriction fragments identical in mobility to fragments detected by the same probes in human genomic DNA digested with the same enzymes. Therefore, gross rearrangement is unlikely to be involved in the human insert in YAC XY-539. Orientation of the human fragment in XY-539 was determined with a YAC contig extending to DXS295 (VK18), a marker known to be distal to DXS296 (VK21). In addition, several human DNA fragments were subcloned from XY-539. This experiment not only generated the restriction map of XY-539, which provided more detailed information for the human genome map in this region, but also facilitated the introduction of techniques which are ultimately essential to the isolation and characterization of the fragile X mutation.

3.2. INTRODUCTION

Linkage study and physical mapping generated a map of the DNA markers at the fragile X region. The order of DNA markers around the fragile X was established as : cen-//-DXS297(VK23)-DXS293(VK16), FRAXA-DXS296(VK21)-DXS295(VK18)-//-Xqter (Suthers, et al., 1990, 1991a, 1991c), with the genetically closest probe VK21 being only 1 cM distal to FRAXA (Suthers, et al., 1989).

The YAC cloning system provided an ideal tool to cover that genetic distance, because YAC vectors had the advantage of carrying DNA fragments of several hundred kilobase pairs, 10 - 20 times larger than that cosmid or lambda vectors could carry.

Based on the availability of the DNA probes which detect markers around the fragile X region, and the YAC cloning system, an initial scheme for cloning the fragile X was proposed. That was 1) screening for YAC clones containing the closest DNA markers; 2) mapping and orientating the human fragments in those YAC clones; (3) establishing the integrity of the human DNA inserts; (4) isolating the end sequences of the human inserts in YACs for chromosome walking until the fragile X region was covered.

XY-539, a yeast clone isolated by VK21, was thought to be a good starting point for "walking" toward the fragile X. The initial study of XY-539 was part of the process to achieve the above goals. In this chapter, the molecular analysis of XY-539 will be described, including the construction of a restriction map, isolation of human sequences, establishment of the integrity and orientation of the human fragment, as well as the experience obtained from these experiments.

3.3. MATERIALS AND METHODS

3.3.1. Yeast Clone

Yeast clone XY-539 was recovered from the Xq YAC library (see section 2.6.9.1.) by hybridisation with VK21 (Abidi et al., 1990), and was kindly provided by Dr. D. Schlessinger (St. Louis, USA) on a collaborative basis.

3.3.2. DNA Probes

Probes YAC-L, YAC-R and VK21D were used to construct the restriction map of XY-539. The origins of YAC-L and YAC-R were described in detail in Chapter 2 (see section 2.6.5., Fig. 2 - 3). VK21D is a subclone of VK21, which has been described elsewhere (Yu et al., 1989).

Probes pS3 (0.9 kb), pS5 (2.1 kb) and pS8 (0.8 kb) are single copy plasmid subclones of XY-539.

For YAC map construction, the human DNA probes cloned in plasmid were purified from the plasmid vector to avoid vector hybridization signal. All DNA probes were radiolabelled by using the multiprime DNA labelling system (Amersham).

3.3.3. Restriction Enzyme Digestion with Infrequent Cutters

To generate the restriction map of XY-539, complete digestion of XY-539 DNA was performed with restriction endonucleases MluI, NruI, NotI, SfiI, SalI, SacII, NaeI, BssHII, PvuI, NarI or RsrII (Biolabs), according to the manufacturer's instructions. SalI and SfiI partial digestions were initially tested with enzyme concentrations ranging from 0.001 to 10 units per 100 µl of agarose beads and finally achieved with enzyme concentrations ranging from 0.05 to 0.5 unit per sample for both enzymes.

In order to verify the DNA integrity in XY-539, DNAs from XY-539 and from a lymphoblastoid cell line constructed from a normal male were double digested with MluI and NruI. The restriction fragments were separated side by side by PFGE and transferred onto membrane. The DNA blot was hybridized with VK 21D. The existence of restriction fragments of common size in both samples was taken as a confirmation of the DNA integrity in XY-539.

3.3.4. Pulsed Field Gel Electrophoresis (PFGE)

Switch intervals of 10 or 15 seconds were applied in PFGE in order to separate DNA fragments in the arrange from 10 kb to 210 kb, since the size of the human insert in XY-539 was determined as 210 kb.

3.3.5. End Rescue and Subcloning

To clone the ends of human insert in XY-539 for chromosome "walking", plasmid rescue and inverse PCR methods (see section 2.6.6.) were tried but were not successful. At the same time, a YAC subcloning strategy was used to isolate human inserts from XY-539 (see section 2.6.8. and Fig. 2 - 5 for technical detail). Briefly, the YAC DNA was isolated from the yeast chromosome background by PFGE and then digested with PstI or PstI/HindIII. The restriction fragments were ligated into pUC19, and then the ligation products transformed into *E. coli* strain MV1190. Transformants were randomly picked and DNAs were prepared on a small scale. The DNA inserts were sized on a minigel and all of the DNA inserts with different sizes were used as probes against blots that contained total human DNA, XY-539 DNA, and DNAs from other YACs. Up to nine inserts with different sizes were used simultaneously as probes for hybridization. The human DNA sequences could be identified by the presence of common size bands in the human and XY-539 DNA tracks.

3.4. RESULTS

3.4.1. Construction of a Restriction Map of XY-539

The restriction map of XY-539 was based on the data obtained from complete and partial enzyme digestions. The enzymes used were NruI, MluI, NotI, SalI, SfiI, ClaI, NaeI, NarI, PvuI, SacII, RsrII and BssHII.

With complete enzyme digestion, YAC-L detected a 20 kb NruI fragment (Fig. 3 - 1A, lane 2), while YAC-R detected a 190 kb NruI fragment (Fig. 3 - 1B, lane 2). This indicated that XY-539 contained only one NruI site which located 20 kb in from YAC-L (or 190 kb in from YAC-R) (Fig. 3 - 9A). Similarly, a single MluI site was mapped 140 kb in from YAC-L (or 70 kb in from YAC-R), and a unique BssHII site was located 25 kb in from YAC-L (or 185 kb in from YAC-R) in XY-539 (Fig. 3 - 9A). For SalI, the situation was slightly different. YAC-L detected a 20 kb SalI fragment (Fig. 3 - 1A, lane 12) while YAC-R and VK21D identified the same 110 kb SalI fragment (Fig. 3 - 1B, lane 12, Fig. 3 - 4, lane 6). It appeared that XY-539 contained at least two

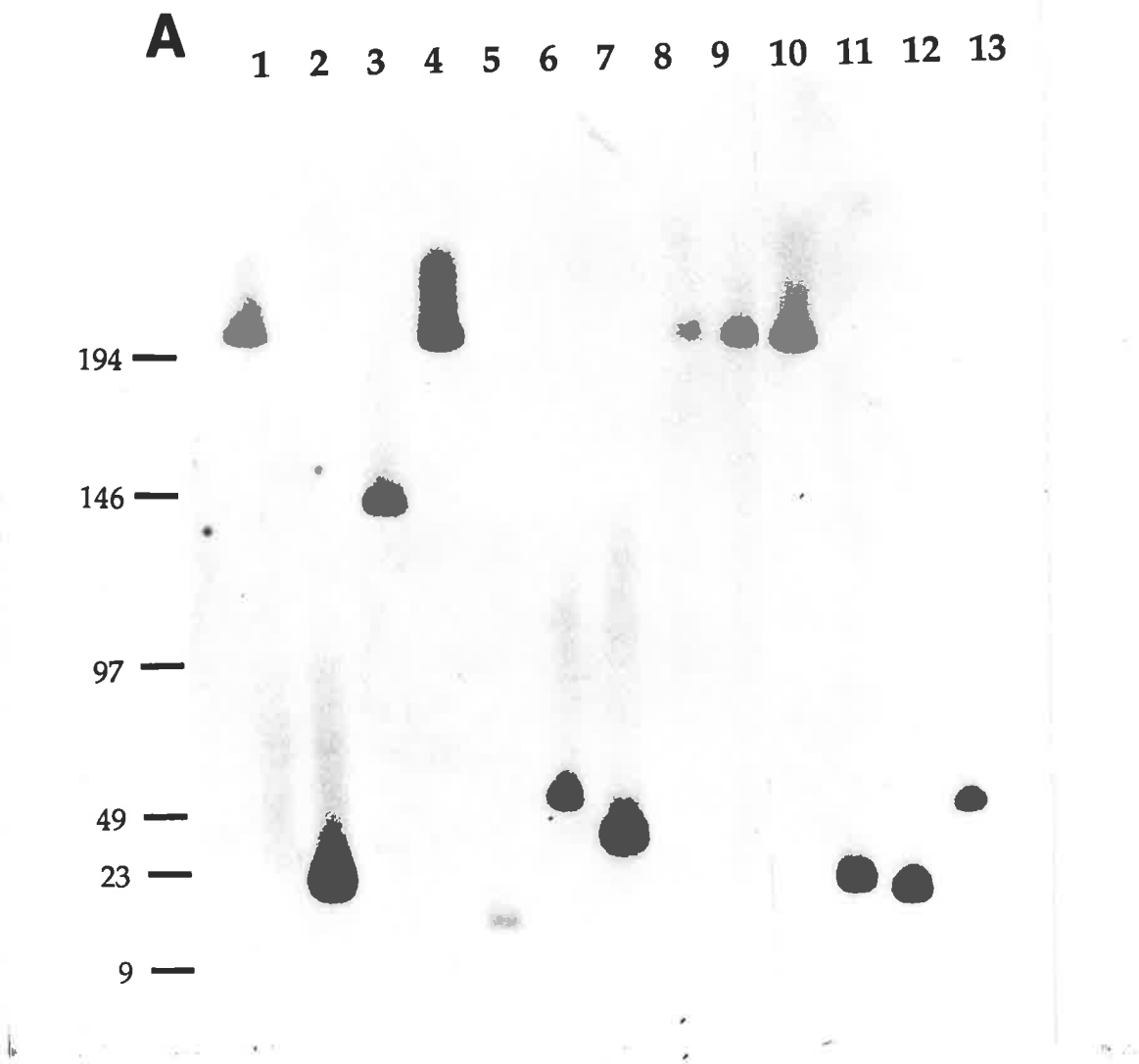


Fig. 3 -1. (**Panel A**) End-label mapping of XY-539 with different rare cutting restriction enzymes. A single blot containing uncut XY-539 DNA (lane 1) and XY-539 DNA digested with NruI (lane 2), MluI (lane 3), NotI (lane 4), ClaI (lane 5), NaeI (lane 6), NarI (lane 7), PvuI (lane 8), SacII (lane 9), RsrII (lane 10), BssHII(lane 11), SalI (lane 12), and SfiI (lane 13) was sequentially hybridized with ^{32}P labelled YAC-L (**Panel A,** this figure) and YAC-R (**Panel B,** the figure on the next page). The sizes (in kb) of DNA markers are indicated on the left of the gel. Faint bands in panel B are indicated with arrows.

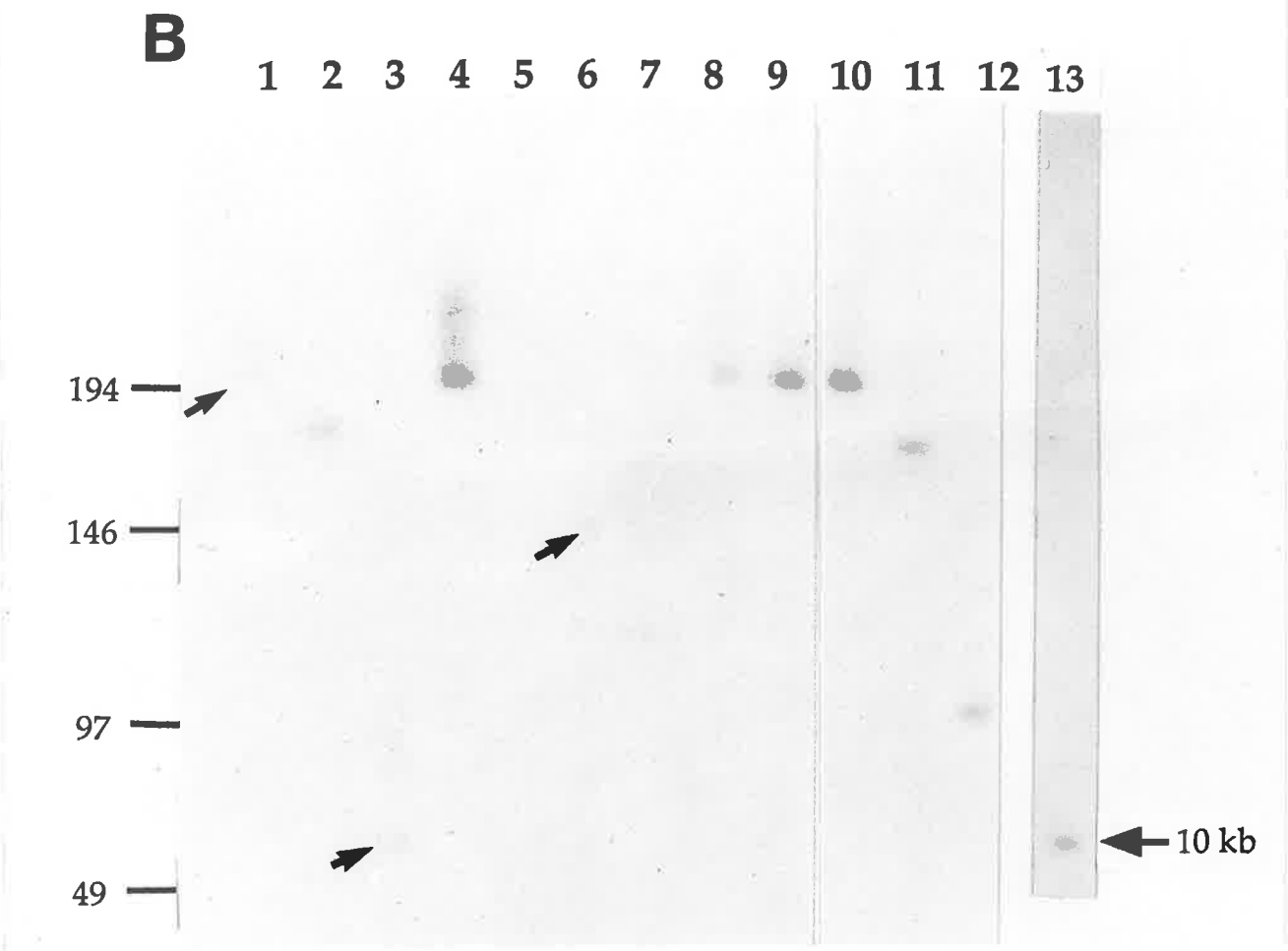


Fig. 3 -1. (**Panel B**) End-label mapping of XY-539 with different rare cutting restriction enzymes. A single blot containing uncut XY-539 DNA (lane 1) and XY-539 DNA digested with NruI (lane 2), MluI (lane 3), NotI (lane 4), ClaI (lane 5), NaeI (lane 6), NarI (lane 7), PvuI (lane 8), SacII (lane 9), RsrII (lane 10), BssHII(lane 11), SalI (lane 12), and SfiI (lane 13) was sequentially hybridized with ^{32}P labelled YAC-L (**Panel A,** the previous figure) and YAC-R (**Panel B,** this figure). Lane 13 in this figure is from a different filter. The sizes (in kb) of DNA markers are indicated on the left of the gel. Faint bands in panel B are indicated with arrows.

SalI sites with one SalI site 20 kb in from YAC-L, and another SalI site 110 kb in from YAC-R. Because these two SalI fragments did not add up to 210 kb, any SalI site(s) between these two SalI sites could not be determined by using this strategy. No size change was observed with enzymes NotI, PvuI, SacII and RsrII when both end probes were used (Fig. 3 - 1A and B, lanes 4, 8, 9, 10). This indicated that the human insert in XY-539 did not contain recognition sites for these enzymes. The size of restriction fragments detected by both end probes are listed in Table 3 - 1.

XY-539 was partially digested with SalI and SfiI to establish a complete map for these two enzymes which have more than two cleavage sites in XY-539. The size of partial-digest fragments produced by SalI and SfiI are listed in Table 3 - 2. With SalI partial digestion, both YAC-L and YAC-R detected three partial-digest bands, as well as the undigested YAC band. The partial-digest bands detected by YAC-L were 20 kb, 50 kb and 100 kb in size (Fig. 3 - 2 A); while those detected by YAC-R were 110 kb, 160 kb and 190 kb (Fig. 3 - 2 B). The results obtained with these two end probes are in accordance with each other, indicating that there are three SalI sites in the human insert in XY-539, and they are 20 kb, 50 kb and 100 kb in from YAC-L (or 110 kb, 160 kb and 190 kb in from YAC-R) (Fig. 3 - 9A).

With the SfiI partial digestion of XY-539, YAC-L detected two bands (50 kb and 75 kb) as well as the undigested YAC band (Fig. 3 - 3A), indicating there are two SfiI sites in XY-539 and that they are 50 kb and 75 kb in from YAC-L. Hybridization with YAC-R detected an additional SfiI site very close (10 kb) to the YAC-R end (Fig. 3 - 3B). Theoretically, YAC-R should detect two more SfiI partial-digest-bands of 135 kb and 160 kb in size, but the 135 kb band is very faint, and the 160 kb band is hardly visible (Fig. 3 - 3B), implying preferential cleavage of the SfiI site which was 10 kb in from YAC-R.

Combining the data of the complete and partial digestions, the "rare cutting" restriction map of XY-539 was determined and is shown in Fig. 3 - 9A. VK21 is located between the MluI and SalI sites (Fig. 3 - 9B) by its hybridization to the 110 kb SalI fragment and the 140 kb MluI fragment (Fig 3 - 4, lane 2, lane 6).

Table 3 - 1. Sizes (kb) of Restriction Fragments of XY-539 Detected by Three DNA Probes

	DNA probe		
Enzyme	YAC-L	YAC-R	VK21D
NruI	20	190	190
MluI	140	70	140
NotI	210	210	210
SalI	20	110	110
SfiI	50	10	125
ClaI	15	-	nd
NaeI	45	150	nd
NarI	30	-	nd
PvuI	210	210	nd
SacII	210	210	nd
RsrII	210	210	nd
BssHII	25	185	nd

- no visible band. nd -- not done.

Table 3 - 2. DNA Fragment Size (in kb) of XY-539 Partially Digested with SalI or SfiI

	DNA probe	
Enzyme	YAC-L	YAC-R
SalI	20	110
	50	160
	100	190
SfiI	50	10
	75	135
	200*	160**

* not resolved from undigested YAC DNA (210 kb).

**hardly visible, possibly due to the preferential enzyme cleavage.

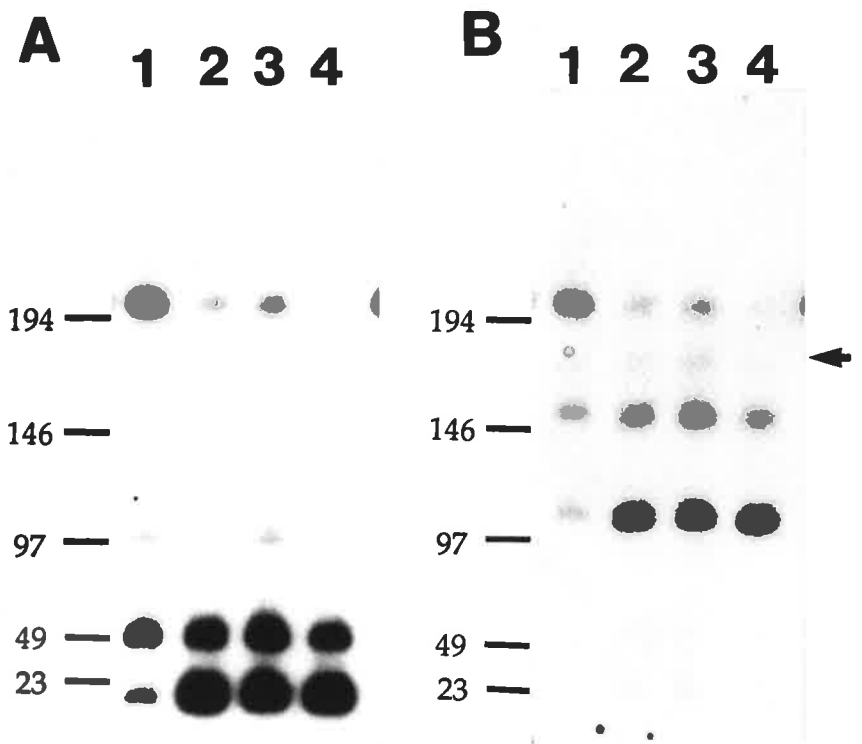


Fig. 3 - 2. End-label mapping of XY-539 with SalI. One hundred µl of agarose beads containing XY-539 DNA was digested with SalI at various concentrations (0.05 unit, lane 1; 0.1 unit, lane 2; 0.3 unit, lane 3; and 0.5 unit, lane 4) to produce partial restriction digest fragments. These were then separated by PFGE. After blotting, the filter was sequentially hybridized with YAC-L (panel A) and YAC-R (panel B). The size of molecular weight markers (kb) is indicated on the left of the autoradiographs. The faint bands in panel B are indicated with an arrow.

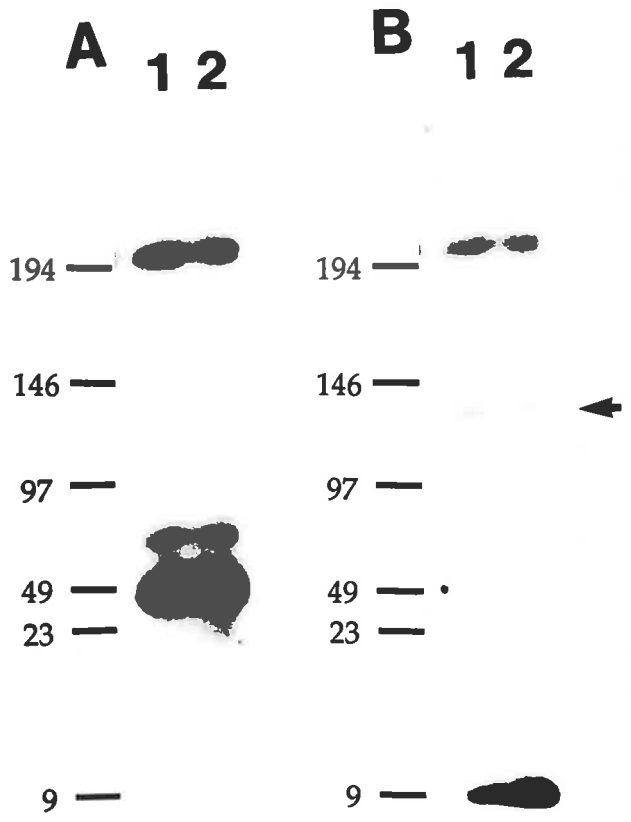


Fig. 3 - 3. End-label mapping of XY-539 with SfiI. One hundred µl of agarose beads containing XY-539 DNA was digested with 0.1 unit of SfiI (lane 1 and 2). The SfiI partial restriction digest fragments were then separated by PFGE. After blotting, the filter was sequentially hybridized with YAC-L (panel A) and YAC-R (panel B). The size of molecular weight markers (kb) is indicated on the left of the autoradiographs. Faint bands in panel B are indicated with an arrow. Interpretation of the restriction pattern is described in the text.

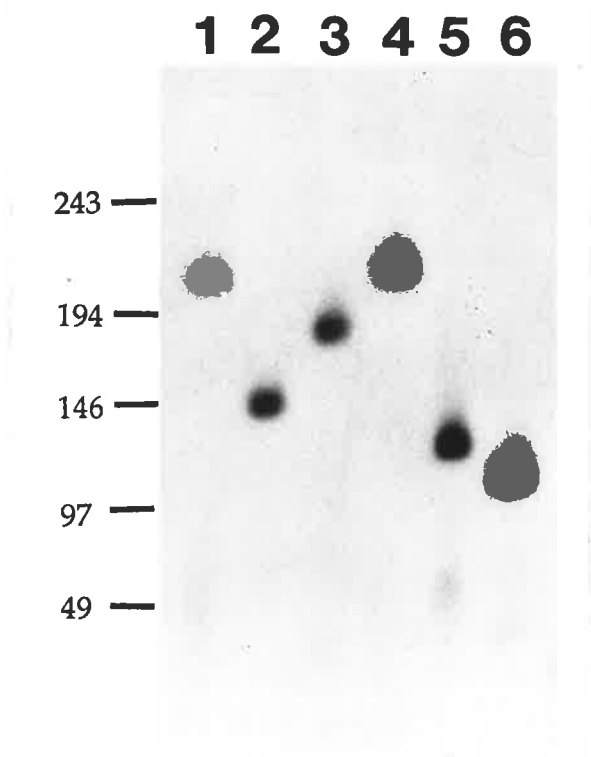


Fig. 3 - 4. Restriction fragments of XY-539 detected by VK21. A blot containing uncut (lane1), MluI (lane 2), NruI (lane 3), NotI (lane 4), SfiI (lane 5), SalI (lane 6) digested XY-539 DNA was hybridised with VK21D (a subclone of VK21). The size of molecular weight markers (in kb) is shown on the left of the autoradiographs.

3.4.2. Verification of DNA Integrity in XY-539

Probe VK21D detected a common band of 120 kb in both normal human genomic DNA and XY-539 DNA double-digested with MluI and NruI (Fig. 3 - 5), suggesting that at least a large proportion of the human insert in XY-539 is truly representative of the corresponding region in the human genome.

3.4.3. Human DNA Fragments Subcloned from XY-539

YAC XY-539 was subcloned into pUC19 in order to isolate unique human DNA fragments. Six subclones containing human DNA fragments (Table 3 - 3) were identified from 62 randomly selected recombinants in a period of three months.

Three of them were single copy human DNA sequences (Fig. 3 - 6), while the other three contained repetitive DNA sequences (one of them, pS4, is shown in Fig. 3 - 7). The three unique human DNA sequences were further localized on the map of XY-539 (Fig. 3 - 9 C), by their hybridization patterns (Fig. 3 - 8).

3.4.4. Orientation of XY-539

The ends of the human insert in XY-539 (XY539-L and XY539-R) were provided by Dr. D. Schlessinger's laboratory (St. Louis, USA). They were used to screen for overlapping YAC clones. One YAC clone, D49G8, isolated by XY-539-R, was later shown to contain both VK21A and VK18 (Kalatzis, 1991). Since VK18 is known to be distal to VK21 by PFGE (Hyland, unpublished observation), the orientation of XY-539 was thus determined with XY539-R being telomeric, and XY539-L centromeric.

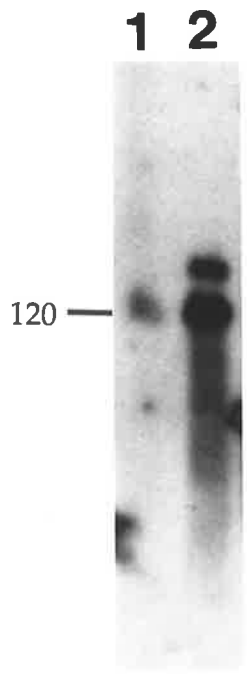


Fig. 3 - 5. MluI and NruI double digests of human (lane 1) and XY-539 (lane 2) DNA probed with VK21D. A 120 kb common band is present in both samples. The additional appoximately 140 kb band in lane 2 is identical in size to the band seen in MluI digest (data not shown), and thus it probably resulted from incomplete NruI digestion.

Table 3 - 3. Human DNA Fragments Subcloned From YAC XY-539

Subclone	Size of DNA insert (kb)	Restriction fragment	Contain repetitive sequences
pS2	1	PstI/HindIII	+
pS3	0.9	PstI/HindIII	-
pS4	5.2	PstI	+
pS5	2.1	PstI	-
pS6	2.2	PstI	+
pS8	0.8	PstI	-

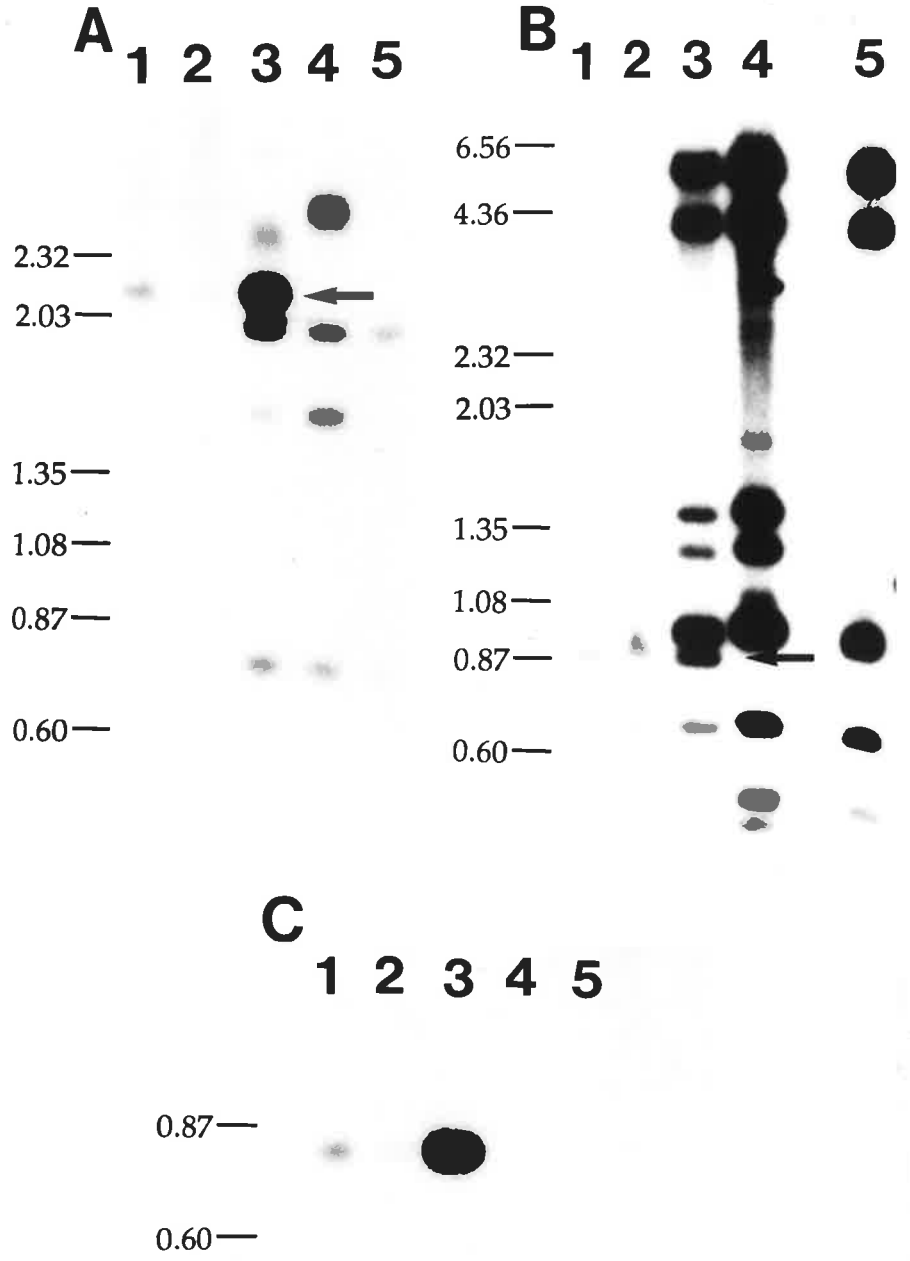


Fig. 3 - 6. Identification of single-copy human DNA fragments from subclones of XY-539. Single-copy human DNA fragments were identified by hybridization of the subclone inserts to a blot containing DNA samples of human (female DNA in lane 1, male DNA in lane 2), XY-539 (lane 3), XY-497(lane 4) and yeast (lane 5). A) four subclone inserts (PstI fragment) were probed to a PstI blot simultaneously. Only one insert (pS5) of 2.1 kb in size hybridized to the DNA of human and XY-539 (pointed with an arrow). The other three were non-human DNA sequences. B) nine subclone inserts (PstI/HindIII fragment) were hybridized to a PstI/HindIII blot simultaneously. Only one insert (pS3) of 0.9 kb in size hybridized to the DNA of human and XY-539 (pointed with an arrow). The other eight are non-human DNA sequences. C) the insert pS8 hybridized to the DNA of human and XY-539.

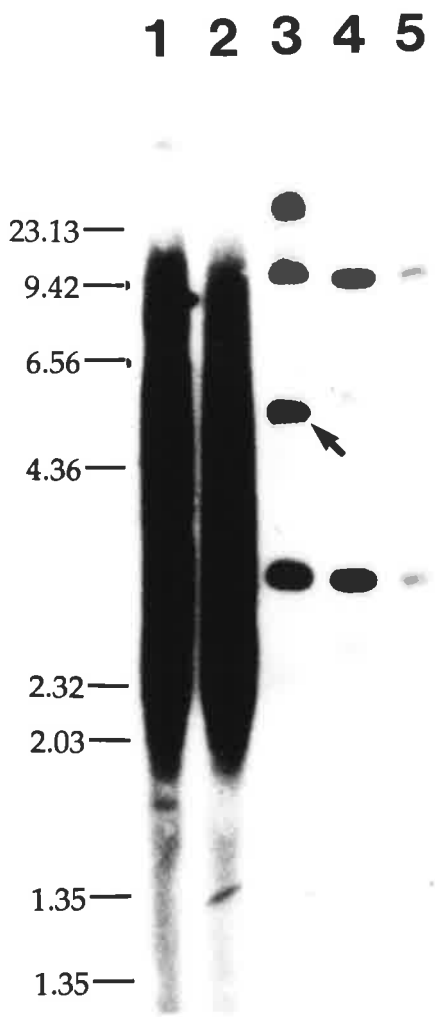


Fig. 3 - 7. Identification of human DNA fragment containing repetitive sequences. Three inserts (PstI fragments) were hybridized to the blot containing PstI digested DNA samples: Lane 1, female DNA; lane 2, male DNA; lane 3, XY539 DNA; lane 4, XY497 DNA; lane 5, yeast DNA. Insert pS4 (5.2 kb) detects a single band in XY-539 (see arrow) and a smear in total human DNA samples, and therefore is likely to be a human DNA fragment containing repetitive sequences.

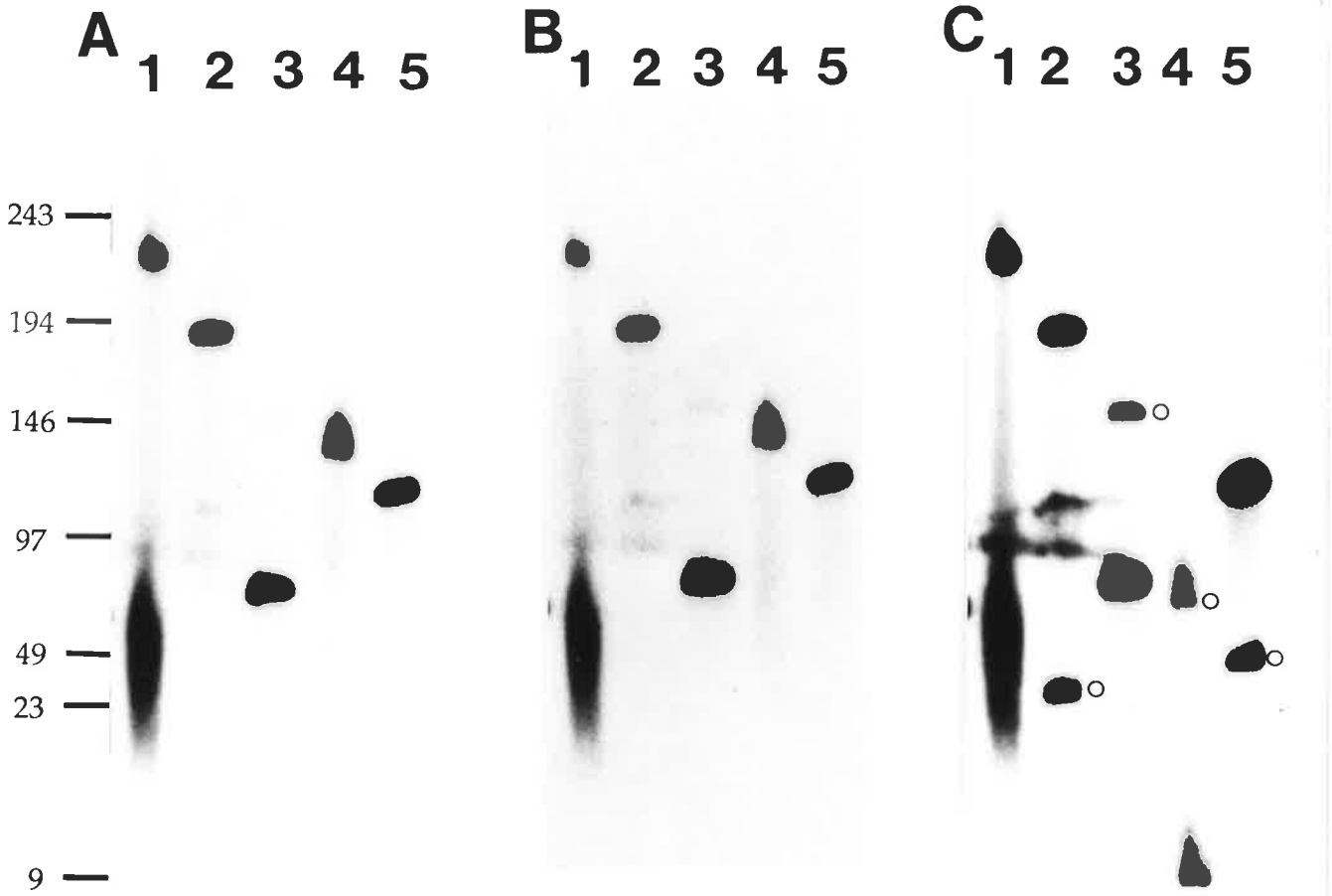


Fig. 3 - 8. Localization of subclones to the map of XY-539. DNA of XY-539 digested with different enzymes (lane 1, uncut; lane 2, NruI; lane 3, MluI; lane 4, SfiI; and lane 5, SalI), was hybridized with ^{32}P labelled pS3 (panel A), pS8 (panel B) and pS5 (panel C). Both pS3 and pS8 were localized to the same region on the XY-539 map (see Fig. 3 - 9C) by hybridization to the 70 kb MluI fragment (panel A lane 3, panel B lane 3) and the 125 kb SfiI fragment (panel A lane 4, panel B lane 4). However, they are not identical because of their different PstI hybridisation patterns (data not shown). pS5 is mapped on the XY-539 restriction map by its hybridization to the 10 kb SfiI fragment (panel C, lane 4). In panel C, the bands with relatively low intensity in lanes 2, 3, 4 and 5 indicated with circles are the hybridization signals of contaminating vector sequences (pUC19).

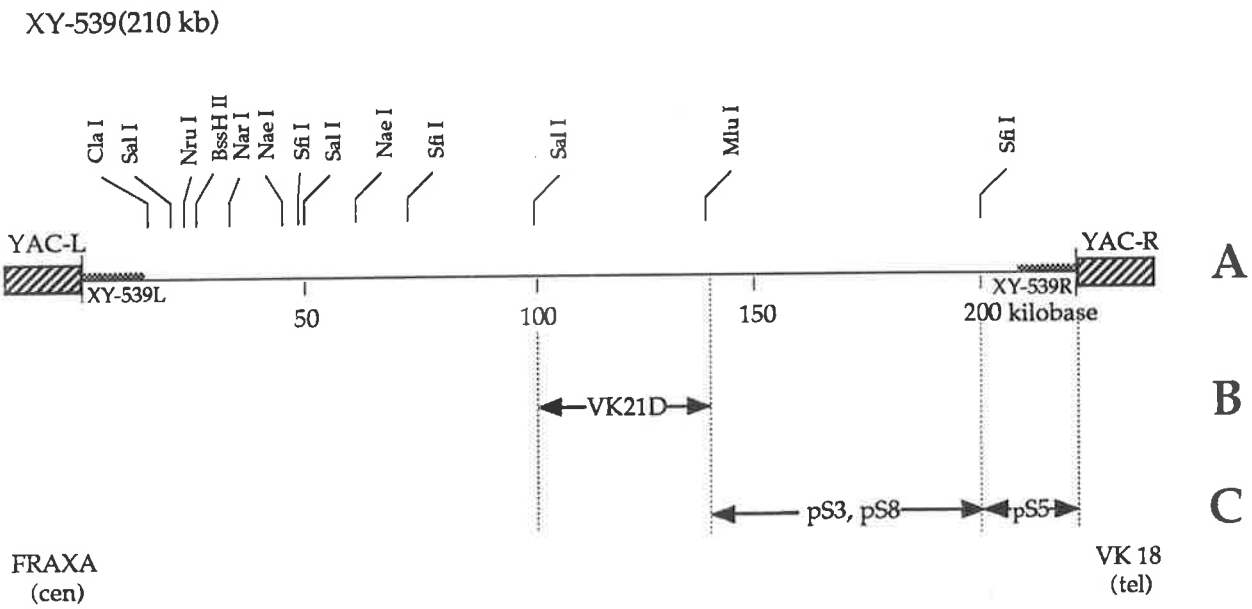


Fig. 3 - 9. "Rare-cutter" restriction map of YAC XY-539 (A) showing locations of VK21D (B) and the three subclones pS3, pS5 and pS8 (C). Subclone pS3 and pS8 hybridized to PstI fragments of different sizes (data not shown), and thus are not overlapping DNA fragments. Complete map sites in human insert DNA are shown only for MluI, NruI, SalI, BssHII and SfiI. Other enzymes may have one or more additional sites.

3.5. DISCUSSION

3.5.1. Experience in YAC Restriction Map Construction

Constructing the restriction map of XY-539 was an initial step in cloning the fragile X by using the YAC cloning system. The experience gained from these experiments laid the foundation for further study on YACs.

During the YAC map construction, some confusing results came from probe contamination. For example, both end probes, YAC-L and YAC-R, were generated from pBR322 (Fig. 2 - 3). When pBR 322 was digested with endonucleases BamHI, PstI and NruI, three DNA fragments were expected: YAC-L (1.1 kb), YAC-R (0.6 kb) and the rest of pBR 322 (2.6 kb) (Fig. 2 - 3). Although these three fragments could be well separated on a gel, YAC-L (or YAC-R) can still be easily contaminated with the rest of pBR322 including YAC-R (or YAC-L) in minute amounts. A contaminated end probe, YAC-L contaminated with YAC-R or vice versa, would generate a confusing hybridization pattern in YAC restriction map construction. Since pUC19 has overlapping DNA sequences with pBR322 (Fig. 2 - 3), it can be used instead of YAC-L to avoid contamination in this case. Apart from the end probes, most of the human DNA probes were cloned in pUC18, pUC19 or pBR322. Thus, when the human DNA inserts were isolated, they were usually contaminated with a trace of vector DNA, which is enough to give a hybridization signal. This kind signal can be recognized by its relatively weak intensity, or by its similar hybridization pattern to that of the vectors.

Theoretically, the protocols for isolating both ends of a human DNA insert in a YAC are straightforward, but in practice they are not. With the plasmid rescue method (see section 2.6.6.), usually no colonies were obtained at all. On one occasion, a few recombinants were obtained, but their restriction maps showed that they did not contain the vector-insert junction region as expected. Alternatively, selective collection of the vector-insert junction fragments for ligation and transformation would increase the chance of success (E. Kremer, unpublished observation). As a variation on the method described in Chapter 2 (see section 2.6.6.), restriction fragments of a YAC were separated on an agarose gel, after restriction endonuclease

digestion. Then the gel region containing vector-insert junction fragments was detected by probing with either YAC-L or YAC-R. The DNA fragments in this region were isolated and cloned in pUC19. Since the vector-insert junction fragments were enriched in this way, the chance of getting transformants containing the vector-insert junction was thus increased.

The inverse PCR method was complicated by its inconsistent results. Most of the time it gave no PCR products at all, but sometimes it generated multiple PCR products under the same conditions. Southern analysis with these PCR products as probes on human DNA revealed that they were not human DNA sequences (data not shown).

Initially, establishment of the integrity of human DNA inserts in YAC clones was a major concern because of the frequent incidence of rearrangements, deletion and chimerization found to occur in previously characterized YACs (D. Schlessinger, St. Louis, USA, personal communication). Some observations have lessened these concerns. For example, in the case of *Caenorhabditis elegans*, restriction maps of YACs have consistently matched the restriction maps previously constructed from mapping cosmid clones (Coulson et al., 1988). In the case of human genome, several genes and anonymous DNA fragments from the Xq24 -Xq28 region have been cloned into YACs, and each of these probes detected identical hybridization patterns between human genomic DNA and YAC DNA (Brownstein et al., 1989; Wada et al.,1990). As for the human DNA fragments in XY-539, the presence of common fragments in the YAC DNA and the genomic DNA for several restriction enzymes suggests that gross DNA rearrangement is unlikely to have occurred.

3.5.2. Relevance to the Human Genome Project

One of the emphases of the Human Genome Project is the physical mapping of the human genome. The physical map is a combination of a restriction map and a contig map (Olson et al., 1989). The end of the long arm of the human X chromosome is of particular interest, since it contains the highest density of the genes associated with human genetic diseases. Accelerated by YAC cloning technology, the human Xq24-Xq28 region has been mapped by YAC contigs

(Wada et al., 1990, Schlessinger et al., 1991), and a long-range restriction map of Xq27.2-qter has also been established (Poustka et al., 1991). Since XY-539 was detected by VK21 (DXS296), which was mapped distal to the fragile X by in situ hybridization (Suthers et al., 1989), the restriction map of XY-539 adds more details to the restriction map of this region since several relatively frequent cutters, such as SfiI and SalI, were used.

In addition, the DNA sequences near DXS296 may modify the X inactivation pattern. Usually, the two X chromosomes in a female are randomly inactivated, but this inactivation pattern is often altered when an X chromosome carries a structural defect. In the case of balanced X/autosome translocation, the rearranged X chromosome is preferentially active, whereas the X chromosome with deletion is always inactive.

However, two exceptional cases have been documented in the literature. In a mentally retarded girl with an interstitial deletion (X) (q27.1-q27.3), Schmidt et al. (1990) have found that the deleted X chromosome is preferentially early replicated (active) in her fibroblasts, and B and T lymphocytes. Clarke et al. (1992) reported a girl with Hunter's syndrome (iduronate sulfatase deficiency) who had a submicroscopic deletion on one X chromosome between the loci DXS297 and DXS296. The deleted X chromosome in this girl is always active. It is interesting to note that the deletions in both patients are overlapped, but the deletion in the girl with Hunter's syndrome is much smaller than that in the patient described by Schmidt et al. (1990). The unbalanced inactivation of the normal X chromosome in these two patients may be due to either a non-random X chromosome inactivation, or a selection procedure for the cells with an inactive normal X chromosome.

Recently, Gedeon et al. (1992) documented that a female, the mother of a boy with fragile X syndrome, had a deletion between the loci DXS297 and B2. The deleted region in this female is also partially overlapped with the deletions seen in above two cases, but is further smaller than that in the Hunter's syndrome patient of Clarke et al. (1992). In this female, however, the two X chromosomes are randomly inactivated (Gedeon et al., 1992). Therefore, if there are DNA sequences at or near Xq27.3 region modifying the X inactivation pattern, they are likely

to locate in the non-overlapping region between the two deletions reported by Clarke et al. (1992) and Gedeon et al. (1992). The restriction map of XY-539 and the region around DXS296 may facilitate to clarify this possibility.

3.6. ADDENDUM

At the early stage of the experiments, an RFLP study was carried out to identify highly informative DNA markers for prenatal diagnosis and carrier detection of fragile X syndrome. Two previously reported RFLPs identified by VK21A [TaqI (A_1:10.9 kb, A_2: 9.9 kb)] and VK21C [MspI (B_1:12.7 kb, B_2: 9.9 kb)], were found to have low heterozygosities (Suthers et al., 1989). An extended search for RFLPs was undertaken in an attempt to increase the informativeness of the DXS296 locus. Four single copy subclones of VK21: VK21A, VK21B, VK21C and VK21D were used as probes to screen for RFLPs. Restriction endonuclease BclI detected a two allele polymorphism with VK21A (Yu et al., 1989). From 27 unrelated individuals (total 45 X chromosomes) allele frequencies were C_1(5.0 kb) = 0.87, C_2 (10.7 kb) = 0.13. All haplotypes were either $A_1B_1C_1$ or $A_2B_2C_2$. No polymorphism was found with the four subclones of VK21 for BanI, BglI, BstXI, BstNI, EcoRV, XbaI and XmnI with DNAs from ten unrelated females. Although the BclI RFLP appears to be in linkage disequilibrium with the TaqI and MspI RFLPs, this BclI RFLP adds flexibility to diagnostic strategies based on reprobing filters since BclI polymorphisms are also detected by other markers near FRAXA, such as DXS52 (F814 and St14), DXS105 (55.E) and DXS304 (U6.2).

CHAPTER 4

CHARACTERIZATION OF A HUMAN DNA SEQUENCE WHICH SPANS THE FRAGILE X

4.1. SUMMARY

A yeast artificial ring chromosome XTY-26 containing a 275 kb human insert was isolated by using probe VK16B3 of the DXS293 locus from a telomere-rescue YAC library made from the X chromosome of a fragile X patient. The human DNA sequence in XTY-26 was shown to span the fragile X by in situ hybridization, and to contain the two closest DNA markers flanking the fragile site. A detailed rare cutting restriction map of XTY-26 was generated by pulsed field gel electrophoresis (PFGE). Comparison of the restriction map of XTY-26 with that of the YACs constructed from a normal X chromosome revealed no large-scale DNA differences involved in fragile X mutation. The restriction map of XTY-26 and the localization of the fragile X within a 160 kb DNA region led to characterization of the fragile site at the molecular level (Chapter 5).

4.2. INTRODUCTION

The chromosomal location of the fragile X mutation has been known for almost two decades, but isolation of the mutation itself has not been achieved. One of the main reasons was the lack of closely linked DNA markers in this region. By the end of 1988, the closest distal DNA marker DXS52 (St14) was 12 cM, and the closest proximal marker DXS98 (4D-8) 7 cM from FRAXA (Brown et al., 1988a). In recent years, great effort has been put into isolation of DNA markers closer to FRAXA. Three DNA markers (DXS369, DXS304 and DXS296) located within 5 cM of FRAXA were isolated, with the closet marker DXS296 being 1 cM distal to FRAXA (Hupkes et al.,1989; Oostra et al., 1990; Dahl et al., 1989a, 1989b; Suthers et al., 1989). By using a panel of cell lines, Suthers et al. (1990) mapped several new loci near FRAXA. The order of these loci was DXS297(VK23)-DXS293(VK16),FRAXA-DXS296(VK21)-DXS295(VK18). DXS293 (VK16) was found to be very close to the fragile X by physical mapping (Suthers et al., 1990). However, the genetic distance between DXS293 and fragile X was unknown, because no RFLP was detected with VK16. More recently, a number of DNA markers were identified to be very close to the fragile site. Probes 2.34 (DXS477) and St677 (DXS463) were mapped proximal, Do33(DXS465) was distal to the translocation breakpoints of the fragile X hybrids (see section 1.4.6.). But probes Do33 and St677 were mapped to a common 2 Mb MluI fragment (Vincent et al., 1991). The availability of these DNA markers combined with the YAC cloning system greatly accelerated the process of cloning the fragile X.

In this project, four DNA probes (VK23, VK16, VK21, VK18) were used to isolate YAC clones. The aim was that chromosome walking from sites known to flank the fragile site would cover the fragile X region efficiently. Nine YAC clones (see Table 2 - 1) were obtained by hybridization to any one of these four probes. One of the YAC clones, XTY-26 (isolated by hybridization to VK16B3), appeared important to us because it was shown to span the fragile site by in situ hybridization, and contained the DNA probes known to flank the fragile site. Since XTY-26 was derived from a fragile X chromosome, this implied that the human insert in

XTY-26 contained the DNA sequences responsible for the fragile X as well as its associated syndrome.

In this chapter, the restriction map of XTY-26 and the evidence that the fragile X is contained in XTY-26 will be presented. The restriction map of XTY-26, derived from a fragile X chromosome, is compared with that of other YACs derived from normal X chromosomes. Since a group effort was put into this project in order to accelerate the process, a complete picture of the research will be given with an indication of the role of each of the contributors.

4.3. MATERIALS AND METHODS

4.3.1. Yeast Clone

XTY-26 was the only positive yeast clone isolated from a telomere-rescue YAC library (see section 2.6.9.2.) by hybridization to VK16B3 (a subclone of VK16) at DXS293 locus. It was provided by Dr. D. Schlessinger and colleagues (St. Louis, USA) on a collaborative basis. The YAC library was derived from hybrid X 3000.11 which contains the Xq24-qter portion of a fragile X chromosome translocated onto a hamster chromosome (Nussbaum et al., 1986a). The fragile site in this hybrid can be induced cytogenetically.

4.3.2. DNA Probes

DNA probes 2.34 and Do33 were obtained on a collaborative basis from Dr. JL Mandel and colleagues (Strasbourg, France). Their isolation has been described elsewhere (Rousseau et al., 1991a). VK16A3 and VK16B3, two subclones of VK16 (Suthers et al., 1990), were obtained from Dr. J. Mulley (ACH, Adelaide).

4.3.3. In Situ Hybridization (performed by E. Baker)

DNA probes VK16, 2.34 and Do33 and the total DNA of XTY-26 were used as probes for in situ hybridization to metaphase chromosomes expressing the fragile X. The protocol for fluorescent in situ hybridization has been described elsewhere (Kremer et al,. 1991b).

4.3.4. Verification of XTY-26 containing DNA probes 2.34 and Do33 (performed by Dr. E. Kremer)

Total human DNA and the DNA from XTY-26 were digested with HindIII, BamHI and TaqI. DNAs cleaved with the same enzyme were separated side by side by electrophoresis on a 0.8% agarose gel. After Southern transfer, the filter was hybridized with probes 2.34 and Do33 sequentially. Common-sized restriction fragment between total human DNA and the DNA of XTY-26 detected by 2.34 or Do33 was taken as a confirmation of the presence of the 2.34 and Do33 sequences in XTY-26.

4.3.5. Construction of the Restriction map of XTY-26 (performed by the candidate)

The map of the yeast artificial ring-chromosome, XTY26, was derived by pulsed-field gel electrophoresis of XTY-26 DNA which was cleaved with the restriction enzymes (Biolabs) shown in Fig. 4 - 11. After Southern transfer, the filter was sequentially hybridized with probes pUC 19 (vector), 2.34 (DXS477), Do33 (DXS465), and two subclones of VK16 (DXS293).

Complete DNA digestion was performed with endonucleases SalI, NruI, SnaBI, EagI, ClaI, NaeI, SfiI, BssHII, NotI and MluI (Biolabs) according to the manufacturer's instructions. Double digestion was carried out with enzymes BssHII/SfiI, SalI/NruI, NruI/SnaBI, NruI/BssHII, ClaI/SnaBI, SfiI/SnaBI, MluI/SalI, NotI/SalI, PvuI/SalI, SacII/SalI, RsrII/SalI, SnaBI/SalI, NaeI/SalI and NaeI/SfiI. The protocol for double digestions has been described in Chapter 2 (see section 2.6.7.). To confirm the restriction map of XTY-26, XTY-26 DNA was partially digested with enzymes NruI or SnaBI. Partial digestion was tested initially with enzyme concentration in the range from 0.001 to 10 units per 80 µl of agarose beads, and eventually accomplished with enzyme concentration ranging from 1 to 10 units per sample for both enzymes. Since the size of XYT-26 was 280 kb, a three-stage pulse program (1 second for 4 hours, 10 seconds for 5 hours and 20 seconds for 9 hours) was used in PFGE in order to get good resolution of DNA fragments in the size range of 20-280 kb. Details of preparation of

HMW DNA in agarose beads, PFGE analysis, Southern blotting, filter hybridization and YAC map construction were described in Chapter 2.

4.4. RESULTS

4.4.1. The Human Insert in XTY-26 Spans the Fragile X (performed by E. Baker and Dr. E. Kremer)

The evidence that the human fragment in XTY-26 spanned the fragile X came from two independent experiments. DNA probes Do33, VK16, 2.34 and DNA of XTY-26 were used for in situ hybridization to metaphases expressing the fragile X. The locations of signal for various probes in relation to the fragile X are summarized in Table 4-1. The majority of the signal for XTY-26 was distal to the fragile site, whereas probe VK16, one of its subclones was used to isolate XTY-26, was proximal to the fragile site. Do33 was mapped distal and 2.34 proximal to the fragile site. Furthermore, both probes Do33 and 2.34 were found to be present in XTY-26 by their restriction patterns for the enzymes BamHI, HindIII and TaqI which were identical in both XTY-26 and total human DNA (data not shown).

4.4.2. Construction of a Restriction Map of XTY-26

The restriction map of XTY-26 was essential for the further characterization of the fragile X and its associated syndrome. Construction of the restriction map of XTY-26 was difficult because the hybridization data was inconsistent and confusing initially. It was found later that XTY-26 did not have the expected structure of a telomere-rescue YAC.

Because XTY-26 was isolated from a telomere-rescue YAC library, initially, it was expected that XYT-26 had one telomere provided by the YAC vector pTYAC1 and the other contributed by human DNA sequences (Fig. 2 - 6). In YACs like this, pBR322 derived end probes (including pUC19) would be able to detect only the centromere end, but not the non-centromere end of the YAC. So that various internal human DNA probes would be required to generate a restriction map.

Table 4 - 1. Location of In Situ Hybridization Signal for Various Probes in Relation to the Fragile X (provided by E. Baker)

	Position of signal (in relation to the fragile site)			
Probe	Proximal	Central	Distal	Proximal and Distal
XTY-26	11	10	39	8
VK16	10	2	0	0
2.34	9	3	0	0
Do33	0	0	10	0

The map construction was initiated by probing various DNA digests of XTY-26 with pUC19 in order to generate a restriction map from the centromere end of XTY-26. As presented in Fig. 4 - 1A. B, pUC19 detected a 30 kb EagI fragment, a 40 kb SnaBI fragment, a 50 kb NaeI fragment, an 80 kb ClaI fragment, a 140 kb NruI fragment, a 160 kb SfiI fragment and a 210 kb BssHII fragment. Based on these results, the restriction map of XTY-26 was initially proposed as shown in Fig. 4 - 1C.

Subsequent data obtained from probing the same filter with 2.34 was confusing. As shown in Fig. 4 - 2, probe 2.34 detected the same 40 kb SnaBI fragment, 50 kb NaeI fragment, 140 kb NruI fragment, 160 kb SfiI fragment and 210 kb BssHII fragment, as detected by pUC19. But 2.34 detected a different EagI fragment of 120 kb. Thus, 2.34 could be located between the EagI and the SnaBI sites as indicated in Fig. 4 - 1C. The only problem was that probe 2.34 did not hybridize to the 80 kb ClaI fragment as expected, instead it hybridized to a 50 kb ClaI fragment.

The results acquired from probing the same filter with Do33 (Fig. 4 - 3) were even more confusing. Do33 revealed the same 30 kb EagI fragment, 80 kb ClaI fragment, 160 kb SfiI fragment and 210 kb BssHII fragment as pUC19. However, it hybridized to a 60 kb SnaBI fragment, a 70 kb NruI fragment and a 140 kb NaeI fragment different from those detected by pUC19. These results rendered the localization of Do33 on the initial map of XTY-26 (Fig. 4 - 1C) impossible.

The hybridization results with VK16B3 further increased the concern that the initial map of XTY-26 (Fig. 4 - 1C) might be incorrect. As shown in Fig. 4 - 4, both VK16B3 and pUC19 hybridize to the same 140 kb NruI fragment and 210 kb BssHII fragment. However, pUC19 hybridized to a 160 kb SfiI fragment, while VK16B3 to a different 120 kb SfiI fragment. These results were not compatible with the proposed map shown in Fig. 4 - 1C.

The size of restriction fragments of XTY-26 detected by the four probes are summarized in Table 4 - 2.

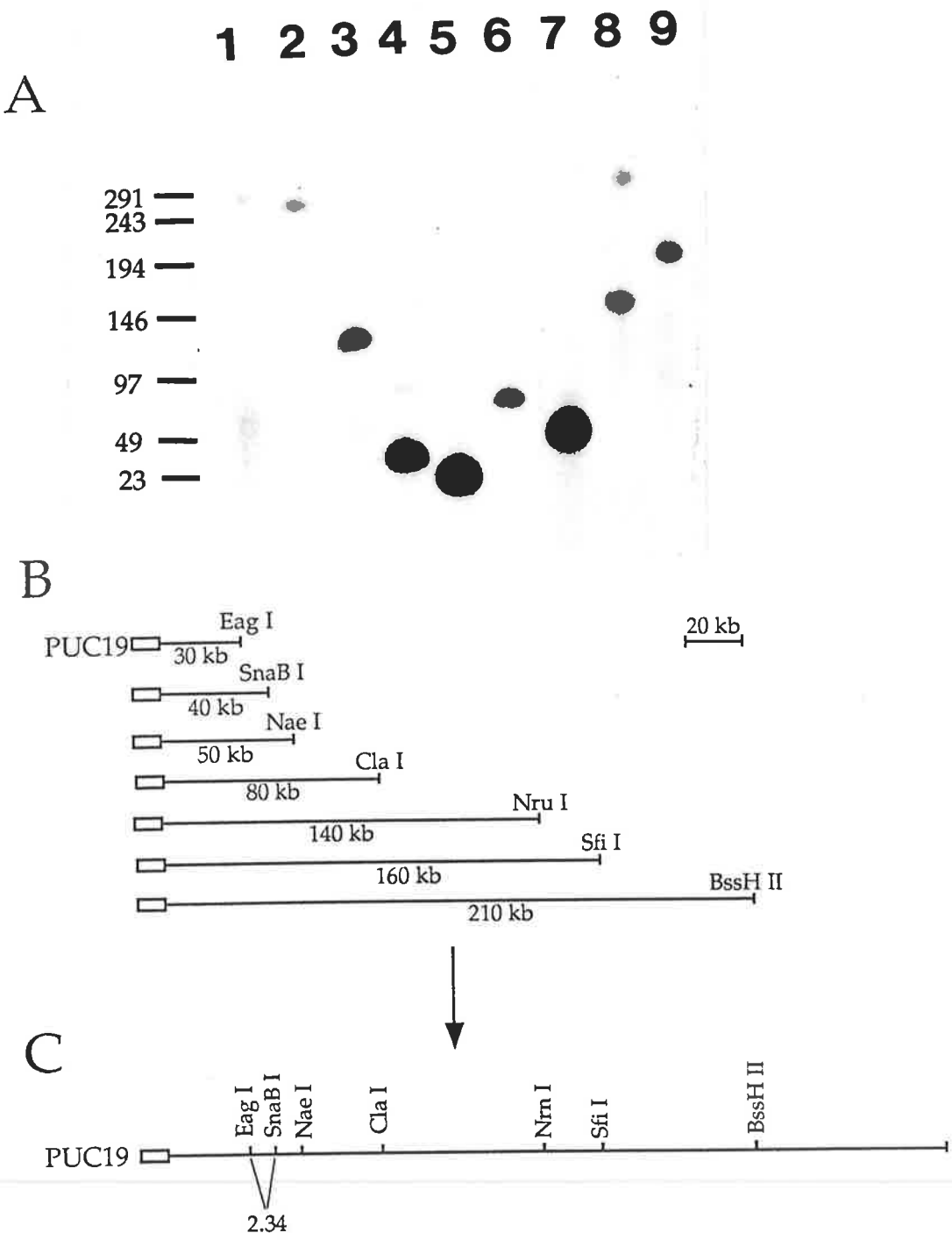


Fig. 4 - 1. Construction of the initial restriction map of XTY-26. A) various restriction fragments of XYT-26 detected by pUC19. Lane 1, uncut; lane 2, SalI; lane 3, NruI; lane 4, SnaBI; lane 5, EagI; lane 6, ClaI; lane 7, NeaI; lane 8, SfiI and lane 9, BssHII. Molecular weight markers (kb) are shown. B) the size of restriction fragments of XTY-26 detected by pUC19. C) The initial map of XTY-26.

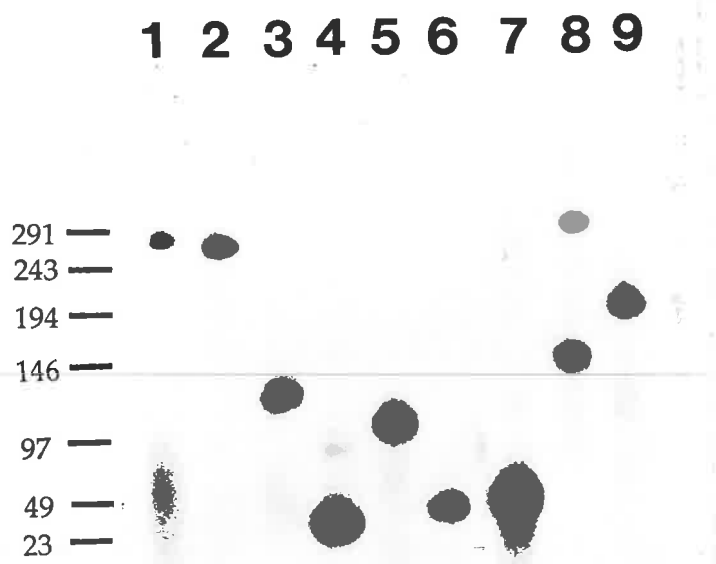


Fig. 4 - 2. Various restriction fragments of XTY-26 detected by probe 2.34 (the same filter as that in Fig. 4 - 1A). Lane1, uncut; lane 2, SalI; lane 3, NruI; lane 4, SnaBI; lane 5, EagI; lane 6, ClaI; lane 7, NeaI; lane 8, SfiI and lane 9, BssHII. The faint band in lane 4 is probably due to incomplete enzyme digestion. Molecular weight markers (kb) are shown on the left.

Fig. 4 - 3. Various restriction fragments of XTY-26 detected by probe Do33 (the same filter as that in Fig. 4 - 1A). Uncut (lane1), SalI (lane 2), NruI (lane 3), SnaBI (lane 4), EagI (lane 5), ClaI (lane 6), NeaI (lane 7), SfiI (lane 8) and BssHII (lane 9). Molecular weight markers (kb) are shown on the left.

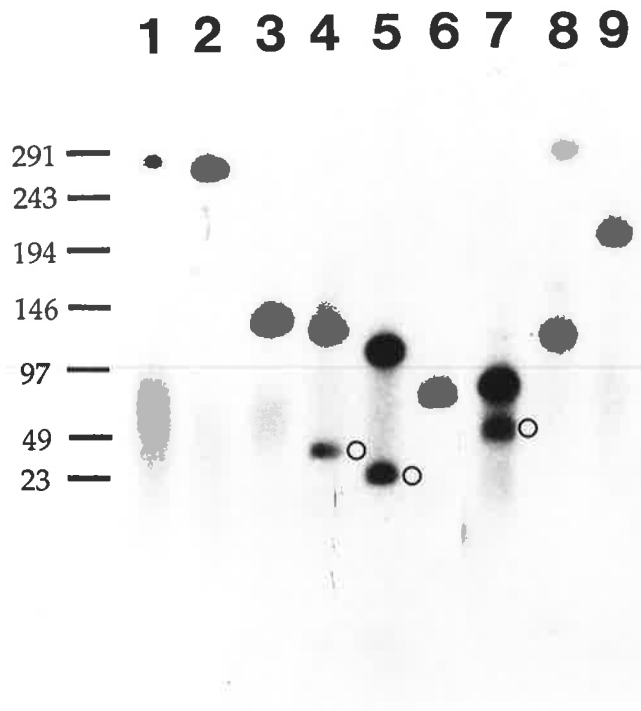


Fig. 4 - 4. Various restriction fragments of XTY-26 detected by probe VK16B3 (the same filter as that in Fig. 4 - 1A). Uncut (lane1), SalI (lane 2), NruI (lane 3), SnaBI (lane 4), EagI (lane 5), ClaI (lane 6), NeaI (lane 7), SfiI (lane 8) and BssHII (lane 9). The faint bands indicated with circles are the hybridization signals of contaminated vector pUC19 sequences. Molecular weight markers (kb) are shown on the left.

Table 4 - 2. Size (in kb) of Restriction Fragments of XTY-26 Detected by Various Probes

(Single enzyme digestion)

Restriction Enzyme	DNA probe			
	pUC 19	VK16B3	Do33	2.34
none	280*	280*	280*	280*
SalI	270*	270*	270*	270*
NruI	140	140	70	140
SnaBI	40	130	60	40
EagI	30	120	30	120
ClaI	80	70	80	50
NaeI	50	80	140	50
SfiI	160	120	160	160
BssHII	210	210	210	210

* different mobility between the circular and linear DNA.

To clarify these confusing results, double-enzyme digestion of XTY-26 was applied to determine the distances between any two restriction sites shown in Fig. 4 - 1C. The sizes of various double-digest restriction fragments detected by four DNA probes (2.34, VK16B3, pUC19 and Do33) are demonstrated in Fig.4 - 5, Fig.4 - 6, Fig. 4 -7, Fig 4 - 8 and are summarized in Table 4 - 3.

Generally, the data were incompatible with the restriction map of XTY-26 shown in Fig. 4 - 1C. For example, 2.34 should detect a 40 kb fragment on either SnaBI/ClaI or SnaBI/SfiI double digestion if the initial map on Fig. 4 - 1C was correct. However, it detected a 20 kb band on SnaBI/ClaI double digestion (Fig. 4 - 5 lane 8) and a 40 kb band on SnaBI/SfiI double digestion (Fig. 4 - 5 lane 10). Similarly, according to the restriction map in Fig. 4 - 1C, probe 2.34 should detect a 140 kb band on either NruI or NruI/BssHII digestion. However, it identified a 140 kb band on NruI digestion (Fig. 4 - 5 lane 7) but a 130 kb band on NruI/BssHII digestion (Fig. 4 - 5 lane 6). Moreover, a single SalI restriction site was verified to be present in the human insert in XTY-26 by double digestion. This site was not observed in the SalI alone digestion of XTY-26. Since the lambda clone VK16 used to isolate XTY-26 contained one SalI site (Hyland, unpublished observation), it was expected that the SalI site was also in XTY-26. However, all four DNA probes, pUC19, Do33, VK16B3 and 2.34, revealed only subtle difference in mobility between the SalI digested and undigested XTY-26 DNA. By contrast, VK16B3 detected a 30 kb SalI/NruI fragment (Fig. 4 - 6 lane 2) and a 140 kb NruI fragment (Fig. 4 - 6 lane 7) suggesting that there was a SalI site 30 kb away from a NruI site.

The data obtained from double digestion of XTY-26 indicated that 1) the map shown in Fig. 4 - 1C was incorrect; and 2) that XTY-26 could not have the structure shown in Fig. 2 - 6. But what structure could it be? To answer this question, a number of model structures were proposed (Fig. 4 - 9) and they were examined with the data from single and double digestion. Among all of the proposed models, only the circular structure fitted all of the data from restriction digestion. The convincing evidence for the circular structure of XTY-26 came from several restriction digests which were only compatible with a circular map. Firstly, the vector

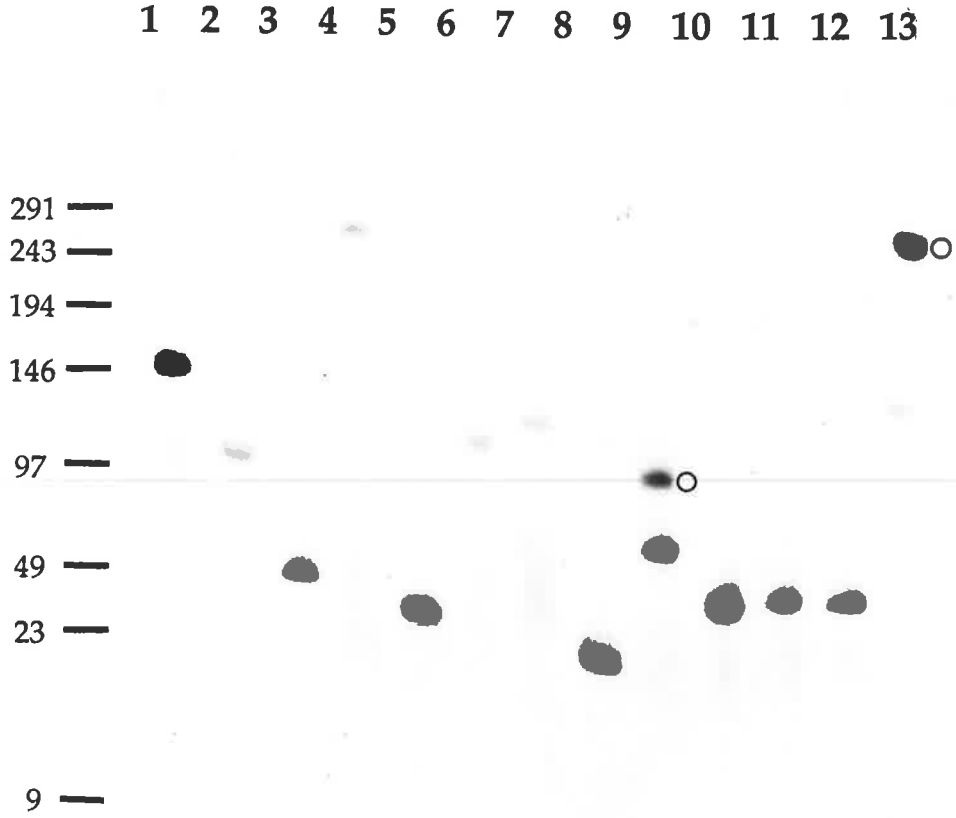


Fig. 4 - 5. Single and double digested restriction fragments of XTY-26 hybridised to probe 2.34. BssHII/SfiI (lane 1), SalI/NruI (lane 2), SalI/SnaBI (lane 3), SalI (lane 4), NruI/SnaBI (lane 5), NruI/BssHII (lane 6), NruI (lane 7), ClaI/SnaBI (lane 8), SfiI/EagI (lane 9), SfiI/SnaBI (lane 10), SfiI/ClaI (lane 11), ClaI (lane 12) and SfiI (lane 13). The bands indicated with circles on lane 9 and lane 13 are due to incomplete SfiI digestion. Molecular weight markers (kb) are shown on the left.

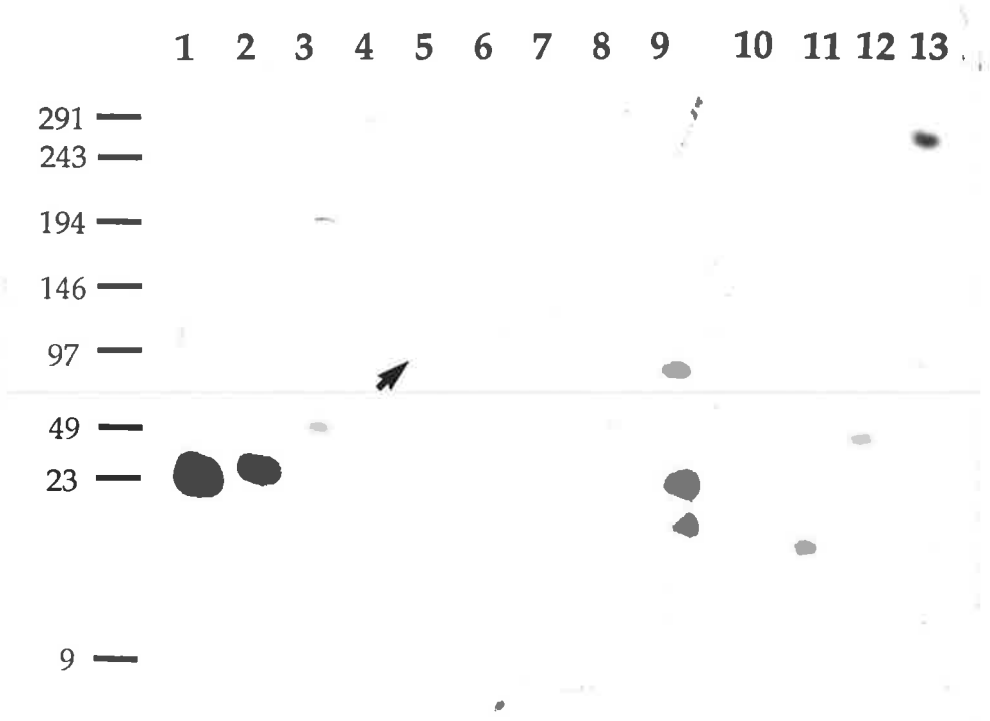


Fig. 4 - 6. Single and double digested restriction fragments of XTY-26 hybridised to probe VK16B3 (the same filter as that in Fig. 4 - 5). BssHII/SfiI (lane 1), SalI/NruI (lane 2), SalI/SnaBI (lane 3), SalI (lane 4), NruI/SnaBI (lane 5), NruI/BssHII (lane 6), NruI (lane 7), ClaI/SnaBI (lane 8), SfiI/EagI (lane 9), SfiI/SnaBI (lane 10), SfiI/ClaI (lane 11), ClaI (lane 12) and SfiI (lane 13). The faint bands are indicated with arrows. Molecular weight markers (kb) are shown on the left.

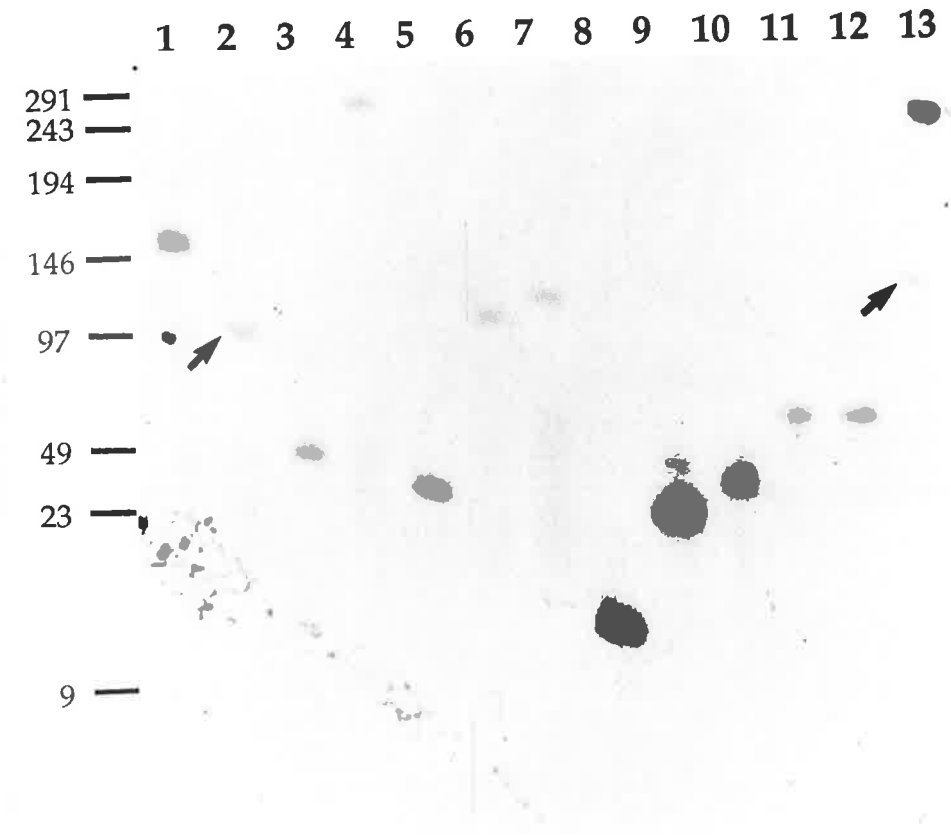


Fig. 4 - 7. Single and double digested restriction fragments of XTY-26 hybridised to probe pUC19 (the same filter as that in Fig. 4 - 5). BssHII/SfiI (lane 1), SalI/NruI (lane 2), SalI/SnaBI (lane 3), SalI (lane 4), NruI/SnaBI (lane 5), NruI/BssHII (lane 6), NruI (lane 7), ClaI/SnaBI (lane 8), SfiI/EagI (lane 9), SfiI/SnaBI (lane 10), SfiI/ClaI (lane 11), ClaI (lane 12) and SfiI (lane 13). The faint bands are indicated with arrows. Molecular weight markers (kb) are shown on the left.

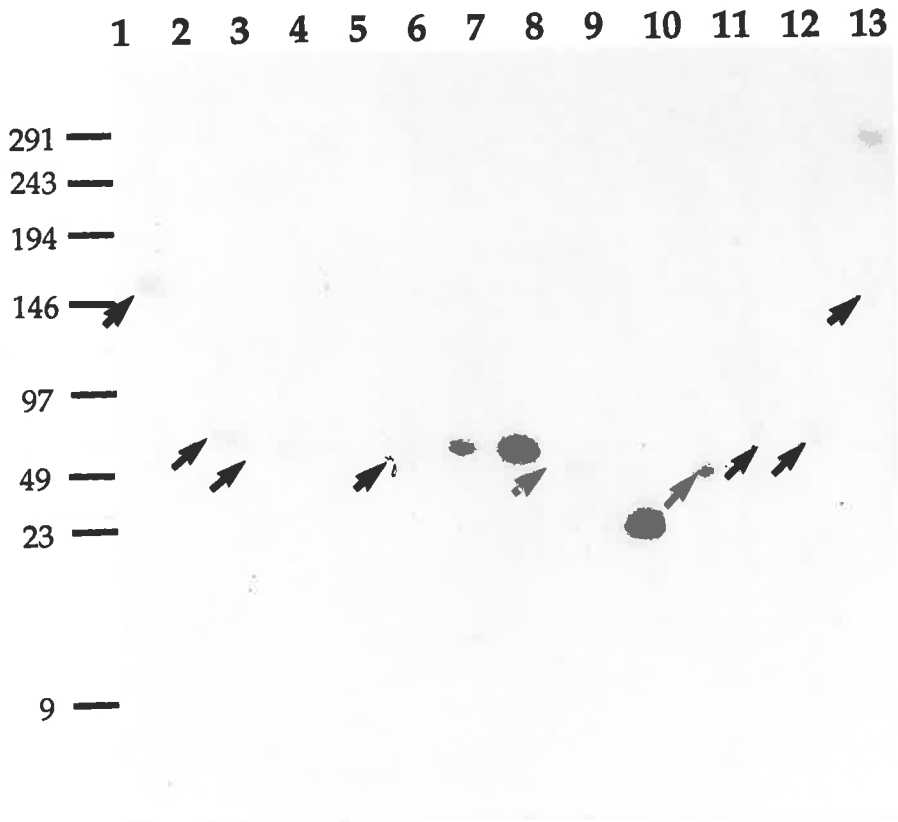


Fig. 4 - 8. Single and double digested restriction fragments of XTY-26 hybridised to probe Do33 (the same filter as that in Fig. 4 - 5). BssHII/SfiI (lane 1), SalI/NruI (lane 2), SalI/SnaBI (lane 3), SalI (lane 4), NruI/SnaBI (lane 5), NruI/BssHII (lane 6), NruI (lane 7), ClaI/SnaBI (lane 8), SfiI/EagI (lane 9), SfiI/SnaBI (lane 10), SfiI/ClaI (lane 11), ClaI (lane 12) and SfiI (lane 13). The faint band are indicated with arrows. Molecular weight markers (kb) are shown on the left.

Table 4 - 3. Size (in kb) of Restriction Fragments of XTY-26 Detected by Various Probes

(Double-enzyme digestion)

Restriction Enzyme	DNA probes			
	pUC 19	VK16B3	Do33	2.34
BssHII+SfiI	160	30	160	160
SalI + NruI	110	30	70	110
SalI+SnaBI	40	50	60	40
NruI+SnaBI	30	110	60	30
NruI+BssHII	130	130	70	130
ClaI+SnaBI	15	65	60	25
SfiI + EagI	30	*	30	**
SfiI + SnaBI	40	***	50	40

* Three bands are visible at 20, 30 and 80 kb.

** Two bands are visible at 80 and 120 kb.

*** Three bands are at 25, 70 and 120 kb.

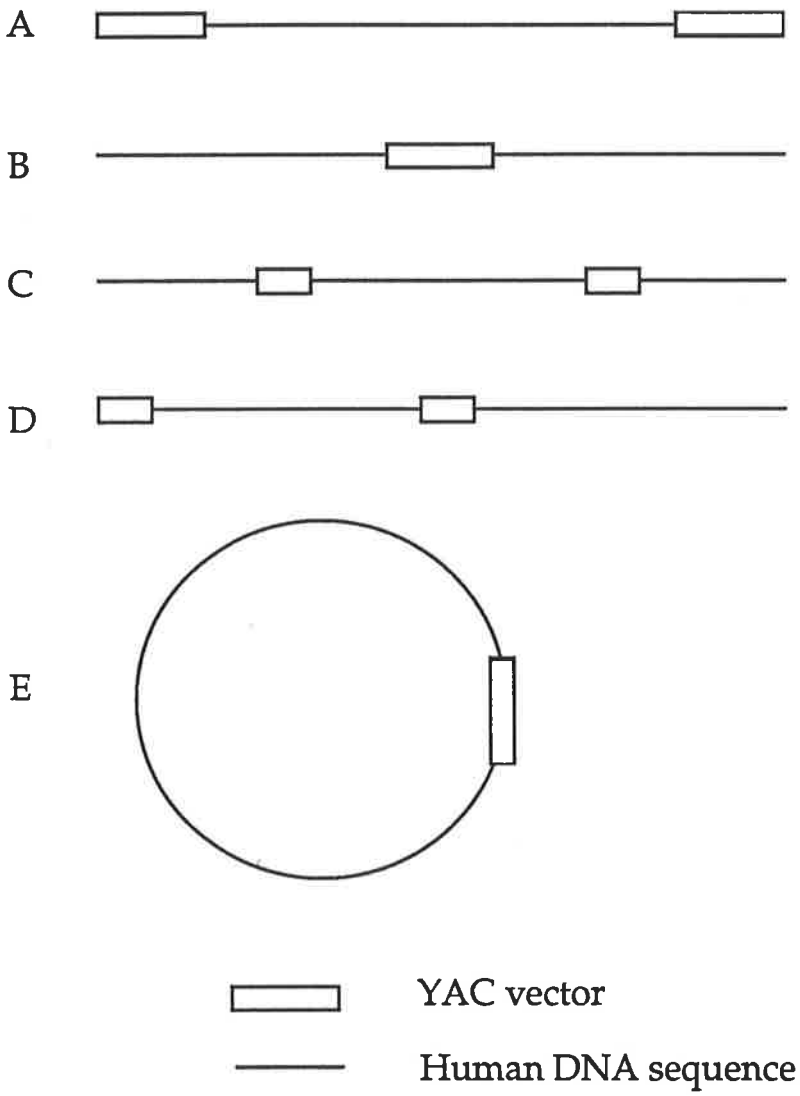


Fig. 4 - 9. Proposed model structures of XTY-26. A. B. C and D shows the linear structures with the YAC vector sequences at different places of the YAC. E is a circular structure.

sequences pUC19 (supposed to be at one end of a linear YAC) and 2.34 mapped to a common 40 kb SnaBI fragment, yet to two different ClaI fragments (80 kb for pUC19 and 50 kb for 2.34). Secondly, vector sequences pUC19 and VK16 detected the same 140 kb NruI fragment, but different SfiI fragment (pUC19 detecting a 160 kb SfiI fragment, VK16 detecting a 120 kb SfiI fragment). Finally, only a minimal alteration in the size of XTY-26 was observed with SalI digestion, in contrast, VK16B3 revealed a 30 kb fragment with SalI and NruI double digestion.

Since XTY-26 was a yeast artificial ring chromosome, any single restriction site in it could not be determined without double-enzyme digestion. No size change in XTY-26 was observed after digestion with enzymes MluI, NotI or PvuI, and minimal alteration with RsrII, or SacII. These enzymes were further used in combination with SalI to digest XTY-26 DNA. No size change was detected by VK16A3 with enzymes MluI/SalI, NotI/SalI and PvuI/SalI (Fig.4 - 10, Table 4 - 5), indicating that the human insert in XTY-26 did not contain restriction sites for enzymes NotI, MluI and PvuI. In contrast, VK16A3 detected a 180 kb RsrII/SalI fragment (Fig. 4 - 10, lane 1) and a 260 kb SacII/SalI fragment (Fig. 4 - 10, lane 2), implying the existence of one SacII site and one RsrII site in the human insert in XTY-26 DNA. Therefore, the minimal alteration observed after single digestion with RsrII, SalI or SacII presumably reflects the differences between the mobility of the circular DNA and that of linear DNA. Combining all the data obtained from various enzyme digestions, the restriction map of XTY-26 was constructed as shown in Fig. 4 - 11.

Results of NruI partial digests of XTY-26 (Fig.4 - 12 and Table 4 - 4) were also compatible with its ring structure.

4.4.3. Confirmation of DNA Integrity in XTY-26

The 120 kb SfiI fragment in XTY-26 was compared with the corresponding fragment in normal human genomic DNA. Fig. 4 - 13 shows that DNA probe VK16A3 detected a common SfiI band of 120 kb in both human genomic DNA and XTY-26 DNA, confirming the integrity of at least a portion of the human DNA sequences in XTY-26.

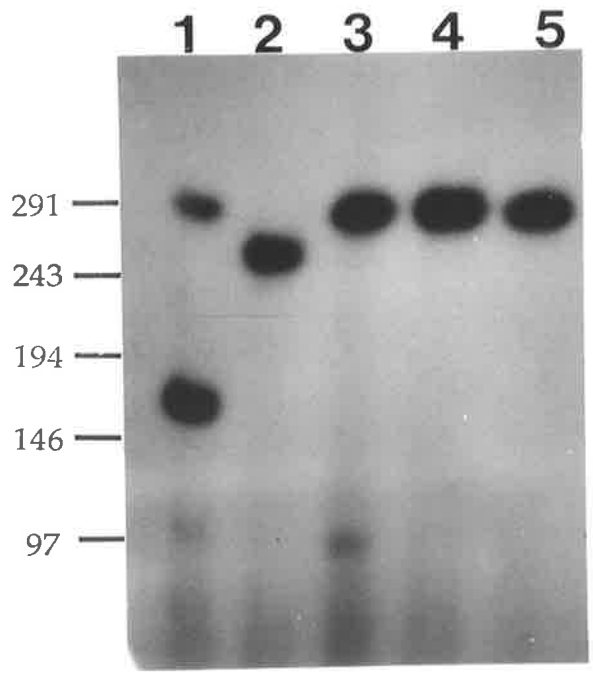


Fig. 4 - 10. Identification of single rare restriction sites on the ring YAC XTY-26 with probe VK16A3. SalI/RsrII (lane 1), SalI/SacII (lane 2), SalI/PvuI (lane 3), SalI/NotI (lane 4) and SalI/MluI (lane 5) was hybridised with probe VK16A3. The 280 kb band in lane 1 is probably due to incomplete digestion. Molecular weight (kb) is shown on the left.

Table 4 - 5. Size of Restriction Fragments of XTY-26 Detected by VK16A3

Restriction Enzyme	Size of band (in kb)
RsrII + SalI	180
SacII+SalI	260
PvuI + SalI	270*
NotI + SalI	270*
MluI+SalI	270*
none	280*

* different mobility between the circular and linear DNA

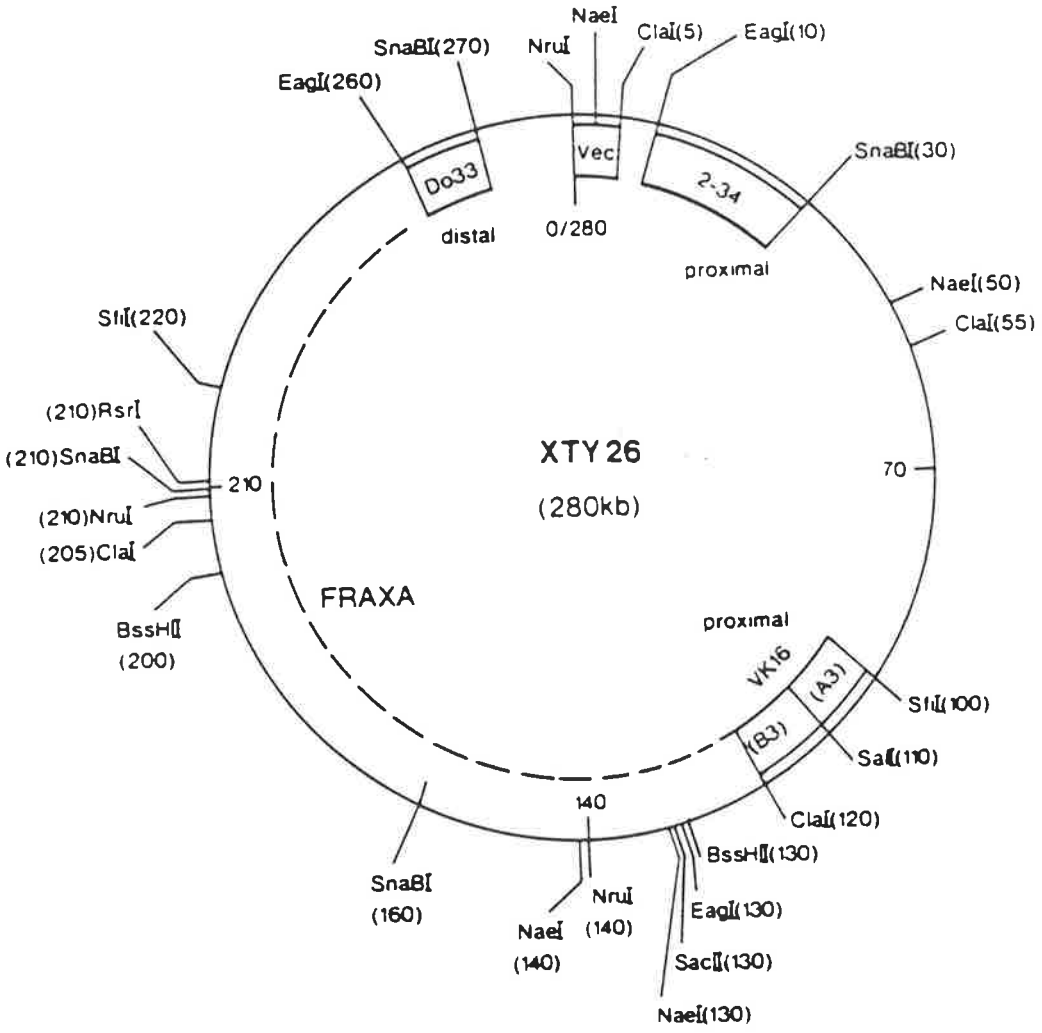
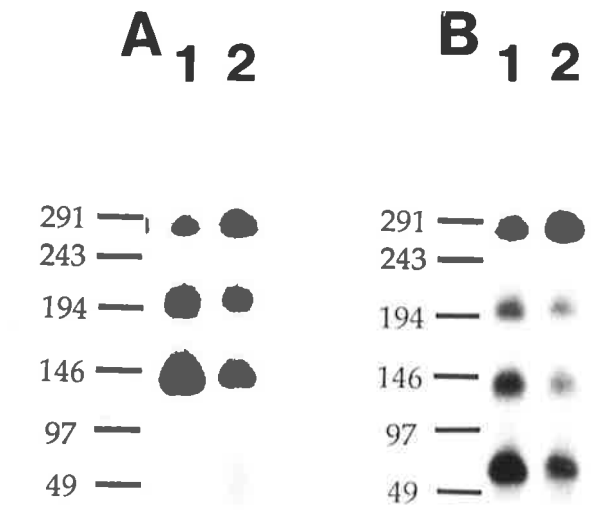


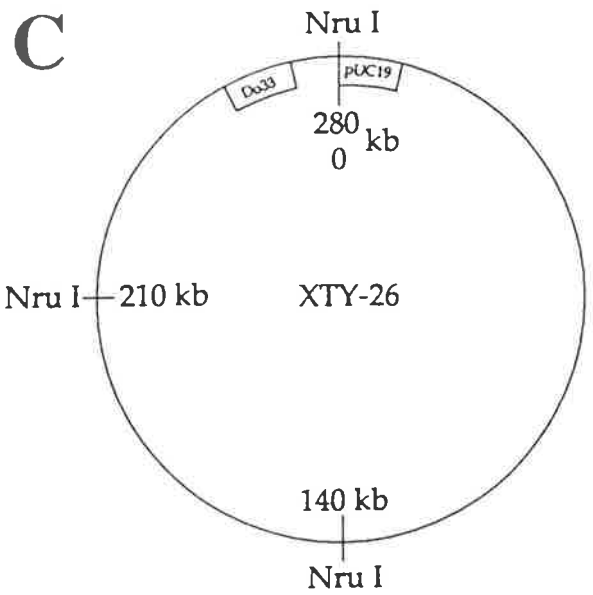
Fig. 4 - 11. Restriction map of XTY-26. Complete map sites in human insert DNA are shown only for SalI, SfiI, NruI, RsrII and SacII. Each of the other enzymes may have one or more additional sites. Multiple additional NaeI sites within the vector sequences are not shown.

Table 4 - 4. Size of NruI Partial Restriction Fragments of XTY-26

DNA probe	Number of band	Size of each band (kb)
pUC 19	3	280
		210
		140
Do33	4	280
		210
		140
		70

Fig. 4 - 12. NruI partial digested XTY-26 DNA hybridized with pUC19 (panel A) and Do33 (panel B). For 100 µls of agarose beads, 10 units (lane 1) or 5 units (lane 2) of enzyme NruI were used. The restriction map of XTY-26 in panel C is simplified with only the NruI sites and the positions of probes pUC19 and Do33 shown. The partial digestion data are interpreted as following: Among the three bands identified by pUC19 (panel A), the 280 kb band is the linear form of XTY-26 (XTY-26 being cleaved once at any NruI site). The 210 kb band is a doublet of partial digested NruI fragments with one from position 0 to 210 kb (clockwise), and the other from position 210 kb to 140 kb (clockwise). The 140 kb band contains a NruI fragment from position 0 to 140 kb. The four partial digestion bands detected by Do33 (panel B) are interpreted in the same way. The 280 kb band is the linear form of XTY-26. The 210 kb band is a partial digested NruI fragment from position 210 kb to 140 kb (clockwise). The 140 kb band is a partial digested NruI fragment from position 140 kb to 280 kb (clockwise). The 70 kb band is a single NruI fragment from position 210 kb to 280 kb.


A
1 2
291
243
194
146
97
49
B
1 2
291
243
194
146
97
49


C
Nru I
Dx33
pUC19
280
0
kb
Nru I
210 kb
XTY-26
140 kb
Nru I

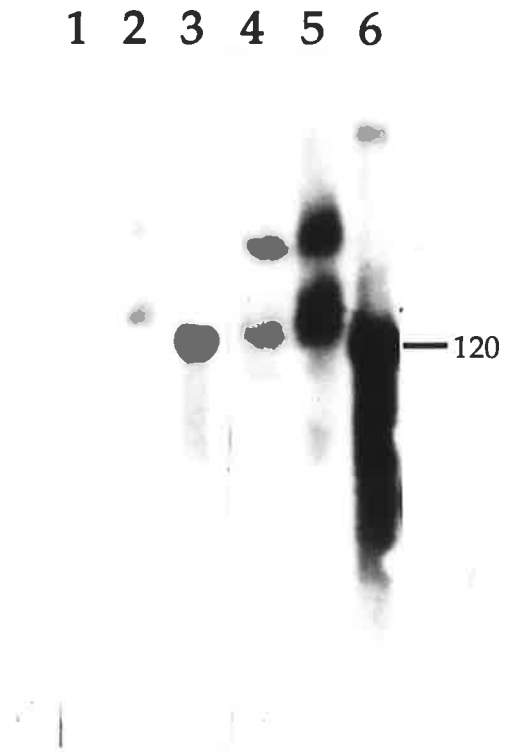


Fig. 4 - 13. SfiI digests of XTY-26 and normal human DNA probed with VK16A3. Lanes 1 and 4, approximately 5ug human DNA. Lanes 2 and 5, approximately 25ug human DNA. Lanes 3 and 6, approximately 1ug DNA from XTY-26. Lanes 1-3 show results after 16 hours exposure, and lanes 4-6 show results for the same filter after 72 hours exposure. The slight differences in mobility of the 120 kb fragment (e.g., between lanes 4, 5 and 6) occur because of the different amounts of DNA loaded in these lanes, as demonstrated by ethidium bromide staining (not shown). The additional approximately 200 kb band in lanes 1, 2, 4 and 5, as well as the 280 kb band in lane 6, are presumably due to incomplete digestion.

4.5. DISCUSSION

4.5.1. Strategies of Cloning the Fragile X

Various strategies have been adopted for molecular cloning of the fragile X. Dr. S. Warren and coworkers established somatic cell hybrids containing human/rodent translocation chromosomes with the breakpoint on human X chromosome at or near the fragile X site (Warren et al., 1987). Then they tried to isolate the human/rodent junctional DNA sequences, since the human portion of the junctional DNA should contain DNA sequences from the fragile X site (Warren et al.,1988).

In recent years, the YAC cloning system has been shown to have the capacity of cloning DNA fragment up to several hundred kilobase pairs, many times larger than that can be cloned in Lambda or cosmid vectors. The YAC cloning system has had great success in accelerating the cloning process of various genes (Wallace et al., 1990; Green et al., 1990).

To isolate fragile X, similar approaches were undertaken in different laboratories, although the initial emphases were different. Generally, DNA markers close to the fragile X were used to identify YAC clones from YAC libraries which were constructed from either normal X chromosome or fragile X chromosome (Burke et al., 1987; Albertsen et al., 1990; Abidi et al., 1990). Then in situ hybridization was used to localize the cloned human DNA fragments in relation to the cytogenetic fragile site. The fragile X hybrids, which were constructed to break at or near the fragile site (Warren et al., 1987, 1988, 1990), were also used as indications for crossing the fragile site. DNA probes characterized previously to be distal or proximal to the fragile X were other independent indications of crossing the fragile X. In this way, at least five laboratories independently obtained a YAC or YACs spanning the fragile X (Heitz et al., 1991, Dietrich et al., 1991; Hirst et al., 1991a; Kremer et al., 1991b; Verkerk et al., 1991). YAC clones that span the fragile site are summarized in Table 4 - 6.

In the present project, the approach to clone the fragile site was started by isolating a large number of DNA probes in the vicinity of the fragile X from a phage library constructed from somatic cell hybrid CY3, which contained human Xq26 to Xqter (Hyland et al., 1989).

These probes were then mapped physically and genetically in relation to the fragile X (Suthers et al., 1989, 1990, 1991a, 1991c). The DNA probes located very close to the fragile X site (such as VK18, VK16, VK21, VK23) were used to isolate YAC clones. To verify whether a YAC clone spanned the fragile site, in situ hybridization was considered to be the most reliable indicator. YAC XTY-26 isolated by VK16 (DXS293) was confirmed to span the fragile site by in situ hybridization and by containing the DNA probes known to flank the fragile site.

Dr. J. Mandel's group initially identified a CpG island with probe Do33, which showed abnormal methylation in fragile X syndrome males (Vincent et al., 1991). They also verified that two probes, St677 (DXS463) and Do33 (DXS465), were within a 2-Mb MluI fragment (Vincent et al., 1991), yet were on either side of the breakpoints of two fragile X hybrids (Rousseau et al., 1991a). Four YAC clones were isolated with these two probes. End probes from one of the YAC clones (209G4) were generated and shown to be separated by the breakpoints of these hybrids. With fluorescent in situ hybridization, YAC clone 209G4 was confirmed to span the fragile site. Another YAC clone 141H5 was found to contain 209G4, thus also spanned the fragile site. The restriction maps of the two YACs were in good agreement in the overlapping region and revealed the CpG island found to be associated with fragile X phenotype (Heitz et al., 1991).

Dr. A. Poustka's group established a 12-Mb physical map of the entire human Xq28 region. On this map, the breakpoints of several fragile X hybrids were located within a region of 700 kb between the loci DXS296(VK21) and DXS477(2.34) (Poustka et al., 1991). Therefore, these two markers as well as DXS465(Do33) were used to screen YACs from a YAC library. Four overlapping YAC clones (Y3, Y4, YF, Y47) were isolated by probes distal to the fragile X region (VK21, 391, A12, Do33). Three YAC clones (Y2, Y1, Y16) were detected by the proximal probe 2.34. YACs Y47 and Y1 were overlapped. End sequences of human inserts were isolated from those YAC clones and subsequently used as probes to screen cosmid libraries. A cosmid contig of 250 kb was established by chromosome walking and the breakpoints of the fragile X hybrids were further localized within a region of 50 kb within the cosmid contig. Two DNA probes (189

and 6.2) from this region identified abnormal methylation patterns in fragile X syndrome patients, indicating that the fragile X associated CpG island was very close to the fragile X hybrid breakpoint region. In situ hybridization with the cosmids from the contig showed that cosmid A6 was distal and cosmid 7172 was either proximal or distal to the fragile site. The cloned region was thus verified to contain the fragile X hybrid breakpoint region, the fragile X associated CpG island and the fragile site (Dietrich et al., 1991).

In Dr. K. Davies's group, a large number of unique DNA probes were generated by microdissection of the fragile X region (MacKinnon et al., 1990). They were used to construct a long-range restriction map covering 7 Mb DNA across the fragile site. In this 7 Mb DNA region, a CpG island was found to be abnormally methylated in fragile X syndrome males (Bell et al., 1991). Using sequence-tagged sites (STSs) derived from two of the microclones (M749 and M759) to screen YAC libraries, a YAC contig was established and shown to span the fragile site by in situ hybridization (Hirst et al., 1991a).

Dr. S. Warren and his coworkers initially studied the fragile X translocation breakpoints in fragile X hybrids. Through regional mapping of YAC clones to the distal human Xq (Nelson et al., 1991), a YAC clone RS46 (80 kb) was mapped to Xq27.3, proximal to the fragile X translocation breakpoints. A subclone of RS46 identified a 600 kb SalI fragment in hybrid Y75-1B-M1, which contained a fragile X chromosome and was a parent cell line of several fragile X translocation hybrids. This subclone, however, detected variant bands in six fragile X translocation hybrids, indicating that the translocation breakpoints of those hybrids were within the 600 kb SalI fragment. Using the primers generated from RS46, YAC clone 209G4, the same clone as that in Dr. J. Mandel's laboratory, was isolated. This YAC (209G4) was shown to contain sequences both distal and proximal to the breakpoints of the fragile X translocation hybrids and to span the fragile site by in situ hybridization (Verkerk et al., 1991).

All YAC clones which spanned the fragile site were particularly important for characterization of the molecular basis of the fragile X and its associated syndrome (Chapter 5; Yu et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Nakahori et al., 1991).

4.5.2. The DNA Region around the Fragile X

The human DNA sequences in XTY-26 were verified to span the fragile site by in situ hybridization and by containing the DNA markers both distal and proximal to the fragile site. In the restriction map of XTY-26, the closest proximal probe VK16B3 and the closest distal probe Do33 are about 140 kb to 160 kb apart, indicating that the fragile X is localized within a region of less than 160 kb. The orientation of the human fragment in XTY-26 was thus determined. In this region, two CpG islands were observed about 80 kb apart (Fig. 4 - 11, position 130 and 210). Since CpG islands are known to be associated with the 5' ends (or the promotor regions) of house keeping genes (Bird, 1986, 1987), the two CpG islands provided clues to search for the gene(s) responsible for the disorder, fragile X syndrome. Of the two CpG islands, the one next to VK16B3 (at position 130) contains all three restriction sites (SacII, BssHII and EagI) which were reported previously to be methylated in the DNA of fragile X syndrome males but not in normal individuals (Vincent et al., 1991; Bell et al., 1991). Therefore, this CpG island is more likely to mark the disease gene. Being only about ten kilobase pairs away from VK16B3, the CpG island at position 130 and its adjacent region can be easily covered by chromosome "walking" from VK16B3. This approach led to the characterization of the fragile X genotype (Yu et al., 1991; Chapter 5). The gap at the fragile site observed under microscope was estimated to be occupied by 10 to 100 kb of DNA by using a 425 kb human sequence (in YAC) as a probe (Heitz et al., 1991). With two probes only 160 kb apart, VK16 and Do33, our results suggested that the gap at the fragile site could contain much less DNA than that proposed by Heitz et al. (1991).

4.5.3. Comparison of the Fragile X and the Normal DNA Region

The unusually high mutation rate postulated for fragile X syndrome (Sherman et al., 1985) suggested that either a large gene or unusual sequences were involved in fragile X mutation. Comparison of the fragile X and the normal DNA region became very important for characterization of the fragile X mutation. This was carried out by comparison between

different combinations of DNA samples: different cloned DNA samples, cloned and genomic DNA samples, or different genomic DNA samples.

In this project, human DNA sequences in XTY-26 (derived from a fragile X chromosome) were compared with those in normal genomic DNA. First of all, DNA probes Do33 and 2.34 were hybridized to XTY-26 DNA and human genomic DNA both digested with enzymes BamHI, HindIII or TaqI. Each probe detected an identical hybridization pattern in both XTY-26 and human genomic DNA. Secondly, the 120 kb SfiI fragment in XTY-26, which contained the fragile X, was compared with the corresponding region in a normal genomic DNA sample. Enzyme SfiI was chosen because it does not contain CpG in its recognition sequences, and thus would not give different cleavage patterns between chromosomal and cloned DNA. The fact that a 120 kb SfiI fragment detected by VK16A3 in a normal genomic DNA sample indicated that the 120 kb region containing the two CpG islands was similar in size between normal and fragile X DNA, thus gross DNA rearrangement appeared unlikely to be involved in fragile X mutation.

In addition, the lack of gross DNA rearrangement in fragile X mutation was tested by comparison of the restriction maps of various YACs derived from a normal or a fragile X chromosome. XTY-26 was derived from a fragile X chromosome, whereas YACs Y1, 141H5 and 209G4 were constructed from normal X chromosomes and their restriction maps have been published (Dietrich et al., 1991; Heitz et al., 1991; Hirst et al., 1991a; Verkerk et al., 1991). Restriction maps of these YACs are in good agreement in the overlapping region (Fig. 4 - 14). The map of XTY-26 showed the best correspondence with the maps of 209G4 and 141H5 proposed by Heitz et al. (1991) and Verkerk et al. (1991), although there were small differences, which were likely due to the relative lack of precision of PFGE. On the restriction map published by Dietrich et al. (1991), only those restriction sites observed both in chromosomal DNA and in cloned DNA were shown. This explains the small number of restriction sites on their map.

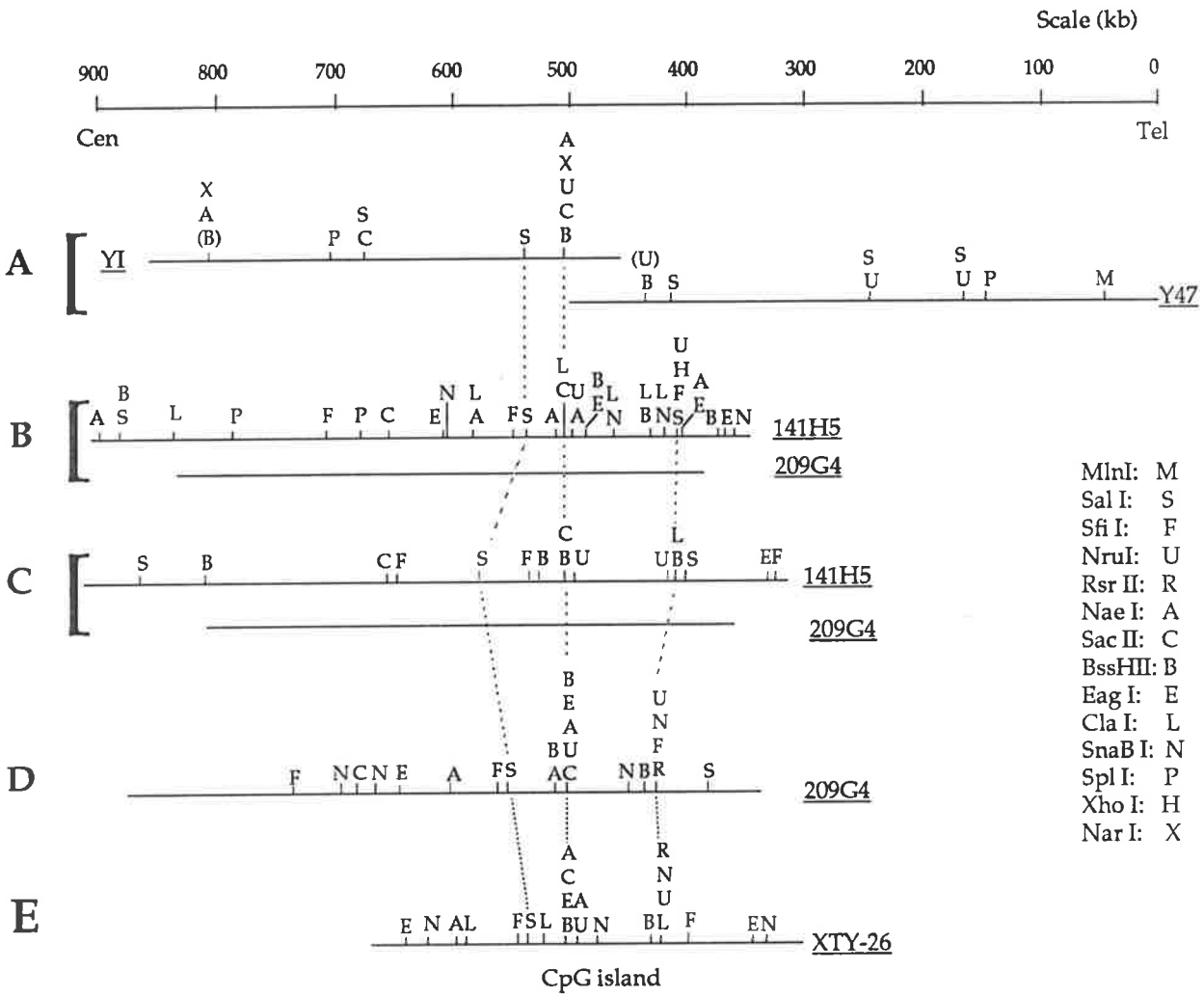


Fig. 4 - 14. Comparison restriction map of YAC clones around the fragile X associated CpG island. The name of the YACs are underlined. Restriction maps of YACs except that of XTY-26 are obtained from publications. The relevant references are: A) Dietrich et al., 1991; B) Heitz et al., 1991; C) Hirst et al., 1991a; D) Verkerk et al., 1991; E) Kremer et al., 1991b.

Higher resolution comparison of the fragile X and normal DNA has been carried out by Dr. A. Poustka's group between the cloned DNA samples and between the uncloned genomic DNA samples. A 170 kb DNA region distal to the fragile X associated CpG island was analysed by comparing restriction digests of clones isolated from libraries GM1416B (constructed from a normal X chromosome) and QIZ (constructed from a fragile X chromosome). Furthermore, DNA sequences in three cosmids, which flank the CpG island, were hybridized to Southern blots of normal and fragile X patient genomic DNA digests. No difference was observed between normal and fragile X DNA samples in all the tests, indicating no gross rearrangement in this area (Dietrich et al., 1991).

The above tests provided no evidence of gross DNA change at the fragile site. Higher resolution comparison between normal and fragile X DNA using subcloned sequences and frequent cutting restriction enzymes revealed the differences between them (Chapter 5).

CHAPTER 5

FRAGILE X GENOTYPE CHARACTERIZED BY AN UNSTABLE REGION OF DNA

5.1. SUMMARY

A lambda clone contig was constructed between the two closest markers which flanked the fragile X. The fragile X was mapped within a 15 kb DNA region by in situ hybridization with lambda clones from the contig. The translocation breakpoints of two fragile X hybrids, which were constructed to break at the fragile site, were found to lie within a common 5 kb EcoRI fragment in the 15 kb region. When the lambda clone, Subclone 5, which contained the 5 kb EcoRI fragment, was used as a probe to hybridize to the DNAs from normal individuals and fragile X syndrome patients, it was found that only in fragile X syndrome patients the 5 kb EcoRI fragment was replaced by a fragment (or fragments) which was larger but variable in size in different individuals.

In fragile X pedigrees, the variable region segregated with the fragile X genotype and differed in length within families, indicating that the region was unstable. This unstable DNA region is characterized by 1) exhibiting a small amplification in transmitting males and normal carrier females, but a large amplification in fragile X syndrome patients; 2) localized very close (250 bp) to the CpG island which was abnormally methylated in affected males; and 3) lying within one of the exons of the gene FMR-1. The DNA probe pfxa3, which hybridizes to DNA sequences in the vicinity of the variable region, detects fragile X mutation regardless of sex or cytogenetic expression, and thus provides an excellent reagent for identification of the fragile X genotype.

5.2. INTRODUCTION

The unusual genetics and cytogenetics of the fragile X syndrome make it particularly important to elucidate the fragile X genotype. Identification of the YAC clone XTY-26 which contains the human DNA sequences spanning the fragile X opened up this possibility (Chapter 4).

So far, many human genes have been cloned by using either direct cloning or positional cloning. Direct cloning is to target the gene based on the knowledge of the gene products. Whereas positional cloning is to target the gene by its known position on a chromosome. Since gross chromosomal rearrangements found in rare cases provide useful cytogenetic landmarks to pinpoint the disease genes, study of the rearrangement breakpoints has greatly facilitated the isolation of many disease genes by positional cloning. This approach has led to the identification of the genes for Duchenne muscular dystrophy (Monaco et al., 1986; Koening et al., 1987), retinoblastoma (Friend et al., 1986; Weinberg, 1989), Wilms tumour (Call et al., 1990; Gessler et al., 1990) and neurofibromatosis (Wallace et al., 1990, Cawthon et al., 1990).

The fragile site at Xq27.3, which is associated with fragile X syndrome, provides a cytogenetic marker for localization of the DNA sequences which are responsible for both the fragile site and the associated syndrome. Furthermore, a number of fragile X hybrids were constructed to break at the fragile site. Study of the breakpoints in these hybrids provides a means of mapping the fragile site at the DNA level.

This chapter presents how the fragile X region was localized and how the instability of this region was identified in fragile X families. The isolation of the DNA probe, pfxa3, a direct diagnostic reagent for fragile X syndrome genotype, is also described.

This project was carried out on a cooperative basis by Drs. M. Pritchard, E. Kremer, M. Lynch, E. Baker and the candidate. Contributions from each will be specified in the text.

5.3. MATERIALS AND METHODS

5.3.1. Somatic Cell Hybrids

5.3.1.1. Fragile X Hybrids

DNA samples from the following three fragile X somatic cell hybrids were provided by Dr. S. Warren (Atlanta, USA) on a collaborative basis. Construction of these somatic cell hybrids has been described elsewhere (Warren et al, 1987, 1988, 1990; see also section 1.4.6.).

Y75-1B-M1 contains a fragile X chromosome as the only human DNA in a Chinese hamster background and is the parental cell line of the following two hybrids .

Micro21D (a derivative of Y75-1B-M1), retains human Xpter-Xq27.3 which has been translocated to a rodent chromosome, with the breakpoint on the X chromosome at, or very close to, the fragile X site.

Q1X (also a derivative of Y75-1B-M1), retains only human Xq27.3-qter that has been translocated to a rodent chromosome, with the breakpoint on the X chromosome at, or very close to, the fragile X site.

5.3.1.2. Hybrid Cell Lines Related to XTY-26

GM4025 is a lymphoblastoid cell line constructed from a fragile X syndrome male. Hybrid 4.12 is a Chinese hamster/human cell line containing a fragile X chromosome from GM4025 (Nussbaum et al., 1983). X 3000.11 is a rodent/human hybrid containing human Xq24-qter derived from hybrid 4.12 (Nussbaum et al., 1986a). XTY-26 was isolated from a telomere-rescue YAC library constructed from X3000.11 DNA.

5.3.1.3. Non-Fragile X Cell Line

CY3 is a mouse-human hybrid cell line containing human Xq26-Xqter that was derived from a non-fragile X syndrome individual (Hyland et al., 1989).

5.3.2. DNA Samples from Fragile X Families

Peripheral lymphocyte DNA samples of 266 subjects from 25 fragile X syndrome families were obtained from the DNA Bank of Adelaide Children's Hospital (Adelaide, South Australia). The carrier status of each subject has been previously determined by cytogenetic examination and linkage analysis with flanking markers (Mulley et al., 1987, 1988; Suthers et al., 1991a, 1991b, Richards et al., 1991a).

5.3.3. Lambda Library Construction (performed by Dr. E. Kremer)

XTY-26 DNA was partially digested with Sau 3AI and was cloned into Lambda GEM-3 (Promega) using the manufacturer's protocols and packaging extracts. A lambda library of 4×10^4 pfu was made with the size of inserts within the range of 9 to 23 kb.

5.3.4. Lambda Library Screening (performed by Drs. M. Pritchard and M. Lynch)

The lambda library of XTY-26 was screened with total human DNA as a probe. One hundred and eleven purified positive lambda clones (known as Subclone 1, 2, ... 111) were obtained and gridded onto Hybond N+ filter (Amersham). From the grid containing 111 lambda clones, overlapping lambda clones across the fragile X were identified (see section 5.4.1.2.).

5.3.5. Generation of Alu PCR Products (performed by K. Holman)

A PCR product, Alu2, was generated by using XTY-26 DNA as template and the Alu consensus sequence oligo (TC)65 (Nelson et al., 1989) as the primer. PCR incubations were performed in 10 µl volumes in a Perkin Elmer-Cetus thermal cycler for 10 cycles and each cycle included incubation at 94°C for 60 seconds, at 60°C for 90 seconds, and at 72°C for 90 seconds, followed by 25 cycles at 94°C for 60 seconds, at 55°C for 90 seconds, and at 72°C for 90 seconds.

5.3.6. Generation of RNA Probes from Lambda Clone DNA (performed by Dr.M. Pritchard and the candidate)

Lambda vector GEM-3 was designed in the way that a T7 promoter lies in one arm and an Sp6 promoter in the other arm. Since both promoters are located near the cloning site, RNA sequences can be generated from the vector-insert junction. To produce DNA templates of appropriate size at the vector-insert junction, DNA from each lambda clone was digested with restriction endonucleases HaeIII, TaqI or HindIII. Then the pooled DNA digests were used as templates to generate RNA probes using an in vitro transcription system kit (Promega) according to the manufacturer's instructions, except using ^{32}P labelled UTP instead of ^{32}P labelled CTP. The following components were added at room temperature: 3.5 µl of nuclease-free H_2O, 4 µl of 5 X transcription buffer, 2 µl of 100 mM DTT, 0.5 µl (20 units) of RNasin ribonuclease inhibitor, 4 µl of ATP-GTP-CTP mix (2.5 mM each), 2 µl (200 ng) of template DNA, 3 µl of ^{32}P-UTP, and 1 µl (20 units) of Sp6 or T7 RNA polymerase. Then the mixture was placed at 37°C for one hour before hybridization (no denaturing was required).

5.3.7. Southern Blot Analysis

Genomic DNA samples were cleaved with endonuclease EcoRI or PstI (Biolabs) according to the manufacturer's instructions.

Five DNA probes, Fragment A, B, C, D and E (pfxa3) (see Fig. 5 - 1G) were derived from a 5.0 kb EcoRI fragment of lambda Subclone 5. Fragment A (0.8 kb), Fragment B (1.05 kb), Fragment C (1.0 kb), and Fragment D (2.3 kb) were produced when the 5.0 kb EcoRI fragment was digested with PstI. Fragment E (known as pfxa3) is a single copy DNA probe derived from Fragment B (Fig. 5 - 1G). The methods for electrophoresis, Southern blotting and hybridization were described in Chapter 2.

The DNA probes containing repetitive DNA sequences were prereassociated with an excess of human DNA to block the repeated sequences in the probes (performed by J. Nancarrow). The DNA probes were radiolabelled with the random primer kit (Amersham) to incorporate ^{32}P-

dCTP. The labelled probe was mixed with 1 mg of human placental DNA (Sigma) (sonicated to an average size of 500 bp), and the reaction was adjusted to 5 x SSC (pH 7.0). The DNA probe was denatured by heating for 10 minutes at 100°C and then incubated at 68°C for one hour before addition to the hybridization solution (Hyland et al., 1989).

5.3.8. Washing Condition for Probe pfxa3

DNA probe pfxa3 is a 536 bp PstI/NheI fragment and its DNA sequence has been previously reported (Kremer et al., 1991a). This DNA probe has a high CG content (66%) and the Tm (the temperature at which the probe is 50% denatured) was calculated to be 77°C*. Therefore, after hybridization with pfxa3, the blots were washed in 0.1 x SSC containing 0.1 % SDS at 70°C for 30 minutes to reduce background signal.

* Tm calculation:

$Tm = 81.5 - 16.6 \times (|\log [Na^+]|) + 0.41 (\%C+G) - (600/N)$

N, the length of the probe (in bp).

$[Na^+]$, sodium concentration in mole.

5.4. RESULTS

5.4.1. Localization of the Fragile X Region

The procedure for localization of DNA sequences at the fragile X region is illustrated in Fig. 5 - 1.

5.4.1.1. Establishment of the Closest Markers Flanking the Fragile X (performed by K. Holman, M. Pritchard and the candidate)

Probe VK16 was shown to be proximal, and Do33 was distal to the fragile X by in situ hybridization. They were 160 kb apart on the restriction map of XTY-26. To establish DNA markers between VK16 and Do33, several Alu PCR products were generated by using XTY-26

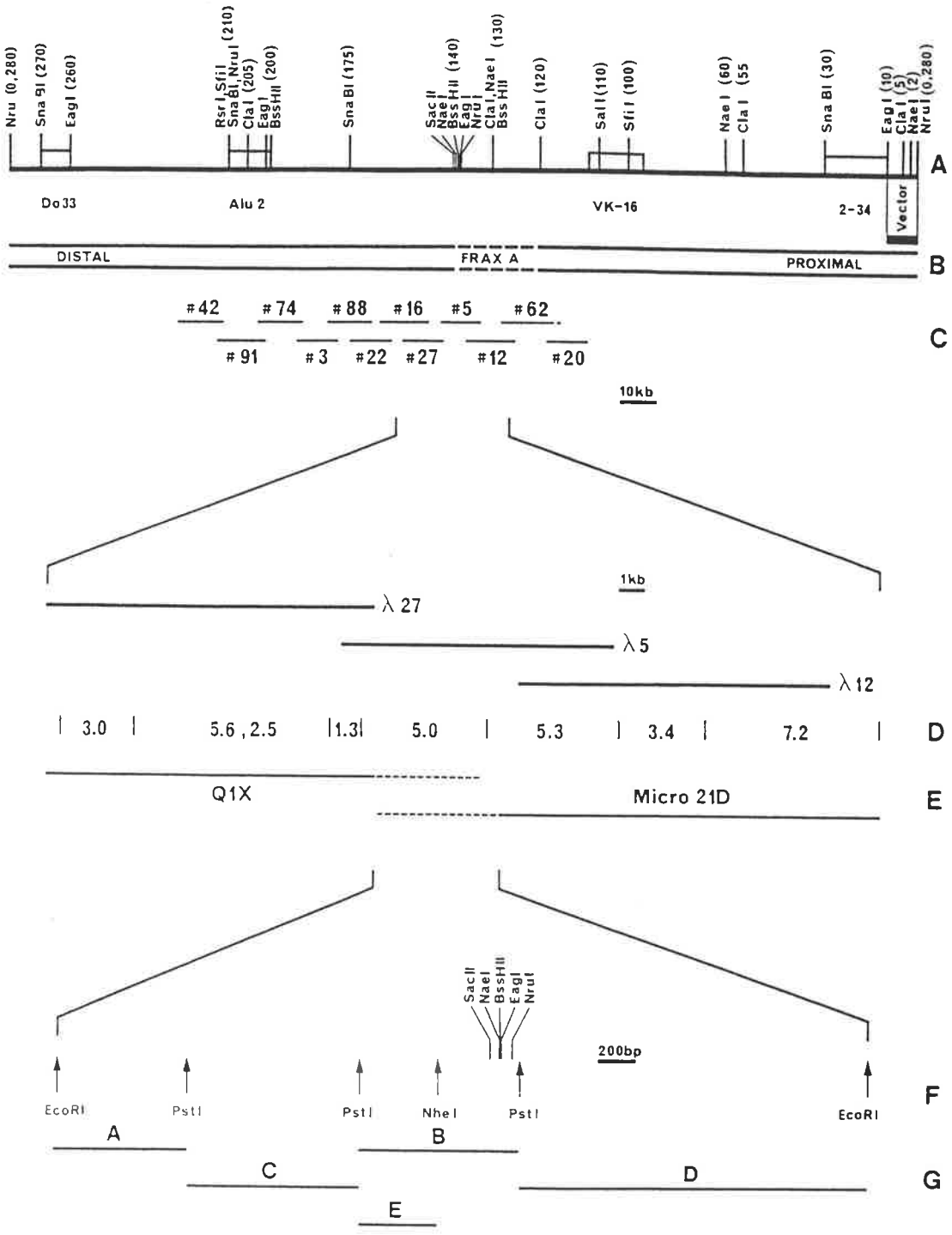


Fig. 5 - 1. A schematic diagram illustrating the steps taken in localization of the fragile X region. Each step has been described in detail in the results section of this chapter (see section 5.4.1.).

DNA as a template. Only one Alu PCR product, known as Alu2, was mapped between VK16 and Do33 on the restriction map of XTY-26. Since Alu2 did not give a clear result from in situ hybridization, Subclone 91 (a lambda clone hybridized to Alu2) was used instead, and was shown to be distal to the fragile X by in situ hybridization. Therefore, VK16 and Alu2 (Subclone 91) were the two closest markers flanking the fragile X and they were 100 kb apart (Fig. 5 - 1A).

5.4.1.2. Establishment of a Lambda Contig across the Fragile X Region (Fig. 5 -1C, performed by Dr. M. Pritchard and the candidate)

From the lambda library of XTY-26 (see section 5.3.5.), 111 lambda subclones containing human DNA fragments were isolated (see section 5.3.6.) and used for further lambda contig construction. A lambda clone contig was constructed between the two closest flanking markers, VK16 and Alu2. RNA probes from both ends of Subclone 91 (hybridized to Alu2) and Subclone 20 (hybridized to VK16) were generated and used to identify overlapping clones from the 111 lambda subclones. The direction of the walk was established by hybridization of those RNA probes to the blots containing various restriction enzyme digests of XTY-26. Moreover, another four lambda clones were selected from the 111 subclones on the basis that they did not hybridize to any of the DNA probes tested (2.34, Do33, VK16 and Alu2), in the hope that some of these lambda clones might lie between VK16 and Alu2. Three of these four lambda subclones, Subclone 3, 22 and 27, were found to lie between VK16 and Alu2. These clones therefore provided multiple points for walking, and thus accelerated the process of contig construction. RNA probes generated from Subclone 3, 22, 27 were hybridized to the grid containing the 111 lambda subclones to isolate overlapping clones. In this way, the lambda contig was extended further until the region between the two flanking markers was covered. All lambda clones from which RNA probes were derived and lambda clones overlapping with each RNA probe are listed in Table 5 - 1. Representative lambda clones between the two closest markers are presented in Fig. 5 - 1C.

Table 5 - 1. Overlapping Lambda Clones

Subclones	RNA probes (T* and S*) generated from lambda subclones of XTY-26												
	20 T-S	91 T-S	55 T-S	3 T-S	22 T-S	27 T-S	45 T-S	102 T-S	79 T-S	5 T-S	62 T-S	53 T-S	8 S**
5						+				+ +			+
8										+	+		+
9		+											
12								+		+	+	+	+
14				+	+								
15		+											
16					+	+							
17	+		+						+		+ +	+	
18		+											
20	+ +		+				+				+	+	+
23					+ +								
24						+ +							
25						+ +							
27						+ +				+			
32				+									
37		+											
38		+											
42		+											
43				+									
44				+									
45	+		+ +				+ +	+ +	+		+	+ +	
52				+	+								
53	+		+ +				+ +	+ +	+		+	+ +	
55	+		+ +				+	+ +			+	+ +	
56				+									
58					+ +								
60		+											
62	+		+ +				+ +	+ +	+ +		+ +	+ +	
63				+	+								
69					+								
72	+		+				+	+	+		+	+	
73		+											
74		+		+									
75		+											
78				+ +									
79	+		+ +				+ +	+ +	+		+	+ +	
80										+	+	+	+
86		+											
88				+	+		+						
91		+ +					+						
96										+			+
97					+ +								
99		+ +											
102	+		+ +				+ +	+	+		+	+ +	
103	+							+ +	+ +		+	+	
111					+ +		+						

* RNA probes generated with T7 promotor (T); or Sp6 promotor (S). ** RNA probes generated from both ends of Subclone 15 and 18 and from one end (T7) of subclone 8 contain repetitive DNA sequences.

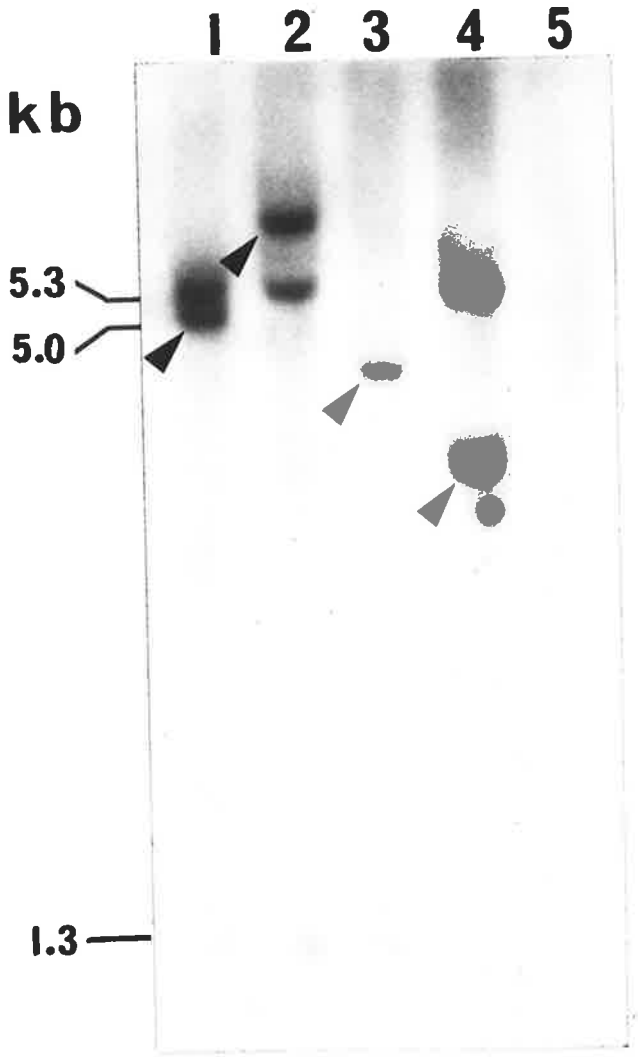


Fig. 5 - 2. Localization of the breakpoints of two fragile X hybrids to a 5 kb EcoRI fragment. A blot containing EcoRI digests of CY3 (lane 1), Y75-1B-M1 (lane 2), Q1X (lane 3), micro21D (lane 4) and A9 (lane 5) was probed with Subclone 5 (provided by J. Nancarrow).

5.4.1.3. Definition of the Fragile X Site by In Situ Hybridization (Fig. 5 - 1B, performed by E. Baker)

Most lambda clones in the lambda contig between Alu2 and VK16 were localized in relation to the fragile X by in situ hybridization. The distribution of in situ hybridization signal for various lambda clones in relation to the fragile site are summarized in Table 5 - 2. Most of these clones were mapped on either side of the fragile site, whereas Subclones 5, 12 and 62 and their overlapping clones had signals on both sides of the fragile X. Therefore, the fragile site was located to a DNA region of 15 kb (shown in dashed lines in Fig. 5 -1B).

5.4.1.4. EcoRI Restriction Map of the DNA Region Bridging the Fragile X (Fig. 5 - 1D, performed by J. Nancarrow, Drs. M. Pritchard and M. Lynch)

EcoRI restriction maps of the human inserts in Subclones 5, 12, 20, 27, 55, and 62 were constructed. Each of these Subclones was then used as probe for Southern blot analysis to establish a EcoRI restriction map in human genomic DNA. The EcoRI restriction map generated from cloned DNA and that from genomic DNA were in agreement. The EcoRI restriction map around the fragile X region is shown in Fig. 5 - 1D.

5.4.1.5. Localization of the Breakpoints of Two Fragile X Hybrids (Fig. 5 - 1E, performed by J. Nancarrow)

Subclones 5, 12 and 62 appeared to span the fragile X site by in situ hybridization. Therefore, they were further tested to see whether they span the breakpoints of the fragile X somatic cell hybrids (see section 1.4.6.). After prereassociation, each of these three lambda clones was hybridized sequentially to the EcoRI digests of hybrids CY3, Y75-1B-M1, Micro21D and Q1X. Subclone 12 and 62 showed different hybridization patterns on the EcoRI digests, but the pattern was identical among the above hybrid cell lines (data not shown). Subclone 5 detected three EcoRI bands (5.3, 5.0 and 1.3 kb) in the non-fragile X cell line CY3 (Fig. 5 - 2, lane 1). In hybrid Y75-1B-M1 which contains a fragile X chromosome, the 5.3 kb and 1.3 kb bands detected by

Subclone 5 were identical to that in CY3. However, the 5 kb band was found to be replaced by a band of 5.9 kb (Fig. 5 - 2 lane 2, indicated with an arrow). In hybrid Q1X (containing DNA distal to the fragile site), the 1.3 kb band was identical to that in CY3, and in hybrid Micro21D (containing DNA proximal to the fragile site), the 5.3 kb band was identical to that in CY3. In both hybrids, however, the 5 kb EcoRI fragments were missing and replaced by bands of different sizes (Fig. 5 - 2 lanes 3 and 4, indicated with arrows). This suggested that the breakpoints in Micro21D and Q1X were located in a common 5 kb EcoRI fragment.

5.4.1.6. Identification of a Variable DNA Region Associated with Fragile X Syndrome (performed by J. Nancarrow and the candidate)

Since Subclone 5 was shown to span the fragile site and to contain the breakpoints of the two fragile X hybrids, it was used as a probe to identify the abnormal DNA region associated with fragile X syndrome. Probe Subclone 5 detected no variations in all the DNA samples from normal individuals. However, in all DNA samples from fragile X syndrome males the 5 kb EcoRI fragment, which contained the breakpoints of the two fragile X hybrids, was replaced by a fragment or fragments of higher molecular weight (Fig. 5 - 3) but varying in size in different patients (Fig. 5 - 3). This 5 kb EcoRI fragment was thus considered to contain the DNA region associated with fragile X syndrome. To further localize the variable region, the 5 kb EcoRI fragment was subcloned into pUC19 (performed by Dr. M. Pritchard), known as Plasmid 5. The restriction map of the 5 kb EcoRI fragment was constructed as shown in Fig. 5 - 4 and Fig. 5 - 1G. (More accurately, the 5 kb EcoRI fragment should be 5.2 kb, as shown in Fig. 5 - 4. To avoid confusing, it will be referred as 5 kb in this Chapter, 5.2 kb in Chapter 6). Restriction fragments (Fragment A. B. C and D) (Fig. 5 - 1G) were generated from the 5 kb EcoRI fragment by PstI digestion and were used as probes on PstI digests of normal and fragile X syndrome individuals. Each of Fragment A, B, C and D detected identical hybridization patterns among those individuals. Fragment B (known as pfxa2) was found to hybridize to repetitive DNA sequences in the human genome. However, Fragment E (known as pfxa3), a 536 bp PstI/NehI fragment

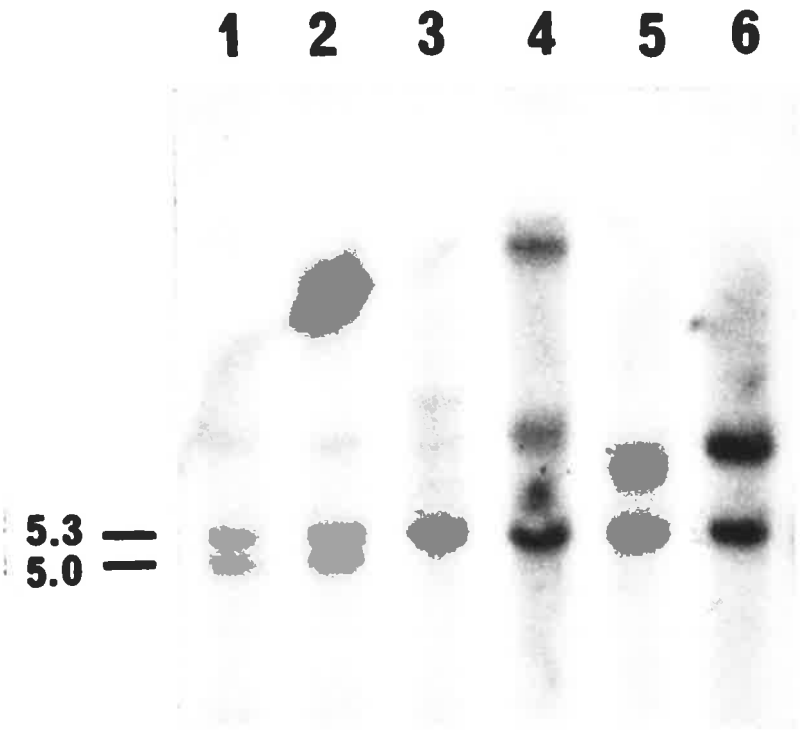
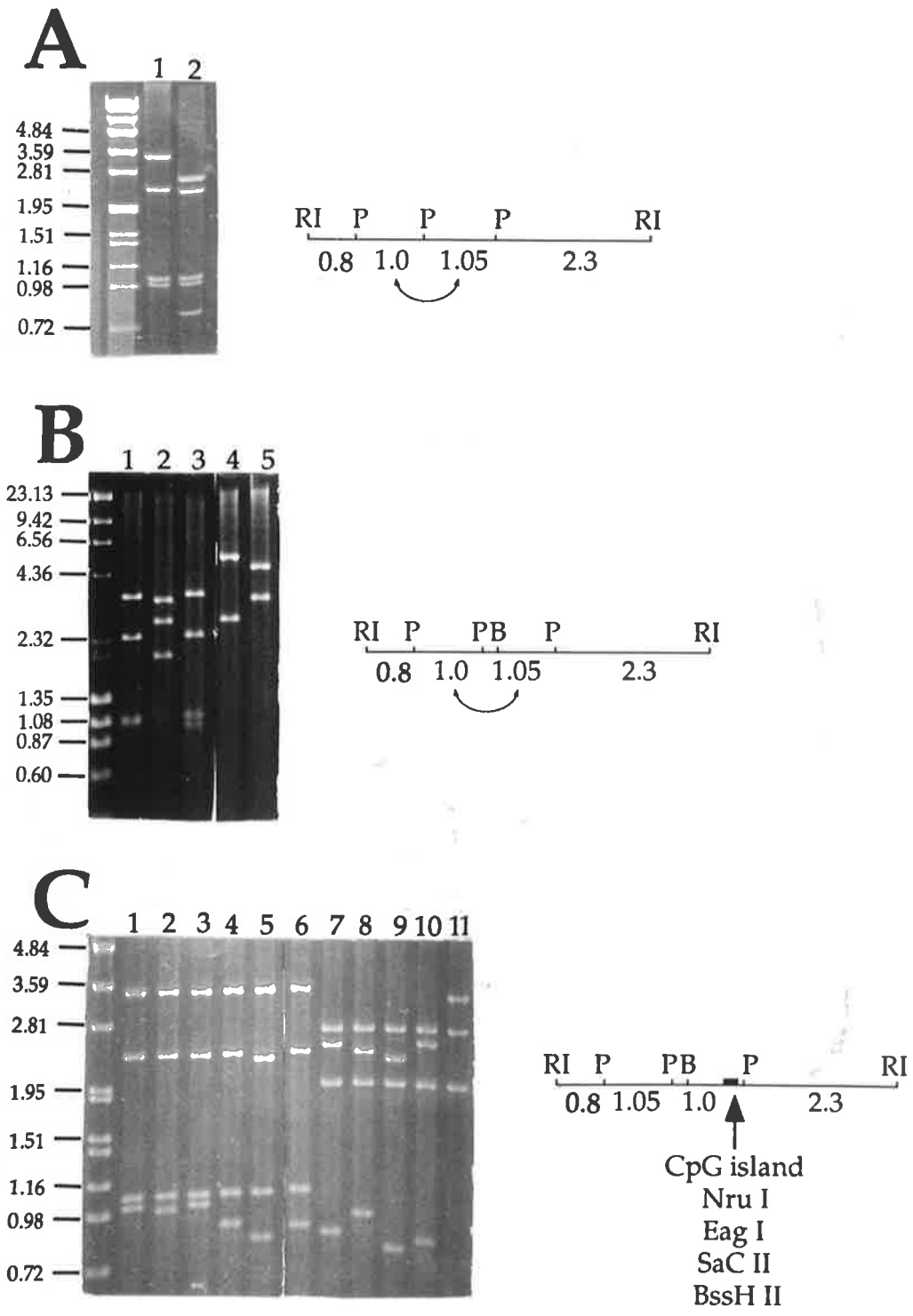


Fig. 5 - 3. Identification of the variable region associated with fragile X syndrome. A blot containing EcoRI digested DNA samples from a normal male (lane 1), a normal male from a fragile X family (lane 2) and four unrelated fragile X syndrome males (lanes 3 to 6) was hybridized with Subclone 5. In two normal males (lanes 1 and 2), the probe detected three bands of 5.3 kb, 5.0 kb and 1.3 kb in size. However, in all four affected males, the 5 kb band was replaced by a band larger but varible in size (lanes 3 to 6) in different individuals. The 5.3 kb and 1.3 kb bands are identical in all six samples (the 1.3 kb band is not shown) (provided by J. Nancarrow).

Fig. 5 - 4. Construction of the restriction map of the 5 kb EcoRI fragment. Plasmid 5 DNA digested with various enzymes was separated on a 1% agarose gel. DNA was stained with ethidium bromide and visualized with UV light. A) a gel containing DNA of plasmid 5 digested with PstI (lane 1) and PstI/EcoRI (lane 2). The PstI map is thus constructed as shown next to the gel (P: PstI restriction site). The order of the two internal PstI fragments (1.0 kb and 1.05 kb) cannot be determined as indicated by the arrows. B) A gel containing DNA of plasmid 5 digested with PstI (lane 1), BamHI/EcoRI (lane 2), BamHI/PstI (lane 3), EcoRI (lane 4) and BamHI (lane 5). One BamHI site (B) is present as shown on the map next to the gel. C). A gel containing DNA of plasmid 5 digested with various enzymes: PstI (lane 1), PstI/NruI (lane 2), PstI/NaeI (lane 3), PstI/EagI (lane 4), PstI/SacII (lane 5), PstI/BssHII (lane 6), BamHI/EcoRI/EagI (lane 7), BamHI/EcoRI/NruI (lane 8), BamHI/EcoRI/SacII (lane 9), BamHI/EcoRI/BssHII (lane 10) and BamHI/EcoRI (lane 11). Four rare restriction sites (NruI, EagI, SacII and BssHII) are located in the 1.0 kb PstI fragment and the order of the two internal PstI fragments is determined as shown on the map next to the gel.


A
1 2
4.84
3.59
2.81
1.95
1.51
1.16
0.98
0.72
RI P P P RI
0.8 1.0 1.05 2.3
B
1 2 3 4 5
23.13
9.42
6.56
4.36
2.32
1.35
1.08
0.87
0.60
RI P PB P RI
0.8 1.0 1.05 2.3
C
1 2 3 4 5 6 7 8 9 10 11
4.84
3.59
2.81
1.95
1.51
1.16
0.98
0.72
RI P PB P RI
0.8 1.05 1.0 2.3
CpG island
Nru I
Eag I
SaC II
BssH II

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derived from Fragment B, hybridized strongly to a single PstI fragment and also detected variation in size between unrelated fragile X syndrome patients. The variable sequence associated with fragile X syndrome was thus localized to the 1kb PstI fragment.

5.4.2. Instability of the 1 kb PstI Fragment in Fragile X Families (performed by the candidate)

The nature of this variable sequence associated with fragile X syndrome was further investigated in fragile X syndrome pedigrees. PstI digested DNA samples of 266 individuals from 25 fragile X families were analysed with probe pfxa3. This study demonstrated that the variable region segregated with the fragile X genotype. Instead of the normal 1kb PstI fragment, a higher molecular weight band(s) was observed in all transmitting males, female carriers, and fragile X syndrome patients. However, the size of the pfxa3 band was different within families even between affected brothers. A representative pedigree is shown in Fig. 5 - 5. The variable sequence was observed to increase in size from generation to generation if the genotype was transmitted by females, but not when the genotype was transmitted by males. This indicated that the variable DNA sequence in the fragile X region was highly unstable during female meiosis in fragile X families.

5.4.3. Fragile X Specific Hybridization Pattern

DNA probe pfxa3 detected a 1kb PstI band in DNA samples from normal individuals. It detected, however, a PstI band(s) larger than 1 kb in DNA samples from all individuals with the fragile X genotype, which indicated an amplification or insertion involved in fragile X mutation. The size of the band was usually larger in affected individuals than that in normal carriers (Fig. 5 - 6). Some fragile X syndrome patients had one to four recognizable bands, varying in size from about 1.6 to 3.5 kb, decreasing in intensity as the number of bands increased. Others had multiple bands that manifested as a smear. The heterogeneous pattern indicated instability of the variable region during mitosis. The number of individuals with different pfxa3 hybridization patterns are summarized in Table 5 - 3.

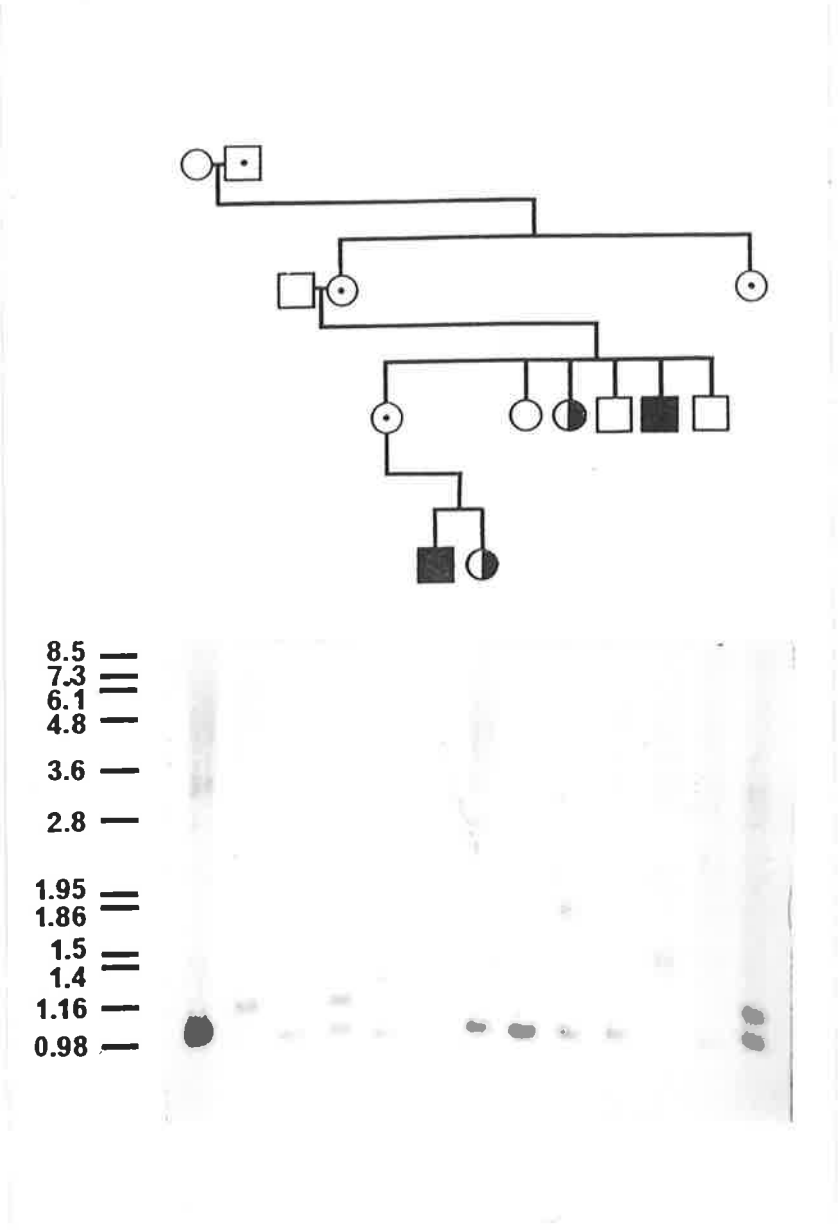


Fig. 5 - 5. Unstable DNA sequences associated with the fragile X genotype in a fragile X syndrome pedigree. DNA from members in one branch of a fragile X family (see F19 in appendix I) was digested with PstI and subjected to Southern blot analysis with probe pfxa3. The corresponding lane is found under each symbol. Pedigree symbols: transmitting males (dot in a square), female carriers not expressing the fragile X (dot in a circle), carrier females expressing the fragile X (half shaded circle), Retarded fragile X syndrome males expressing the fragile site (shaded square), normal females (open circle) and normal males (open square).

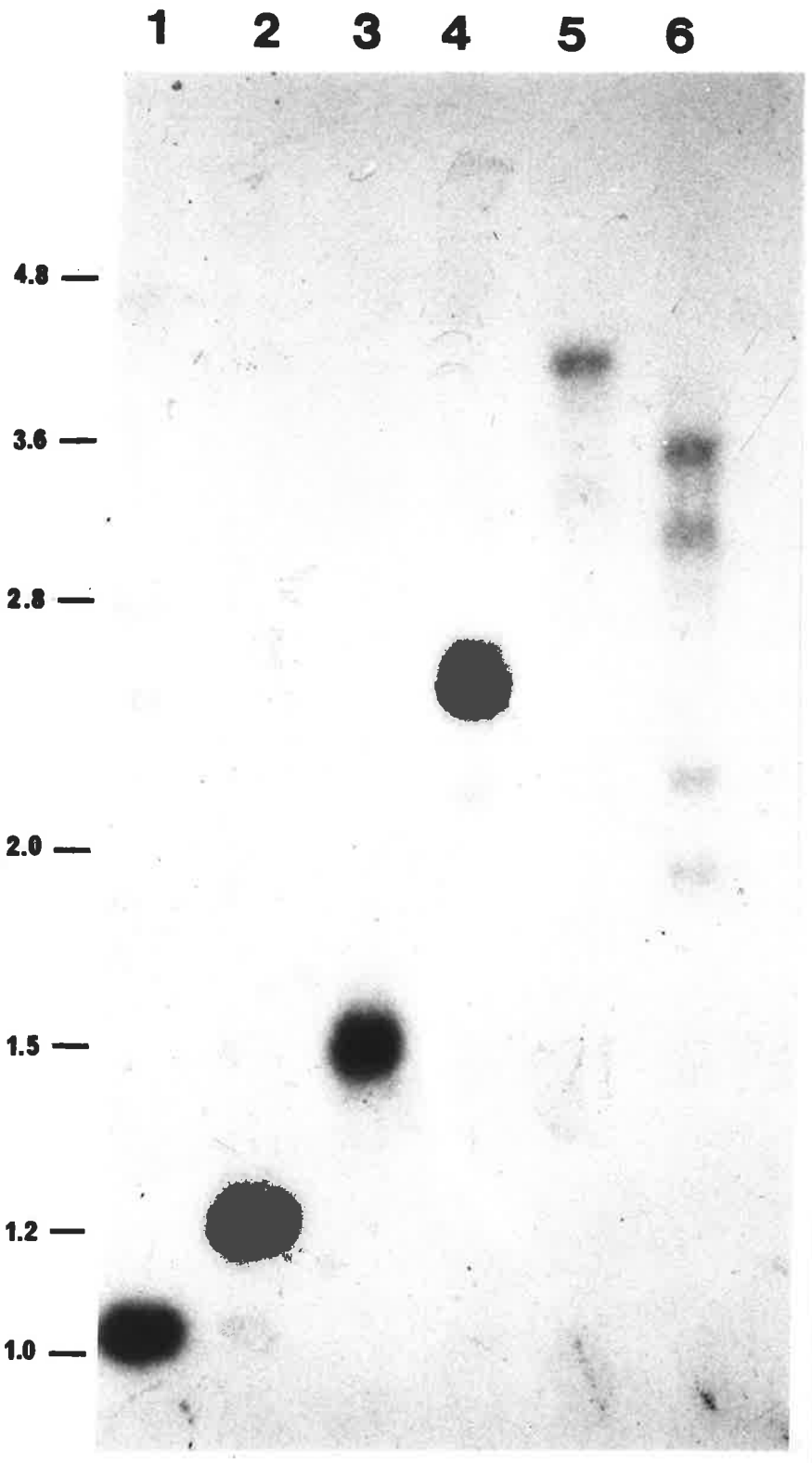


Fig. 5 - 6. Unstable DNA sequences associated with the fragile X genotype. Southern blot of PstI digested genomic DNA probed with pfxa3. A normal male (lane 1) shows a 1.0 kb normal band. Transmitting males (lanes 2 and 3) show replacement of the normal band by a larger band of up to about 1.6 kb. Fragile X syndrome males (lanes 4 - 6) also show replacement of the normal band by either a single band greater than about 1.6 kb in size (lane 4) or multiple bands of decreased intensity which approach a "smear" (lanes 5 and 6).

Table 5 - 3. Number of Individuals With Different Fragile X specific Hybridization Patterns Detected By pfxa3 (PstI digests)

Classification	Normal band	Single band of increased size	2 to 4 bands of increased size	Multiple bands (smear)
		MALES		
Affected	0	18	10	5
Transmitting	0	11	1	0
Normal	65	0	0	0
		FEMALES		
Normal carrier	82	71	5	6
Affected	9	4	3	2
Normal	65	0	0	0

5.4.4. The Variable Region Decreases in Size During Cloning (performed by the candidate)

As mentioned earlier in this chapter, YAC XTY-26 was isolated from a YAC library constructed from the human/hamster somatic cell hybrid X3000.11. Hybrid 3000.11 was made from hybrid 4.12 containing a fragile X chromosome. Hybrid 4.12 was made from a lymphoblastoid cell line, GM4025, from a fragile X syndrome male. In this study, the variable region in XTY-26 was subcloned into a lambda vector (namely Subclone 5) and further subcloned into plasmid vector pUC19 (known as Plasmid 5). To trace if any DNA size change occurred during cloning, PstI digests of GM4025, X3000.11, XTY-26, subclone 5 and plasmid 5 were used for Southern analysis with pfxa3 as a probe (DNA from hybrid 4.12 was not available). As shown in Fig. 5 - 7, a 2.8 kb PstI band is observed in the GM4025 DNA (lane 1), a 1.8 kb PstI band in the X3000.11 DNA (lane 2), and a 1 kb PstI band in DNAs of XTY-26 (lane 3), Subclone 5 (lane 4) and Plasmid 5 (lane 5). The slight difference in mobility of the bands in lanes 3, 4, and 5 is likely due to different amounts of DNA loaded. Obviously, the variable sequence in the fragile X syndrome patient decreases in size during cloning.

5.5. DISCUSSION

5.5.1. Instability of the Fragile X Mutation

Fragile X genotype is characterized by an unstable region of DNA (Yu et al., 1991, Oberlé et al., 1991). Instability of the DNA sequences in the fragile X region has been demonstrated in both meiosis and mitosis. Generally, in the fragile X families, when the fragile X genotype is transmitted by a female, the size of the unstable region increases from generation to generation. However, when the fragile X genotype is transmitted by a male, this unstable region either remains the same size or only slightly increases in size (up to 200 base pairs). Therefore, marked differences in hybridization patterns have been shown within pedigrees, even between sibships, whenever the mother is a carrier. In a proportion of males and females who carry the mutation, multiple bands of increased size, or a smear, are visible. This heterogeneity probably results from instability of the variable region in somatic cells during mitosis.

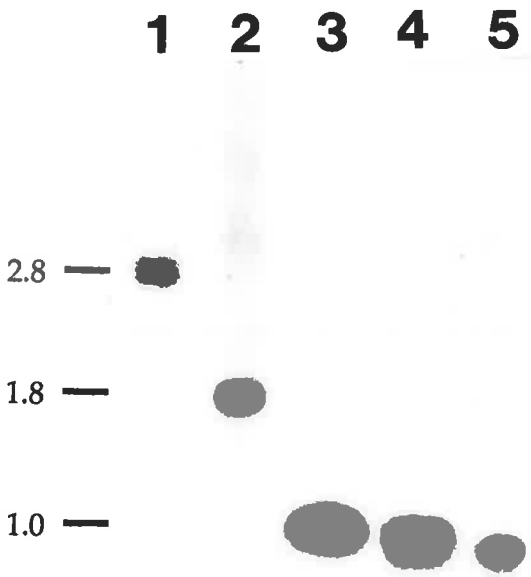


Fig. 5 - 7. The variable region decreases in size during cloning. A blot containing PstI digested DNA from GM2045 (lane 1), X3000.11 (lane 2), XTY-26 (lane 3), Subclone 5 (lane 4) and Plasmid 5 (lane 5) was probed with pfxa3. Approximately equal molar amounts of each DNA sample were added in each lane (lane 1, 10 µg; lane 2, 10 µg; lane 3, 10 µl of agarose beads; lane 4, 0.015 µg; lane 5, 0.0033 µg). Sizes of bands are indicated in kilobase pairs.

The instability at the fragile X region was also observed by Oberlé et al. (1991). They initially noticed abnormal methylation of a CpG island in the vicinity of DXS465 (Do33) in fragile X syndrome males (Vincent et al., 1991). Probes Do33 and St677 were used to isolate four YAC clones (Heitz et al., 1991). One of the YAC clones, 209G4, was shown 1) to contain the breakpoints of fragile X somatic cell hybrids, 2) to span the fragile site by fluorescent in situ hybridization, and 3) to contain the CpG island which was the site of abnormal methylation in patients with fragile X syndrome. Furthermore, a 9 kb DNA region around the CpG island was isolated. DNA probes were generated from the 9 kb region and used to identify fragile X-specific hybridization patterns. One of the DNA Probes, StB12.3 (identical to pfxa7 see Fig. 5 - 8), which localized telomeric to the CpG island, detected fragile X-specific highly variable patterns in BglI or in BglI/EagI digests in almost all individuals carrying a fragile X mutation. The same probe StB12.3 also detected different methylation patterns between normal and affected males in BanI digests. DNA probes StA22 and StX21E (analogous to pfxa5 , see Fig. 5 - 8), which localized centromeric to the CpG island, also detected the highly variable patterns in BanI digests of affected males, in which the normal 1.15 kb BanI fragment was absent or much reduced and was replaced by a larger band. However, transmitting males showed the same pattern as normal males. These probes (StB12.3, StA22 and StX21E) also detected abnormal patterns in EcoRI, HindIII, AvaII and XmnI digests (Oberlé et al. 1991). These authors also observed different hybridization patterns between males from the same family or even between affected brothers, which indicated instability of the variable region. From a study of 49 fragile X families, they concluded that 1) normal transmitting males as well as their daughters always have a small amplification in the 150-500 bp range; 2) when the mutation was transmitted by carrier males, it remained either unchanged or had a small size increase of up to 200 bp, however, when the mutation was passed from the daughters of transmitting males to their next generation, 80% of individuals with the fragile X genotype showed large amplification from 1.5 to 2.5 kb; 3) the abnormal hybridization bands appeared as a faint smear instead of visible bands in some cases, which indicating somatic heterogeneity; 4) a small amplification was always associated

with a lack of clinical symptoms and absence or low levels of fragile X expression. All of these observations are in agreement with those in this study.

5.5.2. Molecular Basis of the Instability at the Fragile X Region

The fragile X region of instability was further localized to a trinucleotide CCG repeat because the DNA sequences flanking the CCG repeat were identical between normal and affected individuals (Kremer et al., 1991a, Fu et al, 1991). Normal individuals had from 6 to 46 copies of CCG, from which carriers had a small amplification of the CCG repeat up to 600 basepairs (bp). However, the boundary (CCG copy number) between normal and carrier is not established. By comparison, affected individuals had an amplification of the CCG repeat larger than that seen in normal carriers. Again, at what CCG copy number a carrier would be affected is not known since the existing PCR methods failed to amplify across a CCG repeat longer than 600 bp (Fu et al, 1991). Based on the above observation, it was proposed that amplification of the CCG repeat unit is associated with the fragile X syndrome, although the mechanism of amplification is poorly understood.

Amplification of the CCG repeat unit represents a novel molecular mechanism of DNA mutation. It is different from the known mutation mechanisms such as DNA point mutations, frame-shift mutations, deletions, insertions and other DNA rearrangements. This new mutation mechanism provides explanations to many of the unusual genetics of the fragile X syndrome. Furthermore, it may account for a wide range of genetic phenomena including anticipation, incomplete penetrance and variable expression, which are not explained by the well known mutation mechanisms (Sutherland et al., 1991a).

It has been documented that CCG repeat exists in other parts of the human genome, such as in the ribosomal RNA gene (Gonzalez et al., 1985) and in the breakpoint cluster region (BCR) gene which is involved in the Philadelphia chromosome rearrangement (Zhu et al., 1990). It would be very interesting to know whether the CCG repeats in these regions also exhibit instability.

A gene (FMR-1) isolated from the fragile X region expresses a 4.8 kb message RNA in human brain and placenta (Verkerk et al, 1991). One of the exons of FMR-1 contains the CCG repeat (Verkerk et al., 1991). The FMR-1 mRNA has been shown to be present in normal individuals and in normal carriers, but absent in most of the males with fragile X syndrome. This strongly suggests that FMR-1 is involved in the development of the fragile X syndrome (Pieretti et al., 1991).

5.5.3. Molecular Diagnosis of Fragile X Syndrome

DNA probes that locate at the vicinity of the CCG repeat, such as pfxa3, are shown to be an excellent indicators in diagnosis of fragile X genotype. Southern analysis using these probes has many advantages over the traditional methods. For example, observation of cytogenetic expression cannot identify transmitting males or about 50% of carrier females. The high specificity of RFLP linkage analysis requires that the family be informative for the available DNA markers, which are so close to FRAXA that crossover between them is unlikely. However, pfxa3 and other similar probes can detect abnormal hybridization patterns in almost all individuals with the fragile X genotype, regardless of sex or cytogenetic expression.

DNA probes in the fragile X region have also been developed in other laboratories (Fig. 5 - 8). DNA probe StB12.3 (identical to pfxa7), StX21 and StA22 (both analogous to pfxa5) can detect the fragile X specific hybridization patterns in EcoRI, HindIII, AvaII and XmnI digests, but not in TaqI, PstI and MspI digests (Oberlé et al, 1991). Apart from that, StB12.3 can detect methylation-specific patterns in BanI digests (Oberlé et al., 1991). Probes Ox0.55 (identical to pfxa3), Ox1.9 (analogous to pfxa7) (Nakahori et al., 1991) and pE5.1 (identical to the 5 kb EcoRI fragment in Plasmid 5) (Verkerk et al., 1991) can determine the fragile X specific hybridization pattern on Southern analysis. Further experience with these probes in fragile X families could conceivably lead to a means of fragile X genotype identification, as well as phenotype prediction and eventually revealed novel genetics of the unstable element (Chapter 6).

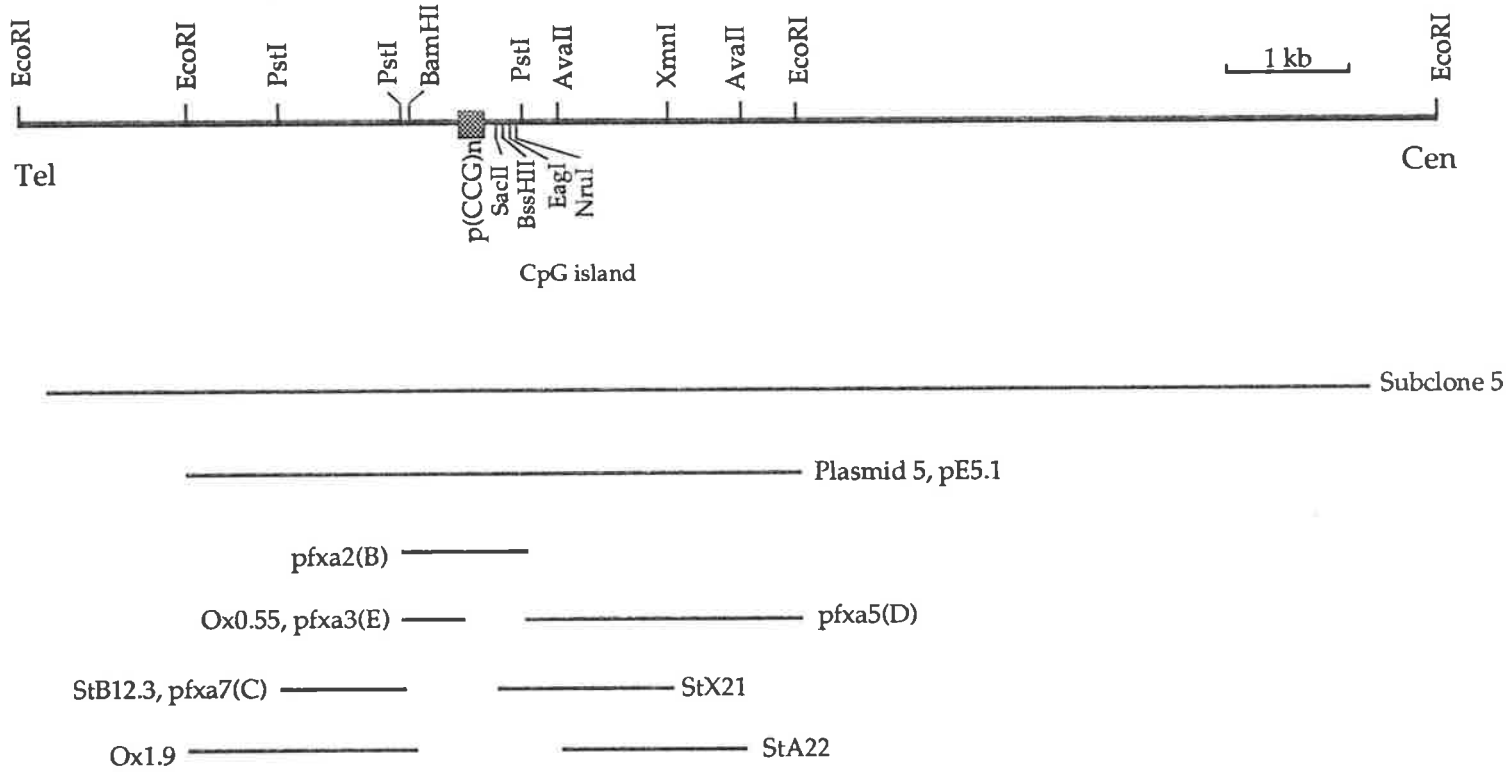


Fig. 5 - 8. DNA probes around the fragile X region. DNA probes Subclone 5, Plasmid 5 and probes with the prefix pfxa were generated in this study. Other probes were isolated in other laboratories. Positions of different DNA probes in relation to the CpG island and the CCG repeat region [p(CCG)n] are indicated. The names of probes in brackets are corresponding to that in Fig. 5-1G. The relevant references are: "pfxa" probes: Kremer et al., 1991; Yu et al., 1991. "St" probes: Oberlé et al., 1991; "Ox" probes: Nakahori et al., 1991; pE5.1: Verkerk et al., 1991.

CHAPTER 6

UNIQUE GENETICS OF THE HERITABLE UNSTABLE ELEMENT

6.1. SUMMARY

The fragile site at Xq27.3 is an amplified CCG repeat sequence. This sequence shows stable polymorphism in the normal population and exhibits variable amplification in fragile X syndrome pedigrees. The length of this sequence correlates with fragile site expression, mental status and methylation status of the SacII site in the CpG island adjacent to the fragile site. There is a direct relationship between increased CCG copy number and propensity for instability: individuals having large amplifications exhibit somatic variation. The instability of the CCG repeat, when transmitted through affected pedigrees, explains the unusual segregation patterns of fragile X syndrome, including the Sherman paradox. All of the patients with fragile X syndrome were found to have a parent who has an amplified CCG repeat. This indicates that all cases of fragile X syndrome are familial. In the fragile X syndrome families, the DNA probe pfxa3 is now routinely used for prenatal diagnosis and for the determination of carrier status.

6.2. INTRODUCTION

Fragile X syndrome, as an X linked disorder, has perplexed clinicians, cytogeneticists and molecular geneticists for more than two decades by its unusual genetics. These include the existence of transmitting males, high penetrance (30%) in female heterozygotes, increasing penetrance in successive generations (referred to as the Sherman paradox), high prevalence (Sherman et al., 1984; 1985) and the association with a fragile site at Xq27.3.

Recently, a CpG island was shown to be abnormally methylated in fragile X syndrome patients (Vincent et al., 1991; Bell et al., 1991). DNA probes near the CpG island detected a variable region which differed in size in fragile X syndrome patients, transmitting males, carrier females and normal individuals (Yu et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Nakahori et al.1991; Rousseau et al., 1991b). This region exhibited instability in meiosis and mitosis in fragile X syndrome families (Yu et al., 1991; Oberlé et al., 1991). The variable region was found to be a CCG trinucleotide repeat of variable copy number (Kremer et al., 1991a; Verkerk et al., 1991; Fu et al., 1991). The CCG repeat is embedded within a gene (FMR-1) whose transcription is associated with the absence of methylation of the CpG island adjacent to the CCG repeat (Verkerk et al., 1991; Pieretti et al., 1991).

To investigate the instability of the CCG trinucleotide repeat during transmission from generation to generation and to assess possible correlations of the fragile X genotype with phenotype, the genotypes of individuals from 49 fragile X syndrome pedigrees were determined by Southern blot analysis with probe pfxa3. This chapter presents the novel genetic properties of the fragile X mutation obtained from family studies, and the utility of DNA probe pfxa3 in direct molecular diagnosis of the fragile X syndrome. Genotyping of fragile X families with pfxa3 were carried out by the candidate with assistance from Andrew Donnelly and Agi Gedeon (ACH, Adelaide).

6.3. MATERIALS AND METHODS

6.3.1. Genomic DNA Samples

6.3.1.1. DNA Samples from Fragile X Families

Peripheral lymphocyte DNA samples from 420 subjects of 39 fragile X families (including the 25 fragile X families studied in Chapter 5) were obtained from the DNA bank of Adelaide Children's Hospital (Adelaide, South Australia). All families had been previously studied for cytogenetic fragile site expression and linkage information had been gathered by using flanking DNA markers. These results, collected in the Department of Cytogenetics and Molecular Genetics of Adelaide Children's Hospital during a period of 15 years, have been reported elsewhere (Mulley et al., 1987, 1988; Suthers et al., 1991a,b; Richards et al., 1991a). The DNA samples and the cytogenetic data from an additional 10 fragile X families were provided by Dr. G. Turner (Sydney, Australia) on a collaborative basis, and seven of them were selected because they contained apparently isolated cases. The mental status of each subject was considered to be that determined by the contributing clinicians. All pedigrees, together with information on mental status, fragile site expression, and the size of the pfxa3 fragment and the SacII site methylation status at the CpG island for each individual are shown in Appendix I.

6.3.1.2. DNA Samples of Somatic Cell Lines

Somatic cell lines (including 23 lymphoblasts and 7 fibroblasts) were established from 23 members of nine fragile X families in the Department of Cytogenetics and Molecular Genetics of Adelaide Children's Hospital (Adelaide, Australia). The cell lines were maintained by Ms. S. Lane, and DNA extraction was performed by Mrs. J. Spence of the Department.

6.3.1.3. DNA Samples of Various Tissues from a Terminated Fragile X Affected Fetus

Multiple tissues from an 11 week fragile X syndrome fetus following suction termination curettage were obtained from Dr. L Kornman (Queen Victoria Hospital, Adelaide, South

Australia). Histological examination of each tissue was performed to reveal the tissue type by R.W. Byard (ACH, Adelaide). The identified tissues included intestine, cartilage, skin, muscle, squamous epithelium, liver, kidney, heart, gonad, osteoid, vertebral body, calvaria, chorionic villus and umbilical cord. DNA was extracted from the various tissues by the candidate using a method modified from that of de Martinville et al. (1984).

Tissue pieces were washed in proteinase K buffer to remove maternal blood and the wet weight of each tissue was recorded. 50 - 70 mg of tissue was put into a screw capped eppendorf tube containing 400 µl of proteinase K buffer, 50 µl of 10% SDS and 50 µl of 10 mg/ml proteinase K. After being well mixed, tubes were put on a rotating wheel at 37°C overnight. After this step, no apparent tissue pieces were seen in tubes. DNA extraction was performed once with an equal volume of phenol, once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and once more with an equal volume of chloroform/isoamyl alcohol (24:1). DNA precipitation was performed by adding 0.1 volume of 3 M sodium acetate (pH 4.6) and two volumes of cold ethanol to each tube. Then the tubes were inverted several times until DNA precipitation was apparent. The DNA was transferred to a fresh tube and washed twice in 70% ethanol and desiccated for 5 minutes before resuspension in TE (3 - 5 µl per mg of wet weight tissue).

6.3.2. DNA Probes and Southern Blot Analysis

DNA probe pfxa3 is a 536 bp fragment, its target sequence is a PstI/NheI fragment which lies immediately distal to the CCG repeat and about 250 bp distal to the fragile X related CpG island (Fig. 5 - 8). The pfxa3 probe detected a 1.0 kb PstI fragment in DNA samples from normal individuals, and higher molecular weight fragment(s) from individuals carrying the fragile X mutation.

Probe pS8 is an 800 bp PstI fragment derived from YAC XY-539 (see Fig. 3 - 9C), and detects an 800 bp PstI fragment of human genomic DNA on Southern blots. It was used as an internal positive control probe in double hybridization with probe pfxa3 on PstI filters.

For Southern blot analysis, 8 μg of each DNA sample was digested with restriction endonuclease PstI (Biolabs) according to the conditions recommended by the manufacturer. The restriction fragments were separated on a 1% agarose gel with molecular weight marker (see section 2.4.3.). After Southern blotting the filters were hybridized with DNA probes pfxa3 and pS8 under the conditions specified in Chapter 2 (see section 2.4.6.) and then washed as described in Chapter 5 (see section 5.3.8.). The hybridization bands were sized visually by comparison with the molecular weight markers and the size increase from the normal 1 kb PstI band was recorded as Δ, adopted from Oberlé et al. (1991). For example, an abnormal band of 3 kb was recorded as Δ = 2 kb.

For better estimation of unstable element band length, genomic DNA was digested to completion with Sau3AI prior to electrophoresis on a 1.3% agarose gel. After blotting, the filter was probed with pfxa3 alone. In normal individual, the Sau3AI band detected by pfxa3 was o.7 kb.

Details of blotting, gel electrophoresis and hybridization conditions were presented in Chapter 2.

6.3.3. Methylation Study

In order to analyze the methylation status of the SacII site in the fragile X associated CpG island, DNA samples from individuals with the fragile X genotype were digested with endonucleases EcoRI and SacII and probed with pfxa3 after blotting. The probe detected a 2.8 kb band in a normal unmethylated X chromosome, and a 5.2 kb band in a normal methylated X chromosome (such as the inactive X chromosome in females). Therefore, a normal male has only the 2.8 kb band; a normal female has both the 2.8 kb and the 5.2 kb bands. Individuals with the fragile X genotype had band(s) larger than these two by their value of Δ. Female heterozygotes would have bands generated from both the normal X and the fragile X chromosome. In a proportion of females with the mutation, the bands derived from the normal X chromosome and from the fragile X chromosome could be distinguished based on the size

differences. However, in those females with a very small amplification (Δ = 0.1 ~ 0.3 kb), the band derived from the normal X chromosome is too close to be distinguished from the band derived from the fragile X chromosome. Methylation study was performed in those female heterozygotes in whom the bands from the two X chromosomes were distinguishable, so that the degree of methylation in the normal X chromosome and in the fragile X chromosome could be determined independently. The degree of methylation was determined by comparing the intensity of the methylated and unmethylated bands from each X chromosome (degree of methylation = intensity of the methylated band/intensity of the methylated band plus the unmethylated band).

6.3.4. Polymerase Chain Reaction across the CCG Repeat (performed by D. Hillen)

Polymerase chain reaction across the CCG repeat was accomplished using a modification of the reaction conditions of Kogan et al. (1987). Because of the exceptionally high CG content of this region, 7-deaza-dGTP was used in place of dGTP. The reactions were performed in a volume of 10 µl with 5 µCi of alpha 32P dCTP and visualized after autoradiography for 72 hours following electrophoresis on 6% polyacrylamide-urea gels. Thermal cycle conditions were as previously described (see section 5.3.5.). The primers, #203 and #213, were on either side of the CCG repeat (Kremer et al., 1991a). The electophoretic mobility of the CCG containing sequence (in certain instances of known length) was inconsistent with the mobility of DNA size markers on both acrylamide and agarose gels, presumably as a consequence of its unusual base composition. This is evident in Fig. 6 - 8B by the large discrepancy between the dystrophin PCR markers (388, 360 and 331 base pair in length) and the 203/213 PCR products using pfxa2 (fragment B in Fig. 5 - 1G) as a template (310 base pairs in length) (Kremer et al., 1991a). Lengths of CCG containing sequences must therefore be considered approximations.

6.4. RESULTS

6.4.1. Correlation Between Size of Amplified CCG Repeat and Phenotype

Including the 25 families studied in Chapter 5, a total of 49 fragile X syndrome pedigrees were studied by Southern blot analysis with probe pfxa3. Genotypes of 420 individuals from these 49 fragile X families were determined (see Appendix I). These included 120 normal and 85 fragile X genotype males and 90 normal and 125 fragile X genotype females. Fig. 6 - 1 shows the distribution of the amplified element size in males (A) and females (B) who carry the mutation. When the phenotype (mental status) of each individual was considered, a correlation between size of the amplified CCG repeat and phenotype was apparent. In all males with fragile X genotype, the normal 1 kb PstI band (Δ = 0 kb) was replaced by one or more bands of increased but variable size. However, the variable bands seen in the affected individuals were larger (Δ > 0.6 kb) than that in 16 transmitting males ($\Delta \leq$ 0.6 kb) (Fig. 6 - 1A). It would thus appear that, in males, small amplification of the unstable element was tolerable as far as the functions of the target tissues were concerned, but once the amplification reached a certain size it interfered with gene function.

Among female heterozygotes, those recorded by clinicians as "affected" usually had moderate to severe mental retardation, whereas those with border line mental retardation were often recognized as unaffected. The proportion of affected females in the present data is likely to be underestimated. Based on the available information in female heterozygotes, those with small amplification ($\Delta \leq$ 0.6 kb) were always not mentally impaired (Fig. 6 - 1 B) and often did not express the fragile site cytogenetically or in rather low percentage (Fig. 6 - 2B); whereas those with larger amplifications (Δ > 0.6 kb) usually had cytogenetic expression of the fragile site, and could be either carriers or mentally retarded (Fig. 6 - 1B; Fig. 6 - 2B). This observation implies that factors other than amplification, for example, methylation of this region of the X chromosome and /or non-random X inactivation in cells of the target tissues, may contribute to the phenotype, especially to intellectual status, in female heterozygotes.

Moreover, the correlation between the size of the amplified element and the degree of cytogenetic fragile X expression was observed in both sexes (Fig. 6 - 2A; B).

6.4.2. Correlation Between Degree of Amplification in Carrier Mothers and that in Their Offspring

Marked amplification of the CCG repeat at the fragile X region was observed when the fragile X genotype was transmitted by females but not when transmitted by males. To further investigate if the degree of amplification of the CCG repeat in mothers related to that in their offspring, 51 female carriers, who have had at least one child with fragile X genotype, were divided into four groups according to their degree of amplification, Δ value. The length of the CCG repeat in the 51 carrier mothers and that in their offspring was determined by Southern blot analysis with pfxa3 on PstI digests. As shown in Fig. 6 - 3, female carriers with a $\Delta = 0.1$ kb usually produce a small increase ($\Delta \leq 0.6$ kb) in the successive generation, while those female carriers with $\Delta = 0.2$ kb may have small ($\Delta \leq 0.6$ kb) or large ($\Delta > 0.6$ kb) amplification in their offspring. In contrast, carrier females with $\Delta = 0.3 \sim 0.6$ kb usually have only large amplification in their offspring. Each type of female transmission is shown in Fig. 6 - 4. As a result, carrier women with $\Delta \leq 0.2$ kb can have either transmitting or affected sons, while those with $\Delta > 0.2$ kb have only affected sons, where those sons inherited the fragile X genotype. This result indicates that the degree of amplification in offspring increases with degree of amplification in their mothers. In other words, the risk of a carrier female having an affected son increases with the length of her amplified CCG repeat sequence.

In contrast to female transmission, when males transmitted the fragile X, the size of the CCG repeat region usually remained either unchanged or had small changes (increase or decrease) limited within 200 bp (Fig. 6 - 5 A. B). The only one (out of 69) fertile fragile X syndrome male, who showed a smear of pfxa3 bands, had two daughters who have inherited an apparently identical pfxa3 band of $\Delta = 0.4$ kb. The pfxa3 hybridization patterns of the male (lane 1) and one of his daughters (lane 2) are shown in Fig. 6 - 5 C.

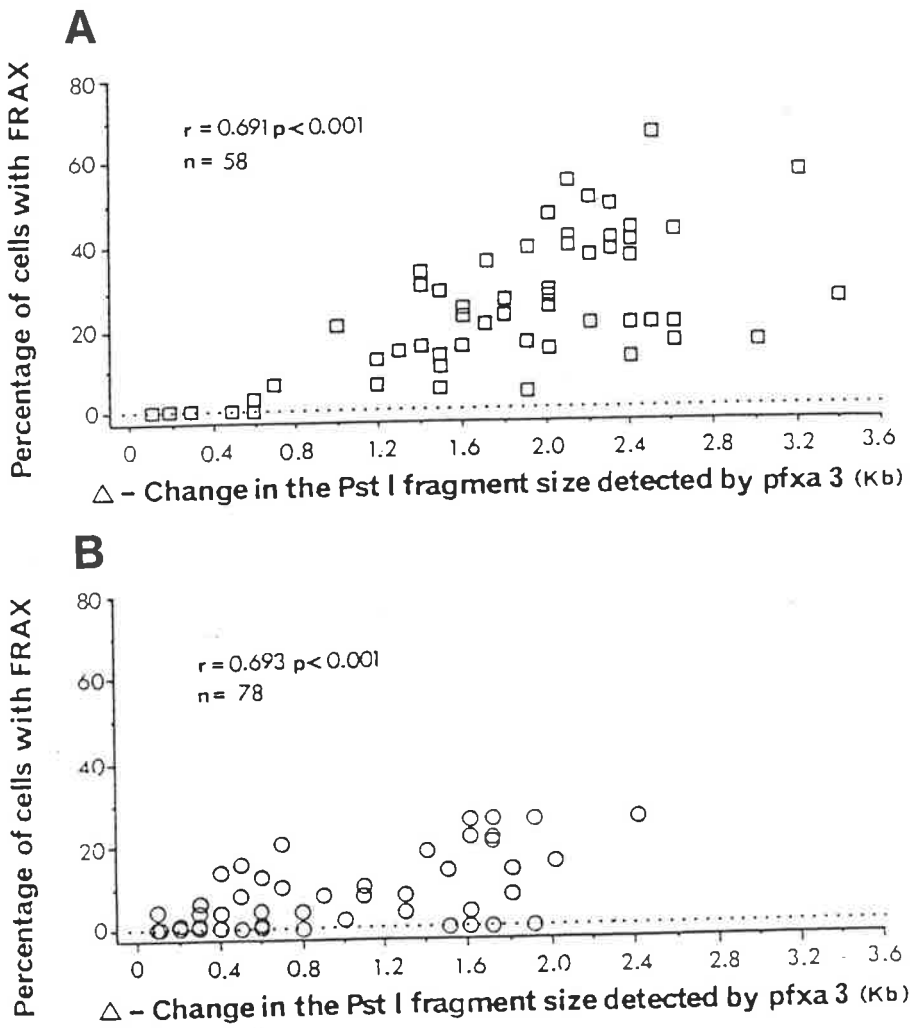


Fig. 6 - 2. Correlation of unstable element length with the percentage of fragile X expression in males (A) and females (B). For the individuals with more than one band only the size of the largest detected band was scored.

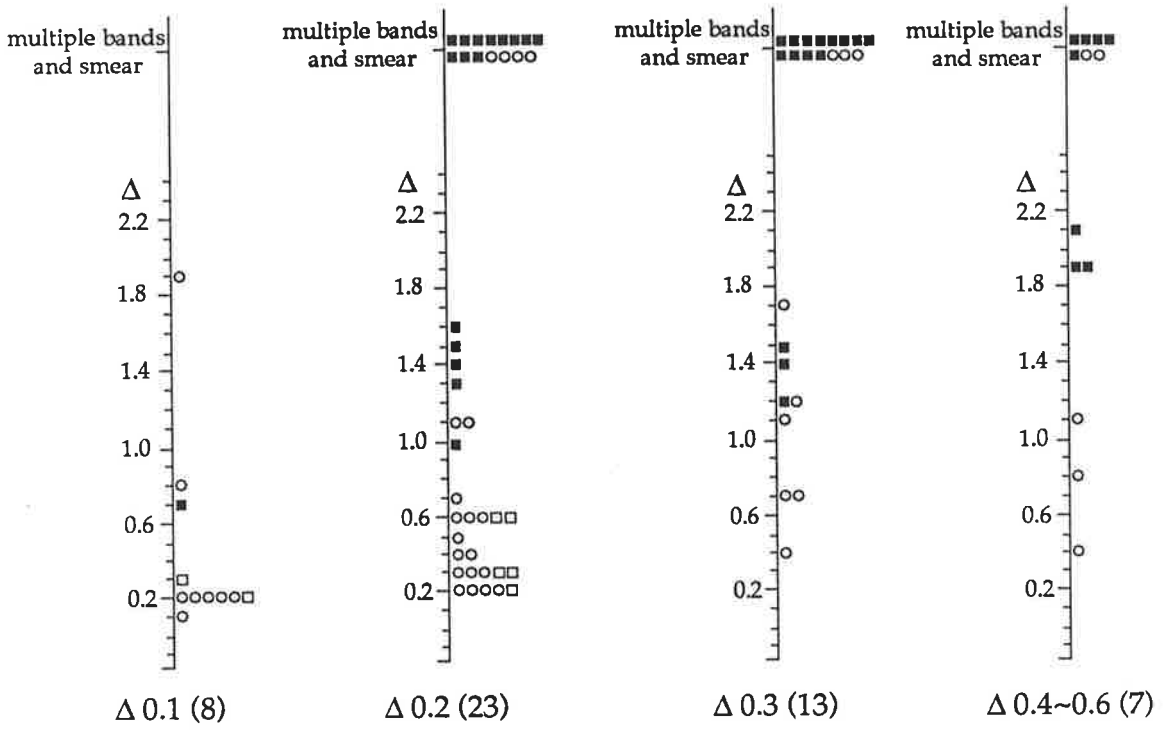


Fig. 6 - 3. Correlation of unstable element length in fragile X carrier mothers with that in their fragile X genotype offspring. Carrier mothers are divided into groups according to their Δ value (indicated beneath each column) determined by the pfxa3 PstI fragment. The Δ value in their offspring is plotted above. The number of carrier mothers in each group is indicated in brackets. Symbols: circle, female carrier; open square, transmitting male; closed square, fragile X syndrome male.

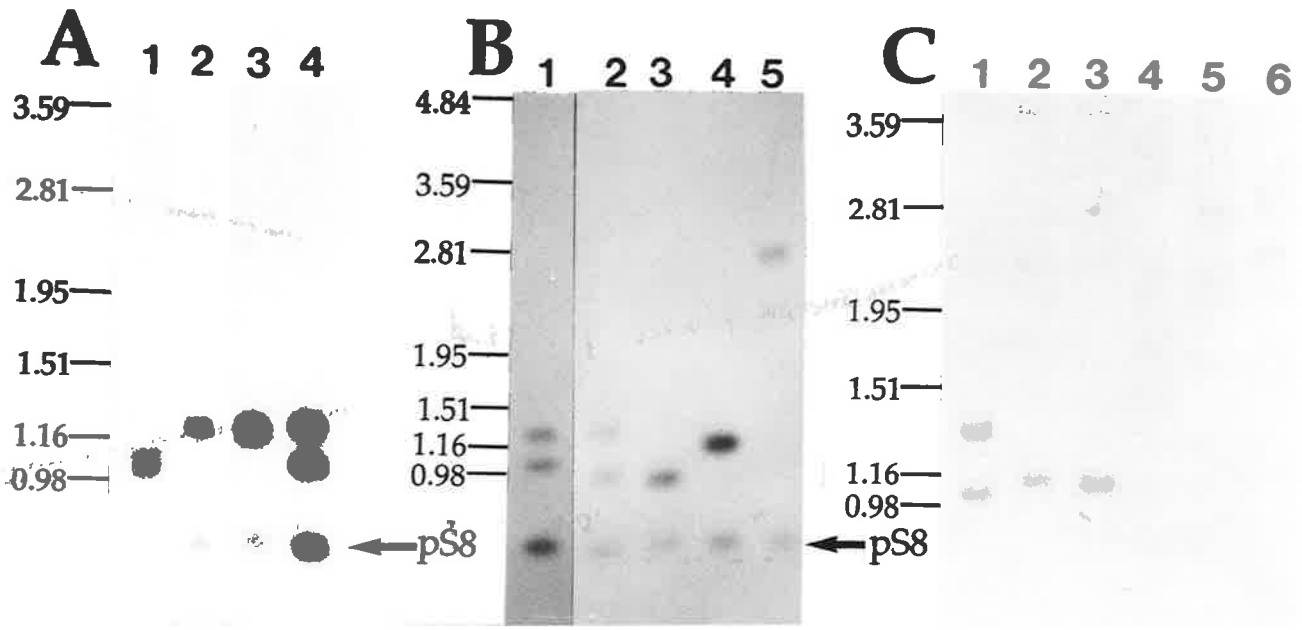


Fig. 6 - 4. Sample pedigrees demonstrating different types of female transmission. PstI digests of genomic DNA were used for Southern analysis with probe pfxa3. Panel A: a carrier mother with Δ = 0.1 kb (lane 1) had two sons (lane 2, 3) and one daughter (lane 4) all with small amplifications. Panel B: a carrier mother with Δ = 0.2 kb (lane 1) had one daughter (lane 2) and one son (lane 4) with small amplification, one affected son with large amplification (lane 5). Panel C: a carrier mother with Δ = 0.3 kb (lane 1) had three affected sons all with large amplification manifesting as smears (lanes 4, 5 and 6). The size of DNA molecular weight markers is in kb.

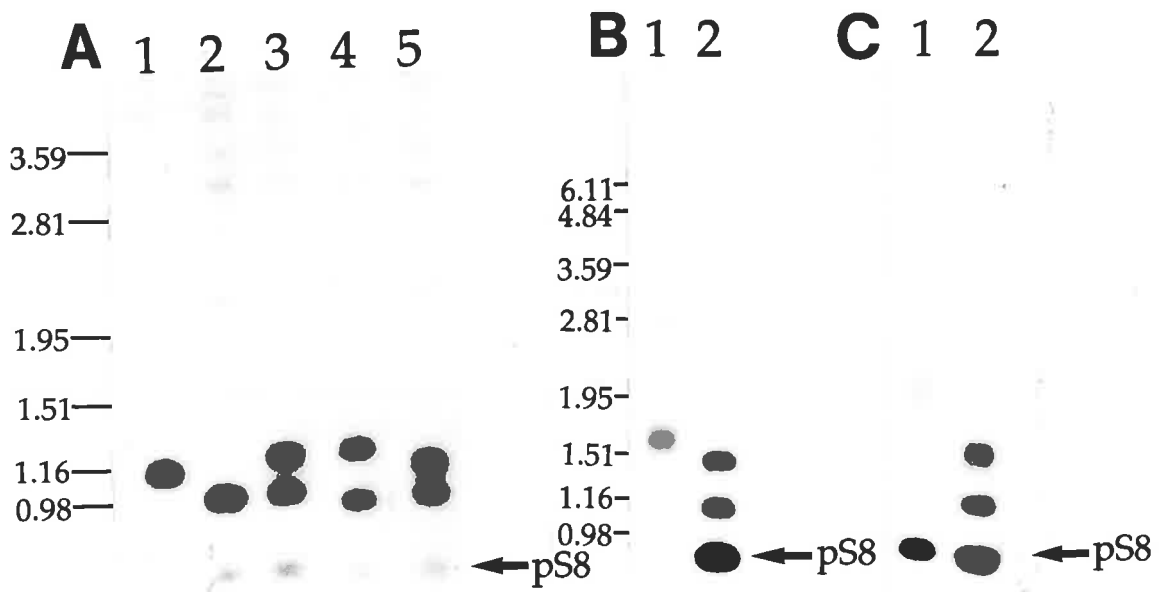


Fig. 6 - 5. Sample pedigrees demonstrating different types of male transmission. PstI digests of genomic DNA were used for Southern analysis with probe pfxa3. Panel A: a transmitting male with Δ = 0.1 kb (lane 1) transmitted the unstable element to his three daughters either unchanged (lane 5) or with small amplification (lanes 3 and 4). Panel B: a transmitting male with Δ = 0.5 kb (lane 1) had two daughters both with Δ = 0.4 kb, only one of his daughters is shown in lane 2. Panel C: an affected male with a faint smear of pfxa3 bands (lane 1) had two daughters both with Δ = 0.4 kb, one of his daughters is shown in lane 2. Panel B and C are from the same filter. The size of DNA molecular markers is in kb.

6.4.3. Methylation Status of the SacII Site and Fragile X Phenotype

Methylation status of the SacII site in the CpG island adjacent to the CCG repeat, was tested in fragile X pedigrees in order to determine the utility of methylation status for phenotype prediction in carrier females and for prenatal diagnosis.

Fig. 6 - 7A shows a clear correlation between the methylation of the SacII site and the length of the unstable element in males. The males with $\Delta \leq 0.6$ kb show no methylation of the SacII site at the CpG island, whereas those with $\Delta > 0.6$ show at least 70% methylation. In females, the methylation status of the SacII sites on both the normal X and the fragile X chromosomes were also analysed. As shown in Fig. 6 - 7B, on the normal X chromosome the SacII site shows $\leq$ 50% methylation, regardless of the size of the amplification on the fragile X chromosome. In contrast, the SacII site on the fragile X chromosome shows $\leq$ 50% methylation if the amplification $\Delta \leq 0.6$ kb, or > 50% if the amplification $\Delta > 0.6$ kb. This clearly indicates a correlation between methylation of the SacII site and the length of the unstable element in females.

In addition, all four rare cutting restriction sites (SacII, BssHII, EagI and NruI) at the fragile X related CpG island were found unmethylated in the chorionic villus (CV) DNA of a fetus with an amplification $\Delta = 1.4$ kb, although the SacII site was methylated to various degrees in different fetal tissues (Fig. 6 - 8). This observation implies that methylation may be established during the early stage of development and may vary from one tissue to another.

6.4.4. Somatic Variation of the CCG Repeat Region

To confirm that the multiple bands observed in some fragile X syndrome patients were due to somatic instability of the CCG repeat, the sizes of the CCG repeat region in various tissues from the same individual were compared. DNA was isolated from lymphocytes, cultured fibroblasts and Epstein-Barr virus transformed lymphoblast cell lines from 23 individuals in 9 unrelated fragile X syndrome pedigrees. Table 6 - 1 summarizes somatic variation between different tissues seen in Southern analysis with pfxa3. In all 11 affected males, regardless of

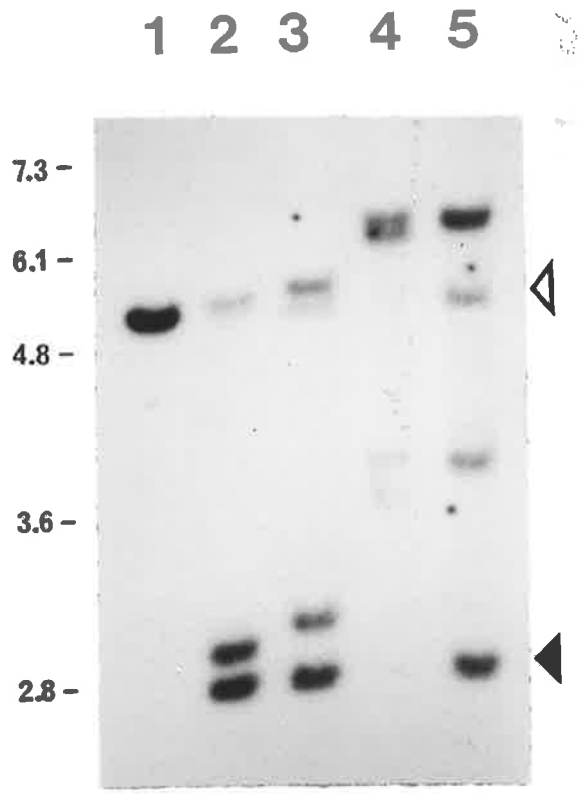
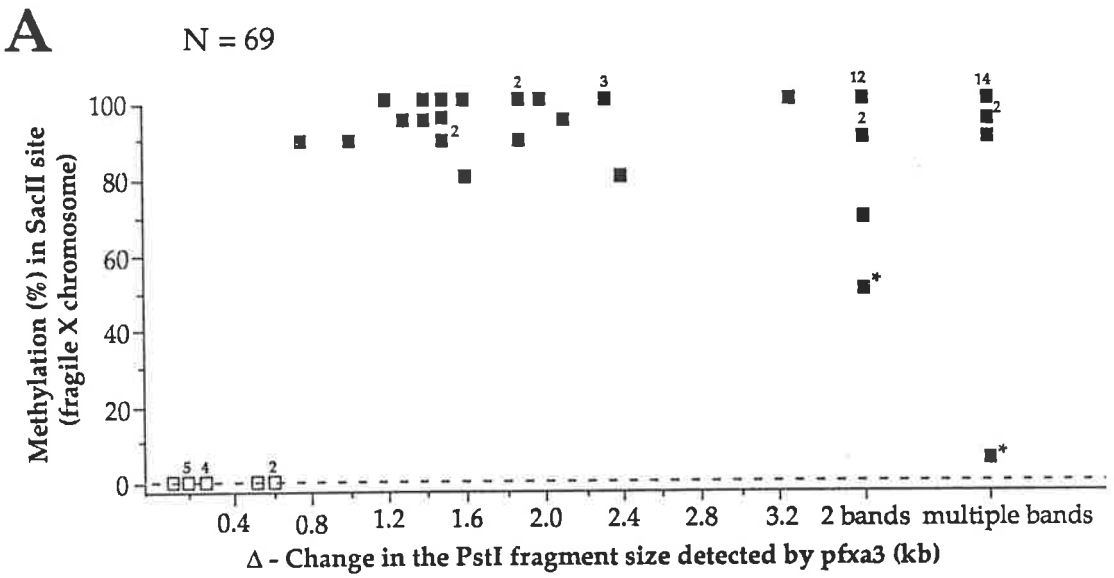
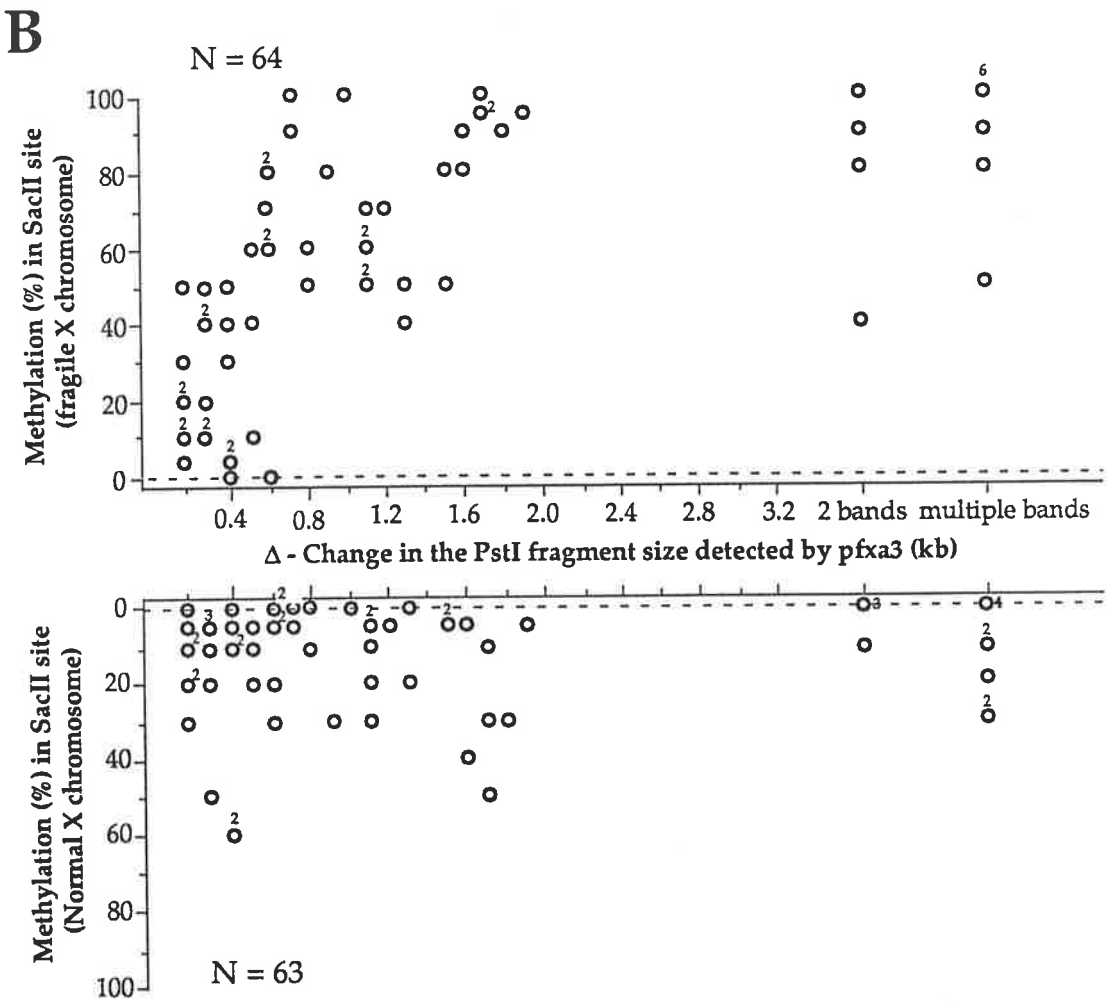


Fig. 6 - 6. Methylation at the SacII site in the CpG island adjacent to the CCG repeat. EcoRI or EcoRI/SacII digests of genomic DNA were used for Southern analysis with probe pfxa3. Lane 1: normal male DNA cleaved with EcoRI only to show the normal size of the pfxa3 hybridising fragment (5.2 kb). Lanes 2, 3 and 5: SacII/EcoRI digests of genomic DNA from three female carriers with different sized amplification. Varying degrees of methylation of the fragile X and the normal X chromosomes are apparent. Lane 4: SacII/EcoRI digests of genomic DNA from a fragile X syndrome male with almost complete methylation of the SacII site. Arrows indicate the positions of the normal methylated band of 5.2 kb (open) and unmethylated band of 2.8 kb (closed) detected by probe pfxa3. The size of DNA molecular markers is in kb.

Fig. 6 - 7. Correlation of methylation at the SacII site with length of the CCG repeat region in males (A) and females (B) with fragile X genotype. Symbols: open square, transmitting male; closed square, affected male; open circle, female carrier. Asterisk indicates affected males with multiple bands, among which only those bands (Δ = 1.9 kb and Δ = 2.0 kb) exhibited methylation at the SacII site, other bands (Δ = 0.3 kb) showed no methylation at the SacII site. Numbers above symbols indicate the number of individuals at the same position.


A
N = 69
Methylation (%) in SacII site
(fragile X chromosome)
100
80
60
40
20
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
2 bands
multiple bands
Δ - Change in the PstI fragment size detected by pfxa3 (kb)


B
N = 64
Methylation (%) in SacII site
(fragile X chromosome)
100
80
60
40
20
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
2 bands
multiple bands
Δ - Change in the PstI fragment size detected by pfxa3 (kb)
Methylation (%) in SacII site
(Normal X chromosome)
0
20
40
60
80
100
N = 63

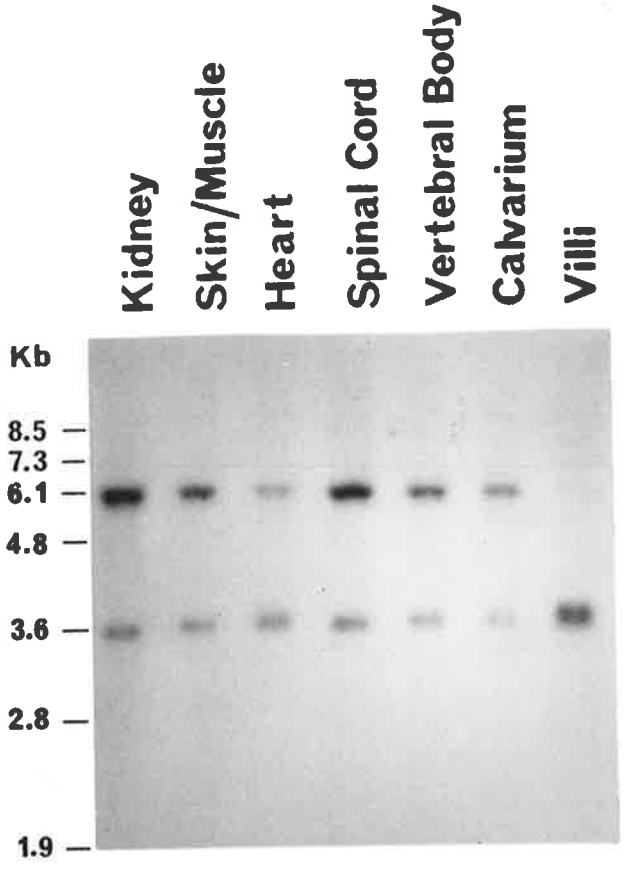


Fig. 6 - 8. Southern blot showing the lack of methylation at SacII site in chorionic villus DNA. Genomic DNA from various tissues was digested with EcoRI/SacII and used for Southern analysis with probe pfxa3. The sources of DNA samples are indicated above each lane. DNA molecular markers are SPP-1 bacteriophage DNA digested with EcoRI.

Table 6 - 1. Somatic Variation

					Δ - Change in size of PstI fragment detected by pfxa3 (kb)		
Family	No.	Name	Sex	%FRAX	Lymphocytes	Lymphoblasts	Fibroblasts
Affected	3	S.L.	M	42	1.1 2.4	1.0 2.3	
	4	A.M.	M	36	1.0 2.2	2.1	2.0 2.4
	4	B.M.	M	46	2.0	2.1	2.4
	4	G.M.	M	48	2.3	1.6 2.4	2.4
	5	D.O.	M	26	1.1 2.3 3.4	smear	
	6	J.G.	M	70	0.4 1.4 2.5	1.3 2.2	
	7	M.E.	M	30	1.5	1.0	
	13	R.M.	M	22	smear	smear	
	13	J.B.	M	38	1.9	1.8	
	14	M.F.	M	42	1.6 2.6	1.5	
	27	S.W.	M	15	1.6	1.5	
Carrier	4	J.M.	F	10	0.7*	1.2 1.4	1.6
	5	K.O.	F	0	0.2	0.2	0.2
	7	F.E.	F	0	0.3	0.3	0.3
	13	G.M.	F	0	0.2	0.2	
Normal	4	C.E.	F	0	0.0	0.0	
	6	S.G.	F	0	0.0	0.0	
	7	D.D.	F	0	0.0	0.0	
	9	A.B.	M	0	0.0	0.0	
	13	C.M.	M	0	0.0	0.0	
	13	S.B.	F	0	0.0	0.0	
	14	A.F.	M	0	0.0	0.0	
	27	G.W.	M	0	0.0	0.0	

* Less than half dosage.

whether instability was observed in the lymphocyte DNA or not, one or more bands of different length were observed in cultured cell lines. One example is shown in Fig. 6 - 9B. No somatic variation was detected in eight normal individuals including four males and four females. Among four carrier females, the three carriers with small amplification (Δ = 0.1 - 0.2 kb) showed no somatic variation, an example is shown in Fig. 6 - 9A. While the carrier with a slightly larger amplification (Δ = 0.7 kb) in lymphocyte DNA exhibited obvious somatic variation between different tissues (Table 6 - 1). All the individuals for whom somatic variation has been observed showed cytogenetic expression of the fragile X (Table 6 - 1).

To examine the developmental stage when the somatic variation occurred, various tissues from a terminated fragile X fetus (11 weeks) were analysed. Probe pfxa3 detected a 2.4 kb PstI band (Δ = 1.4 kb) in different tissues tested, suggesting the existence of amplification but without somatic variation (data not shown). This observation suggests that either the cultured cell lines exaggerate the somatic variation, or the somatic variation could develop at a later stage of embryonic development or even during the life span of an affected individual.

6.4.5. Lack of New Mutation in Fragile X Syndrome Patients

Since a high mutation rate had been proposed for fragile X syndrome, new mutations were expected in fragile X families especially in those with apparently isolated cases. However, no evidence of new mutation was found in the 42 initial families, which included three apparently isolated cases (see F5, F14 and F32 in Appendix I), although as many ancestors as possible were analysed in each family. A four generation family (F19 in Appendix I) is shown in Fig. 6 - 10. In an attempt to identify instances of new mutation, the pedigrees of an additional seven apparently isolated cases of fragile X syndrome (from Dr. G. Turner, Sydney, Australia) were analysed. In all 10 apparently isolated cases (8 affected males and 2 affected females), the pfxa3 probe demonstrated that the mother carried the fragile X genotype; and where DNA was available (in two families), it was found that one of the relevant grandparents was also a carrier. In another pedigree (see F32 in Appendix I), several distant relatives of the affected

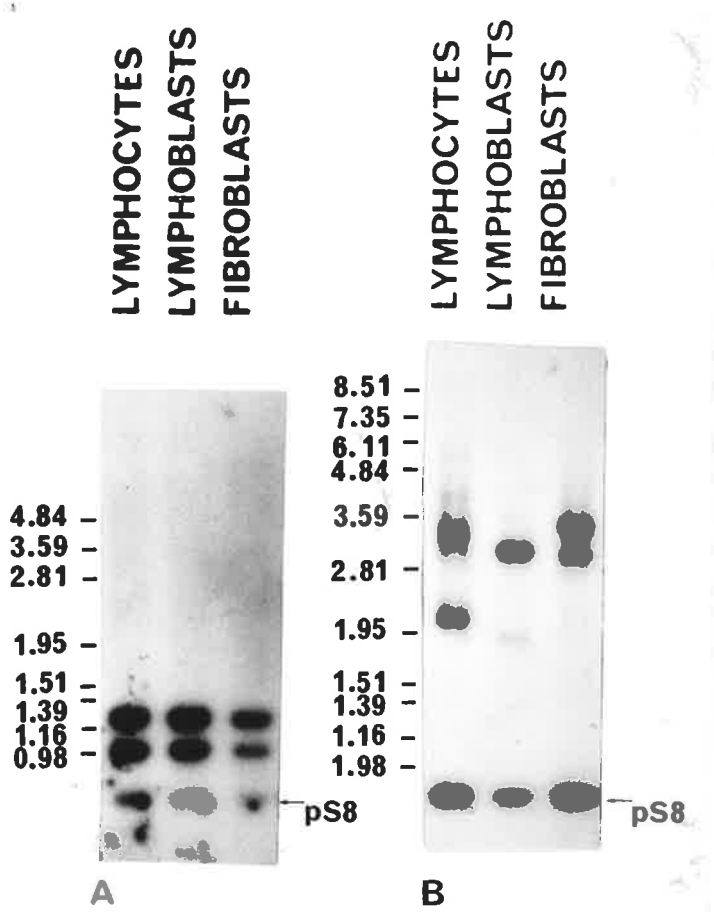


Fig. 6 - 9. Somatic variation at the fragile site. PstI digests of genomic DNA from various cell types were used for Southern analysis probe pfxa3. Probe pS8 was used as a positive control. Size of DNA markers (kb) is shown.

A) DNA from a fragile X female carrier (KO in Table 6 - 1.)

B) DNA from a fragile X affected male (AM in Table 6 - 1.)

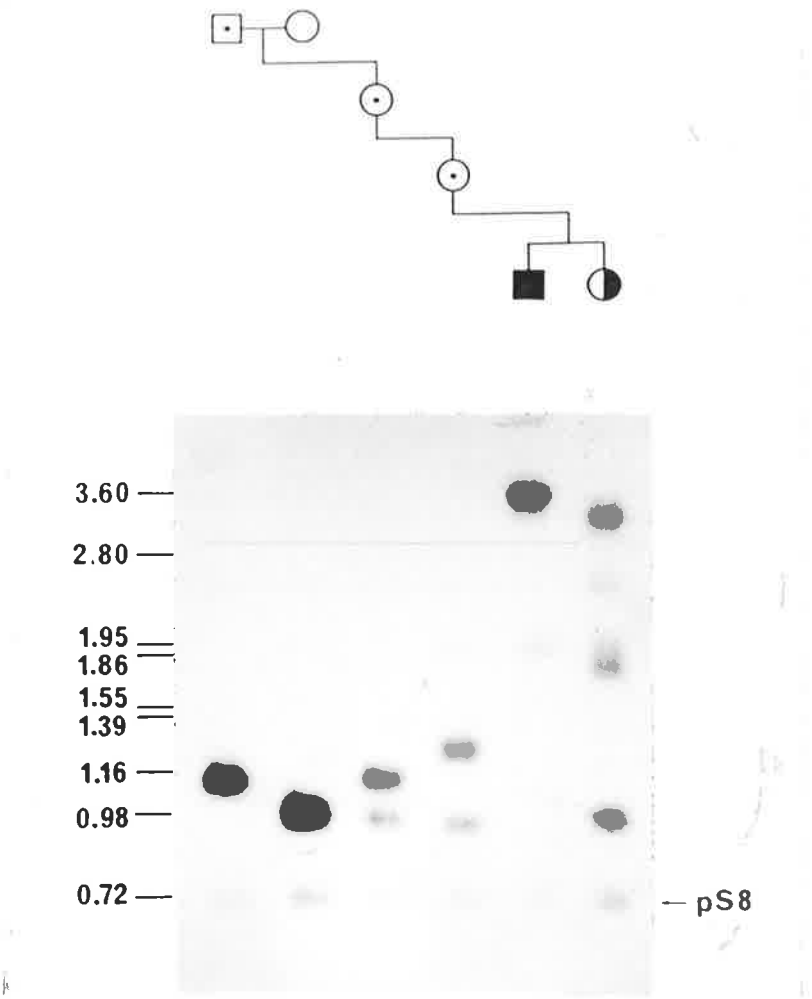


Fig. 6 - 10. Transmission of fragile X mutation in a four generation lineage from a large fragile X syndrome family. Genomic DNA was digested with PstI and probed with pfxa3. The control probe pS8 was included in the hybridization. Lanes correspond to the members of the pedigree indicated above each lane. The pfxa3 result is consistent with that of linkage analysis with flanking markers. Pedigree symbols: dot in square, transmitting male; shaded square, affected male (expressing the fragile X); open circle, normal female; dot in circle, normal female carrier (not expressing the fragile X); half shaded circle, normal female carrier (expressing the fragile X).

girl were found to be carriers, indicating that one of the great-grandparents of the affected girl was an obligate carrier. So far, all affected individuals were found to have a parent, and a grandparent where testing was possible, who had a fragile X genotype. In other words, all the affected individuals, including apparently isolated cases, are familial.

To further investigate the size variation of the pfxa3 band in normal population, DNA samples containing the largest or the smallest size of PstI band were selected from more than 300 normal X chromosomes. They were analysed for the size of their Sau3AI fragments (Fig. 6 - 11A). The largest band in the normal population is very similar in size to the smallest ones in the carriers (Fig. 6 - 11A). The relative sizes of the unstable elements of different individuals detected by either Southern blot or PCR was identical (Fig. 6 - 11B). The wide range of copy number of CCG repeat in normal individuals was demonstrated clearly in the PCR analysis (Fig. 6 - 11B).

6.4.6. Utility of the Probe pfxa3 in Diagnosis

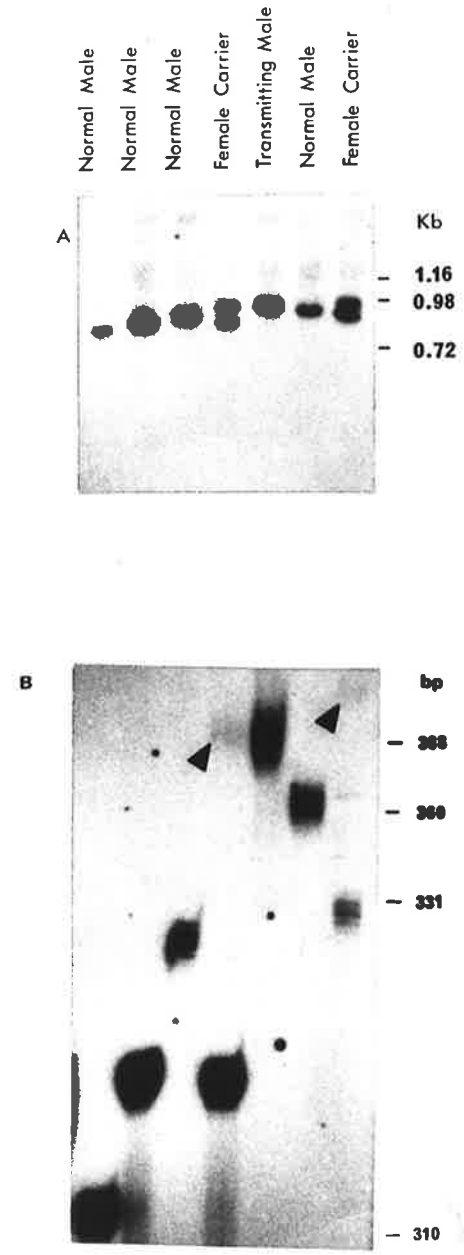
On PstI digests, probe pfxa3 detected a 1 kb band ($\Delta = 0$) in normal males, a slightly larger band ($\Delta \leq 0.6$ kb) in transmitting males and a much larger band(s) ($\Delta > 0.6$ kb) or a faint smear of bands in affected males (Fig. 5 - 5). The 1 kb PstI band detected by pfxa3 in a normal female is from both her normal X chromosomes. In carrier females, however, the probe detected a 1 kb PstI band corresponding to the normal X chromosome and a larger band(s) corresponding to the fragile X chromosome. The latter band resembled that seen in fragile X genotype males. In Chapter 5, it was stated that the PstI hybridization patterns of female carriers without an obvious abnormal band because of the faint smear background looked similar to that of normal females. Further experience revealed that the "smear" could be condensed into "cluster" of bands, which were easier to detect, by using enzymes that generated larger fragments around the CCG repeat region, such as EcoRI (Fig. 6 - 12).

In the 39 fragile X families studied with DNA flanking markers, the results of direct diagnosis using pfxa3 was consistent with that by using linkage analysis. According to the

Fig. 6 - 11. The length variation of the unstable element.

A. Genomic DNA from normal and fragile X genotype individuals was digested with Sau3AI and probed with pfxa3 to give accurate sizing and discrimination of CCG length on Southern blot. The normal male DNAs were chosen from over 100 unrelated individuals to represent the range including the maximum (lane 6) and minimum (lane 1) of the CCG length. DNAs of the three fragile X carriers were chosen from over 200 fragile X genotype individuals studied to represent the smallest CCG length in fragile X genotype. DNA size markers are indicated in kb.

B. Polymerase chain reactions were performed on the same DNA samples as in A. DNA size markers were PCR products from the dystrophin locus (388, 360 and 331 bp in length, Chamberlain et al., 1988) and the 203/213 reaction using pfxa2 DNA as a template (310bp in length, Kremer et al., 1991). Arrows indicate the position of faint products of the affected allele in the two female carriers.


Normal Male
Normal Male
Normal Male
Female Carrier
Transmitting Male
Normal Male
Female Carrier
A
Kb
1.16
0.98
0.72
B
bp
388
369
331
310

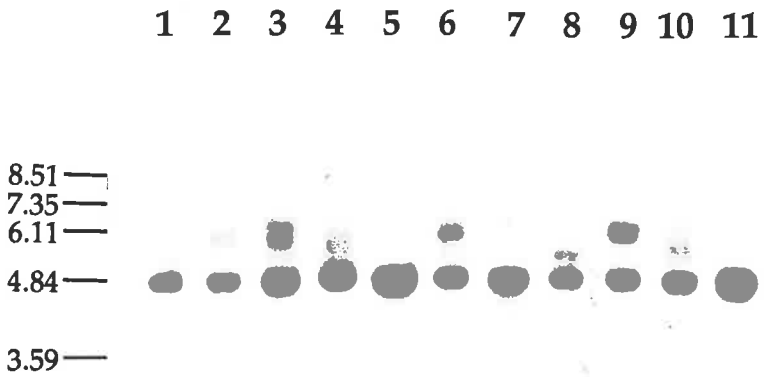


Fig. 6 - 12. Detection of fragile X mutation in EcoRI digests from female heterozygotes. Genomic DNA was digested with EcoRI and probed with pfxa3. The DNA samples of female carriers (lanes 1, 2, 3, 4, 6, 8, 9, 10) were chosen because they showed a faint smear of pfxa3 band on PstI blot. On EcoRI digests, it is apparent that the smear in female carriers is condensed and easily visualised and can be distinguished from normal female DNA samples (lanes 5, 7, 11).

pfxa3 results, fragile X syndrome was excluded from three families. Two families had isolated affected females with low fragile site expression (3% and 5% respectively). No abnormal pfxa3 band was identified in the two affected girls, their sibships or either of their parents, suggesting a misdiagnosis of fragile X syndrome in these families. The third family showed high cytogenetic fragile site expression at Xq27.3 without mental retardation. Probe pfxa3 detected a normal 1 kb PstI band in all of the family members (data not shown). Further in situ hybridization study of the third family revealed a new fragile site, the FRAXE, which is located between DXS465 (Do33) and DXS296 (VK21) (Sutherland and Baker, 1992).

A false positive result was seen in one family, in which a high molecular weight band was "inherited" for three generations. However, no size change was observed when it was transmitted from generation to generation. This feature was not consistent with the instability seen in fragile X mutation transmission. It was later found that the high molecular weight bands seen in this family were due to plasmid contamination. The false hybridization signal in a normal male is shown in Fig. 6 - 13 (lane 3). Also, partial digestion of DNA samples can give high molecular weight band(s) resembling that of fragile X genotype (Fig. 6 - 13, lane 5). Overall, probe pfxa3 is of great utility in the detection of fragile X carriers.

6.5. DISCUSSION

6.5.1. The Sherman Paradox

Sherman et al (1985) observed an unusual and characteristic segregation pattern in fragile X syndrome from investigation of 206 fragile X pedigrees. They found that the penetrance of mental impairment was higher in offspring of intellectually normal daughters of transmitting males (74%) than that in offspring of intellectually normal mothers of transmitting males (18%) (the Sherman Paradox), although mothers and daughters of transmitting males are similar in phenotype (mentally normal and no cytogenetic fragile X expression).

Since the newly isolated DNA probe pfxa3 can detect fragile X genotype (increased length of the CCG repeat), analysis of transmission of the CCG repeat by females in fragile X

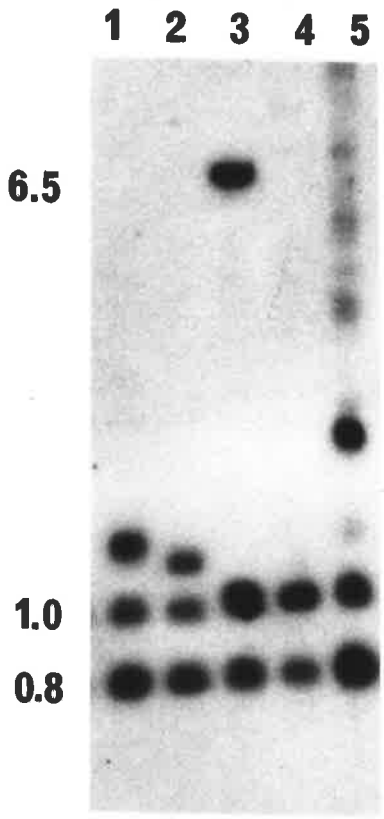


Fig. 6 - 13. Plasmid contamination and incomplete DNA digestion resembling the hybridization pattern of fragile X unstable element. Lane 3 shows a contaminating plasmid band of 6.5 kb in a normal male sample. Lane 5 shows incomplete DNA digestion in a female carrier sample. The size of DNA markers is in kb.

syndrome pedigrees has allowed resolution of the Sherman paradox. Initially, the mothers and daughters of transmitting males were assumed to be of identical genotype, so they should have had a similar ratio of affected and unaffected offspring, but this was not the case. Study of transmission of the CCG repeat indicates that mothers and daughters of transmitting males are at different stages of progression of the mutation. Since the CCG repeat is usually further amplified when transmitted by females (Yu et al., 1991; Oberlé et al., 1991), thus the amplification progressively increases through generations in fragile X pedigrees, the daughters of transmitting males usually should have larger amplifications than do the mothers of transmitting males. Moreover, the risk of a female carrier having an affected son increased with the length of her CCG repeat (Fig. 6 - 3). In other words, carrier females with longer CCG repeat are more likely to have affected sons. Therefore, the daughters of transmitting males are at greater risk of having affected sons than the mothers of transmitting males.

A parallel experiment was undertaken by Fu and co-workers. These authors proposed a similar explanation of the Sherman paradox (Fu et al., 1991). They used PCR to amplify across the CCG repeat region and separated the PCR products through a denaturing DNA sequencing gel for resolution at a single triplet level. Female transmission of 15 alleles containing between 52 and 113 repeat units of CCG were analysed (Fu. et al., 1991). The average frequency of generating large amplification (Δ > 600 bp) was zero (0/7) at 59 repeat units or below, 17% (1/6) in the range of 60 - 69 repeat units. The frequency increased to 71% (10/14) within the range of 70-79 repeat units and 82% (14/17) in the range of 80-89 repeat units, and became 100% (19/19) at 90 repeat units or over. This indicated that the risk of expansion correlates well with the size of the CCG repeat, and fitted very well with the data obtained by Sherman et al. (1985). The 18% penetrance in brothers of transmitting males would predict that mothers of transmitting males are likely to have alleles in the 60 - 69 repeat units range, with a 17% risk of expansion. The 74 % penetrance for the grandsons of transmitting males would predict that

the daughters of transmitting males would have alleles in the 70 - 89 repeat units range, with a 77% (24/31) risk of expansion.

6.5.2. Property of the Fragile X Mutation

The fragile X mutation is characterized by an increased length of trinucleotide CCG repeat. This mechanism, by amplification of a trinucleotide repeat, is completely different from other well documented DNA mutations such as insertions, deletions, substitutions or frame shift mutations, therefore, its genetic properties would be expected to behave differently from the above mutations. Being a member of the microsatellite repeat class of sequences, the CCG repeat identified at the fragile X region are likely to share common properties with other well characterized repeats. Most notably, the extent of polymorphism is directly proportional to the repeat length (Weber, 1990), in other words, longer repeat sequences will have a higher rate of new mutation.

6.5.2.1. Polymorphism of the CCG Repeat Region in the Normal Population

In the normal population, the CCG repeat is highly polymorphic although stable within pedigrees tested at the limit of accuracy of Southern blot analysis (Kremer et al., 1991a). The CCG repeat appears to consist of about 40 ± 25 copies in the normal population as determined by this method. The biggest amplification observed in the normal population is very close to the smallest ones in transmitting males (Fig. 6 - 11). The exact length at which a random individual ought be considered as a mutation carrier is not clear.

In a parallel experiment, PCR analysis across the CCG repeat region revealed 31 distinctly sized alleles among normal individuals, alleles ranged from 6 to 54 repeat units (Fu et al., 1991). Heterozygote frequency was found to be 63% in the normal population analysed, with 29 copies of the CCG repeat units being the most frequent (30%) allele (Fu et al., 1991). The two largest alleles found in the normal population were 46 and 54 copies of the CCG repeat units. The 46 repeat allele showed no size change in three meiotic events, whereas the 54

repeat allele generated alleles with varied size in all five offspring (Fu et al., 1991). The 54 CCG repeat allele found in the normal population is larger than the smallest allele (52 CCG repeat) found in a transmitting male (Fu et al., 1991), suggesting that the individual with 54 CCG repeat identified in the normal population is a potential carrier. A carrier of this kind would not be recognized until he or she has at least one affected descendant.

6.5.2.2. Progressive Amplification of the CCG Repeat Region

The fragile X mutation rate was estimated to be very high, 7.2×10^{-4} (Sherman et al., 1984), one of the highest for a human genetic disease. Several hypotheses have been proposed to explain the unusually high mutation rate, such as, a huge gene or very unusual sequences being involved (Sutherland et al., 1985; Nussbaum et al., 1986). Recent discoveries on fragile X may provide explanation for this phenomenon. A systematic search for new mutations by direct DNA testing in over one hundred fragile X pedigrees revealed no evidence of this (Fu et al., 1991; Rousseau et al., 1991b; Yu et al., 1992). The normal length of CCG sequence does not change to a greatly amplified CCG sequence in one generation (Rousseau et al., 1991b; Yu et al., 1992) and small amplifications can be transmitted for several generations without phenotypic effect (Fu et al., 1991; Yu et al.,1992). Therefore, fragile X mutation appears as a progression of increasing copy number of the CCG repeat rather than a simple mutation or a two-step mutation (a premutation followed by a full mutation). These results predict a high frequency of carriers in the general population. The mutation rate (from normal CCG length to small amplification) still awaits determination.

6.5.2.3. Further Amplification Determined by the CCG Repeat length in Carrier Females

In the female carriers with small amplifications ($\Delta \leq 0.6$ kb), it was observed that longer repeats tend to produce larger amplification in their offspring. Southern blot analysis revealed that carrier females with $\Delta = 0.1$ kb usually have small amplification ($\Delta \leq 0.6$ kb) in the

successive generation, those with Δ = 0.2 kb can have either small or large amplification (Δ > 0.6 kb). In contrast, those female carriers with Δ > 0.2 kb usually have large amplification in their offspring (Fig. 6 - 3). This phenomenon was also confirmed by Fu et al. (1991) by using PCR across the CCG repeat. They observed small increase of up to 73 copies of CCG repeat in the offspring of female carriers with CCG repeat at the range of 52 - 59 repeat units, but no large amplification (Δ > 0.6 kb). The average frequency of producing large amplification (Δ > 0.6 kb) was 17% at 60 - 69 copies of CCG, 71% at 70 - 79 copies of CCG, increased to 82% at 80-89 copies of CCG, and became 100% at 90 copies of CCG or more (Fu et al., 1991). Although with different methods, the results from these two laboratories are consistent in that longer CCG repeat will generate larger amplification through female transmission.

6.5.2.4. Instability of the CCG Repeat in Mitosis

Apart from the meiotic instability mentioned above, the fragile X mutation exhibits instability in mitosis. Mosaicism involving length variation is a common phenomenon in individuals with large amplification (Δ > 0.6 kb) and was found more often in children than in adults (Rousseau et al., 1991b). The latter observation was explained by selection favouring the cells with smaller amplification. The unstable element of different sizes were observed in DNA samples from different tissues of the same individual by Southern blot analysis, regardless of whether instability was observed in the lymphocyte DNA or not (Table 6 - 1; also see Yu, et al., 1992). Normal individuals and three carrier females with amplifications up to Δ = 0.3 kb showed no sign of somatic variation by Southern blot analysis. But one carrier female with Δ = 0.7 kb in her lymphocyte DNA showed obvious somatic variation (Table 6 - 1). Therefore, it seems that the CCG repeat becomes mitotically unstable only when it reaches a certain length.

However, in two rare instances PCR analysis has revealed two alleles of 66 and 80 CCG repeat units in a carrier female, and five alleles of 12, 18, 45, 63 and 116 CCG repeat units in a transmitting male (Fu et al., 1991). It is difficult to be certain that this apparent instability is

not due to a PCR artifact. The conditions required for the CCG repeat to show somatic variation can not be concluded from the limited data available.

In conclusion, a hypothesis on the nature of fragile X mutation is proposed as shown in Fig. 6 - 14 on the basis of the above observations. In brief, the longer the CCG repeat, the more unstable it becomes. In the normal population, individuals can have varying copy numbers of the CCG repeat which is stable in both mitosis and meiosis. However, individuals have higher CCG repeat copy numbers are at a higher risk of transmitting even longer CCG repeats to their offspring. As the repeat gets longer, meiotic instability of the CCG repeat is evident when transmitted by carrier females, but usually no mitotic instability is observed. When the CCG repeat gets even longer, mitotic as well as meiotic instability become obvious. However, meiotic instability is seen only in female carriers, since males with large amplification ($\Delta > 0.6$ kb) usually do not reproduce.

6.5.3. Methylation and Fragile X Syndrome

Methylation of the CpG island adjacent to the CCG repeat was initially proposed to be associated with the fragile X phenotype. The BssHII and EagI site in the CpG island were methylated in affected individuals but not in normal carriers or normal individuals (Vincent et al., 1991; Bell et al., 1991; Oberlé et al., 1991). Moreover, complete methylation of the BssHII site within the CpG island was observed in 80% of fragile X syndrome patients who show absence of FMR-1 gene transcription (Pieretti et al., 1991). In the remaining 20% of patients, presence of the FMR-1 mRNA was associated with incomplete methylation of the site at the CpG island (Pieretti et al., 1991). It appears that methylation of the BssHII site at the CpG island is associated with lack of transcription of the FMR-1 gene.

Laird (1987) and Laird et al. (1990) postulated a role for genetic imprinting in the unusual transmission pattern of the fragile X syndrome. In this hypothesis the passage of the X chromosomes through a female predicts a different pattern of methylation on the inactive

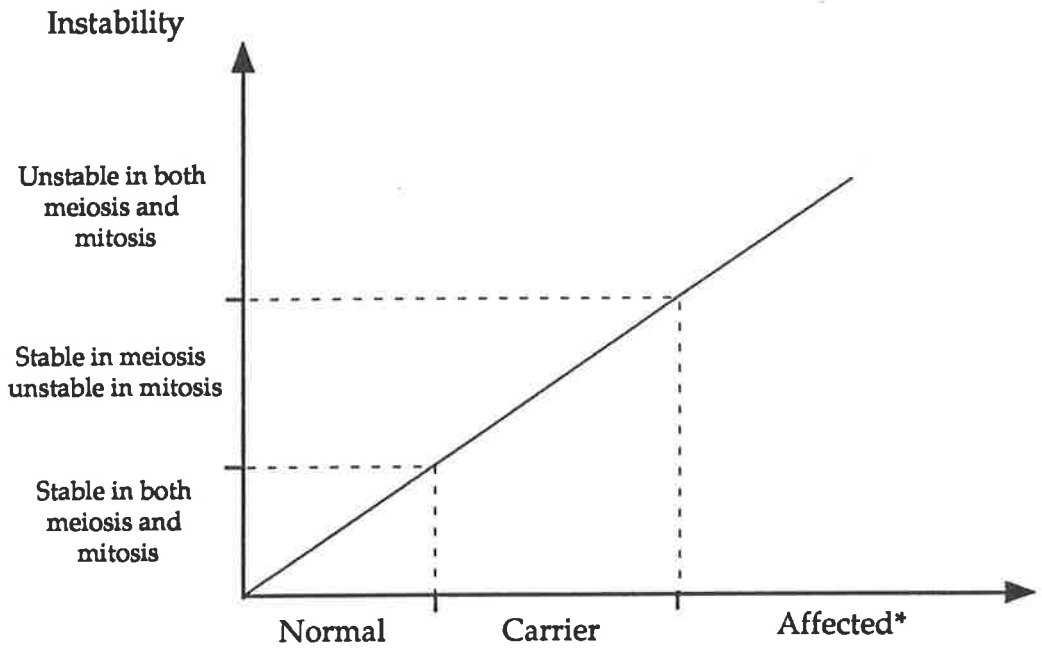


Fig. 6 - 14. The hypothesised correlation between the length of the unstable element and its instability. * some females in this range do not have mental retardation, and males in this range usually do not reproduce.

fragile X compared to an active fragile X chromosome. Whether a male carrying the mutation is affected or not, will depend on whether the fragile X chromosome is inactivated in his carrier mother. This X-inactivation imprinting model predicts 1) absence of gene products in affected individuals, but not abnormal gene products; 2) the number of transmitting males would be the same as that of fragile X syndrome patients. It has been reported that about 20% of the affected individuals have mosaicism, with small unmethylated amplification ($\Delta \leq 0.6$ kb) in some cells and large methylated amplification ($\Delta > 0.6$ kb) in other cells. These individuals were shown to have normal level of FMR-1 mRNA (Fu et al. 1991). These results failed to provide experimental evidence in supporting the imprinting hypothesis. Further testing of the hypothesis can be made on whether equal numbers of transmitting males and affected males are presented in the offspring of normal carrier females.

Amplification of the CCG repeat adjacent to the CpG island was shown to segregate with fragile X genotype (Yu et al.,1991; Oberlé et al.,1991), and is located within the FMR-1 gene (Verkerk et al.,1991). The length of the CCG repeat correlates with the fragile X phenotype (mental retardation) (Rousseau et al., 1991b; Yu et al., 1992), with the methylation status of the CpG island (Yu et al., 1992), and also with the instability of the CCG repeat itself (Fu et al., 1991; Yu et al., 1992). The observation that presence of amplification ($\Delta = 1.4$ kb) but lack of methylation in the chorionic villus of the fetus (Sutherland et al., 1991b), suggested that amplification of the CCG repeat plays a causative role in fragile X syndrome and that methylation is secondary to this, although methylation may be essential to elaborate phenotype.

One of the most active areas of fragile X study has been the fragile X chromosome inactivation pattern, but the previous studies have been inconclusive (Chapter 2). With the new molecular technology, Rousseau et al. (1991c) were able to determine in female carriers the proportion of cells in which the inactive X chromosome had the fragile X mutation. Methylation status of the EagI site at the fragile X associated CpG island was determined by Southern blot analysis of EcoRI/EagI digests with probe StB12.3. In the DNA sample of a

female carrier, the respective intensities of the bands of 2.8 kb and 5.2 kb corresponded to the proportion of cells with a normal active X chromosome or with an abnormal inactive X chromosome. The proportion of cells with fragile X on the inactive X chromosome reflected the proportion of cells with normal active X chromosome (the intensity of the 2.8 kb band/the sum of intensity of the 2.8 kb and the 5.2 kb bands). By analysis of 45 female carriers, they concluded that the fragile X mutation ($\Delta > 0.6$ kb) was preferentially located on the inactive X chromosome in female carriers when compared to the group of female carriers with $\Delta < 0.6$ kb. When the age was considered, this pattern was only seen in adult carrier females but not in younger ones. Such a non-random X inactivation pattern in adult carrier females was explained by a selection process against cells carrying the fragile X mutation on the active X chromosome (Rousseau et al., 1991c). However, in the present study, the SacII methylation patterns of normal X chromosomes in female carriers are similar, regardless of whether a fragile X chromosome is carrying a large or a small amplification (Fig 6 - 7B). This is possibly due to the different restriction site tested since there is another SacII site 270 bp away from the fragile X associated CpG island (J. Mandel and D. Nelson, unpublished observation).

6.5.4. Molecular Diagnosis of Fragile X Syndrome

Cytogenetic detection of the fragile site has been a major method in diagnosis of fragile X syndrome for about two decades, although it is not completely reliable for carrier detection and prenatal diagnosis (Sutherland and Hecht, 1985; Hagerman and Silverman, 1991). This method can detect affected males with high specificity, but it fails to identify almost all transmitting males. Only 56% of female heterozygotes has either cytogenetic expression or varying degrees of mental retardation (Sherman et al., 1984). In recent years, linkage analysis with DNA markers close to FRAXA has been used to identify transmitting males and female carriers without cytogenetic expression (Chapter 1). The accuracy of linkage analysis depends on the distance between the informative DNA markers and FRAXA. The utility of this test in a particular family relies on the family being informative for the DNA marker(s). Key members

of a family need to be analysed before carrier status can be determined, thus this analysis is usually laborious and time consuming. Recently, the methylation of the CpG island was found to be associated with fragile X phenotype. Amplification of the CCG repeat very close to the CpG island segregated with fragile X genotype. The gene, FMR-1, isolated in the CCG repeat region, shows close involvement in the development of the syndrome. These new discoveries made it possible to develop methods of direct molecular diagnosis of the fragile X syndrome.

6.5.4.1. Southern Blot Analysis

Since DNA probes were isolated to identify the unstable element associated with fragile X genotype (Yu et al., 1991; Oberlé et al., 1991; Nakahori et al., 1991), these probes have been used in several laboratories for carrier detection and prenatal diagnosis, and they have proved to be reliable in directly detecting the fragile X mutation.

Yu et al. (1991, 1992) analysed 49 fragile X pedigrees with the DNA probe pfxa3 on PstI blots (Chapter 5 and 6). This probe detected all transmitting males by the replacement of the normal 1 kb PstI band (Δ = 0 kb) with a slightly larger band ($\Delta \leq$ 0.6 kb). It also detected all affected males by the replacement of the normal band with a much larger band or bands ($\Delta >$ 0.6 kb), or apparent absence of the normal band. All carrier females, regardless of fragile site expression and mental status, can be identified by the presence of an additional band(s) of $\Delta > 0$ kb. The multiple abnormal bands (smear) in female carriers could be difficult to detect, especially in the instance when high quality of Southern blots was not obtained. However, using restriction enzymes such as EcoRI or BglII to generate larger fragments than those produced by PstI, can condense the smear to form an easily detectable band with higher molecular weight than the normal band (Fig. 6 - 12; also see Rousseau et al., 1991a). Recent experience showed that dosage analysis with pfxa3, plus using pS8 as confirmation could detect all female carriers on PstI digests (Mulley et al., 1992). Since the length of the CCG repeat is correlated with the phenotype of mental status (Fig. 6 - 1; see also Rousseau et al., 1991a; Yu et al., 1992) and can be determined in chorionic villi and amniotic fluid samples, Southern

analysis of the unstable element has been used for prenatal diagnosis (Surtherland et al., 1991b; Hirst et al., 1991c; Dobkin et al.,1991). On PstI digests, a male fetus with the pfxa3 band $0 < \Delta \leq 0.6$ kb would be expected to be a normal transmitting male, whereas those with a band $\Delta > 0.6$ kb would be affected. For a female fetus, those with a band $0 < \Delta < 0.6$ kb would be a normal carrier, while those with a band $\Delta > 0.6$ kb can be either a normal carrier or affected with a risk probably of 50% (Rousseau et al., 1991b; Mulley et al., 1992; Yu et al., 1992).

Rousseau et al. (1991b) tested the probe StB12.3 (Fig. 5 - 8) on EcoRI or EcoRI/EagI double digests in 530 individuals from 63 fragile X pedigrees for its utility for carrier detection and prenatal diagnosis. The use of EcoRI/EagI double digest allows the CCG length and methylation status to be determined in a single test. Overall, no false positive or false negative results was obtained among the 439 individuals whose carrier status was unambiguously determined previously on the basis of a high level of fragile X expression, linkage analysis, or both (Rousseau et al., 1991a). They also analysed retrospectively 28 chorionic villus DNA samples obtained from fetuses at high risk. The results of direct diagnosis were consistent with those of linkage analysis. DNA samples from eight male fetuses carrying large amplification ($\Delta > 0.6$ kb) had at least partial methylation of the EagI site (Rousseau et al., 1991b). This result is in contradiction with that of Sutherland et al. (1991b), where no methylation was observed in the chorionic villi sample of a fragile X male fetus. Therefore, methylation status cannot be used alone for prenatal diagnosis.

Hirst et al. (1991b) analysed more than 100 X chromosomes (including normal and fragile X chromosomes) on EcoRI, BglI or HindIII digests using probe Ox1.9 (Fig. 5 - 8). They detected amplifications in 56 out of 59 fragile X syndrome males and in all seven transmitting males. The three cases without amplification were explained by the authors to be either cases of misdiagnosis or mosaics (Hirst et al., 1991b). No amplification was observed in 43 normal X chromosomes. Similar observations from several laboratories were summarized by Webb (1991).

6.5.4.2. PCR Amplification Across the CCG Repeat

If CCG repeat of several kilobases could be amplified by PCR reaction, amplification across the CCG repeat would be the most powerful and rapid method to diagnose fragile X syndrome. At present, however, PCR can only amplify CCG repeat with $\Delta < 0.6$ kb (Fu et al., 1991), thus does not fulfil diagnostic requirements.

Fu et al. (1991) used PCR to amplify the CCG repeat, which allowed direct sizing of the CCG repeat ($\Delta < 0.6$ kb). However, this PCR based assay gave inconsistent PCR products when longer CCG repeats ($\Delta > 0.6$ kb) were studied. Therefore, the usefulness of this PCR technique has been limited in diagnosis of fragile X syndrome, because most affected individuals have a large amplification of the CCG repeat ($\Delta > 0.6$ kb) (Yu et al., 1991; Oberlé et al. 1991; Nakahori et al., 1991). The PCR test alone has limited utility even in primary screening of carriers with small amplifications in prenatal diagnosis. Firstly, a proportion of affected males have small amplifications as well as large amplifications on Southern blot analysis (Oberlé et al., 1991; Pieretti et al., 1991; Hirst et al., 1991b), they would be misdiagnosed as transmitting males on the basis of the small size of PCR products. Secondly, the alleles with small amplifications generate less PCR product than the normal alleles, and this effect is exacerbated with increasing allele size (Fu et al., 1991) especially for female samples (Richards et al., 1992). As a consequence, females at the higher end of the range of small amplification (such as $\Delta = 0.6$ kb) would sometimes be mistaken as carriers of large amplification on the basis of lacking visible PCR product from the fragile X chromosome or as normal homozygous for allele length of the CCG repeat.

6.5.4.3. Diagnosis by PCR Based Linkage Analysis

Cloning of the fragile X enabled the identification of a number of highly polymorphic AC repeat markers very close to the FRAXA. Linkage analysis with these markers was very useful in diagnosis of the fragile X genotype. AC repeat markers FRAXAC1 and FRAXAC2 are physically mapped within 10 kb of the CCG repeat with heterozygosities of 44% and 71%

respectively (Richards et al., 1991b). Another AC repeat marker DXS548 is 150 kb proximal to the CCG repeat with heterozygosity of more than 80% (Verkerk et al., 1991). PCR based analysis with these markers has provided a rapid and accurate means of exclusion of non-fragile X genotype in carrier detection and prenatal diagnosis. However, in prenatal diagnosis, once a fragile X genotype is confirmed, Southern blot analysis is required to determined the size of the amplification, thus to predict the phenotype of the fetus (Mulley et al., 1992).

6.5.4.4. Study of Gene Expression

Since a gene (FMR-1) was identified in the fragile X region, expression of this gene may provide valuable information for diagnosis of the associated syndrome. For this purpose, Pieretti et al. (1991) studied the expression of the FMR-1 gene in fragile X syndrome patients, carriers and normal controls. FMR-1 mRNA was absent in the majority (16/20) of fragile X syndrome males, and present in all 14 normal individuals and 10 female carriers (Pieretti et al., 1991). Therefore, diagnostic tests based on the expression of the FMR-1 mRNA or FMR-1 protein product would fail to detect carriers and 20% of the affected males.

6.5.4.5. Isolated Cases of Fragile X Syndrome

The observation of lack of new mutation in fragile X syndrome patients including apparently isolated cases (Rousseau et al., 1991b; Yu et al., 1992) changes the clinical view on families with apparently isolated cases. It become important to determine the origin of the mutation and to identify carriers in those families, since all fragile X syndrome patients are familial. Furthermore, a potential carrier was identified in the normal population (Fu et al., 1991). Searching systematically for potential carriers in the normal population provides a means of preventing isolated cases. More economically, this kind of search can be carried out only in females who are planning to have a child, as the transition from a small amplification to a large amplification does not happen until the fragile X genotype is transmitted by a

female (Yu et al., 1991; Oberlé et al., 1991; Fu et al., 1991; Rousseau et al., 1991b; Mulley et al., 1992).

6.5.4.6. Potential Problems in Diagnosis

Some experimental artifacts or difficulties of interpretation occurred in direct molecular diagnosis of fragile X syndrome (Nakahori et al., 1991; Rousseau et al., 1991b; Mulley et al., 1992; Yu et al., 1992) are summarized as following:

1) Contamination of human DNA samples with plasmid may generate bands of various size which resemble fragile X genotype. Reprobing the blot with plasmid vector sequences can detect the spurious bands (present study, Rousseau et al., 1991b; Mulley et al., 1992).

2) Incomplete digestion of human DNA samples gives hybridization bands similar to multiple abnormal pfxa3 bands. Rehybridization of the same blot with another human probe can detect the DNA incomplete digestion.

3) In DNA samples from some female carriers, pfxa3 detected a faint smear of PstI fragments instead of an abnormal band. This pattern is very similar to that in normal females (Yu et al., 1991). By using enzymes such as EcoRI or BglII, the smear may be condensed and the "cluster" of bands is easier to detect (Fig. 6 - 12; see also Rousseau et al., 1991b). On the other hand, dosage analysis, comparing the signal intensity of pfxa3 with another human DNA probe such as pS8 on the same PstI blot, can confirm homozygosity or heterozygosity of the normal 1kb PstI fragment (Mulley et al., 1992).

4) For those families with good fragile site expression at Xq27.3 but without abnormal pfxa3 fragments (Nakahori et al., 1991; Rousseau et al., 1991b; Mulley et al.,1992), the presence of another fragile site, FRAXE (Sutherland and Baker, 1992) is the most plausible explanation. In situ hybridization with DNA probes slightly distal to FRAXA, such as Do33, to the chromosome spreads expressing the fragile site in these families could clarify the confusion. The signals of such probes would be distal to FRAXA but proximal to FRAXE.

5) Isolated cases diagnosed based on low percentage of fragile site expression but having normal pfxa3 bands usually suggest misdiagnosis (Rousseau et al., 1991b; Mulley et al., 1992). These cases were probably misdiagnosed previously on the basis of the presence of FRAXD at Xq27.2 (Sutherland and Baker, 1990). They are reclassified as mental retardation of unknown cause.

6) In very few families reported, affected individuals who appeared to have fragile X clinical features showed a normal pfxa3 band (Nakahori et al., 1991). This may indicate that different mutations, such as a point mutation, deletion or insertion, interrupt the FMR-1 gene, or that other genes are responsible for the fragile X phenotype. Alternatively, these mentally retarded patients may belong to a different clinical entity. Recently, a male patient, who exhibited typical clinical features of fragile X syndrome but had neither fragile X expression nor pfxa3 band, was found to have a large deletion in the fragile X region (Gedeon et al., 1992). Therefore, cases with apparent fragile X phenotype should be examined with pfxa3 irrespective of whether they express the fragile site.

7) In prenatal diagnosis with pfxa3, detection of a PstI band of 1.5-1.7 kb in chorionic villi (CV) of a male fetus would render prediction of mental status rather difficult, since this size of amplification is at the boundary of affected and unaffected (Rousseau et al., 1991b). At that stage, methylation of the CpG island may not be well established in the CV sample (Sutherland et al., 1991b), therefore, methylation of the CpG island in CV sample cannot be used for prenatal diagnosis. However, complete methylation of the EagI site at the CpG island was observed in an amniotic fluid (AF) sample of a female fetus from an obligate carrier mother (Dobkin et al., 1991). If methylation of the fragile X related CpG island is well developed in AF samples, male fetuses with a 1.5 - 1.7 kb (Δ = 0.5 - 0.7 kb) PstI band in CV sample may have to wait until a methylation study can be undertaken on AF samples. In addition, detection of a large PstI band (Δ > 0.6 kb) in a female fetus would result in a problem of prediction of phenotype because females with amplification at this range can be either carriers or affected (Yu et al., 1991; Rousseau et al., 1991a; Mulley et al., 1992). An overall risk of 50% to be

affected was estimated for female fetus with $\Delta > 0.6$ kb (Rousseau et al., 1991b). Further investigation is needed to evaluate how the mutation evolves in females, however, the basis for this risk is unclear since the data of Rousseau et al. (1991b) were not corrected for ascertainment bias.

In conclusion, Southern blot analysis with DNA probes very close to the CCG repeat provides a direct molecular diagnosis of fragile X syndrome with very high sensitivity and specificity. The sizes of the CCG repeat amplification are distinguishable between normal individual, normal carrier and fragile X syndrome patient, and can be detected from various tissues including chorionic villi. Therefore, the direct molecular diagnosis test is very useful in carrier detection, prenatal diagnosis and differential diagnosis of fragile X syndrome.

In some cases such as female carriers with smearing pfxa3 bands, PCR based linkage analysis in conjunction with Southern blotting would clarify the diagnosis, particularly for laboratories without intensive experience in the interpretation of pfxa3 results.

CHAPTER 7

CONCLUSIONS

This thesis has been written at an exciting time when the molecular picture of the fragile X syndrome and the associated fragile site have just been discovered and the work described herein is itself part of that process. Positional cloning using the YAC cloning system led to the isolation of the fragile site, FRAXA (Chapter 3, 4), which is coincident with the mutation underlying the fragile X syndrome. Fragile X genotype is characterized by an unstable DNA region (Chapter 5): a variable length of CCG repeat (Kremer et al., 1991a). The genetic features of this sequence are novel in that it is unstable both in mitosis and meiosis. In fragile X families, the CCG repeat sequence usually increases in size through generations when the fragile X genotype is transmitted by a female. No or little size change of the CCG repeat is observed in male transmission. The unusual genetics of this unstable element explain many puzzling aspects of the fragile X syndrome, including the existence of transmitting males, lack of new mutant in affected individuals and the increasing penetrance in successive generations (namely the Sherman Paradox) (Chapter 6).

The work presented in this thesis is of direct clinical significance. The isolated DNA probe pfxa3 is a reagent for direct diagnosis of the fragile X genotype and is already in use in identifying transmitting males and cytogenetically normal carrier females. This probe can be used to differentiate fragile X syndrome from the other forms of non-specific X linked mental retardation. The correlation of the size of the pfxa3 fragment with the phenotype of mental retardation also allows prenatal diagnosis of fragile X syndrome, although some female fetuses will be problematic in terms of prediction of their mental status. The finding that all mothers of affected individuals are carriers does change the clinical view towards the families with apparently isolated cases of fragile X syndrome. All isolated cases should be handled as familial, not as possible new mutations. Identification of potential female carriers in fragile X families as well as in the population provides a means of prevention of fragile X syndrome.

The significance of cloning the fragile X is far beyond understanding of the fragile X syndrome itself. Cloning of the fragile X revealed a new mechanism of genetic mutation in humans: amplification of a trinucleotide repeat. Indeed, this mechanism has also been identified

in myotonic dystrophy (Harley et al., 1992; Buxton et al., 1992; Aslanidls et al., 1992) and in Kennedy disease (La Spada et al., 1991). In both disorders, amplification of an AGC repeat sequence was identified as the molecular basis, although transcription is from different strands of DNA (La Spada et al., 1991; Brook et al., 1992). In myotonic dystrophy, the size of the amplification is in parallel with increasing severity and earlier onset of the disease, a genetic phenomenon termed "anticipation" (Harley et al., 1992; Buxton et al., 1992), analogous to the "Sherman paradox" in fragile X syndrome. Understanding of the new mutation mechanism provides a means to study those genetic disorders exhibiting unusual genetic characteristics. After the DNA region between flanking markers of a disorder is cloned by positional cloning, the disease causing mutation can be pinpointed by probing with a panel of microsatellite repeat sequences (Richards and Sutherland, 1992). The existence of heritable unstable DNA sequences of this kind may provide new explanations for many old genetic questions that can not be explained by the classical genetic mechanisms.

Revelation of the unstable elements in fragile X syndrome and in myotonic dystrophy is but a beginning to the understanding of this new mutation mechanism. Searching for cDNA clones containing a CCG repeat should isolate additional genes which contain it. The mechanism of amplification itself is unknown. The function of this sequence in fragile X syndrome is still a mystery. Since the CCG repeat sequence is most likely located in the 5' untranslated region of the FMR-1 gene and has at least six copies in normal individuals, this sequence appears to have a function unrelated to the FMR-1 protein. It may act as the binding site for a DNA binding protein and thus play a role in gene regulation. Following the haplotypes of two highly polymorphic DNA markers flanking the fragile X in normal and fragile X chromosomes, it has been possible to demonstrate that the mutation is transmitted from a few ancestral founders by asymptomatic carriers and spread though the population (Richards et al., 1992). In fragile X syndrome, whether methylation or amplification play a causative role is still inconclusive. However, in myotonic dystrophy, amplification of a AGC trinucleotide without methylation in the vicinity of the repeat implies a causative role for amplification rather than for methylation. The fact

that the CCG repeat, but not the AGC repeat, is associated with a folate sensitive fragile site awaits explanation. Since the CCG repeat can be methylated but not the AGC repeat, a question arises as to whether methylation of the CCG repeat is essential to produce a fragile site. Cloning of fragile sites other than FRAXA will allow better understanding of the molecular basis for the cytogenetic phenomenon.

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APPENDIX I

FRAGILE X PEDIGREES

Notes for Appendix I

This Appendix lists the detailed data used in the present project, for the members of 49 fragile X syndrome families.

The data for each individual include:

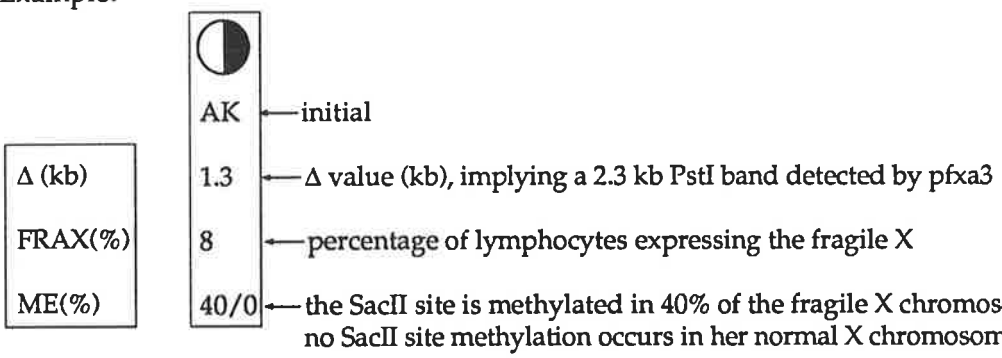
1. initials of the person.
2. Δ value: the increase in size from the normal 1 kb Pst fragment (for example: a 2 kb pfxa3 band is recorded as Δ = 1 kb). M: multiple pfxa3 bands. S: a smear of pfxa3 bands.
3. Cytogenetic fragile X expression.
4. Degree of methylation of the SacII site at the fragile X associated CpG island.

$$\text{Degree of methylation} = \frac{\text{Intensity of methylated band}}{\text{Intensity of methylated + unmethylated bands}}$$

For female carriers, methylation status of the SacII site on both fragile X and normal X chromosomes are recored separately (fragile X chromosome/normal X chromosome).

na, data not vavailable.

Example:



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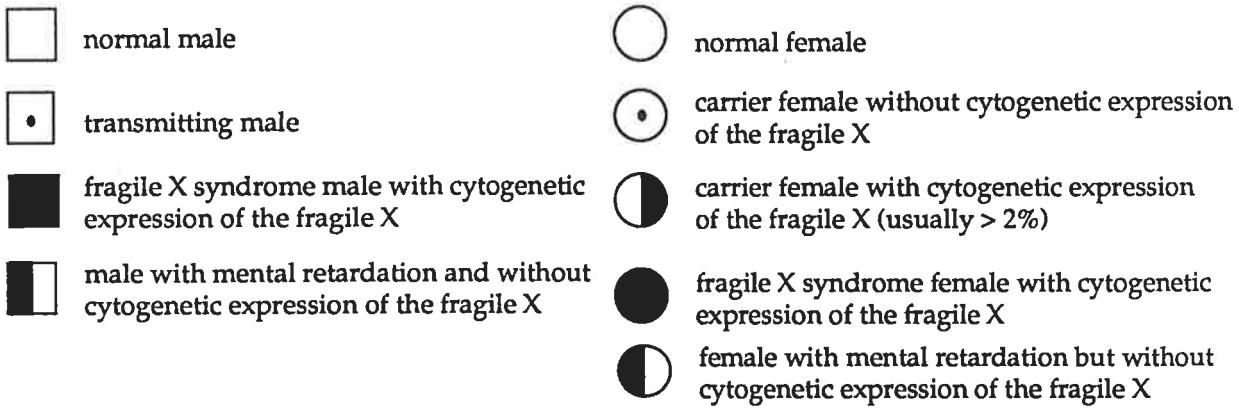

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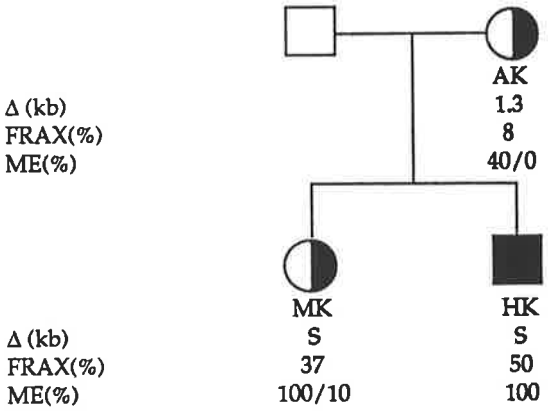

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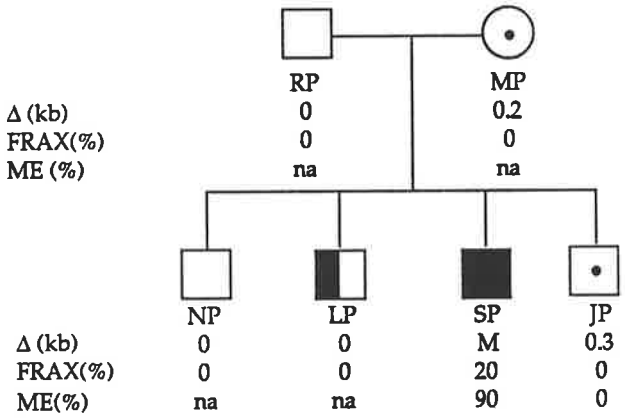
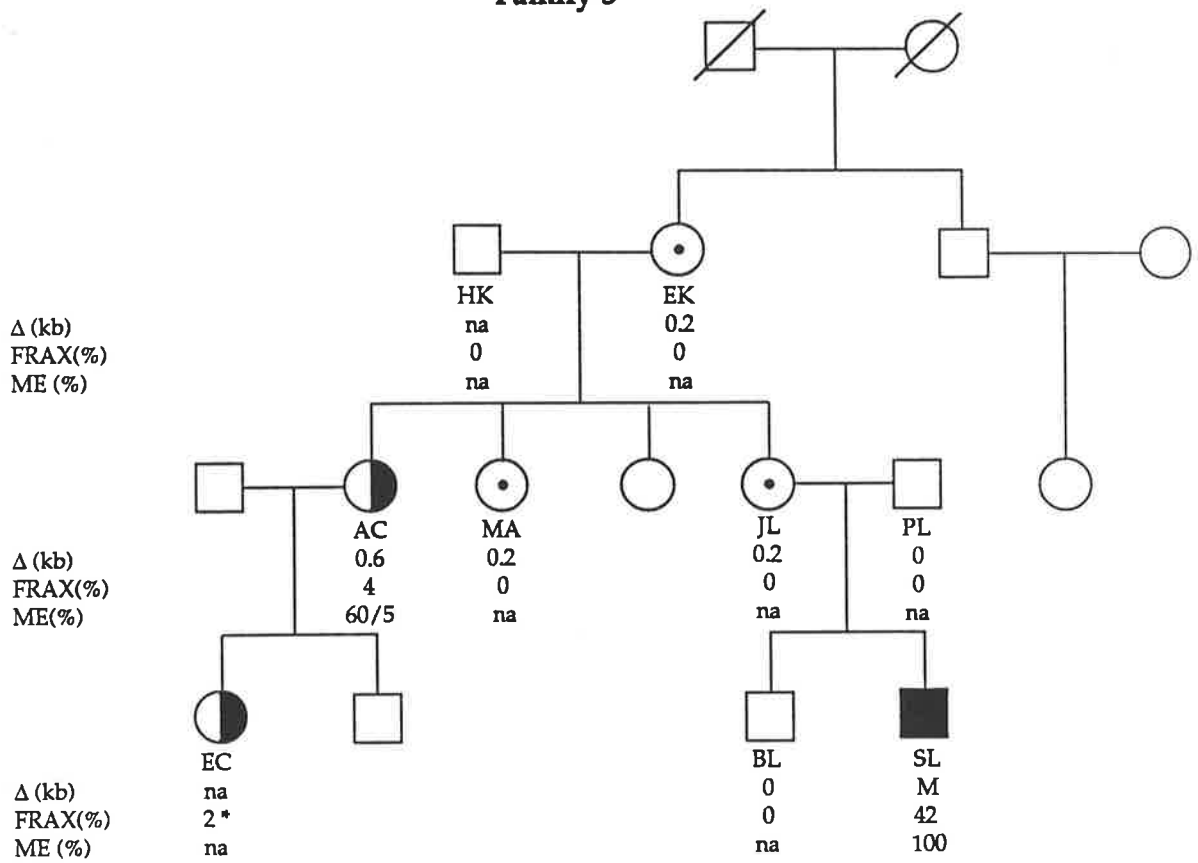
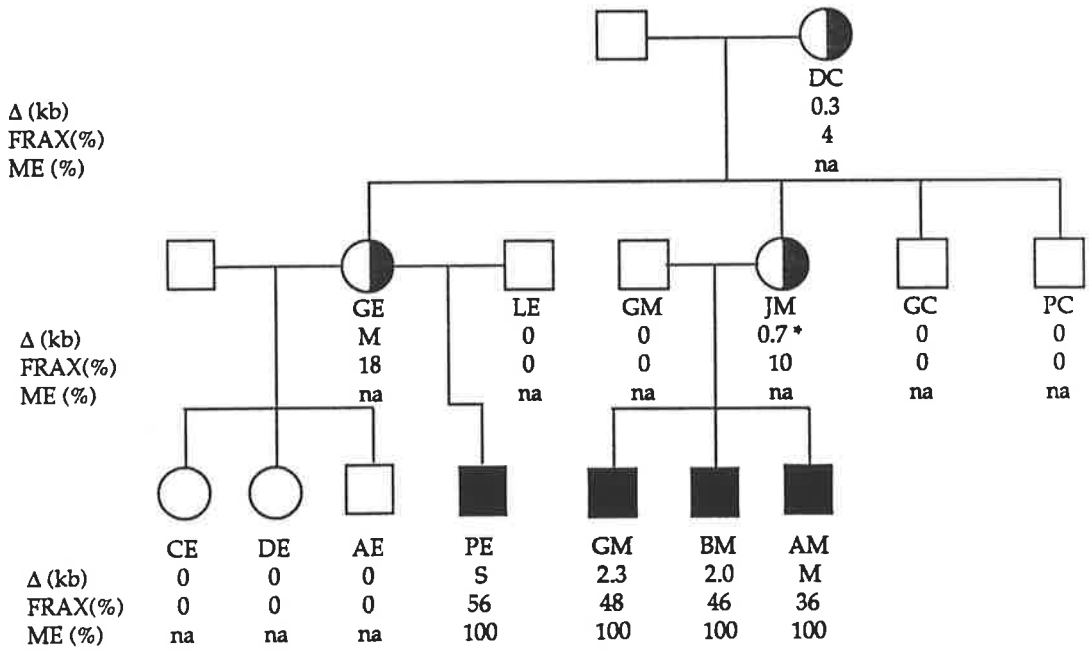


Family 3



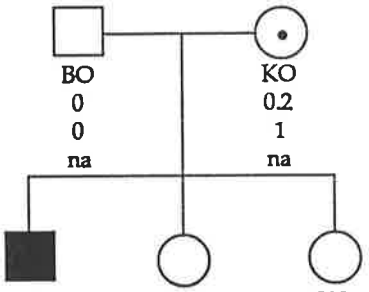
* Fragile X expression in chorionic villi cells.

Family 4

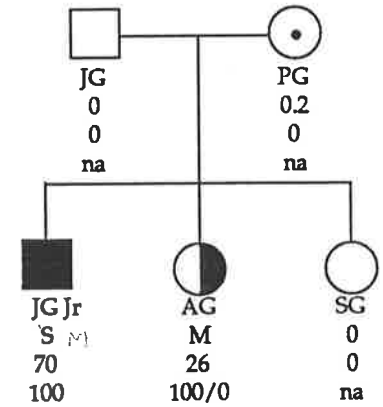


* The intensity of the band is less than half dosage.

Family 5



Family 6



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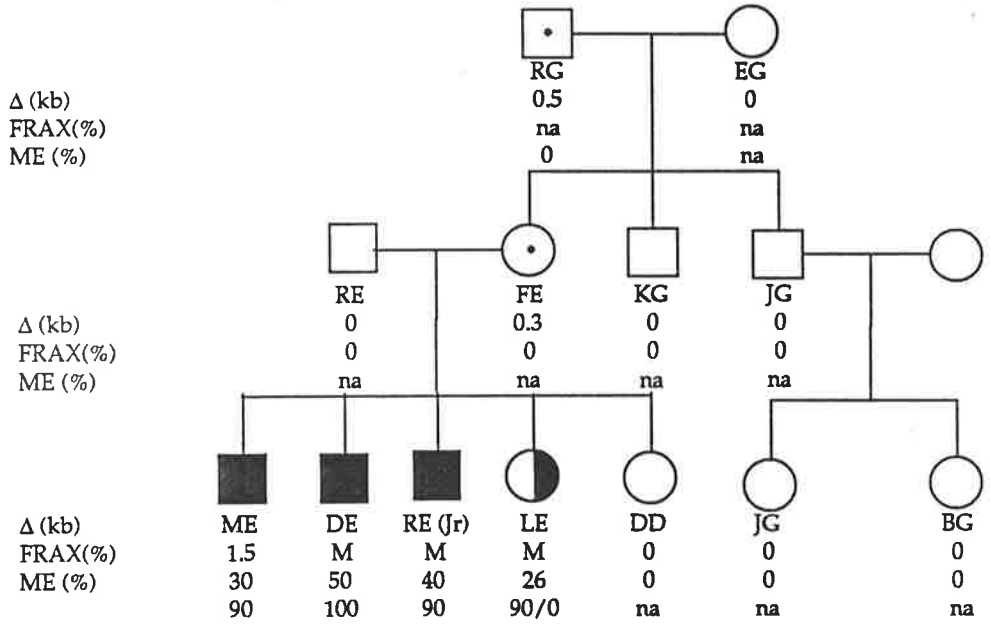

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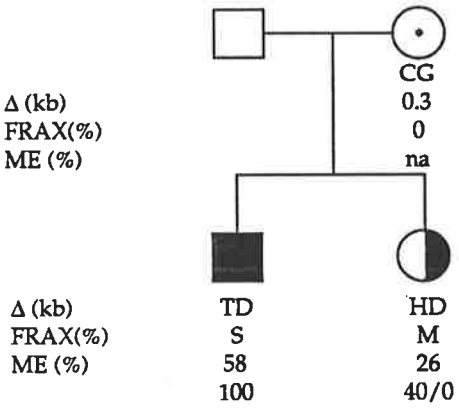

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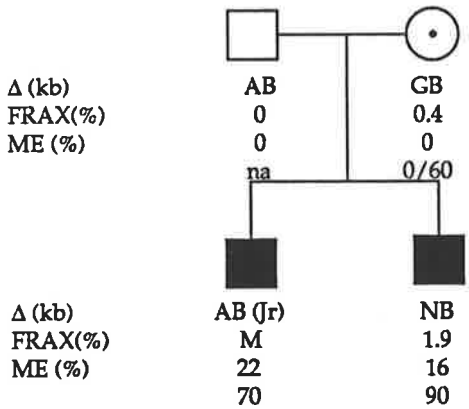

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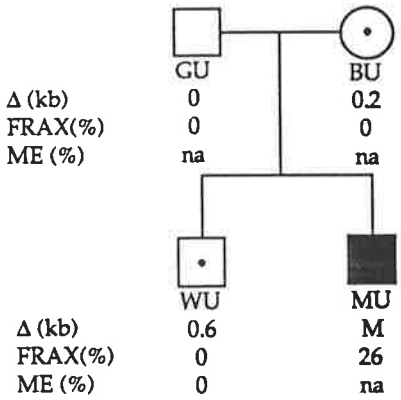

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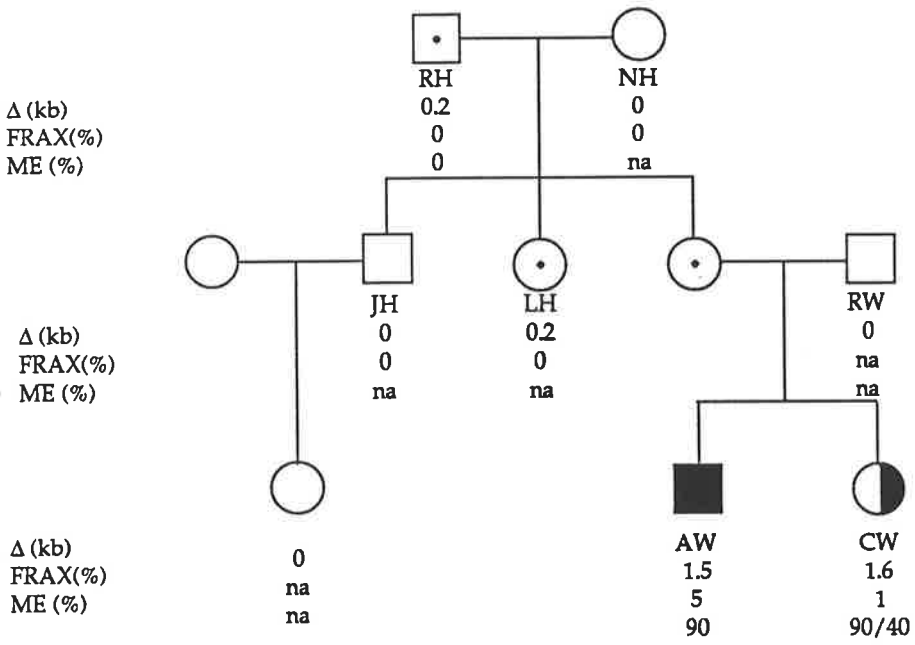

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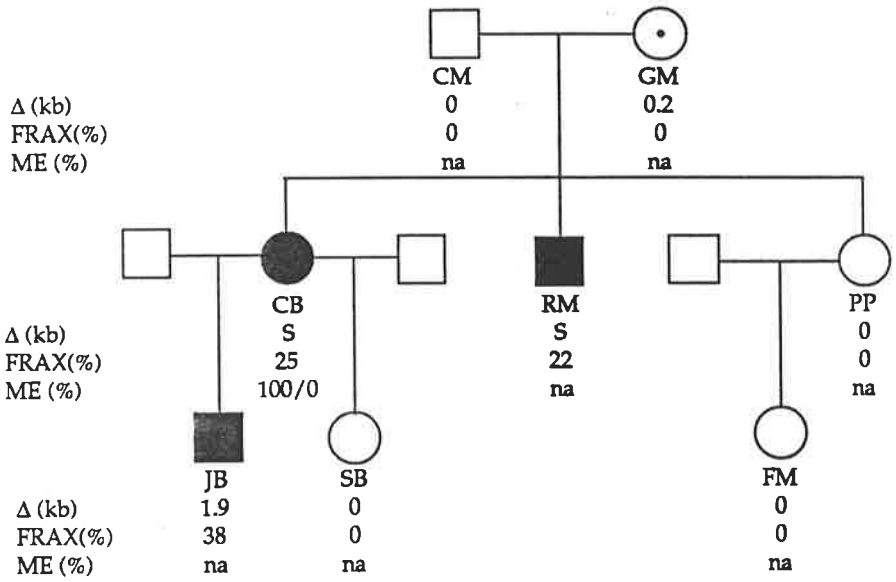

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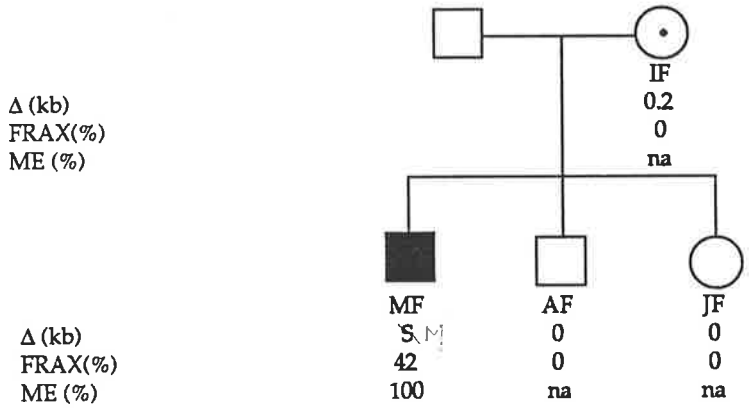

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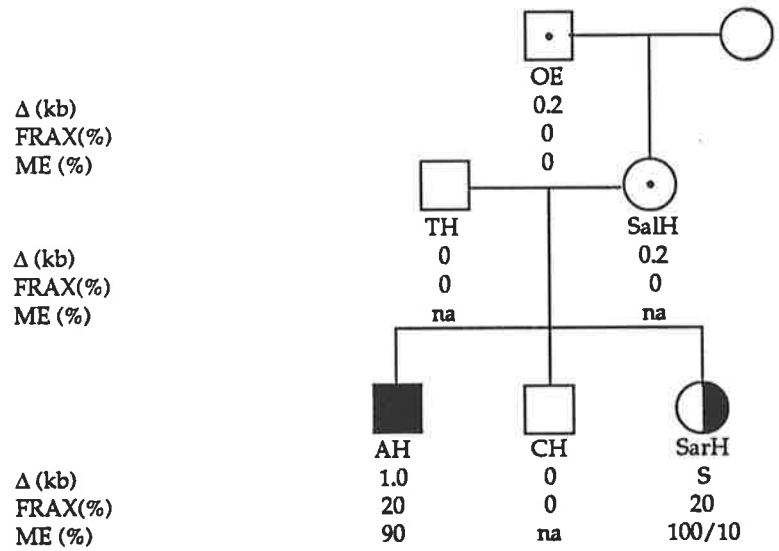

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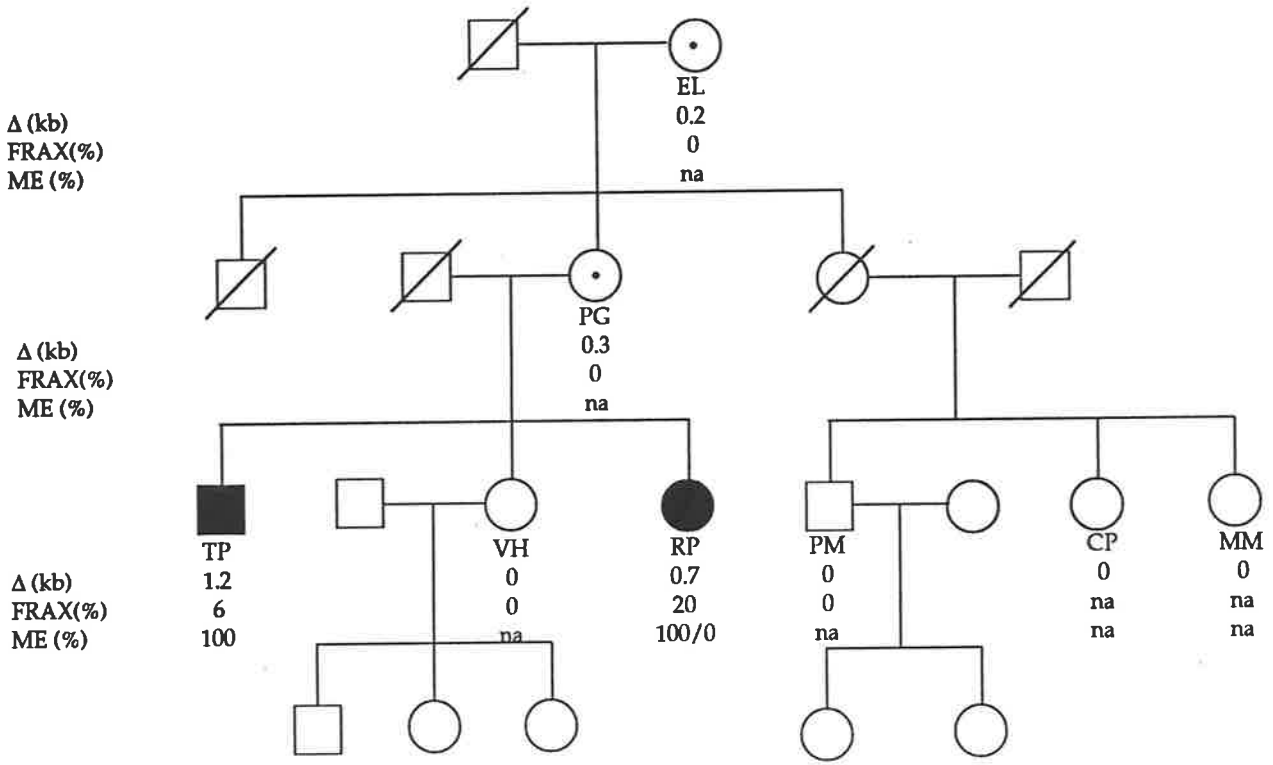
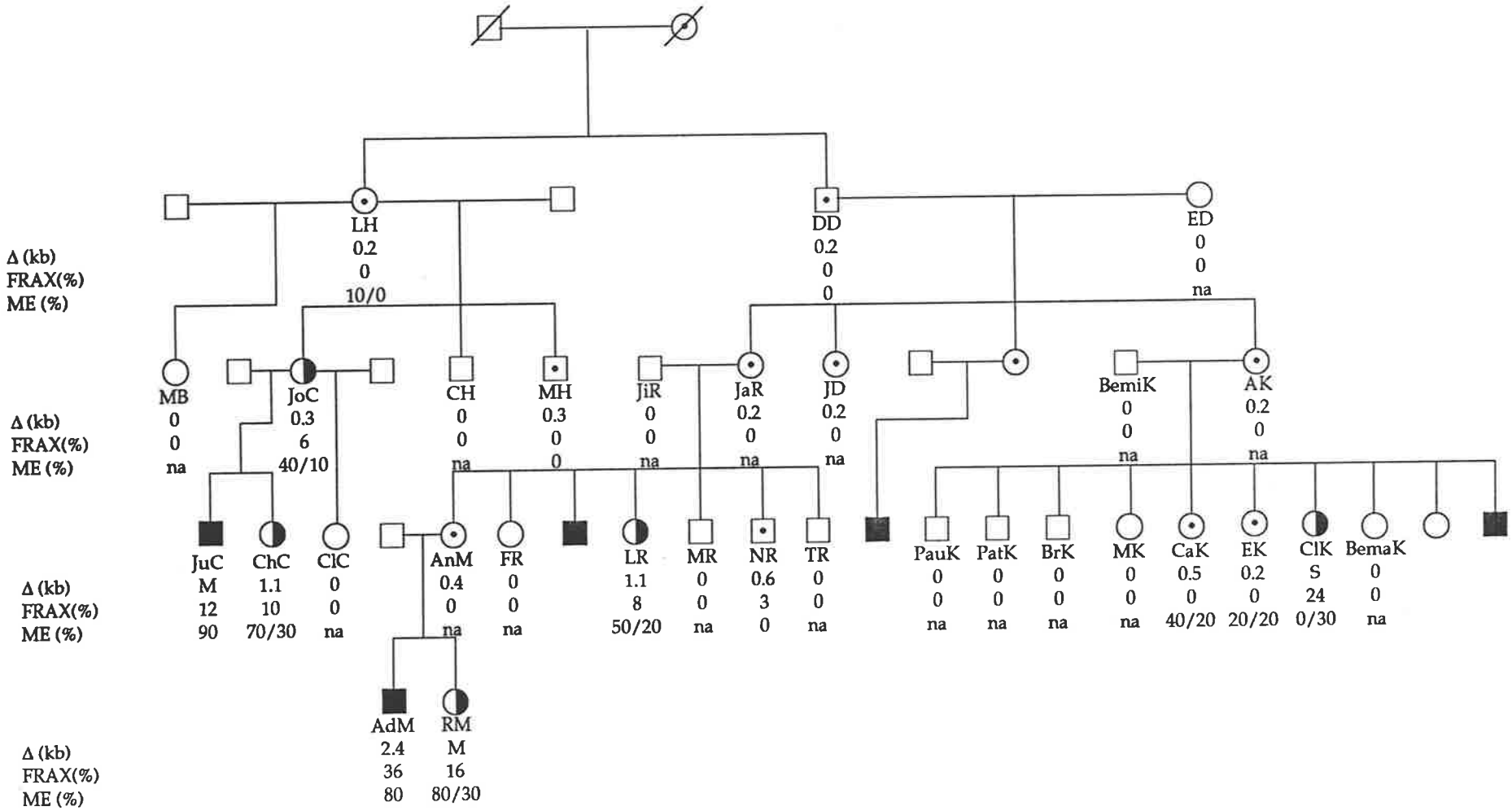
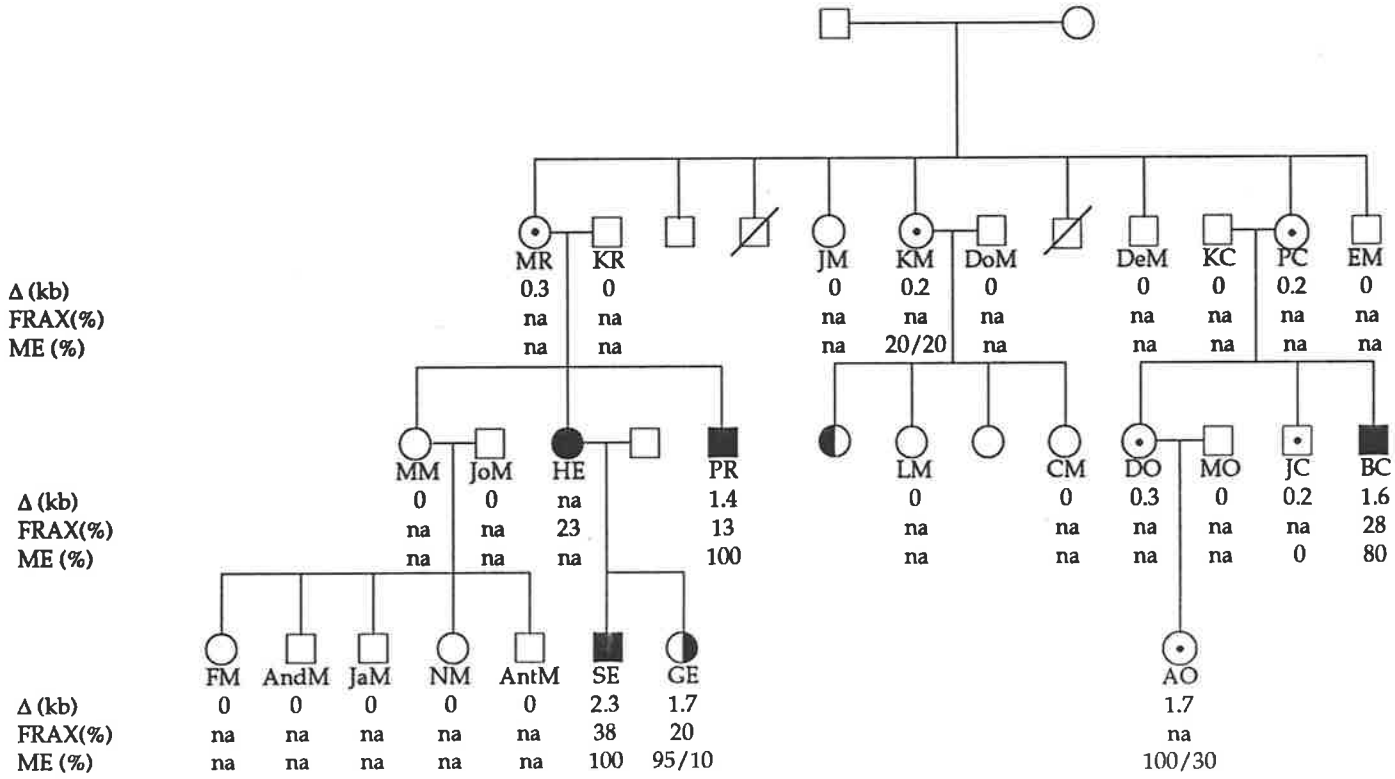

Δ (kb)
FRAX(%)
ME (%)
EL
0.2
0
na
PG
0.3
0
na
Δ (kb)
FRAX(%)
ME (%)
TP
1.2
6
100
VH
0
0
na
RP
0.7
20
100/0
PM
0
0
na
CP
0
na
na
MM
0
na
na
Δ (kb)
FRAX(%)
ME (%)

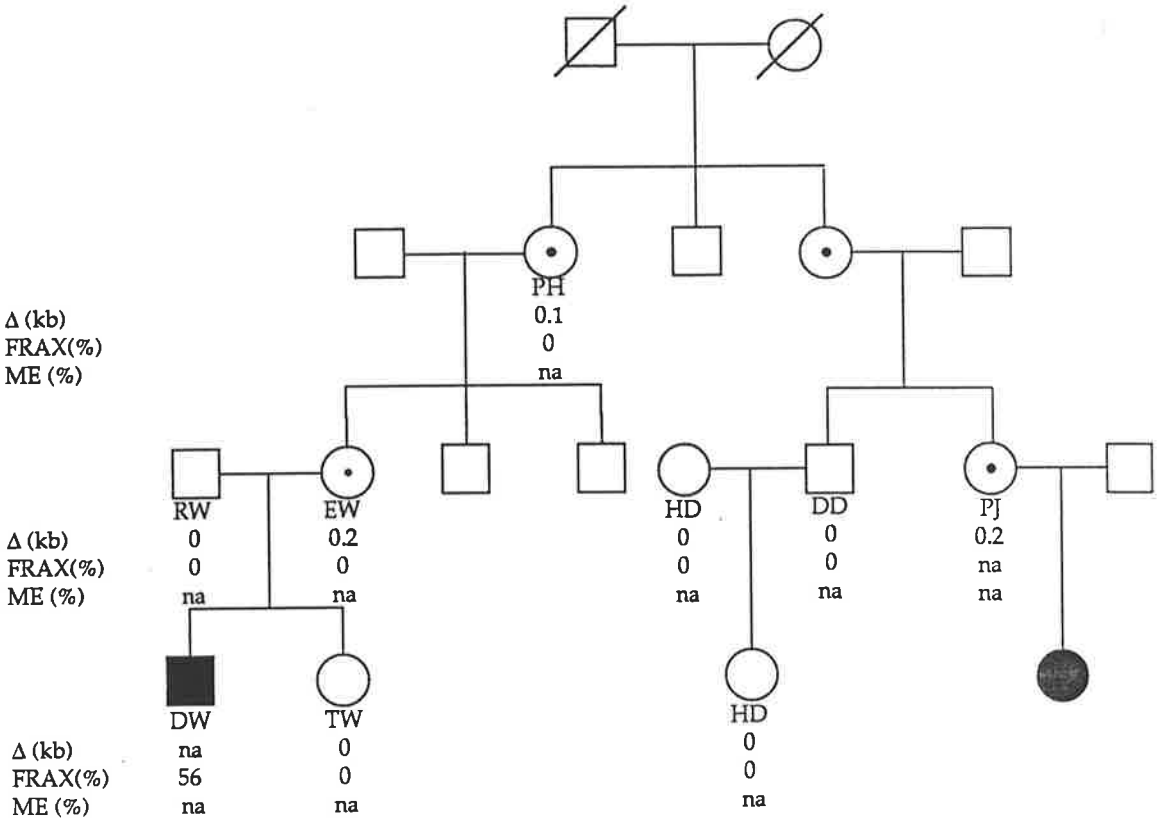
Family 19


Δ (kb)
FRAX(%)
ME (%)
LH
0.2
0
10/0
DD
0.2
0
0
ED
0
0
na
MB
0
0
na
JoC
0.3
6
40/10
CH
0
0
na
MH
0.3
0
0
JiR
0
0
na
JaR
0.2
0
na
JD
0.2
0
na
BemiK
0
0
na
AK
0.2
0
na
JuC
M
12
90
ChC
1.1
10
70/30
ClC
0
0
na
AnM
0.4
0
na
FR
0
0
na
LR
1.1
8
50/20
MR
0
0
na
NR
0.6
3
0
TR
0
0
na
PauK
0
0
na
PatK
0
0
na
BrK
0
0
na
MK
0
0
na
CaK
0.5
0
40/20
EK
0.2
0
20/20
ClK
S
24
0/30
BemaK
0
0
na
AdM
2.4
36
80
RM
M
16
80/30

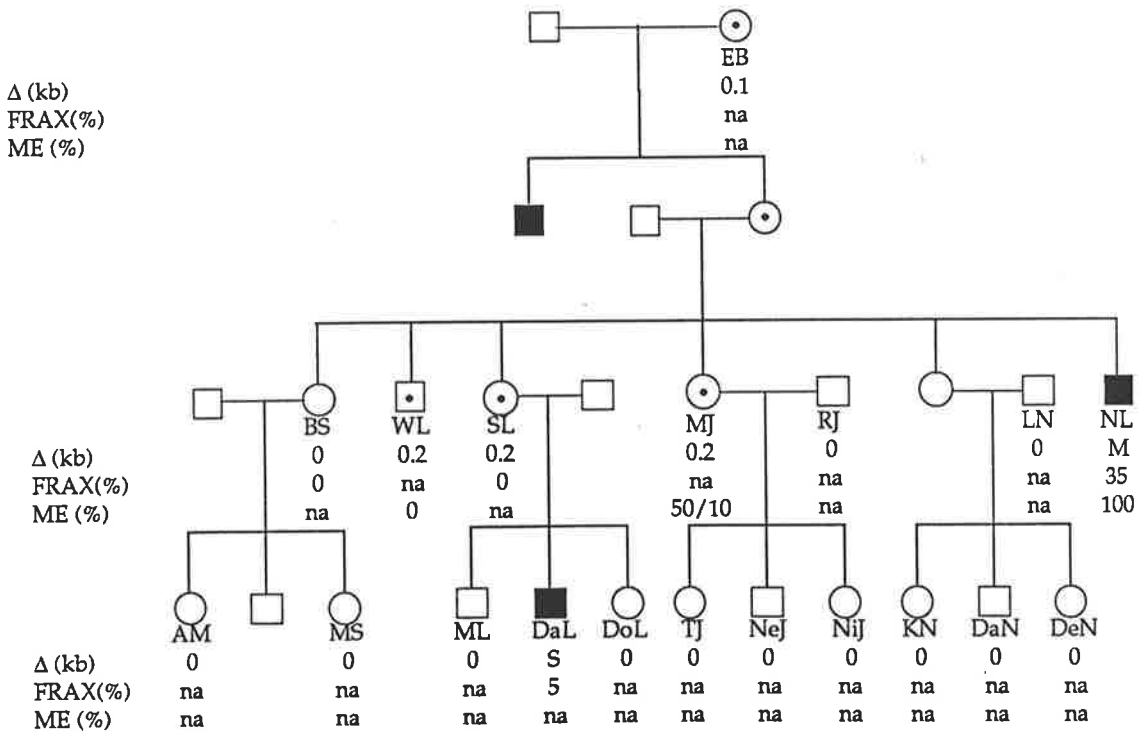
Family 20


MR KR JM KM DoM DeM KC PC EM
Δ (kb) 0.3 0 0 0.2 0 0 0 0.2 0
FRAX(%) na na na na na na na na na
ME (%) na na na 20/20 na na na na na
MM JoM HE PR LM CM DO MO JC BC
Δ (kb) 0 0 na 1.4 0 0 0.3 0 0.2 1.6
FRAX(%) na na 23 13 na na na na na 28
ME (%) na na na 100 na na na na 0 80
FM AndM JaM NM AntM SE GE AO
Δ (kb) 0 0 0 0 0 2.3 1.7 1.7
FRAX(%) na na na na na 38 20 na
ME (%) na na na na na 100 95/10 100/30

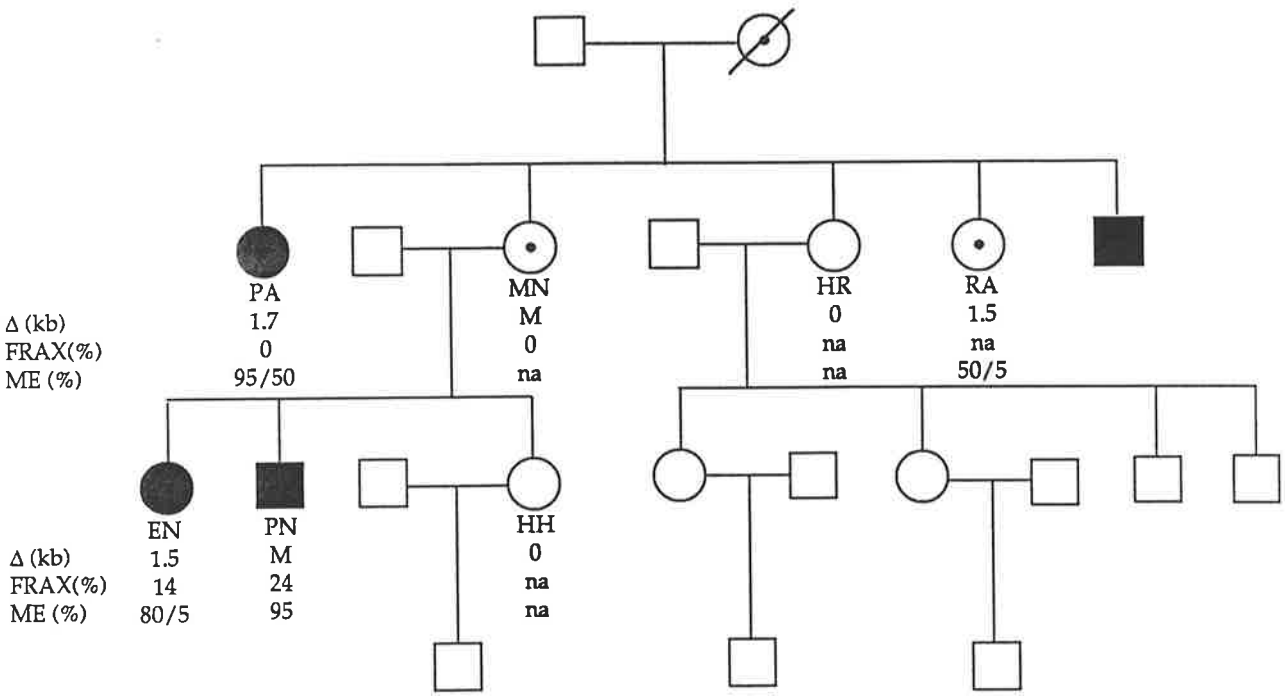
Family 21



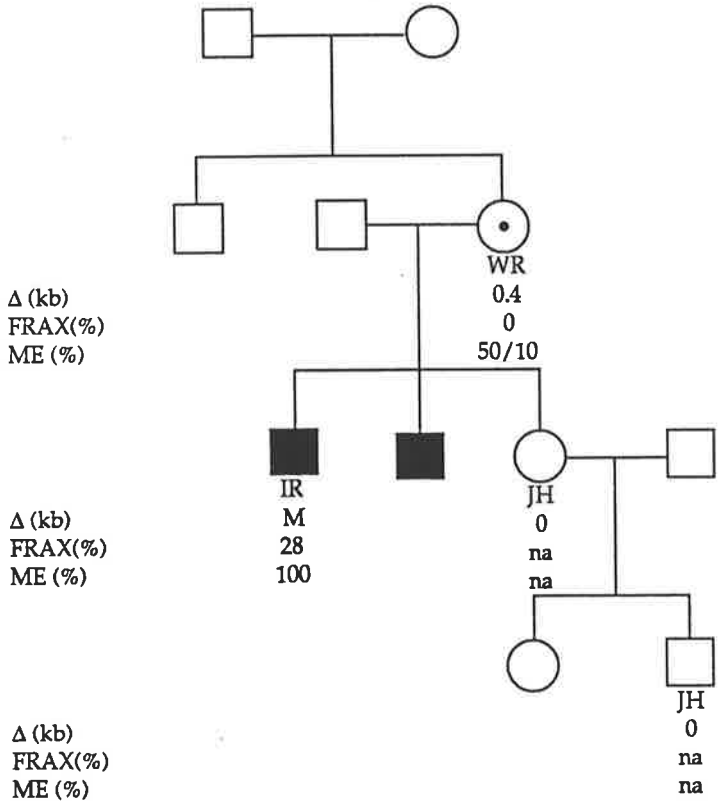
Family 22



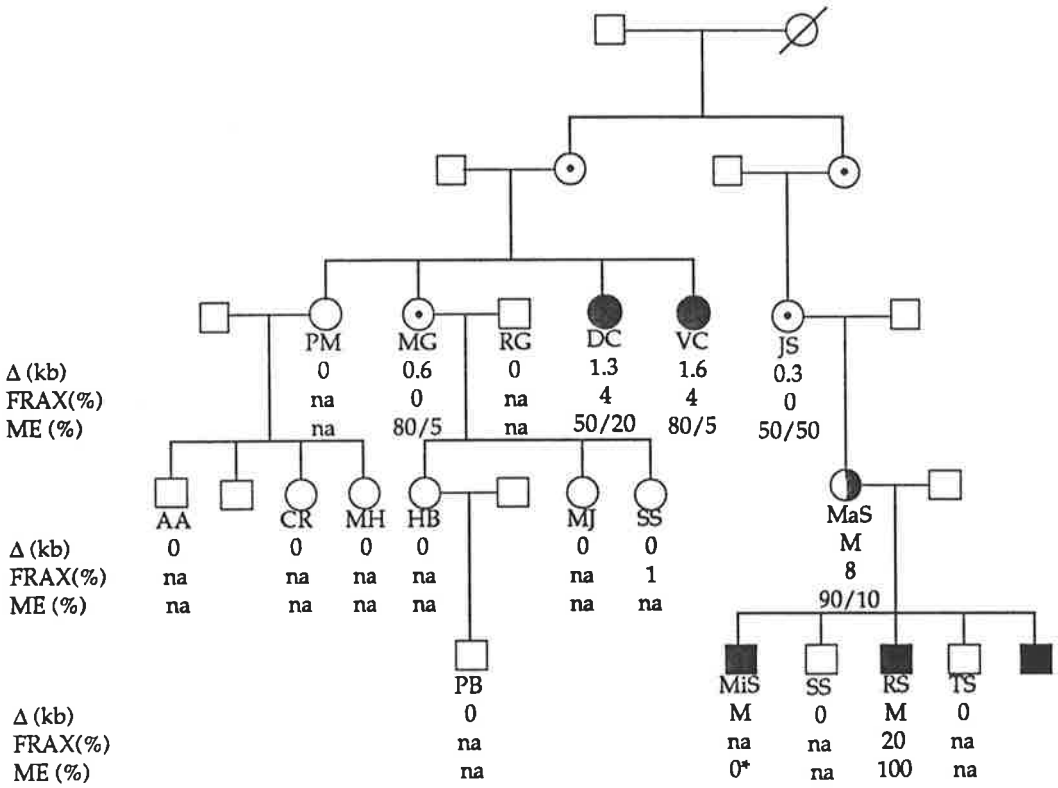
Family 23



Family 24

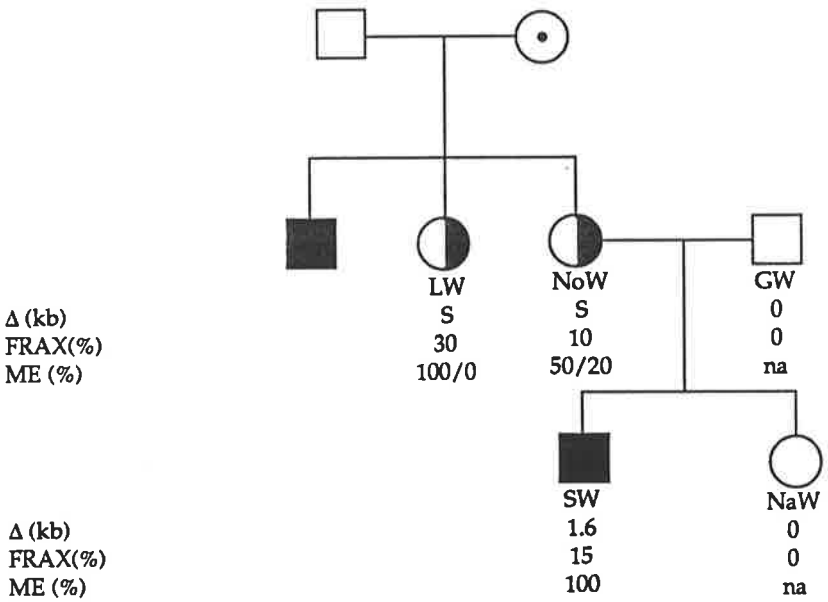


Family 26

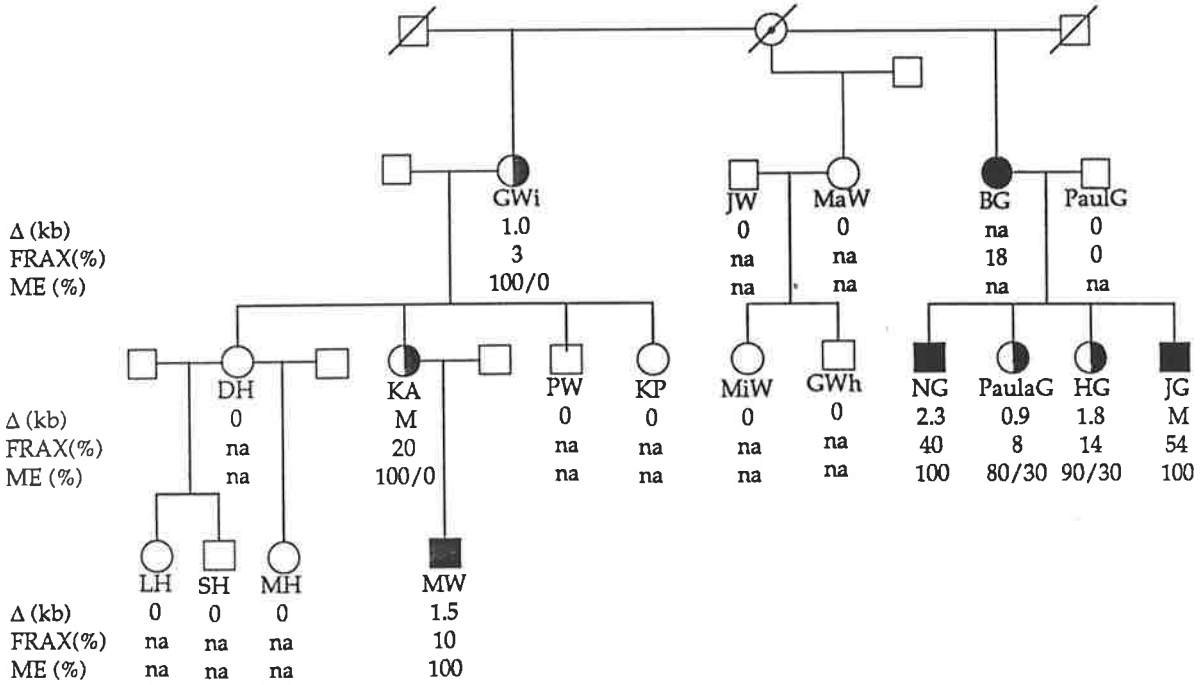


* Only one of the three hybridization bands, the 2.9kb band, is methylated.

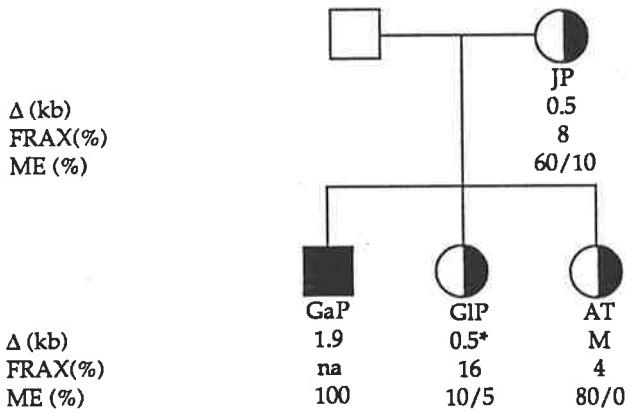
Family 27



Family 28

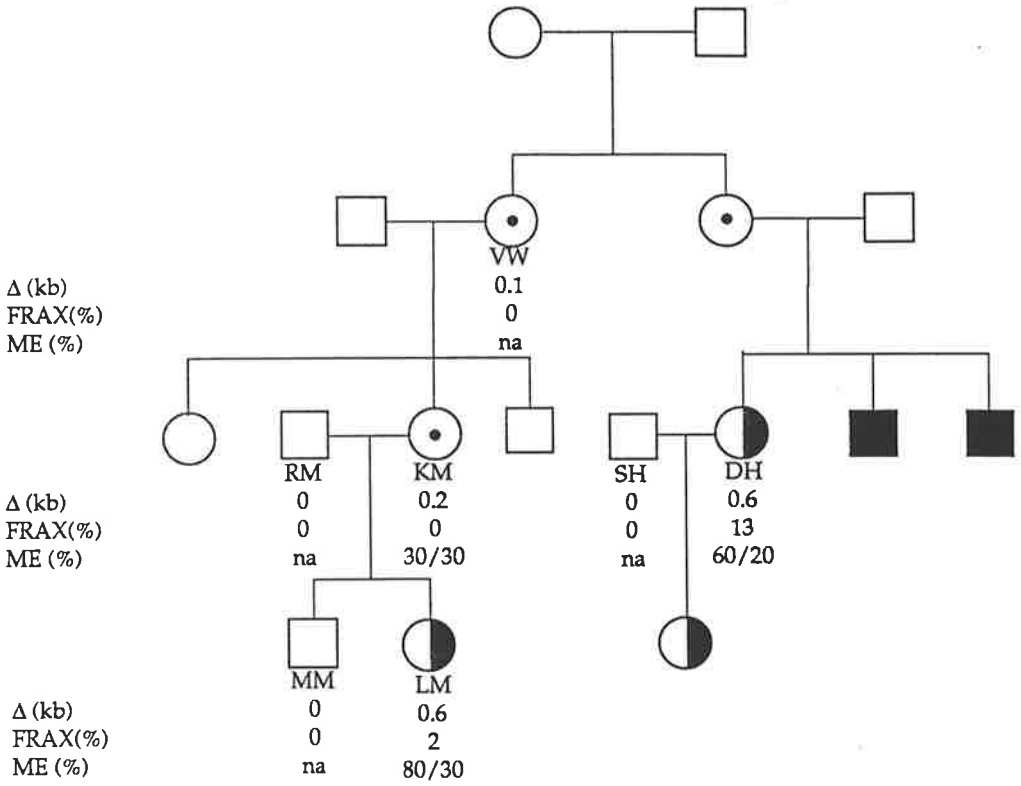


Family 29

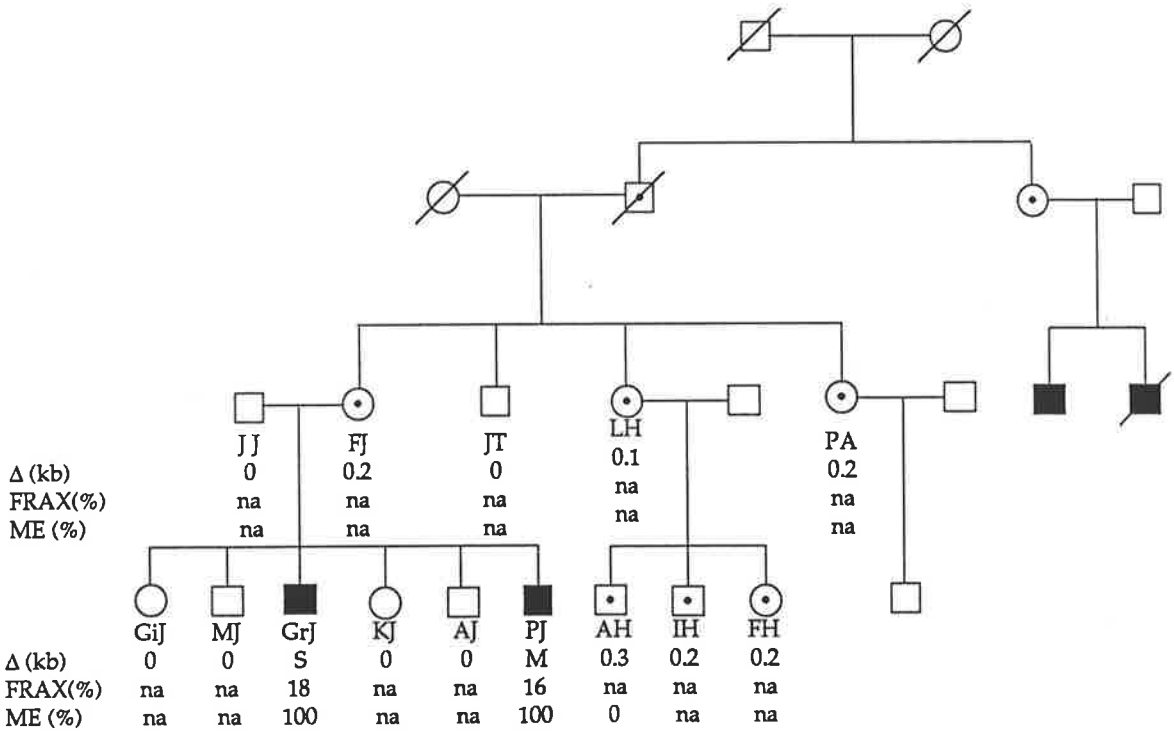


* The intensity of the band is less than half dosage.

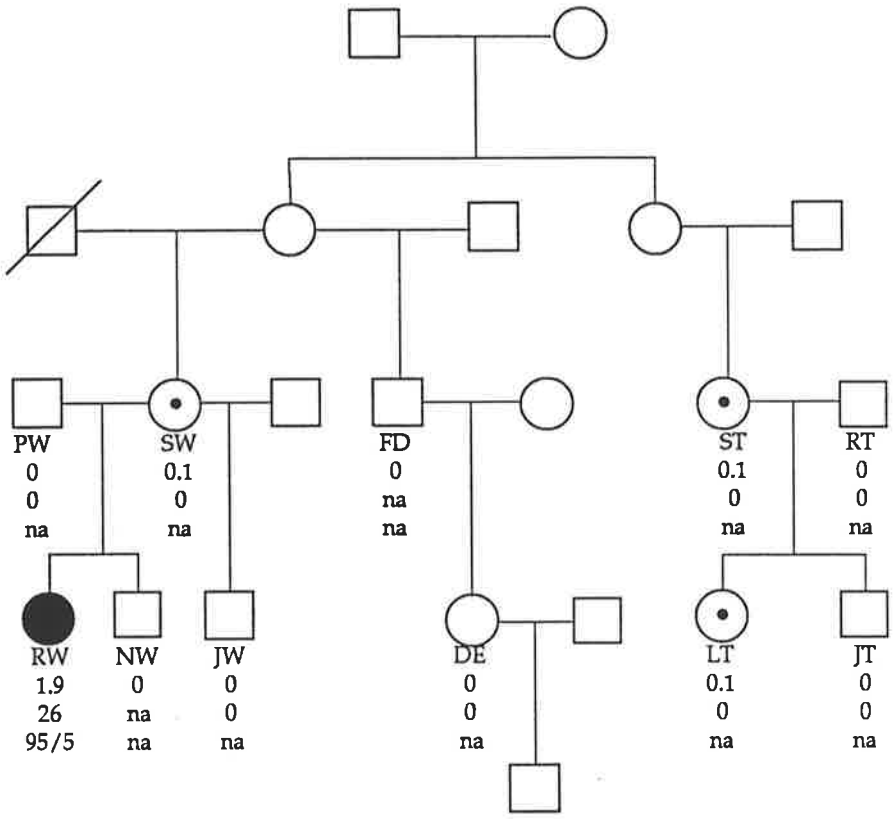
Family 30



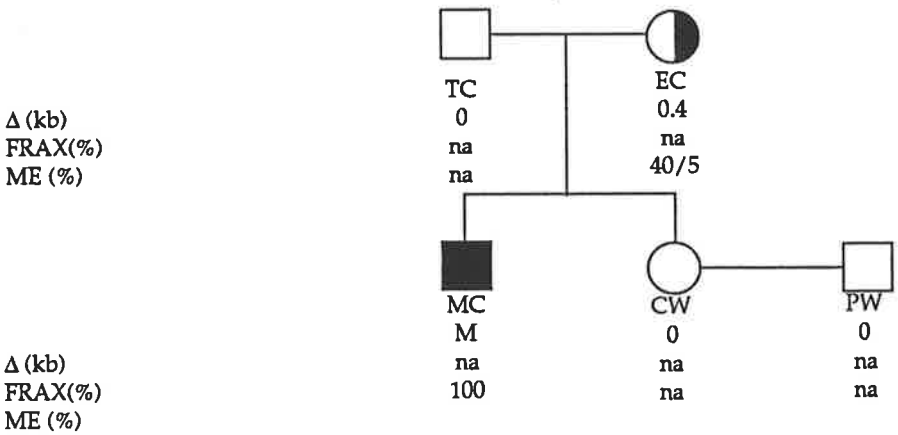
Family 31



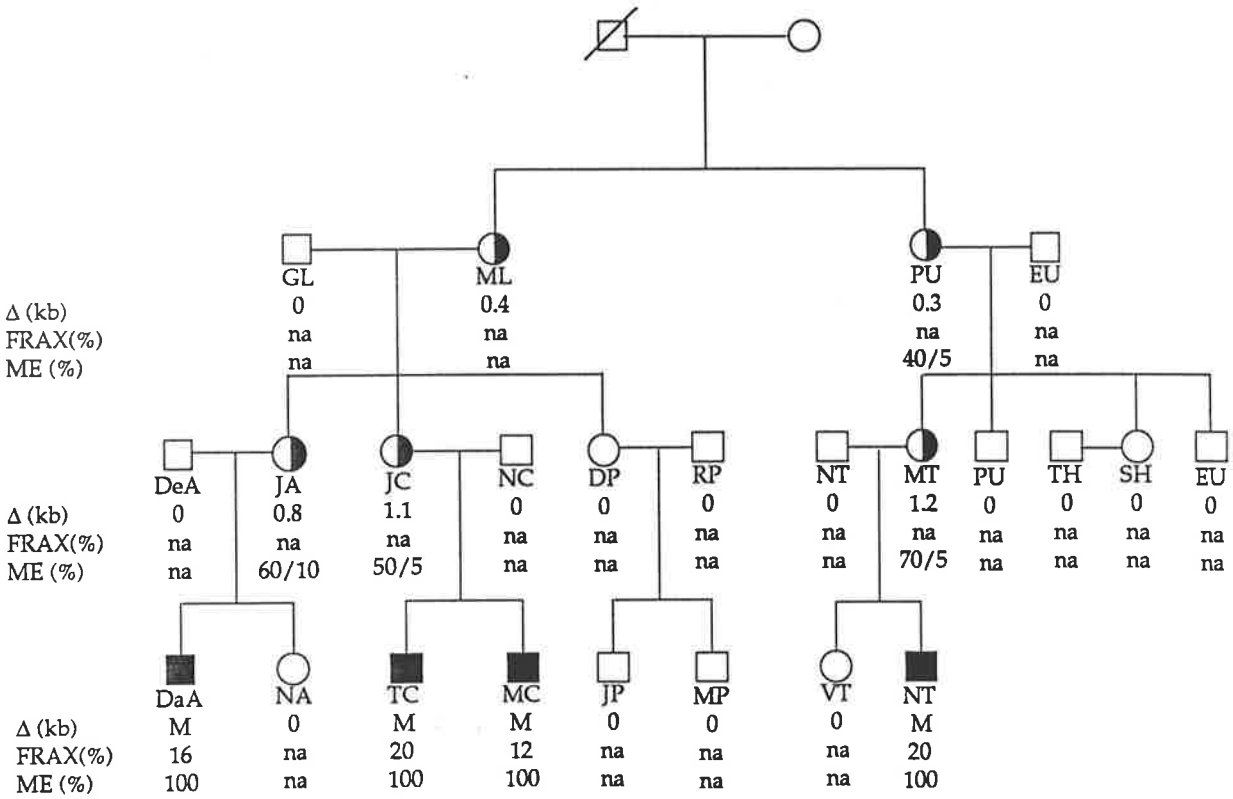
Family 32



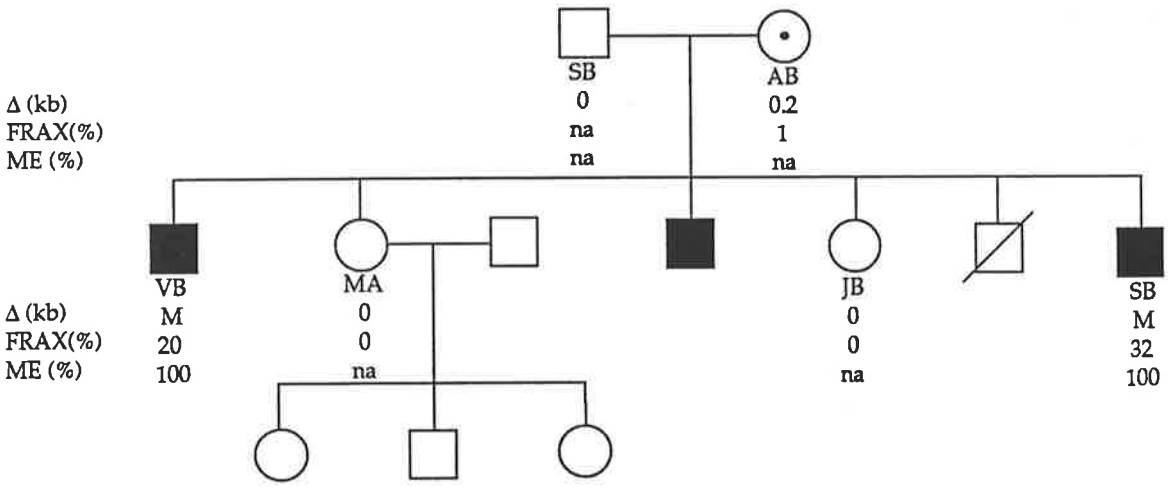
Family 33



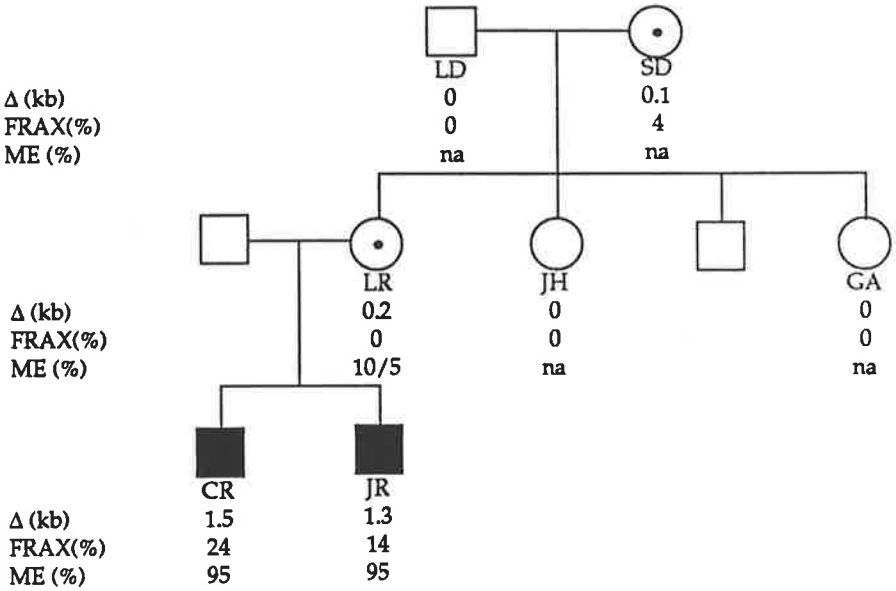
Family 34



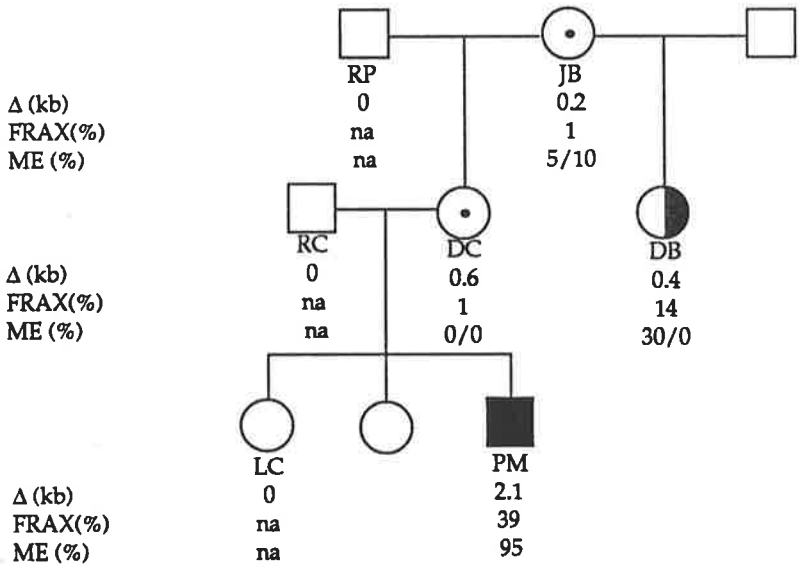
Family 35



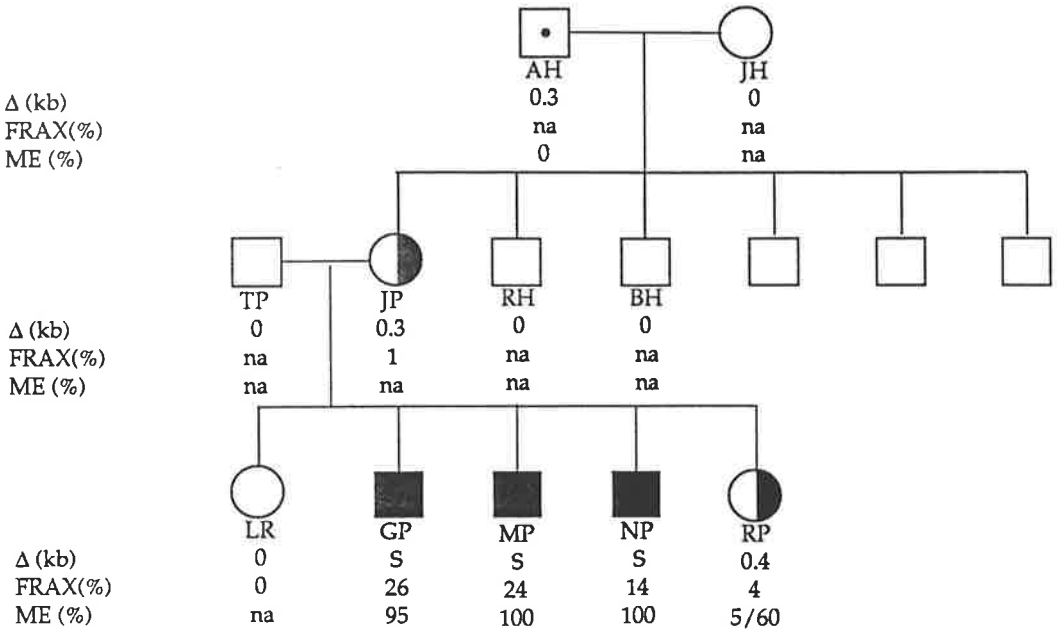
Family 37



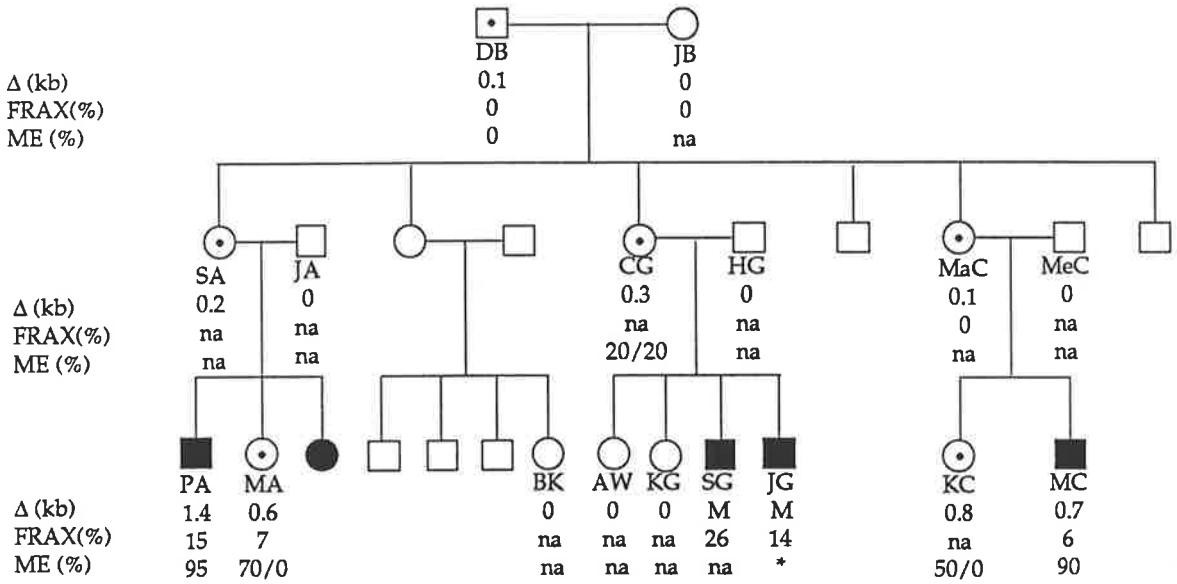
Family 38



Family 39

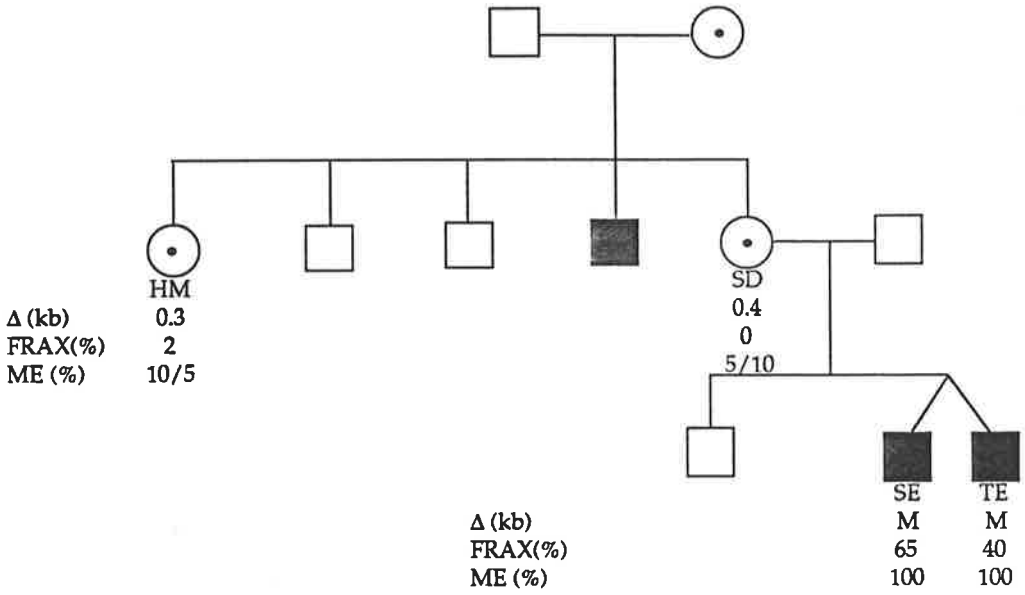


Family 40

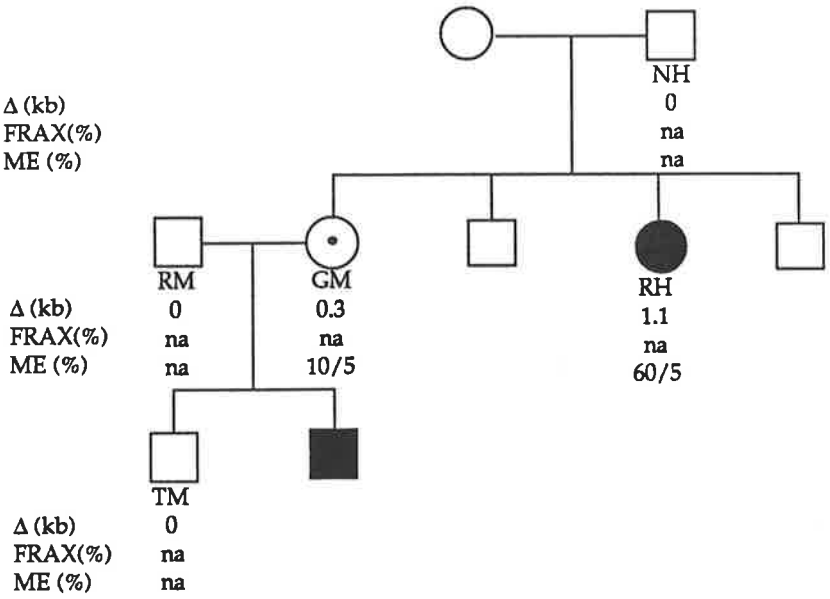


* The SacII site is not methylated in the band with Δ = 0.3 kb, but methylated in the band with Δ = 2.0 kb.

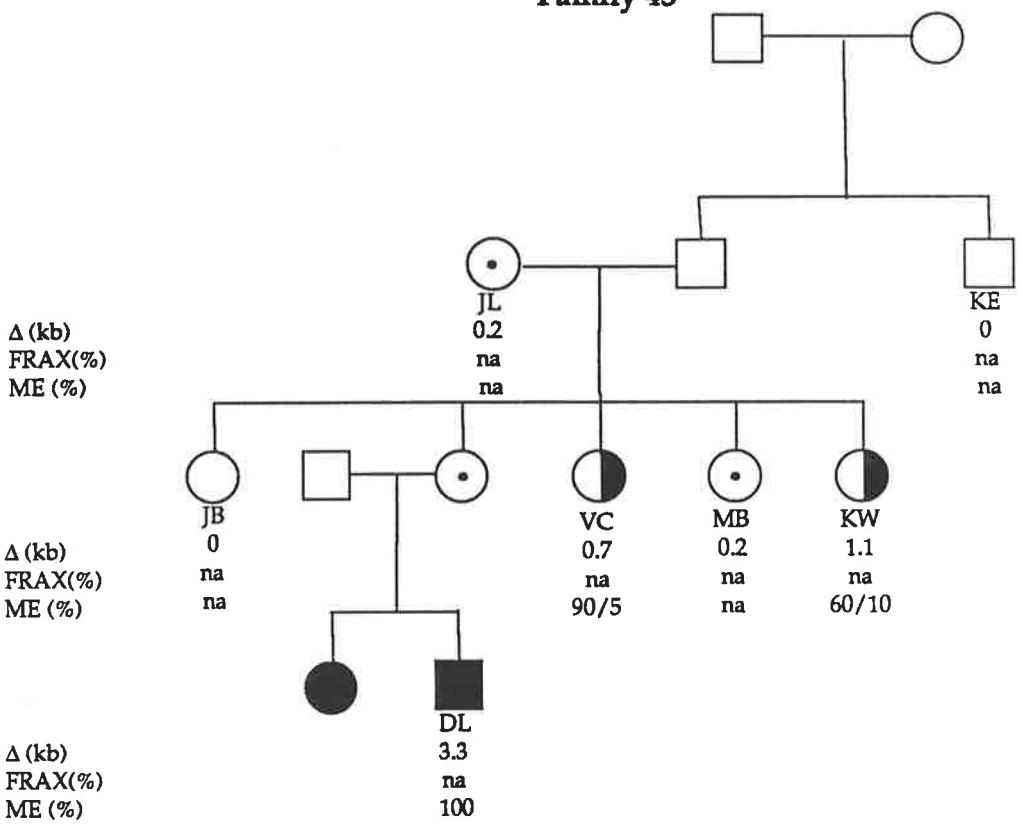
Family 41



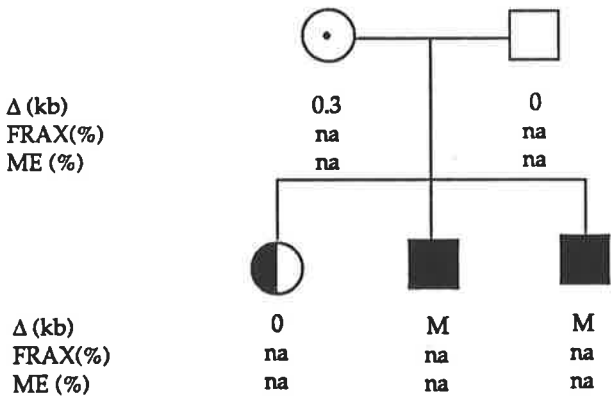
Family 42



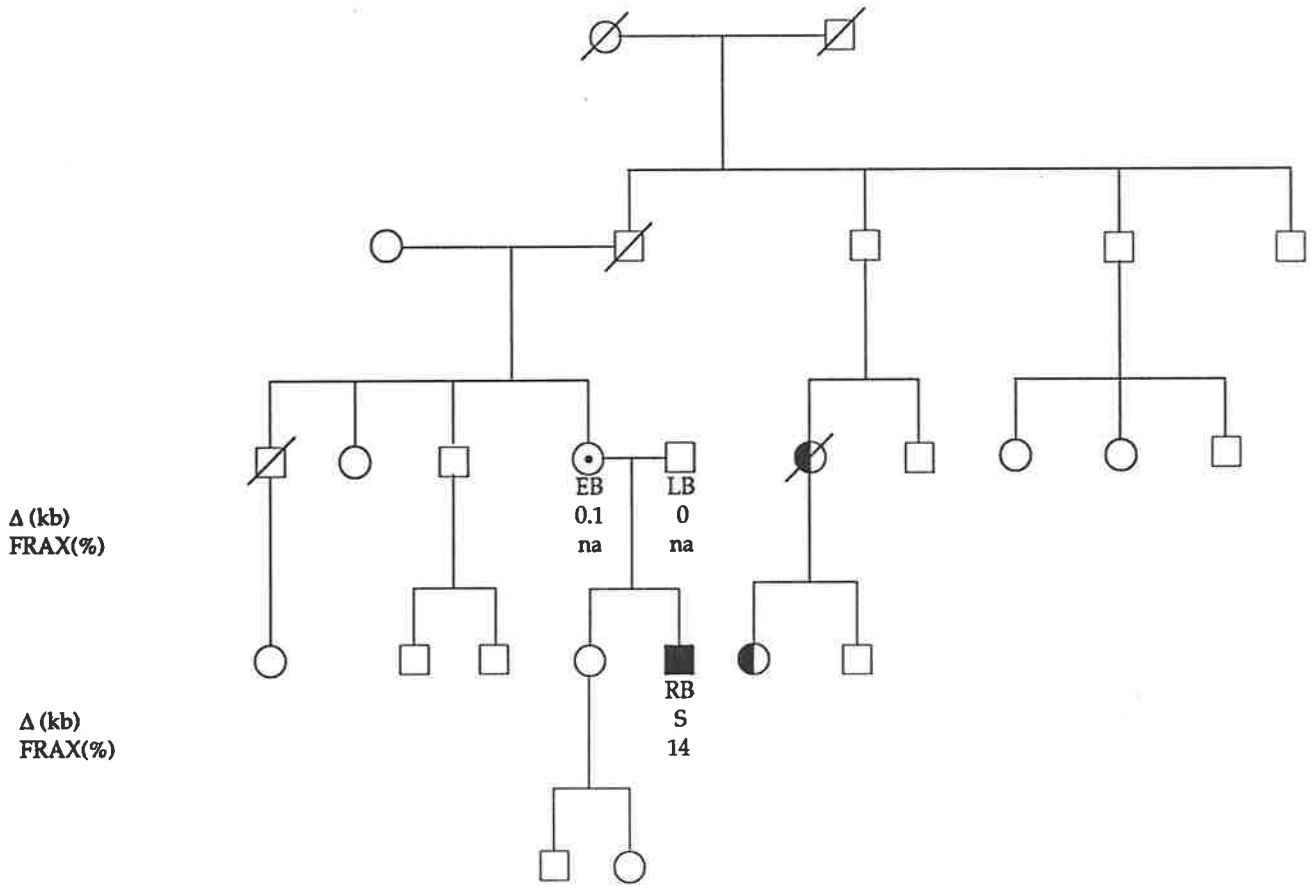
Family 43



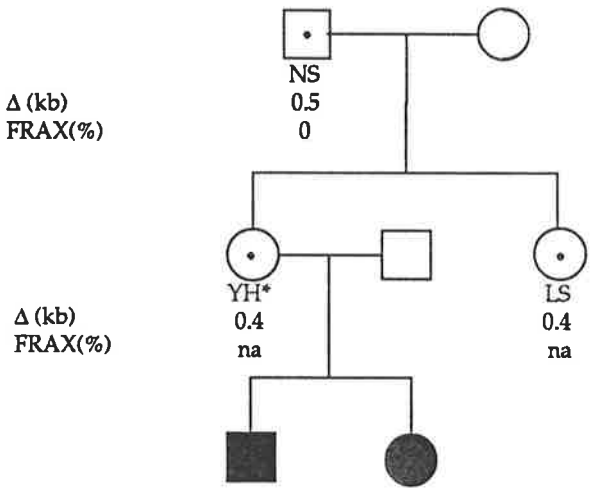
Family 44



FX-1

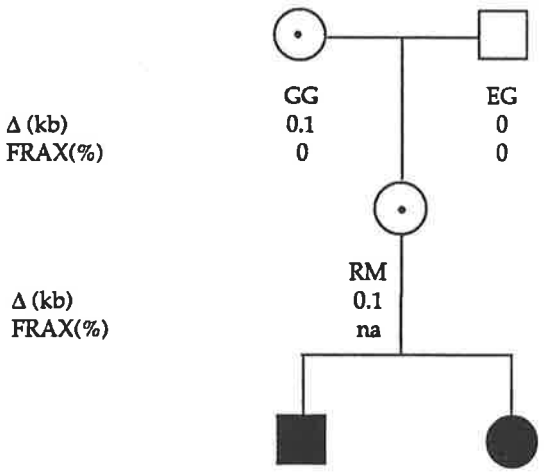


FX-2

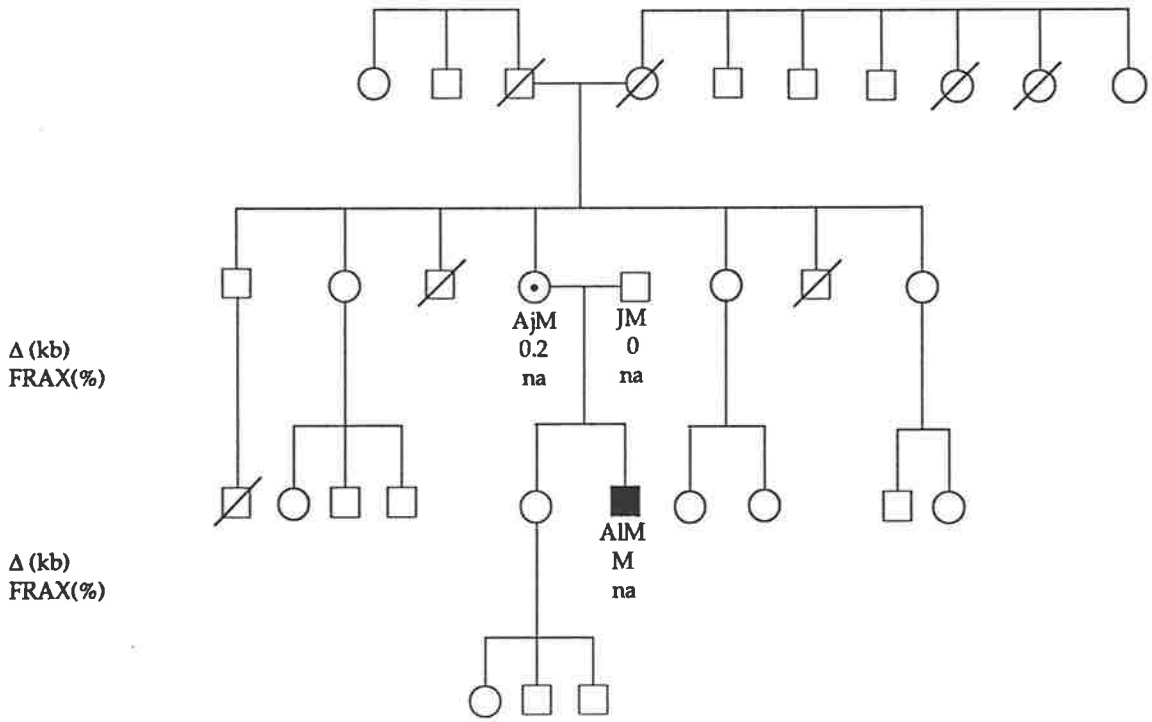


* Psychiatric problem.

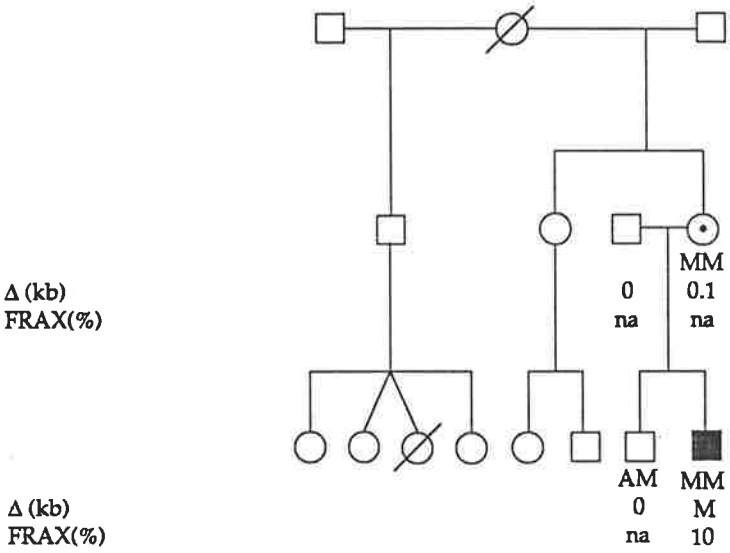
FX-3



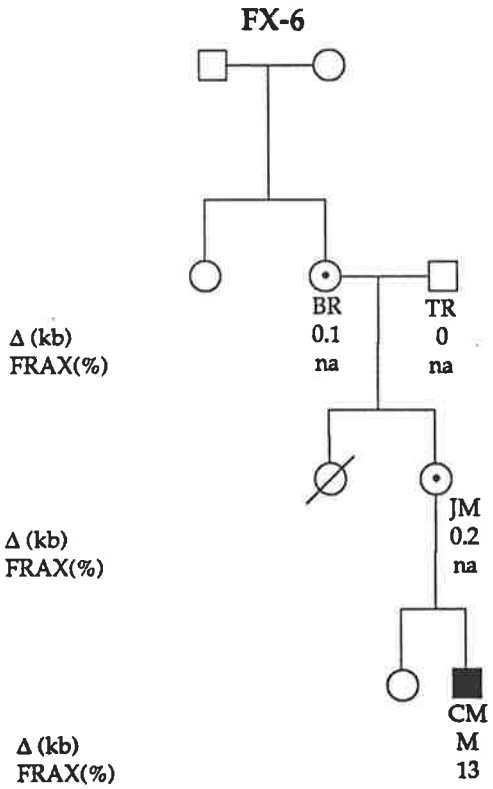
FX-4



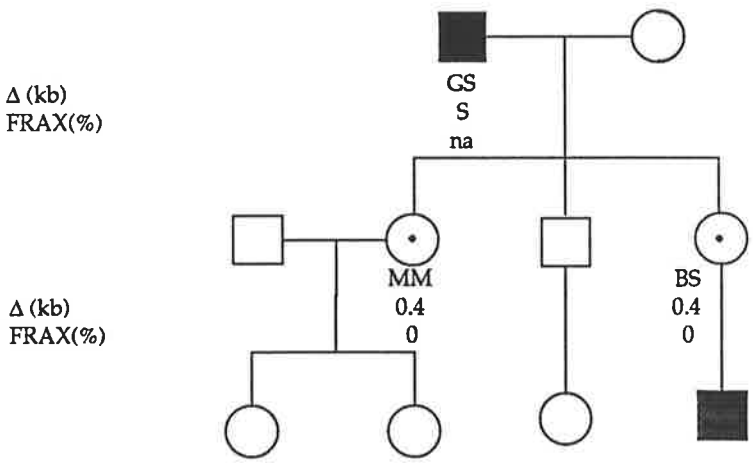
FX-5


Δ (kb)
FRAX(%)
0
na
MM
0.1
na
Δ (kb)
FRAX(%)
AM
0
na
MM
M
10

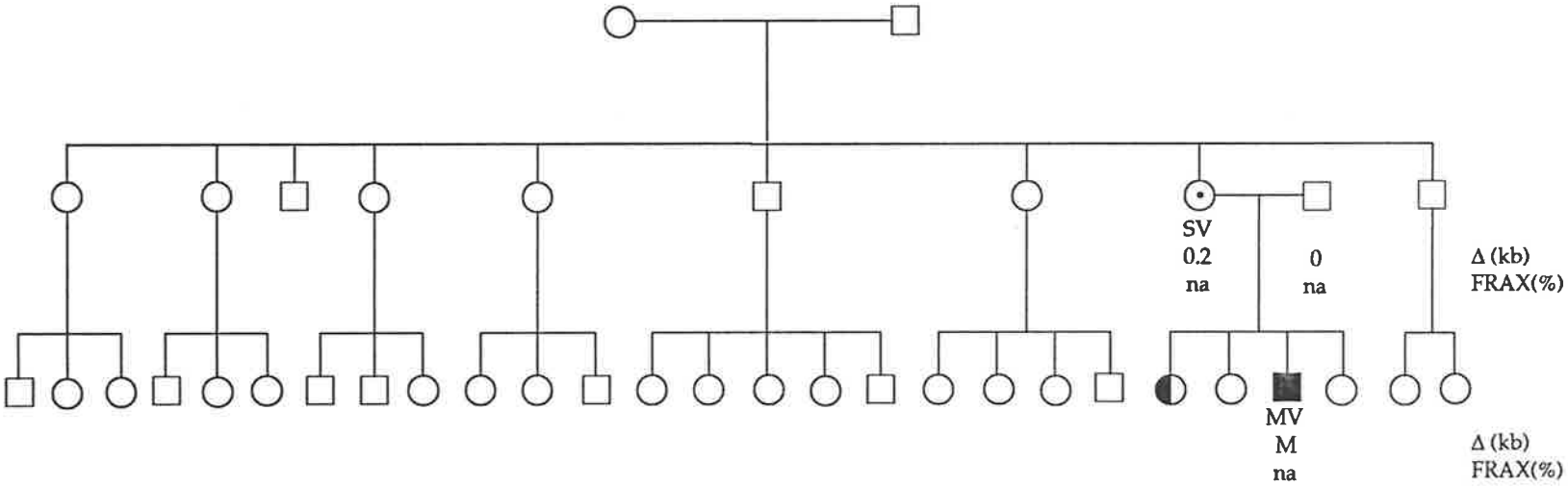
FX-6


BR
0.1
na
TR
0
na
Δ (kb)
FRAX(%)
JM
0.2
na
Δ (kb)
FRAX(%)
CM
M
13
Δ (kb)
FRAX(%)

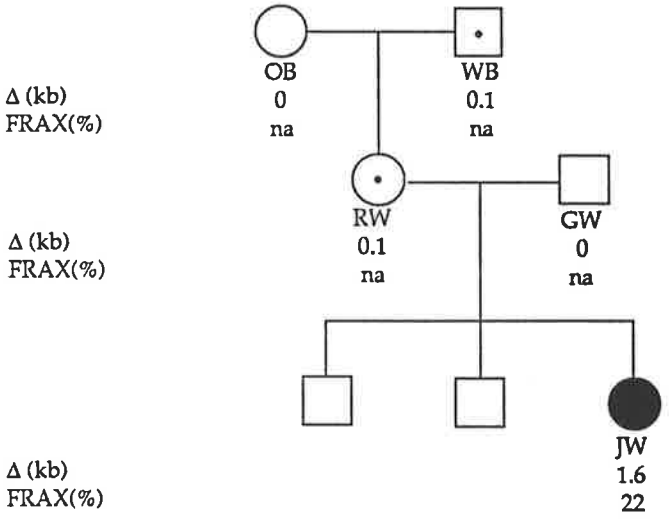
FX-7



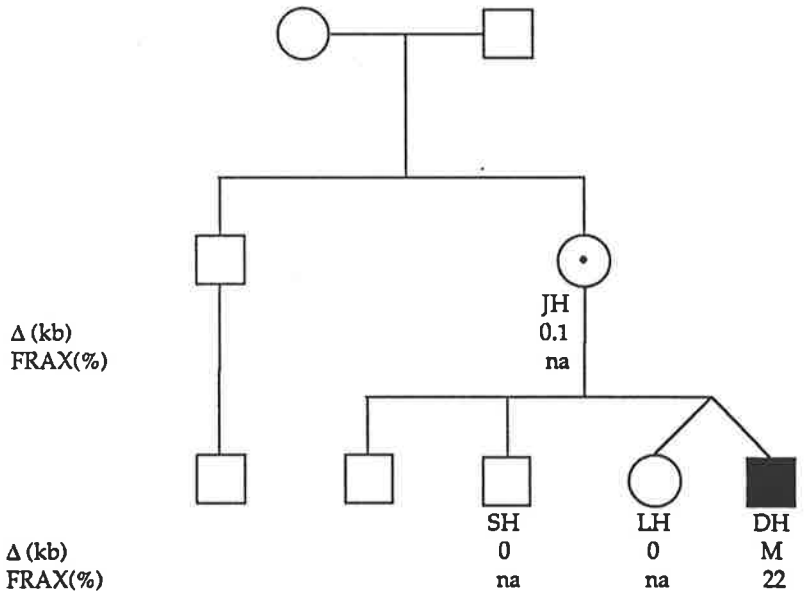
FX-8



FX-9


Δ (kb)
FRAX(%)
OB
0
na
WB
0.1
na
Δ (kb)
FRAX(%)
RW
0.1
na
GW
0
na
Δ (kb)
FRAX(%)
JW
1.6
22

FX-10


Δ (kb)
FRAX(%)
JH
0.1
na
Δ (kb)
FRAX(%)
SH
0
na
LH
0
na
DH
M
22

APPENDIX II

PUBLICATIONS

Most of the material presented in this thesis has been published or is 'in press'. The roles of the candidate in each publication are specified; followed by copies of the papers and manuscripts. Reference to the appropriate chapter in the thesis is indicated.

1. S Yu, GK Suthers, JC Mulley (1989) A BclI RFLP for DXS 296 (VK21) near the fragile X. Nucl Acid Res 18: 690.

The candidate documented the RFLP and wrote the paper under Dr. Mulley's supervision (Chapter 3, Addendum). This publication was included in the PhD thesis of Dr. G. K. Suthers, University of Adelaide, 1990.

2. S Yu, M Pritchard, E Kremer, M Lynch, J Nancarrow, E Baker, K Holman, JC Mulley, ST Warren, D Schlessinger, GR Sutherland, and RI Richards (1991) Fragile X genotype characterized by an unstable region of DNA. Science 252: 1179 - 1181.

The candidate generated the restriction map of XTY-26, established part of the lambda contig, located the fragile X mutation to the 1 kb PstI fragment, isolated the DNA probe pfxa3, identified the instability in fragile X pedigrees with pfxa3, and documented the differences in instability between male and female transmissions (Chapter 5).

3. EJ Kremer, S Yu, M Pritchard, R Nagaraja, D Heitz, M Lynch, E Baker, VJ Hyland, RD Little, M Wada, D Toniolo, A Vincent, F Rousseau, D Schlessinger, GR Sutherland and RI Richards (1991) Isolation of a human DNA sequence which spans the fragile X. Am J Hum Genet 49: 656 - 661.

The candidate generated the circular restriction map of the YAC XTY-26 and confirmed the integrity of the 120 kb SfiI fragment in XTY-26 (Chapter 4).

4. GR Sutherland, EA Hann, E Kremer, M Lynch, M Pritchard, S Yu and RI Richards (1991) Hereditary unstable DNA: a new explanation for some old genetic questions? Lancet 338: 289 - 292.

The candidate and coworkers characterized the molecular basis of the fragile X syndrome and thus for this hypothesis.

5. GR Sutherland, A Gedeon, L Kornman, A Donnelly, RW Byard, JC Mulley, E Kremer, M Lynch, M Pritchard, S Yu and RI Richards (1991) Prenatal diagnosis of fragile X syndrome by direct detection of the unstable DNA sequence. N Engl J Med 325: 1720 - 1722.

The candidate isolated probes pfxa3 and pS8, extracted DNA from various tissues of the fragile X fetus, documented the amplification of the unstable DNA sequences in various tissues of the fetus and the absence of methylation in DNA from chorionic villi.

6. RI Richards, K Holman, H Kozman, E Kremer, M Lynch, M Pritchard, S Yu, J Mulley, GR Sutherland (1991). Fragile X syndrome: genetic localization by linkage mapping of two microsatellite repeats FRAXAC1 and FRAXAC2 which immediately flank the fragile site. J Med Genet 28: 818 - 823.

Dr Pritchard and the candidate constructed the lambda contig, from which the two microsatellite repeats were isolated.

7. EJ Kremer, M Pritchard, M Lynch, S Yu, K Holman, E Baker, ST Warren, D Schlessinger, GR Sutherland, RI Richards (1991) Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n. Science 252: 1711-1714.

The candidate documented the normal CCG repeat range in the population and localized the breakpoints of the two somatic fragile hybrids to the CCG repeat by Southern analysis and identified the instability of the CCG repeat during cloning.

8. S Yu, Mulley J, Loesch D, Turner G, Donnelly A, Gedeon A, Hillen D, Kremer E, Lynch M, Pritchard M, Sutherland GR and Richards RI (1992) Fragile X syndrome: unique genetics of the heritable unstable element. Am J Hum Genet (in press).

The fragile X families studies were carried out by the candidate with assistance from A Donnelly and A Gedeon. The candidate analysed the family data and revealed the correlation between the length of the unstable element and mental status, the fragile X expression and methylation status of the CpG island. The candidate proposed the explanation of the Sherman paradox, identified that all fragile X syndrome cases are familial. The candidate wrote part of the materials and methods, results and discussion sections of the paper (incorporated in Chapter 6).

9. JC Mulley, S Yu, AK Gedeon, A Donnelly, G Turner, D Loesch, CJ Chapman, RJM Gardner, RI Richards and GR Sutherland (1992) Experience with direct molecular diagnosis of fragile X. J Med Genet (in press).

The candidate isolated probes pfxa3 and pS8, established the utility of the pfxa3 probe for direct molecular diagnosis of fragile X syndrome by examineing large number of fragile X families and discovered that the new fragile site FRAXE is not associated with mental retardation.

An anonymous DNA probe E20 [D4S143] on chromosome 4 detects a TaqI polymorphism

L.Warnich, E.Dietzsch, J.S.Herbert, J.Brusnický, L. du Plessis and A.E.Retief
MRC Cytogenetics Research Unit, Faculty of Medicine, P.O. Box 63, Tygerberg 7505, South Africa

Source and Description: A 13kb fragment cloned into the EcoRI site of phage lambda L47.1, isolated from a human genomic library.

Polymorphism: TaqI identifies a two allele polymorphism (A1: 4.7kb; A2: 7.2 kb) with constant fragments at 1.75 and 2.15kb.

Frequency: Studied in 79 parents of CEPH families.
A1: 0.5570
A2: 0.4430

Not Polymorphic for: EcoRI, HindIII, BamHI, and PstI with DNA of 5 unrelated individuals.

Chromosomal Localization: The probe was localised to 4q21−24 by means of *in situ* hybridization.

Mendelian Inheritance: Co-dominant segregation has been observed in 21 informative CEPH families.

Probe Availability: Available for collaboration.

Other Comments: The probe was pre-associated with an excess of sonicated total human DNA prior to hybridization.

Acknowledgments: This work was supported by the Medical Research Council, Cape Provincial Administration and the University of Stellenbosch.

A *BclI* RFLP for *DXS296* (VK21) near the fragile X

S.Yu, G.K.Suthers and J.C.Mulley
Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, 5006 South Australia

Source/Description: Four single copy plasmid sub-clones of VK21:VK21A (1.3 kb), VK21B (2.2 kb), VK21C (2.5 kb), and VK21D (3.2 kb) were used as probes

Polymorphism: *BclI* (New England Biolabs) detected a polymorphism with VK21A, in addition to previously reported polymorphisms identified by VK21A with *TaqI* and VK21C with *MspI* (Suthers, *et al.*, 1989).

TaqI	10.9 kb A1	*BclI*	10.7 kb C1
	9.9 kb A2		5.0 kb C2
MspI	12.7 kb B1	(present study)	
	9.9 kb B2		

(Suthers *et al.*, 1989)

Frequency: From 27 unrelated individuals (total 45X chromosomes): C1=0.87, C2=0.13. All haplotypes were either A1B1C1 or A2B2C2.

Not Polymorphic for: *BanI*, *BglI*, *BstN*, *BstXI*, *EcoRV*, *XbaI*, *XmnI* in addition to previously reported restriction enzymes (Suthers *et al.*, 1989).

Chromosomal Localization: Distal but closely linked to FRAXA (Suthers *et al.*, 1989).

Mendelian Inheritance: Codominant inheritance was demonstrated in Utah Families 981 and 983. In both families the male parent was hemizygous for the C1 allele and the female parent heterozygous C1C2. From a total of 15 offspring there were 7 males hemizygous for the C2 allele, 4 males hemizygous for the C1 allele, 2 females heterozygous C1C2 and 2 females homozygous C1C1.

Probe Availability: Available on a collaborative basis from Dr.G.R.Sutherland, Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

Other Comments: The VK21 probes are useful for diagnosis in families with the fragile X. This extended search for RFLPs was undertaken in an attempt to increase their informativeness. Although the new *BclI* marker appears to be in linkage disequilibrium with the existing *TaqI* and *MspI* markers, the availability of a *BclI* RFLP adds flexibility to diagnostic strategies based on reprobing of filters since *BclI* also defines other markers near FRAXA: *DXS52* (F814 and St-14), *DXS105* (55.E), and *DXS304* (U6.2).

Reference: G.K.Suthers, D.F.Callen, V.J.Hyland, H.M.Kozman, E.Baker, H.Eyre, P.S Harper, S.H.Roberts, M.C.Hors-Cayla, K.E.Davies, M.V.Bell and G.R.Sutherland. A new DNA marker tightly linked to the fragile X locus (*FRAXA*). (1989) *Science* **246**, 1298−1300.

Yu, S., Pritchard, S., Kremer, E., Lynch, M., Nancarrow, J., Baker, E., et al., (1991)
Fragile X genotype characterized by an unstable region of DNA.
Science, v. 252 (5009), pp. 1179-1181.

NOTE:
This publication is included in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1126/science.252.5009.1179

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