CHROMOSOMAL FRAGILITY AND HUMAN GENETIC DISORDERS

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ABSTRACT

The first report of X-linked mental retardation correlated with the presence of marker chromosome came in 1940. It was in 1990 that the molecular basis of fragile X syndrome was deciphered. This elucidation marked the discovery of a novel process of mutation designated as dynamic mutations, resulting in the expansion of a triplet repeat sequence within the human genome. Subsequently several human genetic disorders involving triplet repeat expansion have been discovered. Almost all the disorders are known to affect the nervous system and/or the brain. This review presents an overview of fragile sites in the genome and the molecular genetics of fragile X syndrome.

A fragile site on a chromosome is seen under the microscope as a nonstaining gap or constriction. The fragile sites are usually present in only a small proportion, of cells in metaphase. But when expressed, fragile sites are present at exactly the same chromosomal position in all cells from an individual or kindred (1). Fragile sites are classified on the basis of their frequency in the population and conditions under which they are expressed in cells in culture. Rare fragile sites are seen at a specific location in 1 in 40 metaphases but Common fragile sites are seen on almost all the chromosomes in the general population. Most fragile sites are not expressed spontaneously but can be induced. When they are expressed spontaneously, the proportion of metaphases in which they are seen can usually be greatly increased by appropriate conditions of induction, such as the addition of methotrexate and FudR. The classification based on the induction conditions are shown in Table 1. Most of the erstwhile known fragile sites are listed in Table 2.

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Table 1. Classification of fragile site. Taken from (2).

Group	Description
	<i>Rare</i>
Group 1	Folate-sensitive fragile sites
Group 2	Distamycin A inducible (a) Also inducible by BrdU (b) Not inducible by BrdU, and recorded only in Japanese subjects
Group 3	BrdU-requiring
	<i>Common</i>
Group 4	Aphidicolin-inducible
Group 5	5-Åzacytidine-inducible
Group 6	BrdU-inducible

The clinically significant fragile sites that have been characterised are FRAXA, FRA11B and FRAXE. FRA11B is located in the 5' untranslated region of a gene designated, CBL2 oncogene. Fragility is seen in some carrier mothers whose progeny are affected with mental retardation and malformation condition as in Jacobsen's syndrome. In some cases the fragile sites result in deletion of the chromosome fragment distal to the locus; for example, Jacobsen syndrome is a clinical condition that occurs due to the deletion of a part of

Table 2. A list of fragile sites observed on human chromosomes. Table is modified from (3). Clinically significant fragile sites presently characterised are shown in bold font. R-rare, C-common, aph-aphidicolin, dist-distamycin, BrdU-bromodeoxyuridine.

Gene Symbol	Chr. location	Type	Gene Symbol	Chr. Location	Type
FRA1A	1p36	C,Aph	FRA8A	8q22.3	R,folate
FRA1B	1p32	C,Aph	FRA8C	8q24.1	C,aph
FRA1C	1p31.2	C,Aph	FRA8E	8q24.1	R, dista
FRA1L	1p31	C,aph	FRA8D	8q24.3	C,aph
FRA1D	1p22	C,aph	FRA9A	9p21	R, folate
FRA1M	1P21.3	R,folate	FRA9C	9p21	C,BrdU
FRA1E	1p21.2	C,aph	FRA9F	9q12	C,5azaC
FRA1J	1q12	C,5azaC	FRA9D	9q22.1	C,aph
FRA1F	1q21	C,aph	FRA9B	9q32	R, folate
FRA1G	1q25.1	C,aph	FRA9E	9q32	C,aph
FRA1K	1q31	C,aph	FRA10G	10q11.2	C,aph
FRA1H	1q42	C,5azaC	FRA10C	10q21	C,BrdU
FRA1I	1q44	C,aph	FRA10D	10q22.1	C,aph
FRA2C	2p24.2	C,aph	FRA10A	10q23.3	R, folate
FRA2D	2p16.2	C,aph	FRA10B	10q25.2	R,BrdU
FRA2E	2p13	C,aph	FRA10E	10q25.2	C,aph
FRA2L	2p11.2	R,folate	FRA10F	10q26.1	C,aph
FRA2A	2q11.2	R,folate	FRA11C	11p15.1	C,aph
FRA2B	2q13	R,folate	FRA11I	11p15.1	R,dista
FRA2F	2q21.3	C,aph	FRA11D	11p14.2	C,aph
FRA2K	2q22.3	R,folate	FRA11E	11p13	C,aph
FRA2G	2q31	C,aph	FRA11H	11q13	C,aph
FRA2H	2q32.1	C,aph	FRA11A	11q13.3	R, folate
FRA2I	2q33	C,aph	FRA11F	11q14.2	C,aph
FRA2J	2q37.3	C,aph	FRA11B	11q23.3	R,folate
FRA3A	3p24.2	C,aph	FRA11G	11q23.3	C,aph
FRA3B	3p14.2	C,aph	FRA12A	12q13.1	R,folate
FRA3D	3q25	C,aph	FRA12B	12q21.3	C,aph
FRA3C	3q27	C,aph	FRA12E	12q24	C,aph
FRA4A	4p16.1	C,aph	FRA12D	12q24.33	R,folate
FRA4D	4p15	C,aph	FRA12C	12q24.2	R,BrdU
FRA4B	4q12	C,BrdU	FRA13A	13q13.2	C,aph
FRA4E	4q27	C,Uncl	FRA13B	13q21	C,brdU
FRA4C	4q31.1	C,aph	FRA13C	13q21.2	C,aph
FRA5E	5p14	C,aph	FRA13D	13q32	C,aph
FRA5A	5p13	C,BrdU	FRA14B	14q23	C,aph
FRA5B	5q15	C,BrdU	FRA14C	14q24.1	C,aph
FRA5D	5q15	C,aph	FRA15A	15q22	C,aph
FRA5G	5q35	R,folate	FRA16B	16q22.1	R,dista
FRA6B	6p25.1	C,aph	FRA16C	16q22.1	C,aph
FRA6A	6p23	R,folate	FRA16D	16q23.2	C,aph
FRA6C	6p22.2	C,aph	FRA17A	17p12	R,dista

Table 2 contd.

Gene Symbol	Chr. location	Type	Gene Symbol	Chr. Location	Type
FRA6D	6q13	C,BrdU	FRA17B	17q23.1	C,aph
FRA6G	6q15	C,aph	FRA18A	18q12.2	C,aph
FRA6F	6q21	C,aph	FRA18B	18q21.3	C,aph
FRA6E	6q26	C,aph	FRA19B	19p13	R,folate
FRA7B	7p22	C,aph	FRA19A	19q13	C,5AzaC
FRA7C	7p14.2	C,aph	FRA20B	20p12.2	C,aph
FRA7D	7p13	C,aph	FRA20A	20p11.23	R,folate
FRA7A	7p11.2	R,folate	FRA22B	22q12.2	C,aph
FRA7J	7q11	C,aph	FRA22A	22q13.1	R,folate
FRA7E	7q21.2	C,aph	FRAXB	Xp22.31	C,aphid
FRA7F	7q22	C,aph	FRAXC	Xq22.1	C,aph
FRA7G	7q31.2	C,aph	FRAXD	Xq27.2	C,aph
FRA7H	7q32.3	C,aph	FRAXA	Xq27.3	R,folate
FRA7I	7q36	Co,aph	FRAXE	Xq28	R,folate
FRA8B	8q22.1	C,aph	FRAXF	Xq28	R,folate

the long arm of the chromosome 11 and the deletion breakpoints in some cases are at or very close to the fragile site. This is the only known autosomal fragile site associated with a syndrome (4). Another fragile site FRA16A is detected without any phenotypic association. FRAXA is associated with fragile-X syndrome, the most common cause of inherited mental retardation next to Down syndrome in frequency of occurrence. The frequency of occurrence is estimated to be 1 in 1000–2500 births (5). FRAXE is located 600bp distal to FRAXA. Both FRAXA and FRAXE are folate sensitive fragile sites at Xq27.3 and Xq28 respectively. Cytologically these two are indistinguishable from each other. The incidence of FRAXE associated MR appears to be 4% of mental retardation (MR) due to FRAXA i.e., 1/50,000 (6). There is still a great deal of uncertainty about a genotype-phenotype relationship in FRAXE-associated MR and this is mainly due to the mild phenotypic effect, low incidence and low detection of new FRAXE cases from large candidate population studies (6).

The presence of another fragile site distal to FRAXE was demonstrated by fluorescence *in situ* hybridisation (FISH) analysis using cosmid probes

close to iduronate sulphatase gene at Xq28, 600 kb distal to FRAXE locus (7). The inheritance of the fragile site at the FRAXF locus does not correlate with the presence of mental impairment. One of the well studied systems is the fragile sites on the X chromosome at Xq27 and 28; mainly because of the high incidence of mental retardation. The following sections of the review will discuss the molecular and medical genetics of fragility of the X chromosome.

THE CHARACTERIZATION OF A FRAGILE X INDUCTION SYSTEM

It was discovered by Sutherland in 1977(1) that a folate deficient culture medium was necessary to induce fragile site at Xq27.3. Folate is a one-carbon donor in nucleotide biosynthesis and its deficiency results in altered thymidine pool within the cell. Excess or deficient thymidine seems to interfere with the DNA helicity. It was seen that the antimetabolite FudR interferes with thymidylate synthase activity by limiting or blocking the formation of TMP from UMP. Increasing the thymidine concentration by including methotrexate and FudR also induces fragile sites. In order to unambiguously visualize the fragile site most

laboratories use multiple induction system, specifically 'a double induction' that includes both FudR and thymidine (8).

FRAGILE X SYNDROME: BACKGROUND

As early as 1938, Penrose noted an increased incidence of mental retardation in males compared to females (9). Subsequently in 1943 a family reported by Martin and Bell (10) became the first account of familial X-linked mental retardation (XLMR). The proband and the affected males in the family reported by Martin-Bell showed 17 to 50% of fragile sites in their cells - the first report of Fragile X syndrome. Independently it was reported by Escalante *et. al.* in 1971 that there exists a class of X-Linked Mental Retardation associated with macroorchidism in males (11). In 1969, Lubs reported a three generation family with affected males and a marker chromosome with a constriction in the distal arm of the X-chromosome (12). The syndrome is seen in all ethnic/racial groups and has an estimated frequency of 1 in 1250 males and 1 in 2000 females. Some of the common features of fragile X patients are the following (13);

- (a) Macroorchidism
- (b) Prominent, large ears
- (c) High arched palate
- (d) Narrow, midfacial diameter
- (e) Narrow intereye distance
- (f) Long facial distance
- (g) Large Head Circumference
- (h) Prominent forehead
- (i) Facial asymmetry
- (j) Hyperextensible joints
- (k) Mitral valve prolapse

AUTISM AND FRAGILE X

Autism is a severe developmental disorder that is characterised by marked social deficits, delay in language development and a restricted range of stereotyped repetitive behaviour. The ratio of affected males to females is 4:1. The precise cause of autism and the mode of transmission is unknown but it does not follow the classical Mendelian inheritance. Several disorders of known genetic aetiology have

been reported to be associated with autism. Among these, fragile X linked mental retardation is the most common. The incidence of the association of fragile-X with autism ranges from 14% or less to 47% (14,15).

CAUSES OF FRAGILE X SYNDROME

Genetic linkage studies as well as the analysis of somatic cell hybrids that carry the fragile X chromosome in a rodent background has shown that the mutation leading to fragile-X syndrome is cis-acting and located at or very close to the fragile site. Subsequently the discovery of a CpG island, a land mark for protein coding sequences, was followed by the identification of an unstable region containing the CGG triplet repeat. Further studies revealed the occurrence of the CGG triplet repeat at the 5' untranslated region (UTR) of a gene which was designated as fragile-X mental retardation, FMR-1 (Fig 1). The most prevalent cause of the fragile X syndrome is the expansion of the CGG triplet at the 5' untranslated region of FMR-1. The size of the CGG repeat is between 1 and 50 in normal individuals while in patients it can be greater than 200. In a range between 50 and 200 the allele becomes unstable and has a high probability of expansion to greater than 200 repeats in the next generation. The syndrome itself seems to be manifested due to a lack of expression of the functional Fragile X Mental Retardation Protein (FMRP). The causative role of FMR1 gene in fragile X syndrome has been confirmed by the detection of deletions and point mutation in the gene from patients suffering from mental retardation and a clinical phenotype similar to the fragile X syndrome. So far the mutations reported at the locus range from deletions of small but critical regions to deletions resulting in removal of the entire gene sequence (16,17,18), small insertions and deletions that lead to loss of function of FMR-1 (19). Point mutation leading to conversion of isoleucine to aspartic acid that results in loss of function of FMR-1 lead to fragile-X syndrome (20). Molecular study of FRAXE has shown that the fragility is due to an amplification of a GCC repeat adjacent to a CpG island. A candidate gene, FMR2 was identified by the positional cloning. FMR2 was confirmed as the FRAXE-associated gene when

Fig. 1. A schematic representation of FMR 1 gene. The vertical bars represent exons, 17 in all. ▽ represents the location of CGG repeats within the gene. The alphabets stand for sites restriction endonucleases, E-Eco R₁, P-Pst₁, B-Bss H2. O represents CGG repeats of 25 each. Representative range of repeats for the three states are indicated.

cytogenetically positive individuals with 100% methylation of the FRA XE CpG island were found to have transcriptional silencing (21). As far as the repeat expansions are concerned, in FRA XA there are three distinct status of repeat, a normal 5-50, a premutation 50-200 and a full mutation range of >200 copies while in the case of FRA XE, a normal range of 4-39 repeats directly seems to expand to a level of around 150-180 copies associated with mild mental impairment along with speech and learning difficulty

FRAGILE X MENTAL RETARDATION PROTEIN (FMRP)

The FMR1 gene consists of 17 exons spanning 38kb of the genome and the corresponding 4.8 kb transcript is alternatively spliced resulting in

several isoforms of the protein. Northern blot analysis of RNA from several human tissues indicated the presence of a 4.8kb FMR1 mRNA. The sequence predicts a full-length protein of 614 amino acids with an expected mass of 69 kDa (22). Antibodies against the human FMRP, fragile X mental retardation protein detected the presence of a similar protein expressed in mice in a wide variety of tissues, particularly in brain, testes, ovaries, thymus, oesophagus and spleen (23). Moderate to low levels have been detected in kidney, liver, colon, uterus, thyroid and lung. No FMRP was detected in muscle, heart and aorta. FMRP is highly conserved among vertebrates. The human FMRP exhibits 97% homology to the murine protein and early mouse embryos exhibited an enrichment of FMRP in brain and gonads. There are several interesting points about its expression pattern. Immunostaining

experiments have shown that FMRP is expressed intensely in nucleus basalis, magnocellularis and hippocampus. These are centres for cholinergic innervation for cortical and limbic development and control motivation, learning, memory and behaviour (24). RNA *in situ* hybridizations have shown that markedly enhanced expression is found in Type A1 spermatogonia. Expression in the fetal testis is observed and it reaches the highest level in immature testis and declines early in adult life. In the mature ovary no FMR1 expression signal is found but enhanced levels were seen in fetal ovary (25). FMR1 expression studied in primary culture of epithelial cells derived from young mouse kidney shows maximum levels when the cells reach the quiescent G₀ stage, signifying terminally differentiated cells. However, the FMR1 transcript levels decrease suggesting a post-transcriptional autoregulatory mechanism. When the same cells are transformed with SV40 and the proliferation is resumed, a significant increase in FMRP levels is detected (23). Transgenic mouse model carrying FMRP fused with lac Z showed strong staining patterns in interstitial cells and leydig cells of the testis and granulosa cells of the ovarian follicles and adrenal cortex- all the cell types that are involved in androgen biosynthesis (26). Thus the pattern of expression of FMR1 gene closely correlates with the tissues or organs whose function is affected in the diseased state. However the exact function of FMRP is not known. In a fragile X patient with an expansion of the CGG repeat stalling of the 40s ribosomal subunit occurs during scanning prior to the translational initiation because of the extensive potential secondary structure of the GC rich sequence. Hence no functional FMRP is expressed and the FMR1 transcription is also down regulated (27).

In all the tissues in a normal individual FMRP localizes to the cytoplasm. The exact function of the protein is not known, but it shares extensive homology with certain proteins containing RNA binding domain such as the Rev protein of HIV, hnRNP A1, a protein found ubiquitously and two autosomal proteins FXR1 and FXR2. FMR-1 forms a heteromer with FXR1 and FXR2 (28). About 4% of all the mRNAs in fetal tissues is bound by FMRP. Apart from the RNA binding domains, FMRP has a N-terminal KKXKP, a nuclear localization signal

(NLS) and a 17 amino acid sequence in the exon 14 that is a nuclear export signal (NES). The isoform resulting from alternative splicing of exon 14 lacking the nuclear export signal localizes in the nucleus. So under physiological conditions the NES (also known as the Cytoplasmic Retention Signal) seems to override the effects of NLS. Since FMRP possesses both the NLS and NES, it is proposed to be involved in nucleocytoplasmic transport (29). Any mechanism such as point mutation that leads to impaired FMR1 function results in an imbalance in the nucleocytoplasmic shuttle. A hypothesis explaining the probable function of FMR1 is that it might be involved in binding RNA transcripts within the nucleus and transporting them across the nuclear membrane for translation in the cytoplasm. In the case of the point mutation in addition to its inability to bind effectively to the transcripts generated, the mutant FMRP forms abnormally small mRNP particles which fail to be transported across the nucleus and therefore are not selected for translation (30). The fact that FMR1 is expressed in actively dividing cells, cosediments with 60s ribosomes and strongly interacts with Rab40, a protein found in the nuclear pores seem to support its postulated function (31). FMRP also interacts with the autosomal proteins with identical RNA binding motifs, FXR1 and FXR2. The non-lethal effects of the lack of FMR1 may be due to the redundancy of its function (28).

INHERITANCE OF FRAGILE X SYNDROME

Fragile X Syndrome is an X-linked disorder but has an unusual pattern of inheritance (Fig 2). The general features of the inheritance pattern is known from the analysis of over 200 pedigrees (32). It deviates from the Mendelian pattern of inheritance such that about 30% of the carrier females are mentally retarded although they are often less severely affected than males. There are two groups of affected individuals,

- a) a population of around 20% males and females who carry the mutation but do not manifest any phenotypic effects. These are the non-transmitting males. Female progeny of the non-transmitting males also turn out to be carrier females.
- b) individuals, predominantly males who inherit the mutation from the carrier mothers and manifest the clinical effects of the syndrome.

In normal transmitting males and their daughters no fragile site at Xq27.3 is detected. Thus the disease phenotype itself is found in the offspring only after the transmission of mutation through a female. In other words the syndrome exhibits 'skipping of generation', along with 'genetic anticipation', i.e., the severity of the disease increases in subsequent generation. Collectively these unusual attributes are referred to as '**Sherman Paradox**'. A typical pedigree for fragile X syndrome is depicted in fig 2. The variability in the degree of mental impairment in females and the existence of males who are not affected but pass on the fragile X gene indicates a complex pattern of heredity. Taken into consideration, all the observations, fragile X syndrome is described as an X linked dominant disorder with incomplete penetrance.

IMPRINTING AND FRAGILE - X SYNDROME

The existence of mentally normal males who can transmit the mutation to their daughters who

again are asymptomatic and the observation that only grandsons of the non-transmitting males (NTM) are affected at high frequency has led to the hypothesis that fragile -X syndrome might be a two step process involving an imprinting mechanism (34). Genomic imprinting is a mechanism in which there is non-equivalence of the parental genetic contribution to the progeny which leads to differential expression of homologous genes. The discovery of expansion of CGG repeats at the 5' untranslated region of FMR1 gene as the most common molecular basis of fragile -X syndrome and the establishment of three distinct states of the alleles corresponding normal, carrier and full mutation has provided an explanation for some of the unusual attributes of the genetics of fragile X syndrome. Consistent with X inactivation model the expansion seems to occur only when the premutation allele is passed through meiosis in the female germ line. Inappropriate methylation and unusual DNA structure are proposed as possible explanation for

Fig 2.0: Inheritance of Fragile X Syndrome: A hypothetical pedigree to illustrate the different levels of penetrance of the fragile X mutation. The carrier I-I has an 18% chance (approximately one in 5 risk) that her sons are affected, while the carrier III-I has a 76% chance (3 in 4 risk) that her fragile X sons are affected. The size of the amplification of the fragile X p(CGG)_n repeat is given as Δ i.e., for a carrier individual Δ is equal to the number of the repeat increased over the normal. Values for Δ indicated are those expected hypothetically for individual 1 in each generation. Filled squares indicate individuals with fragile X syndrome; black dots indicate premutation. Adapted from (33). This illustrates the "Sherman Paradox".

the imprinting phenomenon. However, the exact mechanism of the repeat expansion and the late replication of fragile X locus still remain elusive.

DIAGNOSIS OF THE FRAGILE X SYNDROME

Cytogenetic testing was one of the most common tools used for the diagnosis of Fragile X syndrome. Whole blood from the patient is seeded in a folic acid deficient medium and the lymphocytes are cultured for 72 hours. The lymphocytes are further processed and the metaphase spreads are stained and examined for the fragile site at Xq27.3 (Fig3) (35). The frequency of a fragile X chromosome observed in the karyotypes of males positive for fragile X syndrome varies from 4 to 40% (1) However the variability and subtlety of the classical phenotypic features along with the peculiar inheritance pattern lowers the chances of recognising the fragile site. To increase the reliability of the detection, more than one method of induction, such as FudR, Thy and Methotrexate induction is used. Such a combination or the most often used 'double induction' system consisting of FudR and Thy was found to exhibit marginal high frequencies of fragile X expression. However this also proved to be unreliable since more often than not the cultures oscillated between poor growth in thymidine containing medium to about 5 times higher expression of fragile site in media with

FudR alone(6). In India cytogenetic analysis in low folate media has been the main course of induction to visualize the fragile X syndrome (36).

Though cytogenetic testing is done on a routine basis it is highly unreliable because not all the cells in an affected male exhibits the fragile site, both transmitting males and carrier females have no abnormality and a significant percentage of the carrier females are cytogenetically normal. It is also tedious and unreliable for prenatal diagnosis. During the 1980, linkage analysis with DNA markers close to the fragile X locus was used in conjunction with the cytogenetic analysis for prenatal diagnosis and genetic counselling in families with the fragile X syndrome. But such analysis requires the testing for many markers not to mention the availability of informative families. The situation is also complicated by recombination involving the fragile X locus. All things considered molecular diagnosis with the use of a DNA probe mapping at FMR1 gene proved to be the most reliable and direct method in both the carriers of the fragile X syndrome as well as the affected individuals, regardless of their sex (37). It is also reliable and applicable for prenatal diagnosis.

Cloning of the 5'UTR of FMR-1 and its further characterisation has resulted in the discovery of the

Fig.3: A representative metaphase plate showing fragile site at Xq27.3 from a patient. On southern analysis his DNA from this patient was found to have a CGG repeat number of around 600.

CpG island upstream of the (CGG) repeat loci. In premutations (CGG) n where $n=50-200$ and full mutation where $n>200$ abnormal methylation of the CpG island is detected. Using restriction enzymes that are inhibited by DNA methylation it is possible to detect the sequences that are abnormally methylated on the X chromosome in females that can be distinguished from the fragments corresponding to the premutation or the normal sequence which remain unmethylated only on the active X chromosome. Direct diagnosis of the fragile X syndrome by the digestion with restriction endonucleases such as EcoRI, HindIII, PstI and BclI has greatly improved the chances of detection of fragile X syndrome (37). These enzymes can cleave the DNA sequences on either side of the region where CGG repeat is present. Therefore the length of the fragment derived from this region of the FMR1

gene will vary depending upon the number of the CGG repeats present (Fig 4). By Southern hybridization, using probes derived from sequences 3' or 5' to the (CGG) repeat that are part of the larger fragments large deletions involving CGG repeat and flanking sequences as well as the methylation status of the FMR1 in an individual can be detected (37). Reagents for DNA based analysis of the FMR1 have been generated and utilised in the diagnosis of fragile-X patients and analysis of polymorphism at the FMR1 allele in the Indian population (38). Southern blotting are inconvenient when large number of individuals have to be tested for fragile X syndrome. The use of monoclonal antibodies against FMRP offers a rapid means to discriminate the normal from the affected individuals. However detection of the borderline premutation status that does not affect the expression cannot be detected

Figure 4.0: Schematic representation of the pattern observed in the case of a methylated full mutation when subjected to EcoRI and EagI digest. Each circle represents 30 CGG repeats. On expansion the size of the fragment increases, and methylation of the EagI site results in resistance to EagI. The increase in size is depicted as delta. The inset shows a schematic representation of Southern hybridisation probes from FMR1 will detect a fragment of 5.2kb, 2.4 kb and 2.8kb in a normal female, in a carrier with premutation a fragment larger than 5.2kb derived from the inactive X is seen along with 2.4 and 2.8 kb fragments. In patients a smear derived from the methylated and expanded FMR1 is generally seen along with the 2.4 and 2.8kb fragments from the normal allele. In the example shown here a female profile is chosen And the normal allele is assumed to on the inactive X

(39). Point mutations leading to the manifestation of the syndrome can be detected by amplifying the exons of FMR1 by PCR followed by sequencing the PCR product.

ANIMAL MODELS

The FMR1 gene is highly conserved among the species and the murine homolog, *fmr1* shows almost 97% homology at the amino acid sequence level. The expression pattern of FMR1 at the mRNA and the protein level is very similar in different tissues of humans and mice. Mouse also has the CGG repeat stretch at the 5'UTR though the repeat numbers show no polymorphism (40,41). This makes mouse a good model system to study fragile X syndrome. The initial attempts to create a mouse model for fragile X syndrome was the FMR1 knockout mice. The knock out mice lack the normal *Fmr-1* RNA and protein and show enlarged testes, impaired cognitive function and aberrant behaviour reflecting the phenotype exhibited by affected individuals (42). Three independent attempts have been made to create mouse models carrying the trinucleotide repeat sequences. Transgenic mice with [(CGG)₂₂TGG(CGG)₄₃TGG(CGG)₂₁] (43) transmitted the sequence without detectable changes. Transgenic mice containing 32, 76 and 120 triplets including 9, 53 or 97 uninterrupted CGGs respectively at the 3' end of the repeat array were created. Analysis of the repeat stretch upto 4 generations showed that there was no difference in the size from the microinjected construct (44). Transgenic mouse models carrying the repeat sequences even in premutation range showed no instability. This suggests that apart from being a human-specific phenomenon, the position of the interruptions, the flanking sequences and several other factors relating to the genome organisation influence the repeat expansion. Taking genome organisation of the human FMR1 into consideration, recently a mouse model for repeat expansion has been generated. This transgenic system shows limited intergenerational instability of the CGG repeats (41). The utility of the model system is immense in resolving certain unique attributes of the fragile X syndrome. For instance the developmental timing of repeat expansion is debated

due to the occurrence of monozygotic twin pairs showing discordant repeat numbers at FMR1 locus (41,45) on one hand and the results with abortuses from fragile X patients which showed full mutation alleles suggesting that expansions are perhaps prezygotic event and that the variability in repeat number occurs during early embryonic divisions (46).

CONCLUDING SECTION

Among the normal population the polymorphic CGG repeat varying between 6-50 is interspersed with one to three single AGG sequence approximately every 10 CGG repeat. The AGG sequence is proposed to have an anchoring effect on the repeat sequence and to confer stability. Point mutations in the AGG interspersions could result in sequence configurations which lack AGG or have just one AGG interspersions as in (CGG)₉AGG(CGG). Such sequences with a rare AGG interspersions towards the 3' end are considered to be predisposed towards replication slippage. Additionally there seems to be a strong association between long (CGG) repeats at the 3' end of the AGG interspersions with configurations such as (CGG)₉AGG(CGG)_{≥20}, (CGG)₁₀AGG(CGG)_{≥20} and their tendency to assume premutation status. Such alleles are also called protomutations (47). These observations have led to the hypothesis that imperfections within repeats are responsible for stabilizing the size of the repeat and the absence of imperfections contributes to the instability and the repeat expansion. The molecular mechanism resulting in triplet repeat mutational expansions are still unclear. A working hypothesis regarding the evolution of fragile X full mutations can be defined in the following 4 stages (57). Initial occurrence of ancestral events leading to the formation of predisposing alleles which have large total repeat length (e.g. between 35-50) but no AGG or one AGG interspersions. This event could be the point mutation leading to the loss of AGG, a gradual slippage of these predisposing alleles to small premutations (S alleles), followed by conversion from S alleles to larger premutations (Z). Finally, the Z alleles can undergo massive expansions to form full mutation (L) alleles, resulting in fragile X syndrome. Evidence for this hypothesis comes from the fact that PCR

amplification of the CGG repeat region in the normal control as well as the premutation alleles followed by sequence analysis shows the highest variability of the pure repeat lengths among the controls occurring at the 3' end. In one of the studies all the premutation alleles showed ≥ 50 pure CGG repeats while a majority of the controls hardly had ≥ 40 repeats (28).

The discovery of molecular basis of several neurological disorders highlight the functional potential of repetitive sequences in the genome. The high degree of polymorphism in such sequences seen in the population has been effectively utilised in molecular genetic analysis of human genome and its functions. This is also important in deciphering the genetic basis of variability in phenotypes

between individuals; even in such simple phenotypes as levels of hemoglobin. Thus the molecular basis of fragile X syndrome has drawn attention to the importance of untranslated sequences within genes in disease processes. The knowledge gained over the last decade has contributed greatly to fundamental biology and the fragile X syndrome in particular, however the therapeutic value of this knowledge is still to be fully perceived and practiced. The immediate application would be restricted to prenatal diagnosis and genetic counseling. Therapy in terms of gene replacement or intervention in disease causing mutations is presently at an entirely academic level. It is important to note that a beginning at therapy at the primary level cannot even be contemplated if the molecular anatomy of the disease is unknown.

REFERENCES

1. Sutherland, G.R. (1977) Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. *Science* 197, 265-266.
2. Sutherland, G.R. (1991) The detection of fragile sites on human chromosomes. In: *Advanced techniques in chromosome research*. Ed. K.W. Adolph. Dekker, New York. p. 203-266.
3. Sutherland, G.R. (1993) Human fragile sites. In *genetic maps*. Eds. J.O'Brien. Cold Spring Harbour Laboratory Press, New York. Vol IV, p. 6.264-6.267.
4. Jones, C., Penny, L., Mattina, T., Yu, S., Baker, E., Voullaire, L., Langdon, W.Y., Sutherland, G.R., Richards, R.I. and Tunnacliffe, A. (1995) Association of a chromosome deletion syndrome with a fragile site within the proto-oncogene *1CBL2*. *Nature* 376, 145-149.
5. Nelson, D.L. (1995) The fragile X mental retardation syndrome. In: *Molecular genetics of human inherited disease*. Eds Duncan J Shaw. John Wiley and Sons Ltd. Baffins, England. p. 91-116.
6. Brown, W.T. (1996) The FRAXE syndrome: Is it time for routine screening? *Amer. J. Hum. Gen.* 58, 903-905.
7. Parrish, J.E., Oostra, B.A., Verkerk, A.J.M.H., Richards, S., Reynolds, J., Spikes, A.S., Shaffer, L.G. and Nelson, D.L. (1994) Isolation of a GCC repeat showing expansion in FRAXF, a fragile site distal to FRAXA and FRAXE. *Nat. Genet.* 8, 229-235.
8. Howard Peebles, P.N. (1991) Fragile X expression: Use of double induction system. *Amer. J. Med. Genet.* 38, 445-449.
9. Penrose, L.S. (1938) A clinical and genetic study of 1280 cases of mental defect (Special report series No.299) London Medical Research Council.
10. Martin, J.P. and Bell, J.J. (1943) A pedigree of mental defects showing sex linkage. *Neurol. Neurosurg. Psychiatry* 6, 154-157.
11. Escalante, J.A., Grunspun, H. and Frosa-Pessoa, O. (1971) Severe sex linked mental retardation. *J. Hum. Genet.* 19, 137-140.
12. Lubs, H.A. (1969) A marker X chromosome. *Amer. J. Hum. Genet.* 21, 231-244.
13. Brown, W.T. (1989) The fragile X syndrome. *Neurologic Clinics* 107-121.

14. Fryns, J.P. (1984). The fragile X syndrome: A study of 83 families. *Clin. Genet.* 26, 497-528
15. Hagerman R., Jackson A., Levitas, A., Rimland B. and Braden M. (1986). An analysis of autism in 50 males with fragile X syndrome. *Amer.J.Med.Genet.* 23,359-374.
16. Gronskov, K., Hjalgrim, H., Bjerager, M.O. and Brogdum-Nielsen, K. (1997) Deletion of CCG repeats plus flanking sequences in FMR-1 does not abolish gene expression. *Amer. J. Hum. Genet.* 61, 961-967.
17. Tarleton, J., Ritchie, R., Schwartz, C., Rao, K., Aylsworth, A.S., and Lachiewicz, A. (1993) An extensive *de novo* deletion removing FMR1 in a patient with mental retardation and the fragile X syndrome phenotype. *Hum. Mol. Genet.* 2, 1973-1974.
18. Gedeon, A.K., Baker, E., Robinson, H., Partington, M.W., Gross, B., Manca, A., Korn, B., Poustka, A., Yu, S., Sutherland, G.R. and Mulley, J.C. (1992) Fragile X syndrome without CCG amplification has an FMR1 deletion. *Nat.Genet.* 1, 341-344.
19. Lugenbeel, K.A., Peier, A.M., Carson, N.L., Chudley, A.E. and Nelson, D.L. (1995) Intragenic role of FMR1 in fragile X syndrome. *Nat. Genet.* 10, 483-485.
20. De Boule, K., Verkerk, A.J.M.K., Reyniers, E., Vits, L., Hendrikx, J., Van Roy, B., Van den Bos, F., de Graaf, E., Oostra, B.A. and Willems, P.J. (1993) A point mutation in the FMR-1 gene associated with fragile X mental retardation. *Nat. Genet.* 3, 31-35.
21. Gecz, J., Gedeon, A.K., Sutherland G.R. and Mulley J.C. (1996). Identification of the gene FMR2, associated with FRAXE mental retardation. *Nat.Genet.* 13,105-108.
22. Ashley C.T., Sutcliffe J.S., Kunst C.B., Leiner, H.A., Eichler E.E., Nelson,D.L. and Warren, S.T. (1993) FMR-1 protein: conserved RNP family domains and selective RNA binding. *Science* 262, 563-566.
23. Khanjian, E.W., Fortin,A., Thibodeau, A., Tremblay, S., Cote, F., Devy, D., Mandel, J.L. and Rousseau, F. (1995) A heterogeneous set of FMR 1 protein is widely distributed in mouse tissues and is modulated in cell culture. *Hum. Mol. Genet.* 4, 738-790.
24. Abitbol, M., Menini, C., Delezoide, A-L., Rhyner, T., Vekemans, M., Mallet, J. (1993) Nucleus basalis magnocellularis and hippocampus are the major sites of FMR-1 expression in the human fetal brain. *Nat.Genet.* 4, 147-152.
25. Bachner D., Manca, A., Steinbach, P., Wohrle, D., Just, W., Vogel, W., Horst, H. and Poustka, A (1993) Enhanced expression of the murine FMR1 gene during germ cell proliferation suggests a special function in both the male and the female gonad. *Hum. Mol. Genet.* 12, 2043-2050
26. Hergersberg, M., Matsuo, K., Gassmann, M., Schaffner, W., Luscher, B., Rulicke T. and Aguzzi, A. (1995) Tissue specific expression of a FMR-1/b-galactosidase fusion gene in transgenic mice. *Hum. Mol. Genet.* 4, 359-366.
27. Feng, Y., Zhang, F., Lokey, L.K., Chastain, J.L., Lakkis, L., Eberhart, D. and Warren S.T. (1995) Translational suppression by trinucleotide repeat expansion at FMR1. *Science.* 268, 731-734.
28. Zhang, Y., O'Connor P.J., Siomi, M.C., Srinivasan, S., Dutra, A., Nussbaum, R.L. and Dreyfuss, G. (1995) The fragile X mental retardation syndrome protein interacts with novel homologs FXR1 and FXR2. *EMBO*, 14, 5358-5366.
29. Eberhart, D.E., Malter, H.E., Feng, Y. and Warren, S.T. (1996) The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. *Hum. Mol. Genet.* 5,1083-1091.
30. Feng, Y., Absher, D., Eberhart, D.E., Brown, V., Malter, H.E. and Warren S.T. (1997) FMRP associates with polyribosomes as an mRNP and the I304N mutation of severe fragile X syndrome abolishes this association. *Mol. Cell.* 1, 109-118.

31. Fridell R.A., Benson, E.R., Hua, J., Bogerd, H.P. and Cullen B.R. (1996) A nuclear role for the fragile X mental retardation protein EMBO. 15, 5408-5414.
32. Sherman, S.L., Morton, N.E., Jacobs, P.A. and Turner, G. (1984) The marker (X) syndrome: a cytogenetic and genetic analysis. Ann.Hum.Genet. 48, 21-37.
33. Sutherland, G.R. and Richards, R.I. (1995) Simple tandem DNA repeats and human genetic disease.Proc. Natl. Acad. Sci. USA. 92, 3636-3641.
34. Laird, C.D. (1991) Possible erasure of the imprint on a fragile X chromosome when transmitted by a male. Amer. J. Med. Genet. 38, 391-395.
35. Seabright, M. (1971) A rapid banding technique for human chromosomes. Lancet 11, 971-972.
36. Chetan, G.K. (1996) Systematic survey of the frequency of fragile X chromosome among subjects with non-specific mental retardation: A prospective study. Ph.D. thesis. Bangalore: National Institute of Mental Health and Neuro Sciences (Deemed University).
37. Rousseau, F., Heitz, D., Biancalana, V., Blumenfeld, S., Kretz, C., Boue', J.,Tommerup, N., Gilgenkrantz, S., Jalbert, P., Voelckel, M-A., Oberle', I. and Mandel, J.L (1991) Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. New Engl. J. Med. 325: 1673-1681.
38. Sujatha, B., Naseerullah, M.K., Manjunatha, K.R., Chetthan, G.K., Arthi, R., Bhaskar, G.V., Girmaji, S.R., Shobha, S., Sheshadri, S., Radha, R.D. and Vani, B. (1998) Triplet repeat polymorphism and fragile X syndrome in the Indian context. Ind. J. Med. Res. 107, 29-36.
39. Willemsen, R., Los, F., Mohkamsing, S., Van Den Oue A., Deelen, W., Galjard, H. and Oostra, B. (1997) Rapid antibody test for prenatal diagnosis of fragile X syndrome in amniotic fluid cells: a new upraisal. J. Med. Genet. 34, 250-251.
40. Eichler, E.E., Hammond, H.A., Macpherson, J.N., Ward, P.A. and Nelson, D.L. (1995) Population survey of the human FMR1 CGG repeat substructure suggests biased polarity for the loss of AGG interruptions. Hum. Mol.Genet. 4, 2199-2208.
41. Sujatha, B. (2000) A mouse model for dynamic mutation and fragile syndrome. Thesis submitted for Ph.D degree, Indian Institute of Science, Bangalore, India.
42. Bakker, C.E., Verheij, C., Willemsen, R., Vamnderhelm, R., Oerlemans, F., Vermey, M., Bygrave, A., Hoogeveen A.T., Oostra, B.A., Reyniers, E., Debouille, K., Dhooge, R., Cras, P., Van Velzen, D., Nagels, G., Martin, J.J., Dedeyn, P.P., Darby, J.K. and Willems, P.J. (1994) Fmr-1 knock-out mice: a model to study fragile X mental retardation. Cell 78, 23-33.
43. Lavedan, C.N., Garrett, L., and Nussbaum, R.L. (1997). Trinucleotide repeats (CGG)22TGG (CGG)43TGG, (CGG)21 from the fragile X gene remain stable in transgenic mice. Hum. Genet. 100, 407-414.
44. Bontekoe, C.J., de Graaf, E., Nieuwenhuizen, I.M., Willemsen R., and Oostra B.A. (1997) FMR-1 premutation allele is stable in mice. Eur. J. Hum. Genet. 5, 293-298.
45. Kruyer, H., Mila, M., Glover, G., Carbonell, P., Ballesta, F. and Estivill, X. (1994) Fragile X syndrome and the (CGG)*n* mutation: Two families with discordant monozygotic twins. Am. J. Hum. Genet. 54, 437-442.
46. Malter, H.E., Iber, J.C., Willemsen, R., de Graaff, E., Tarleton, J.C., Leisti, J., Warren, S.T. and Oostra B.A. (1997) Characterisation of the full fragile X syndrome mutation in fetal gametes Nat.Genet 15, 165-169.
47. Snow, K., Tester, D.J., Kruckeberg, K.E., Schaid, D.J. and Thibodeau, S.N. (1994) Sequence analysis of the fragile X trinucleotide repeat: implications for the origin of the fragile X mutation. Hum.Mol.Genet. 3, 1543-1551.