The fragile X syndrome

Complex behavior of a simple repeat

Esther de Graaff

The fragile X syndrome
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Het fragiele X syndroom
Complex gedrag van een eenvoudige repeat

Proefschrift

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H. van Veen

To you

Contents

Chapter 1

General introduction

Approximately 2-3 % of the population in industrialized countries is thought to be mentally retarded (1,2). Most patients are mildly retarded (IQ<70) and only 3-5 per 1000 people have a severe form of mental retardation (IQ<50). Mental retardation can be either acquired due to for instance toxic insults, traumas or infections, or caused by genetic factors. The genetic contribution to mental retardation is thought to be important, as over 300 genetic disorders are known to have mental retardation as (part of) the clinical phenotype (3).

In 1938, Penrose was the first to notice the excess of mentally retarded males in institutes for mentally disabled, suggesting that aberrations in X-linked genes are likely to contribute to mental impairment (4). It is now believed that 30-50 % of inherited mental retardation in human is caused by mutations in genes on the X-chromosome (5). One form of X-linked mental retardation associated with specific facial appearances was detected in a family described by Martin and Bell in 1943 (6). Much later in 1969, Lubs *et al.* (7) detected a fragile site on the long arm of the X chromosome in four mentally retarded males. After the discovery that the fragile site could be induced by culturing cells in folic-acid deficient medium a link was established between the expression of the fragile site and the clinical phenotype thus far known as the Martin-Bell syndrome (8,9).

The fragile X syndrome as it is known now, appears to be the most frequent form of inherited mental retardation. Initially a prevalence of 1 in 1200 males was reported (10) but a more recent estimate is approximately 1 in 4000 males (11). The discovery of the responsible gene and the mutational mechanism (12-14) enabled the development of reliable tests for the diagnosis of fragile X patients. The use of a recently developed, simple protein test (15) in large scale screening tests in the near future will undoubtedly provide more accurate data about the incidence of affected males.

* 1.1 Clinical phenotype

1.1.1 The classical fragile X phenotype in male patients

The term 'fragile X syndrome' indicates that the patients exhibit several clinical features. The classical fragile X syndrome consists of a combination of four features: facial appearance, connective tissue dysplasia, macroorchidism and characteristic behavior and learning abnormalities. The clinical features of fragile X patients are extensively described by Hagerman (16).

Facial features

The most characteristic facial appearances are the broad and high forehead, long face, and prominent chin. The ears are generally quite broad, soft and long, mostly longer than 6 cm and prominent. The shape is usually normal (16).

Connective tissue dysplasia

The joints can be loose and hyperextensible, and flat feet are reported in 50 % of the patients (17). The skin of fragile X patients is soft and smooth, possibly the result of a

decrease of elastin fibrils in the deep dermis (18).

Macroorchidism

One of the most consistent physical signs of the fragile X syndrome is macroorchidism (enlarged testes; 40-60 ml as compared to an average of 25 ml in control males), present in approximately 75-90 % of the fragile X males (16,19). This testicular enlargement mostly develops during puberty, since only approximately 20 % of the prepubertal boys have macroorchidism, i.e. a testicular volume larger than 3-4 ml (20).

The cause of the enlarged testes remains unclear, but only a few studies have been performed. The levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid hormone, influencing the number of Sertoli cells, are reported to be normal (21,22). The results of a small number of histological studies, involving only few fragile X patients, are contradicting. The germinal epithelium and spermatogenesis appears to be normal but the interstitial tissue is reported to be either normal (21) or augmented, which is probably due to edema (23,24). However, reduced spermatogenesis (25) has also been described. Some fragile X males are known to have children (26-28).

Behavioral pattern and neurological features

Fragile X males exhibit a broad spectrum of impaired cognitive functioning: 5.1 % of the patients is of normal or borderline intelligence, 15.7 % is mildly mentally retarded, 27.3 % moderately, 30.6 % severely, and 21.3 % is profoundly mentally retarded (29).

The most frequent behaviors described in fragile X patients are hyperactivity, decreased attention span, speech and language problems, and autistic features like handflapping, poor eye contact and gaze avoidance. The autistic features are present mainly in the more severely mentally retarded males and several groups have suggested an association between autism and the fragile X syndrome (30-32). Other investigations, however, failed to find a correlation between the fragile X syndrome and autism. Cohen *et al.* (33) found that the mechanism of some of the autistic features in fragile X patients is distinct from that in non-fragile X autistic males. In a large comparative study, combining the results from a large number of previous studies, Fisch (34) concluded that there is no association between autism and the fragile X defect.

Epileptic seizures, already described in the original report of the fragile X marker chromosome in 1969 (7), are quite common in fragile X patients, with a prevalence of 13-23%. Various forms of seizures have been observed (35,36) with the onset mostly before adulthood. In most cases anti-epileptic drugs are efficient.

Neuropathological studies have thus far shown some nonspecific features such as abnormal dendritic spine morphology (37,38), a reduction in the length of synapses (35) and mild ventricular dilatation (39). Using magnetic resonance imaging, Reiss *et al.* (40-42) detected an increase of the hippocampal volume, a reduction in size of the posterior cerebellar vermis, an enlarged fourth ventricle and an increased caudate nucleus in fragile X patients as compared to age- and sex matched normal and mentally retarded individuals. It

should be noted that the studies involved a small number of patients and have never been confirmed by others.

1.1.2 Female carriers

Females expressing the fragile X clinical phenotype have also been reported, with a 2-fold less frequent incidence than males. These females display some degree of mental retardation, ranging from mild to severe. The characteristic facial features, including a high, broad forehead, long face and protruding lower jaw and behavioral problems are detected, though less pronounced and with a lower prevalence than in affected fragile X males (43-45).

1.1.3 Subphenotypes

Besides the classical phenotype as described by Martin and Bell (6), two less frequent occurring subphenotypes of the fragile X syndrome have been reported. Patients have been described resembling either Prader-Willi (46,47) or Sotos (cerebral gigantism) patients (48,49). Since these subphenotypes are present in only a small number of individuals within fragile X families displaying the classical phenotype, it is likely that other (genetic) factors contribute to these subphenotypes. The gene mutation in both subphenotypes is identical to that present in the classical phenotype.

1.1.4 The fragile X mouse

Recently, a mouse model was generated in which the gene known to be involved in the fragile X syndrome was inactivated (50). The clinical abnormalities of this mouse are similar to these of the human condition in that the mice show mild macroorchidism, learning deficits and hyperactivity. Microscopic and macroscopic examination of the brain and testis did not reveal any difference between mutant mice and there normal littermates (50).

* 1.2 Cytogenetic expression

The Martin Bell syndrome is associated with the cytogenetic expression of a fragile site on the long arm of the X chromosome on Xq27.3 in cultured lymphocytes or fibroblasts (7,51,52). The fragile site is visible as a constriction or gap in chromatids and chromosomes, but only when cells are cultured under specific conditions before the chromosome spreads are made. Expression of the fragile site can be induced by using culture media with a lack of folate, with an excess of thymidine or with inhibitors of replication. Due to these components in the culture medium there will be a shortage of the DNA synthesis precursors thymidine and cytidine, which may lead to the expression of the fragile site (53).

The presence of a fragile site on the tip of the long arm of the X chromosome does not always coincide with the classical fragile X phenotype (53,54). A more accurate determination

of the location of the fragile sites revealed that two other folic acid sensitive fragile sites can be present on the long arm of the X chromosome, on Xq28. The three different fragile sites were subsequently called FRAXA, FRAXE and FRAXF. FRAXA is associated with the Martin-Bell phenotype. FRAXE is located 600 kb distal to FRAXA (55-57) and expression of this site coincides with a non-specific form of mental impairment, without any further characteristic features (57,58; *Publication 4.5*). Expression of FRAXF, 1-2 Mb distal to FRAXA site does not appear to correlate with the presence of mental impairment (59,60; see also *Chapter 1.7*).

* 1.3 Isolation of the fragile X gene

Identification and cloning of a gene of which the protein product is unknown is usually performed according to so-called positional cloning strategies. The presence of the fragile site on Xq27 made it very likely that the gene responsible for the fragile X syndrome was localized in this region. Many years after the discovery of the fragile site, linkage was reported between the fragile X locus and coagulation factor 9 and G6PD (61,62). Subsequently, linkage analysis narrowed the region of interest down to a 5-7 cM region flanked by the distal markers U6.2 and VK21 (63,64) and the proximal markers RN1 and VK23 (65,66). The construction of somatic cell hybrids, containing portions of the X chromosome located either exclusively proximal or exclusively distal to the fragile site (67), helped in the isolation of new markers (68,69) and YACs spanning the fragile site (12,70,71). Within this region a CpG island was detected, which was methylated in fragile X patients and not in control individuals (72,73). The detection of larger fragments, containing the CpG island, in fragile X patients on Southern blots, suggested an insertion within this fragment (13). The region of interest was narrowed down to 5 kb and contained a CGG repeat (12,74). The length variation was subsequently located within a 1 kb PstI fragment; more specifically within an CGG trinucleotide repeat (14,75).

Concurrently, cosmids spanning the fragile site were used to screen a human fetal brain cDNA library, resulting in the isolation of the cDNA which contained the CGG repeat. The gene was designated *FMR1* (fragile X mental retardation 1; 12). The cloning of the *FMR1* gene has been described in detail in the thesis of Verkerk (76).

The *FMR1* cDNA consists of 4362 nucleotides and contains an open reading frame of approximately 1.9 kb, encoding a polypeptide of 631 amino acids (77,78). The CGG repeat, identified as the unstable fragment in patients expressing the fragile X chromosome, is located in the first exon. The ATG startcodon of translation lies 69 bp downstream of the CGG repeat, indicating that the CGG repeat itself is not translated (77,79,80).

The genomic sequence of the *FMR1* gene encompasses approximately 38 kb and contains 17 exons (81). Splicing of the *FMR1* mRNA does not result in one specific mRNA but could result in over 20 different mRNAs (Fig. 1.1; 77,78). This alternative splicing involves exons 12, 14, 15 and 17. Only the exclusion of exon 14, by joining of exon 13 to one of three splice acceptor sites in exon 15, leads to a frame shift in the open reading frame, resulting in *FMR1* isoforms with a different C terminal amino acid sequence.

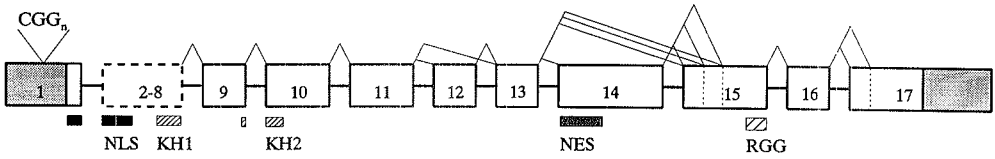

Figure 1.1 Schematic depiction of the genomic structure of the *FMR1* gene. The exons (1-17) are represented by boxes. The upper lines connecting the exons represent (alternative) splicing. The RNA-binding domains, KH1, KH2 and RGG, and the nuclear localization (NLS) and export (NES) signals are depicted below the gene. The 5′ and 3′ untranslated regions are shaded (Adapted from Sittler *et al.*, (108)).

The *FMR1* gene is highly conserved among different species, including yeast and nematodes (12) and displays a significant homology between both human and mouse: 95 % based on nucleotide identity in the open reading frame and 97 % based on amino acid identity (77) and human and chicken (85 % on nucleotide sequence and 92 % on amino acid identity; 82). Although located in the 5′ untranslated region, the CGG repeat is also evolutionary conserved in many species (83-85), suggesting a possible role of the repeat in the regulation of the gene. Specific nuclear proteins are known to be able to bind to the CGG repeat and other simple repeats, although their function is still unknown (86,87).

Molecular basis of the fragile X syndrome

The cloning of the FRAXA site and the associated *FMR1* gene unwrapped a new mutational mechanism, namely that of an expansion of a trinucleotide repeat. The CGG repeat in the fragile X syndrome, located in the promoter region of the *FMR1* gene (12-14,75), is highly polymorphic in humans and in different primate and non-primate mammals (14,83,85). Besides the normal sized repeat lengths, two configurations of expansion are present: the premutation and the full mutation (Fig. 2.1).

In the normal allele, found in unaffected individuals, the CGG repeat length ranges from 6 to 54 CGGs. The majority of normal arrays have 29-30 repeats, and this is found in different populations. Over 95 % of the normal sized alleles are below 40 repeats, with only 1-2 % of the alleles being over 46 CGGs. Transmission of the normal allele to offspring does not involve changes in repeat length, indicating that this allele size is usually inherited stably. In order to explain the presence of expanded alleles in the population, occasionally an alteration must occur in the normal allele (see *Chapter* 3). This appears to be an infrequent event, as alterations in a CGG repeat in the normal range have been described only twice: in a Japanese family a decrease from 29 to 21 repeats was recorded (88) and Macpherson *et al.* described a case in which a normal 29 CGG allele was enlarged to 39 CGGs (89).

The two expanded configurations of the CGG repeat are unstable upon transmission. A premutation allele (90) is detected in unaffected male and female carriers. The length of the premutation allele ranges from 43-200 CGGs (13,14). Transmission of the allele to offspring usually results in an increase of the repeat length, being either a small increase, usually resulting in the maintenance of a premutation sized allele, or an excessive amplification to

over 200 CGGs. This latter amplification occurs only if the expanded repeat passes a female meiosis. Repeat lengths over 200 CGGs are generally associated with methylation of the CpG island, the CGG repeat itself and flanking sequences and are called the full mutation (13,91-93). Males with a methylated full mutation do not express *FMR1* mRNA (94) resulting the fragile X phenotype described in *Chapter 1.1.*

*** 1.4 Mode of inheritance**

Although the genetic defect of the fragile X syndrome is associated with the X chromosome, the mode of inheritance differs from other Mendelian X-linked traits. The identification of two categories of mutations of the unstable CGG repeat of *FMR1* resolved this discrepancy.

In X-linked disorders, all males carrying a mutation are affected. However, in families with the fragile X syndrome, males are known who transmit the mutated X chromosome but who do not express the fragile X clinical phenotype (6,43,95). They are called normal transmitting males (Fig. 1.2, III-1). These males do not express the FRAXA site cytogenetically (96) and are now known to have the premutation sized allele (13,14).

The presence of affected females is also remarkable. Approximately 50-75 % of the females who carry a methylated full mutation are mildly to moderately mentally retarded (97), despite the presence of a second normal X chromosome. It is likely that this is due to a non-random, or skewed, X-inactivation, which will be described in more details in *Chapter 3.2.*

A third peculiar observation in the mode of inheritance is the phenomenon known as the 'Sherman paradox' (14,95). The risk of expressing the fragile X clinical phenotype depends on the position of an individual in the pedigree (Fig. 1.2). In Mendelian X-linked traits, 50 % of the sons and no daughters of a female carrier are affected and 50 % of the daughters are carrier. This Mendelian X-linked transmission is seen in both fragile X affected and unaffected females carrying a full mutation (Fig. 1.2, IV-5). This full mutation is passed to half of their offspring, resulting in 50 % affected sons and 50 % female carriers, of whom 50-75 % is also affected. In contrast, male and female offspring of unaffected carrier females have a risk of being affected of only 38 % and 16 %, respectively. This is due to the presence of premutation alleles in female carriers, that can be passed to their offspring as either a full mutation or a premutation. Transmission of a full mutation by carriers of a premutation depends on the size of the premutation; the longer the CGG repeat, the higher the change of a full mutation in the offspring. All premutation alleles containing 90 CGGs or more expand to a full mutation (Fig. 1.2, IV-3), whereas approximately 80 % of the premutation alleles in the range of 70-90 CGGs expand to a full mutation. Only 20 % of the alleles ranging from 60-69 CGGs become a full mutation (Fig. 1.2, II-2; 14,98,99). Expansion to full mutation also depends on the sex of the offspring; premutations expanding to a full mutation occurs 1.5 times more frequently in male offspring than in female offspring (100).

Another remarkable finding has been resolved only partially. Offspring of affected males are never affected and are also not a carrier of the cytogenetically expressed fragile site

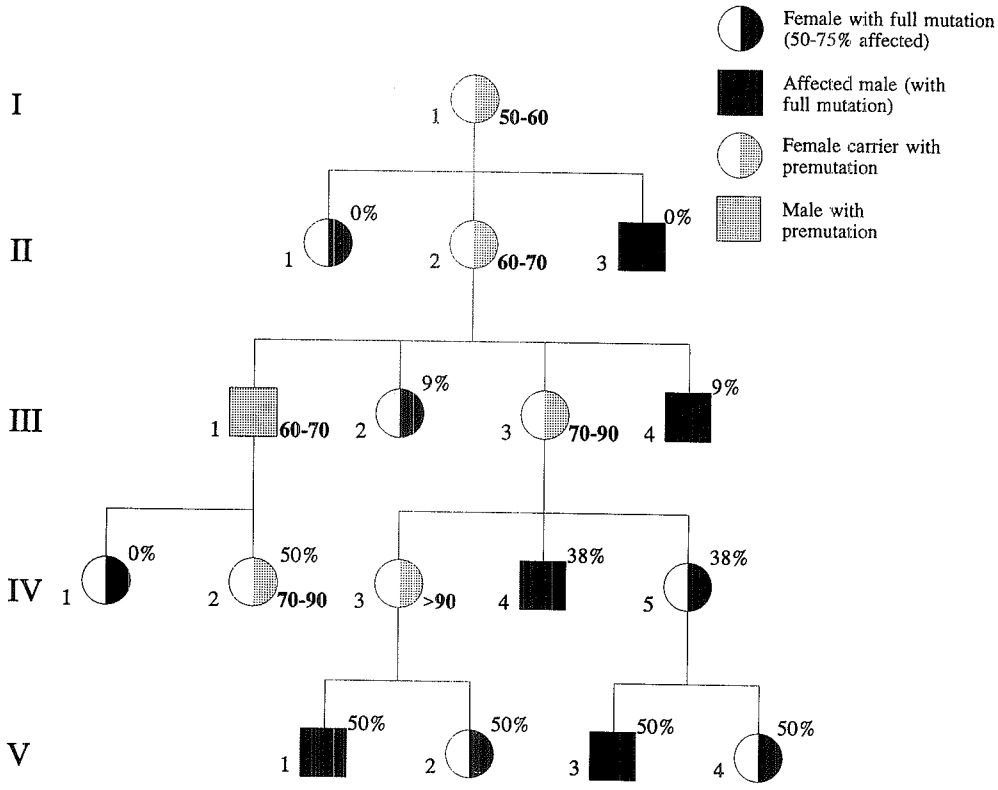

Figure 1.2 Empiric risk of carrying a full mutation varies with the position in the pedigree. The risk of carrying a full mutation allele in the offspring is indicated in percentages above each individual; 50-75 % of the females carrying a full mutation is affected. Suggested CGG repeat numbers are given in bold to the right of transmitting parents. The variability in risk of expansion to full mutation (and affected status) is dependent upon size of the premutation allele and accounts for the variation in risk based on the position in the pedigree. Individuals with normal alleles only (50 %) were omitted from this pedigree.

(13,26-28). Molecular analysis revealed that males only transmit a premutation (Fig. 1.2, IV-1 and IV-2) and not a full mutation due to the fact that their sperm contain a premutation only, irrespective of the length of the repeat in their somatic tissues (101,102). Mechanisms explaining the absence of a full mutation in sperm will be discussed in *Chapter 3*.

* 1.5 The *FMR1* gene product

The molecular basis of the fragile X syndrome is now known to be restricted to the expansion of a CGG repeat, which, when over 200 CGGs, is associated with methylation of the *FMR1* promoter region. This impairs the transcription of the *FMR1* gene, which is likely

to result in the absence of the *FMR1* protein (FMRP) in male fragile X patients. In order to both verify this and to be able to study FMRP, antibodies have been raised against the predicted amino acid sequence of the *FMR1*. Using these antibodies it was shown that, as expected, no FMRP is present in cells from fragile X patients (79,80).

In lymphoblastoid cell lines from control individuals, several isoforms of FMRP were detected, ranging from 67 kD to 80 kD as a result of alternative splicing (79,80). These different FMRP isoforms are not only detected in human lymphoblastoid cells but also in different tissues of humans, monkeys and mice. Low molecular weight proteins, of 39-41 kD, have been detected in several tissues and are likely to be the result of carboxy-terminal proteolytic cleavage. The significance of the different low and high molecular weight isoforms remains to be elucidated (103,104).

Despite the presence of a sequence encoding a putative nuclear localization signal (NLS; 105) in the C-terminal part of the *FMR1* cDNA (12), FMRP is predominantly localized in the cytoplasm (79,80). However, *in vitro* experiments have demonstrated that cells expressing the N-terminal half of FMRP, thus lacking the putative NLS, FMRP was localized in the nucleus (80), indicating the presence of an additional signal in the N terminal part of FMRP that directs FMRP to the nucleus. This signal is suggested to be present in the initial 166 amino acids residues of FMRP (S.T. Warren, *pers. comm.*), and this region contains a large number of arginine and lysine residues. Regions rich in basic amino acids have been described to have a NLS function in several RNA binding proteins. It is also possible that the NLS in FMRP is a unique signal associated with nuclear transport as has been identified in several other proteins (106,107).

The nuclear localization of alternatively spliced isoforms of FMRP, lacking exon 14, has lead to the suggestion that a cytoplasmic retention domain is present in exon 14 (108). Expression studies with different deletion constructs revealed that the initial 17 amino acids of exon 14 are required for the export of FMRP from the nucleus (S.T. Warren, *pers. comm.*). These 17 amino acids show high similarity to recently described nuclear export signals (NES; 109,110). The presence of both a NLS and a NES in the FMRP indicate that FMRP can shuttle continuously between the nucleus and cytoplasm, suggesting a function as nucleocytoplasmic transporter. FMRP is only rarely detected in the nucleus, which suggests that the export from the nucleus must occur very quickly.

1.5.1 Expression pattern of FMRP

Different studies, using either *in situ* hybridization or immunohistochemistry, have shown that *FMR1* is a housekeeping gene: the protein is detected in all tissues, although the expression levels are quite different. The highest expression of FMRP is found in testis and brain, the two main tissues affected in fragile X patients. In addition, high expression of *FMR1* is detected in esophagus, eye, thymus, ovarian follicles and thymus. The expression pattern of FMRP is identical in men and mice.

The *FMR1* gene is widely transcribed during fetal development, and *FMR1* mRNA can be detected in a variety of tissues, including brain, testis, spinal cord, eye, liver and kidney

(111). *In situ* hybridization studies of *FMR1* mRNA expression during early development revealed a high *FMR1* expression in the neural tube, and a strong, ubiquitous labelling of the whole embryo. In the subsequent stages of embryonic development, this widespread expression of *FMR1* mRNA decreases and *FMR1* expression becomes more localized to the specific tissues mentioned above (112,113).

In adults, the highest expression of FMRP is detected in specific regions of the brain: in the neurons of the granular layer of the hippocampus and the cerebellum and in the gray matter of the cortex (80,112,113). The high expression of FMRP in the normal cerebellum might be correlated to the decrease in size of the cerebellar vermis found in fragile X males who lack FMRP (41,42). Likewise, it can be speculated that the autistic like behavior that is sometimes seen in fragile X patients, may be associated with the high expression in the normal hippocampus (16,112,113).

In testis, FMRP is highly expressed in early spermatogonia, which has led to the suggestion that FMRP may be involved in spermatogenesis (80,114). However, studies with the knock out mice, which completely lack FMRP, have shown that FMRP is not essential for sperm development (50). In addition, in man, a fragile X male with a deletion in the *FMR1* promoter region, thus lacking FMRP expression, was reported to have a progeny (115; *Publication 4.1*). Only very low levels of FMRP are expressed in the connective tissue, including the Leydig cells, of the testis (R. Willemsen, *pers. comm.*). Studies of the testis of a fragile X male revealed expression of FMRP in the spermatogonia (*Chapter 2.6; Publication 4.4*), which is likely due to the spermatogonia containing an unmethylated premutation allele (101).

1.5.2 RNA binding capacity of FMRP

In 1993, highly conserved sequence motifs were described in the protein hnRNP K, a pre-mRNA binding K protein. These domains, termed KH domain for K protein homology domain, consist of 40 to 50 amino acids (116) and are often found in proteins that are capable of binding RNA (116,117). In FMRP two KH domains are present, in addition to two RGG boxes, another domain often found in RNA binding proteins (118-120). Subsequent *in vitro* and *in vivo* binding studies have demonstrated that FMRP is capable of binding with high affinity to its own mRNA and to approximately 4% of other mRNAs expressed in brain (118,119).

Interestingly, a fragile X patient has been described with a CGG repeat in the normal range and thus without the hypermethylation of the promoter as is found in the majority of fragile X patients. In the open reading frame of this patient a point mutation was detected (121). This point mutation, Ile304→Asn, is located within the second KH domain at a position which is highly conserved among KH domains of different species. In cells of this patient, neither translation, processing or localization of FMRP were found to be impaired (103). However, this mutated protein seems to bind to mRNA less stable than the wild type protein, possibly due to an impaired stability of the KH domain (103,122).

Interaction of FMRP with the ribosome was recently demonstrated. The association of

FMRP to the ribosome is easily removed by washing with a high salt buffer (123), indicating that FMRP is not part of the ribosomal protein complex, but is associated instead. This association is likely to occur through binding to RNA, as treatment with Micrococcus Nuclease dissociates FMRP from the ribosomal complex (124).

1.5.3 FMRP related proteins

Recently two other genes have been cloned, designated *FXR1* and *FXR2*, for FMR1 crossreacting relatives, 1 and 2 (84,125,126). *FXR1* and *FXR2* are autosomal genes and are mapped on chromosome 3q28 and 17p13.1, respectively (125,126). The molecular masses of the gene products are 70 kD and 74 kD, respectively. *FXR1*, *FXR2* and *FMR1* are highly homologous, with an overall amino acid identity of approximately 60 %. There are large differences in homology for different regions of the genes (Fig. 1.3), with the highest homology in the KH domains and an absence of homology in the carboxy terminal parts. Like *FMR1*, the *FXR1* and *FXR2* genes contain two KH domains, and the encoded proteins are capable of binding RNA (84,125). In addition, all three gene products are found in the cytoplasm and have been found to interact tightly with each other, forming either homo- and/or heterodimers or larger complexes. Cells from fragile X patients, completely lacking FMRP expression, have a normal level of *FXR1* and *FXR2*. This makes it unlikely that *FXR1* and *FXR2* are redundant genes, that are able to take over the function of FMRP.

In contrast to *FMR1* mRNA, which is expressed at the highest level in early spermatogonia (114,127), the *FXR1* gene product is only expressed in postmeiotic spermatids (126). *FXR1* protein is absent in neurons (R. Willemsen, *pers. comm.*), whereas FMRP is highly expressed in these cells (80,112,113). Expression of the *FXR2* protein has thus far only been studied in brain, where it is expressed, like FMRP, in neurons of both cerebellum and cortex. *FXR2* expression in neurons is unaltered in fragile X males and in *FMR1* knock out mice lacking FMRP (R. Willemsen, *pers. comm.*).

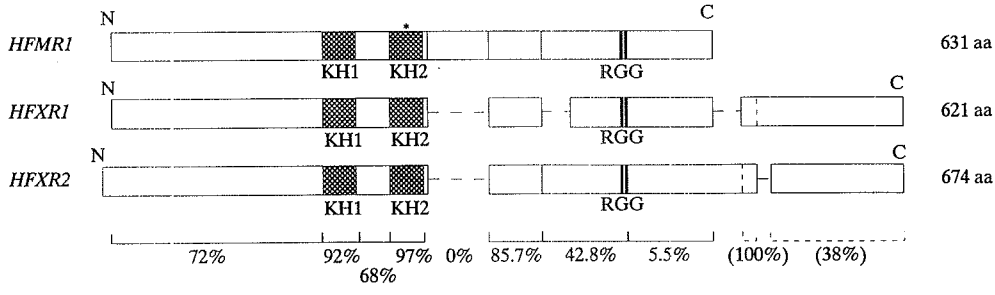

Figure 1.3 Homology comparison between human *FMR1*, *FXR1* and *FXR2*. Black boxes depict the KH domains, the blocked boxes represent the RGG boxes. The percentages of identity of the different domains in the three genes are depicted. The asterix denotes the Ile304->Asn point mutation.

* 1.6 Fragile X diagnostics

The identification of the *FMR1* gene and the finding that the fragile X mutation resides within the CGG repeat in the 5′ untranslated region have resulted in the development of reliable DNA diagnostic methods. Using either PCR or Southern blot analysis on DNA derived from blood cells, a prediction can be made of the fragile X status of an individual by determining the length of the CGG repeat and the methylation pattern of the *FMR1* promoter (14,71,97,128).

With PCR analysis the exact length of the CGG repeat within the normal and premutation range can be determined (14). This is useful in both identifying the carrier status in females and in the identification of changes in repeat length in successive generations, thereby revealing unstable alleles within families, which is helpful in genetic counseling. Though not impossible, PCR amplification of a full mutation is very difficult (129). Therefore, the most reliable detection of a full mutation allele is by Southern blot analysis. Digestion with either *Eco*RI or *Hind*III, and hybridization with a probe near the CGG repeat (Fig. 1.4; 97,128), results in a normal 5.2 kb fragment, containing the promoter and the first exon of *FMR1*, including the CGG repeat. Premutation alleles, varying from 43-200 repeats display a fragment in the 5.3-5.8 kb range. Full mutation alleles usually do not display a single band but are frequently visible as a smear of over 6 kb, indicating the presence of multiple repeat lengths within an individual. Double digestion of *Eco*RI (*Hind*III) and one of the methylation-sensitive enzymes *Bss*HII or *Eag*I cleaves the normal, unmethylated 5.2 kb fragment in 2.4 kb and 2.8 kb fragments, the latter containing the CGG repeat. In premutation alleles the 2.8 kb fragment has increased to 2.9-3.4 kb. Since the majority of full mutations is methylated, and therefore resistant to cleavage by *Bss*HII and *Eag*I, double digestion of full mutation alleles display a similar pattern as with digestion by *Eco*RI (*Hind*III) alone.

Determination of the CGG repeat length can also be reliably performed in prenatal diagnosis. In chorionic villi and amniotic fluid cells both the full mutation and the premutation can be easily identified using Southern blot analysis (93,130-135). Methylation analysis of chorionic villi revealed a discrepancy in methylation pattern between the embryonic and extra embryonic tissue in a number of cases. The abnormal methylation pattern, detected in fetal tissue is not always present in chorionic villi of 10-11 week old fetuses (93,97,130,132). In another report, chorionic villi sampled at 11 and 12 weeks of gestation were shown to be methylated (136). This suggests that methylation of chorionic villi is delayed as compared to the fetal tissues and may occur around the 11th week after gestation. Absence of methylation seems to be a common phenomenon in chorionic villi (137,138). In fetal tissue, methylation of the *FMR1* CpG island on the inactive X chromosome of normal females is already present at week 6 after gestation (139), whereas this methylation is frequently absent (or only partial) in chorionic villi.

The correlation between the presence of an allele of full mutation size and the clinical expression of the fragile X syndrome is not absolute, since not all full mutation size alleles are methylated. Unmethylated full mutations have been reported in unaffected males (140; B.B.A. De Vries, *pers. comm*) and in mildly retarded fragile X males (102,141,142). This

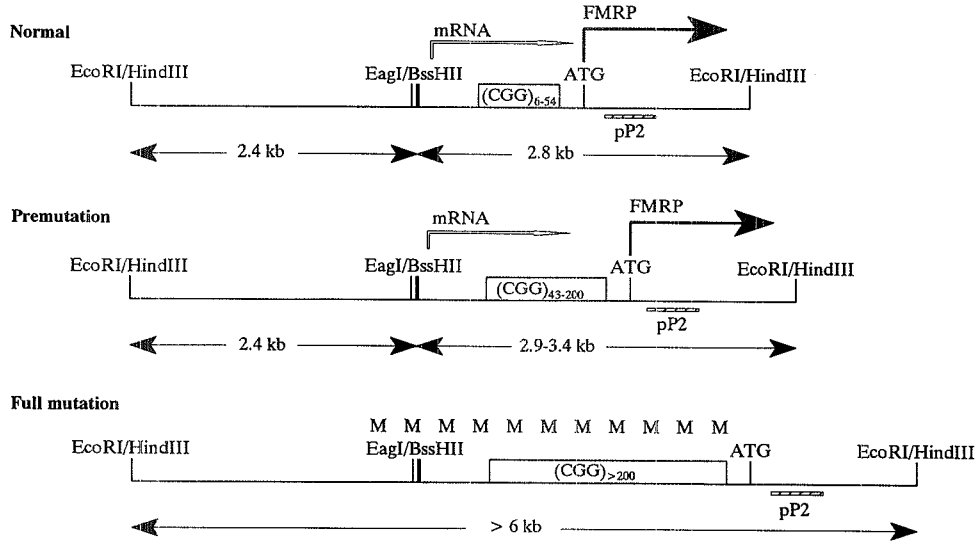

Figure 1.4 Schematical representation of the CGG repeat containing region of the *FMR1* gene and the molecular basis of the fragile X syndrome. Expansion of the repeat results in increased sizes of the DNA fragments obtained after digestion (depicted in kb). Methylation (M) of the promoter region on full mutation alleles results in the absence of *FMR1* transcription and translation. The probe pP2 is frequently used in determining the CGG repeat length in Southern blot analysis (128).

emphasizes the importance of analyzing the methylation pattern in the molecular diagnosis of the fragile X syndrome.

Recently, a new diagnostic method has been described, based on the absence of FMRP in fragile X patients. Using an antibody test on blood smears, FMRP can be detected in the lymphocytes of control individuals and not of fragile X patients (15). This test can not be used for premutation carrier detection, since premutation alleles produce normal amounts of FMRP (143). However, female carriers of the full mutation can be detected, since approximately 50 % of the cells will not express FMRP due to the (random) X-inactivation of the normal X chromosome (15).

The antibody test has also been performed successfully in prenatal diagnosis on chorionic villi of a 12 week old pregnancy. Cells in the cytotrophoblast, the second most outer layer of the chorionic villi have a very strong FMRP expression in controls. Chorionic villi of affected fetuses remain unstained due to the absence of FMRP as a result of the methylation of the *FMR1* promoter region (R. Willemsen, *pers. comm.; unpublished results*). Larger numbers of samples, taken at different times after gestation, need to be tested to both validate the method as well as to determine the time after conception at which the chorionic villi should be taken for a reliable prenatal diagnosis. This latter is related to the exact timing of methylation in the chorionic villi (93,130,132,136).

* 1.7 Other trinucleotide repeat disorders

The dynamic mutation, the expansion of a trinucleotide repeat from one generation to the next, is not unique for the fragile X syndrome. Since the identification of the *FMR1* gene and the CGG repeat amplification in 1991, several other disorders have been found to be associated with an expansion of a trinucleotide repeat. Interestingly, thus far only triplet repeat expansions are found to be associated with disorders as opposed to di- or tetranucleotide repeats. The various disorders can be distinguished into different categories according to the nature of the triplet repeat that is amplified, and its location within the gene: 1) the fragile sites, with an CGG repeat; 2) neurodegenerative disorders, with an CAG repeat; 3) Friedreich's ataxia with an GAA repeat and 4) myotonic dystrophy with an CTG repeat (see Figure 1.5 and Table 1.1). One of the most characteristic features of the neurodegenerative disorders and myotonic dystrophy is the phenomenon known as anticipation. Anticipation is the increase in severity of the clinical features and a decrease in the age of onset of the disease in successive generations within a family. In the fourth category, the fragile sites, most clear for the fragile X syndrome, anticipation is not a very distinct feature, although the risk of being affected increases in successive generations in a fragile X family; a phenomenon designated the 'Sherman paradox', which was explained in *Chapter 1.4.*

1.7.1 Fragile sites

Besides the fragile site at Xq27.3, four other rare folate-sensitive fragile sites have been cloned (see Table 1.1). Two are located on the X chromosome in the proximity of FRAXA and are designated FRAXE and FRAXF, and two are autosomal: FRA11B and FRA16A. Besides the fragile X syndrome, FRAXE is the only other fragile site associated with a specific clinical phenotype, namely mild mental retardation (55-58).

FRAXE

FRAXE is located approximately 0.6 Mb telomeric from FRAXA, (55,56) and has been found to contain a GCC repeat. Normal individuals have 6-25 copies of the repeat, whereas fragile X expressing individuals have over 200 repeats, resulting in methylation of the CpG island (57).

The gene associated with the CpG island is called *FMR2*, as it is the second gene associated with fragile X expression-linked mental retardation. The 9.5 kb mRNA transcript is mainly detected in placenta and brain, more specifically in the hippocampus and amygdala (144-146). *FMR2* encompasses over 600 kb, and the first intron is extremely large, approximately 150 kb. The GCC repeat is present in the 5'untranslated region of *FMR2* transcript (146). The *FMR2* protein is relatively large (1,276 amino acids) and is highly enriched in serine, proline and threonine. The gene bears no homology with the *FMR1* gene but is homologous to a gene thought to have transcriptional activity, the *AF-4* gene. In fibroblasts of individuals with a methylated GCC repeat, and associated CpG island,

transcription of the *FMR2* mRNA is absent (145,146).

FRAXF

FRAXF is located 1-2 Mb distally from the FRAXA locus (59) and a GCC repeat was detected in a 5 kb *Eco*RI fragment (60,147). Normal alleles encompass 6-29 repeats, fragile site containing chromosomes have a 300-500 repeat size, coinciding with methylation of the GCC repeat containing region. An intermediate, unmethylated form can also be detected. The GCC repeat was located in a cDNA that contained an open reading frame in both orientations, one of which translates into a polypeptide with similarity to the mouse homeobox protein *engrailed*. The expression of this polypeptide has not yet been studied further.

FRA16A

The first cloned autosomal fragile site, FRA16A is located at 16p13.11. The site is associated with an unstable CCG repeat, located near a CpG island of a yet unknown gene (148). Normal alleles vary from 16-49 repeats. Individuals with cytogenetic expression of the

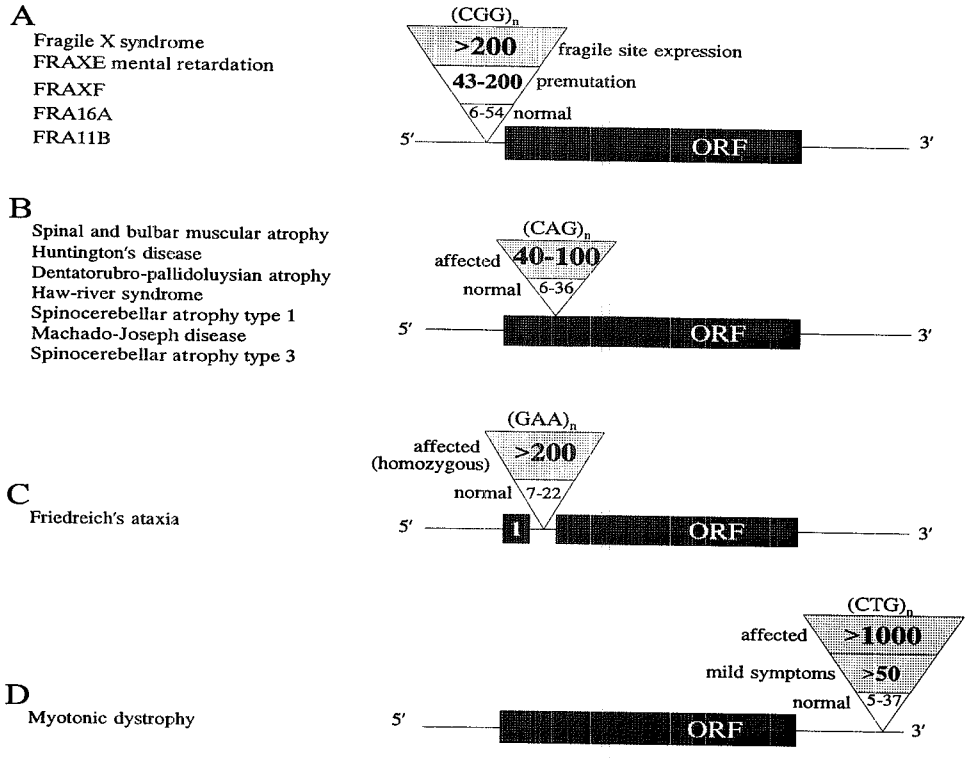

Figure 1.5 Genetic disorders associated with trinucleotide repeats. Location and different sizes of the trinucleotide repeats in the fragile site syndromes (A), neurodegenerative disorders (B), Friedreich's ataxia (C) and myotonic dystrophy (D) are schematically depicted. (Adapted from Oostra and Halley (189)). For more details see table 1.1.

Table 1.1. Some characteristic features of trinucleotide repeat disorders.

Disease	OMIM[§]	Gene	Location	Triplet	Site	Repeat length normal	Repeat length affected	P/M[$]	Effect[¶]	References
FRAXA	309550	*FMR1*	Xq27.3	CGG	5'UTR	6-54	>200-m[@]	M	loss	(12,16,189)
FRAXE	309548	*FMR2*	Xq28	GCC	5'UTR	6-35	>200-m[@]	M	loss	(55,57,58,190)
FRAXF	300031	?	Xq28	GCC	?	6-29	>300-m[@]	?	?	(59,60)
FRA16A	-	?	16p13.11	CCG	?	16-49	>1000-m[@]	?	?	(148,191)
FRA11B	600651	*CBL2*	11q23.3	CCG	5'UTR	8-14	>100-[@]	?	?	(149)
SBMA	313200	*AR*	Xq12	CAG	ORF	12-30	40-62	P	?	(150,173)
HD	143100	*huntingtin*	4p16.3	CAG	ORF	6-34	36-100	P	?	(151, 174-177)
DRPLA	125370	*atrophin*	12p12	CAG	ORF	7-25	49-75	P	?	(178-181)
HRS	140340	*atrophin*	12p12	CAG	ORF	7-25	63-68	-	?	(153,179)
SCA1	164400	*ataxin*	6p22-p23	CAG	ORF	25-36	40-81	P	?	(182-184)
SCA3	183085	*MJD1a*	14q24.3-32.1	CAG	ORF	12-28	67-78	?	?	(185,186)
MJD	109150	*MJD1a*	14q24.3-32.1	CAG	ORF	13-36	68-82	P	?	(154,187)
FRDA	229300	*X25*	9q13	GAA	intron	7-22	>200	-	loss	(162)
DM	160900	*DMPK/*	19q13.3	CTG	3'UTR	5-37	>50 />1000	M	?	(163-167, 188)
		DMAHP	19q13.3	CTG	5'UTR	5-37	>50 />1000	M	?	(168)

§: OMIM number (3), -: absent, ?: not known, m[@]: methylation; $: transmission of the (most severe phenotype of) the disease occurs preferably either paternally (P) or maternally (M), ¶: effect of expanded repeat on the function of the gene product.

FRA16A site have an increased repeat of 3-5.7 kb (1000-1600 repeats), coinciding with methylation of the adjacent CpG island. One non-expressing carrier has been detected with an amplification of 160 repeats and without methylation.

Expression of the fragile site is not associated with a clinical phenotype, but this might be due to heterozygosity; homozygotes for the FRA16A expression have not yet been detected. In contrast to the other fragile sites, transmission does not appear to be maternally biased, as males can transmit a full mutation.

FRA11B

The CCG repeat associated with FRA11B is located at chromosome 11q23.3, in the *CBL2* gene, an oncogene involved in a receptor tyrosine kinase signal transduction pathway, (149). Over 70 % of the normal alleles consist of 11 CCGs. A premutation allele below 100 repeats was detected in an individual without cytogenetic expression. All individuals tested with over 100 CGGs had cytogenetical expression of the FRAX11B fragile site, and this coincides with methylation of the CpG island.

A chromosomal deletion, resulting in Jacobson's syndrome (11q$^-$) is located in the FRA11B region. This deletion starts within 20 kb proximal to the repeat, but not in the CCG repeat itself. In two families studied, the deletion was found on the chromosome 11 containing the expanded CCG repeat. This suggests an association between the presence of an expanded repeat and the formation of the deletion (149).

The finding of only CGG (GCC) repeats in all fragile site disorders and the lack of association with a fragile site of the other triplet (CAG/CTG) repeats, implies that the fragile site is induced by specific properties of the CGG repeat. The cytogenetic expression of the FRAXA site in two brothers with an unmethylated full mutation sized allele indicates that the expression of the fragile site is not dependent on methylation (140).

1.7.2 Neurodegenerative disorders

Thus far, seven neurodegenerative disorders have been identified to have an expansion of an CAG repeat: spinal and bulbar muscular atrophy (SBMA), Huntington's disease (HD), dentatorubro-pallidoluysian atrophy (DRPLA), which is allelic to Haw-River syndrome (HRS), spinocerebellar ataxia type 1 (SCA1) and 3 (SCA3), which is allelic to Machado-Joseph disease (MJD). All these disorders are progressive and are mainly characterized by the degeneration of specific groups of neurons. Some characteristics of these disorders are summarized in Table 1.1 and figure 1.5

In all seven disorders there is a correlation between the length of the CAG repeat and both the age of onset and the severity of the disease: the longer the repeat, the earlier the age of onset in the different disorders (150-154). The increase in repeat length in successive generations in a family, explaining the phenomenon of anticipation.

Remarkably, in all five genes involved in the disorders mentioned above, the CAG repeat resides in the coding region, more specifically in the same reading frame, resulting in a poly-

glutamine stretch. In affected individuals normal protein translation is encoded by the unaffected allele, but also the abnormal genes are translated and result in a protein of higher molecular weight because of the CAG repeat expansion. Poly-glutamines are found in several transcription factors, but, since most of the proteins involved in these neurodegenerative disorders, are detected in the cytoplasm rather than in the nucleus, it seems unlikely that they have a regulating function (155).

The neurodegenerative disorders associated with an expanded CAG repeat are likely to be caused by a dominant effect of the mutated protein. Absence of the androgen receptor (*AR*) does not result in the clinical phenotype of SBMA but leads to androgen resistance (156). Similarly, individuals, lacking one normal copy of the *huntingtin* gene as a result of a translocation do not develop HD, despite a 50% reduction in the amount of *huntingtin* (157). This implies a dominant effect of the mutated protein. The same is postulated for the other neurodegenerative disorders with expanded CAG repeat.

The different disorders are characterized by the degeneration of specific groups of neurons. As all these disorders are characterized by an expanded CAG repeat, this may suggest that the protein with an enlarged poly-glutamine stretch causes this cell death. The exact mechanism by which this occurs is unknown. Different protein-protein interactions, as a result of the expanded polyglutamine stretch have been proposed. A huntingtin associated protein 1 (*HAP1*), was found to bind to the mutated form of huntingtin, with a positive correlation between repeat length and the strength of binding (158). Although nothing is known about the function of the *HAP-1* gene product or on the effect of the expanded CAG repeat on *HAP-1*, either activation or inactivation, it is remarkable that *HAP-1* expression is restricted to brain. This might explain that the mutated huntingtin protein, expressed in several tissues, only has an effect in brain cells, as these are the only cells expressing the *HAP-1* gene (158).

Recently another protein, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), was found to bind to the polyglutamine stretch of the normal *HD*, *DPRLA*, and *SCA1* protein. Additional studies demonstrated that GAPDH enzyme activity decreased as a function of disease-associated polyglutamine lengths. GAPDH is a multifunctional enzyme involved in energy metabolism. As impairment of energy generation has been considered to be a plausible mechanism for the pathogenesis of HD, GAPDH is considered a good candidate protein for involvement in the neurodegenerative disorders (159,160).

The finding that the poly-glutamine stretch is translated into protein facilitates the search for other genes that may be associated with neurodegenerative disorders, especially those that display anticipation. In line with this, Trottier *et al.* generated antibodies directed against a glutamine stretch, which specifically detects proteins containing an expanded glutamine stretch. It was demonstrated that in patients with ScaII and ADCA type II, another autosomal dominant cerebellar ataxia with retinal degeneration, a protein is recognized by antibodies directed against an expanded glutamine stretch, suggesting that these disorders are associated with an expanded CAG repeat as well (161).

1.7.3 Friedreich's ataxia

Recently a new trinucleotide repeat was identified in another spinocerebellar degenerative disorder (162). Unlike the other disorders, the GAA repeat is located in the first intron of the gene *X25* (Figure 1.5). Expansion of the repeat to over 200 repeats is likely to result in an abnormal transcription or RNA processing. Patients, homozygous for the expanded repeat, show extremely low amounts of *X25* mRNA, whereas individuals heterozygous for the expanded repeat are unaffected. Because of the mutation being recessive, anticipation is not present in this disorder.

1.7.4 Myotonic dystrophy (DM)

DM is so far the only disorder of which the molecular basis resides in an expanded CTG repeat (see Table 1.1, Figure 1.5). The mechanism by which the expanded CTG repeat results in the clinical phenotype of DM is unknown. The CTG repeat resides in the 3' UTR of a putative protein kinase gene, designated *DMPK* (163,164). The role of the *DMPK* gene in the clinical manifestation of DM is unclear since both decreases and increases in *DMPK* mRNA levels in different cells have been described (165-167).

Recently, a second gene was isolated immediately 3' of *DMPK*, in the opposite orientation (168). The gene, *DMAHP* for DM-associated homeodomain protein, has a large CpG island in which the unstable CTG repeat resides. However, the effect of the expanded CTG repeat on expression levels of this *DMAHP* has not yet been determined.

Another protein has been identified that can bind to the mRNA, containing the CUG repeat. It is postulated that binding of this CUG binding protein (*CUG-BP*) to the expanded CUG repeat, in the mRNA of either *DMPK* or *DMAHP* alters the function of the *CUG-BP*, resulting in the DM phenotype (169).

Trinucleotide repeats are not uncommon in the human genome. Both database searches and screening of cDNA libraries revealed the existence of numerous polymorphic trinucleotide repeat containing cDNAs (170,171). The presence of expanded repeats in the genome has also been demonstrated: using RED, for repeat expansion detection, a long CTG repeat, located on chromosome 18, was identified in three independent families (172). Although this expansion was not yet related to a clinical phenotype, it is likely that in the future more human inherited diseases, not only those exhibiting anticipation but possibly also recessive disorders, will be identified to be caused by the expansion of a trinucleotide repeats.

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Chapter 2

Characterization of the CGG repeat containing region

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The identification of the *FMR1* gene elucidated the peculiar mode of inheritance in the fragile X syndrome and provided new opportunities in genetic counseling, carrier detection and (prenatal) diagnosis. The discovery that a new mutational mechanism, a trinucleotide expansion, causes the fragile X syndrome, however, has also raised many new questions on how and when this CGG repeat expansion occurs. The scope of this thesis was to study this mutational mechanism that leads to the mutated *FMR1* gene.

* 2.1 Mutations in the *FMR1* gene other than the expansion of the CGG repeat

One of the questions during and after the cloning of the *FMR1* gene was whether the fragile X syndrome is caused by the absence of expression of the *FMR1* gene only, or whether other genes could be involved as well. The methylation, associated with the full mutation, might not be restricted to the promoter region of the *FMR1* gene, but might also affect other genes in the vicinity as well. The identification of different point mutations and small deletions in the *FMR1* gene in patients expressing the fragile X clinical phenotype but without the full mutation allele, strongly suggested that the fragile X syndrome is a single gene disorder (115,121,192,193; *Publication 4.1).*

Although most of the fragile X patients with a deletion or point mutation in the *FMR1* gene were detected in sporadic fragile X patients, we had the opportunity to study a family, in which 11 individuals carried a 1.6 kb deletion (115; *Publication 4.1*). In this family, all (four) males with the deletion did not express the *FMR1* mRNA and all displayed the fragile X clinical phenotype. Sequencing the breakpoint of this deletion revealed that the deletion was localized immediately 5′ of the CGG repeat with the 3′ endpoint of the deletion falling within the CGG repeat. We demonstrated that the remaining repeat consisted of approximately 45 CGGs, and was not interrupted by AGG triplets, as was found in the originally published *FMR1* cDNA (12). Considering the size and configuration of the CGG repeat in this family, it seemed likely that prior to the occurrence of the deletion an expansion of the repeat had occurred. Transmission of the deleted allele was demonstrated to be unstable, suggesting that the remaining repeat was in the premutation range (*unpublished results*).

Besides the 1.6 kb deletion, eleven other deletions of the *FMR1* gene have been found in male fragile X patients, varying in size from 0.4 kb (193) to over 13 Mb (194). Three different kinds of deletions have been described; encompassing only a part of the *FMR1* gene (193), part of the *FMR1* gene and additional flanking sequences (115,195-198), or encompassing the entire *FMR1* gene plus flanking sequences (194,199-201). A schematic representation of the deletions and point mutations detected so far in fragile X patients is depicted in figure 2.1.

Although most patients with an *FMR1* deletion have the classical fragile X phenotype, some atypical clinical features were present in the patients in which over 9 Mb was deleted, suggesting that in these cases one or more other genes were deleted as well (194,201). A number of deletions encompassing the Xq27.3 region have also been described in females. It has been shown that these individuals had a skewed X inactivation pattern, with the deleted X chromosome being preferentially active. Two females were found to have a mild fragile X

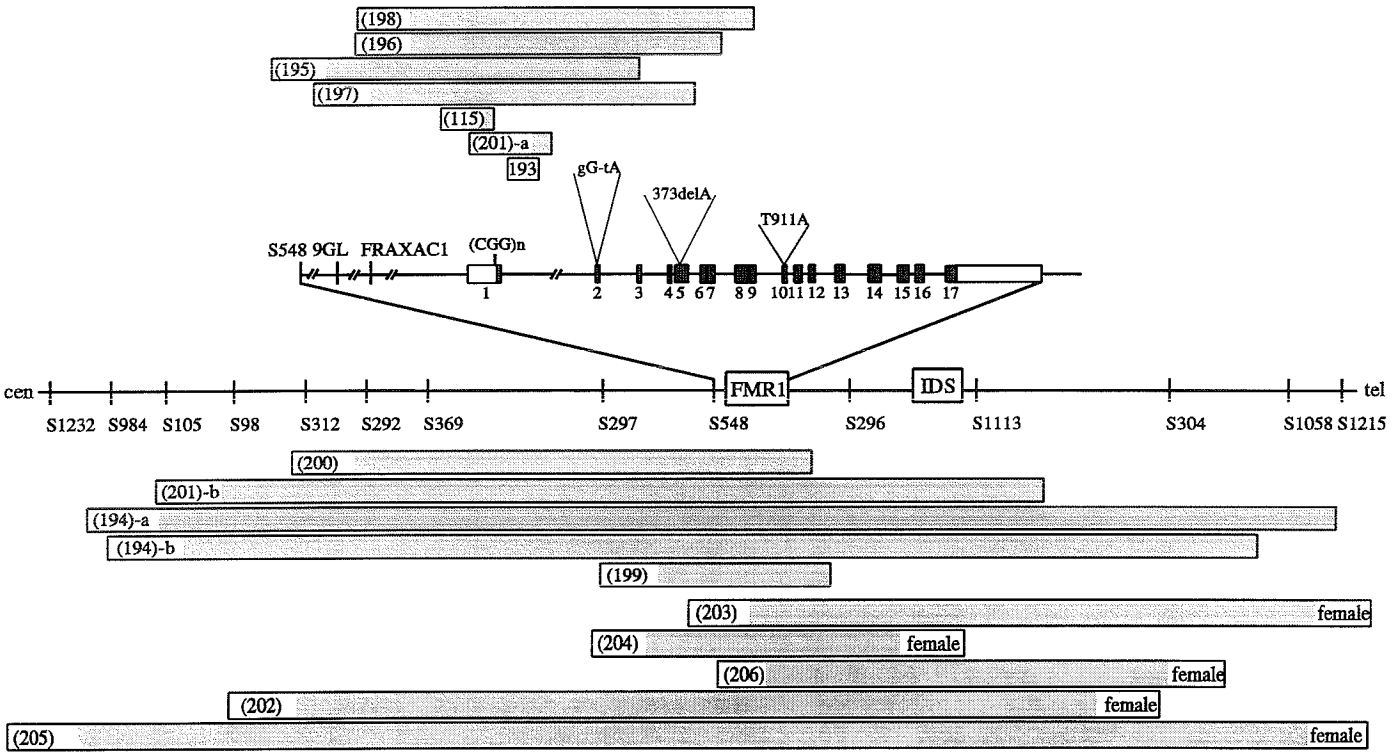

Figure 2.1 Mutations in the *FMR1* gene. Schematic representation, not drawn to scale, of the *FMR1* gene and the flanking sequences at Xq26-Xq28. Numbers 1-17 denote the exons; the S-numbers refer to markers. Deletions are depicted as shaded boxes, with the number between brackets representing the references.

clinical phenotype (202,203). In three other reports (204-206), females expressed symptoms of either Hunter syndrome as a result of the deletion of the *Idunorate Sulfatase* (*IDS*) gene, or myotubular myopathy (MTM). Nothing was mentioned about the presence of clinical features of the fragile X syndrome.

So far, three fragile X patients have been described with an unexpanded CGG repeat and normal *FMR1* mRNA expression but with a point mutation within the *FMR1* gene instead (Fig. 2.1). A deletion of an adenosine residue at position 373 in the open reading frame (when counted from the translation start codon) in exon 5 altered the reading frame, which resulted in a premature translation stop 66 amino acids later (192). A GG->TA substitution at the genomic position 23714-23715 (GSDB Acc# L69074) occurred in a splice acceptor site in exon 2 and resulted in the splicing out of exon 2 alone or together with exon 3. The absence of exon 2 resulted in a frame shift and a premature translation stop 3 amino acids into exon 3, whereas the lack of both exon 2 and 3 kept the reading frame intact but removed 49 amino acids. Protein analysis revealed that both patients completely lacked FMRP; no truncated form of FMRP could be detected (192). The third point mutation within the *FMR1* gene, a T911A substitution, is the only mutation known to result in the expression of FMRP (121). The T911A alteration results in a substitution of Ile304 by an Asn, in the second KH domain leading to a protein with a reduced RNA binding capacity (103,122).

In addition to the mutations within the open reading frame of the *FMR1* gene, recently two mutations in the promoter region of the *FMR1* gene have been described (207): 1) a C-G substitution at position 2436 of the pE5.1 (208), resulting in the elimination of the *Eag*I restriction site, and 2) an GGC insertion, at position 2432. The GGC insertion was demonstrated to impair the expression of FMRP, whereas the effect of the C->G substitution has not yet been tested. It is unlikely that this latter mutation is a polymorphism, since it has not been found in several thousands of patients analyzed for diagnostic purposes.

* 2.2 Mitotic instability

Alterations in the length of the CGG repeat do not only occur during transmission from parents to offspring (germline instability), but also within an individual (somatic instability). Patients with a full mutation have different repeat lengths in different cells within the same tissue, which is clearly visible as a smear on Southern blot analysis. Several studies have indicated that this mosaic pattern is not caused by a permanent mitotic instability. Firstly, the mosaicism is established early in embryonic development, as the mosaic pattern is already found to be identical in different tissues derived from fetuses (132,195,209). This identical pattern can already be detected as early as 13 weeks after gestation.

Secondly, a number of monozygotic twins have been shown to have an identical pattern of full mutation repeat lengths (132,210), although Kruyer *et al.* described a monozygotic twin with a full mutation, and the twin brothers displayed a clear difference in repeat length (211). This discrepancy can be explained by a different timing of separation of the monozygotic twins. In the first reports the twins may have separated after the establishment of the mosaic

pattern, whereas the monozygotic twin with the difference in repeat lengths may have been separated before the mitotic expansion in the somatic tissue occurred. As the latest day at which twins can be separated is 13 days after gestation, the mosaic pattern is likely to established before that time.

Thirdly, we characterized the instability of the CGG repeat length in an adult fragile X male and detected that the mosaic pattern in several different tissues were similar (212; *Publication* 4.2). This indicates that the mosaic pattern, established early in embryonic development does not change during adult life.

A fourth argument in favor of a non-permanent mitotic instability of the CGG repeat resulted from *in vitro* studies. The progeny derived from one single cell had the same repeat length after at least 22 rounds of DNA replication, thereby demonstrating mitotic stability (195). This is in striking contrast to the DM CTG repeat: in a similar *in vitro* study, the CTG repeat increased in length after 15 to 45 cell doublings, thereby displaying a continuous mitotic instability (213). This difference in mitotic instability between the CTG and CGG repeat was also observed early in embryonic development. A difference in mosaic pattern of the CTG repeat in different fetal tissues could only be demonstrated in a 16 week old fetus, and not in the different tissues from a 13 week old fetus (213), this in contrast to the *FMR1* CGG repeat. In addition, the mosaic pattern of the expanded CTG repeat (214), but not the expanded CGG repeat (*Publication* 4.2) differs more between different the tissues with an increasing age of the patients.

It can be postulated that the difference in somatic instability is caused by differences in methylation. Methylation of the *FMR1* full mutation was suggested to result in a somatically stable CGG repeat, whereas this stabilizing effect of methylation is absent on the DM CTG repeat (213). It is not known at which gestational age full mutation alleles in fragile X fetuses are methylated.

Variation in repeat length within an individual has also been described for DRPLA and HD. Especially in sperm cells but also in specific regions in the brain a larger CAG repeat was detected as compared to other tissues of the same patient. Whereas the severity of the diseases is correlated with repeat length, no correlation could be found between the observed variation between the different regions in brain and the neuropathological involvement (180,215).

Besides the extensive somatic instability within the full mutation range, a second form of mosaicism exists. In more than 40 % of the patients with a full mutation in blood lymphocytes a premutation is present as well, as can be detected by either Southern blot or PCR analysis (13,14,212,216; *Publication* 4.2). It is possible that the number of mosaic patients is even higher but that the percentage of cells with a premutation is below the detection level of the techniques used. We have reported a fragile X male with a full mutation in all tissues as determined by Southern blot analysis (212; *Publication* 4.2). However, by immunohistochemical staining for the *FMR1* protein approximately 1% of the cells was found to be positive. Subsequent, extensive, PCR analysis, confirmed the immunohistochemical finding, which appeared to be due to the presence of a premutation in a small percentage of cells.

Remarkably, the size of the CGG repeat in somatic cells with a premutation is clearly distinguishable from that in sperm cells of this patient, being 160 and 60 repeats, respectively (*Publication* 4.2).

Mosaicism is not only observed with full mutation alleles, but is also detected within the premutation alleles. Fu *et al.* described both a normal transmitting male in his blood lymphocytes, with at least 5 different repeat lengths, ranging from 12 to 116 CGGs, and a female with two different premutation alleles, containing 66 and 80 CGGs. This indicates that alleles in the premutation range may also be somatically unstable (14). Additionally, patients have been described to be mosaic for a full mutation in combination with either a normal allele (217,218), or deletions of the CGG repeat and approximately 100-500 bps of flanking sequences (198,219-223; *Publications 4.3 and 4.4*). The mechanism(s) resulting in the presence of either a premutation, normal allele, or deletion in combination with a full mutation, will be discussed in *Chapter* 3.3.

Instability in the CGG repeat containing region

The existence of deletion mosaics, and of patients with a deletion in the CGG repeat containing region only, indicates that the instability of the repeat is not restricted to the CGG repeat itself, but can extend to the flanking sequences as well. Thus far, 14 deletions in the CGG repeat containing region have been described and they are schematically depicted in figure 2.2. Most of these deletions [1-4, 6, 7, 8, 11, 12, 14, and 15] were found in males, mosaic for a deletion in 5-40 % of their blood lymphocytes and a full mutation in their remaining lymphocytes. Deletion 13 was detected in 85 % of a patient's fibroblasts (222). The mildly mentally retarded patient, in which deletion 10 was identified, was also mosaic with a normal allele in 60 % of the cells (198). Deletion 9 was detected in a family, in which all 4 males with the deletion were found to be affected (115; *Publication 4.1*). The two deletions on both sites of the CGG repeat depicted as deletion 5, were the result of a cloning artefact; the YAC XTY26 was subcloned from a hybrid cell line (X.3000.1), that contained a fragile X chromosome with a repeat length of approximately 350 triplets. Sequence analysis of the YAC revealed only 43 CGGs, indicating that most of the repeat had been deleted (75), in addition to some flanking sequences (14). Thus the deletion may also occur in yeast.

Interestingly, the 5′ endpoint of 10 out of 15 deletions reside within an a 52 bp interval, approximately 75 bp upstream of the CGG repeat. We therefore suggested a putative hotspot for deletions 5′ of the CGG repeat (*Publication 4.3*). An 8 bp Chi-like sequence (5′-GGTGGAGG-3′) is located immediately adjacent to the 5′ hotspot region (Fig. 2.2). The Chi-octamer (5′-GC[A/T]GG[A/T]GG-3′), is frequently found within hypervariable mini satellites and in the vicinity of translocations and is thought to be involved in recombination and replication slippage (224,225). This sequence could cause a DNA break near the CGG repeat, and subsequent repair of this break could be hampered by the presence of an expanded CGG repeat leading to mispairing and subsequently to a deletion.

At the 3′ part of the repeat another hotspot appears to be present, with 9 out of 15 deletions having the 3′ breakpoint residing within 48 bp. At the 3′ site of the CGG repeat another Chi-like sequence is present (5′-GCTGGTGGT3′), located 20 bp more downstream of

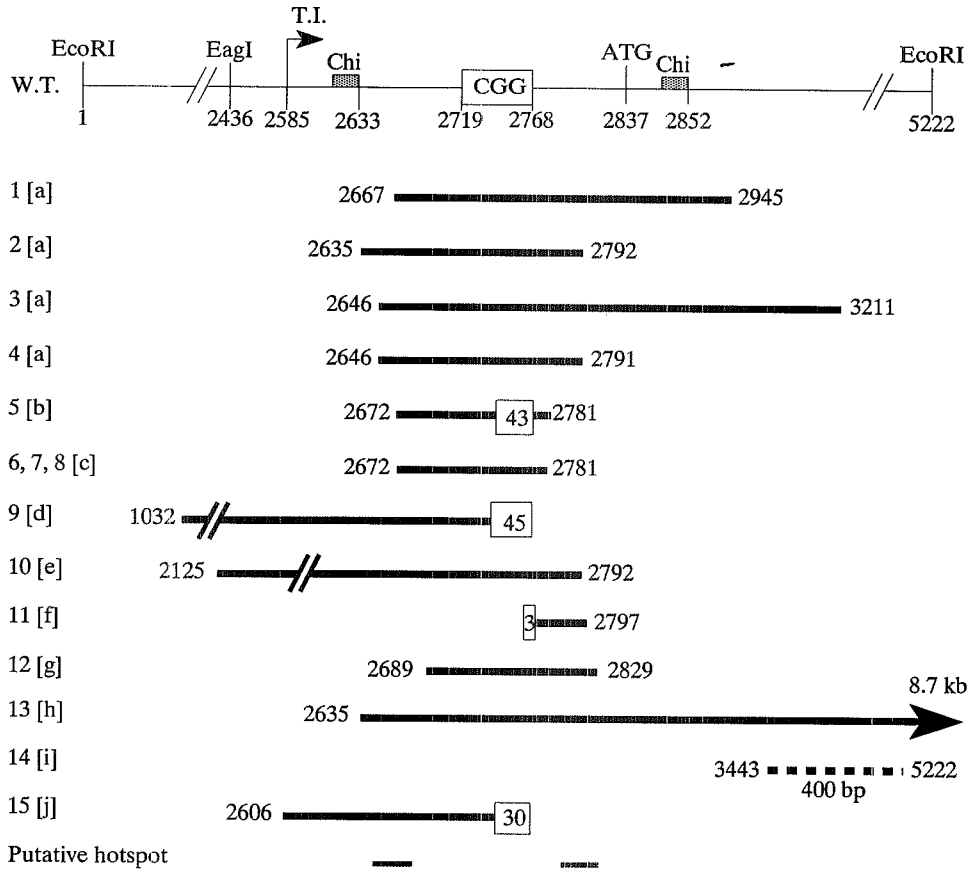

Figure 2.2 Schematic representation, not drawn to scale, of the deletions in the CGG region described thus far. Numbers indicate the position in the pE5.1 sequence (208). Boxed numbers boxes denote the number of CGGs. WT: wildtype; T.I.: translation initiation site; ATG: startcodon for translation. The deletions were obtained from the following references: [a]:(220); [b]:(75); [c]:(234); [d]:(115); [e]: (198); [f]:(223), [g]:(221), [h]:(222), [i]:(193), [j]:(218).

the 3′ hotspot region. Although the significance of Chi-like elements is unclear, it might be speculated that the presence of the two Chi-like elements on both sites of the CGG repeat result in an increased instability in this CGG repeat containing region.

The exact mechanism by which the deletions of the CGG repeat and flanking sequences have occurred is not clear. The presence of short repeats in the majority of patients is similar to previously described 2-6 bp repeats flanking deletions (226,227), and it is assumed that mispairing between the repeats during replication, due to the presence of an expanded, unstable CGG repeat or the Chi-like element, results in a single stranded loop formation. Excision of this loop removes one of the repeats and the intermediate sequence, resulting in the deletion. Good examples of this slippage mispairing mechanism are deletions 5 (5′ part)

and 6-8, were the deletion breakpoint are flanked by repeats of 11 bp and 6 bp, respectively. The other deletions are flanked by either 4 [deletion 13], 3 [deletions 10, 11], or 2 bp [deletions 2, 3, 9] repeats, whereas deletions 1, 12, and 15 are not flanked by any repeated sequence.

One deletion [4] is flanked by an inverse repeat: 9 out of 10 bp are complementary, which may align together in a quasi-palindromic manner, resulting in the formation and excision of the loop (228,229).

* 2.3 *FMR1* promoter activity

Not much is known about the promoter of the *FMR1* gene, besides the finding that methylation of the promoter region, associated with the full mutation in fragile X patients, results in the silencing of the *FMR1* gene. Hergersberg *et al.* (230) used a fusion gene, containing a *Eco*RI-*Nhe*I fragment (position 1-2820 of the pE51 sequence) fused to a LacZ reporter gene in order to study the expression pattern of the *FMR1* gene in mice. The expression pattern was identical to that in previous *in situ* hybridization studies (112,113) indicating that the 2.8 kb fragment contains all sequences required for normal *FMR1* mRNA expression. Protein and/or mRNA analysis of patients with deletions in this region may help to identify important regulatory sequences. Of the patients with a deletion in the *FMR1* promoter region, only three could be tested on the expression of *FMR1* mRNA. One patient [del. 11] (Fig. 2.2) was found to have expression of *FMR1* mRNA, and using immunocytochemical staining of a blood smear, FMRP was detected in 28 % of his blood cells. Based on the Southern blot analysis, the deletion was estimated to be present in approximately 25-30 % of the patient's blood cells. As this amount is very similar to the percentage of DNA with the deleted fragment, it is very likely that the cells expressing FMRP are the cells with the deletion (223; *Publication 4.4*). In two other patients, [deletions 9 and 10] no *FMR1* mRNA could be detected (115,198; *Publication 4.1*), which may be explained by the deletion resulting in the absence of the putative transcription initiation site of the *FMR1* gene (231).

In three patients, the deletion encompasses the translation initiation codon ATG [deletions 1, 3, 13] and their cells are therefore unable to synthesize FMRP. It would be interesting to determine whether the other deletions can result in normal production of *FMR1* mRNA and protein. Using an *in vitro* expression system with the luciferase reporter gene and different fragments from the *FMR1* promoter region, Hwu *et al.* studied the promoter activity of *FMR1* (231). A 291 bp fragment, position 2311-2602, (208) was the minimal fragment which still had promoter activity. Determining whether *FMR1* mRNA is present in cells of the patients 2, 6-8, and 12 could verify whether the *in vitro* results can be extrapolated to the *in vivo* situation. The presence of normal protein expression in cells containing deletion 12 (223; *Publication 4.4*) confirmed that immediately 3' of the CGG repeat no regulatory sequences are present (223,231).

Expression of the *FMR1* gene is not only determined by the absence or presence of regulatory sequences in the promoter region, but is also influenced by the pattern of methylation. Until recently, an absolute correlation was detected between expression of *FMR1* mRNA and the absence of methylation of the restriction sites *Eag*I and *Bss*HII that represent the CpG island. We recently identified the only exception to this correlation thus far. Southern blot analysis of DNA derived from different tissues from a male fragile X patient who had died from a lung tumor has been performed (*Publication 4.2*). The tumor in this patient was found to have a premutation consisting of 160 CGGs and additional methylation analysis revealed that the *Eag*I, *Bss*HII and *Sac*II sites were methylated like the full mutation allele, present in the other tissues of this patient. Immunohistochemical analysis revealed that despite the methylation in the tumor cells, FMRP was expressed (212). Since the methylation sensitive restriction sites, used in determining the methylation status, may not represent the sites essential for silencing the *FMR1* gene, a high resolution methylation analysis is required to detect whether there are differences between the methylation pattern of the tumor cells and that of other cells with the full mutation allele. It is expected that in the tumor the essential CpGs are not methylated. This is currently under investigation.

* 2.4 The CGG repeat in germ cells

The timing of the repeat amplification from premutation to full mutation allele, remains one of the major unexplained issues in the fragile X syndrome. In order to answer the question whether the expansion occurs during the germ cell meiosis or early in embryonic development, germ cells needed to be studied.

Molecular analysis of fragile X families revealed that all males, with either a premutation or a full mutation allele in their blood cells, transmitted only a premutation to their daughters (13,28,102). Analysis of blood and sperm samples of several adult fragile X males demonstrated that, despite the presence of a full mutation allele in their somatic tissues, only a premutation was present in their sperm cells (101).

As the full mutation is only transmitted through females, it is possible that the expansion of premutation to full mutation allele occurs during female meiosis. To verify this hypothesis, female oocytes needed to be tested. However, for obvious reasons, it is difficult to obtain oocytes from female carriers. Recently, an ovary of a 26-week-old female fetus was studied (S.T. Warren, *pers. comm.*), after prenatal diagnosis had shown a methylated full mutation. In several tissues a methylated full mutation and a normal allele, derived from the normal second X chromosome were detected. At 26 weeks gestation, the cellular percentage of oocytes in an ovary is estimated to be approximately 30 % (139). Considering this percentage of oocytes in the ovary, the possible presence of a premutation allele in the oocytes should be detected by Southern blot analysis, as are premutation alleles in mosaic fragile X males. In DNA derived from the fetal ovary only the full mutation allele, and the normal allele derived from the second X chromosome, were found, whereas a premutation allele was not detected. The full mutation was found to be unmethylated in a percentage of cells equivalent to the

percentage of oocytes present in the ovary (S.T. Warren, *pers. comm.*). This suggests that in the oocytes an unmethylated full mutation is present.

We studied a second fetal ovary derived from a younger female fetus (17th week of gestation) that, by prenatal diagnosis, was shown to have a methylated full mutation. Again an unmethylated full mutation was detected, which was most likely derived from the oocytes (*unpublished results*), thus confirming that in contrast to sperm cells, a full mutation can be present in oocytes.

The two fetuses with a full mutation in their oocytes, were also found to have a full mutation in their somatic tissue, and is therefore not a proper representation of the situation in adult females carrying a premutation. In order to really study the timing of expansion of a premutation to a full mutation, oocytes are needed from a female carrying a premutation in her somatic cells, who can pass a full mutation to her offspring. Since these oocytes are difficult to obtain, we had to develop another (animal) model.

We generated transgenic mice, containing the fusion gene, consisting of the 2.8 kb *FMR1* promoter region and the reporter gene LacZ, as described by Hergersberg *et al.* (230). The repeat of this initial construct, consisting of 16 CGGs, was replaced by a repeat in the premutation range. Assuming that the mouse model resembles the human situation, expansion of the repeat from the premutation to a full mutation is expected to be followed by methylation of the promoter region of the *FMR1* gene (91,92). It may therefore be expected, that if the repeat in these transgenic mice expands to a full mutation, expression of the LacZ gene will be absent due to the methylation of the *FMR1* promoter.

In order to clone an expanded repeat, a premutation allele of a carrier was amplified. However, due to the high instability of an expanded CGG repeat in both PCR analysis and in *E. coli* bacteria, resulting in many regressions, cloning of a large repeat is very difficult. The CAG triplet, though present in some mammals (85), has never been detected in human CGG repeats (232-234). It is therefore likely that this CAG triplet originated during the cloning procedure, either in the *E. coli* bacteria, or as a PCR artefact. Two different repeat lengths were cloned into the promoter region:

A: $[(CGG)_{11}AGG(CGG)_{29}CAG(CGG)_8]$

B: $[(CGG)_{11}AGG(CGG)_{60}CAG(CGG)_8]$

In total, 5 transgenic mouse lines were obtained with these two constructs, two with the 29 pure CGGs and three with the stretch of 60 pure CGGs. However, after three and two generations, for the short and long constructs respectively, no alteration in repeat size has been detected (*unpublished results*). This indicates that these constructs are stably inherited in mice. Thus far, this mouse model cannot be used for studying the timing of the repeat amplification.

* 2.5 FRAXE mental retardation

We performed molecular studies in a large family in which a mild mental retardation, without an additional specific clinical phenotype, cosegregated with the FRAXE site. The

mode of inheritance of FRAXE is very similar to that of the fragile X syndrome. Repeat lengths in the normal range of 6-25 are stable, whereas the expanded repeats are unstable upon transmission. In addition, the fragile site is expressed cytogenetically when the repeat length exceeds 200 GCCs. Repeat lengths above 130 repeats are methylated in FRAXE patients (58; *Publication 4.5*).

Transmission of an expanded GCC repeat through females usually results in increases in repeat length, though reductions in size have been described in approximately 20 % of the transmissions. This in contrast to the fragile X syndrome were regression through female meiosis only occurs occasionally. Transmission through a male has thus far always resulted in a reduction of repeat length. In one FRAXE mentally retarded male the CCG repeat length in sperm cells was smaller than the repeat length in blood cells, though the presence of large expansions in a low percentage of sperm cells could not be excluded (*unpublished results*).

There are two striking differences in mode of inheritance between the fragile X syndrome and FRAXE mental retardation. The first discrepancy is the apparent absence of premutation alleles in FRAXE mental retardation. Thus far, no normal transmitting males and no allele sizes between 25 and 200 repeats have been described (58,190; *Publication 4.5*). It should be noted though, that FRAXE mental retardation occurs less frequent than the fragile X syndrome and that analysis of more families may lead to the identification of premutation alleles and normal transmitting males.

The second, more striking distinction between the fragile X syndrome and FRAXE, is that FRAXE mentally retarded males can have affected daughters with a methylated repeat above 130 GCCs, although in these daughters cytogenetic expression of the FRAXE site could not be detected (58; *Publication 4.5*).

Chapter 3

Discussion

In general, the identification of a gene, associated with a certain disorder, answers several questions about molecular genetics issues, but at the same time new questions may arise, as is illustrated after the cloning of the *FMR1* gene. Although the past few years a lot of information has been acquired about the nature of the *FMR1* protein and its subcellular localization, very little is understood about the exact function of FMRP. Therefore, the pathology of the fragile X syndrome, and the role of FMRP in this process is also still unknown.

The discovery of the expanded CGG repeat in the 5'UTR of the *FMR1* gene resolved the peculiar mode of inheritance of the fragile X syndrome, but raises the questions how and when the CGG repeat expands. In this chapter the recent experimental data and the relation to the remaining questions will be discussed.

* 3.1 Function of FMRP

Based on the identification of both a nuclear localization signal (NLS) as well as a nuclear export signal (NES) in FMRP, combined with the RNA-binding properties of FMRP and association of FMRP to ribosomes, a model for the function of FMRP may be postulated (S.T. Warren, *pers. comm.*). According to this model, FMRP is transported to the nucleus due to binding of an NLS receptor to the NLS in the N-terminal part of the protein. In the nucleus, FMRP assembles with newly synthesized RNA into ribonucleo-protein (RNP) particles and following recognition of the NES by the NES receptor, the complex is shuttled back to the cytoplasm. In the cytoplasm the FMRP-RNA complex assembles with the ribosome via RNA binding with the 60 S subunit (123,124; Dreyfuss, *pers. comm.*). FMRP might therefore play a role in the (initiation of the) translation machinery.

What remains unexplained, however, is why absence of FMRP is not associated with lethality, although one of the most important processes in the cell, the translation machinery is suggested to be impaired. Instead, the main effect of the lack of FMRP appears to be mental retardation, the pathogenesis of which is little understood. In normal individuals, FMRP is widely expressed and binds to approximately 4% of the total mRNA in brain (118,119). It is possible that the function of those mRNAs, that can be bound by FMRP, is only important in distinct cell types. The lack of transport and/or translation of these specific mRNAs due to the absence of FMRP, would then lead to mental retardation and other clinical manifestations of the fragile X syndrome, and not in a similar way to other pathological features in other tissues/organs.

Another explanation for the non-lethality of a lack of FMRP, might be that the function of FMRP can be taken over by other proteins in those cell types that are not involved in the pathological features of the fragile X syndrome. The *FMR1* gene is highly homologous with *FXR1* and *FXR2*, and appears to be a member of a new gene family (84,125,126). The three gene products bind tightly to each other, and are able to form homodimers and heterodimers, and it is also possible that FMRP forms multiprotein complexes with the two others proteins (125). Recently, it was demonstrated that FMRP is part of a 240 kD complex

(124), perhaps also consisting of *FXR1* and/or *FXR2*. Interestingly, both *FMR1* and *FXR2*, but not *FXR1* are expressed in brain of control individuals (126). It might be speculated that the absence of FMRP alone in this complex does not impair the functional capacity of the remaining complex, whereas the absence of the *FXR1* gene product in brain in combination with lack of FMRP due to the full mutation of fragile X patients, might result in a reduction, or total absence, of functional capacity of the complex. It should be noted that the complementation of FMRP by *FXR1* and *FXR2* is not associated with an enhanced expression of either gene; the expression level of both genes is unaltered in fragile X males lacking FMRP (84,126).

Males have been described with a fragile X clinical phenotype without a detectable alteration of the *FMR1* gene (235). Considering the interactions between the *FMR1*, *FXR1*, and *FXR2* gene products, it is possible that these fragile X patients have a mutation in either the *FXR1* or *FXR2* gene. On the other hand, the fragile X phenotype might be caused by a mutation in one of the target mRNAs that is bound by FMRP. If so, the absence of FMRP might cause an altered stability or functioning of one (or more) of these mRNAs, resulting in the fragile X phenotype. The availability of a knock-out mouse model (50) may offer new experimental possibilities to study the pathogenesis of the fragile X syndrome as a result of the lack of FMRP.

* 3.2 Genotype and phenotype

Before discussing the relationship between genotype and phenotype, it should be noted that the analysis of the CGG repeat containing region of fragile X patients (and normal individuals) is generally performed on blood lymphocytes, and not in brain, the relevant tissue affected in mental retardation. The situation in blood cells, and thus also the available experimental data, might not be representative for the situation in brain. For obvious reasons this is difficult to verify. Several studies have shown that the mosaic pattern, especially the methylation pattern, is very much alike in different tissues. It should be noted that in these studies DNA isolated from blood was not included (80,195,212; *Publication* 4.2).

In the brain of a fragile X male, with a premutation in approximately 1 % of his cells, we demonstrated that approximately 1 % of his neurons expressed FMRP, suggestive of the presence of a premutation allele in these neurons (*Publication* 4.2). Due to this similarity in DNA pattern in the different somatic tissues, the following discussion assumes that the DNA analysis as performed on blood lymphocytes resembles the situation in other somatic tissues, such as brain.

It is generally accepted that the absence of FMRP results in the fragile X clinical phenotype. A complete correlation exists between the presence of a methylated full mutation, the absence of FMRP and the clinical features. Unexplained, however, remains the occurrence of mosaic males, who carry both a full mutation and a premutation. Premutation alleles have been shown to result in a normal transcription and translation of the *FMR1* gene (79,80,94,143). As a consequence, these mosaic males express FMRP, though in a lower

number of cells than individuals with normal or premutation repeat sizes in all cells. The clinical phenotype of these males is comparable to males with a full mutation only (94,97,219,236), suggesting that in these mosaic patients the number of cells with a premutation is too low for normal cognitive functioning. In keeping with this, a male patient, mosaic for a full mutation and a deletion in 28 % of his blood lymphocytes, was recently described (*Publication 4.4*). Despite the fact that 28 % of these blood cells still express FMRP, the patient was found to be mentally retarded. Assuming that the situation in lymphocytes reflect the situation in brain, this would suggest that 28 % of the cells expressing FMRP is not sufficient for normal cognitive functioning.

In contrast, mosaic males have been described with an unmethylated and a methylated full mutation, and either a normal or less severe cognitive functioning. Rousseau *et al.* reported on a cognitive normal male with an unmethylated full mutation in 60% of his lymphocytes (102). Although no data were presented on the expression of FMRP, it is likely that these cells produce FMRP. Also normal males have been described with an unmethylated full mutation of up to 1500 CGGs, with FMRP production in all cells, though at a reduced level (140,237). It should be noted though that conflicting results, however, were reported by Feng *et al.* (238). Fibroblast subclones, derived from a (mildly affected) 19-month-old male, were demonstrated to have repressed FMRP translation if the repeat length was beyond 200 CGGs, despite the absence of methylation. However, since several methylation mosaic males were reported to display a less severe phenotype (102,141,142), it is most likely that alleles with an unmethylated full mutation can produce FMRP, though probably at a reduced level. These findings suggest that normal cognitive functioning requires either a normal expression of FMRP in a high number of cells, or a reduced expression of FMRP in all cells.

In this respect it is interesting to note that approximately 50-75 % of the females carrying a full mutation have some degree of mental impairment (97,239). In contrast, in other X-linked disorders, females are only occasionally affected. As this is usually caused by a skewed X inactivation with the majority of cells containing an active mutated X (202,204,206), it was suggested that a similar mechanism would result in affected fragile X females. In mildly mentally retarded females with a full mutation, the percentage of blood cells containing an active normal X chromosome was reported to be higher as compared to the percentage in affected fragile X females (239,240). This may suggest that random X inactivation, leading to 50 % of the cells expressing FMRP may not be sufficient for proper cognitive functioning. Therefore a relatively high percentage of females carrying a full mutation display some degree of mental impairment. This again confirms the necessity of a relatively high number of cells expressing FMRP for normal cognitive functioning. In contrast, in other X linked disorders only a low percentage of cells expressing the normal gene product might be required for normal functioning, resulting in only a low number of affected females. Since the degree of mental impairment in fragile X females is highly variable, some gradual effect may be present, with a correlation between the cognitive functioning and the percentage of cells still expressing FMRP.

The finding that, both normal or reduced FMRP expression in a high number of cells

(over 50 %) appears to be required for proper intellectual functioning has some important implications for therapy. In somatic gene therapy, or when it would be possible to administer FMRP directly, a large number of neurons will need to be targeted, to perhaps attain a proper cognitive functioning. As this will be very difficult, other forms of treatment need to be investigated.

* 3.3 Amplification of the CGG repeat

3.3.1 Timing of repeat amplification

One of the intriguing features of the mode of inheritance of the fragile X syndrome is the amplification of a normal sized CGG repeat to a premutation and a full mutation. The question how and when the repeat expands from premutation to full mutation is still unanswered. Two interesting observations have been made. First, in sperm of fragile X males with a full mutation in their somatic cells, only a premutation is present (101). Second, females with a full mutation can transmit a premutation to their children, though always in a mosaic way, i.e. in combination with a full mutation. Based on the presence of either a full mutation or a premutation allele in the female oocyte, two models have been postulated (Fig. 3.1).

The first model assumes that expansion of the CGG repeat occurs before or during female meiosis, resulting in an oocyte that contains a full mutation. After fertilization, the zygote divides, proliferates and differentiates to finally result in an embryo with a full mutation in all cells. However, regression of the repeat size may occur both in somatic tissue, thereby explaining the occurrence of mosaic fragile X males, and in male germ cells. In order to explain the presence of premutation alleles only in male germ cells, a selection mechanism must be present, favoring the spermatogonia with a premutation over those with a full mutation allele.

Indications for the existence of a selection mechanism were presented by Hansen *et al.* (241). They identified an allele of 200 repeats in a lymphoblastoid cell culture derived from a mosaic fragile X patient, and infer that this allele was derived from a subpopulation of lymphoblasts that were somehow selected during cell culture. Replication of full mutation alleles has been demonstrated to be delayed as compared to normal or premutation alleles (241). Male germ cells containing a premutation allele may therefore proliferate faster than those with a full mutation, thereby overgrowing the germ cells containing a full mutation.

Selection could also be based on another mechanism. Considering the high expression of FMRP in spermatogonia (80,114), it was postulated that FMRP is required for spermatogenesis. However, two findings argue against this hypothesis. First, a fragile X male with a deletion in the promoter region of *FMR1* resulting in the absence of *FMR1* mRNA was found to have children, with all daughters carrying the deletion (115; *Publication 4.1*). Second, *FMR1* knock-out mice, completely lacking FMRP, are fertile, indicating that FMRP is not essential for sperm development (50). What remains unclear, is why the selection mechanism is restricted to the male germ cells, and is not present in the female germ cells.

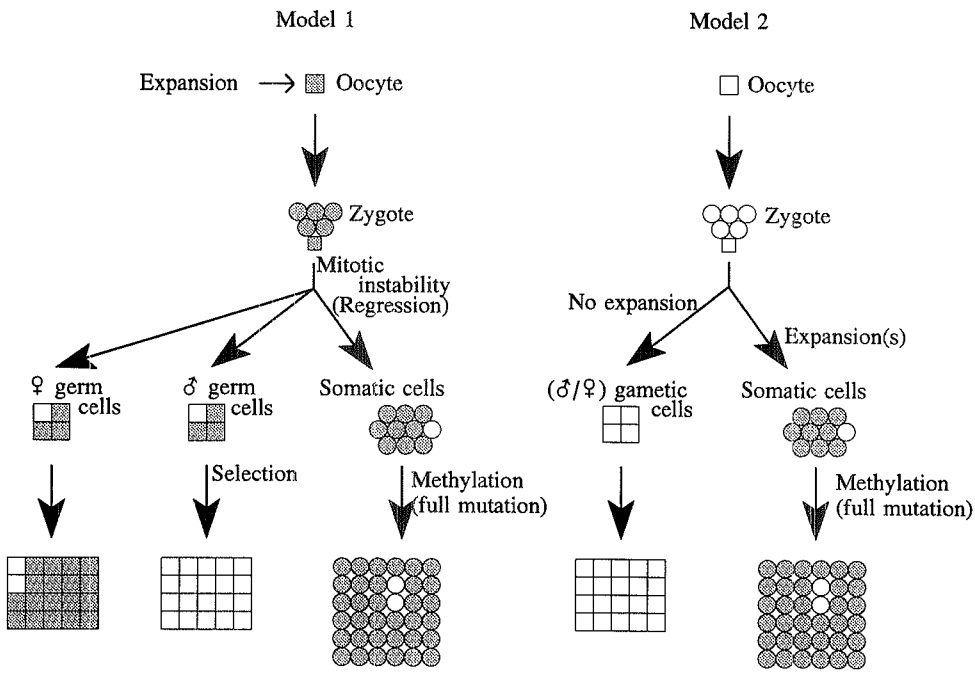

Figure 3.1 Schematic depiction of the two models for the timing of repeat amplification. Somatic cells are represented by circles, germ cells by squares. Grey symbols indicate cells with a full mutation, whereas open symbols represent cells containing a premutation.

The second model on the timing of the repeat amplification assumes the presence of a premutation in the oocyte, which expands to a full mutation during the early stages of embryonic development (Fig. 3.1). This expansion to a full mutation occurs after separation of the germ line and only in the somatic cells. Therefore in (male and female) germ cells a full mutation will be absent.

A number of findings are difficult to explain according to either model. A first problem is the parental bias observed in the transmission of the full mutation. This bias, with full mutations being transmitted through females only, is better explained by the first model, in which there is a specific selection against male germ cells containing a full mutation. According to the second model, in which both male and female germ cells contribute a premutation allele to the zygote, there has to be an imprinting mechanism that distinguishes a maternally derived premutation from a paternally derived premutation. However, such an imprint has not been identified yet.

A second issue of debate is the existence of fragile X males, who are mosaic for a full mutation and a premutation, as can be detected in the patients blood lymphocytes. Both these premutations and the premutation detected in germ cells of male patients is mostly present as a distinct band, indicating the presence of a single premutation repeat length.

According to the second model, this phenomenon can be explained by the lack of expansion to a full mutation in one, or a few, somatic cells, that contain a premutation. If this premutation allele is the result of regression of a full mutation allele (corresponding to the first model), this regression should be a single event.

Interestingly, in a male fragile X patient the repeat length in somatic tissue was different from the repeat length detected in sperm, being 160 and 60 CGGs, respectively (212; *Publication 4.2*). This implies that in this patient the regression mechanism occurred as two separate events, with a larger regression in the male germ cells than in the somatic tissue. A higher number of mosaic patients need to be investigated to determine whether this difference in repeat length in this patient is a common finding in the fragile X syndrome.

The identification of patients, mosaic for a full mutation and a deletion of the CGG repeat and flanking sequences (*Publications 4.3 and 4.4*) also supports the occurrence of regression in somatic tissue. However, in these patients the regression is not restricted to the CGG repeat, but extends to the flanking sequences as well. It should be noted though, that the deletion in these individuals could also have occurred in a premutation allele.

The two hypotheses on the timing of amplification were based on the presence of either a full mutation or a premutation allele in the female germ cell. Recently, the oocytes from two female fetuses (respectively 17 and 26 weeks after gestation) were demonstrated to contain an unmethylated full mutation (see *Chapter 2.7*), thereby favoring the first model. Although it should be noted that only two female fetuses have been studied, it is evident that a full mutation can be present in oocytes, indicating that the postulated regression mechanism has to exist.

Fetal testes from fragile X patients have also been studied. Interestingly, part of the primordial germ cells of these fetal testes (17.5 weeks after gestation), was demonstrated to express FMRP. It can be speculated that this may be due to the presence of a premutation allele in these precursor cells (R. Willemsen, *pers. comm.*), which would suggest that the regression, responsible for the presence of the premutation, had already occurred. The absence of FMRP in the other spermatogenic precursor cells, might be postulated to be the result of the presence of a full mutation allele, confirming the first model on the timing of the repeat amplification. It should be noted though, that the absence of FMRP may also be due to a more general mechanism in the cells, resulting in the silencing of FMRP. In order to verify whether this is indeed a common mechanism in primordial sperm cells, control testes will need to be studied.

Although the results obtained thus far favor the first model, the exact timing of the expansion of premutation to full mutation allele is still unknown. More knowledge can only be obtained by studying the oocytes of a female carrying a premutation in her somatic cells that transmits a full mutation to her offspring. For obvious reasons, it will be difficult to obtain these oocytes.

To overcome this problem, transgenic mice have been acquired, containing a fusion gene of the *FMR1* promoter region with repeat of either 29 or 60 pure CGGs, and the reporter

gene LacZ (*Chapter* 2.7). After two generations, however, the repeat was still unaltered. There are several explanations for the observed stability of this CGG repeat in the transgenic mice. A first possibility is that the CAG interruption that was likely to be the result of a PCR or cloning artefact, stabilizes the expanded repeat (see also *Chapter* 3.3.2). Introduction of a perfect CGG repeat in transgenic mice would overcome this artificially introduced stability.

A second explanation may be that expanded triplet repeats in general are (more) stable in mice, due to species differences. Three other studies have reported that transgenic mice containing an expanded CAG repeat, unstable in human, were found to transmit this repeat stably (242-244).

A third possibility is, that the sequences flanking the triplet repeat are involved in the repeat instability. The studies described thus far introduced an expanded triplet repeat as a transgene, with the transgene integrating at random in the genome. Therefore, the sequences normally flanking the triplet repeat present at the human locus are not near the transgene. In order to mimic the human situation as much as possible, expanded repeats will need to be introduced at the proper locus through homologous recombination.

Other trinucleotide repeats

In FRAXE mental retardation, the full mutation sized GCC repeat is mainly (57,190), but not exclusively, transmitted through females (58; *Publication 4.5*). In contrast to the fragile X syndrome, FRAXE males can have affected daughters. These affected daughters do not express the FRAXE site cytogenetically, and only have a small, methylated full mutation. This suggests that the full mutation transmitted by their fathers is rather small. Thus far, sperm of only one FRAXE male has been studied, and, like in fragile X males, a smaller repeat length was present, though the presence of full mutation alleles in a small percentage of sperm cells could not be excluded (*unpublished results*).

Similar to the fragile X syndrome, the congenital form of DM, associated with repeat lengths of 1000-2000 CTGs, is only transmitted through females (163,164). DNA analysis revealed that in sperm of DM males repeat lengths beyond 1000 CTGs, are absent. This suggests that, similar to the situation with the fragile X CGG repeat, the presence of a certain selection mechanism against repeat lengths above a specific size in germ cells of DM males. In contrast to the fragile X syndrome, the CTG repeat length in sperm of DM males is highly variable: males with a small or intermediate repeat length (<70 CTGs) have a similar and higher number of CTGs in sperm. Males with large allele expansions in somatic tissue (>700 CTGs) have similar or smaller repeat lengths in sperm (245).

The timing of amplification in the trinucleotide repeats in the neurodegenerative disorders may be different. The repeat lengths in sperm of HD and SCA1 male patients have been reported to be larger and more mosaic than that in somatic tissue (215,246). This is in agreement with the finding that in these disorders the more severe phenotype is paternally transmitted (151,176,247). The paternal bias in transmission of the more severe phenotype is also seen in the other five neurodegenerative disorders SBMA, SCA3, MJD, DRPLA and HRS, suggesting that male patients with these disorders are also gametically mosaic.

The observed parental sex differences in transmission of the expanded SCA1 or HD allele has been proposed to be caused by difference in number of mitotic divisions during oogenesis and spermatogenesis between oogonia and spermatogonia. As a result of the much greater number of cell divisions, the mutant allele is more likely to be expanded in spermatozoa than in oocytes (246). The selection mechanism, favoring the smaller repeat lengths as in the fragile X syndrome and DM, appears to be absent.

3.3.2 *Repeat configuration*

The originally cloned cDNA of *FMR1* did not consist of a pure CGG repeat stretch, but was reported to be interspersed by AGGs (12). Detailed analysis of the CGG repeat of over 400 normal alleles revealed that the AGG interspersion is very common, occurring once every 9-10 CGGs (232-234,248-251). The majority of normal alleles were reported to contain 2 AGGs (67,4 % (250)), whereas the total repeat length of the most frequent allele in controls is 29-30 repeats (14,252). Only a small minority (4 %) of the alleles of controls consists of a perfect CGG repeat, with the longest uninterrupted stretch being 34 CGGs. In contrast, 55 % of the premutation alleles lack the AGG interspersion.

Most of the length variation occurs at the 3′ part of the CGG repeat, the part in which the longest pure CGG stretches are found (232-234,248,249,251). In premutation alleles the 3′ stretch of pure CGGs is expanded, indicating polarity in repeat expansion. The threshold for instability has been proposed to be approximately 34 pure CGG repeats (248), though both stable and unstable alleles have been found in the range of 30-46 pure CGG repeats (234,248). The loss of the most 3′ AGG has been suggested to predispose human alleles to the development of the fragile X syndrome.

The increase in stability of interruptions in a simple repeat is a common phenomenon in di- and trinucleotide repeats. SCA1 imperfect repeats were found in 98 % of the unaffected individuals, whereas a perfect repeat consisting of 43-81 CAGs has been detected in all affected individuals. All normal, stably inherited alleles, consisting of 23-36 repeats, were interrupted by 2 CAT triplets, resulting in the configuration $[(CAG)_n\text{-}CATCAGCAT(CAG)_n]$. The HD CAG repeat is flanked by one CAA triplet in both the general population as in affected alleles. In a family in which a new HD mutation was identified, this CAA triplet was lost and was reported to be associated with decreased stability (244). The CAG repeat in the *MJD1* gene, has been found to be interrupted by 1 AAG triplet and 2 CAA triplets, in the 5′ part of the repeat. These interruptions however, were detected in all affected and unaffected individuals studied (154).

Recently, in the CTG repeat an allele of 37 repeats was described with an altered configuration $[(CTG)_4(CCGCTG)_{16}CTG]$, which was demonstrated to be more stable than a smaller allele consisting of 27 pure CTGs (253).

The CCG repeat at FRA16A is a complex polymorphism, interrupted by several CCT and/or CTG triplets. The normal length repeats can be divided into 4 main configurations (191); whereas the configuration of full mutation alleles has not been reported yet.

Cytogenetic expression of the FRA16A site has thus far only been detected in the European population and is completely absent in the Japanese and Indian populations. Interestingly, the configuration containing the smallest number of interruptions is found in 25 % of the European, only in 1.5 % of the Indian and completely absent in Japanese population. This suggests that this allele might be more prone to expansion, which would confirm that the lack of interruptions results in a more unstable allele.

Increased stability by the interruption in a repetitive tract by a variant unit has also been demonstrated in microsatellites (254). Variations in a repeat sequence have been proposed to result in an increased recognition by DNA mismatch repair systems, thus leading to an increased stability (255). Indeed, mutations in one of the mismatch repair genes *MSH2, MLH1, PMS1* and *PMS2* resulted in an increased instability of microsatellites in hereditary nonpolyposis colon cancer (HNPCC; 256-258). However, the involvement of the mismatch repair genes appears to be more important in the stability of dinucleotide repeats and not in triplet repeats (255).

All trinucleotide repeats described thus far, have the same threshold level of approximately 35 uninterrupted repeats, above which instability increases extremely (259). This suggests that a common mechanism might cause the expansion of the repeats.

Polarized variability

The majority of alterations in repeat length occur at the 3' part of the CGG repeat. This polarity can be explained by two different mechanisms. Polarity of gene conversion events in minisatellites was indicated to be the result of sequence differences in flanking sequences (260). On the other hand it might be possible that the polarity is caused by different mutation potentials for the leading and lagging strand (259). Noteworthy in this respect are the findings that deletions and other mutations in *E. coli* and yeast have been found to occur more frequent on the lagging than on the leading strand (261,262). The importance of the direction of replication on the alterations of a CGG repeat in *E. coli* was recently demonstrated (263). The CGG repeat was maintained more stably if the CGG strand was in the leading template strand with respect to direction of replication. Similar results were demonstrated for CTG repeats in *E. coli* (264; for review see 265).

3.3.3 Mechanism of repeat expansion

Several mechanism for the expansion of the CGG repeat have been proposed (13,14,266). One possible mechanism assumes expansion occurring through recombination during the female meiosis. Recombination events, however, would result in recombinations of the markers flanking the CGG repeat, which is not detected. Furthermore, recombination results in an equal amount of increases and decreases in repeat length, whereas in the fragile X syndrome mainly increases in repeat length are found. In addition, expansion through recombination is expected to result in the larger alleles containing more interruptions by AGGs. Recombination within the CGG repeat is likely to result in a cassette-like arrangement of the $[(CCG)_{9\text{-}10}AGG]$ repeat unit. Premutation alleles however, tend to lack the AGG

interspersions, making it unlikely that recombination causes the expansion of the repeat.

Two other mechanisms possibly causing the repeat expansion are unequal sister chromatid exchange and gene conversion, which have also been suggested to cause alterations in minisatellites (260). A gene conversion event involving the CGG repeat and sequences flanking the CGG repeat, has been described in a fragile X family (267). The vast majority of expansions of the CGG repeat do not involve changes in flanking sequences, which implies that the gene conversion would have to be restricted to the CGG repeat.

Another, more likely, possibility is that expansion is the result of slippage during replication (Fig. 3.2). There are two possible mechanisms of replication slippage that may result in the repeat instability. *In vitro* replication studies of expanded CTG and CGG repeats (17-160 repeat units) have demonstrated that DNA polymerase pauses at 29-31 pure CGGs (Fig. 3.2A; 268), which is near the instability threshold of 34 CGGs (248). This pausing only occurs with the CCG strand as a template and not with the complementary CGG strand again indicating the importance of the direction of replication in the mutational mechanism. The assumed block can be either nucleosomes (found to preferably bind to expanded CTG repeats in DM (269)), triplet repeat binding proteins (86,87), or a secondary structure that is formed due to the presence of an expanded C-G rich repeat. As the replication study was performed *in vitro*, hence in the absence of nucleosomes or putative CGG binding proteins, it is more likely that the block is caused by the formation of secondary structures (268,270-274).

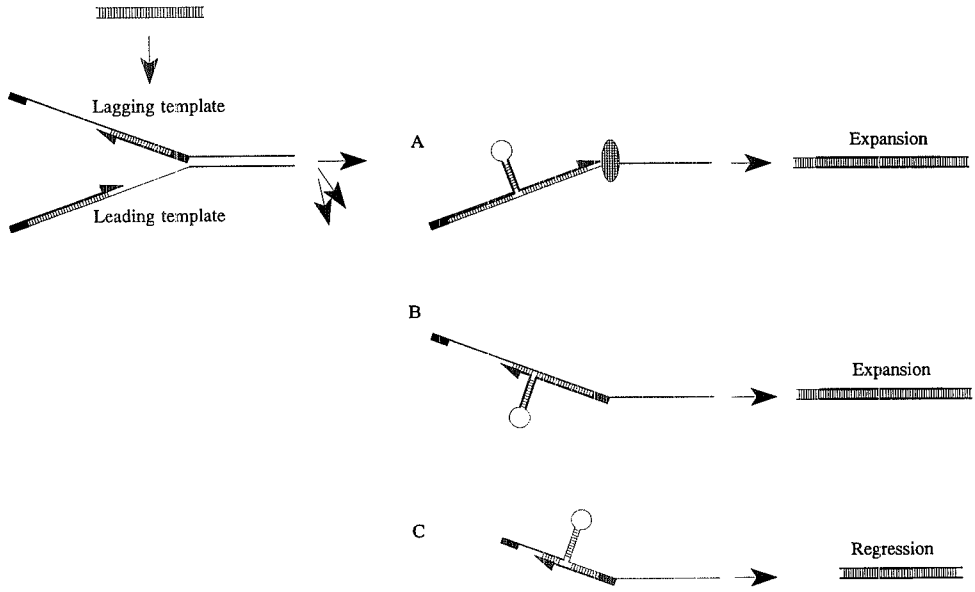

Figure 3.2 Model for repeat expansion and regression due to replication slippage. (Adapted from Wells (265)). (A) Hairpin structures are formed due to a block, resulting in the pausing of DNA polymerase and (B) the single stranded, newly synthesized strand can form a hairpin structure, both resulting in an elongation of the newly synthesized strand; (C) The single stranded template strand can from hairpin structures, leading to regression of the repeat length.

Due to this block and subsequent pausing of replication, multiple slippages might occur, resulting in misalignment and the formation of hairpin structures leading to an expanded strand (Fig. 3.2A). It seems possible that when the CGG repeat is larger the block formed by the hairpin is more stable, resulting in a more extensive increase in repeat length.

A second possibility is, that either the newly synthesized strand or the template strand on which the new DNA strand is synthesized, forms a stable hairpin, resulting in the slippage and misalignment (Fig. 3.2B and C; 270-274). Hairpin structures in the newly synthesized strand will result in expansion of the repeat (Fig. 3.2B), whereas hairpin structures in the template will lead to a regression (Fig. 3.2C; 265,275). Repeat lengths above the expansion threshold (approximately 70 pure CGGs) might form multiple hairpins, resulting in more slippage, thus leading to a more extreme expansion. For a review on the molecular basis of triplet repeat instability see Wells (265).

3.3.4 Origin of the FMR1 mutation

Affected fragile X males and females hardly reproduce (95), resulting in the continuous loss of mutated chromosomes. Considering the high population frequency of the fragile X syndrome a high mutation rate was expected. However, thus far no new mutation involving the CGG repeat has been described in fragile X families; all mothers of affected individuals were found to be fragile X carriers (97,276).

Haplotype studies, using microsatellite markers closely linked to the *FMR1* gene revealed differences in haplotype frequencies between fragile X and normal chromosomes (266). This linkage disequilibrium has been detected in different populations (277-282). Only a small number of haplotypes are detected in the majority of fragile X chromosomes, suggesting that the present day fragile X chromosomes are derived from a limited pool of independently mutated ancestral haplotypes. This might explain the finding that a mutated allele has been transmitted silently through at least 6 generations (283).

It has been postulated that initial chromosomes predisposed to become fragile X chromosomes can be transmitted by numerous generations, maybe up to 100, before a full mutation or high risk premutation is obtained (284,285). This suggests that a large amount of premutation alleles has to be present in the population. The frequency of females carrying an allele in the premutation range has been estimated to be approximately 1/400 (250,286-288).

Linkage disequilibrium has also been described for DM (289) and HD (290) in which the existence of alleles predisposed toward repeat amplification have been suggested. The almost exclusive presence of DRPLA in the Japanese population, also suggests a founder effect (178). A possible founder effect in the other trinucleotide repeats has not yet been reported, but due the high similarity in mutational mechanism of the disorders a founder effect is likely to be present in all disorders.

Based on the finding that the mutated *FMR1* allele can be transmitted silently through numerous generations, it is thought that the expansion of the CGG repeat is a multistep process (Fig. 3.3; 234,248,281). The first step would involve the loss of the most 3' AGG, either through a deletion, or by an A to C transversion, creating a larger stretch of

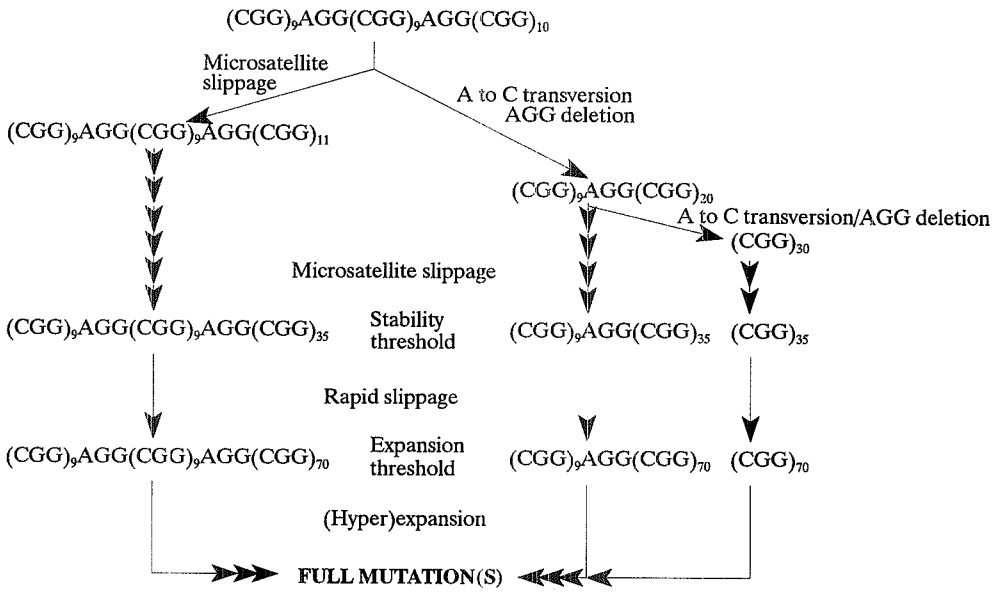

Figure 3.3 Schematic representation of the multiple alterations in the repeat that may lead to a full mutation (adapted from Eichler *et al.* (248)). Loss of an AGG results in an increased rate of microsatellite slippage.

(approximately 24) perfect CGGs (232), thus predisposing normal alleles to become the precursors of a premutation. Subsequently, gradual slippage during replication at a low mutation rate, would result in relatively small alterations in repeat length. After many generations (and a number of slippage events) the stability threshold of 34 pure CGGs is reached, after which in subsequent generations, slippage is more frequent. Again a few generations later, the length of the pure stretch of CGGs has reached yet another threshold, the expansion threshold consisting of approximately 70 CGGs (thus a total repeat length of approximately 90 repeats). Repeats above this threshold have been shown to expand extensively, resulting in a full mutation sized allele, provided it passes a female meiosis (14). Further, mitotic instability of the repeat during early embryonic development, involving both small and large, expansions, as well as small regressions of larger full mutation alleles, will subsequently lead in a mosaic pattern.

* 3.4 Concluding remarks

The identification of the *FMR1* gene and the finding of an unstable trinucleotide repeat in the 5′ UTR of the *FMR1* gene, elucidated the molecular basis of the fragile X syndrome. The development of reliable diagnostic methods enabled the analysis of the CGG repeat in a large number of patients and their close relatives. Although this has led to a better insight in the instability of the CGG repeat, it remains unclear when and how the repeat expands.

The introduction of a perfect, expanded CGG repeat through homologous recombination at the murine *Fmr1* locus, keeping the sequences flanking the triplet repeat intact, may result in a valuable mouse model, containing an unstable expanded CGG repeat comparable to the human situation. Analysis of the repeat length and the methylation pattern in the early embryonic stages in such transgenic mice may give more definite answers on the questions how and when the repeat expands and how and when the CGG repeat containing region becomes methylated. In addition, expressing FMRP in specific cell types, or in different proportions of cells, in the *Fmr1* knock out mouse, is likely to give a better insight in the number of cells expressing FMRP that is required for normal functioning. Ultimately these studies will lead to a better understanding of the molecular mechanism resulting in the fragile X phenotype.

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Chapter 4

Publications

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A deletion of 1.6 kb proximal to the CGG repeat of the *FMR1* gene causes the clinical phenotype of the fragile X syndrome.

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Abstract

The vast majority of individuals with the fragile X syndrome show expanded stretches of CGG repeats in the 5'non-coding region of FMR1. This expansion coincides with abnormal methylation patterns in that area resulting in the silencing of the FMR1 gene. Evidence is accumulating that this directly causes the fragile X phenotype. Very few other mutations in FMR1, causing the fragile X phenotype have been reported thus far and all concerned isolated cases. We, however, report a family, in which 11 individuals have a deletion of 1.6 kb proximal to the CGG repeat of the FMR1 gene. Although fragile X chromosomes were not detected, all 4 affected males and 2 of the carrier females show characteristics of the fragile X phenotype. Using RT-PCR we could demonstrate that FMR1 is not expressed in the affected males, strongly suggesting that the FMR1 promoter sequences 5' to the CGG repeat are missing. The deletion patients have approximately 45 CGG repeats in their FMR1 gene, though not interspersed by AGG triplets that are usually present in both normal and expanded repeats. It is hypothesized that prior to the occurrence of the deletion, an expansion of the repeat occurred, and that the deletion removed the 5' part of the CGG repeat containing the AGG triplets. Transmission of the deletion through the family could be traced back to the deceased grandfather of the affected males, which supports the hypothesis that the FMR1 gene product is not required for spermatogenesis. Finally, the data provide additional evidence that the fragile X syndrome is a single gene disorder.

Introduction

The fragile X syndrome, which affects approximately 1 in 1250 males, is the most common form of inherited mental retardation (1). The condition is transmitted as an X-linked dominant trait with reduced penetrance (80% in males and 30% in females) (2), and derives its name from the expression of a folate-sensitive fragile site at Xq27.3. Typical morphological features of affected males include large, prominent ears and jaw; broad forehead; highly arched palate and macroorchidism (3). Affected individuals can exhibit a broad scala of behavioral and psychological problems, including poor eye contact, hand biting, hyperactivity, learning disabilities, tactile defensiveness and autistic behaviour. However, mental retardation is the most pronounced feature in affected males (4-6). Characterization of the FMR1 gene (7-10) demonstrated that the fragile X syndrome, and the risk of transmitting the disease phenotype, is correlated with the size of a [CGG]n trinucleotide repeat in the 5' untranslated region of the FMR1 gene (11). Normal individuals carry allele size between 6 and approximately 50 repeat units that are stable upon transmission. CGG repeat numbers between 50 and approximately 200, representing the premutation range of fragile X, are observed in normal transmitting males and most unaffected carrier females, and it has been reported that this degree of size expansion is not associated with cognitive or physical abnormalities (8). When the premutation is passed through female meiosis, it may greatly expand in size over 200 repeats. Several studies have shown that this full mutation coincides with an abnormal methylation of the CGG repeat and a CpG island 250 bp proximal to the

CGG repeat (8,12-15). This DNA methylation is suggested to be the major cause of the repression of FMR1 transcription occurring in tissues highly affected in fragile X (e.g. several regions of the brain and the tubules of the testes (16-19).

Recently, rare fragile X negative patients with *de novo* deletions involving the proximal part of the gene or the complete FMR1 gene has been reported (20,21,22). In another fragile X negative patient a missense mutation in the coding region of the FMR1 gene was observed (23). These studies have confirmed that the absence or modification of the FMR1 gene product results in the fragile X syndrome. In the present paper we report an exceptional family in which individuals with fragile X syndrome characteristics have a deletion of 1.6 kb proximal to a CGG repeat of the FMR1 gene and we demonstrate that the deleted DNA sequences causes the silencing of the expression of the FMR1 gene in these patients.

Results

The pedigree of the investigated family is shown in fig. 1. Based on the clinical findings of affected individuals, as described in the clinical report and illustrated in fig. 2 for the index patient, the fragile X syndrome was clinically suspected.

Cytogenetic analysis of white blood cells of affected males and carrier females, however, did not reveal any karyotypic abnormalities or X chromosomes expressing the fragile site. Molecular analysis was initiated with Southern blot analysis on *Eco*RI digested genomic DNA, probed with pPX6 or pP2, in order to reveal possible expanded CGG repeats in the FMR1 gene. As is obvious from fig. 3A, no bands or smears representing fragile X pre- or full mutations were visible. Most surprisingly, however, was the fact that the 5.1 kb *Eco*RI allele in all affected males and one of the 5.1 kb alleles of the heterozygote females had disappeared and was substituted by a distinct band of 3.5 kb. This finding was suggestive for a deletion of approximately 1.6 kb within the 5.1 kb *Eco*RI fragment containing the 5' part of the FMR1 gene.

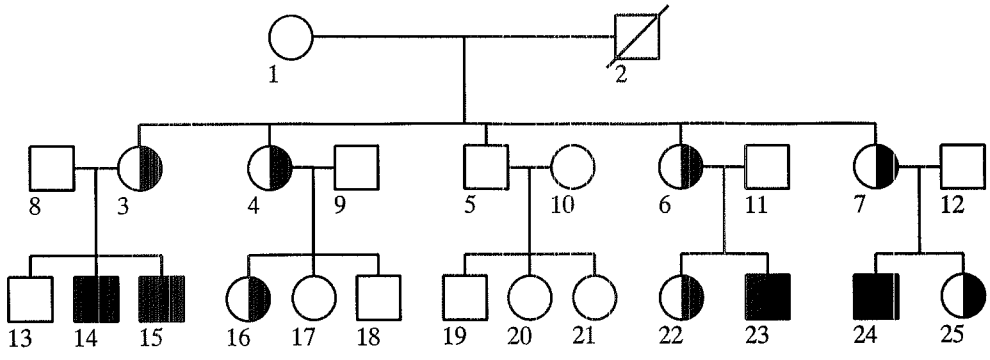

Figure 1. Pedigree of the investigated family. Males and females with the deletion in the FMR1 gene are represented by black or half-black symbols, respectively.

Figure 2. Features of the index patient's face (number 24 in the pedigree).

Double digestion with *Eco*RI/*Bss*HII showed the same 3.5 kb fragments in patients and female carriers (Fig. 3B). This could be either due to methylation of this *Bss*HII site, or due to the absence of the site caused by the deletion. To exclude the first possibility we performed double digests with *Eco*RI and the methylation sensitive enzyme *Xho*I. *Xho*I was able to digest the 3.5 kb fragment (data not shown), suggesting that no methylation occurred in this fragment as is found in fragile X patients with an expanded CGG repeat. Therefore it is most likely that the *Bss*HII site is located within the deletion. The 1.8 kb band in the *Eco*RI/*Pst*I blot, present in the patient and his mother (Fig. 3C) can be explained by assuming that the *Pst*I site at position 3 (Fig.4) is missing, while the *Pst*I site at position 2 is still intact. These initial combined results placed the deletion proximal to the *Xho*I site. The absence of the deletion in the grandmother in combination with the finding that all four daughters carry the deletion strongly suggests that the deletion was transmitted by the grandfather. To confirm that the trait associated with the deletion found in this family is not linked to the grandmaternal X-chromosome, we analyzed the polymorphic CA repeat RS46 (DXS548), located 140 kb proximal to the fragile X locus (9). As is shown in fig. 5A, the disease locus segregates with the 204 bp allele in all affected males and is also present in single doses in all carrier females. This allele is not present in the grandmother and is therefore most likely derived from the deceased grandfather. It is obvious that normal males 5 and 13 both have inherited a grandmaternal allele, with sizes of 196 bp and 206, respectively. This again suggests that the deleted allele originates from the grandfather.

In order to elucidate the precise location of the deletion we performed PCR analysis

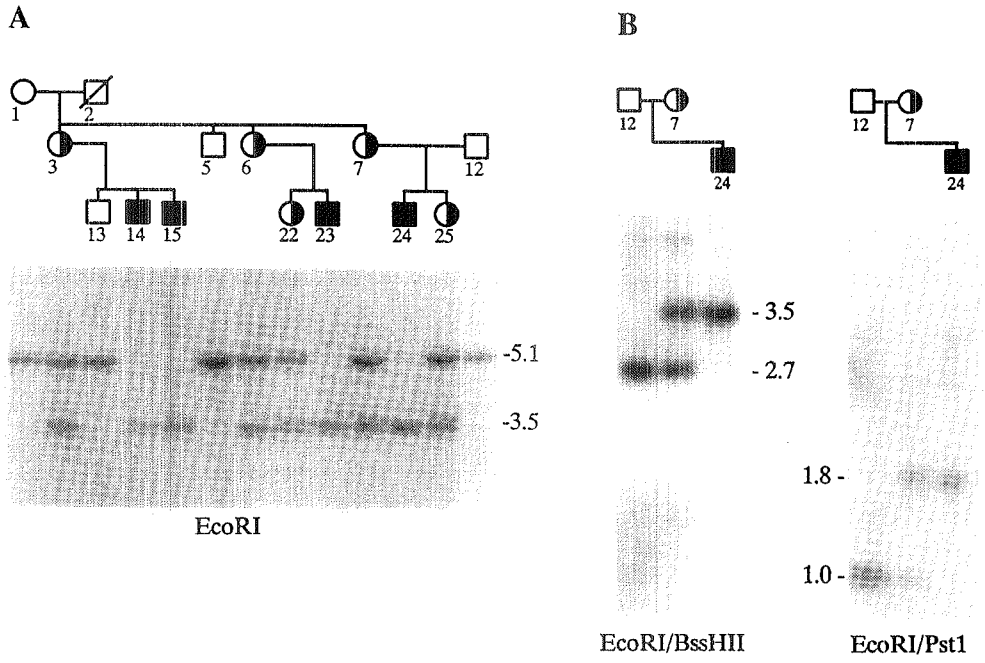

Figure 3. Southern blot analysis of the major branches of the pedigree. A: DNA was digested with *Eco*RI and following electrophoresis, fragments were blotted on filters and probed with pP2. B/C: DNA of the core family (the index patient and his parents) was double digested with *Eco*RI and *Bss*HII (B) or with *Eco*RI and *Pst*I (C) and following electrophoresis fragments were blotted on filters and probed with pPX6.

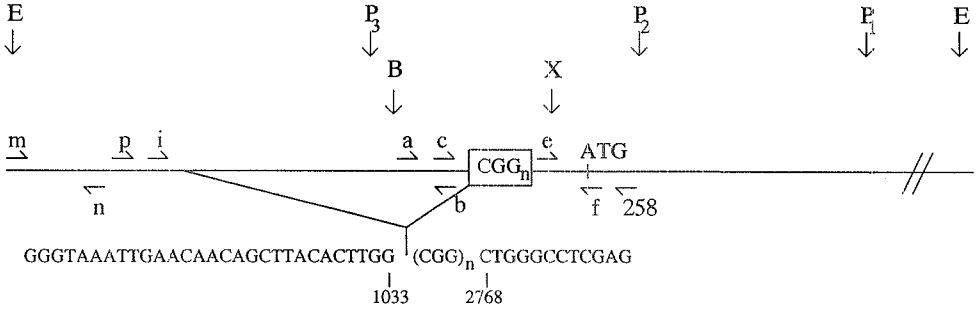

Figure 4. Schematic map of the 5.1 kb *Eco*RI fragment (pE5.1) (ref 26). Horizontal arrows indicate the location of the primers used for amplification and sequencing (not on scale). The deletion covers position 1034 to 2719 according to the pE5.1 sequence published by Fu *et al.* (26). Vertical arrows indicate restriction sites: E: *Eco*RI; B: *Bss*HI; P: *Pst*I; X:*Xho*I.

of the sequences surrounding the CGG repeat. The results obtained by the *Eco*RI/*Bss*HII double digestions made it clear that the 3′ deletion-endpoint is located between the *Bss*HII and the *Xho*I restriction site. This was confirmed by the absence in the affected males of a PCR product using primers c-f (Fig. 4) that bridge the CGG repeat (data not shown). Amplification with primerset a-b at the 5′ site of the repeat showed that the 105 bp product is also absent in the affected males (Fig. 5B). In contrast, a PCR product of 99 bp obtained by amplification with e and f at the 3′ site of the CGG repeat, including the *Xho*I site is amplified in all individuals (Fig. 5C). To exclude the possibility that the deletion was much larger than 1.6 kb, thereby including the proximal *Eco*RI restriction site, amplification with primers m and n (Fig. 4), located at the most 5′ end of the 5.1 kb *Eco*RI fragment was performed. This amplification resulted in the expected 430 bp product (data not shown), indicating that the 1.6 kb deletion was indeed completely located within the 5.1 kb *Eco*RI fragment. We concluded that the deletion should be located between primers n and e.

Junction sequence of the deletion

In order to sequence the junction of the deletion, DNA of patient 14 was amplified with primerset p-258. This resulted in a PCR product of approximately 650 bp in the patient and

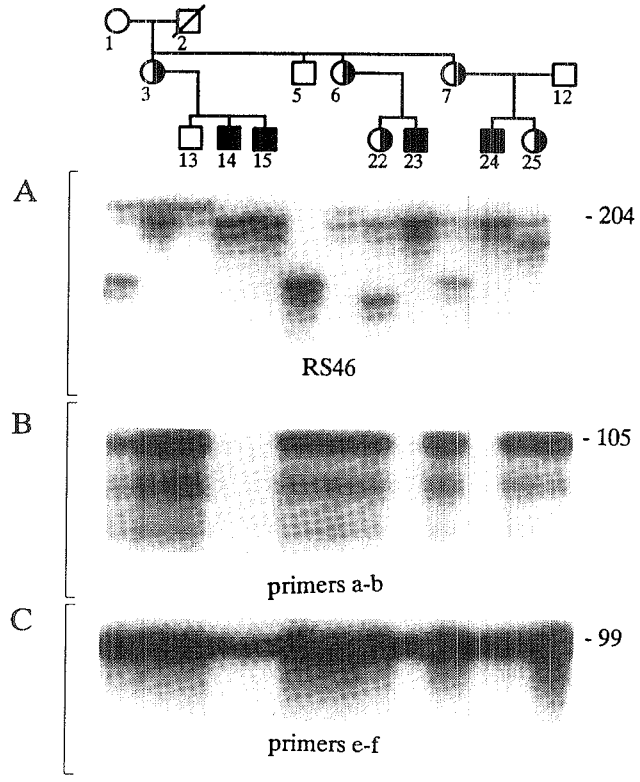

Figure 5. PCR products of DNA samples from the major branches of the pedigree. **A**: locus DXS548 (RS46) located 140 kb proximal to the fragile-X region. Primers RS46-f and r amplify fragments ranging between 190 and 206 bp. **B**: Primers a and b amplify a DNA sequence of 105 bp, located proximal to the CGG repeat in the FMR1 gene. **C**: Primers e and f amplify a DNA sequence of 99 bp, located distally to the CGG repeat of the FMR1 gene.

not in a control sample. (In control DNA a band of 2.2 kb was expected but this appeared to be too large to be amplified efficiently). The 650 PCR product of the patient was purified by Centricon-100 (Amicon) and sequenced directly with primer i using the BRL cycle sequencing kit. Part of this sequence is shown in fig. 6. The deletion in this patient covers a total of 1686 bp, from bp 1034 to 2719 according to the published sequence of the published sequence of the *Eco*RI fragment (26).

Length of the CGG repeat

The size of the PCR product obtained by amplification with primerset p and 258 was approximately 650 bp. By comparing this with the total length of the published sequence surrounding the deletion in the patients, excluding the CGG repeat, we conclude that approximately 45 CGG repeats are present in the FMR1 gene in these patients.

Expression of *FMR1*

The characterized deletion in the FMR1 gene compromised the promoter region of the gene. The first ATG of FMR1 however, is still present. It could not be excluded that transcription of FMR1 could start from another promoter nearby. We therefore performed RT-PCR on total RNA isolated from EBV or CMV transformed lymphocytes from our male patients, controls, and fragile X patients with an expanded CGG repeat. As is shown in fig. 7, the upper band corresponds to the amplification product of the internal control HPRT, which is present in all lanes. In control cell lines an additional 150 bp product derived from amplification of the FMR1 transcript is present (lanes 3-5, Fig. 7). This product is absent in both fragile X patients with an enlarged CGG repeat (lanes 6 and 7) and the two patients with the deletion (lanes 1 and 2). Thus, no FMR1 expression is present in the patients with the 1.6 kb deletion.

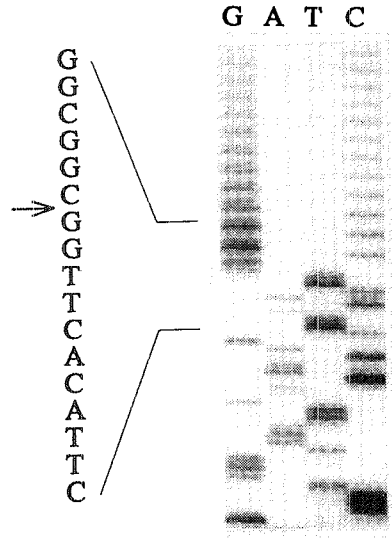

Figure 6. Partial DNA sequence of (pE5.1 of patient 14). The junction is indicated by the arrow. The sequence shown covers position 1023 (most 5') to 2725 of the pE5.1 fragment (Fig 4). The PCR product was sequenced using primer i.

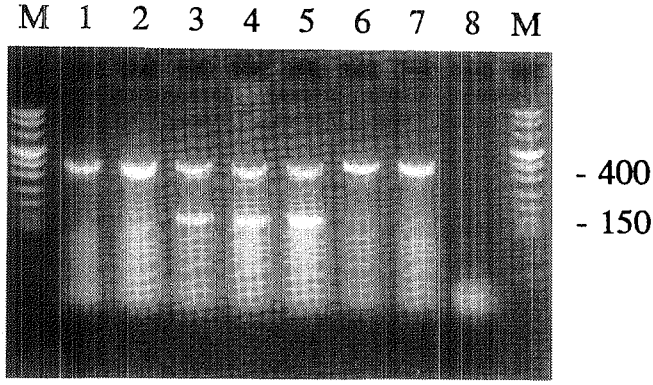

Figure 7. Expression of FMR1 mRNA. RT-PCR products from the 2 affected brothers (lanes 1 and 2), controls (lanes 3-5) and fragile X patients with an expanded CGG repeat (lanes 6-7). Lane 8: no DNA; M: marker. The upper band corresponds to the amplification product from the HPRT transcript, the lower band results from amplification of FMR1 mRNA.

Discussion

The crucial role of the FMR1 gene in the development of the fragile X syndrome has been convincingly demonstrated by various studies appearing after the first reports describing the gene (7,9,10). In order to determine the exact role of the FMR1 gene in the pathogenesis of the fragile X syndrome, more must be known about regulation and about functional domains in FMR1. One strategy in achieving the identification of these domains may be studying the FMR1 gene in fragile X patients lacking the expanded CGG repeat. Thus far only a few of these patients have been identified. Two patients have been described in which the entire FMR1 gene has been deleted, as well as either 2.5 Mb (22) or 3 Mb of flanking sequences (21). In another patient a deletion of 250 kb was found. Due to this deletion the FMR1 CpG island and at least 5 exons including the CGG repeat were missing (20). Although these results indicate the importance of FMR1 in the development of fragile X syndrome, it cannot be excluded that other genes are disturbed by the deletions, thereby causing the fragile X phenotype.

In patients, methylation of the FMR1 promoter region causes the down-regulation of FMR1 expression (12,18), resulting in the lack of the FMR1 protein and thus causing the fragile X phenotype (17,27). It is possible however, that the hypermethylation affects other genes in the proximity of FMR1, thereby causing clinical abnormalities which are part of the fragile X phenotype. Clarke *et al.* (28) found a reduction in the expression of the IDS gene, located approximately 1 Mb distal to FMR1, in affected fragile X males. A more direct proof of the role of FMR1 in the fragile X syndrome was given by De Boulle *et al.* (23). They identified a patient with the fragile X phenotype, but without the CGG expansion. Instead, a single point mutation was found resulting in an Ile367Asn alteration, which was suggested to cause the fragile X phenotype in this male. However, it should be noted that this male is thus far the only known individual with the fragile X phenotype due to a point mutation. A possible effect of the Ile367Asn mutation on the function of FMR1 was reported by Siomi *et al.* (29) and Ashley *et al.* (30). they demonstrated that FMR1 contains two types of sequence motifs also found in RNA binding proteins: an RGG box and two KH domains. This Ile367Asn mutation is thought to influence the RNA binding capacity of FMR1, since it

changes the most highly conserved residue in one of the KH domains (29). Further studies should be performed to test the effect of this point mutation on the function of the FMR1 protein.

We had the unique opportunity to study a family, of which 4 males and 7 females had a deletion of 1.6 kb proximal to the CGG repeat of the FMR1 gene. All 4 males with the deletion have clinical and/or behavioral symptoms, characteristic for the fragile X syndrome. We have shown in this report that males with the deletion do not express the FMR1 mRNA. Additionally we have shown that the *XhoI* site (which is methylated in FRAXA patients (14)), is not methylated in our patients, suggesting that no methylation occurs in the FMR1 region. Therefore, it seems unlikely that the deletion affects other genes in the FMR1 region via methylation. The finding in this family strongly support the hypothesis that the fragile X is a single gene disorder.

The sequence of the regions flanking the deletion showed that the distal endpoint of the deletion falls within the CGG repeat (Fig. 6). Sequencing the repeat with primers i and f (Fig. 4) enabled us to read approximately 30 CGGs from either side. Remarkably no AGG triplet was found in this repeat. In the originally published FMR1 cDNA the CGG repeat was interspersed by two AGG triplets resulting in the following sequence: $(CGG)_{10}AGG(CGG)_9AGG(CGG)_n$ (9). In most CGG repeats interspersed AGGs are found in the 5′ end of the repeat and it is assumed that the expansion of the repeat would occur at the 3′ end of the repeat (S.T. Warren, personal comm.). Since no AGGs were found in the CGG repeat following the deletion and approximately 45 triplets were present, we suggest that prior to the occurrence of the deletion an expansion of the CGG repeat had occurred. The deletion removed the 5′ part of the expanded CGG repeat containing the AGG triplets.

Based on interviews with members of the family, it was retrospectively concluded that the deceased grandfather had a normal appearance, intelligence and behaviour. However, considering the short distance between the DXS548 locus and FMR1 -140 kb- we concluded from the linkage analysis with 99.9 % certainty that the deletion was originating from the grandfather. An explanation for the normal phenotype of the grandfather could be that he was a transmitting male with a premutation allele in the somatic cells. This allele is detected early in the grandfather's development, leaving him a germline mosaic for the premutation and the deletion. This fits with the fact that there are no cryptic AGGs found in the CGG repeat in this family. A similar germline mosaicism for a mutation in an unaffected male is described by Arveiler *et al.* (33) in a family with Wiskott Aldrich.

No transcription was found in EBV transformed cell lines of the four male patients. It might be assumed that there is also no transcription of FMR1 in the testes, although this could not be tested directly. The grandfather had 5 children, which demonstrates that he was fertile despite the fact that he probably had no FMR1 mRNA expression. Several authors (17,31) have looked at FMR1 expression in testes and they could demonstrate high expression in male germ cells and low expression in Sertoli cells. These studies, however, have been controversial, since a third study (18) showed low FMR1 expression in spermatogonia and enhanced expression in Sertoli cells. Nevertheless, from our own family study we conclude that FMR1 expression is not required for the development of sperm cells.

Reijniers *et al.* (19) reported on the presence of a premutation and not a full mutation in male gametes in patients that have an expanded CGG repeat in their blood cells. They postulated two models on the possible timing of the expansion of the CGG repeat. The first model assumes that a full mutation, present in all somatic cells, can regress to a premutation in germcells. Due to a strong selection for cells expressing FMR1, only those cells may be able to proliferate which carry a premutation. The second model proposed that the expansion of the premutated repeat to full mutation length is a postzygotic event, which may take place after the separation of the germ line (day 5-6 p.c.) and is restricted to somatic cells.

Our finding that FMR1 expression is not required during spermatogenesis rejects the first model. The second model therefore, appears to be more likely.

In conclusion, we have identified a 1.6 kb deletion in the promoter region of the FMR1 gene in 11 individuals of a family. We have shown that in 4 males in this family this deletion causes the lack of FMR1 mRNA expression, resulting in the clinical phenotype of the fragile X syndrome.

Acknowledgements

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Subjects, materials and methods

Clinical report

The index patient in this family (see pedigree fig. 1, male 24) was seen by one of us (J-P Fryns) at the genetic clinic at the age of 3 10/12 years. He was referred for diagnostic evaluation because of marked delay in speech development and hyperactive behavior. He was the first child of healthy, normal parents. At his birth the mother was 26 years old. His younger sister (25, fig. 1) is slightly retarded with a mental age of 20 months at the chronological age of 26 months (Baily Developmental scale).

Pregnancy and delivery (at 39 weeks), were normal. Birth weight was 3.250 kg. No medical problems were noted during the first years of life. The boy started to walk without support at the age of 18 months. From that age on a marked delay in verbal development was noted and at the age of 3 1/2 years he could only express a few, single words. Episodes of hyperactive behaviour, aggressivity and hand fluttering were noted. Clinical examination revealed a hyperactive boy with relative macrocephaly (length 105 cm (75th percentile for age), weight 16 kg (50th percentile for age), head circumference 52 cm (90th percentile for age)). We further noted a large and high forehead (Fig. 2), large ears (6 cm in length, 97th percentile for age) and enlarged testes (2.5/1 cm, >97th percentile for age). Psychodiagnostic evaluation revealed a moderate, general development delay (IQ=52 Therman-Merril scale) with hyperactivity, low stress tolerance, hand biting and fluttering.

Further examination of the family showed that the mother (female 7, see fig. 1) was the youngest in a family of 5 children. She and her brother and three sisters (5, 3, 4 and 6 respectively, see fig. 1) are physically and mentally normal. The oldest sister (3) has three sons, one normal 19 years old boy (13) and two moderately mentally handicapped, 17 and 9 years old, respectively (14 and 15), lacking facial and other clinical features usually associated with FraX. Both are extremely shy with infantile behaviour. The second sister (4) has three children, two daughters (16,17)and one son (18), who are physically and mentally normal. The third sister (6) has two children. the oldest is a borderline intelligent (IQ(t)=81, IQ(v)=89, IQ(p)=78 WISC-R), 12 years old daughter (22) with all growth parameters above the 97th percentile for age, relative macrocephaly, myopia and a very shy behaviour. The youngest is a moderately mentally retarded 7 years old boy (23) with normal phenotype.

Cytogenetic analysis

Peripheral lymphocytes of all affected males and obligate carrier females were cytogenetically analyzed using GTG banding protocols. In addition, folate-deprived culturing conditions were applied to promote fragile sites at Xq27.3. In this case 100 metaphases were screened for fra(x) in affected males and 200 metaphases in females.

Southern blot hybridization

DNA was extracted from white blood cells of participating members of the family according to standard procedures (24). Samples of 5-10 µg DNA were digested with various restriction endonucleases or combinations (*Eco*RI, *Pst*I, *Xho*I, *Bss*HII), were electrophoresed through 1 % or 1.5 % agarose gels, and were blotted onto Hybond N+ membranes. Blot hybridization was carried out using ^{32}P-αdCTP random-primed labelled probes pPX6 or pP2. Clone pP2 is a 1 kb *Pst*I fragment of PE5.1 recognizing a 5.1 kb *Eco*RI fragment which includes the (CGG)n repeat and the CpG island in the 5' untranslated region of the FMR1 gene. Probe pPX6 is a 0.6 kb *Xho*I/*Pst*I fragment of PE5.1 recognizing a 1 kb *Pst*I fragment including the CGG repeat. Expanded CGG repeats representing fragile X premutations or full mutations are expected to be seen as discrete bands or smears above the normal 1 kb *Pst*I and 5.1 kb *Eco*RI fragments, respectively. Details of the procedures have been previously described (25).

PCR analysis and sequencing

Amplification of the polymorphic locus DXS548 was performed as described (9). Amplification reactions used for determining the localisation of the deletion more precisely were performed as previously described (11), with minor modifications. One primer of each primerset was end-labelled with ^{32}P-γdATP instead of using ^{32}P-αdCTP in the PCR mix. The total volume of the reaction was 15 µl. The PCR products were analyzed on 6-8% PAGE gels containing 6 M urea according to standard procedures and were subsequently autoradiographed. The primersets used were a-b, c-f, e-f and m-n (Fig. 4). In order to amplify the sequence that bridges the deletion we used a different PCR protocol. An amount of 150 ng DNA was amplified in a total volume of 25 µl consisting of 0.2 mM of each dNTP, 12.5% DMSO, 20 mM Tris-Cl pH8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 100 µg/ml BSA, 0.4 µM of each primer and 1.25 U of exo (-) *Pfu* polymerase (Stratagene) as described (32). The reactions were initially denatured for 5 minutes at 98°C, followed by 35 cycles of 1 minute at 98°C, 1 minute at 65°C and 3 minutes and 30 seconds at 75°C. A final extension of 10 minutes was carried out on 75°C. Cycle conditions were performed on a 9600 thermocycler (Perkin-Elmer). The PCR products were analyzed on a 1% agarose gel, stained with ethidium bromide. After a Centricon-100 purification step (Amicon), PCR products were directly sequenced with primer *i* using the BRL cycle sequencing kit. The following primers were used (the position in the pE5.1 sequence covered by the primers is indicated between brackets):

m: 5′ AGCTAGAGATCAGAGTAAGGCTGAGACTC (39-67)
n: 5′ GTTACTGCCAACTCCCTGAAGTCATACTG (419-474)
p: 5′ TGCAGCTTGTGTCTAGAAGAATGAGAGTGGG (668-698)
i: 5′ TGACAATGTGCTGAAAATTGAGGAGCAAAG (773-802)
c: 5′ GCTCAGCTCCGTTTCGGTTTCACTTCCGGT (2599-2628)
e: 5′ TGGGCCTCGAGCGCCCGCAGCCCACCTCTC (2768-2797)
f: 5′ AGCCCCGCACTTCCACCACCAGCTCCTCCA (2838-2867)
258: 5′ GGGGCCTGCCCTAGAGCCAAGTACCTTGT (2883-2911)
RS46-f:5′ AGAGCTTCACTATGCAATGGAATC (ref 9)
RS46-r:5′ GTACATTAGAGTCACCTGTGGTGC (ref 9)

mRNA expression

RNA was isolated from EBV or CMV transformed lymphocytes as described (23). Five μg total RNA was reverse transcribed as previously described (16), with minor modifications. Instead of precipitating the cDNA, 2 μl of the reverse transcribed reaction was used directly for PCR. The FMR1 mRNA was quantitated by comparison with mRNA expression of the hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene. The PCR products were analyzed on a 1.5% nusieve + 1.5% regular agarose gel, stained with ethidium bromide.

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Instability of the CGG repeat and expression of the *FMR1* protein in a male fragile X patient with a lung tumor.

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Abstract

The molecular mechanism of the fragile X syndrome is based on the expansion of an CGG repeat in the 5′ UTR of the *FMR1* gene in the majority of fragile X patients. This repeat displays instability both between individuals and within an individual. We studied the instability of the CGG repeat and the expression of the *FMR1* protein (FMRP) in several different tissues derived from a male fragile X patient. Using Southern blot analysis, only a full mutation is detected in 9 out of the 11 tissues tested. The lung tumor contains a methylated premutation of 160 repeats, whereas in the testis, besides the full mutation, a premutation of 60 CGG repeats is detected. Immunohistochemistry of the testis revealed expression of *FMR1* in the spermatogonia only, confirming the previous finding that, in the sperm cells of fragile X patients with a full mutation in their blood cells, only a premutation is present. Immunohistochemistry of brain and lung tissue revealed that 1% of the cells are expressing the FMRP. PCR analysis demonstrated the presence of a premutation of 160 repeats in these *FMR1*-expressing cells. This indicates that the tumor was derived from a lung cell containing a premutation. Remarkably, despite the methylation of the *Eag*I and *Bss*HII sites, FMRP expression is detected in the tumor. Methylation of both restriction sites has thus far resulted in a 100% correlation with the lack of *FMR1* expression, but the results found in the tumor suggest that the CpGs in these restriction sites are not essential for regulation of *FMR1* expression. This indicates a need for a more accurate study of the exact promoter of *FMR1*.

Introduction

The fragile X syndrome is the most frequent cause of inherited mental retardation, affecting approximately 1/1,250 males (Brown and Jenkins 1992, see Oostra et al. 1993*b* for review). Besides mental impairment, the fragile X phenotype includes the presence of large, everted ears, elongated face with mandibular prognathism, and macroorchidism (Hagerman 1991). In the majority of patients, the disorder is based on an expansion of a CGG repeat present in the first exon of the *FMR1* gene (Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991). In the normal population, this repeat varies between 6 and 53 CGGs and is stable on transmission (Fu et al. 1991; Snow et al. 1993; Reiss et al. 1994). A premutation allele of 43-200 repeats can be found in unaffected carriers. This allele is unstable and can expand to a full mutation of >200 repeats, when transmitted through a female. The presence of a full mutation coincides with hypermethylation of the CGG repeat and the preceding CpG island (Hansen et al. 1992; Sutcliffe et al. 1992; Hornstra et al. 1993). This results in the repression of expression of *FMR1* mRNA (Pieretti et al. 1991) and of *FMR1* protein (FMRP; Devys et al. 1993; Verheij et al. 1993) and thus in the fragile X clinical phenotype. Premutation alleles are not methylated and therefore give a normal expression of FMRP. Methylation of the CGG repeat region in fragile X patients is usually studied using the methylation-sensitive restriction enzymes *Eag*I or *Bss*HII (Rousseau et al. 1991*a*; Oostra et al. 1993*b*). Thus far, a 100 % correlation is observed between methylation of these sites and the absence of *FMR1* mRNA expression.

FMRP has been suggested to play a role in RNA metabolism. However, although the two RNA-binding motifs present in the *FMR1* protein (an RGG box and two KH domains) have been demonstrated to be involved in RNA binding (Ashley et al. 1993; Siomi et al. 1993, 1994), the exact function of the FMRP is still not fully understood. Localization studies in human and mouse tissues, using either in situ hybridization or immunohistochemistry, have demonstrated a high *FMR1* expression in brain and testis, the two major organs affected in fragile X patients (Abitbol et al. 1993; Bächner et al. 1993; Devys, et al. 1993; Hinds et al. 1993). In the brain, *FMR1* was mainly located in the neurons of the hippocampus and the grey matter of the cortex (Hinds, et al. 1993; Devys, et al. 1993; Abitbol, et al. 1993). The exact localization in testis remains unclear. Low expression in germ cells and enhanced expression in Sertoli cells has been reported (Hinds, et al. 1993). In contrast, two other studies demonstrated enhanced *FMR1* expression in spermatogonia and not in Sertoli cells (Bächner, et al. 1993; Devys, et al. 1993).

The CGG repeat is not only unstable during transmission from carriers to their offspring but also within an individual. Most patients are mosaic: they display an extensive smear on Southern blot analysis, indicating the presence of a wide range of full mutations (Oberlé et al. 1991; Rousseau, et al. 1991*a*, 1994). Besides mosaicism within the full mutation range other forms of mosaicism are also known. Approximately 20%-40% of the patients have a premutation allele in combination with the full mutation (Pieretti, et al. 1991; Nolin et al. 1994). Moreover, it has been demonstrated that male patients with a full mutation in their blood have a premutation in their sperm cells (Reyniers et al. 1993). A patient has been reported to have two normal alleles in addition to a full mutation (Van den Ouweland et al. 1994). Finally, we recently described four unrelated patients, mosaic for a full mutation and a deletion of 150-300 bp including the CGG repeat and flanking sequences (De Graaff et al. 1995). The instability is established in early fetal life: in both a 13-wk-old fetus and a monozygotic twin, identical patterns of mosaicism were found in the different fetal tissues (Devys et al. 1992; Wöhrle et al. 1993).

In this paper, we describe both the mitotic instability of the CGG repeat and the expression of FMRP in different tissues of a male fragile X patient. We demonstrate that, although in various tissues only a full mutation was detectable on Southern blot analysis, approximately 1% of the cells showed expression of the FMRP. *FMR1* expression is also seen in the spermatogonia in the testis and in a lung tumor that carries a methylated premutation.

Results

Southern blot analysis

The stability of the CGG repeat in different tissues of the patient was studied by Southern blot analysis. DNA derived from 11 different tissues was subjected to *Hin*dIII digestion. Hybridization of the Southern blots with the probe pP2, which recognizes the CGG repeat and the preceding CpG island (Oostra, et al. 1993*a*), resulted in a 5.2-kb fragment in control individuals (fig. 1, lanes 1 and 2). In the patient, a smear of >6 kb was present in the majority of the tested tissues (fig.1, lanes 3-13). This indicates the presence of a full mutation of >300

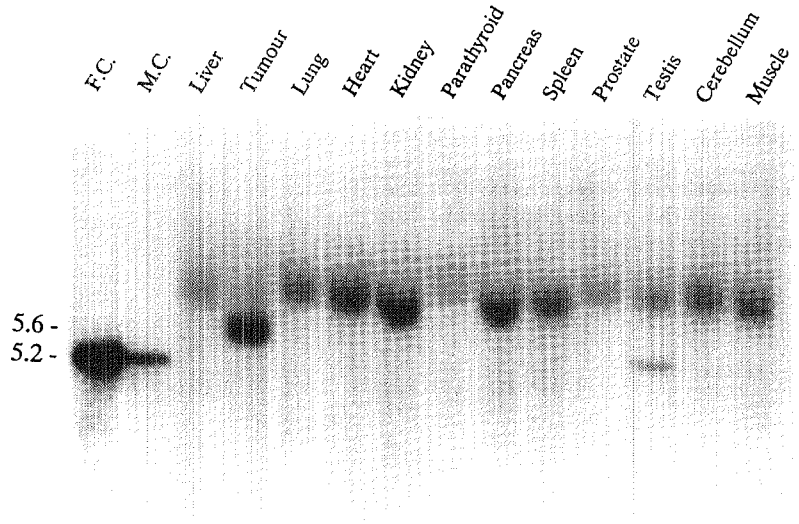

Figure 1 Southern blot analysis of 11 different tissues of the patient. Genomic DNA was cut with *Hin*dIII, and, following electrophoresis, fragments were blotted and probed with pP2. F.C.= female control; M.C.= male control. The length of the normal fragment (5.2) and the tumor (5.6) are indicated in kilobases.

CGG repeats. In DNA isolated from the testis an additional band of 5.3 kb was seen. This premutation, estimated to be approximately 60 repeats, is likely to be derived from the sperm cells present in the testis. Since no additional fragments were detected in the other tissues, we concluded from the Southern blot analysis that the patient is not mosaic for a full mutation in combination with a premutation.

Interestingly, the full mutation was not present in the DNA derived from the tumor. Instead, a 5.6-kb *Hin*dIII fragment was visible, representing a premutation of approximately 160 repeats, 2.5 times larger than the premutation found in testis. To exclude the presence of possible rearrangements or deletions in the tumor, the filter was reprobed with the entire pE5.1 probe (Fu et al. 1991). Again, only a 5.6-kb *Hin*dIII fragment was detected in the tumor. No additional bands were detected with either probe, indicating that the FMR1 promoter, including the start codon, is unchanged.

Methylation analysis using an *Eco*RI-*Eag*I double digest revealed that the full mutation, present in all tissues, was methylated (data not shown), whereas the premutation in the testis was unmethylated. Methylation analysis of the premutation in the tumor gave a surprising result. Digestion of an unmethylated allele with *Eco*RI in combination with either *Bss*HII or *Eag*I resulted in a 2.8-kb fragment (fig 2, lanes 6 and 9, respectively). The full mutation found in the lung (lanes 4 and 7), as well as the premutation in the tumor (lanes 5 and 8) remained, respectively, 6-kb and 5.6-kb *Eco*RI fragments, indicating they were not digested by either

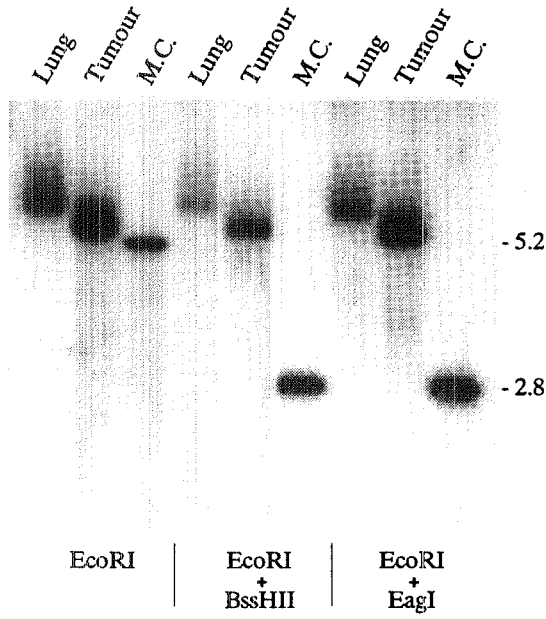

Figure 2 Methylation analysis of the tumor and lung of the patient. Genomic DNA from tumor, lung and blood from a male control (M.C.) was digested as indicated. Following electrophoresis, the fragments were blotted and probed with pP2. The length of the normal fragments are indicated in kilobases.

*Eag*I or *Bss*HII. A possible unmethylated fragment, expected to be approximately 3.4 kb, was not detected in the tumor. To ensure that both enzymes had digested the DNA to completion the same blot was reprobed with OxE20, which recognizes the GCC repeat located in the *FRAXE* region (Knight et al. 1993). In lung tissue, tumor and a control sample, both *Bss*HII and *Eag*I were found to be digested to completion (data not shown).

The lack of digestion with both *Eag*I and *Bss*HII could not only be due to methylation but could also be caused by a deletion, removing the two restriction sites. To exclude this possibility, we amplified the sequences flanking the CGG repeat. Using primer sets *e-f*, immediately 3′ of the CGG repeat (Fu et al. 1991), and *t-d*, 5′ of the CGG repeat encompassing the *Eag*I and *Bss*HII sites, a normal PCR product was obtained in DNA from both lung and tumor (data not shown). Therefore, none of the sequences flanking the CGG repeat is lacking. In addition, we found that the PCR product could be digested with *Eag*I, showing that there was no point mutation in this site that could have erased the restriction site. These results clearly indicate that the lack of digestion is caused by methylation of the restriction sites. When a third methylation-sensitive enzyme, *Sac*II, was used, again no difference in methylation pattern between tumor and lung was found (data not shown).

Immunohistochemistry

In blood, unmethylated premutation alleles of the size found in the testis in this patient do result in *FMR1* mRNA expression (Pieretti et al. 1991). To verify expression in the testis, we performed localization studies on cryosections of the testis and some other tissues of the patient using the monoclonal antibody 1A (Devys et al. 1993), directed against an N-terminal

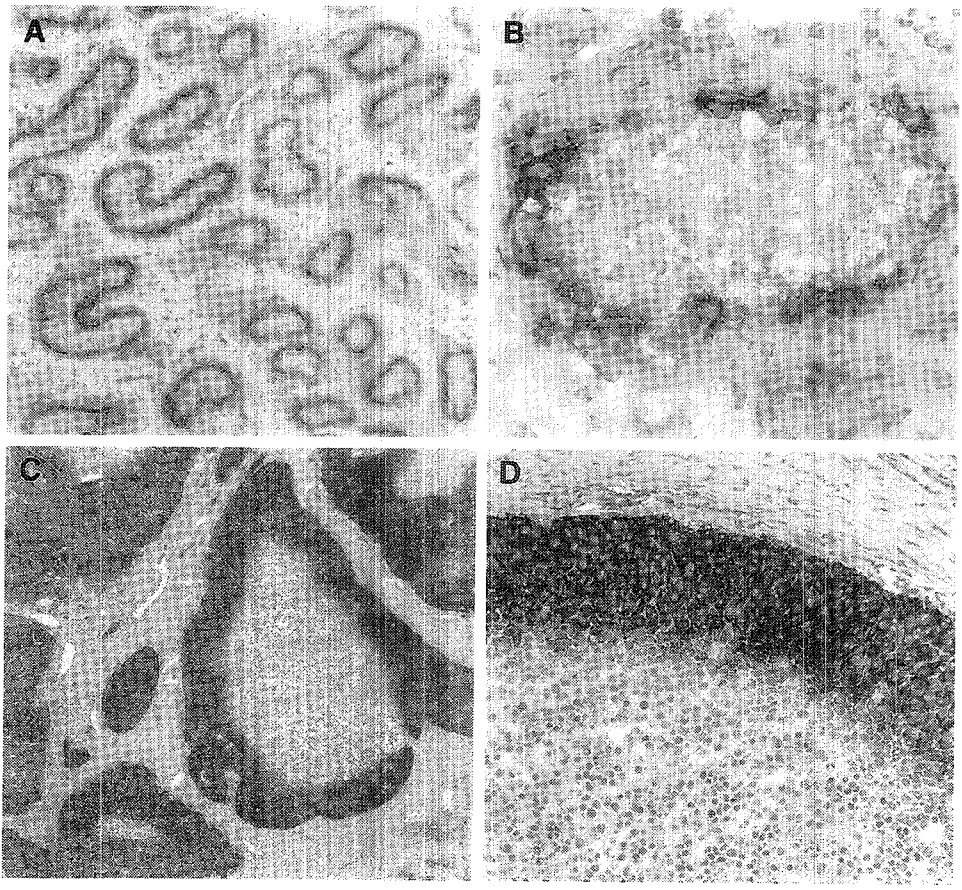

Figure 3 Light-microscopic micrographs of cryostat sections from testis (A and B) and lung tumor (C and D) of the fragile X patient. Sections were immunostained for FMRP with the monoclonal antibody 1A using an indirect immunoperoxidase technique.

epitope of the FMRP. In testis, a clear staining at the bases of the seminiferous tubuli in the spermatogonia was seen (fig. 3A and 3B). This staining was absent in the Sertoli cells, the connective tissue and the more mature sperm cells. Thus, in agreement with the presence of a premutation in sperm cells only, expression of the *FMR1* gene in the testis is limited to the spermatogonia.

Immunohistochemical analysis of the tumor gave a surprising result: despite the observation that the premutation in the tumor is methylated, a high expression of FMRP was present in approximately 30%-40% of the total tumor tissue (fig. 3C and 3D). Both the centre of the tumor, consisting of necrotic cells as based on histological analysis, and the connective tissue surrounding the tumor lacked *FMR1* expression, whereas the (still dividing) outer layer of the tumor clearly expressed the FMRP.

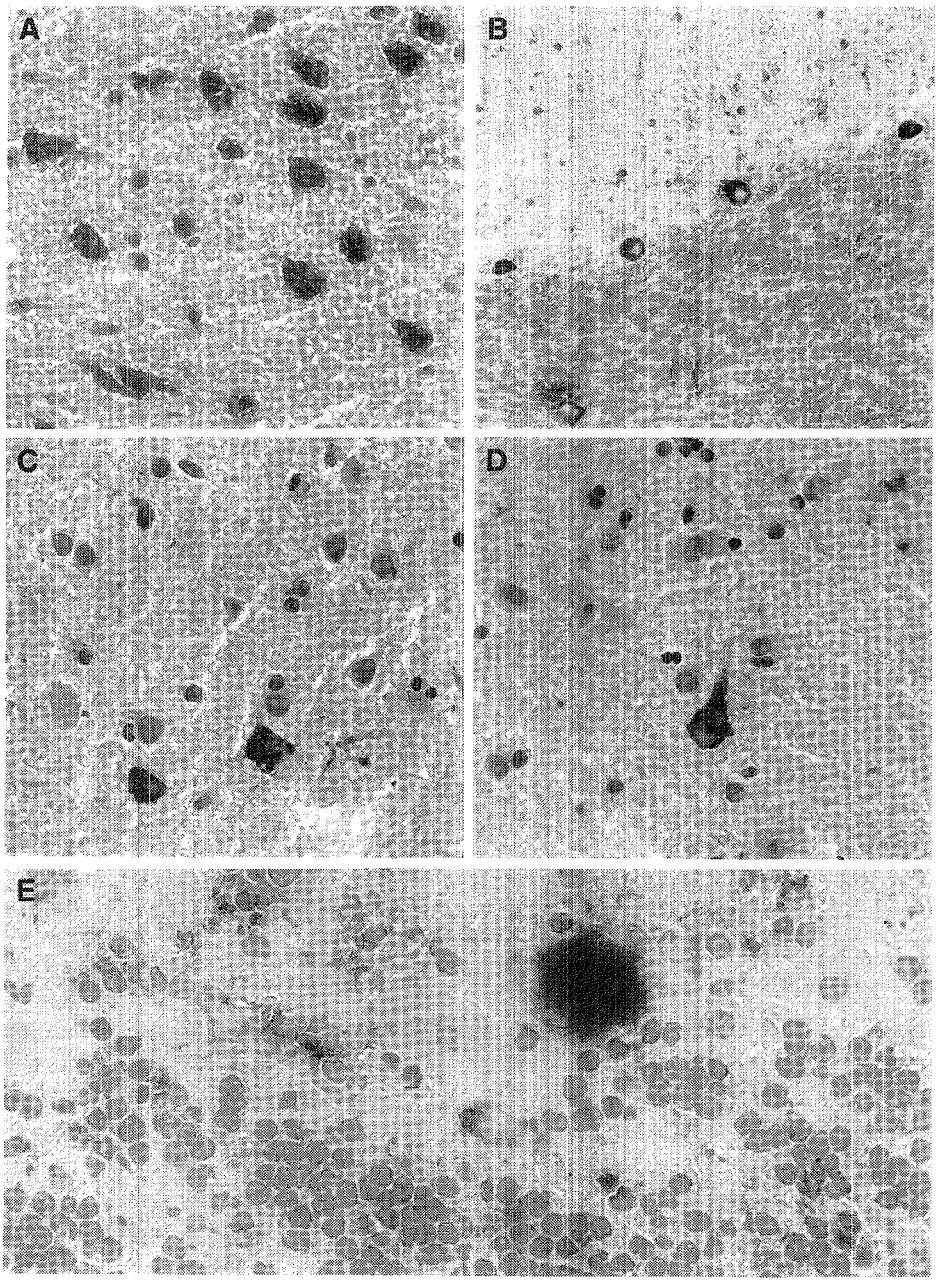

Figure 4 Light-microscopic micrographs of cryostat sections from cortex (A) and cerebellum (B) of a normal individual and cortex (C), hippocampus (D) and cerebellum (E) of the fragile X patient. Sections were immunostained for FMRP with the monoclonal antibody 1A, using an indirect immunoperoxidase technique.

Expression of FMRP was also investigated in brain tissue. *FMR1* is known to be highly expressed in the neurons of unaffected individuals (fig. 4A and B). However, in this patient, on the basis of the presence of a methylated full mutation only, as determined by Southern blot analysis, the brain was expected to lack FMRP expression. Surprisingly, some labelling was present in the brain tissue. In the cortex, approximately 1 % of the neurons showed a cytoplasmic labelling in the neurons similar to the expression found in neurons of unaffected individuals (fig. 4C). This is likely due to the presence of a premutation allele instead of a full mutation in these neurons, suggesting that the patient is mosaic. Expression of FMRP was also detected in 1% of the neurons in the hippocampus (Fig. 4D) and occasionally in Purkinje cells in the cerebellum (fig. 4E), two brain structures known to have a high expression of FMRP in unaffected individuals. Control cryosections of the tissues, not incubated with the monoclonal antibody 1A but otherwise treated identically, were found to be completely negative.

PCR-analysis

We needed to verify whether the immunohistochemical staining observed in 1% of the neurons was the result of the presence of a premutation in these cells. Southern blot analysis is not sensitive enough to detect a possible premutation in only 1% of the cells. We therefore PCR-amplified DNA derived from cerebellum, kidney, lung, tumor and testis. A predominant premutation size of 60 repeats in testis was amplified along with a fainter 37 repeat size suggesting some mosaicism. In the tumor, a distinctly larger premutation size of approximately 160 repeats was amplified along with several fainter and smaller sized bands. The other three tissues -lung, kidney and brain- showed mosaic patterns with a predominant premutation allele size of 160 repeats but with secondary alleles ranging in size from normal to the full mutation, confirming the immunohistochemistry and Southern blot analysis. The *MnlI* partial digestion analysis of the predominant premutation bands of both testis and tumor did not reveal any AGGs interspersed within the CGG repeat region (data not shown).

Discussion

The lack of *FMR1* expression is widely accepted to be the cause of the fragile X syndrome. However, this does not explain the presence of mosaic males having a premutation (and thus *FMR1* expression) in a low percentage of their leukocytes. It is possible that the percentage of cells that do express *FMR1* is too low, indicating that expression of *FMR1* is required in a higher number of cells. Another possible explanation could be that the leukocytes, in which the mosaicism is detected, are not representative for the tissues affected in the fragile X syndrome (Pieretti, et al. 1991; Rousseau, et al. 1991*a*; De Vries et al. 1993). In line with this assumption, Wöhrle et al. (1992) demonstrated that, in an affected fetus, both length and methylation pattern of the CGG repeat region varied between different tissues. In the patient described in this paper, however, there is only a minor variation in fragment length *Hin*dIII digests between the different tissues. All tissues, except two, were found to be completely methylated; none of the tissues showed a partially unmethylated fragment. Therefore, in this

patient there appears to be a good similarity between the methylation of the patient's different tissues.

Two tissues, the testis and tumor, are clearly different from the others. In the testis, besides the full mutation, a premutation is detected, caused by the presence of sperm cells in the testis. Reyniers *et al.* (1993) reported that fragile X males with a full mutation in blood have a premutation in their spermatogonia.

The premutation seen in the tumor seemed to be more difficult to explain. The premutation was not visible in DNA derived from the lung on the Southern blot (fig. 1). However, both immunohistochemistry and PCR analysis revealed that approximately 1% of the cells in lung and brain tissue of the patient have a premutation in stead of a full mutation. It is therefore very likely that the tumor has originated from a lung cell with a premutation. A second possibility, regression of a full mutation to a premutation in the tumor seems less likely, because the length of the CGG repeat of the premutation in the tumor, 160 CGG repeats, is identical to that found in 1% of the lung cells.

Instability of microsatellites is a common phenomenon in hereditary nonpolyposis colon cancer, and is caused by mutations in one of the mismatch repair genes *MSH2*, *MLH1*, *PMS1*, and *PMS2* (Leach et al. 1993; Bronner et al. 1994; Nicolaides et al. 1994). Whether the same instability is found in lung carcinomas is not yet known. The finding that the repeat length in the tumor is identical to that in the lung implies that the CGG repeat seems to be stable. Two additional CA repeats in the vicinity of *FMR1*, DSX548 and FRAXAC2, were found to be identical in all tissues, including the tumor (data not shown). Therefore, this tumor does not appear to exhibit microsatellite instability.

The presence of FMRP-positive cells in the brain (fig. 4C-E) demonstrates that the patient is mosaic; approximately 1% of the patients cells contain a premutated allele instead of a full mutation. In contrast, this patient did not appear to be mosaic according to the Southern blot analysis, which is due to the difference in sensitivity of the two methods. This finding strongly suggests that the percentage of mosaic male patients is much higher than the 20% proposed elsewhere (Fu, et al. 1991; Pieretti, et al. 1991; Rousseau, et al. 1991). Recently, Nolin *et al.* (1994) reported mosaicism in 41% of the fragile X males. In line with this, by using PCR we found approximately 50% of the male patients with a full mutation to be mosaic (data not shown). Using an even more sensitive method such as immunohistochemistry, that enables detection at single cell level, it might eventually be possible to detect mosaicism in all fragile X males.

Although many studies have described the instability of the CGG repeat, the exact mechanism by which the CGG repeat expands from premutation to full mutation and the timing of this expansion is still unclear. The finding that sperm cells of male patients with a full mutation in their blood cells contain a premutation only, has lead to two hypotheses on the possible timing of the amplification of the CGG repeat (Reyniers et al. 1993). In the first hypothesis the amplification is assumed to occur during oogenesis, leading to a full mutation in all somatic cells. In a low number of cells, both sperm and somatic cells, the full mutation regresses to a premutation and because of a selection mechanism, only the sperm cells with a premutation can proliferate. In the second hypothesis, the extensive amplification of the

repeat occurs during postzygotic proliferation, after separation of the germ line, which occurs early in the blastocyst stage. This selection mechanism is not based on the absence or presence of FMRP, since recent breeding studies with *Fmr1* knockout mice have demonstrated that *Fmr1* protein is not required for spermatogenesis (Bakker et al. 1994). In addition, Meijer *et al.* (1994) described a 1.6-kb deletion that resulted in the absence of *FMR1* mRNA in all affected males in a family. This deletion was originally derived from a male with a progeny of five children, confirming that expression of FMRP is not required for spermatogenesis. Another selection mechanism, however, is still possible. Hansen *et al.* (1993) showed that full mutation alleles are replicated later than normal or premutation alleles. Therefore, sperm cells containing a premutation might replicate and divide faster than sperm cells carrying a full mutation. This then results in the detection of a premutation present in the majority of sperm cells. Selection for blood lymphocytes carrying small expansion has been described twice. Rousseau *et al.* (1991*b*) reported the positive correlation between age and the proportion of inactive X chromosome with a full mutation in lymphocytes of female carriers. In addition, they found an inverse correlation between age and percentage of cytogenetically expressed fragile X sites, suggesting a decrease in heterogeneity of full mutations in female carriers.

A similar decrease in heterogeneity appears to occur in lymphoblast cell culture. Hansen *et al.* (1993) identified an allele of 200 repeats in a lymphoblast cell culture of a mosaic fragile X patient. They infer that this allele was derived from a subpopulation of lymphoblasts that were somehow selected during cell culture. Hypothetically, a similar selection mechanism can explain the presence of a premutation in the tumor. Although it seems very likely that the tumor originated by chance from a cell with a premutation, it is possible that the unregulated replication leading to the formation of the tumor was facilitated by the presence of a premutation, thus implying the presence of a selection mechanism. It would therefore be interesting to study the CGG repeat length in other tumors and the tissues from which the tumors arose, in fragile X patients.

A remarkable finding in this patient is that the premutation in the sperm cells differs from the premutation found in the 1% of the cells in lung brain and kidney. How does this fit with the two hypotheses on the timing of the amplification? According to the first hypothesis, regression from full mutation to a premutation would occur in both sperm cells and somatic cells as two independent events. The second hypothesis would suggest a two-step, expansion in the somatic cells, after the separation of the germ line. In the first step the premutation expands mildly, resulting in a repeat length of 160 CGGs. The second step involves a expansion to a full mutation in the majority of cells, leaving approximately 1% of the cells with the premutation of 160 CGGs. In order to distinguish between these two hypotheses, we wanted to study the origin of the premutation in both tumor and testis. The CGG repeat is interspersed with AGGs (Verkerk et al. 1991). The position of these AGGs varies between different alleles (Eichler et al. 1994; Hirst et al. 1994; Kunst and Warren 1994; Snow et al. 1994). Thus, studying the origin of the premutation in tumor and testis was performed by investigating the distribution of AGGs. Both in tumor and testis, the *FMR1* repeat consists of CGG triplets only, which could be due to a deletion within the full mutation. A deletion

within a full mutation CGG repeat was elsewhere suggested to cause regression to a normal allele size in a mosaic fragile X patient (Van den Ouweland, et al. 1994). However, premutation alleles lacking AGG interspersion have been described (Eichler, et al. 1994; Snow, et al. 1994) leaving the possibility that in the allele from which both premutation and full mutation possibly originated, the AGGs were already absent. Elucidation of this problem by determining the AGG interspersion in either the other tissues or in the mother or other relatives of the patient was not possible. Therefore, the origin of the premutation sized allele remains unclear in this patient.

The expression of FMRP in the tumor remains an interesting finding. The presence of small premutation alleles in the PCR analysis could lead to the assumption that these alleles result in the expression of FMRP in the tumor. However, considering the percentage of FMRP expressing cells, estimated to be approximately 30-40%, the smaller alleles should also have been detected on Southern blot analysis. In addition, the intensity of the 5.6-kb EcoRI fragment of the tumor is similar to that of the lung (Fig 2), indicating that the majority of the tumor cells contain the methylated premutation allele of 160 CGGs. The finding that the premutation in the tumor is methylated is not completely uncommon. Abnormal methylation is a common phenomenon in cancer and includes both hypomethylation, widely spread over the entire genome, and hypermethylation of specific regions (see Laird and Jaenisch 1994 for review). In fragile X patients with a full mutation, the whole CGG region is methylated, including the CGG repeat and the CpG island (Hansen et al. 1992; Hornstra et al. 1993). Methylation of the CGG repeat region is usually determined using the restriction enzymes *Eag*I and *Bss*HII. Methylation of these restriction sites is completely correlated with the lack of *FMR1* mRNA expression (Pieretti, et al. 1991; Sutcliffe, et al. 1992; Hagerman et al. 1994). In the tumor of this patient this correlation is absent: although both the *Eag*I and the *Bss*HII site are methylated, FMRP is expressed in the tumor. A plausible explanation for this discrepancy may be that, whereas in fragile X patients the whole CGG region is methylated, the CGG repeat region in the tumor is only methylated partially. This could be due to either partial demethylation, coinciding with the regression of the full mutation to the premutation, or methylation of only a number of CpGs of the premutation allele in the tumor. The resulting unmethylated CpGs may be essential for the regulation of *FMR1* expression. This assumption is in line with in vitro methylation studies described by Hwu *et al.* (1993). They demonstrated that *Sss*I methylase, methylating all CpG dinucleotides, completely destroys the FMR1 promoter activity. In contrast, *Hpa*II methylase (methylating only CCGG sites), did not impair the promoter activity. Since the *Eag*I restriction site encompasses 2 *Hpa*II sites, methylation of the *Eag*I site is likely to have no effect on the FMR1 promoter activity. The importance of methylation of individual CpGs has previously been suggested by Wölfl *et al.* (1991). The exact position of methylated and nonmethylated CpGs in the tumor could not be determined.

In conclusion, we demonstrated the localization of FMRP in the spermatogonia, the outer layer of a lung tumor and 1% of the cells, in a male fragile X patient. The presence of a premutation in the tumor illustrates the instability of the CGG repeat. Furthermore, the discrepancy between the expression of FMRP in the lung tumor and the methylation of the

*Eag*I and *Bss*HII sites indicate the need for a more accurate study of the exact promoter of *FMR1*. Future studies on the repeat length in both tumors and the tissues from which the tumors are derived, in fragile X patients, may shed a light on the mechanism and timing by which the amplification of the CGG repeat occurs.

Acknowledgements

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Subject, Materials and Methods

The patient displayed the typical characteristics of the fragile X syndrome: mental retardation, large ears, elongated face with mandibular prognathism, large hands and feet, and macroorchidism. Fra (X) cytogenetic expression was found in 6% of the cells. The patient died at 66 years of age of a large cell carcinoma in his lung, with advanced necrosis. The testes were found to be atrophic with impaired spermatogenesis.

Immunohistochemistry

Tissues were snap frozen in liquid nitrogen. Cryostat sections (7 µm) were fixed with 4% paraformaldehyde followed by a methanol step. Subsequently, sections were incubated with monoclonal antibodies (mAb) 1a (kindly provided by J.-L. Mandel) directed against an N-terminal epitope of the FMRP (Devys et al. 1993), followed by an indirect immunoperoxidase technique using 3,3'-diamino-benzidine.HCl (Serva) as a substrate. Endogenous peroxidases were inhibited by a 30-min incubation in PBS-hydrogen peroxide-sodium azide solution (Li et al. 1987). The sections were counterstained with hematoxylin.

Southern blot analysis

Genomic DNA was isolated from the various tissues, using standard procedures (Miller et al. 1988). Seven µg of DNA were digested to completion with various single endonucleases or combinations, electrophoresed on a 0.7% agarose gel and transferred to a Hybond N+ blotting membrane. pP2, a 1-kb *Pst*1 fragment derived from pE5.1 that detects the fragment containing the $(CGG)_n$ repeat and the preceding CpG island (Oostra et al. 1993*a*), was used as a probe after labelling by the random priming method (Feinberg and Vogelstein 1983). After overnight hybridization at 65°C, the filters were washed to 0.3 x SSC, 0.1% SDS at 65°C prior to exposure to X-ray film.

PCR

Genomic DNA was PCR amplified using the robust *Pfu*-method described by Chong et al. (Chong et al. 1994). Primers used for amplifying the sequences flanking the CGG repeat were *c, d, e, f* (Fu, et al. 1991), and *t* (5'-CGCGGAATCCCAGAGAGGCCGAACTG-3', position 2243-2268 of pE5.1, EMBL X16378). The dinucleotide repeat markers DXS548 and FRAXAC2 were analyzed as described by Verkerk et al. (1991) and Richards et al. (1991), respectively.

Determination of the exact repeat length in tumor, testis and FMR1-positive cells was made according to the method of Zhong et al. In short, genomic DNA (25-50 ng) was PCR amplified in a 50-µl reaction volume containing 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 50 pmol of primer *A* (Fu et al. 1991) and primer *3* (Brown et al. 1993), and 2.5 units Taq polymerase (Perkin Elmer Cetus). The cycles were initiated with 4-min denaturation at 95°C, followed by 30 cycles of 65°C for 1 min, 72 °C for 2 min and 95°C for 1 min with a final extension for 10 minutes at 72°C. The PCR product was extracted once with phenol/chloroform, ethanol precipitated, and digested in 10 µl with 5 U *Mnl*I at 37°C for 60 min. The DNA was sized by PAGE on Sequagel-6 (National Diagnostics), semidry electroblotted on to Nitron+ membrane (S&S), and probed with a 5'-labelled oligonucleotide (GCGTGCGGCAGCGCGGCGGCG) in rapid-hyb buffer (Amersham) at 65°C for 60 minutes in Hyb-

oven (National Labnet). The membrane was washed at a final stringency of 65°C, 0.1 x SSC twice for 10 min with 0.1% SDS and autoradiographed for 3-17 h at -70°C.

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Hotspot for deletions in the CGG repeat region of *FMR1* in fragile X patients.

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Abstract

The fragile X syndrome is the most frequent cause of inherited mental retardation. The molecular mechanism of the disorder is based on the expansion of a CGG repeat in the 5′ UTR of the *FMR1* gene in the majority of fragile X patients. The instability of this CGG repeat containing region is not restricted to the CGG repeat itself but expands to the flanking region as well. We describe 4 unrelated fragile X patients that are mosaic for both a full mutation and a small deletion in the CGG repeat containing region. Sequence analysis of the regions surrounding the deletions showed that both the $(CGG)_n$ repeat and some flanking sequences were missing in all 4 patients. The 5′ breakpoints of the deletions were found to be located between 75 to 53 bp proximal to the CGG repeat. This suggests the presence of a hot spot region for deletions in the CGG repeat region of the *FMR1* gene and emphasizes the instability of this region in the presence of an expanded CGG repeat.

Introduction

The fragile X syndrome is the most common inherited form of mental retardation (1) and the disorder is associated with the expression of a fragile site at Xq27.3. Besides mental impairment, the patients are characterized by typical morphological features such as large, prominent ears, elongated face with mandibular prognathism and macroorchidism (2). The incidence of fragile X syndrome is estimated to be approximately 1 in 1250 males and 1 in 2500 females (for review see 3).

In the majority of the patients, the molecular mechanism of the disorder is based on an unstable $(CGG)_n$ trinucleotide repeat in the 5′ UTR of the *FMR1* gene (4,5,6,7,). In the normal population this trinucleotide repeat is polymorphic, varying between 6 and 52 repeat units, and is stable upon transmission (7). Premutation alleles, ranging from 50 to 200 repeats, exhibit instability, usually resulting in increases in repeat number in the offspring when compared to the parents. Premutations are not associated with a clinical phenotype and are found in female carriers and normal transmitting males. During or after passage through female meiosis, a premutation can expand to a full mutation of over 200 repeats resulting in methylation of the CGG repeat and the CpG island 250 bp proximal to the CGG repeat (5,8-10). This methylation is associated with repression of *FMR1* transcription, thereby resulting in the severe reduction of the level of *FMR1* protein leading to the fragile X phenotype (11,12). Although the function of the *FMR1* protein is not yet fully understood, evidence is accumulating that the protein plays a role in RNA metabolism. Two motifs have been identified that are present in RNA binding proteins: a RGG box and a KH domain (13,14).

Transmission of an expanded CGG repeat mostly results in increase in repeat length, although occassionally, regression of the CGG repeat has been reported as well. Reduction of a premutation to a smaller premutation has been detected (6,7,15,16,17) as well as reduction of a premutation to a repeat length within the normal range (18). Decreases from a full mutation to a premutation has been reported only once (15), whereas the reverse mutation, from full mutation to a normal allele has not yet been described. This in contrast to the CTG

repeat in myotonic dystrophy, in which a number independent reverse mutations have already been described (19-21).

Several deletions in the *FMR1* gene resulting in the fragile X syndrome are now known. Two large deletions of 2.5 Mb (22) and 3 Mb (23) have been described including the entire *FMR1* gene plus flanking sequences. In a third patient a 250 kb deletion resulted in the absence of the *FMR1* gene associated CpG island and the first 5 exons of the gene (24), whereas in a fourth patient approximately 100 kb, including the first 8 exons and upstream sequences, were missing (25). Recently a small deletion of 1.6 kb immediately 5′ of the CGG repeat was detected, removing regulatory sequences of the gene. This deletion resulted in 4 affected males within a fragile X family (26).

The instability of the CGG repeat is not only seen between generations, but it can also be seen within one individual. Patients with a full mutation display a variety of repeat lengths, visible as a smear in Southern blot analysis. In addition, approximately 20% of the affected males is mosaic for both a full mutation and a premutation allele (5,7,11,15).

In this paper we describe 4 unrelated fragile X patients that are mosaic for a full mutation in the majority of their lymphocytes and a deletion of the CGG repeat and flanking sequences in approximately 5-10% of their blood lymphocytes. Sequence analysis of the deletion junctions revealed that all 5′ breakpoints are located within 30 bp of each other. This emphasizes the instability of the CGG repeat region of *FMR1*.

Results

We studied the length of the CGG repeat and the methylation pattern of the CpG island proximal of the *FMR1* gene using Southern blot analysis. In normal males and females digestion of DNA with *Eco*RI results in a 5.2 kb fragment. The majority of fragile X males exhibit a smear of over 5.8 kb, due to expansion of the CGG repeat to over 200 repeats. In 4 male patients, digestion with *Eco*RI not only resulted in this smear but in addition a fragment slightly smaller than the normal 5.2 kb band was found (data not shown). These novel fragments were estimated to be derived from approximately 5-10% of the cells in patients 1, 2 and 4 and 15% in patient 3. Double digestion of DNA of normal males with *Eco*RI and the methylation sensitive enzyme *Eag*I leads to a 2.8 kb fragment (Fig. 1, lanes 6 and 9). In females both the 2.8 kb fragment and the methylated 5.2 kb fragment are detected, due to normal X chromosome inactivation (Fig. 1, lanes 7 and 8). In fragile X males the smear of over 5.8 kb remains due to methylation of the *Eag*I site. However, in the 4 male patients we detected both this smear as well as a smaller fragment (Fig. 1, lanes 1, 3, 5, 10). These fragments varied in size between 2.7 kb in patient 1 (lane 1) to 2.2 kb in patient 3 (lane 5) again indicating the presence of deletions that varied from 150 to 600 bp, all falling within the 2.8 kb *Eco*RI-*Eag*I fragment. The *Eag*I restriction site could clearly be digested, which implies the absence of hypermethylation of the novel fragment. DNA derived only from the mothers of patient 1 and 2 was also subjected to Southern blot analysis (Fig. 1, lanes 2 and 4, respectively). Both females were carrier of a premutation and did not have the smaller fragment present in their sons. This indicates that the deletions in the patients were derived

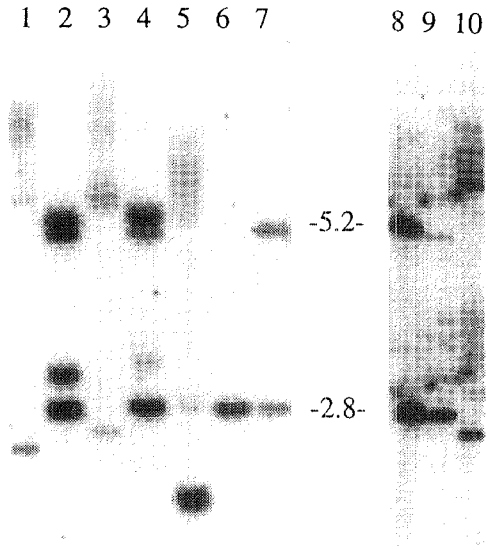

Figure 1. Southern blot analysis of the four patients. DNA was digested with *Eco*RI-*Eag*I and following electrophoresis, fragments were blotted on filters and hybridized with pP2. Lane 1, 3, 5 and 10 represent patients 1, 2, 3 and 4 respectively. Lane 2: mother of patient 1; lane 4: mother of patient 2; lane 6 and 9: control male; lane 7 and 8: control female. The normal length of the fragments are indicated.

from a *de novo* event.

In order to determine the exact junction of the 4 deletions we PCR amplified the region surrounding the CGG repeat. The deletion in the *Eag*I-*Eco*RI fragment was only present in a low percentage of their cells. Therefore, in order to get a sufficient amount of PCR product, we performed two cycles of amplification standardly. After a Centricon-100 purification, the PCR products were sequenced using the same primers as were used for amplification. A schematic representation of the deletion breakpoints of the 4 patients described are shown in figure 2. This figure also includes the deletion described by Meijer et al (26; pat. 24) and Kremer et al. (32, XTY26). The exact sequence of the junctions of all deletions are shown in table 1.

Discussion

The instability of the expanded CGG repeat in the *FMR1* gene is a well known phenomenon. Transmission of a premutation to the offspring can result in increase and decrease of the CGG repeat, whereas increase to a full mutation only occurs after transmission by a female (6,7,28). The variation of repeat length is not only found between generations but is also present within individual fragile X patients and is called somatic instability or mosaicism. Two different types of mosaicism have been observed. Firstly, there is the wide range of repeat lengths of the full mutation, visible as a smear on Southern blot analysis (7,29,30). Secondly, 20% of the fragile X patients are mosaic for a premutation allele in addition to the full mutation (7,11,15). Mosaicism is established in early fetal life. Identical patterns of mosaicism were demonstrated in different patients are mosaic for a premutation

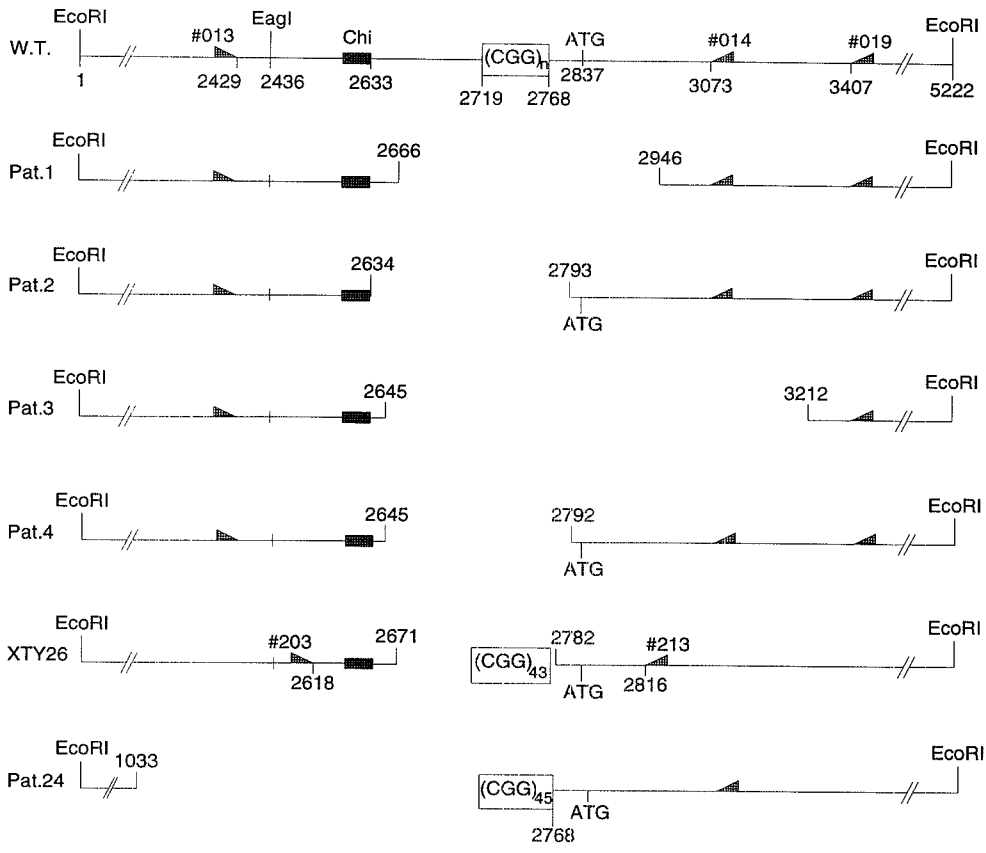

Figure 2. Schematic representation, not drawn to scale, of the deletions found thus far in the CGG region. The position of the primers in the pE5.1 sequence are indicated (32) as triangles. WT: wild type sequence; ATG: startcodon. Patients (Pat.) 1 to 4 are described in this paper; XTY26 and Pat.24 were described by Kremer et al.(27) and Meijer et al. (26), respectively.

allele in addition to the full mutation (7,11,15). Mosaicism is established in early fetal life. Identical patterns of mosaicism were demonstrated in different fetal tissues of monozygotic twins (30) and a 13 week old fetus (31). In vitro studies on proliferation of single fibroblasts from adults resulted in several clones, each carrying only one repeat length (29). This indicates that once mosaicism is established early in development it remains stable during life.

In this paper we describe a third kind of mosaicism; in 4 unrelated fragile X males we detected both a full mutation and a deletion of the CGG repeat and flanking sequences. The deletions in patients 1 and 3 encompasses the first ATG of *FMR1*. Therefore these patients will not produce a functional protein. The other two patients may have *FMR1* mRNA and protein expression but this could not be tested. However, this may be irrelevant, for it has

Table 1 Junction sequences of seven deletions in the CGG repeat region. Patients 1-4 are described in this paper; deletions 24 and XTY26 were reported previously (26,27)

Patient	deletion breakpoint@ 5′ 3′
Patient 1	CCGACGGCGAgcgcgggcgg-----tttcttcttgGTGTCGGCGG
Patient 2	C*GGTGGAGGG*ccgcctctga-----ccgcagcccaCCTCTCGGGG
Patient 3	GCCTCTGAGCgggcggcggg-----gggccgaaatGGCGCTAAGT
Patient 4	**** ***** ***** **** GCCTCTGAGCgggcggcggg-----cccgcagcccACCTCTCGGG
Pat. 24	TTACACTTGGaggggtataa-----gcggcggcggCGGCGGCGGC
XTY26 5′	GGCGAGCGCGggcggcggcgg----gcggcagcgcGGCGGCGGCGG
XTY26 3′	CGGCGGCGGCtgggcctcgagcgcCCGCAGCCCAC

@ The wild type sequence is shown. The deleted sequences are indicated by lower case letters. Short regions of homology at the deletion termini are underlined. The asterisks denote complementary regions. The Chi-like element is indicated in bold italics.

already been shown that mosaic males, with both a full mutation and a premutation, have some mRNA expression but do not have a significantly different clinical phenotype than patients with a full mutation only (11,15).

The deletions reported in this paper are not the first deletions known in the region flanking the CGG repeat. In 1991, Kremer et al. (27) sequenced the 1 kb *Pst*I fragment, that was found to contain the unstable CGG repeat. This sequence was obtained from a YAC (XTY26) that was subcloned from a hybrid cell line (X.3000.1) expressing a fragile X chromosome. Southern blot analysis had shown that this cell line contained approximately 350 triplets. However, only 43 CGGs were detected in the YAC, indicating that a large part of the repeat had been deleted during cloning. Sequence analysis of this region in DNA of both fragile X patients and unaffected individuals performed independently by Fu et al. (7) revealed that in the YAC some of the CGG flanking sequences were lacking as well. Remarkably, the 5′ endpoint of the deletion in the YAC, at position 2671 of the pE5.1 fragment, is also located within the 35 bp interval in which the 5′ endpoints of the other 4 deletion are found (Fig.2; 32).

Recently, a deletion in the CGG region has been described in a fragile X family (26). The 3′ endpoint of this 1.6 kb deletion was located within the CGG repeat and the deletion was assumed to be caused by an expanded, unstable CGG repeat. Previously, 4 large deletions, spanning 2.5 Mb, 3 Mb, 250 kb and 100 kb respectively, in the fragile X gene have been described (22,23,24,25). The CGG repeats of the mothers of these patients were in the normal range suggesting that these deletions were not caused by an unstable CGG repeat. The 4 deletions described in this paper appear to be directly derived from an expanded, unstable CGG repeat; all 4 patients expressed a full mutation smear on Southern blot analysis besides

the deletion fragment (Fig. 1).

An intriguing question remains: when did the deletion take place? In 1993, Reyniers et al. (33), showed the presence of a premutation and not a full mutation in sperm of patients with a full mutation in their blood cells. From this they postulated two hypotheses on the possible timing of the CGG repeat amplification. The first model assumed expansion during meiosis of the oocyte, resulting in the presence of a full mutation in all somatic cells. In sperm cells this full mutation regressed to a premutation and due to a selection mechanism only cells with a premutation will proliferate. In the second model a premutation expands to a full mutation during postzygotic proliferation of the somatic cells, after the separation of the germ line (day 5-6 p.c.). Recent breeding studies with fragile X knock out mice demonstrated that Fmr1 gene expression is not required for gametogenesis (34). In addition, Meijer et al. (26) showed that the 1.6 kb deletion resulted in the absence of *FMR1* mRNA in the affected males of the family and that this deletion was originally derived from a male with a progeny of 5 children. This confirms that the expression of *FMR1* is not required for spermatogenesis, thus excluding the presence of a selection mechanism in sperm. The second model therefore seems more likely.

The deletions of the 4 patients described in this paper could originate from either a premutation or a full mutation. In the first case the premutation allele expands, resulting in a full mutation in the offspring. However, due to a certain error, in a few cells instead of expansion a deletion takes place. In the latter case, the premutation allele of the mother expands to a full mutation. This full mutation, which is somatically much more unstable than premutations, later, during subsequent proliferation and development of the zygote, results in a deletion in a low percentage of the cells. It is possible that all premutations found in mosaic patients are the result of regression of a full mutation indicating that reduction of the CGG repeat is a more frequent event than expected. Female carriers of a full mutation can have children with either both a full and a premutation or a full mutation alone. In these mosaics the premutation may well be caused by a deletion of a large number of CGGs in the full mutation of the mother. A similar deletion mechanism could have resulted in a patient described by Van den Ouweland et al. (35). This patient had, in addition to a full mutation, 2 alleles within the normal range. The repeat length of these normal alleles, 21 and 33 respectively, did not correspond to the mother's normal allele of 29 repeats. The sequences flanking the repeat were found to be normal suggesting that regression within the mother's expanded CGG repeat resulted in the two normal alleles present in this patient. In most cases the deletion that causes the reduction from full mutation to premutation will be restricted to the CGG repeat meaning that on Southern blot analysis either a normal or a premutated fragment will be detected. These deletions will not be noticed in diagnostic screening, resulting in an underestimation of the total percentage of deletion events. Occasionally, the deletion will also involve sequences flanking the CGG repeat, resulting in the 4 deletions described in this paper, now estimated to be below 1 %. It should be noted that these latter deletions will not be detected if the diagnostic analysis is performed by PCR using primers immediately flanking the repeat.

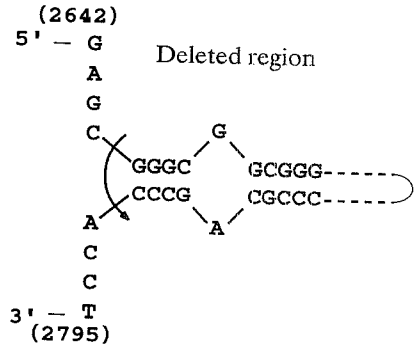

Figure 3. Possible stem-loop DNA structure formed at the deletion junction of patient 4. Single DNA strand from position 2642 to 2795 (32) is shown. Alignment of the complementary sequences at the junction results in the formation of a stem-loop. The cleavage site is indicated by the arrow.

Regression of a full mutation to a premutation would involve two mechanisms: the reduction in number of CGG repeats and demethylation. Both the premutations found in mosaic patients and the deletions described in this paper, are unmethylated fragments (Fig.1). Demethylation may be explained by that methylation of the CpG island is an active process, maintained by the presence of a full mutation. Disappearance of the full mutation would then results in passive demethylation. Methylation being an active mechanism might explain the occurrence of unmethylated full mutations (36). The possibility that demethylation is an active process in itself, causing the deletion by for instance nicking, can not be excluded.

Several studies on the mechanism of deletions have shown that approximately 40 % of the large deletions in human disorders are flanked by very short direct repeats of 2-6 bp (37-39). It is proposed that these short repeats slip and mispair during replication, resulting in the formation of a loop between the two repeats (37,38). Subsequent excision of this loop removes both the sequences between the repeats as well as one of the repeats. Of the 7 deletions in the CGG region of *FMR1* now known 5 have such a short region of homology (Table 1). Patient 3 and the deletion described by Meijer et al. (26) have a GG flanking the deletion. The deletion of XTY26 at the 3′ site of the repeat is flanked by a CG and in patient 2 the deletion is flanked by a CC. It should be noted however, that the CGG region of *FMR1* is very C-G rich and that both GG, GC and CC are present rather often in this region. It can therefore not be excluded that the presence of the 2 bp repeats represents a chance occurrence. The deletion at the 5′ site of the CGG repeat of XTY26 is a better example of this slippage mispairing mechanism. This deletion is flanked by a sequence repeat of 11 bp (Table 1).

Another mechanism may account for the deletion in patient 4. This deletion is flanked by a short inverted repeat; 9 out of 10 bp are complementary (Table 1). These sequences can align together in a quasi-palindromic manner, resulting in the formation and subsequent excision of the loop, as previously described (40-41, Fig. 3).

Neither of the two mechanism can explain the close vicinity of the 5′ endpoint of the different deletions. Recently Jeffreys et al. (42) demonstrated that both gain and loss of repeat

units in a minisatellite mainly occurred at one end of the repeat. They postulated the presence of a specific mutation initiator element adjacent to the repeat that would cause the instability by inducing a double strand break. Such an element could be a Chi element, a short sequence that is often located in the vicinity of breakpoints. Chi or Chi-like elements are thought to play a major part in recombination processes (43) and may also be involved in replication slippage (44). At position 2626, immediately 5 ′ of the breakpoint of patient 2, a Chi-like sequence is present (GGTGGAGG; Table 1) and could cause a DNA break. Subsequent misrepair of this break, possibly caused by the presence of a large CGG repeat would then result in a deletion.

Our results, that only the 5′ and not the 3′ breakpoints of the deletions are located within a hotspot, correlate well with the previous findings of Kunst and Warren (45). They demonstrated that expansion of the CGG repeat occurs at the 3′ end of the repeat. Both studies thus indicate that variability of the CGG repeat is mainly present at the 3′ site, suggesting that the replication of this region goes in one direction.

In conclusion we have described a hot spot of deletions approximately 70 bp upstream of the $(CGG)_n$ repeat. These findings may help in future unraveling of the mechanism of the CGG repeat expansion.

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Subjects, materials and methods

Patients

Patient 1 has a Prader-Willi like phenotype and was previously described by de Vries et al (46). The three other patients have the classical Martin-Bell phenotype.

Southern blot analysis

Genomic DNA was isolated from blood leukocytes according to standard procedures (47). Seven µg DNA was digested to completion with *Eco*RI and *Eag*I, electrophoresed on a 0.7% agarose gel and transferred to Hybond N+ blotting membrane. Probes StB12.3 (5) or pP2, a 1 kb *Pst*I fragment derived from pE5.1 which identifies the (CGG)n repeat and the preceding CpG island (48), were used after labelling by the random oligonucleotide priming method (49). After overnight hybridization the filters were washed to 0.1xSSC, 0.1% SDS at 65°C prior to exposure to X-ray film.

PCR and sequencing analysis

Amplification was performed using 200 ng of genomic DNA in a total volume of 50 µl in the presence of 0.2 mM of each dATP, dCTP and dTTP, 0.05 mM dGTP, 0.15 mM 7-deaza-dGTP, 10 mM Tris-Cl pH8.3, 50 mM KCl, 10% DMSO, 1 mM $MgCl_2$, 2.5 U Taq polymerase (BRL) and 1 µM of each primer. The reactions were first denatured for 5 minutes at 95°C, followed by 30 cycles of 2 minutes at 95°C, 2 minutes at 61°C and 2 minutes at 72°C. A final extension of 10 minutes was carried out at 72°C. In order to get enough product to use for sequencing, a sample of 2.5 µl of the PCR product was additionally amplified another 30 cycles using the same conditions. Products were analyzed on a 1% agarose gel, stained with ethidium bromide. After a Centricon-100 purification (Amicon), the PCR

products of the second amplification were directly sequenced using the BRL cycle sequencing kit. The primers used for the PCR reactions and the sequencing protocols as well as their position in the pE5.1 sequence are indicated (between brackets) (32):
#013: 5'- CGCGTCTGTCTTTCGACCCG (2409-2429)
#014: 5'- TAGGCGCTAGGGCCTCTCGG (3073-3093)
#019: 5'- TTCACAGCCCTCGCCCAGAACAG (3408-3430)
#203: 5'- GCGCTCAGCTCCGTTTCGTTT (2597-2619, ref.27)
#213: 5'- CTCCATCTTCTCTTCGCCCTGCTAG (2816-2842, ref.27)

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Publication 4.4

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The fragile X phenotype in a mosaic male with a deletion showing expression of the *FMR1* protein in 28% of the cells.

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Abstract

The instability of the CGG repeat region of *FMR1* is not restricted to the CGG repeat but expands to flanking sequences as well. A mosaic fragile X male is reported with a deletion of part of the CGG repeat and 30 bp immediately 3′ of the repeat, thus confirming the presence of a hotspot for deletions in the CGG region of FMR1. The deletion, detected in 28 % of his lymphocytes, did not impair the transcription and translation of *FMR1*, suggesting that regulatory elements are not present in the deleted region. The patient has the characteristic fragile X phenotype and assuming that the mosaic pattern detected in the lymphocytes reflects the mosaic pattern in brain, 28 % expression of FMRP may not be sufficient for normal cognitive functioning.

Introduction

The fragile X syndrome is the most common form of inherited mental retardation. In the majority of patients, the disease is associated with a large expansion of a CGG trinucleotide repeat (>200 CGGs) in the 5′ untranslated region of the *FMR1* gene [Verkerk et al., 1991; Oberlé et al., 1991; Yu et al., 1991]. This full mutation coincides with hypermethylation of both the CGG repeat and the *FMR1* CpG island [Hansen et al., 1992; Sutcliffe et al., 1992; Hornstra et al., 1993], resulting in the repression of *FMR1* mRNA [Pieretti et al., 1991] and *FMR1* protein (FMRP) expression [Verheij et al., 1993; Devys et al., 1993]. A smaller expansion of 50-200 CGGs, called a premutation, can be detected in unaffected male and female carriers and is not associated with abnormal methylation [Fu et al., 1991a; Reiss et al., 1994].

Instability of the expanded CGG repeats occurs both meiotically, leading to differences in repeat lengths between parents and offspring, and mitotically, resulting in a variety of repeat lengths within an individual. The full mutation in patients is visible as a smear on Southern blot analysis, thus displaying a variety of repeat lengths. Some patients are mosaic for a full mutation in combination with either a premutation [Oberlé et al., 1991; Rousseau et al., 1991; Nolin et al., 1994], normal allele [Van den Ouweland et al., 1994] or deletion [De Vries et al., 1993; De Graaff et al., 1995a] in a proportion of their cells. Moreover, it has been demonstrated that in male patients with a full mutation in their blood cells, a premutation is present in their sperm cells [Reyniers et al., 1993].

The absence of FMRP expression is generally accepted to be the cause of the fragile X syndrome. Indeed, all males with a methylated full mutation are mentally retarded, whereas in approximately 60 % of the female carriers mental impairment is observed [Rousseau, et al., 1991; Rousseau et al., 1994a]. Since the premutation, which is not methylated, results in a normal FMRP expression, a less severe phenotype in mosaic patients is expected. However, males mosaic for either a full mutation and a premutation do not have a significantly different clinical phenotype than patients with a methylated full mutation only [Rousseau et al., 1994a; De Vries, et al., 1993]. Recent reports have described methylation mosaics having an IQ ranging from normal to mental retardation [McConkie-Rosell et al., 1993; Hagerman et al., 1994; Rousseau et al., 1994b; Smeets et al., 1995].

In this paper we describe a male fragile X patient mosaic for a methylated full mutation and an allele with a deletion of part of the CGG repeat and 30 bp immediately 3′ of the CGG repeat. The deletion did not impair transcription and translation of FMRP, indicating the absence of regulatory elements in the deleted region. Despite the presence of FMRP in 28 % of the blood lymphocytes, this male displayed the clinical phenotype of the fragile X syndrome.

Results

Patient J. was encountered during an ongoing screening program in which the incidence of the fragile X syndrome in institutes for the mentally disabled is evaluated. Screening for the fragile X mutation was initially performed by PCR with primers c and f. If no normal band was detected, the screening was continued using standard Southern blot analysis with probe pP2 and *Hin*dIII and *Hin*dIII-*Eag*I digests. In normal individuals, digestion of the DNA with *Hin*dIII alone results in a 5.2 kb fragment (Fig.1, lane 2). In patient J. a mosaic pattern was found: beside a full mutation smear of over 6.5 kb an additional fragment was detected representing 20-30 % of the DNA (Fig.1 lane 1). This fragment appeared to be slightly smaller than 5.2 kb. The reduction in size was confirmed by using *Hin*dIII-*Eag*I double digests. Whereas the normal unmethylated *Hin*dIII-*Eag*I band is 2.8 kb (lane 4), the fragment in the patient was approximately 2.7 kb, indicating the presence of a 100 bp deletion within this fragment. The finding that the fragment was digested by *Eag*I implies that the fragment was not methylated, this in contrast to the full mutation which remained a smear of over 6.5 kb (Fig. 1, lane 3).

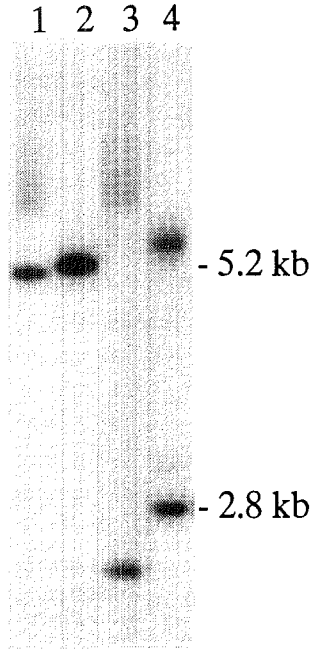

Figure 1. Southern blot analysis of the patient. DNA was digested with *Hin*dIII alone (lanes 1,2) or in combination with *Eag*I (lanes 3,4). Following electrophoresis, fragments were blotted on filters and hybridized with pP2. Lanes 1 and 3 represent patient J, lane 2: female control; lane 4: female carrier of a premutation. The normal lengths of the fragments are indicated.

To study the location of the deletion, the region surrounding the repeat was amplified. Using primerset *a-258* a PCR product of 470 bp, approximately 100 bp shorter than the expected product, was found, indicating that the deletion fell within this primerset. Likewise, amplification with primers *c* and *f* resulted in a PCR product of 160 bp in stead of the expected 260 bp. The exact breakpoint of the deletion was determined by sequence with *Hin*dIII alone results in a 5.2 kb fragment (Fig.1, lane 2). In patient J. a mosaic pattern was found: beside a full mutation smear of over 6.5 kb an additional fragment was detected representing 20-30 % of the DNA (Fig.1 lane 1). This fragment appeared to be slightly smaller than 5.2 kb. The reduction in size was confirmed by using *Hin*dIII-*Eag*I double digests. Whereas the normal unmethylated *Hin*dIII-*Eag*I band is 2.8 kb (lane 4), the fragment in the patient was approximately 2.7 kb, indicating the presence of a 100 bp deletion within this fragment. The finding that the fragment was digested by *Eag*I implies that the fragment was not methylated, this in contrast to the full mutation which remained a smear of over 6.5 kb (Fig. 1, lane 3) analysis. After a Centricon-100 purification step, the PCR-products obtained by amplification with primers *a-258*, were sequenced directly with primers *c* and *f*. The deletion was found to encompass part of the CGG repeat in addition to 30 bp 3' of the CGG repeat (position 2768 to 2797 of pE5.1 [Fu et al., 1991b]. The exact sequence of the junction of the deletion is depicted in figure 2A. Figure 2B shows a schematic representation of the deletion, in comparison with previously described deletions in the CGG repeat region.

Considering the absence of hypermethylation of the fragment with the deletion, normal mRNA expression could be expected in the cells containing the deletion as would be in line with unmethylated premutation alleles. Additionally, the finding that the deletion only included part of the untranslated region suggested that the cells containing the deletion could still produce the FMRP. To verify this, we performed immunocytochemistry on cultured blood lymphocytes and blood smears. Figure 3A illustrates the FMRP expression in the cytoplasm of cultured lymphocytes derived from a healthy individual. Fragile X patients with a methylated full mutation only, lack expression of FMRP in blood lymphocytes (Fig. 3B). In agreement, the majority of lymphocytes (72%) derived from this patient were negative (Fig. 3C), demonstrating the silencing of the *FMR1* gene. However, normal cytoplasmatic expression of FMRP could be detected in 28% of the cultured lymphocytes. These cells are likely to contain the 100 bp deletion in stead of the methylated full mutation, thus indicating that the deletion did not impair FMRP translation. In a blood smear of patient J. a similar percentage of the lymphocytes was found to produce the FMRP (data not shown), indicating that both blood smears as cultured lymphocytes can be used for the detection of FMRP in blood cells.

Discussion

Little is known about regulatory elements in the promoter region of *FMR1*. Recently, using a *FMR1*/ß-galactosidase fusion gene in transgenic mice, a 2.8 kb fragment in the 5' region of *FMR1* has been demonstrated to be sufficient for normal *FMR1* expression [Hergersberg et al., 1995]. Therefore, this 2.8 kb *Eco*RI-*Nhe*1 fragment, including the CGG

A

(2627)

GGAGGGCCGCCTCTGAGCGGGCGGCGGGCCGACGGCGAGGCGGGCGGCGGCGGTGACGGAGGCGCCGCT

GCCAGGGGGCGTGCGGCAGCGCGGCGGCGGcggcggcggcggcggcggcggaggcggcggcggcggcggc

ggcggcggcggctgggcctcgagcgcccgcagcccacctctCGGGGGCGGGCTCCCGGCGCTAGCAGGGC

TGAAGAGAAGATGGAGGAGCTGGTGGTGGAAGTGCGGGGCTCCAATGGCGCTTTCTACAAGGTACTTG

(2894)

B

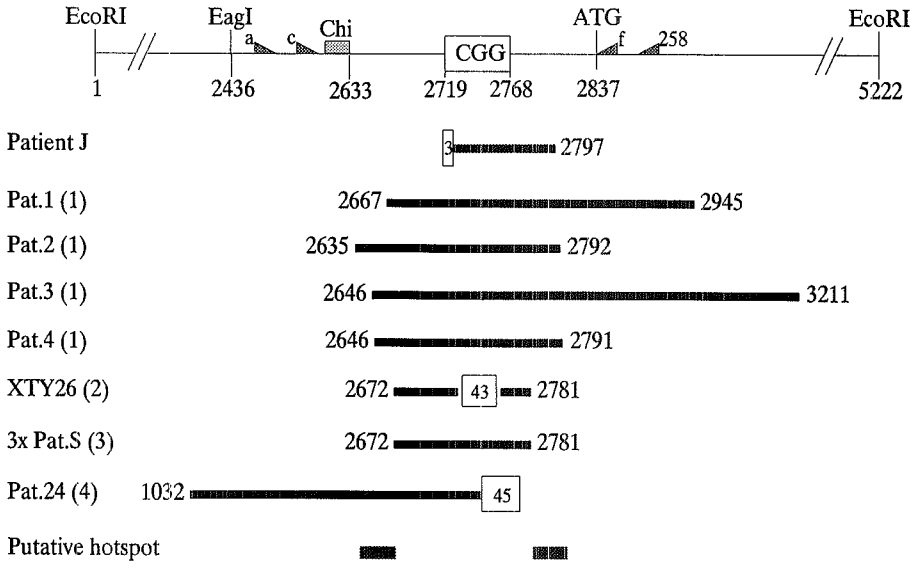

Figure 2. (A) Junction sequence of the deletion in patient J. The deleted sequence is given in bold lowercase. The short direct repeat flanking the deletion is underlined, the startcodon is double underlined. Depending on which short CGG repeat is deleted either 3 or 4 CGG copies remain. (B) Schematic representation of the deletions found thus far in the CGG region (not drawn to scale). Triangles represent the primers, boxed numbers represent the number of remaining CGG copies; ATG: start codon for translation; Chi:Chi-like element. Deletions described previously are as follows: (1): De Graaff et al. [1995a], (2): Kremer et al. [1991], (3): Snow et al. [1993] and (4): Meijer et al. [1994], respectively.

repeat and a large part of the first exon, contains all regulatory elements for normal *FMR1* gene expression. In addition, a 1.6 kb deletion, coinciding with the 2.8 kb fragment, has been described in a fragile X patient lacking *FMR1* mRNA expression [Meijer et al., 1994], indicating that regulatory elements reside in this fragment.

The absence of expression of FMRP is completely correlated to methylation of the CGG repeat and the preceding CpG island of *FMR1*. In the majority of fragile X patients with a full mutation all CpG dinucleotides in the promoter region are methylated [Hansen et al., 1992; Hornstra et al., 1993]. Recently, we described a lung tumor with a premutation allele that expressed the FMRP despite the methylation of the *Eag*I, *Bss*HII and *Sac*II restriction sites [De Graaff et al., 1995b]. It is very likely that not all CpG dinucleotides in this tumor are methylated. Hwu et al. [1993] performed in vitro methylation studies using different methylases. Methylation of all CpGs, using *Sss*I methylase resulted in the total absence of the *FMR1* expression, whereas methylation with *Hpa*II methylase did not hamper the promoter activity. Thus, certain CpG dinucleotides in the promoter region appear to be essential for promoter activity of *FMR1* and these are presumably unmethylated in the tumor.

Since the deletion, observed in part of the cells in this patient, did not impair the production of FMRP, the 30 bp immediately 3′ of the CGG repeat does not contain any regulatory sequences. We have recently described four unrelated patients, also mosaic for a full mutation and a deletion encompassing the entire CGG repeat plus flanking sequences [De Graaff et al., 1995a]. In two patients, patient 2 and 4, the 3′ breakpoint of the deletion is located near the observed breakpoint in the current case (Fig.2B). However, these two patients lacked respectively 85 bp and 74 bp immediately 5′ of the CGG repeat as well.

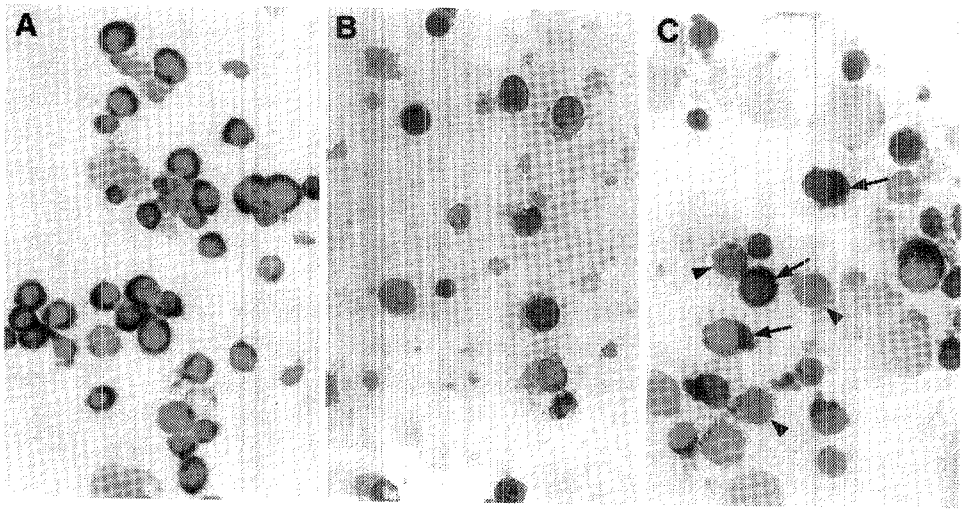

Figure 3. FMRP expression in cultured lymphocytes in a healthy individual (A), a fragile X patient with a methylated full mutation (B) and patient J. (C). Arrows denote FMRP positive lymphocytes, whereas the arrowheads indicate some unlabelled lymphocytes.

Unfortunately the expression of FMRP could not be tested in these patients, for this could indicate whether regulatory sequences are present immediately 5′ of the CGG repeat.

The instability of the expanded CGG repeat is a well described phenomenon and is present both meiotically and mitotically. Carriers of a full mutation display a large variety in repeat length, visible as a smear on Southern blot analysis [Yu et al., 1991; Oberlé et al., 1991; Wörhle et al., 1992; Devys et al., 1992]. Patients have been described to be mosaic for a full mutation in combination with either a premutation [Oberlé et al., 1991; Rousseau et al., 1994a; Nolin et al., 1994], normal allele [Van den Ouweland et al., 1994] or deletion [De Vries et al., 1993; De Graaff et al., 1995a]. The occurrence of deletions in the CGG repeat region of *FMR1* indicate that the instability is not restricted to the repeat itself, but it expands to the flanking region [Kremer et al., 1991; Meijer et al., 1994; De Graaff et al., 1995a].

Although the CGG repeat length of the mother is unknown, it is very likely that the deletion occurred within an expanded repeat. The patient has a full mutation indicating that the mother will be a carrier of either a premutation or a full mutation. In addition, the occurrence of repeat sizes below 10 CGGs is very rare, with 6 repeats being the smallest allele found thus far [Fu et al., 1991a]. Whether this deletion occurred in a full mutation or a premutation allele depends mainly on the timing repeat expansion of premutation to full mutation.

The finding that sperm cells of male patients, with a full mutation in their blood cells, only have a premutation, resulted in two hypotheses on the possible timing of the repeat expansion [Reyniers et al., 1993]. In the first hypothesis, the repeat expands during postzygotic proliferation in the somatic cells, after the separation of the germ line. Occasionally somatic cells escapes the CGG repeat expansion, thus resulting in a mosaic genotype. In this hypothesis, the deletion could occur both before amplification, thus in a premutation, or after amplification of the repeat, in a full mutation.

The second hypothesis assumes expansion of the repeat in the oocyte, during meiosis, resulting in a full mutation in all somatic cells. In some cells gametic and somatic, regression to a premutation can occur and due to a strong selection mechanism only sperm cells with a premutation can proliferate. Although FMRP is highly expressed during gametogenesis [Devys et al., 1993; Bächner et al., 1993], FMRP is not essential during gametogenesis [Bakker et al., 1994; Meijer et al., 1994]. Therefore, absence or presence of FMRP can not be the basis of a selection mechanism. A possible selection mechanism could be based on the finding that replication of a full mutation allele is delayed as compared to premutation alleles [Hansen et al., 1993]. Sperm cells carrying a premutation may therefore proliferate faster than sperm cells carrying a full mutation. Regression of the full mutation can not only delete CGGs but sometimes some flanking sequences as well, resulting in deletions as is found in this patient and previous reports [Kremer et al., 1991; Meijer et al., 1994; De Graaff et al., 1995a].

Regression from a full mutation to the deletion, not only involves deletion of DNA sequences but also demethylation. Methylation of the CGG repeat and CpG island may be an active process, maintained by the presence of a full mutation. Disappearance of the full mutation would result in passive demethylation. Absence of this active methylation process can thus explain the occurrence of unmethylated full mutations. On the other hand, the

possibility that demethylation is an active process in itself, may not be excluded. Active removal of the methyl-groups might then result in damaging of the DNA, thus causing the regression.

The fragile X syndrome is widely accepted to be caused by the absence of expression of FMRP. Evidence is now accumulating that a certain threshold of FMRP expression is required for normal cognitive functioning. Premutations are not methylated and will therefore result in a normal expression of *FMR1* [Feng et al., 1995a]. Nevertheless, fragile X patients, mosaic for a full mutation and a premutation, were not less mentally impaired as patients with a full mutation only [Rousseau, et al., 1991; De Vries, et al., 1993; Rousseau, et al., 1994a], suggesting that the percentage of cells expressing the FRMP is too low in these patients. In keeping with this patient J, described in this paper, was found to be moderately retarded, despite 28% of the blood lymphocytes still expressing FMRP. Therefore 28% of the cells expressing *FMR1* appears to be insufficient for normal cognitive functioning. It should be noted that the analysis of the CGG repeat is generally performed in blood cells, which may not be a proper indication for the number of cells expressing FMRP in brain [Rousseau, et al., 1991; Devys, et al., 1993]. However, for obvious reasons this will be difficult to verify.

In contrast, Rousseau et al. [1994b] described a cognitive normal male with an unmethylated full mutation in 60% of the lymphocytes. Although no data was presented on *FMR1* expression, it is likely that the 60% cells containing an unmethylated full mutation, FMRP is expressed. Apparently this amount is sufficient for a normal cognitive function. Normal males were found to have a unmethylated full mutation of up to 1500 CGGs in all cells [Smeets, et al., 1995]. In contrast to the finding by Feng et al. [1995b], who described the absence of FMRP translation of alleles with CGG repeats above 200, Smeets et al. [1995] found protein expression in all cells, although at reduced levels. This may suggest that both a reduced expression of FMRP in all cells and normal expression of FMRP in a high percentage of cells do not impair cognitive functioning. Noteworthy in this respect is the finding that approximately 60% of the females carrying a full mutation have some degree of mental impairment. This is in striking contrast to other X-linked disorders. A possible explanation for this phenomenon is, at least 50 % of the cells need to express the FMRP for a normal cognitive function. Hence in females, skewed X inactivation, with over 50% of the active X chromosomes containing the methylated full mutation, may lead to less than 50% of the cells expressing FMRP, thus leading to mental impairment. This would also imply that, if in future gene therapy is considered, one should keep in mind that a very high percentage of cells need to be targeted.

We recently described a putative hotspot region for deletions 5′ of the CGG repeat, suggesting that at the 5′ end of the CGG repeat some elements are present that might lead to the instability [De Graaff et al., 1995a]. In addition Snow et al. [Snow et al., 1994] mentioned to have detected a deletion in three unrelated patients coinciding with the deletion of Kremer et al. [1991], thus supporting the possible presence of such a hotspot. Indeed immediately 5′ of the deletions a Chi-like element was found (Fig. 2B). At the 3′ site of the CGG repeat the breakpoints appeared to be more scattered; only 3 out of 5 deletion described were located within 10 bp. However, the 3′ breakpoint of the currently described patient is also in the

vicinity of these 3 breakpoints, suggesting that there might be an additional hotspot for deletions 3' of the CGG repeat.

In line with the deletion reported previously, the deletion in patient J. is flanked by a short direct repeat, 'CGG' (Fig. 2A). Short direct repeats of 2-6 bp often flank deletions and it has been suggested that these short repeats slip and mispair during replication, resulting in the formation of a loop. Subsequent excision of this loop removes both the sequences between the repeats as well as one of the repeats [Canning et al., 1989; Thacker et al., 1992]. Depending on which 'CGG' repeat was deleted, either 3 or 4 CGG copies of the repeat remained.

Initially, blood smears could not be made within 6 hours after bleeding, making it impossible to perform the immunolabeling directly on the blood smears [Willemsen et al., 1995]. To overcome this problem, lymphocytes were first cultured according to standard conditions for cytogenetic expression [Verkerk et al., 1992]. Later, a second blood sample was taken from the patient, which was immediately used for making a blood smear. An equal percentage of FMRP expressing lymphocytes was found in both cultured lymphocytes and the blood smear. This implies that if blood smears can not be made within 6 hours after bleeding, the antibody test can be performed on cultured lymphocytes.

In conclusion, we have described a male fragile X patient, mosaic for a full mutation in combination with a deletion of the CGG repeat and 30 bp immediately flanking the 3' site of the CGG repeat. This deletion did not impair the production of FMRP, indicating that the deleted region did not contain regulatory elements required for expression of *FMR1*. Assuming that the pattern of mosaicism in brain is comparable to the pattern in lymphocytes, it can be concluded that expression of FMRP in 28 % of the cells will be insufficient for normal cognitive functioning.

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Materials and patient

Clinical report

The 68 years old male patient J was moderately retarded. Physical examination revealed a normally proportioned male with a height of 168 cm (10th centile) and a head circumference of 54 cm (25th centile). He had several features of the fragile X syndrome: long face with large everted ears, blue pale irises, flat feet, broad halluxes, a soft velvety skin and macroorchidism (50ml/50ml). Further he showed the typical behavior, like gaze avoidance, preservative speech and echolalia, as is frequently observed in fragile X patients.

Southern blot

Genomic DNA was extracted from blood leukocytes according to standard procedures [Miller et al., 1988]. Seven µg DNA was digested to completion with *Hin*dIII alone or in combination with *Eag*I, electrophoresed on a 0.7 % agarose gel and transferred to Hybond N+ blotting membrane. Probe pP2, a 1 kb *Pst*I fragment derived from pE5.1 which detects the (CGG)n repeat and the preceding CpG

island [Oostra et al., 1993] was used after labelling by the random oligonucleotide priming method [Feinberg and Vogelstein, 1983]. After overnight hybridization the filters were washed to 1xSSC, 0.1% SDS at 65°C prior to exposure to X-ray film.

PCR analysis

Amplification was performed using 200 ng of DNA in the presence of 10 % DMSO, 0.2 mM each of dATP, dCTP and dTTP, 0.05 mM dGTP, 0.15 mM 7-deaza-dGTP, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 1 mM MgCl, 1.25 U *Taq* polymerase (BRL) and 1 µM of each primer *a* and *258*. The reactions were first denatured for 5 min at 95 °C, followed by 32 cycles of 45" 95°C and 2½ min 68°C. A final extension of 5 min was carried out at 68 °C. After a Centricon-100 purification step (Amicon), the PCR products were directly sequenced with primer *c* and *f* using the Exo(-) Pfu Cyclist DNA Sequencing Kit (Stratagene). The following primers were used (positions in the pE5.1 sequence are indicated between brackets [Fu 1991b]:

a: 5' GGAACAGCGTTGATCACGTGACGTGGTTTT (2524-2553)
c: 5' GCTCAGCTCCGTTTCGGTTTCACTTCCGGT (2599-2628)
f: 5' AGCCCCGCACTTCCACCACCAGCTCCTCCA (2838-2867)
258: 5' GGGGCCTGCCCTAGAGCCAAGTACCTTGT (2883-2911)

Immunocytochemistry

Blood smears were made from one drop of blood within 6 hours after bleeding. Slides were air-dried and fixed with 3% paraformaldehyde for 10 minutes followed by 20 minutes permeabilization in 100% methanol. Immunolabeling was performed with monoclonal antibodies 1a1 against FMRP (kindly provided by J.L. Mandel) as described recently for blood smears [Willemsen et al., 1995]. In every preparation 100 lymphocytes were examined and scored for the presence of FMRP.

If blood smears could not be made within 6 hours after bleeding, blood samples were stored overnight at 4°C. Lymphocytes were cultured for three days according to standard procedures [Verkerk et al., 1992] and washed with PBS. Cytospins were made on slides, which were subsequently fixed and immunolabeled as described above.

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Segregation of FRAXE in a Large Family: Clinical, Psychometric, Cytogenetic and Molecular Data

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Abstract

During an ongoing study on X-linked mental retardation we ascertained a large family in which mild mental retardation was cosegregating with a fragile site at Xq27-28. Clinical, psychometric, cytogenetic and molecular studies were performed. Apart from mild mental retardation, affected males and females did not show a specific clinical phenotype. Psychometric assessment of four representative affected individuals revealed low academic achievements, with verbal and performal IQs of 61-75 and 70-82, respectively. Cytogenetically the fragile site was always present in affected males and was not always present in affected females. With FISH the fragile site was located within the FRAXE region. The expanded GCC repeat of FRAXE was seen in affected males and females either as a discrete band or as a broad smear. No expansion was seen in unaffected males, whereas three unaffected females did have an enlarged GCC repeat. Maternal transmission of FRAXE may lead to expansion or contraction of the GCC repeat length, whereas in all cases of paternal transmission contraction was seen. In striking contrast to the situation in fragile X syndrome, affected males may have affected daughters. In addition, there appears to be no premutation of the FRAXE GCC repeat, since in the family studied here all males lacking the normal allele were found to be affected.

Introduction

The fragile X-syndrome is the most common form of inherited mental retardation (Frijns 1989). It is associated with a fragile site at Xq27.3 and at the molecular level it is characterized by an unstable CGG repeat at the 5′ end of the FMR1 gene (Fu et al. 1991; Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991; for review see Oostra et al. 1993*b*). The mechanism of mutation is expansion of the CGG repeat in patients and subsequent hypermethylation of the adjacent CpG island, resulting in silencing of the FMR1 gene expression (Bell et al. 1991; Pieretti et al. 1991; Vincent et al. 1991). Diagnosis of the fragile X syndrome is now based on the determination of the number of CGG repeats: normal alleles have a repeat length <50, premutation alleles have 50-200 copies and in affected individuals full mutation alleles have more than 200 repeats (Fu et al. 1991). In the majority of individuals with both a cytogenetic expression of a fragile site at Xq27.3 and mental retardation, the fragile X syndrome is confirmed by identifying an increased CGG repeat in the FMR1 gene. However, some families have been ascertained with fragile X expression, but without CGG amplification. Refined cytogenetic methods using FISH have allowed differentiation of two other fragile sites, called "FRAXE" (Sutherland and Baker 1992; Flynn et al. 1993) and "FRAXF" (Hirst et al. 1993). Recently, the fragile site FRAXE was cloned and in individuals with cytogenetic FRAXE expression amplification of a GCC repeat was found (Knight et al. 1993). In normal individuals 6-25 copies of the GCC repeat were present with an average of 15 copies. In patients expressing FRAXE >200 copies of the GCC repeat were found. In these patients a CpG island proximal to the GCC repeat was methylated, suggesting that methylation plays a role in the inactivation of a gene in the FRAXE region. This CpG island is located 600 kb distal to the CpG island proximal to the FMR1 gene.

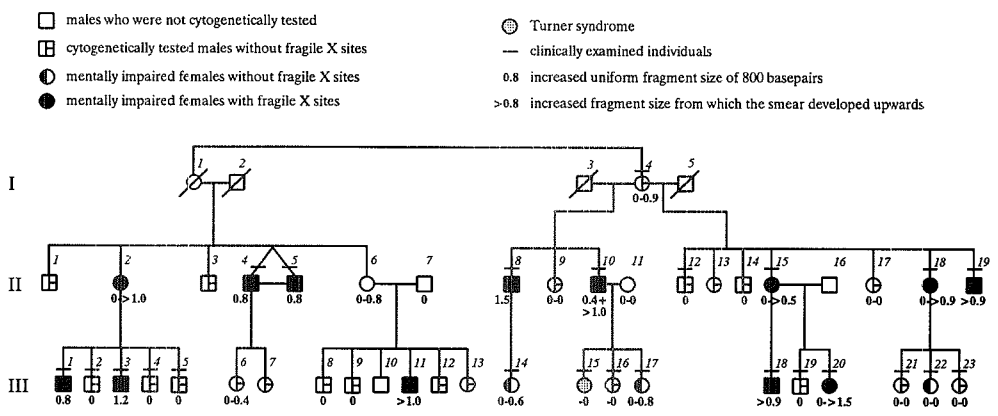

Figure 1 Pedigree

Very little is known about the clinical phenotype of FRAXE positive individuals. In the first paper describing FRAXE (Sutherland and Baker 1992) fragile site expression was reported in mentally normal individuals. In the families described by Knight et al. (1993) almost all males who did express the fragile site FRAXE were mildly mentally retarded. Carrier females were mentally normal. The expanded GCC repeat of FRAXE was seen in affected males and carrier females and was unstable when passed through both the male and female lines. A contraction of the expanded repeat was found when it was passed from an affected father to his daughter, whereas expansion was found when it was passed from a carrier mother to her affected son (Knight et al. 1993). In this paper we describe a large FRAXE family in which FRAXE is cosegregating with nonspecific mild mental retardation.

Results

Phenotype

Though some affected males had mild craniofacial dysmorphism anomalies, there was no consistent clinical phenotype present in the affected individuals. Clinical information on the affected family members is given in tables 1 and 2.

Patient III-1 (fig. 2) is a representative example of this family (fig.1) and will be described in more detail. He was born at term, after an uneventful pregnancy and delivery. Motor milestones were reached within normal limits; speech development, however, was retarded. He attended a special school for children with severe learning difficulties. Presently, he reads and writes with difficulty, but is unable to complete forms (e.g. insurance). He works in a sheltered environment. He is healthy, is married and has three healthy sons. At the age of 34 years his height is 195 cm (97th centile), armspan 196 cm, weight 105 kg (>97th centile), occipito-frontal circumference (OFC) 60,0 cm (>97th centile), ears 72 mm (97th centile), and

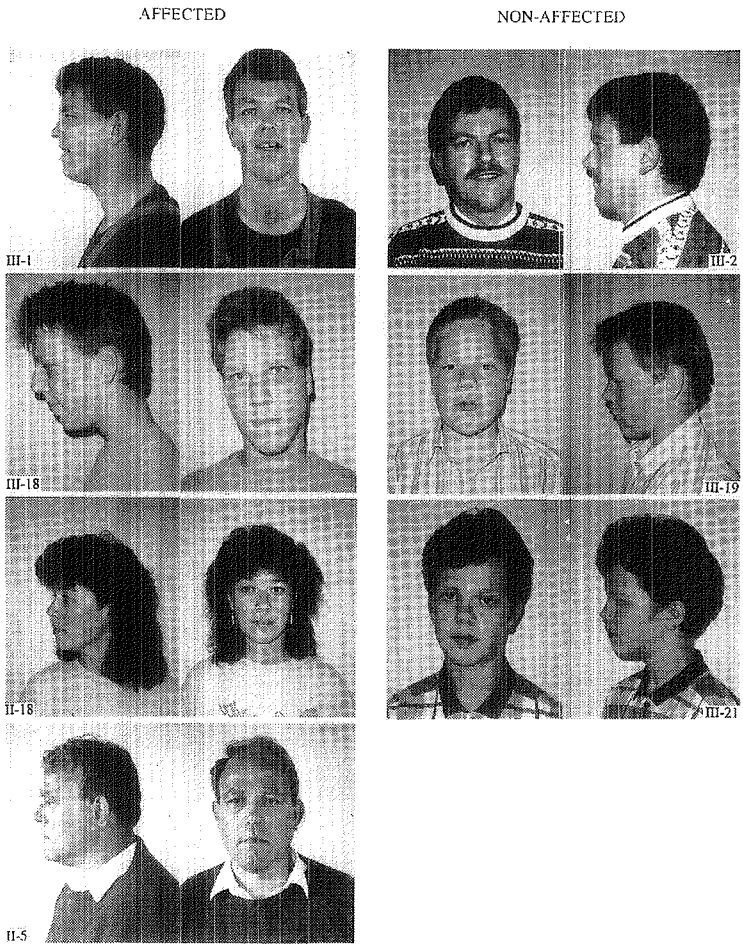

Figure 2. Patients III-1, III-18, II-18, and II-5 and unaffected family members III-2, III-19, and III-21.

testicular volume 25 ml (50-90th centile). He has a long and narrow face, mild midfacial hypoplasia, long and narrow ears and a high arched palate. His neck is long. No other abnormalities were found, in particular no macro-orchidism and hyperlaxity. The male patients II-4, II-5, II-19, III-1, III-3 and III-18 show some resemblance to each other. However, patient III-1 also resembles his normal brothers (III-2, III-4 and III-5). For comparison, clinical information on some unaffected males is given in table 3, while in figure 2 are seen the affected males II-5, III-1 and III-18, the affected female II-18, and for comparison, the unaffected males III-2 and III-19 and the unaffected female III-21. Patient III-15 showed features of Turner syndrome (see below). The only living member of generation I (I-4) is mentally normal.

Table 1 Selected clinical features (centiles) of affected males.

	II-4	II-5	II-8	II-10	II-19	III-1	III-3	III-18
Age (years)	54	54	51	46	32	34	31	16
Height	175 (10-50)	177 (10-50)	169 (<10)	178 (50-90)	188.5 (50-90)	195 (>90)	194 (>90)	176 (10-50)
OFC	58 (97)	58.5 (97)	57 (90-97)	58 (97)	59.1 (>97)	60 (>97)	59.2 (>97)	54.5 (10-50)
Testes (ml)	25 (50-90)	20 (50)	25 (50-90)	25 (50-90)	20 (50)	25 (50-90)	25 (50-90)	20 (50)
Long, narrow face	+	+	-	-	+	+	+	+
Midfacial hypoplasia	-	+	+	+	+	+	+	+
High-arched palate	-	+	-	-	+	+	+	+
Long neck	-	-	-		+	+	+	+
Prognathism	-	-	-		-	+	-	-
Miscellaneous	Obese	Obese	-	Obese	Obese	Obese	-	-

Table 2 Selected clinical features (centiles) of affected females.

	II-15	II-18	III-14	III-15	III-17	III-20	III-22
Age (years)	37	33	16	21	17	8	9
Height	166 (10-50)	165.5 (10-50)	161 (<10)	153.5 (<3)	172 (50-90)	134 (50-90)	140.5 (90)
OFC	54 (10-50)	54.4 (10-50)	53.5 (10-50)	54 (10-50)	55 (50)	134 (50-90)	50.8 (10-50)
Long, narrow face	-	-	-	-	+	-	-
Midfacial hypoplasia	-	-	-	+	-	-	-
High-arched palate	-	-	-	+	-	-	-
Long neck	-	-	-	-	-	-	-
Prognathism	-	-	-	-	-	-	-
Miscellaneous	Obese	Obese	-	45,X/46,XrX	-	-	-

Table 3 Selected clinical features (centiles) of unaffected males.

	II-12	III-2	III-4	III-5	III-19
Age (years)	44	33	29	27	10
Height	172.5 (<10)	186 (50-90)	187 (50-90)	185 (50-90)	152 (>90)
OFC	54.5 (10-50)	59 (>97)	59.5 (>97)	60 (>97)	54.5 (50)
Testes (ml)	25 (50-90)	25 (50-90)	not done	20 (50)	3-4 (50)
Long, narrow face	-	+	-	+	-
Midfacial hypoplasia	-	+	-	-	-
High-arched palate	-	+	+	-	-
Long neck	-	-	-	-	-
Prognathism	-	+	-	+	-
Miscellaneous	-	-	-	-	-

Table 4 Psychological and academic achievement

	Intelligence classification (IQ)						Academic achievement[a]		
Case (Age)	Verbal	Performance	Total	Attention[a]	Visual/ motor skills	Prerequisites[a]	Reading	Writing	Arithemetic
III-18 (16)	66	70	64	+/-	-	+/-	-	-	-
II-32 (32)	61	76	65	-	-	-	unable	unable	unable
II-18 (34)	72	78	72	-	-	-	-	-	-
III-22 (10)	75	82	76	+/-	+/-	+/-	+/-	-	-

[a]: -= Severely impaired (SD≤-2); +/-= mildly impaired (-2<SD≥-1); += unimpaired (SD>-1)

Psychometry

The overall intelligence of the tested patients was below average (total IQ<85). Verbal intelligence was not statistically significantly lower than performal intelligence. Three patients (II-18, II-19, III-18) could be classified as severely impaired, on all tested psychological functions as well as on the academic achievements (reading, writing and arithmetics). The fourth patient (III-22) performed, in comparison with the other patients, relatively well on the psychological functions and reading, whereas writing and arithmetic skill were severely impaired (table 4).

Cytogenetic analysis

A folate-sensitive fragile site at Xq27-28 was shown in all nine affected males (range 1%-40%) and in none of the unaffected. Of the 7 affected females, 4 expressed a folate-sensitive site at Xq27-28 (range 4%-46%), and all 15 unaffected females were fragile X negative. Patient III-15 has a 45,X/46,X,r(X)(92/8) karyotype. FISH analysis of r(X) with X-specific probes showed the presence of Xp and the centromeric region (data not shown).

FISH analysis with cosmids C1/C10 located between FRAXA and FRAXE (Oostra et al. 1993*b*) gave a signal proximal to the fragile site (fig.3*a*), whereas probe VK21, which is located distally from FRAXE, showed a signal distal from the fragile site (fig.3*b*). From these results it can be concluded that the fragile site detected in this family is FRAXE.

DNA analysis

The CGG repeat in the FRAXA region was within the normal range in both unaffected and affected members of this family. Southern analysis of the GCC repeat in the FRAXE region was performed by *Hin*dIII digestion and subsequent hybridization with the probe OxE20 (Knight et al. 1993). In unaffected individuals a band of 5.2 kb was detected (fig. 4). In affected individuals the expansion in the GCC repeat results in an enlarged *Hin*dIII fragment visible either as a discrete band or as a smear. All nine affected males had smears with increases in size that were 800 bp (II-4 and II-5) to far >1,000 bp (III-11). One of the males (II-10) appeared mosaic; besides the smear an additional band of 5.6 kb was visible after longer

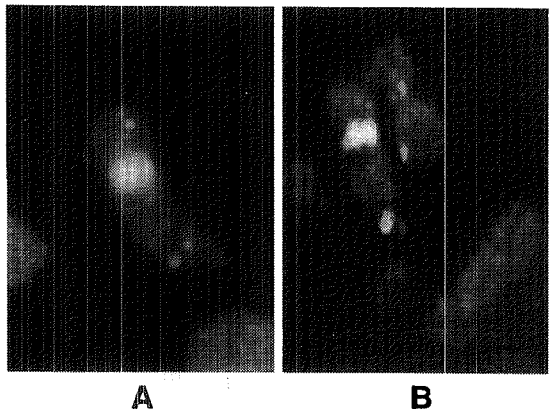

Figure 3 FISH analysis. In situ hybridization of cosmid C1/C10 (A) and cosmid Vk21 (B) to chromosome preparations of an affected member of the family. The X chromosome-specific centromere probe pBAMX5 was used for X chromosome identification. Slides were either recorded on film (A) or digitized (B).

exposure. In females both discrete bands and smears were seen (fig.1). Interestingly, only the four females who showed a smear on the Southern blot (II-2, II-15, II-18, and III-20) had cytogenetic expression of the FRAXE site, whereas females who showed a discrete band (I-4, II-6, III-6, III-14, III-17) did not express this fragile site. All individuals with an expanded GCC repeat were found to be mildly mentally retarded, with the exception of the normal females I-4, II-6 and III-6, who showed an increase of 900bp, 800 bp, and 400 bp respectively. In contrast, the affected female III-22 appeared to have a normal 5.2-kb *Hin*dIII fragment. The intensity of this fragment was equal to the band found in her two normal sisters, indicating that she had received two normal alleles. No smear was detected after longer exposure. Linkage analysis with the CGG repeat in FRAXA, marker St14 and DXS1691 located 2.5-5.3 kb distal from the FRAXE CpG island (S.J.L. Knight, unpublished results), showed that she had received the risk allele from her mother (data not shown). Analysis of the GCC repeat of FRAXE by PCR showed that she had both a normal allele consisting of 17 GCCs, derived from her father, and a second allele consisting of 25 GCCs. Thus the enlarged repeat of approximately 400 copies in the mother had decreased to only 25 copies in the daughter.

The expanded GCC repeat was found to be unstable when transmitted to the offspring. Transmission through a female resulted in an expansion of the repeat in 12 of 15cases, whereas after transmission through males a decrease in length was found in all 3 cases tested.

In one branch of this family (father II-10 and his daughters III-15, III-16, and III-17) molecular findings were particularly remarkable. The affected female III-17 is the only daughter to receive an expanded GCC repeat from her mentally retarded father (II-10). Daughter III-15 had Turner's syndrome; her X chromosome was found to lack the GCC-repeat expansion and was therefore likely to have derived from her mother. In DNA of the unaffected daughter III-16, only the normal 5.2 kb *Hin*dIII fragment was detected; no additional smears or bands were seen, even after a long exposure. The intensity of this normal band was equal to the band found in her sisters, both known to possess only one normal allele. This suggested the presence of only one allele. To determine whether the daughter III-16 had received the risk allele from her father, we tested DXS1491, a (CA)n repeat 2.5-5.3 kb distal to the FRAXE GCC repeat. The risk allele, transmitted by the father to his affected daughter, was transmitted neither to his daughter with Turner syndrome nor to his normal daughter. Instead only one allele appeared to present in III-16, suggesting that on the paternal X chromosome the DXS1491 locus had been deleted. No abnormalities were detected in her karyogram. Amplification of the CGG repeat of the FMR1 gene 600 kb upstream of the FRAXE site revealed both a maternal and a paternal allele, indicating that the deletion did not extend into the CpG island of the FRAXA region. Preliminary results have thus far indicated that the size of the deletion is 7.4 kb, beginning in the region of the FRAXE HTF island and extending distally (S.J.L Knight, unpublished results).

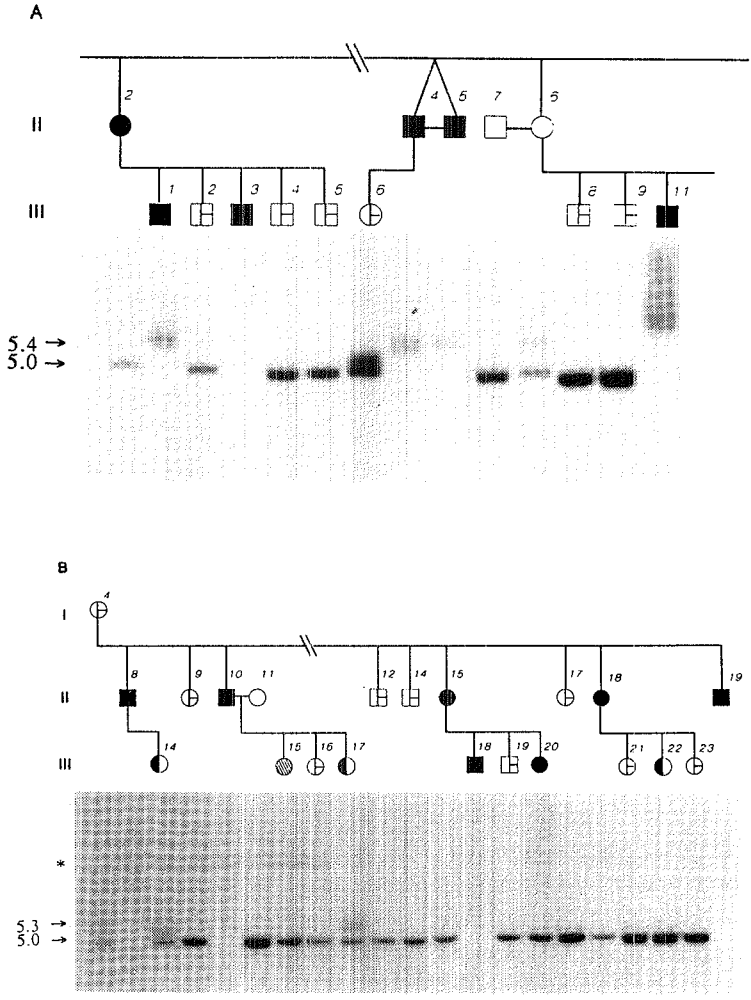

Figure 4 Southern blot analysis of two branches of the FRAXE family. DNA was digested with *Hin*dIII, and, after electrophoresis and subsequent blotting, the filters were hybridized with the probe OxE20. The asterisk indicates a constant, aspecific band, visible in all lanes after longer exposure.

Discussion

In 1981 Daker et al. reported on two mentally normal brothers with fragile site expression at Xq27-28. Since then, several other fragile X-positive probands and families without the CGG amplification of the FMR1 gene have been reported (see table 5, which includes the here-reported family K). FISH analysis has demonstrated that the fragile site in the families C, D, F, H, J and K was FRAXE and in families D, J and K the FRAXE GCC amplification indeed was found; in family F and I the fragile site appeared to be FRAXF. Only in families J and K is FRAXE associated with mild mental retardation, whereas in family H all fragile X positives are mentally normal. In family C 5 out of 10 fragile X positives were mentally retarded, whereas in family D only the proband showed mild mental retardation. The

Table 5 Published families with fragile site at Xq 27.3 other than FRAXA

Family	Clinical status		Fragile site (%)	FISH	DNA analysis	
	Proband	Others			FRAXA CGG	FRAXE GCC
A[a].....	No MR[b]	No MR	6-22	not done	not done	not done
B.....	No MR	No MR	10-75	not done	normal	not done
C.....	Mild MR	Mild MR[c]	13-42	FRAXE	normal	not done
D.....	Mild MR	No MR	26-35	FRAXE	normal	amplification
E.....	Mild MR	No MR	14-40[d]	not done	normal	not done
F.....	No MR	No MR	5-14	FRAXF[e]	normal	not done
G.....	Mild MR[f]	No MR	12-40	not done	normal	not done
H.....	No MR	No MR	14-28	FRAXE	normal	not done
I.....	Moderate MR	Moderate MR	2-26[h]	FRAXF	normal	not done
J.....	Mild MR	Mild MR	1-24	FRAXE	normal	amplification
K.....	Mild MR	Mild MR	1-46	FRAXE	normal	amplification

[a] References are as follows: A—Daker et al. (1981); B—Voelckel et al. (1989) and Oberlé et al. (1992, family 3); C—Nakahori et al. (1991, family 5c), Dennis et al. (1992, family 1), and Flynn et al. (1993); D—Nakahori et al. (1991, family 5b), Dennis et al. (1992, family 2), Flynn et al. (1993), and Knight et al. (1993, family 2); E—Oberlé et al. (1991, PC family; 1992 family 1) and Rousseau et al. (1991); F—Roman and Chapman (1992) and Sutherland and Baker (1992); G—Oberlé et al. (1992, family 2); H—Sutherland and Baker (1992); I—Hirst et al. (1993); J—Knight et al. (1993, family 1); and K—present study. [b]MR=mental retardation; [c]Of 10 fragile X positives, 5 had MR; [d]Folate-insensitive fragile site; [e] Reference: J. Mulley (personal communication); [f]XYY karyotype; [g]Of five fragile X positives, two had MR; [h]Possibly a folate-insensitive fragile site.

location of the fragile sites in two additional families, E and G, remains to be determined. In our family K no specific and consistent clinical phenotype was found, apart from mild mental retardation; this is in contrast to the fragile X syndrome, with its Martin-Bell phenotype.

FRAXE seems to be rare. We have found 1 FRAXE family among >80 families with fragile site expression. In general, FRAXE patients are not in need of medical care, and so they do not come to our attention. Besides, with the present molecular-diagnostic practice, fragile X positives other than FRAXA will be missed. This all makes it, at present, impossible to estimate its frequency in the general population. On formal testing of four representative patients, there appeared to be a tendency for verbal IQ to be lower than performance IQ, whereas in the Martin-Bell phenotype the opposite is found (Brainard et al. 1991).

The expanded GCC repeat was found to be unstable on transmission, similar to the situation in transmission of the CGG repeat in the fragile X syndrome. Reyniers et al. (1993) demonstrated that in fragile X males who have a full FRAXA mutation in their lymphocytes a premutation and not a full mutation is present in their sperm cells. By analogy, it is very

likely that in FRAXE-expressing males a smaller GCC repeat is present in sperm as compared with lymphocytes. (Preliminary results indicate that, in FRAXE, affected males indeed have a smaller *Hin*dIII fragment in sperm cells, although the additional presence of a full mutation could not be excluded.) In striking contrast to the situation in the fragile X syndrome, however, FRAXE-expressing males may have affected daughters. These daughters were found to lack cytogenetic expression of the FRAXE site, indicating that their reduced repeat length did not allow expression of the fragile site.

Because of the size of this family, we could determine the transmission of the GCC repeat by one individual to several children. We found that transmission through the same person can result in both an increase and decrease in repeat length. The passage of the GCC repeat by the FRAXE-expressing female II-20 (330 copies) resulted in an increase to 400 copies in one affected son (III-3) and in a decrease to 265 GCC copies in another son (III-1).

Knight et al. (1993) suggest that the mechanism of silencing in the FRAXE region is the same as that in FRAXA: as soon as a repeat number reaches a critical level, methylation occurs resulting in lack of mRNA and thereby causing the clinical phenotype. In the family that we studied we found that, similar to the FRAXA mutation, all GCC repeats with a length >130 copies were methylated (data not shown). In the fragile X syndrome a premutation can be transmitted through normal transmitting males. In striking contrast to the fragile X syndrome, however, there appears to be no premutation in the FRAXE GCC repeat, since in the family that we studied, all males lacking the normal allele were found to be affected.

We identified a mosaic male (II-10) possessing both a small expansion of 120 GCCs and a large expansion of >760 copies. In contrast to the clinically unaffected mosaic male with a small amplification of 133 copies and a large amplification of 866 copies, reported by Knight et al. (1993), this male was affected. A likely explanation for the observed difference is the finding that both expanded repeats in the affected male are methylated (data not shown), whereas the small fragment in the mosaic described by Knight et al. (1993) is unmethylated.

There were two peculiar phenomena in this family. First, in the mentally impaired female III-22 a fragment of 25 GCCs was present. Its length is at the upper end of the normal range of 6-25 GCCs. The methylation pattern in this female appeared to be normal and could therefore not be used to account for the observed mental impairment. It is noteworthy that in the psychometry this patient had the highest scores; her mental impairment might as well have another cause. Second, there is a remarkable branch in this family, in which the instability in the FRAXE region is clearly shown. An affected, mosaic male (II-10) has three daughters and all were different at the molecular level: one showed an expansion of the GCC repeat of the paternal allele; a second had Turner syndrome, lacking the paternal allele; and the third appeared to have a deletion, containing the GCC repeat of the paternal allele. Despite the deletion, this female was mentally normal. There might be two explanations for this peculiar phenomenon. First, it is possible that the presence of one normal allele results in normal development. This may also explain the three mentally normal females (I-4, II-6 and III-6) with an expanded GCC repeat. However, other females who also carry a normal allele apart from the expanded GCC repeat are mentally retarded. The mental retardation in these

females with an expansion may be caused by skewed X inactivation. Methylation analysis of the DNA isolated from their blood leukocytes revealed that there was no skewed X inactivation (data not shown), but one should be aware that the methylation pattern in blood may not be an accurate representation of other tissues such as brain. A second possibility is that the deletion found in this patient does not affect the promoter of the gene that is otherwise silenced by the amplification of the GCC and the subsequent methylation. Further studies will be required to determine the exact length and location of the deletion which in turn will enable us to learn more about the mechanism by which mental retardation is caused in patients with an expanded GCC repeat.

In conclusion we have describe a family in which amplification of a GCC repeat in the FRAXE region is associated with a mild mental retardation without a distinct clinical phenotype. Remarkably, affected males may have affected daughters, and the absence of normal transmitting males suggests the absence of a premutation in FRAXE. Familial mild mental retardation warrants a specific search for FRAXE.

Note added in proof: Knight et al. (1994) reported in families B and C (table 5); both exhibit GCC repeat extension at the FRAXE locus, and the only mentally retarded patient in family B is a fragile X-negative male with a 550-bp increase in size.

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Subjects, materials and methods

The family (fig.1) was ascertained from >80 families with fragile X expression. The index patient II-5 was admitted for low backpain at the neurology department and a fragile X screening was requested because of familial mild mental retardation. Eight affected males, five unaffected males, seven affected females and four unaffected females were examined by two of us (B.C.J.H. and A.P.T.S.). Clinical photographs were taken and blood sampling was performed. Affected family members were those who attended special schools for children with learning difficulties, while the unaffected individuals received regular education.

Psychometry

Four patients (II-18, II-19, III-18, and III-22) were psychometrically assessed by using standardized tests.These patients were thought to be representative in terms of schooling and intellectual and social functioning. For the intelligence test the WAIS-R (Stinissen et al. 1970) was used for adults, and the WISC-R (Van Der Steene et al. 1986) for children. Attention was scored with the test for sustained attention (Bourbon and Vos 1988). The Bender Gestalt Test
(Koppitz 1964) and Visual Motor Integration Test (Beery 1989) were used to assess the visual/motor skills. The academic achievements, including the prerequisites for reading and writing, were scored with aspects of the Groninger School Onderzoek (Kema and Kema-van Leggelo 1987) and with tests that are specifically designed to assess the reading (Wiegersma 1971; Van Den Berg and Te Lintelo 1977; Brus and Voeten 1979), writing (Struiksma et al. 1986), and arithmetic skills (Heeseb et al. 1974; Ojeman 1977).

Cytogenetics

For cytogenetic analysis, peripheral lymphocytes were cultured for 92 hours in medium TC 199, supplemented with 5% FCS. Chromosome slides were made according to routine procedures. One hundred metaphases of each individual were examined for the presence of a fragile X chromosome after solid Giemsa staining. Potential fra(X) chromosomes were photographed, destained, and subsequently GTG-banded for evaluation.

In situ hybridization was performed according to Kievits et al. (1990) and Verkerk et al. (1992). Whole cosmid DNA, c4.1 (Verkerk et al. 1991), C1/C10 (containing marker Do33), and VK21 (Oostra et al. 1993b) were labeled with the Bio-Nickkit (BRL). Biotinylated DNA specific for the X centromere pBamX5 (Willard et al. 1983) was co-hybridized for X chromosome identification. Each hybridization mix contained 2-4 ng/µl for the cosmid's probe, and 0.1 ng for pBamX5/µl and a 50-fold excess of competitor Cot-1 human DNA (BRL). This mix was denaturated and preannealed for 1 hour at 37 °C, followed by an overnight hybridization at 37 °C. After the slides were washed in 1 x SSC at 65 °C the probes were detected by alternate layers of fluorescein-conjugated avidin (DCS Vector) and biotinylatd anti-avidin antibody (Vector) both diluted to 5 µg/ml in 4 x SSC with 0.5 % Blocking milk (Boehringer).

Slides were washed in 4 x SSC with 0.05 % Tween 20. Slides were rinsed in PBS and mounted in antifading solution (2% DABCO/glycerol; Sigma) containing 0.03 µg propidium iodide/ml and 0.6 µg/ml DAPI. Microscopic analysis was performed with a Leica Aritoplan microscope. For the slides stained with C1/C10, a Kodak Ektachrome 400 ASA daylight film was used, while the slides stained with VK21 were captured by a cooled CCD camera in combination with Macprobe software (Probenaster unit; PSI).

DNA analysis

Genomic DNA was isolated from leucocytes as described elswhere (Miller et al. 1988) and 8 µg was digested to completion with either *Eco*RI (FRAXA) or *Hin*dIII (FRAXE). The samples were separated on a 0.7% agarose gel and were subjected to Southern analysis using the probe pP2 (Oostra et al. 1993*a*) and OxE20 (Knight et al. 1993) for characterizing the FRAXA and FRAXE region, respectively. The probes were labelled by the random oligonucleotide-priming method (Feinberg and Vogelstein 1983). Before hybridization the labelled probe OxE20 was incubated with 100 µg total human DNA for 2 hours at 65°C. After 2 h prehybridization and overnight hybridization, the filters were washed to 0.1 x SSC, 1% SDS at 65°C, prior to exposure to X-ray film. Amplification of the GCC repeat was performed as described elsewhere (Knight et al. 1993). PCR analysis of the FRAXA CGG repeat was performed according to the procedure described by Fu et al. (1991). In order to study DXS1691, a (CA)n repeat located 2.5-5.3 kb distal to the FRAXE CpG island, 60 ng genomic DNA was amplified in a total volume of 10 µl consisting of 1 mM $MgCl_2$, 0.2 mM of dCTP, dTTP and dGTP, 0.025 mM dATP, 10 mMTris-CL pH8.3, 15 mM KCl, 0.01 % gelatin, 4 µCi ^{32}P-dATP, 2.5 U *Taq* polymerase (BRL) and 0.25 µM of primers F322 and F010 as described (S.J.L. Knight, unpublished results). The reactions were initially denatured for 4 minutes at 95°C, followed by 33 cycles of 1 minute at 95°C, 1 minute at 65°C and 1 minute at 72°C. A final extension of 4 minutes was carried out at 72°C. The amplifications were performed on a 9600 thermocycler (Perkin-Elmer). The primer sequences were F322, 5'-GCAATGATAATGTTGAGTTCTACC; and F010, 5'-CTCAAGACCAAACTTGAAGAAACC.

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SUMMARY

The fragile X syndrome is the most frequently encountered form of inherited mental retardation in human. In 1991, the gene associated with the fragile X syndrome was identified and was named *FMR1*. The cloning of the *FMR1* gene revealed a new mutational mechanism, namely that of an expanding trinucleotide repeat. The CGG repeat, located in the 5'UTR of the *FMR1* gene can be present in two mutated configurations: a premutation, detected in unaffected individuals, and a full mutation which leads to the lack of FMR1 protein and the fragile X clinical phenotype. The scope of this thesis was to study the mutational mechanism leading to the mutated *FMR1* gene.

The finding that the fragile X syndrome was caused by an expanded CGG repeat resulted in the development of reliable diagnostic methods using direct DNA analysis. The subsequent use of DNA analysis revealed that the majority, but not all, of the fragile X patients had a methylated full mutation allele. Thus far, three patients with a point mutation in the *FMR1* gene have been described. In addition, twelve patients were reported to have a deletion, encompassing (part of the) *FMR1* gene.

Although most of the point mutations and deletions were detected in sporadic fragile X patients, we identified a family in which a 1.6 kb deletion, immediately 5' of the CGG repeat, was carried by 11 individuals. The four affected males with this 1.6 kb deletion were found to lack *FMR1* mRNA expression, confirming that the fragile X syndrome is a single gene disorder. Interestingly, the deletion, resulting in the absence of FMRP, was derived from a male with normal progeny. This indicated that FMRP is not essential for spermatogenesis.

All but one mutation described thus far, resulted in the absence of FMRP. Mosaic fragile X males, carrying both a full mutation and a premutation allele, are clinically not distinct from males with a full mutation only. This suggested that the number of cells expressing FMRP due to the presence of the premutation allele, was too low for normal cognitive functioning. We identified a patient that was mosaic for a full mutation and a deletion in approximately 30 % of his blood cells. The deletion removed 39 bp immediately 3' of the repeat and did not impair transcription and translation of *FMR1*. Despite the presence of FMRP in 30 % of his cells, the patient was clinically affected, suggesting that even 30 % of the cells expressing FMRP is not sufficient for normal cognitive functioning.

Little is known about the promoter region of the *FMR1* gene. In the patient described above, *FMR1* transcription was not impaired, indicating that the 3' sequences do not have a regulatory function. The 1.6 kb deletion, immediately 5' of the repeat was demonstrated to impair *FMR1* transcription, indicating that (some) regulatory sequences were missing. Methylation of the CpG island is known to play an essential role in the regulation of *FMR1* transcription. Methylation of the CGG repeat containing region is completely correlated with the absence of *FMR1* mRNA. However, we detected FMRP expression in a tumor in a fragile X male, despite the presence of a methylated premutation. Both the *Eag*I and *Bss*HII sites, sites generally used to determine the methylation pattern of the *FMR1* gene, were completely methylated. This suggests that the CpG dinucleotides in these restriction sites are not essential for regulation of *FMR1* expression. We proposed that the CpGs, required for normal

transcription are unmethylated in this lung tumor, which is currently under investigation.

The timing of the repeat expansion is one of the puzzling features of the fragile X syndrome. In sperm of fragile X males only a premutation allele is found, despite the presence of a full mutation allele in the patients blood cells. In the testis of both an adult and a fetal fragile X male, we detected expression of FMRP only in spermatogonia, suggesting the presence of a premutation allele. The difference in repeat length between sperm and somatic tissue, has led to the postulation of a model on the timing of amplification of a premutation allele to a full mutation. This model assumes a full mutation allele in the oocyte, resulting in an embryo with the majority of cells containing a full mutation. Regression and a yet unidentified selection mechanism favoring the proliferation of spermatogonia with a premutation, would then result in a pool of spermatogonia containing premutation alleles only. The finding of a full mutation in the oogonia of two female fetuses indicates that a full mutation can be present in oocytes. This implies that regression and selection can occur.

The regression is not restricted to the male germ cells, but is in some extend also present in the somatic tissue. Over 40 % of the fragile X males is mosaic for a full mutation and a premutation. The regression is not always restricted to the CGG repeat, but can extend to the flanking sequences as well. Mosaic males have been described with both a full mutation and a deletion, encompassing the CGG repeat and flanking sequences. Remarkably the 5′ and 3′ endpoints, of respectively 10 and 9 deletions out of 14, were located in a small interval, indicating that two hotspots for deletions are located in the 5′UTR of the *FMR1* gene. This suggests the presence of a specific sequence which may lead to enhanced instability in the CGG repeat containing region.

In the vicinity of FRAXA, a second fragile site is located, designated FRAXE. In this fragile site a GCC repeat was located which displays very similar characteristics with FRAXA. We characterized this repeat in a large family displaying mild mental retardation. Like in FRAXA, all males and a number of females with an expanded GCC repeat were affected, whereas all unaffected males were found to carry a normal allele. This indicated that in FRAXE the premutation appears to be absent. Sperm of an affected FRAXE male contained a smaller allele as compared to the CCG repeat in the somatic tissue, as was already detected in the fragile X syndrome. However, in striking contrast to the situation in fragile X syndrome, we identified affected males with affected daughters, although cytogenetic expression of the FRAXE site in the daughters could not be detected.

In conclusion, analysis of the CGG repeat in fragile X patients has led to a gain in knowledge concerning the repeat instability. However, in order to answer questions on how and when the repeat expands exactly, what causes the parental bias and when the expanded repeat becomes methylated, further studies will be required. As these studies are difficult in human, new models needed to be developed. We thereto introduced an expanded CGG repeat in transgenic mice. This repeat was stably inherited which might have been caused by interruption of the repeat in the 3′ part of the repeat. In future the introduction of an expanded, perfect CGG repeat, possibly at the FRAXA locus, thus conserving the flanking sequences, might resolve the exact mechanism and timing of the CGG amplification.

SAMENVATTING

Het fragiele X syndroom is de meest voorkomende vorm van erfelijke mentale retardatie in de mens. Het gen, geassocieerd met het fragiele X syndroom, is in 1991 gecloneerd en kreeg de naam *FMR1*, voor fragiele X mentale retardatie. De ontdekking van het *FMR1* gen onthulde een nieuw mutatie mechanisme, namelijk dat van een verlengde trinucleotide repeat. De CGG repeat, die in het 5' onvertaalde gebied van het *FMR1* gen ligt, bestaat in twee gemuteerde vormen: de premutatie, gevonden in niet aangedane individuen, en de volledige mutatie, die resulteert in de afwezigheid van het *FMR1* eiwit en als gevolg daarvan, in het klinische fenotype van het fragiele X syndroom. Het doel van het onderzoek, beschreven in dit proefschrift, was het bestuderen van het mechanisme wat leidt tot het veranderde *FMR1* gen.

De ontdekking dat het fragiele X syndroom veroorzaakt wordt door een verlengde CGG repeat, resulteerde in de ontwikkeling van betrouwbare diagnostische methoden die gebaseerd zijn op directe DNA analyse. Uit veelvuldig gebruik van de DNA analyse bleek dat de overgrote meerderheid, doch niet alle, fragiele X patiënten een allel met een gemethyleerde volledige mutatie hebben. Tot nu toe zijn drie patiënten beschreven, die een puntmutatie hebben in het *FMR1* gen. Daarnaast zijn inmiddels twaalf patiënten bekend waarin (een deel van) het *FMR1* gen ontbreekt.

De meeste puntmutaties en deleties zijn gevonden in sporadische, niet familiale fragiele X patiënten. Wij hebben echter een familie beschreven, waarin een deletie van 1,6 kb direct aan 5' kant van de CGG repeat gevonden is in elf individuen. De vier aangedane mannen met deze 1,6 kb deletie, hadden geen *FMR1* mRNA expressie, wat bevestigt dat het fragiele X syndroom een aandoening is die met één enkel gen geassocieerd is. De deletie, die resulteerde in de afwezigheid van FMRP, is naar alle waarschijnlijkheid doorgegeven door een man die meerdere nakomelingen heeft. Dit geeft aan dat FMRP niet noodzakelijk is voor de spermatogenese.

Met uitzondering van één puntmutatie, resulteren alle mutaties in de afwezigheid van FMRP. Fragiele X mannen die mozaïek zijn voor een volledige mutatie en een premutatie allel zijn klinisch niet te onderscheiden van mannen die alleen een volledige mutatie hebben. Dit suggereert dat in deze mozaïeke mannen het aantal cellen dat FMRP tot expressie brengt als gevolg van de aanwezigheid van een premutatie allel, te laag is voor een normaal cognitief functioneren. We hebben een patiënt beschreven die mozaïek was voor een volledige mutatie en een deletie in ongeveer 30 % van zijn cellen. De deletie van 39 bp, direct aan de 3' kant van de CGG repeat, had geen invloed op de transcriptie en translatie van het *FMR1* gen. Ondanks de aanwezigheid van FMRP in 30 % van zijn cellen, had deze man de klinische verschijnselen van het fragiele X syndroom. Dit suggereert dat normale FMRP expressie in 30 % van de cellen, niet voldoende is voor een normaal cognitief functioneren.

Er is erg weinig bekend over het promotor gebied van het *FMR1* gen. In de hierboven beschreven patiënt was de *FMR1* transcriptie niet veranderd, wat aangeeft dat de sequentie direct na de CGG repeat geen regulerende functie heeft. In de patiënten met de 1,6 kb deletie, direct aan de 5' kant van de CGG repeat werd geen *FMR1* transcriptie gevonden, wat

impliceert dat (een deel van) de regulerende sequentie ontbrak. Methylering van het CpG eiland, in het promotor gebied voor het *FMR1* gen, heeft een grote regulerende invloed op de expressie van het *FMR1* mRNA. Tot nog toe was er een complete correlatie tussen de methylering van het gebied waarin de CGG repeat ligt en de afwezigheid van *FMR1* mRNA. In een tumor van een fragiele X patiënt echter, hebben we FMRP expressie gevonden ondanks de aanwezigheid van een gemethyleerde premutatie. Zowel de *Eag*I site, als de *Bss*HII site, restrictie sites die normaliter gebruikt worden bij het bepalen van het methylerings patroon van het *FMR1* gen, waren volledig gemethyleerd. Dit suggereert dat de CpG dinucleotiden die in deze restrictie sites liggen geen invloed hebben op de regulatie van de *FMR1* transcriptie. Wij veronderstellen dat de CpGs, die benodigd zijn voor normale expressie, in deze tumor ongemethyleerd zijn. Dit wordt momenteel onderzocht.

Eén van de grote puzzels van het fragiele X syndroom, is het tijdstip waarop de repeat verlenging plaatsvindt. Fragiele X mannen hebben, ondanks de aanwezigheid van een volledige mutatie in hun bloedcellen, alleen een premutatie allel in hun sperma. In de testis van een foetale en volwassen fragiele X man, hebben we alleen FMRP expressie gevonden in de spermatogonia, wat suggereert dat deze cellen een premutatie hebben. Het gevonden verschil in repeatlengte tussen sperma en somatisch weefsel heeft geleid tot een model dat het tijdstip waarop het premutatie allel verlengt tot een volledige mutatie mogelijk verklaart. Dit model veronderstelt dat er in de oocyt een volledige mutatie zit, wat uiteindelijk resulteert in een embryo met in de meeste cellen, een volledige mutatie. Door regressie en een nog ongedefinieerd selectie mechanisme dat de proliferatie van spermacellen bevoordeelt, ontstaat een grote pool van alleen sperma cellen met een premutatie. In de oogonia van twee vrouwelijke foetussen hebben we een volledige mutatie aangetoond. Dit geeft aan dat een volledige mutatie in oocyten kan zitten, wat betekend dat de regressie en selectie plaats moet vinden.

De regressie, die volgens dit model plaats vindt, beperkt zich niet tot het sperma, maar gebeurt ook in het somatische weefsel. Meer dan 40 % van de fragiele X mannen is mozaïek voor een volledige mutatie en een premutatie allel. Regressie blijft hierbij niet beperkt tot de CGG repeat, maar kan zich ook uitbreiden tot de flankerende sequentie. We hebben fragiele X mannen beschreven die mozaïek waren voor een volledige mutatie en een deletie van de CGG repeat en flankerende sequentie. Opvallend hierbij was, dat de 5′ en 3′ breekpunten van respectievelijk 10 en 9 (van de 14) deleties dicht bij elkaar lagen, waardoor er een 'hotspot' voor deleties in het 5′ ongetransleerde gebied van het *FMR1* gen lijkt te zijn. Dit suggereert dat er specifieke sequenties in dit gebied aanwezig kunnen zijn, die tot een verhoogde instabiliteit kunnen leiden.

Vlakbij FRAXA ligt een tweede fragiele site, die FRAXE genoemd wordt. In deze fragiele site is een GCC repeat gevonden, die dezelfde karakteristieke eigenschappen heeft als FRAXA. In een grote familie waarin mentale retardatie voorkwam, hebben wij deze GCC repeat bestudeerd. Net als in het fragiele X syndroom waren alle mannen en een deel van de vrouwen met een verlengde repeat aangedaan, terwijl alle niet aangedane mannen een normaal allel hadden. In FRAXE mentale retardatie lijkt er derhalve geen premutatie allelen voor te komen. Net als in het fragiele X syndroom, werd in het sperma van een aangedane

man uit deze familie een kleinere GCC repeat gevonden vergeleken met het somatische weefsel. Echter, in grote tegenstelling tot het fragiele X syndroom, kunnen FRAXE mentaal geretardeerde mannen aangedane dochters hebben. Hierbij dient opgemerkt te worden dat in deze dochters geen cytogenetische expressie van de fragiele site aangetoond kon worden.

Door het bestuderen van de CGG repeat in fragiele X patiënten weten we nu meer over de instabiliteit van de repeat. Desondanks zijn vervolg studies nodig, om precies te bepalen hoe en wanneer de repeat zich verlengt, wanneer de repeat gemethyleerd wordt en waarom alleen vrouwen de volledige mutatie kunnen doorgeven. Aangezien deze experimenten moeilijk (tot onmogelijk) zijn om in de patiënt te bestuderen, hebben we een ander model ontwikkeld, waarin een verlengde CGG repeat in een transgene muis geïntroduceerd werd. Deze repeat werd stabiel doorgegeven aan de volgende generaties, wat mogelijk veroorzaakt werd door de onderbreking van de repeat aan de 3' kant. In de toekomst zal de introductie van een verlengde, perfecte CGG repeat, mogelijk op het FRAXA locus, het exacte mechanisme en het precieze tijdstip van de repeat verlenging kunnen oplossen.

Abbreviations

AR	Androgen Receptor
Asn	asparagine
DM	myotonic dystrophy
DNA	deoxyribonucleic acid
DRPLA	dentatorubro-paalidoluysian atrophy
FMR1	fragile X mental retardation 1
FMRP	FMR1 protein
FRDA	Friedreich's ataxia
FXR1(2)	FMR1-crossreacting relative gene 1 (2)
HAP1	huntingtin-associated protein
HD	Huntington's disease
HRS	Haw-River syndrome
Ile	isoleucine
kb	kilobase
kD	kilodalton
MJD	Machado-Joseph disease
NES	nuclear export signal
NLS	nuclear localization signal
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
SBMA	spino and bulbar muscular atrophy
SCA	spinocerebellar ataxia
UTR	untranslated region
YAC	yeast artificial chromosome

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Stellingen behorende bij het proefschrift

The fragile X syndrome: Complex behavior of a simple repeat

I

Een normale CGG repeat is niet *altijd* stabiel.
Morton (1992), Proc Natl Acad Sci USA 89: 4215-4217; Eichler (1996), Hum Mol Genet 5: 319-330.

II

Niet alle aandoeningen, die geassocieerd zijn met een trinucleotide repeat verlenging, worden gekenmerkt door anticipatie.
Campuzano (1996), Science 271: 1423-1427.

III

Naast 'multiple-gene disorders' bestaan er ook 'multiple-disorder genes'.
Van Heyningen (1994), Nature 367: 319-320; Sugier-Veber (1994), Nature Genet 6: 257-261; Burke (1994), Nature Genet 7: 521-524; Schöls (1995) Hum Mol Genet 4: 1001-1005; Bellus (1995), Nature Genet 10 :357-359; Lloyd (1996), Nature 379: 445-449.

IV

De genetica levert een bijdrage aan de (her)classificatie van complexe syndromen.
Rutland (1995), Nature Genet 9: 173-176; Pilia (1996), Nature Genet 12: 241-247.

V

PCR is een methode op bijbelse grondslag.
Genesis

VI

Niet-coderend DNA is niet per definitie niet-functioneel.
Campuzano (1996), Science 271: 1423-1427. Lugenbeel (1995), 10: 483-485; Tycowski (1996), Nature 379: 464-466.

VII

Het amplificeren van een verlengde repeat vereist veel herhaling.

VIII

MAO (mono-amine oxidase) beïnvloedt de hoeveelheid vrije radicalen.
Reiter (1995), Faseb J. 9: 526-533.

IX

Een intelligent persoon heeft hersens, een slim persoon gebruikt ze ook.

X

Promoveren is als keepen: je moet je doel verdedigen.

XI

Uit de discussie over homo-huwelijk en adoptie door homo-paren blijkt dat in het 'ruimdenkende' Nederland tolerantie niet direct leidt tot acceptatie.

Rotterdam, 26 juni 1996
Esther de Graaff