Chromosoma Focus

Review:

The fragile X syndrome: Isolation of the FMR-1 gene and characterization of the fragile X mutation

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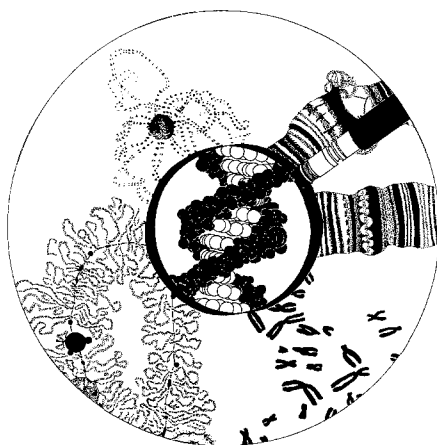*Fragile X syndrome, associated with the fragile X chromosome, is the most common cause of familial mental retardation. A breakthrough has been made in molecular biological research into the fragile X site. In this review we describe the molecular investigations that have led to the isolation of the FMR-1 gene. The nature of the fragile X mutation as well as the implications of the DNA test for the mutation are discussed.*

Introduction

Inherited forms of mental retardation that result from defects on the X chromosome affect 1/500 males and leave about 1/200 females at risk of being carriers (Opitz and Sutherland 1984). To determine whether the same form of X-linked mental retardation is present in two or more unrelated families, there must be a good characterization of the phenotype, which is found in all males. Genetic studies of families with the same phenotype will determine the chromosomal localization of the disease, followed by the search for the gene and the mutation. In this paper we review the research on fragile X syndrome, the search for the gene and the mutation causing the phenotype.

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Description of fragile X syndrome

In 1943, Martin and Bell (1943) described the first pedigree clearly demonstrating an X-linked form of mental retardation. In metaphases of cells from Martin-Bell syndrome patients a fragile site was noted at the tip of the long arm of the X chromosome (Lubs 1969). The fragile sites appear as an unstained gap or break at a defined point on the X chromosome and are inherited in a Mendelian fashion. More than 64 other fragile sites have been described, but none of these other sites has been linked to a genetic disease (Sutherland and Ledbetter 1989).

The phenotype of the fragile X syndrome is mental retardation, usually with an IQ in the 40–70 range (Sutherland and Hecht 1985; Fish et al. 1991), and a number of dysmorphic features: long face, large everted ears and large testicles. Not every patient shows all the physical symptoms, which are generally more apparent after childhood. The fragile X syndrome is the most frequently encountered form of inherited mental retardation in humans, with a prevalence estimated to be 1 in 1,250 males (Gustavson et al. 1986; Webb et al. 1986). Distinction between the fragile X syndrome and other forms of X-linked mental retardation is based on the identification of the fragile site at Xq27.3 in cultured lymphocytes, fibroblasts and amniocytes (Opitz and Sutherland 1984; Sutherland 1977). The exact mechanism underlying fragile site expression is unknown, but it appears to involve
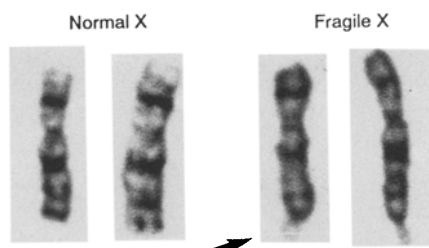
Normal X   Fragile X

Fig. 1. Chromosome preparation of normal and fragile X chromosome

the availability of DNA synthetic precursors. Adequate concentrations of thymidine and/or cytidine are necessary. Fragile X expression is obtained by culturing cells in medium deficient in folic acid and thymidine or with excess thymidine (Sutherland and Baker 1986; Jacky et al. 1991), with 2% to 60% of the cells examined expressing the fragile site (Fig. 1). The frequency of fragile X positive cells seems to be somewhat characteristic of an individual. Although affected males virtually always express the fragile site, only about 50% of obligate carrier females can be detected using this test (Nielsen et al. 1983). The cytogenetic test is not reliable in detecting clinically normal carriers, a fact that has limited its value in genetic counselling.

Genetics

The fragile X syndrome shows unusual genetic characteristics. About one-third of carrier females are mentally retarded but they are often less severely affected than males. One most unusual feature for an X-linked disorder is the existence of normal carrier males. In the original family described by Martin and Bell (1943) two unaffected brothers had passed on the gene for the fragile X syndrome through their healthy daughters to the following generations. Sherman et al. (1985) have shown that approximately 20% of males who are known on the basis of genealogic data to carry the fragile X chromosome are phenotypically normal but they can pass the trait on to their daughters, who are also asymptomatic. But members of the next generation are often mentally impaired. Sherman determined that the risk of having the phenotypic effect for individuals in fragile X families is dependent upon the position of the individual in the pedigree (the so-called Sherman paradox). The grandsons of normal transmitting males have a risk of 40% of intellectual handicap and great-grandsons are at 50% risk; brothers of normal transmitting males are at 9% risk. If the carrier mother has signs of the fragile X syndrome, the risk that the sons will be mentally handicapped becomes 50%.

To explain these unique features several models have been proposed. One model was put forward by Pembrey et al. (1985). A normal transmitting male would harbour a "premutation" on his X chromosome with no phenotypic effects by itself. In a daughter this premutation could undergo meiotic recombination with an "enhancing sequence" on the homologous region of the other X chromosome during oogenesis. Then a full mutation with phenotypic effects would be generated. The nature of this enhancing sequence on the normal chromosome is unidentified. The model predicts that recombination should be found systematically, which is clearly not the case; double recombinants have to be postulated.

A second more attractive model put forward by Laird (1987) proposed a mechanism involving inheritance of fragile X based upon DNA methylation. A normal transmitting male carries an X chromosome with a (pre)-mutation without phenotypic effects. If this chromosome is inactivated in the next generation as part of the process of dosage compensation in females having two X chromosomes, the reactivation of this chromosome is blocked in the fragile X region. DNA methylation is presumed to be involved in X inactivation. The fragile X region has become imprinted: one or several genes in the vicinity of Xq27 are turned off. However, this does not appear to explain the altered segregation ratios of affected to unaffected offspring described by Sherman et al. (1985).

Markers and linkage analysis

The first DNA marker shown to be linked to the fragile locus was Factor 9 (Camerino et al. 1983). Although no recombination was seen in the large family described, several families were reported in which recombination was seen between F9 and the fragile X locus. Brown et al. (1985, 1988) noted differences between families with tight linkage of fragile X to F9 and families with a loose linkage; he suggested genetic heterogeneity as a possible explanation for this discrepancy. This could not be confirmed by others (Suthers et al. 1991a) and the apparent heterogeneity may reflect uncertainties due to limited statistics and mistyping of individuals in families.

Several DNA markers were soon reported for the detection of loci close to the fragile X locus (Fig. 2A). Dahl et al. (1989) and Suthers et al. (1989) described clones U6.2 and VK21, which are located distal to the fragile site. On the proximal side clones RN1 (Oostra et al. 1990) and VK23 (Suthers et al. 1991b) were obtained, which were placed by genetic mapping within 5 cM of the fragile X syndrome locus. These flanking markers, spanning a region of 6–7 cM, have been very useful for carrier testing and prenatal diagnosis in a number of families (Suthers et al. 1991b). More recently, a series of markers were isolated that were shown to map in this interval (Rousseau et al. 1991a; Hirst et al. 1991a; Hulsebos et al. 1991; Riggins et al. 1992) (Fig. 2A). The genetic mapping of these probes limited the region of the fragile site to 1 or 2 Mb. The markers closest to the fragile site were used as starting points for the isolation and characterization of the fragile X mutation.
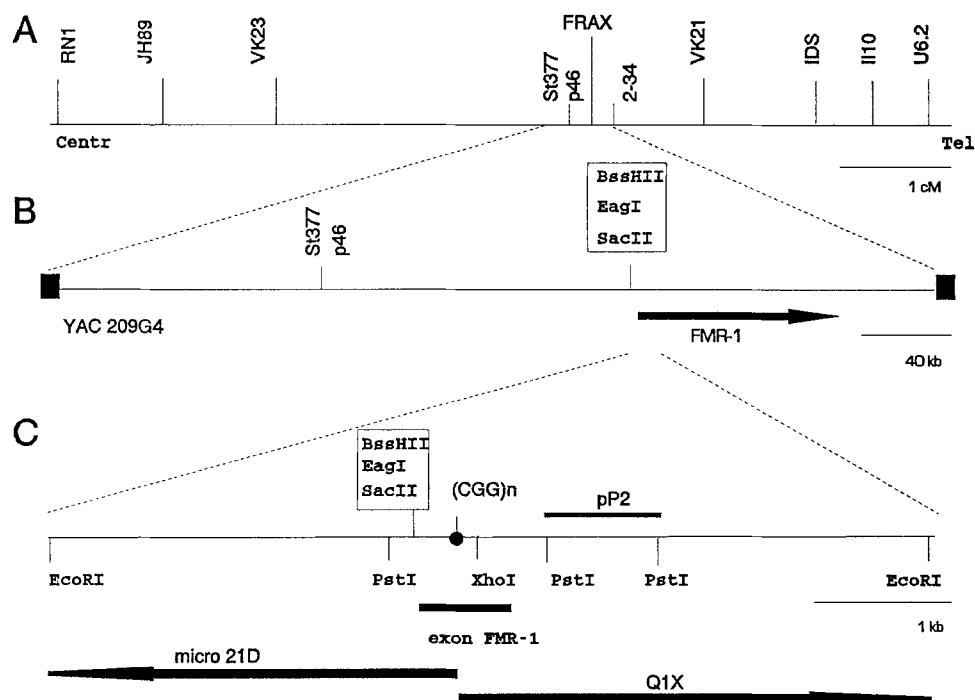
Fig. 2A–C. Map of the fragile X region at Xq27.3. **A** Genetic map of DNA markers flanking the fragile site (*FRAX*). **B** Partial physical map of YAC 209G4 with the location of the FMR-1 gene CpG island shown *boxed*. **C** Fragment containing CpG island, CGG repeat and exon of FMR-1. Schematic representation of hybrid cell lines micro 21D and Q1X (Warren et al. 1987)

Molecular approaches to the isolation of the fragile X locus

An approach to the isolation of the fragile X locus was the strategy designed by Warren et al. (1987). A somatic cell hybrid was isolated that contained a human X chromosome from a fragile X patient in a hamster background. By culturing this cell line under conditions for induction of fragile X expression and selection for expression of genes on either side of the fragile site, several somatic cell hybrids were isolated that contained translocations between human and hamster material. The intention of these experiments was that the fragile site should be a preferential location for the occurrence of such translocation events. DNA marker analysis showed that the breakpoints coincide with a region close to or within the fragile site (Rousseau et al. 1991a; Hirst et al. 1991a). Efforts to clone these breakpoints directly into cosmids have not been successful; this is most likely caused by the sequence composition of the fragile X mutation. These hybrid cell lines have proved to be beneficial in the isolation of the fragile X locus.

A different approach to the isolation of the fragile X region has been chromosome microdissection. The fragile X region was dissected from human metaphase chromosomes followed by cloning of the dissected DNA after amplification by polymerase chain reaction (PCR) methods (Mackinnon et al. 1990). A slightly different version of the technique was performed using laser microdissection of X chromosomes displaying the fragile site (Djabali et al. 1991). A number of clones were isolated, two of which were shown to map in the 1 Mb interval around the fragile site (Mackinnon et al. 1990).

Cloning of human DNA in yeast artificial chromosomes (YACs) made it possible to isolate DNA frag-

ments up to 1 Mb. YAC libraries became available from normal X chromosomes as well as from a fragile X chromosome. By using markers tightly linked to the fragile site YACs were isolated with insert sizes between 275 and 520 kb (Heitz et al. 1991; Verkerk et al. 1991; Kremer et al. 1991a; Hirst et al. 1991b). The YACs contained breakpoints from the cell hybrids isolated by Warren (1987), with breakpoints in the fragile region. Fluorescence in situ hybridization with these YAC clones was performed on metaphase chromosomes displaying the fragile site. Fluorescent signals were scored on the proximal and on the distal side of the (fragile) gap (Heitz et al. 1991; Verkerk et al. 1991; Kremer et al. 1991a; Hirst et al. 1991b; Verkerk et al. 1992) indicating that the YAC clones indeed span the fragile site (Fig. 3). Hybridization of a subclone of YAC 209G4 to a fragile X chromosome is shown on the cover. The fluorescent signal falls on the fragile site. The size of the fragile site has been suggested to be limited to less than 20 kb (Kremer et al. 1991b; Verkerk et al. 1992). The presence of well-separated signals on both sides of the fragile site indicates that the DNA is locally highly unfolded. Another explanation could be that a break has occurred in one of the chromatids.

The FMR-1 gene and the nature of the fragile X mutation

Physical studies of the distal region of the long arm of the X chromosome using pulsed field gel electrophoresis (PFGE) disclosed that the fragile site is located approximately 9 Mb from the telomere (Poustka et al. 1991). Detailed PFGE mapping of the fragile X region showed that rare-cutter restriction sites (rich in CpG dinucleotide sequences) were resistant to digestion by
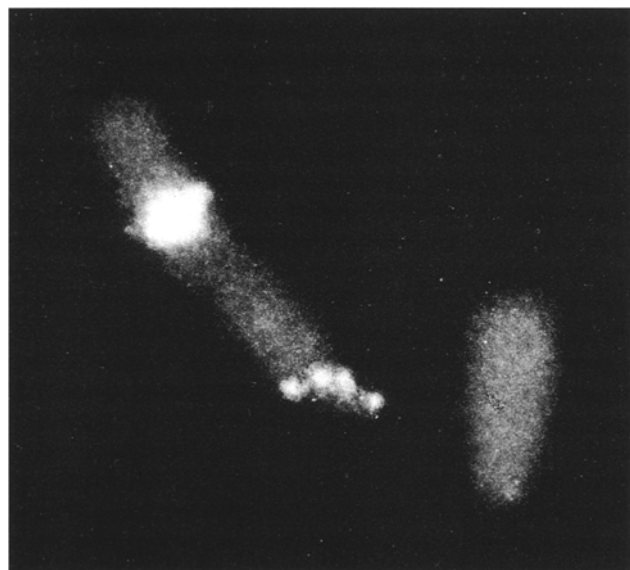
Fig. 3. Fluorescence in situ hybridization of YAC 209G4 (see Fig. 2) to a chromosome preparation from a fragile X patient. A centromere probe specific for the X chromosome confirmed the identity of the chromosome examined

methylation sensitive restriction enzymes in fragile X males, but not in normal males (Vincent et al. 1991; Bell et al. 1991) (Fig. 2). Cytosine methylation of a CpG island containing a cluster of rare-cutter sites is indicative of the promoter region of a gene whose expression is subsequently shut off. Normal DNA methylation was observed on X chromosomes of normal transmitting males but abnormal methylation of the X chromosome was observed in their affected grandsons. This is in agreement with the proposed change in methylation after passage through a carrier female who shows a normal methylation pattern (Laird 1987). A CpG island is localized on the YACs spanning the fragile site.

Subclones of the sequences around the CpG island were used as probes and confirmed the abnormal methylation patterns in affected males (Oberlé et al. 1991; Yu et al. 1991). Clones mapping adjacent to the CpG island detected a 550 bp DNA segment that was unstable in fragile X chromosomes. A small insertion (premutation) of 50 to 500 bp was detected in normal transmitting males, which was inherited by their daughters. In the next generation fragile X positive individuals had much larger inserts (full mutation). The mutated allele appeared unmethylated in normal transmitting males and totally methylated in most fragile X positive males. The size increase was generally heterogeneous among siblings and even within a given individual, indicating somatic mutation.

A different approach was taken by Verkerk et al. (1991). A cosmid clone from YAC 209G4 (Fig. 2), identified overlapping clones from a fetal brain complementary DNA (cDNA) library. The cDNA clone detects an mRNA of 4.4 kb, normally expressed in brain and other tissues; the corresponding gene was designated FMR-1 (fragile X mental retardation-1). A 5' exon of the gene contains a repetitive sequence (CGG)n that is

located 250 bp from the CpG island showing specific methylation in fragile X patients (Fig. 2). In this CGG repeat are located a number of the breakpoints of the somatic cell hybrids with induced breakpoints in the fragile site (Kremer et al. 1991b; S. Warren, personal communication). The CpG island as well as the CGG repeat are located on a 5.2 kb EcoRI fragment (Fig. 2). In patients a dramatic expansion of this band is seen; often marked heterogeneity of the bands results in a diffuse smear (Fig. 4). In normal transmitting males and in daughters of transmitting males (lanes 1, 6 and 10) a small expansion of between 50 and 500 bp is observed. When this premutation passes through female meiosis it can either expand to a full mutation (lanes 2–4, 8 and 9) or not expand, resulting in a normal transmitting male (lane 11).

In lymphoblasts, leucocytes and fibroblasts of most fragile X males the FMR-1 gene is transcriptionally silent, suggesting direct involvement of this gene in the manifestation of the fragile X syndrome (Pieretti et al. 1991). The loss of expression of the FMR-1 gene correlates with methylation of the CpG island. It appears likely that the methylation of the region down-regulates FMR-1 expression resulting in the disease. In a few patients expression of FMR-1 can be demonstrated. These patients show a mosaic DNA pattern with a near normal-size band together with a smear of bands of increased size. Partial methylation of the CpG island is observed. Several explanations could account for the discrepancy of expression in these patients. Firstly, is: the level of expression does not reach the necessary threshold to provide normal levels of protein. Secondly, mRNA expression does not necessarily assure a normal FMR-1 protein. Thirdly, since lymphoblasts and leucocytes were studied mRNA expression could be diminished in the appropriate tissue or at a stage crucial to the development of the fragile X phenotype.

No protein product has been identified as yet. An amino acid sequence has been deduced from the determined nucleotide sequence. The sequence is very basic and contains a nuclear translocation signal suggesting a DNA-binding nuclear type protein; preliminary experiments with synthetic antibodies have shown a localization in the nucleus. It is still uncertain whether or not the CGG repeat is translated into the predicted polyarginine domain (Verkerk et al. 1991).

Variation in the number of CGG repeats was found on normal X chromosomes with a range of from 6 to 46 with a mean repeat number of 29; these normal alleles are stable during meiosis (Fu et al. 1991). A different number was reported by Kremer et al. (1991a) but this was the result of a cloning artefact (Fu et al. 1991). No differences between normal and fragile X chromosomes were found in the CGG flanking region. The variation in the length of DNA at the fragile site therefore appears to be restricted to changes within the CGG repeat. Affected males and females show large (500–5,000 bp) inserts (Oberlé et al. 1991; Yu et al. 1991) in the CGG repeat. The nature of the expansion cannot be determined directly due to difficulties in cloning the expanded repeat region; the expansion is very CGG-rich suggest-

ing that the insert is built up of CGG repeats. Normal transmitting males and their daughters show a premutation with a small expansion. Premutation alleles range in size from 52 to 193 CGGs (Fu et al. 1991). The premutation alleles, in contrast to normal alleles, change in size with each meiotic transmission. It will be necessary to analyse a considerable number of alleles to establish whether the boundary between stable alleles (the largest allele 46) and unstable alleles (the smallest premutation allele 52) is really the cut-off point between normal and premutation.

The risk of expansion of the premutation allele is dependent on the size of the allele (Fu et al. 1991). The risk for a female of having a mentally retarded son depends on the size of her CGG repeat. If the repeat number is low (50–70 CGGs) the risk of a large expansion is low; if the repeat number is high (above 90 CGGs) the risk of expansion to a full mutation is 100%. This can explain the Sherman paradox: the risk that grandsons of normal transmitting males with be mentally retarded is 40% and not 50%. The mother of a normal transmitting male (with a low repeat number) has only a 9% risk of having a mentally retarded son.

Diagnostics

Scoring for the expansion has already proved to be a simple diagnostic test for the fragile X syndrome (Oberlé et al. 1991; Yu et al. 1991; Rousseau et al. 1991b; Fu et al. 1991). Patients with a full mutation can be diagnosed by Southern blot analysis (see Fig. 4). The insert size is always above 500 bp (Oberlé et al. 1991; Fu et al. 1991). In males a band of near normal size is sometimes

seen in addition to the diffuse band; the subject is then mentally retarded. An accurate prediction of mental retardation in female carriers is not possible.

Premutation alleles can be studied by analysing the number of CGG repeats. The insert size ranges from 50–500 bp. DNA analysis has not detected an expansion in a number of cases diagnosed previously as fragile X positive; this is most likely the result of misdiagnosis or the existence of a different fragile site (Rousseau et al. 1991b; Pieretti et al. 1991).

The DNA test for the unstable region is very useful in testing carriers and for prenatal diagnosis (Hirst et al. 1991c; Sutherland et al. 1991). If the prenatal test is carried out on chorionic villi special care has to be taken. In DNA of villi in contrast to fetal DNA no methylation of the CpG island in front of the CGG repeat was observed. Thus methylation cannot be used as a diagnostic test in villi. In patients a heterogeneous DNA pattern is often observed indicating a considerable somatic mutation rate. If the prenatal test is carried out on villi in a male pregnancy of a female carrier and a premutation is detected this is not sufficient proof that the status of the mutation in the DNA of the villi is indicative of the mutation status in the fetus. More data from (retrospective) tests are needed to solve this problem.

The DNA diagnostic test can be used to screen systematically population of men and women with (unexplained) mental retardation. Before population screening for carriers can be set up the cut-off points that distinguish between the repeat size of the normal allele, the premutation and the full mutation have to be determined in more detail. The ethical implications of such a diagnosis should also be examined.
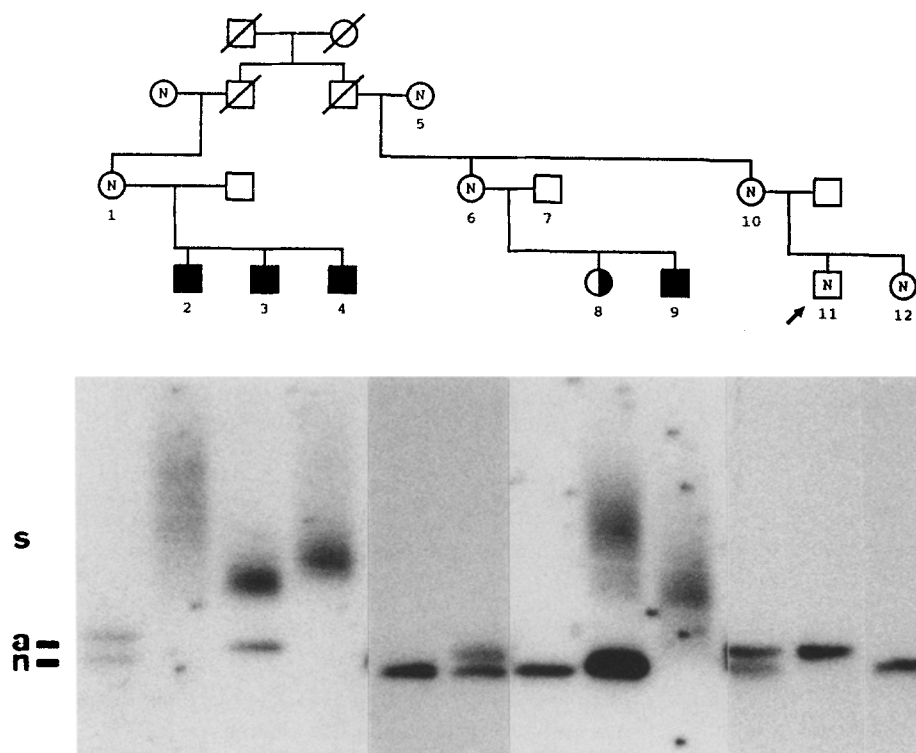

Fig. 4. Southern blot analysis of EcoRI digested DNA of a fragile X family. *Squares* and *circles* represent male and female subjects, respectively. *Open symbols* indicate normal subjects; *N* no cytogenetic fragile X expression. *Filled symbols* represent subjects that are mentally retarded and show fragile X expression. *Half-filled symbols* indicate a subject who is normal but shows fragile X expression. Hybridization was performed with probe pP2 (Fig. 2). *n* normal 5.2 kb band; *a* small insert of between 50 and 500 bp; *s* smear of bands

Mechanism of mutation

Since fully penetrant males rarely reproduce, a very high mutation rate (1/3,000) has been suggested to maintain the frequency of the disease (Brown 1990). The number of CGG repeats is highly polymorphic suggesting a high mutation rate. Direct testing of the DNA mutation has not revealed any new mutations. This is probably caused by a bias in selecting families with an affected member. This suggests that the transition of a (pre)mutation to a full mutation most likely takes several generations. The transmission of a premutation over five generations has been noted (A. Smits, personal communication). A high mutation rate is seen in the premutation allele (Fu et al. 1991). Other high mutation rates have been reported in small repeats (Jeffreys et al. 1988). An explanation could be a pause or stop occurring during DNA replication at the CGG repeat, with subsequent slippage of the polymerase resulting in a change in the number of CGGs. Another mechanism is termination of replication followed by reinitiation at an earlier CGG repeat. Oberlé et al. (1991) have suggested that the mutation could be due to unequal sister chromatid exchange or to unequal meiotic crossing over.

Normal transmitting males with an expanded CGG repeat never show methylation of the CpG island. Thus, the region is unstable before methylation takes place. It has been suggested that X inactivation (Laird 1987) is involved since the expansion of premutation to full mutation is never observed in daughters of transmitting males. If this chromosome is inactivated in the daughter as part of the process of dosage compensation, the reactivation of this chromosome is blocked in the fragile X region. Sutherland et al. (1991) have observed an expanded CGG repeat of the same full mutation size in both chorionic villi and fetal cells, while the methylation was limited to the fetal DNA. This suggests that first expansion has taken place and that the FMR-1 methylation in the fragile X chromosome is acquired during fetal developmental in response to the expansion of the CGG repeat.

Conclusion

Rapid progress has been made in the analysis of the fragile X syndrome during 1991. Different groups have discovered that fragile X chromosomes are preferentially methylated. In these X chromosomes an insertion has been found in the methylated region.

The FMR-1 gene, the transcription of which is shut off in patients, has been isolated. The expansion found in fragile X chromosomes is localized in the coding region of the FMR-1 gene. The fragile X syndrome results from mutations in a (CGG)n repeat found in the coding region of the FMR-1 gene. It will be crucial to determine the FMR-1 protein product in order to learn more about the function of the gene.

Diagnosis of the unstable region by DNA analysis is now available as an efficient and reliable test for the diagnosis of carriers, as well as for prenatal diagnosis.

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