Characterization of the *FMR1* protein involved in the fragile X syndrome

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Karakterisering van het *FMR1* eiwit betrokken bij het fragiele X syndroom

Proefschrift

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Chapter 1

Introduction

1.1 From Martin-Bell to fragile X syndrome

The fragile X syndrome or Martin-Bell syndrome is the most frequent genetic cause of mental retardation after Down's syndrome (61, 192). The syndrome is X-linked and has an incidence of one in 4000 males and approximately 1 in 6000 in females (177).

In 1943 Martin and Bell described a family in which eleven mentally retarded boys were born to normal intelligent mothers (111). They hypothesized that the mental defect of the boys was due to a sex-linked recessive gene which was suppressed in the two grandfathers. In 1969 Lubs found the syndrome to be associated with a fragile site at Xq27.3, called FRAXA, in cultured lymphocytes (102). These results could only be reproduced by culturing cells under folic acid free conditions (167). Since that observation by Sutherland in 1977 the cytogenetic visualization of the fragile site was used as a diagnostic tool for the Martin-Bell syndrome. The mentally retarded individuals in the pedigree described by Martin and Bell indeed showed a fragile site at Xq27.3 and the Martin-Bell syndrome now is referred to as the fragile X syndrome (137).

1.2 Fragile X phenotype

1.2.1 Physical characteristics

The fragile X syndrome is associated with the following clinical characteristics: moderate to severe mental retardation, macro-orchidism, long face with prominent forehead and protruding jaw and large everted ears (Reviewed by Fryns (49) and by Hagerman (63)). This specific combination of symptoms, also called the classical Martin-Bell phenotype, is present in about 60% of the fragile X positive males. Macro-orchidism is present in about 85% of the fragile X males (88, 168, 175). The facial features become more prominent after puberty (63). A typical fragile X patient is shown in Figure 1.1.

Several other physical features are seen. A high percentage of fragile X patients shows connective tissue abnormalities including hyperextensible joints (66 1984), smooth skin (175), high arched palate, pectus excavatum, flat feet (126), mitral valve prolapse and aortic root dilatation (187). In skin biopsies of fragile X patients a decrease or absence of elastin fibres is observed as well as a decreased arborization of the fibres (187).

Other physical features include growth abnormalities such as increase in birth weight and head circumference. Overgrowth observed during early development might be related to abnormalities in the hypothalamic region (47). Increased volumes of specific brain parts are observed as well. The volumes of the caudate nucleus and the lateral ventricle are inversely correlated with IQ and both these volumes are increased in fragile X males (136).

Fragile X patients have a normal lifespan, despite the frequently observed cardiac abnormalities (47).

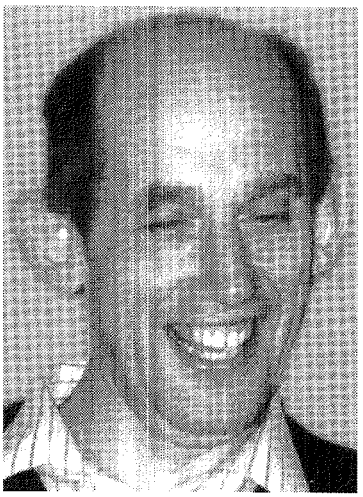


Figure 1.1
Fragile X patient with typical facial characteristics.

1.2.2 Behaviour

Fragile X patients show behavioural characteristics such as abnormal response to stimuli, repetitive motor behaviour like hand flapping and rocking, poor eye contact and shyness (63). Although these behavioural features are shared with autistic patients the fragile X syndrome is not associated with autism (42) as no significant difference in autistic-like behaviour is observed when fragile X patients are compared with other mentally retarded individuals matched for age, sex and IQ (37, 42, 43).

Fragile X patients may show general short term memory deficits (32, 33), but others (106) found that the performance of memory tasks depends on the type of information which had to be reproduced. Abstract information is less well recalled than information which is concrete like an object or a meaningful story. Knowledge which is acquired by fragile X patients is less well used in solving new problems, indicating that patients have difficulties in applying problem solving strategies which were learned in earlier situations (106).

The majority of the fragile X boys are hyperactive, but this is an unspecific feature. Part of the hyperactivity probably originates from learning deficits, in this case a slower and less efficient learning of the environmental setting. After puberty a decline in hyperactivity is observed (47).

1.3 Fragile X females

Female carriers of a recessive X linked disease usually do not manifest clinical features. Females having cytogenetic expression of the fragile site, however, may exhibit the fragile X

syndrome, though the clinical features are less severe than in male fragile X patients (63, 96). Fragile X female patients are learning disabled or mildly retarded and exhibit shyness and social anxiety (12, 22, 64, 65). Female fragile X carriers furthermore may be at increased risk of having dizygous twinning and (48, 149, 176) early menopause (176).

1.4 Normal transmitting males

In the pedigree described by Martin and Bell (111) two normal brothers passed on the fragile X mutation to their likewise normal daughters. Some of the grandsons of these two males, however, were mentally retarded. About 20% of the males carrying the fragile X mutation are unaffected and these males are referred to as normal transmitting males (NTM). Daughters of normal transmitting males inherit the fragile X mutation from their fathers, but are always unaffected: only female carriers can have offspring with the fragile X syndrome. The phenomenon of normal transmitting males can be explained by the existence of two types of mutations, discussed in paragraph 1.6.1.

1.5 The cloning of the *FMR1* gene

Initially, the only way to confirm the diagnosis of the fragile X syndrome was by inducing the fragile site at Xq27.3 in cell culture (Fig. 1.2A). The gene defect was unknown and neither DNA or protein tests were available for diagnosis. Cloning of the gene became essential for improved diagnostic accuracy and understanding the mechanism leading to the fragile X syndrome.

As the protein defect underlying the fragile X syndrome was unknown, positional cloning was the only available strategy to isolate the gene. First the syndrome had to be linked to a precise region in the X chromosome. Genetic markers with known localization on the X chromosome were tested for cosegregation with the syndrome. Hybrid cell lines with translocation breakpoints in the fragile site proved to be very useful in both developing new probes and determining their localization. Two cell lines, Micro21D and Q1X (Fig. 1.2A), played an important role in the isolation of the gene lying within the region linked to the fragile X syndrome. Cell line Micro21D contained the Xpter-Xq27.3 part and cell line Q1X contained the Xq27.3-Xqter part of the fragile X chromosome (189, 190, 191).

Probe DXS465 was the fist probe to show a difference between normal controls and fragile X patients, namely the absence of certain *BssHII* and *SacII* restriction fragments in DNA digests of fragile X patients. The decreased intensity or absence of these fragments was due to

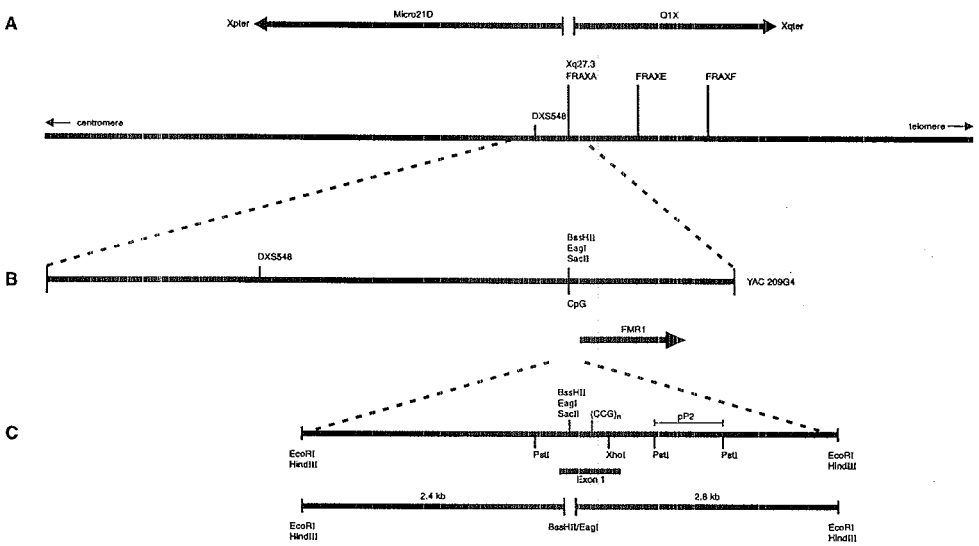


Figure 1.2

Map of the fragile X region at Xq27. A) Localization of the fragile sites FRAXA, FRAXE and FRAXF and DNA markers at the long arm of the X chromosome at Xq27-28. Regions of the X chromosome present in the cell hybrids Micro21D and Q1X are indicated at the top. B) Map of YAC 209G4 containing a region overlapping the fragile site FRAXA. A cluster of restriction sites indicates the CpG island which is located in the FRAXA region. The *FMR1* gene is located downstream of the CpG island. C) *EcoRI* fragment containing the CpG island, CGG repeat and exon 1 of *FMR1*. pP2 is a probe which is used for diagnostic purposes. The fragment sizes obtained after *EcoRI* digestion or *EcoRI-EagI* or *EcoRI-BssHII* double digestion are indicated at the bottom. Similar fragments are obtained with *HindIII-(EagI/BssHII)* double digests.

methylation of the restriction sites (8, 185).

The isolation of YAC 209G4 (Fig. 1.2B) was the key to the isolation of the gene. The YAC spanned the fragile site and contained the region with the CpG island showing differences between controls and patients. A cDNA clone was isolated from a human fetal brain library with the use of cosmids representing part of the insert of YAC 209G4. The isolated gene was designated Fragile X Mental Retardation 1 (*FMR1*) (183). A trinucleotide repeat (CGG) is present in the first exon of *FMR1*. The two translocation breakpoints of Micro21D and Q1X lie in this exon which shows length variation between controls and fragile X patients.

For a detailed description of the isolation of the *FMR1* gene is referred to Verkerk *et al.* (184). An overview of the fragile X region is depicted in Figures 1.2 A and B.

1.6 Gene defects

1.6.1 CGG repeat amplification and the fragile X syndrome

A 5.2 kb *EcoRI* fragment observed in normal X chromosomes is replaced by a much larger *EcoRI* fragment in fragile X chromosomes (122, 183, 200). The first exon, containing the CGG repeat, lies within this 5.2 kb *EcoRI* fragment (Fig. 1.2C). It was suggested by Verkerk *et al.* that the observed length difference was due to CGG repeat amplification (183). Two observations supported this hypothesis. First, the sequences surrounding the CGG repeat were identical in normal and fragile X chromosomes (50). Second, PCR analysis showed CGG repeat amplification in fragile X chromosomes (50). The CGG repeat is located 250 bp distal to the CpG island which is methylated in fragile X patients (in contrast to controls), indicating that there is a relationship between repeat amplification and methylation, in the fragile X syndrome.

The CGG repeat was found to be polymorphic. About 6 to 54 repeats are found in *FMR1* of the normal population (50). Fragile X patients, however, carry a so called full mutation (50, 122, 141, 142, 182, 183) which is characterized by amplification of the CGG beyond 200 copies and methylation of both the repeat and the CpG island (classical fragile X mutation) (68, 75, 138). The methylated promoter region of the full mutation blocks the transcription of the *FMR1* gene in fragile X patients (129, 166). The fragile X syndrome thus results from the lack of *FMR1* expression (Fig. 1.3).

The full mutation does not originate directly from the amplification of a normal repeat, but stems from so called premutation alleles. The normal repeat is stably transmitted from parent to child, without changes in the repeat length. However, premutations containing 43 to 200 CGG repeats are unstable upon transmission as they are able to grow to a full mutation in successive generations (50, 93). Premutation alleles, in contrast to full mutations, have an unmethylated promoter region and are transcribed (129). Amplification from premutation to full mutation can only occur when the premutation is transmitted through a female (50, 122). The larger the premutation, the higher the risk for transformation to a full mutation (50). Normal transmitting males (NTM), who carry a premutation, always transmit a premutation to their daughters, never a full mutation (122). This explains why daughters of NTMs are always unaffected.

The full mutation is somatically unstable as different repeat lengths are found in distinct cells (122, 142). DNA digests of fragile X patients thus usually show a smear in the full mutation range on Southern blots. About 20% of the fragile X patients show a premutation band in addition to the full mutation (27, 122). These patients who carry a premutation in a small number of their cells are often referred to as mosaic patients.

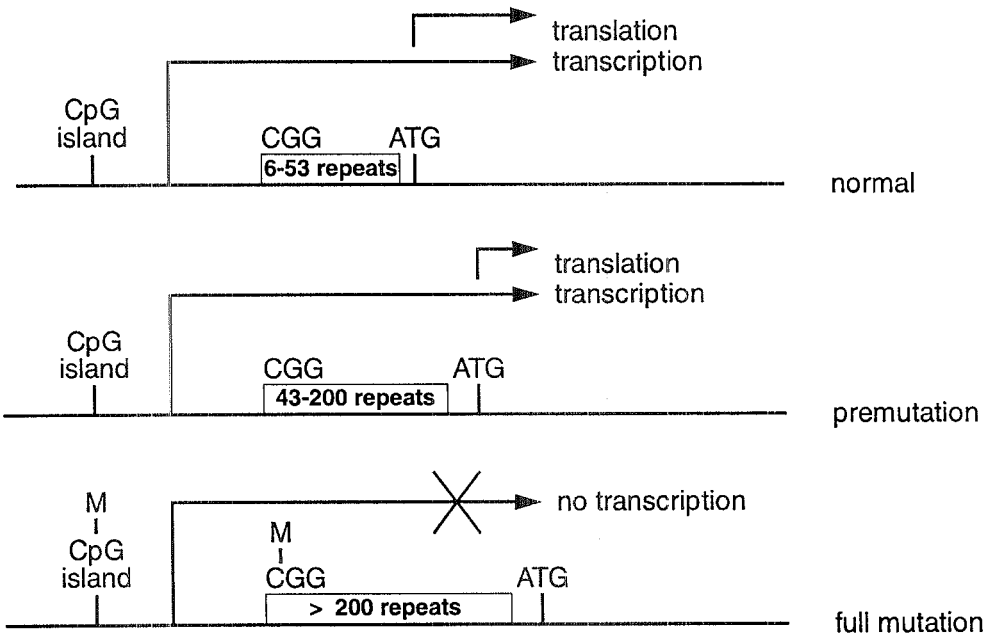


Figure 1.3
Transcription of normal and premutation alleles. Hypermethylation of the promoter region prevents transcription of the full mutation allele.

The normal CGG repeat does not consist of a stretch of pure CGGs. The majority (85%) of the normal CGG repeats in the range of 27-45 repeats carry two AGG interruptions. Sequence variation is found at the 3' end of the repeat and it is suggested that the AGG triplets stabilize the repeat during replication (94). By screening different alleles for the presence of AGG codons, a correlation was found between the composition of the repeat and its stability. Loss of an AGG triplet plus an uninterrupted sequence of 34-38 CGGs seems to be a threshold for instability (35).

1.6.2 Other mutations

The fragile X syndrome is usually caused by repeat amplification and hypermethylation of the promoter region of the *FMR1* gene. Until now only one mutation has been described which

leads to an altered *FMR1* protein product (FMRP). A point mutation changing a conserved amino acid in a functional domain of the *FMR1* protein (24) alters the behaviour of FMRP (discussed in more detail in 3.4.1 and 3.4.2). All other mutations described thus far lead to the absence of FMRP expression.

Several patients have been described in which the fragile X syndrome is caused by the deletion of the *FMR1* promoter region or the deletion of the complete *FMR1* gene and flanking sequences. At least two macrodeletions have been described which erase the *FMR1* gene and flanking sequences (54, 132). Most of the microdeletions occur proximal to the CGG repeat, removing the CpG island and surrounding sequences (26, 60, 114, 172, 197). For some of these cases it has been suggested that the deletions occurred in *FMR1* full mutation alleles (26, 114). De Graaff *et al.* (26) described four patients being mosaic for both a full mutation and a deletion. The deletions in these patients had variable sequences at the 3' end, but the 5' border of the deletions were located in a region 70 base pares upstream of the repeat, in an interval of 35 base pares. This region of 35 base pares might be considered as a hotspot for deletions.

Two patients have been described in which mutations in the coding sequence lead to a premature translation stop (103). One patient carried a single nucleotide deletion in exon 5 (nucleotide 373) causing a frame shift which resulted in a translational stop 3' from the deletion. The *FMR1* protein was not expressed in this patient although the gene was transcribed. The second patient carried a mutated splice acceptor site in exon 2 which resulted in the skipping of exon 2 or both exon 2 and 3. Splicing of exon 2 resulted in a frameshift and premature translation stop and in this patient FMRP expression could not be detected either. These two patients are thus far the only patients in which the *FMR1* gene is silenced because of mutations within the coding region of the gene.

1.6.3 Molecular diagnosis

The clinical diagnosis of the fragile X syndrome based on physical characteristics is rather difficult, especially in young children (1.1). Since the elucidation of its gene defect, the syndrome can be relatively easy identified by DNA testing as the disease is caused by mainly one mutation: the amplification of the CGG repeat.

The cytogenetic test has been replaced by a DNA test (reviewed by Rousseau (141) and Oostra (125)) in which the repeat length and methylation status of the promoter region are determined. For this purpose double digests are performed with methylation sensitive and insensitive restriction enzymes on DNA isolated from blood, chorionic villi or other tissues. A 5.2 kb *EcoRI* fragment of the 5' end of the *FMR1* gene, containing the CpG island and the CGG repeat, is increased in length in fragile X chromosomes (1.5). The *EcoRI* restriction sites coincide with *HindIII* sites and the latter enzyme is used instead for diagnosis. The length of

this fragment and the methylation status of the CpG island can be determined through digestion of the DNA with both *HindIII* and a methylation sensitive restriction enzyme such as *BssHII* or *EagI* (Fig.1.2C). The 5.2 kb *HindIII* fragment is cut by the latter enzymes into fragments of 2.4 and 2.8 kb in case of an active X chromosome, which carries an unmethylated CpG island. If the CpG island is methylated, however, a fragment of 5.2 kb is found, representing the undigested *HindIII* fragment. DNA derived from male fragile X patients thus show enlarged 5.2 kb fragments in this test.

Normal transmitting males, who carry a premutation, have enlarged 2.8 kb *HindIII-EagI* fragments, due to the unmethylated CpG island. DNA of females shows a more complicated pattern as they carry both an active and an inactive X chromosome. Digestion of normal DNA with *HindIII-EagI* therefore results in fragments of 5.2, 2.4 and 2.8 kb. Females carrying a full mutation have *HindIII* fragments larger than 5.2 kb in addition to the three normal bands; females carrying a premutation allele, however, have a combination of normal sized and enlarged *HindIII-EagI* bands.

The difference in size between normal and mutated target fragments is often expressed in Δ basepairs. Premutations have a Δ between 60 and 600 bp, whereas full mutations have a Δ of more than 600 bp. Full mutations are somatically instable as a result of which different repeat lengths are found. The normal 5.2 kb *HindIII* fragment therefore is replaced by a smear above the 5.2 kb region.

PCR analysis has been proven useful in determining the repeat length in the normal and premutation range. CGG repeats within the full mutation range, however, are very hard to amplify which limits the use of PCR in fragile X diagnostics. Analysis of females carrying a full mutation and analysis of mosaic males and females is disturbed by preferential amplification of the allele with the smallest repeat length. Southern blot analysis has to be performed in addition to PCR tests in order to distinguish between premutations and full mutations and in case of doubt about the possible presence of a full mutation in samples showing homozygous PCR results.

The diagnosis of the fragile X syndrome has been partly facilitated by the development of a test for the presence or absence of the *FMR1* protein (196). The protein can be detected in lymphocytes using monoclonal antibodies developed by Mandel and coworkers (29); the antibody test is an easy, inexpensive and quick test as results are obtained within a day. The test is especially useful for the detection of male fragile X patients, as FMRP is absent or expressed at such a low level that it can easily be distinguished from controls. Female patients, however, often show a preferential inactivation of the fragile X chromosome in their lymphocytes, which might cause a false negative result in this test. DNA analysis is thus still the most reliable method for diagnosing the fragile X syndrome in females. As reliable results are obtained with the protein test in diagnosing males for the fragile X syndrome it is likely to

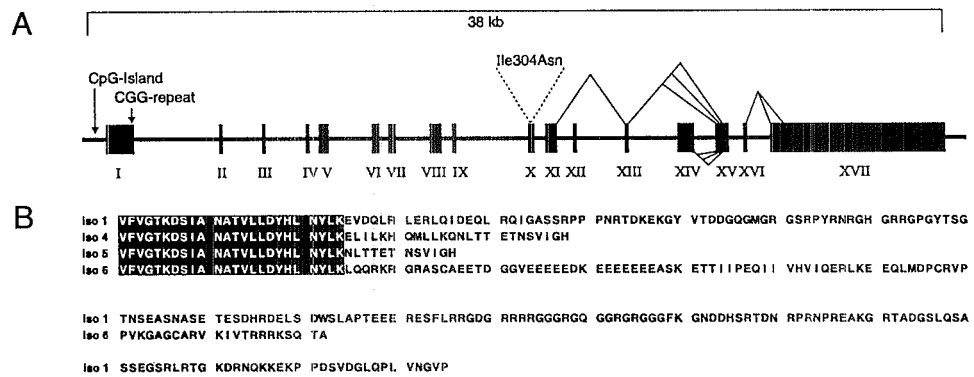


Figure 1.4
Organization of the *FMR1* gene. A) The *FMR1* gene consists of 17 exons and has a genomic size of approximately 38 kb. Alternative splicing of exon 12, 14, 15 and 17 as well as the position of the point mutation described in 1.6..2 are indicated. B) Alternative splicing of exon 14 results in novel C-termini. Iso 1 represents the normal C-terminus. Iso 4 to iso 6 represent the novel C-terminal sequences which result from the use of different splice acceptor sites in exon 15. The boxed region indicates the sequence of exon 13, which are identical in iso 1 and iso 4 to 6. (after Ashley *et al.* (5))

be a very useful test for the screening of larger populations. The detection of patients with a full mutation and absence of FMRP may lead to further investigation of the families involved using DNA diagnosis.

1.7 Organisation of the *FMR1* gene

The *FMR1* gene consists of 17 exons and has a genomic size of approximately 38 kb (Fig. 1.4A) (36). The length of the transcript is 4.4 kb, of which 1.9 kb is coding sequence, predicting a protein product of 631 amino acids (181, 183).

The CGG trinucleotide repeat is present in the 5' part of the transcript. A stopcodon present in the 5' part of the gene is located in front of the repeat. The first startcodon after this stop codon lies 3' of the repeat, indicating that the CGG repeat is part of the 5' untranslated region. The sequence surrounding the start codon, in both human and mouse, corresponds to the Kozak consensus sequence for translational start sites (5).

The promoter region needed for the correct expression of *FMR1* was determined with the use of mice transgenic for the lacZ reporter gene and a putative promoter region. A region containing the *FMR1* startcodon and 3 kb of the 5' sequence was sufficient to generate a lacZ

reporter gene expression which was comparable with *FMR1* expression in control embryonic and adult mice. The regulatory elements necessary for tissue specific expression, although not yet identified, have to be present within this region (70).

The sequence of the *FMR1* gene is conserved in evolution; organisms as divergent as human, *C. elegans, X. laevis* and chicken express *FMR1* (5, 131, 157, 183), indicating that the gene has a similar function in different organisms. The human and murine *FMR1* sequence is almost identical, 95% homology at the nucleotide level and 97% at the amino acid level (5). Both the human and murine 5' untranslated region are CG rich, an aspect that has been linked to transcriptional control. The 3' UTR of the human and murine gene contain regions with strong sequence identity of which the function is unknown. Human and *X. laevis FMR1* are 86% identical at the amino acid level. The CGG repeat as well as exon 11 and 12 are missing in the *X. laevis* and chicken sequences.

1.8 Alternative splicing

The *FMR1* transcript is alternatively spliced in both men and mice, generating as many as 24 splice variants (Fig. 1.4), the largest splice variant encoding a protein of 631 amino acids, the smallest variant encoding a protein of 436 amino acids (5, 181). Four exons, exon 12, 14, 15 and 17, can be alternatively spliced in man (Fig. 1.4A). Exons 12 and 14 can be spliced as a whole, in contrast to exon 15 and 17. Exon 15 contains three splice acceptor sites which cansubsequently result in three possible splice variants. In human the first 51 bp of exon 17 can be spliced out. Murine *Fmr1*, however, lacks this part of exon 17 and subsequently no exon 17 splice variants could be detected in murine tissue (181).

The reading frame is maintained in all splice variants, except for exon 14 splice variants. Exon 14 consists of 196 bp and splicing out exon 14 consequently gives rise to a +1 frame shift. The use of three different splice acceptor sites in exon 15 finally results in three mRNAs encoding proteins with novel C-termini (Fig. 1.4B). No significant homology of these novel C-termini was found in the database. The novel C-termini are less hydrophilic than the full length C-terminal and this might have an influence on both the function and the localization of the exon 14 splice variant encoded proteins. However, only a minority of the splice variants lack exon 14 (5).

No difference was observed concerning the expression of the different splice variants in distinct tissues. Similar ratios were found in fetal brain, liver and pancreas, adult testis and lymphoblastoid cell lines (5, 181).

1.9 *FMR1* mRNA expression

The *FMR1* mRNA expression was studied in embryonic and adult mice in order to determine the expression pattern of the gene. *Fmr1* in situ hybridisation revealed a high expression level of *Fmr1* transcripts in all tissues of mouse embryos at day ten of gestation. The expression decreases and becomes more heterogenous in the subsequent embryonic stages, giving hybridisation patterns comparable to the adult expression pattern (71).The *FMR1* mRNA expression remains high in adult brain and testis, the tissues which are affected in fragile X patients. In addition high expression levels are found in ovaries, thymus, oesophagus and spleen. A moderate to low expression is seen in kidney, liver, colon, uterus, thyroid and lung. The adult heart, muscle and aorta do not show expression at all (71).

The expression of *FMR1* in the developing nervous system was studied in more detail in human fetuses at week 9 and 25 of gestation. High *FMR1* expression levels were seen in the brain, spinal cord, ganglia and neural retina. Proliferating and migrating cells of the nervous system express *FMR1* mRNA, but it is unclear whether the migrating cells are neurons or glial cells. *FMR1* transcripts were found in the ventricular and subventricular zones of the brain of a 25 weeks old fetus, but in differentiating neurons of cerebral structures such as nucleus basalis magnocellularis, hippocampus, neocortex, striatum, thalamic nuclei and cerebellum an even higher expression level was found (2).

Chapter 2

Disorders caused by trinucleotide repeat expansions

2.1 Dynamic mutation

The fragile X syndrome was the first disorder described to result from the amplification of a trinucleotide repeat. Since 1991, the year the cloning of the *FMR1* gene was published, several other disorders have been described which are caused by the amplification of a trinucleotide repeat. The list of disorders caused by this 'dynamic mutation' is still growing. The majority of the trinucleotide repeat disorders show an increase in severity of the disease in successive generations, a phenomenon called anticipation. It is the expansion of the trinucleotide repeat in successive generations that is the cause of the observed anticipation. In fragile X syndrome another phenomenon is observed, called the Sherman paradox which describes the correlation between the increase in chance of offspring with the fragile X syndrome and the CGG repeat length of the mother.

2.2 Subdivision of the trinucleotide disorders

The trinucleotide repeat disorders can be divided into three different classes (Table 2.1) based on the effect the amplification has on gene expression. Class 1 is characterized by the absence of the gene product. In class 2 CAG repeat amplification results in proteins with enlarged polyglutamine stretches. The third class represents just one disorder, correlated with a CTG amplification within the 3' UTR of the DM-PK gene (14).

2.3 Class 1

The disorders in class 1 are associated with absence of the gene product (Table 2.1). This class can be divided into two subclasses. The first subclass consists of folate sensitive fragile sites which are associated with CGG or CCG repeat amplification. The second subclass is represented by Friedreich's ataxia, which is caused by a GAA repeat amplification (19).

Only three of the five folate sensitive fragile sites of the first subgroup have been linked to specific genes. FRAXA and FRAXE are both associated with mental retardation and the genes involved have been named *FMR1* (183) and *FMR2* (21, 53, 59), respectively. Amplification of the repeat beyond a certain limit results in the methylation of the promoter region, preventing the expression of the genes and thereby causing mental retardation. It has to be noted that patients with the fragile X syndrome and FRAXE associated mental retardation have a different phenotype and that the *FMR1* and *FMR2* gene do not show any homology. The possible function of the *FMR1* protein is discussed in Chapters 3 and 4. The predicted protein sequence of *FMR2* shows some resemblance to the proto oncogene AF-4 (21, 53, 59), which

Table 2.1 Disorders caused by trinucleotide repeat amplification.

	#	repeat	region	gene	normal	affected	function	protein localization	tissue	age of onset	phenotype	reference
Class 1												
fragile X syndrome	Xq27.3	CGG	5' UTR	*FMR1*	3-50	≥200	RNA binding protein	cytoplasm (179, 29)	-	birth	mental retardation	50, 122, 129,183
FRAXE	Xq28	GCC	5' UTR	*FMR2*	6-35	≥200	--	-	-	birth	mental retardation	21, 53, 59, 89
FRAXF	Xq28	GCC		-	-	-	-	-	-	-	-	128
FRA16B	16	CGG		-	-	-	-	-	-	-	-	119
FRA11B	11q23	CGG	5' UTR	CBL2	-	-	proto-oncogene	-	-	-	-	82, 169
Friedreich' s ataxia	9q13-9q21.1	GAA	intron 1	χ25	10-21	≥200	-	-	loss of sensory neurons, heart, pancreas	≤25	ataxia	19
Class 2												
spinal bulbar muscular atrophy (SBMSA)	Xq11-12	CAG	exon 1	androgen receptor	17-26	40-52	transactiv-ation	cytoplasm and nucleus	motor neuron degeneration	mid age	muscular atrophy	95
Huntington's disease	4p16.3	CAG	exon 1	huntingtin	8-35	≥36	-	cytoplasm (62, 74)	neuronal death in the striatum and in cortex	mid age	involuntary movements, dementia and personality changes	77, 92
Spinocerebellar ataxia 1 (SCA1)	6p22	CAG	exon	ataxia-1	6-40	≥40	-	cytoplasm (150)	loss of Purkinje cells, neurodegeneration in brain stem and spinocerebellar tracts	mid age	ataxia	127
SCA3/Machado Joseph disease	14q24.3	CAG	exon	MJD1	13-36	68-79	-	-	neurodegeneration	mid age	ataxia	83, 148
Dentatorubral and pallidoluysian atrophy (DRPLA) and Haw River syndrome	12p13.1-p12.3	CAG	exon	DRPLA	7-23	49-75	-	cytoplasm (199)	neurodegeneration in cerebellum and pallial efferent outflow tracts	mid age	myoclonus epilepsy, dementia, ataxia	91, 98
Class 3												
myotonic dystrophy (DM)	19q13.3	CTG	3' UTR	DM1	5-37	≥200	protein kinase	cytoplasm (105, 195)	muscular and testicular atrophy	congenital and mid age	myotonia and muscle weakness	14, 107

is a putative transcription factor. Whether *FMR2* has a function similar to AF-4 remains to be investigated.

The third gene is the proto-oncogene CBL2 which is associated with a fragile site at 11q23 (82, 169). The CBL2 gene is localized within a region (11q23→qter) truncated in patients with Jacobsen syndrome, a disorder characterized by specific facial dysmorphisms and moderate mental retardation (79, 146). The mother of one of the patients described by Jones *et al.* carried a repeat amplification. Thus far there are no indications that repeat amplification in general precedes deletion in Jacobsen syndrome (82). The folate sensitive fragile sites FRAXF (72) and FRA16B (119) are associated with CGG repeat amplification and methylation of the CG rich region. Methylation of this region suggests the silencing of a gene promoter. Thus far neither a specific phenotype has been linked to these fragile sites nor has the gene affected been identified.

Friedreich's ataxia is an autosomal recessive disorder with onset in puberty (140, 159). The majority of the FRDA patients (98%) are homozygous for a GAA repeat amplification in the first intron of the χ25 gene (19). This mutation most likely disturbs the correct splicing of intron 1, interfering with processing of precursor mRNA. This eventually leads to a lack or absence of mRNA expression (9).

FRDA is exceptional within this class of trinucleotide repeat disorders. First, FRDA is a recessive disorder; both χ25 alleles have to be silenced in order to obtain a disease phenotype. Second, the repeat is present in an intron instead of in an exon. Furthermore the expression of χ25 mRNA is restricted to the tissues which are affected in FRDA patients, which is different from the ataxia's present in class 2 where the affected genes are ubiquitously expressed (7, 83, 145, 173). FRDA is the first recessive disorder associated with repeat amplification and it is very likely that other recessive disorders might be caused by a loss-of-function trinucleotide repeat expansion as well (19).

No natural occurring animal models for the Class 1 disorders have been found. Mouse models for the class 1 disorders, however, can be easily made by silencing the endogenous gene as was demonstrated for the fragile X syndrome. The *Fmr1* knock out mouse shows a phenotype similar to human fragile X patients. For a detailed description the reader is referred to Chapter 3 and Publication 5.2.

2.4 Class 2

2.4.1 Identification of new genes

The disorders in class 2 are characterized by a CAG repeat amplification in the coding region of the gene which leads to enlargement of a polyglutamine stretch. A remarkable observation is that the diseases in class 2 are all neurodegenerative disorders (Table 2.1). Other neurodegenerative disorders showing anticipation might be caused by CAG repeat

amplification as well. These new genes could be identified at both the DNA and protein level. The gene involved in spinocerebellar ataxia 1 (SCA1) in fact was isolated by screening of SCA1 families for CAG repeat amplification with cosmids representing the candidate gene region (127). A second method has been shown to be successful as well. Monoclonal antibodies raised against the polyglutamine stretch of the transcription factor TBP (TATA binding protein) are able to detect mutant huntingtin, ataxin-1 and spinocerebellar ataxia 3 (SCA3) proteins (173). Only proteins with enlarged glutamine stretches were detected. The strength of the signal obtained in Western blots was related to the length of the polyglutamine stretch: the longer the polyglutamine tract the stronger the signal. The monoclonal antibodies were able to detect specific protein products in two other neurodegenerative disorders, spinocerebellar ataxia 2 (SCA2) and autosomal dominant cerebellar ataxia II (ADCA II), of which the corresponding genes have not been identified. The monoclonal antibodies might not only facilitate the isolation of the corresponding genes of SCA2 and ADCA II, but also the isolation of genes which are possibly involved in other disorders showing anticipation.

2.4.2 Loss or gain of function of the protein

The presence of the enlarged polyglutamine stretch in the protein might either result in a loss or gain of function of the mutated proteins. A loss of function is not very likely as there are at least two examples in human which show that the loss of one allele does not result in a phenotype similar to the one caused by the amplification of the CAG repeat. First, individuals who have lost one of the wildtype Huntington (HD) alleles do not develop Huntington's disease (3, 77). Second, males who have lost the androgen receptor gene do not develop SBMA symptoms, but show testicular feminization (133). Gain of function, a term which defines the alteration of a protein function or protein characteristic in general, is a more likely cause for the disorders in this class.

Several suggestions have been made in which the enlargement of the polyglutamine stretch might affect the functioning of the proteins. The polyglutamine stretches in these proteins resemble those present in some transcription factors, like the TATA binding protein (56). It was suggested that the enlargement of the glutamine stretch affected the transcription factor activity of the proteins within class 2. Some of the proteins, however, have a cytoplasmic localization (references in Table 2.1) and it is therefore not very likely that all the proteins within this class are transcription factors. Another suggestion has been that the enlarged polyglutamine stretches of the mutant proteins would be good substrates for transglutaminases (58). Some of these enzymes have been described to be involved in cross linking of proteins, like in blood clotting and extracellular matrix formation. The mutated proteins in class 2 might be cross linked to other proteins by these enzymes, causing aggregates. However, aggregates have not been identified in the affected tissues of the patients.

Thus far the most favoured model is that the enlarged polyglutamine stretch might cause a pathological interaction with a tissue specific protein. This could at least be true for Huntington's disease as a rat brain specific huntingtin associating protein (rHAP1) was isolated (100). Wild type huntingtin with 19-22 repeats binds only weakly to rHAP1, whereas the mutant protein binds more strongly as the length of the polyglutamine stretch increases from 44 to 82 glutamines. Interaction of huntingtin with the human homologue of rHAP1 (hHAP1) has only been demonstrated *in vitro*. A similar interaction of huntingtin and hHAP1 has not been demonstrated in post-mortem brains of Huntington's disease patients yet. The possible existence of such an interaction might explain why the repeat amplification in the HD gene only results in brain specific damage.

2.4.3 Class 2 animal models

Animal models are being developed to study the functioning or malfunctioning of the proteins with enlarged polyglutamine stretches. To gain insight in the function of huntingtin several groups generated a Hdh knock out mouse (31, 120, 201). These studies revealed that huntingtin has an important function during embryogenesis, as it was impossible to generate mice with both Hdh genes silenced. Mice with only one Hdh gene silenced did not develop a true Huntington pathology. The latter observation supports the hypothesis which suggests that the pathological enlargement of the polyglutamine repeat leads to a gain of function of the protein product, rather than a loss of function, which is in agreement with the observation that patients lacking one of the wild type HD alleles do not develop Huntington disease (previous paragraph).

Knock out mouse models might be helpful in determination of the function of a gene, but mice transgenic for the gene of interest with amplified CAG repeat will give better insights into the pathogenesis. This is illustrated by the development of a mouse model for SCA1. As human SCA1 patients show loss of Purkinje cells, transgenic mice were generated expressing the human SCA1 gene solely in Purkinje cells using a cell specific promoter. Two mice strains were obtained, expressing the SCA1 gene with either 30 or 82 CAG repeats. The latter strain had a phenotype similar to the SCA1 patients: loss of Purkinje cells and ataxia. However, the SCA1 protein (ataxin-1) could not be detected in these mice. The authors suggested that the protein might be unaccessible for the antibodies used in these experiments due to modification of the protein (17).

Although it remains to be clarified why ataxin-1 in the mice transgenic for the 82 CAG repeats cannot be detected it seems very likely that the only way to simulate the class 2 pathology in mice is through the introduction of an expanded CAG repeat instead of knocking out the gene.

2.5 Class 3

The only disorder in the third and last class of disorders is myotonic dystrophy (DM). Myotonic dystrophy is characterized by myotonia and muscular weakness, cardiac conduction defects, cataracts, hypogonadism and male type of baldness (69) (Table 2.1). The disease is caused by the amplification of a CTG repeat present in the 3' UTR of the myotonic dystrophy protein kinase gene localized to 19q13.3, which encodes a putative seronine-threonine protein kinase of 70 kD (14). The expansions seen in DM patients vary between 200 bp and 10 kb and a negative correlation is found between repeat length and severity of the disease. The longest expansions are found in the congenital form of the disease which is nearly exclusively maternally transmitted (76, 134, 174).

Inconclusive data have been reported about the effect of the CTG expansion on the expression levels of myotonic dystrophy protein kinase (DM-PK) mRNA or protein in DM patients. Decreased mRNA and protein levels have been observed (20, 51, 73, 90, 121) as well as increased DM-PK mRNA levels (144). Both DM-PK knock out mice as well as transgenic mice overexpressing DM-PK were developed to obtain more insight in this matter (80). Neither of the two mice strains, however, had a DM phenotype. This may either indicate that the mouse is not suitable as an animal model for DM, or the DM-PK gene is not defective or it is not the only gene involved.

As the influence of the repeat expansion on the gene expression is inconclusive it was suggested that the repeat might have an influence on the expression of other genes in the DM region. The CTG amplification increases the nucleosome formation efficiency, which could repress the transcription of other genes within the gene rich DM region (188). Upstream of the DM-PK gene a gene is localized called 59 gene in humans (151) and DMR-N9 in mouse (80, 108). This gene is preferentially expressed in brain and testis and aberrant expression of this gene might be responsible for some of the phenotypical characteristics of DM patients. Downstream of DM-PK a gene has been described called DM associated homeobox protein (DMAHP) (13). Amplification of the CTG repeat might influence the expression of this gene as the repeat is part of the CpG island localized in the 5' region of this gene. The influence of the repeat amplification on the expression levels of the genes surrounding the DM gene remains to be determined.

2.6 Concluding remarks

Disorders caused by the amplification of trinucleotide repeats van be divided into three different classes. As has become clear from the previous paragraphs the division into three different groups is based on the effect of the repeat amplification on the gene expression.

Expansion of a trinucleotide repeat either leads to a loss or a gain of function in class 1 and 2, respectively; the effect of repeat amplification in the third class is not yet clear.
Mouse models are being developed to study the pathology of the disorders. It is clear that mouse models for class 1 abnormalities can be obtained by silencing the endogenous gene. This method does not seem suitable for the making of mouse models for class 2 and 3 abnormalities and the introduction of an expanded repeat seems to be the only alternative, as was demonstrated for SCA1.

Whether there is a general mechanism for trinucleotide repeat expansion is unknown. For some of the repeats it has been found that they might be stabilized by interruption with other triplets. The CGG repeat in the *FMR1* gene for example is interrupted by AGG triplets. Loss of such a triplet would predispose the repeat for amplification. Loss of an interrupting triplet is found to be associated with repeat instability in other genes as well, for instance for SCA1 which is a member of the class 2 type of disorders. Although it seems unlikely that all different repeats are amplified by a similar mechanism, some aspects of the amplification might be comparable. For a recent review of this subject the reader is referred to E. de Graaff (25).

Chapter 3

The *FMR1* protein

3.1 Aim of the experimental work

The identification of the *FMR1* gene led to a better understanding of the molecular basis of the fragile X syndrome and to a reliable diagnostic method (Chapter 1). To fully understand the pathology of the fragile X syndrome it is necessary to elucidate the function of the *FMR1* protein. The experimental work described in this chapter was aimed at the characterization of FMRP (Publications 5.1, 5.3 and 5.4) and at the development of an animal model for the fragile X syndrome (Publication 5.2), which would facilitate the studies on the pathology.

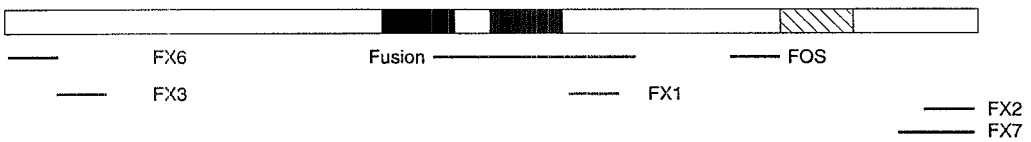
3.2 Antibodies as a helpful tool in the characterization of the *FMR1* protein

Positional cloning led to the identification of the Fragile X Mental Retardation 1 (*FMR1*) gene. The protein sequence, which was deduced from the DNA sequence, showed no homology to other protein sequences at the time the *FMR1* gene was cloned. A possible protein function could therefore not be deduced. The development of antibodies was a first necessary step for the isolation and characterization of the *FMR1* protein and eventually for the determination of the protein function. Different antigens representing specific parts of FMRP were generated to raise specific antibodies.

The amino acid sequence of FMRP was used to predict which parts of the protein were exposed. Hydrophilicity plots were used to select such regions and some of these parts were reproduced as synthetic peptides, depicted in Fig. 2.1A. The peptides were coupled to a carrier protein (keyhole limpet haemocyanin=KLH) in order to raise antibodies in rabbits. An advantage of synthetic peptides is that any part of the protein can be reproduced. However, synthetic peptides consist of 6-30 amino acids and the raised antibodies recognize only limited number of epitopes. A fusion protein was made in order to raise antibodies directed against a much larger part of FMRP. A fragment of the *FMR1* gene, containing nucleotides 940 to 1,325 of the coding sequence and 3,557 to 3,765 of the non-coding sequence, was fused to the glutathione S transferase (GST) gene (161). The (GST)-*FMR1* fusion protein was isolated after overexpression in bacteria, making use of the affinity of GST for glutathione. Polyclonal antibodies against this fusion protein were raised in rabbits as well.

Antisera with high titres were obtained for the different antigens, but not all of the antibodies were successful in detecting the *FMR1* protein. The antibodies raised against the (GST)-*FMR1* fusion protein were successful in immunoprecipitations, Western blots and immunohistological studies. The peptide antisera α1076, α766 and α1079 (Fig.2.1A)

A



B

Code	aa	Sera
FX1	318-333	454
FX2	617-632	459
FX3	33-47	461
FX6	2-23	1076
FX7	606-632	1079
FOS	516-531	766
Fusion	248-400	734, 765, 709

Figure 3.1
A) Positions of the synthetic peptides and the (GST)-*FMR1* fusion protein are indicated, as well as the position of the KH domains and the RGG box. B) The table refers to the amino acid (aa) position of the synthetic peptides and fusion protein (Code) and the antisera which were raised against these antigens (Sera).

detected FMRP in Western blots (Publication 5.3). However, these peptide antibodies did not detect FMRP under non-denaturing conditions such as immunoprecipitations, which is in general a limitation of peptide antisera.

Monoclonal antibodies directed against the N-terminal part of FMRP (Fig. 2.1A) generated by the group of Mandel (29) were also used in experiments described in Publication 5.3. These antibodies also form the basis of a simple immunocytochemical test (196) described in 1.6.3.

3.3 Characterization of the *FMR1* gene product

3.3.1 FMRP isoforms

The antibodies raised against the GST-*FMR1* fusion protein were used successfully in identifying FMRP in lymphoblastoid cell lines. At least four protein products of 67-80 kD were found in Western blots. All FMRPs were absent in cell lines derived from fragile X patients with the full mutation (179) (Publication 5.1). Cell lines carrying a premutation express FMRP with a molecular mass identical to FMRP of control cell lines and it was therefore concluded that the CGG repeat is not translated. The translation begins at the first

start codon after the CGG repeat, at methionine 66 (5, 29, 179) (Publication 5.1).
The different protein products were most likely isoforms of FMRP, derived from the translation of *FMR1* splice variants (5, 181). This hypothesis was tested by transfecting *COS-1* cells with constructs representing two different splice variants. The expression of stable proteins with molecular masses similar to some of the proteins found in the immunoprecipitations of lymphoblastoid cell lines supported the hypothesis of the origin of the isoforms being the translation of different splice variants (179) (Publication 5.1). A second confirmation of this hypothesis came from the fact that all FMRP isoforms could be detected with antibodies directed to the N- and C-terminal portion of the protein, indicating that the difference in molecular weight originated from a change in the more central part of the protein (180) (Publication 5.3).

The discovery of at least four different FMRPs raised the following questions: do these isoforms have different functions and are these isoforms differentially expressed. The expression of FMRPs was studied in several human, murine and monkey tissues (85, 180) (Publication 5.3). The tissue distribution of FMRP was almost identical to the distribution found for *FMR1* mRNA in murine tissues (71). High expression levels of all isoforms of 67 to 80 kD are found in human, monkey and murine brain and testis. Low FMRP expression levels were found in murine muscular tissues like heart, skeletal and abdominal muscle (85). High expression levels of FMRP were also found in murine kidney, which has a low mRNA expression level. The differences observed for the *FMR1* protein and mRNA expression might be explained by a difference in protein and mRNA stability (180) (Publication 5.3).

The low molecular mass isoform observed in the murine kidney cannot be a result of exon 14 splice variants, because higher molecular weights were predicted for these isoforms. Whether the observed low molecular mass proteins result from specific or aspecific proteolysis remains to be determined (180) (Publication 5.3).
In general a high FMRP expression level is found in cells which have a high protein synthesis activity, such as proliferating cells and neurons (29, 85, 180) (Publication 5.3).Thus far no indications have been found for a tissue specific or cell stage specific expression of distinct FMRP isoforms (85, 180).

3.3.2 Intracellular localization

Immunocytochemical studies revealed that FMRP is a cytoplasmic protein (27, 29, 179) (Publication 5.1). The protein is expressed in many tissues, but the strongest FMRP expression is found in brain and testis, the tissues which show predominant clinical symptoms in fragile X patients. In brain the FMRP expression is restricted to neurons and Purkinje cells. Strong labelling was found in the Purkinje cells of the cerebellum and in neurons of the granular layer and the cortex (27, 29). The localization seems to be confined to the perikaryon.

In testis FMRP expression in the tubuli seminiferi is restricted to the early spermatogonia (29) (R.Willemsen, personal communication).

A cytoplasmic localization of FMRP was also found in *COS* cells transfected with two splice variants either lacking exons 12, 15 and 17 or lacking exons 12 and 17 (179) (Publication 5.1). Devys *et al.* (29) described that FMRP, truncated for the C-terminal half, localized to the nucleus in transfected *COS* cells. Exon 14 contains sequences which have been described by one group as a putative cytoplasmic retention domain (CRD) (158) and as a nuclear export signal (NES) by another group (S.T. Warren, personal communication). The N-terminal half of the protein contains sequences which are involved in the nuclear translocation. FMRP lacking the CRD or NES is localized to the nucleus. Exon 14 splicing occurs rarely, explaining why the majority of FMRP is localized in the cytoplasm in immunohistochemical studies. The possible role of the presence of these domains is discussed in Chapter 4.

3.3.3 Influence of the CGG repeat on the translation

The CGG repeat does not contribute to the amino acid sequence of FMRP, because the translation of the messenger starts distal to the repeat at Met66 (3.3.1). An increase in repeat length, like in premutation alleles, therefore does not affect the structure and/or function of the protein. An increased repeat length in premutation alleles could have an influence on the translation efficiency of the *FMR1* mRNA. However, premutation alleles, characterized by a CGG repeat length in the 60-200 range and absence of methylation, are translated normally (29, 40, 179), indicating that repeat lengths within this range do not disturb the efficiency of translation (Publication 5.1).

An interesting family was studied where a grandfather and a grand-uncle of a fragile X patient carried *FMR1* genes with repeat lengths of 100-1500 and 170-340, respectively, in blood cells (160) (Publication 5.4). These repeats were unmethylated and the grandfather and grand-uncle had a normal phenotype and intelligence. A normal *FMR1* transcription level was found in their peripheral blood leucocytes, whereas the *FMR1* transcription in the fragile X patient was strongly decreased.

Slightly lower than control FMRP levels were found in peripheral blood lymphocytes (PBL) and in EBV transformed cell lines of the grandfather and grand-uncle, which was due to an overall lower level of FMRP rather than expression of *FMR1* protein in a limited number of cells. The expanded repeats in the transcripts of these two males thus do not have a great influence on the translation efficiency.

Feng *et al.* (41) reported the influence of the CGG repeat length in clonal fibroblast cell lines derived from a mildly retarded fragile X patient with repeat lengths between 57 and 285 repeats in an unmethylated state. *FMR1* mRNA levels in the clonal fibroblasts were normal,

indicating that the repeat length did not hinder transcription. The translation of transcripts with more than 200 repeats, however, was severely impaired and it was suggested that the migration of the 40S subunit was hindered by conformational changes in the 5' UTR, most likely caused by the enlarged CGG repeat.

The expression of FMRP in EBV transformed cell lines derived from the grandfather and grand-uncle (160) (Publication 5.4), however, showed that *FMR1* translation is not severely hindered by repeat lengths of more than 200 repeat units. It cannot easily be explained why Feng *et al.* found that the presence of more than 200 repeats decreases the translation rate (41), whereas we did not find a strong influence of the repeat length on the translation (160) (Publication 5.4). The different observations made on the influence of the repeat length on the translation of the *FMR1* mRNA might reflect a difference in origin of the cells (patient versus normal) or a difference in cell types (fibroblasts versus lymphocytes).

The normal phenotype of the two males with expanded, but unmethylated repeats could be explained by the presence of a certain amount of FMRP per cell, as FMRP expression was seen in all peripheral blood lymphocytes. In our department De Vries *et al.* (to be published) observed a normal male, carrying an unmethylated full mutation in 90% of his alleles in blood cells, which showed FMRP expression in leucocytes comparable to control levels. The expression per cell, however, was lower than seen in controls.

Rousseau *et al.* (143) described a male who carried an unmethylated full expansion in 60% of his leucocytes. *FMR1* mRNA and protein expression were not studied, but it was assumed that the unmethylated *FMR1* gene accounts for an FMRP expression sufficient for normal functioning. Whether a certain FMRP expression level in all cells is needed, as suggested by the observations made by Smeets *et al.* (160) and De Vries *et al.* (to be published), or whether a certain percentage of cells have to express FMRP for normal functioning, as suggested by the results of Rousseau (143), is an unsolved question. In terms of understanding the molecular pathogenesis of mental retardation it is also clear that the results of studies on the FMRP expression level in blood cells may not be extrapolated to brain cells.

3.4 FMRP is an RNA binding protein

3.4.1 FMRP and RNA binding domains

Initially no clear homologies of FMRP to other proteins (183)) were observed. In 1993 Siomi *et al.* were the first to report the resemblance of FMRP to RNA binding proteins (156). Two K homology domains (KH domains) and an RGG box (a region with RGG repeats, where R stands for arginine and G for glutamine) are present in the central and more C-terminal part of the *FMR1* protein. These domains were thought to play important roles in RNA binding

Tabel 3.1 Proteins containing K homology domains.

gene product	*species*	*Mw in kD*	*KH domains*	*RGG*	*localization*	*DNA/RNA binding*	*function*	*reference*
hnRNP K	human X. laevis	66 47	2 2	+ +	nucleus	RNA/DNA poly (C)	RNA processing	112, 155
MER-1	yeast	31	1				meiotic specific splice factor	38
FMR1	human mouse X. laevis	71	2 2 2	+	cytoplasm	poly (G), poly (U))		4, 156, 183
FXR1	human mouse	73	2	+	cytoplasm	poly (G), poly (U)		157
FXR2	human mouse	74	2	-	cytoplasm	RNA		202
PSI	D. melanogaster	97	3	-	nucleus	RNA	blocks splicing of P element	152
S3	E. coli	~240	1	-	ribosome	rRNA	ribosomal protein	139
PNPase	E. coli	86	1	-		3' RNA	degradation RNA from 3'→ 5'	135
H16	monkey	70	3	-	nucleus	ssDNA≥RNA		52
HX	yeast (homologue of vigilin)	70	6					28
vigilin	chicken	142	14					147
hnRNP grp33	A. salina	33	1			RNA		23
P62	human	62	1	+		RNA	mRNA stability and localization, cell cycle regulation	198

NusA	E.coli B. subtilus	495aa 371aa	2 2			RNA	transcription elongation factor	78
αCP-1 αCP-2	human	43 45	3		cytoplasm	poly (C)	stabilization α-globin mRNA	87
gld-1	C. elegans	47	1		cytoplasm			81
Nova	human	55	3	-	nuclear			15
PCBP1	human clone2.3	37	3	-	nuclear/cytoplasm	poly (C)		1, 97
PCBP2	human homologue of mouse hnRNP X	38	3	-	nuclear/cytoplasm	poly (C)		67, 97
Bic C	D. melanogaster	102	5		cytoplasm		localization specific mRNAs, required for migration of follicle cells	109
quaking	mouse		1	+				34

because of the presence of these domains in RNA binding proteins. The KH domains are named after conserved sequences found in the pre-mRNA binding protein K, a member of the family of hetergenous nuclear RNA binding proteins, collectively called hnRNPs (112). Members of this protein family play a role in (pre-)mRNA metabolism (30). The hnRNP K protein contains two KH domains, located in the N- and C-terminal end (155). Several other proteins involved in RNA binding contain KH domains (listed in Table 3.1), e.g. MER1 a meiosis specific splicing regulator of MER2 in S. cerevisiae (155), and it was therefore suggested that KH domains play an important role in RNA binding. The RGG box present in the C-terminal part of FMRP was initially found in the hnRNP U protein. The RNA binding activity of this protein was confined to a region containing RGG repeats interspersed with aromatic amino acids which might represent a minimal RNA binding domain (86).

An indication for the importance of the KH domains for the functioning of FMRP was the finding of a point mutation in the second KH domain as the sole mutation in the *FMR1* gene of a severely affected fragile X patient. This patient was diagnosed earlier for X-linked glycogenosis (Xp22). The phosphorylase kinase deficiency, however, is not the cause of the fragile X phenotype as 29 relatives with X-linked glycogenosis do not exhibit the fragile X features. The mutation in the *FMR1* gene changed an isoleucine at position 304 into an asparagine (24). This isoleucine is conserved in the KH domains of several proteins (156) and a mutation affecting this amino acid as found in the fragile X patient may well interfere with the KH domain and thereby with the functioning of FMRP.

3.4.2 RNA binding capacity of FMRP

The hnRNA binding proteins have been isolated with the use of RNA homopolymer affinity chromatography, a method which is also used to classify the hnRNP family members as they show different affinities for the RNA homopolymers. This technique was used by Siomi *et al.* (156) to study the RNA binding activity of FMRP. FMRP, made *in vitro*, bound specifically to poly(G) and poly(U). FMRP is not only able to bind RNA homopolymers, but it also binds to *FMR1* mRNA and to 4% of the human brain mRNAs (4). As for hnRNPs it was found that FMRP binds single stranded DNA to a lesser extent (4). The function of FMRP could possibly be deduced from the specific class of transcripts it is binding, but thus far none of the transcripts and corresponding genes has been characterized.

Since the point mutation in the severely affected fragile X patient (discussed in 3.4.1) changes a conserved amino acid in the second KH domain an altered RNA binding activity could be expected. However, *in vitro* made FMRP containing the point mutation bound with equal strength to the RNA poly(G) and poly(U) under low salt conditions as the wild-type

FMRP. A difference in RNA binding was observed only under high salt conditions (153). In this study only one FMRP isoform was studied and the question was whether the natural occurring FMRPs carrying the point mutation would also show a difference in RNA binding under these same conditions.

Homogenates derived from a lymphoblastoid cell line from the patient described in 3.4.1 (24) were used in the RNA homopolymer binding assay. No difference in RNA binding with controls was observed under low salt conditions. Point mutated FMRPs only did show decreased RNA binding under high salt conditions (180) (Publication 5.3), similar to the observations with FMRP made *in vitro* (153).

The same FMRP isoforms were detected in a cell lines carrying the point mutation as in cells carrying the wild-type FMRP, indicating that there were no differences in messenger or protein stability (180) (Publication 5.3). The point mutation did not change the cytoplasmic localization either. So it is unlikely that the mutation affects the processing and localization of FMRP.

The only observed difference between wild-type and mutated FMRP seems to be the RNA homopolymer binding under high salt conditions. This method might be too insensitive to detect the subtle changes in RNA binding due to the point mutation under more physiological conditions.

Another point is that not only the KH domains are responsible for RNA binding, because the RGG box is indispensable for binding as well (153). Truncated FMRP made *in vitro*, containing both KH domains but lacking the RGG box does not bind RNA. The low molecular weight FMRPs isolated from murine kidney, containing both KH domains but lacking the RGG box did not bind either. It is therefore likely that the KH domains and the RGG box work together in the RNA binding (180) (Publication 5.3). It is not yet clear how a point mutation affecting a KH domain in the *FMR1* protein results in the phenotype of the fragile X syndrome. However, that the point mutation affects the RNA binding activity of FMRP has become more likely since the description of mutations in the KH domains of other proteins and their effect on RNA binding and phenotypical consequences (the reader is referred to paragraph 4.1).

3.5 *Fmr1* knock out mice

3.5.1 Animal model

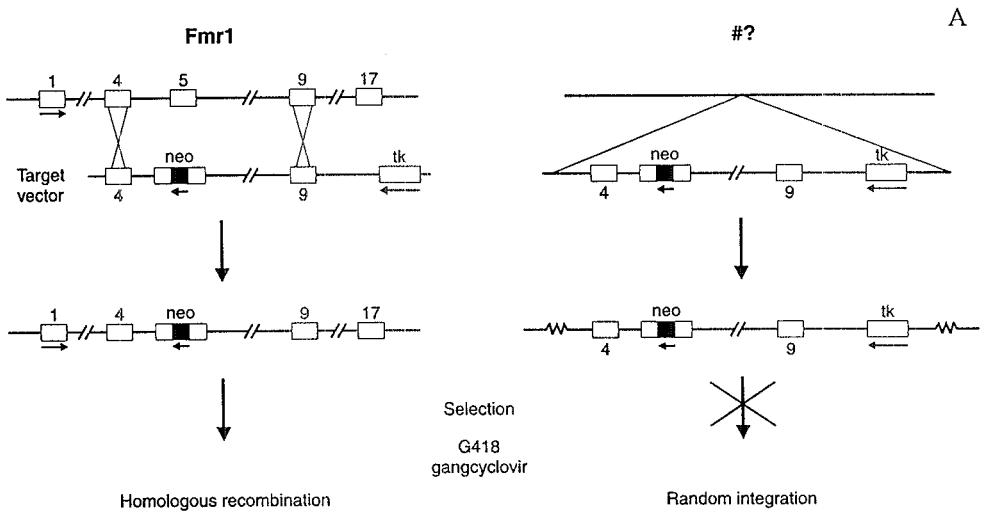
Available tissues of fragile X patients, such as blood cells, cultured skin fibroblasts and (rarely) post-mortem material do not easily allow studies on the molecular pathogenesis of the disease. An animal model could help in understanding the effects of the lack of FMRP expression or the effects of the expression of a mutated FMRP. Since there is no natural model available for the fragile X syndrome an animal model was generated in our laboratory

(Publication 5.2). The development of an animal model has major advantages. First, the unlimited supply of tissue gives the opportunity to study the effects of the lack of FMRP expression on the morphological and molecular level. Second, the behaviour of the knock out mice can be studied in order to understand the mechanisms involved in learning. Third, the knock out mice can be used in crossing experiments with mice transgenic for different mutations or with different expression patterns.

An animal model can either be made by inactivation of the endogenous gene (knock out) or by introducing a (point-) mutation causing the phenotype in humans. The animal chosen must carry a homologue of the human gene of interest. The mouse was chosen as the murine *Fmr1* gene is strongly homologous to the human *FMR1* gene: 97% homologous at the protein level and 95% homologous at the DNA level (5). The function of the *FMR1* gene is therefore thought to be similar in both species. The amplification of the CGG repeat causes the silencing of the *FMR1* gene in patients. Cloning an expanded CGG repeat in the full mutation range is extremely difficult because of technical restrictions and therefore an *FMR1* knock out mouse was developed. The *FMR1* knock out mouse does not express a proper FMRP and therefore mimics the situation in fragile X patients.

3.5.2 The generation of the Fmr1 knock out mouse

Fmr1 knock out mice were made by using the homologous recombination technique. The protein expression of the endogenous *Fmr1* gene can be blocked by the introduction of a neo resistance cassette in the coding region of the gene (110). A targeting vector was constructed containing part of the mouse genomic *Fmr1* gene, exon 4 to 9, interrupted by a neo resistance cassette in antisense orientation in exon 5 (Figure 2.2 A). A thymidine kinase cassette was placed in antisense orientation after exon 9. The neo resistance gene and the thymidine kinase gene are used to select for homologous recombination in the embryonic stem (ES) cells which are transfected with this construct. Selection for the presence of the neo gene and for the absence of the thymidine kinase gene leads to the isolation of ES clones in which homologous recombination has occurred (Figure 2.2. A). These positive clones were injected into blastocysts of C57BL/6J mice, which were transferred to pseudopregnant C57BL/6J females (Figure 2.2 B). Chimeric mice were borne which contained both the cells originating from the ES cells (with the knock out gene) and cells from the original blastocysts. The chimeric males were crossed with wild-type C57BL/6J females to determine possible germ line transmission of the mutant genotype. Two of the twelve chimeric males transmitted the transgene to part of their female offspring. Heterozygote F1 females were crossed with wildtype males and chimeric males (of the F2) to obtain knock out males and females homozygous for the knock out allele. The absence of FMRP does not influence the reproduction or viability of the *Fmr1* knock out mice (6) (Publication 5.2).



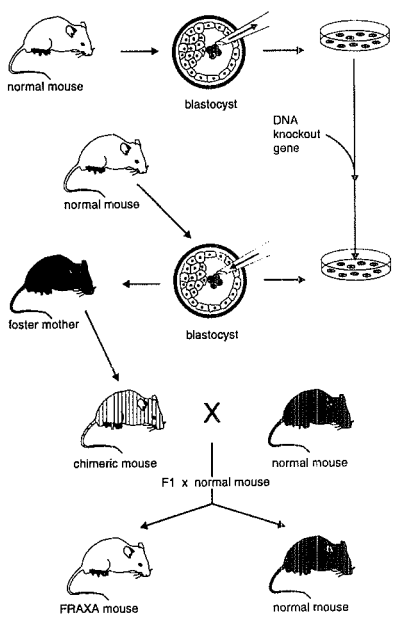


Figure 3.2
Schematic representation of the method used for the silencing of the endogenous mouse *Fmr1* gene in ES cells. A) Homologous recombination of the target vector with the mouse *Fmr1* gene leads to the integration of the neo cassette in the *FMR1* gene. Random integration of the target vector into the mouse genome leads to the integration of both the neo and tk cassette. The addition of G418 and gangcyclovir to the culture medium eventually leads to the selection of clones where homologous recombination has occurred. B) The ES cells in which the *Fmr1* gene is silenced, through the introduction of the neo cassette, are introduced in blastocysts of normal mice. The blastocysts are transferred to foster mothers. *Fmr1* knock out mice are obtained by crossing of the chimeric F1 mice with normal mice.

3.5.3 *Physical and behavioural characteristics of the Fmr1 knock out mouse*

The *Fmr1* knock out mice show some physical and behavioural characteristics which resemble those found in fragile X patients. The only physical difference between the knock out mice and their normal littermates was macroorchidism. Except for the size no further testicular abnormalities were observed. The knock out mice had normal interstitial mass and normal spermatogenesis. The behaviour of the knock out mice was tested with the use of exploratory behaviour and motor activity tests as well as with Morris water maze tasks. The latter test was performed to study whether the knock out mice had deficits in visual short term memory and visual spatial abilities, functions which are impaired in fragile X patients and which might be related to hippocampal dysfunction. The tests revealed that the knock out mice were hyperactive and impaired in the acquisition of novel spatial information. They were not impaired, however, in the retrieval of spatial and non-spatial information. For a more detailed description of physical and behavioural characteristics the reader is referred to Publication 5.2.

Chapter 4

Discussion

4.1 The FMR1 protein since the cloning of the gene

The cloning of the *FMR1* gene facilitated the diagnosis of the fragile X syndrome and paved the way for a better understanding of the molecular pathogenesis. Since the cloning of the gene in 1991, the *FMR1* protein has been under investigation. Many characteristics of FMRP have been determined since then, but we still know little of the function of the protein. However, as more data accumulate, speculations are starting on possible functions of the protein and the relation between the absence of FMRP and the pathogenesis of the fragile X syndrome.

4.2 RNA binding of FMRP

The features which are most important for the functioning of the protein are the RNA binding domains consisting of two KH domains in the central part and an RGG box in the C-terminus of FMRP (155). The functional importance of KH domains (Chapter 3, Table 3.1) has been demonstrated by mutation analysis both *in vivo* and *in vitro* as has been shown for the Ile304Asn mutation in the second KH domain of FMRP (24, 153, 180) (Publication 5.3). Mutations in KH domains of other proteins have been demonstrated to have phenotypical consequences as well. The gld-1 gene of *C. elegans* is a tumour suppressor gene which is essential for oogenesis (45, 46). Mutations of the conserved amino acids in the KH domains of this protein result in the formation of germ cell tumour (81). The Bic-C protein of *D.melanogaster* plays a role in the follicle cell migration and anterior posterior patterning. Mutations in the KH domains of Bic-C disturb these processes (109).

The functional importance of KH domains is not only shown by the phenotypical effects caused by mutations in the KH domains. Individuals with paraneoplastic opsoclonus-myoclonus ataxia (POMA) suffer from an autoimmune neurological syndrome (104). Breast or lung tumours in these patients express the neuron specific Nova-1 gene which encodes a protein containing three KH domains (15, 16). The antibody response to Nova-1 not only results in suppression of tumour growth but also in neurodegeneration. A common phenomenon of autoimmune antibodies is that they are directed to functional domains in proteins, thereby inhibiting the function of the protein (171). The epitope of the autoimmune antibodies in POMA patients corresponds to the third KH domain of Nova-1. The neurodegeneration was suggested to result from the inhibition of the Nova-1 gene product in brain through binding of the autoimmune antibodies to the KH domain.

The effect of the KH domain mutations *in vivo* is not yet understood, but *in vitro* analysis of

these mutations have shown that they affect the RNA binding activity. This has been demonstrated for hnRNP K, FMRP and Nova-1 (16, 153, 180) (Publication 5.3). A recent study on the three dimensional structure of the KH domains of several proteins revealed a potential RNA binding surface, which complements earlier observations on the RNA binding ability of the KH domain (117). In addition it was demonstrated that introduction of the Ile304Asn mutation in the second KH domain of FMRP destroys the structure of the KH domain. Although the RNA binding ability was demonstrated *in vitro* it has been suggested that the mutation of the KH domains interfered with *in vivo* functions of these proteins as mRNA stabilization, transport and translation (16, 81, 109).

While great emphasis has been placed on the KH domains as the elements responsible for the RNA binding of FMRP, the role of the RGG box in RNA binding should not be neglected. RGG boxes have been found in several hnRNPs and other proteins involved in RNA processing (86). The RGG box is less well conserved than the KH domain, as the number of RGG units in these boxes differs. Truncation analysis has not only demonstrated that this domain is involved in the RNA binding activity of hnRNP U, but also of proteins as EBNA-1 (162) and EWS (123). FMRP truncated for the C-terminal part containing the RGG box is unable to bind RNA, even if the two KH domains are still present (156, 180) (Publication 5.3). The exact mechanism by which the KH domains and the RGG box are involved in RNA binding remains to be determined.

The RGG box is not only involved in RNA binding, but also in protein-protein interaction (101). FMRP is able to form homodimers and heterodimers with *FXR1* and *FXR2* proteins (157, 202). The latter proteins are highly homologous to FMRP and have been demonstrated to interact with FMRP *in vitro*. Whether the RGG box plays a role in the formation of these complexes remains to be studied. The arginine residues within the RGG box can be methylated by specific enzymes (101). It is assumed that the methylation of the arginines influence the binding of the RGG box to RNA or to other proteins. The RGG box of FMRP can be methylated as well (101) and it might be interesting to study the influence of this modification on the RNA binding activity or possible protein-protein interaction.

The RNA binding of FMRP has not only been shown in RNA homopolymer binding assays. First, Ashley *et al.* demonstrated that FMRP is able to bind to 4% of the human fetal brain mRNAs. More recently, association of FMRP to the 60S ribosomal subunit has been reported (84). The binding of FMRP to the ribosomes can be easily disturbed with detergents and salt, indicating that FMRP is rather a protein which is associated to the ribosomes than an integral ribosomal protein (84). The association most likely occurs via RNA binding, as incubation of ribosomal preparations with micrococcal nuclease abrogated the binding of FMRP to the ribosomes (170). Another indication that FMRP is bound to the ribosomes via RNA is the observation that FMRP carrying the Ile304Asn mutation has a decreased ability to bind to the ribosomes (170). The association to the ribosomes via the KH domains has also

been reported for the *E. coli* ribosomal protein S3 (178).

The function of the association to the ribosomes is unknown. Recently the protein was visualized in the nucleolus of COS cells transfected with *FMR1* cDNA (previous paragraph) showing that the association of FMRP occurs during the formation of the ribosomal subunits (R. Willemsen, personal communication). The protein is furthermore associated to the ribosomes throughout translation (S.T. Warren, personal communication). These observations thereby exclude a possible role of FMRP as initiation or elongation factor as these factors are associated to the ribosomes only during specific phases of translation during the formation of the ribosomal subunits (R. Willemsen, personal communication). Whether FMRP belongs to a new type of factors involved in the fidelity of translation, as suggested by Khandjian *et al.* (84), remains to be determined.

4.3 Intracellular localization of FMRP

FMRP is expressed in many tissues (paragraphs 1.9 and 3.3.1) and has been localized initially to the cytoplasm of different cell types (29, 84, 158, 179). In COS cells transfected with full length *FMR1* cDNA a normal cytoplasmic localization was observed, but COS cells transfected with a truncated protein consisting of the first 11 exons of *FMR1* (29) showed a nuclear localization. This can be explained by the presence of localization signals in FMRP, which direct the protein either to the cytoplasm or to the nucleus.

It has been demonstrated that a fusion protein consisting of the β-galactosidase reporter gene fused to the first 167 amino acids of FMRP (exons 1 to 5) localizes to the nucleus (158). Since no classical nuclear translocation signal has been identified within this region this either means that FMRP contains a novel type of NLS sequence or that FMRP is targeted to the nucleus through interaction with proteins containing NLS sequences. The first 13 amino acids of exon 14 are referred to as cytoplasmic retention domain (CRD) by these authors (158). This part of exon 14 shows sequence homology to the CRD of Xenopus nuclear factor 7 (Xnf7) (99) and cyclin B (130). The intracellular localization of Xnf7 in the oocyte depends on the developmental stage of the oocyte. The protein is phosphorylated during maturation, which results in the exposition of the CRD and localization of the protein to the cytoplasm (99). The cytoplasmic localization of cyclin B is changed into a nuclear localization upon the onset of mitosis (130).

Warren *et al.* showed that the first 17 amino acids of exon 14 are able to transport a reporter protein from the nucleus to the cytoplasm (S.T. Warren, personal communication). Mutation of Arg430Leu within this sequence makes the transport to the cytoplasm less efficient as the majority of the fusion protein remains in the nucleus. In this way it was shown

that the sequence in exon 14 has characteristics of a nuclear export signal, rather than a cytoplasmic retention domain. The first 13 amino acids of exon 14 of *FMR1* indeed show homology to the nuclear export sequence (NES) found in proteins as Rev (HIV) (44, 194) and PKI. The NES sequence in Rev is important for interaction with cellular components which are involved in nuclear export (NES-receptor) (11, 55, 165).

The finding of both an NLS and NES in FMRP might indicate that FMRP shuttles between the nucleus and the cytoplasm. FMRP has been compared with hnRNP A1, a protein which contains RNA binding motifs (118) and a sequence which acts as a nuclear localization as well as a nuclear export signal (154, 193). This protein binds, like all hnRNP proteins, to nascent transcripts (30) and is supposed to be involved in pre-mRNA splicing (18, 113). The protein is localized predominantly to the nucleus, but has been postulated to shuttle between this compartment and the cytoplasm. This has indeed been demonstrated for the *D. melanogaster* homologue of hnRNP A1, called hrp 36, which transports mRNA to membrane bound ribosomes in the cytoplasm (186).

Recently it has been found that FMRP is shuttling as well. The protein has been localized to the nucleolus in *COS* cells transfected with a cDNA construct containing both the nuclear localization signal and the nuclear export signal (R. Willemsen, personal communication). The majority of FMRP was localized in the cytoplasm and confined to the free and membrane bound ribosomes in electron microscopic sections. These studies provide evidence for both the shuttling of FMRP between the nucleus and the cytoplasm and of the association of the protein to the ribosomes.

4.4 FMRP and the fragile X syndrome

The memory and learning deficits of the fragile X patients are clearly linked to the absence or low levels (in case of mosaic patients) of FMRP in neurons of the brain. The characteristics of FMRP might give a clue to which functions in the neurons are disturbed. The most important clue so far comes from the RNA binding ability of FMRP.

FMRP has been demonstrated to bind 4% of the human fetal brain mRNA *in vitro* (4). A function of proteins binding to mRNA might be the transport of the messenger to specific regions in the cytoplasm, which has several advantages. If a protein product is necessary at a specific place within the cell, it is much more efficient to transport mRNAs than proteins. In addition, transport of mRNAs can also prevent harmful ectopic protein expression. Binding of mRNA to proteins can have functions other than transport as well. mRNAs bound by proteins can be docked in specific regions within the cell. Such a pool of mRNAs would allow the cell to respond quickly to specific stimuli. Transport and docking of mRNAs is thus a way of regulating gene expression and has been demonstrated to exist in neurons (124, 163, 164). If

FMRP is involved in such processes, one can imagine the harmful effect of the absence of the protein in neurons. A less efficient response to stimuli of the neurons might have great effects on the normal functioning of the neurons and thus of the brain. It remains to be studied, however, whether FMRP is involved in processes involving mRNA transport or docking.

Thus far, most indications point to a role of FMRP in translation, as FMRP is found to be associated to ribosomes. *FMR1* is highly expressed in neurons (29) and in proliferating cells (29, 85), groups of cells which are both translationally active. Regulation of translation of specific mRNAs is just as important for the response of neurons to stimuli, as transcription, transport and docking of the mRNAs. Disturbance of translation through the lack of FMRP might have great effects on the normal functioning of the brain.

The disturbance of the functioning of the neurons in fragile X patients through the lack of FMRP leads to learning and memory deficits. Memory and learning are linked to the hippocampus, a structure which shows a high *FMR1* expression level (2). Part of the learning abilities of the *Fmr1* knock out mice was studied with the Morris water-maze test. *Fmr1* knock out mice performed less well than control mice in this test (6) (Publication 5.2), which depends on hippocampal functioning and especially on long term potentiation (LTP) (115, 116). As FMRP is highly expressed in neurons of the hippocampus it has been suggested that FMRP might have a function in LTP.

LTP involves an increase in efficiency of synaptic transmission and can be divided in three phases (10). The first phase (LTP1) is independent of transcription and translation, whereas the latter two phases are dependent on translation (LTP2) or both transcription and translation (LTP3), respectively. The maintenance of LTP depends on the expression of so called early genes which are specifically expressed in LTP3, the phase of LTP which depends on both transcription and translation.

Thus far, preliminary results neither indicate that there are gross differences in LTP1 between normal and *Fmr1* knock out mice (57) nor do they indicate that *Fmr1* is an early gene expressed in LTP3. Only few aspects of LTP have been studied and it is therefore still possible that the lack of FMRP results in more subtle changes (on translation) in LTP.

Absence of *FMR1* expression is associated with macroorchidism, the major clinical characteristic after mental retardation, but the mechanism underlying this abnormality is not clear. During puberty, macroorchidism becomes more pronounced, which might be due to a change in hormonal levels caused by the absence of FMRP. However, no differences in hormonal levels have been described. Another possibility might be that the lack of FMRP in testis results in an aberrant response to normal hormone levels.

After immunohistological staining for FMRP, a strong signal is observed in early spermatogonia of adult testis, whereas a low signal is found in all other cells. Fragile X patients carry a premutation in their sperm cells and FMRP indeed has been detected in early

spermatogonia. The overall low expression level as seen in control adult testis, however, is not detected (R. Willems, personal communication).

The growth of the testis, however might also be regulated by specific regions in the brain and it might be possible that the enlargment of the testis is due to the lack of FMRP in brain. Whether the enlargement of the testis is correlated to the lack of FMRP in cells of the testis other than the spermatogonia or to the lack of FMRP in brain remains to be studied. The generation of transgenic mice expressing *Fmr1* in either specific cells of the testis or in brain might shed light on this question.

4.5 Concluding remarks

Knowledge on the function of FMRP will not be sufficient for the development of a treatment of the fragile X syndrome. It is also necessary to determine whether the effects of the lack of FMRP during (embryonic) development is reversible. Treatment would be almost impossible if FMRP is essential during early development. It has to be established as well what *FMR1* expression levels are needed for normal functioning as well as the percentage of cells which have to express *FMR1* in order to allow normal intellectual development. These problems can be addressed by using the *Fmr1* knock out mouse for the generation of transgenic mice which express *FMR1* at certain developmental stages or which express *FMR1* at different levels.

Depending on the outcome of the investigations future treatment might consist of medication which would compensate for the lack of FMRP. Other possibilities would be cell specific administration of the *FMR1* protein or of an active *FMR1* gene; activation of the silenced gene might be an option as well. None of these putative treatments are within reach, as the function of FMRP is not understood yet and too many technical problems have to be overcome concerning the administration of proteins or genes to specific regions or cells in the brain.

However, research on the processes in which FMRP is involved in, will not only lead to a better understanding of the pathogenesis of the fragile X syndrome and eventually to a possible better treatment, but also to more insight in the processes involved in memory and learning.

References

1. Aasheim, H.C., Loukianova, T., Deggerdal, A. and Smeland, E.B. (1994). Tissue specific expression and cDNA structure of a human transcript encoding a nucleic acid binding [oligo(dC)] protein related to the pre-mRNA binding protein K. *Nucleic Acids Res,* **22**, 959-964.
2. Abitbol, M., Menini, C., Delezoide, A.L., Rhyner, T., Vekemans, M. and Mallet, J. (1993). Nucleus basalis magnocellularis and hippocampus are the major sites of FMR-1 expression in the human fetal brain. *Nature Genet,* **4**, 147-153.
3. Ambrose, C.M., Duyao, M.P., Barnes, G., Bates, G.P., Lin, C.S., Srinidhi, J., Baxendale, S., Hummerich, H., Lehrach, H., Altherr, M. and et, a. (1994). Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somat Cell Mol Genet,* **20**, 27-38.
4. Ashley, C., Jr., Wilkinson, K.D., Reines, D. and Warren, S.T. (1993). FMR1 protein: conserved RNP family domains and selective RNA binding. *Science,* **262**, 563-568.
5. Ashley, C.T., Sutcliffe, J.S., Kunst, C.B., Leiner, H.A., Eichler, E.E., Nelson, D.L. and Warren, S.T. (1993). Human and murine FMR-1: alternative splicing and translational initiation downstream of the CGG-repeat. *Nature Genet,* **4**, 244-251.
6. Bakker, C.E., Verheij, C., Willemsen, R., Vanderhelm, R., Oerlemans, F., Vermey, M., Bygrave, A., Hoogeveen, A.T., Oostra, B.A., Reyniers, E., Deboulle, K., Dhooge, R., Cras, P., Van Velzen, D., Nagels, G., Martin, J.J., Dedeyn, P.P., Darby, J.K. and Willems, P.J. (1994). Fmr1 knockout mice: A model to study fragile X mental retardation. *Cell,* **78**, 23-33.
7. Banfi, S., Servadio, A., Chung, M., Kwiatkowski Jr, T.J., McCall, A.E., Duvick, L.A., Shen, Y., Roth, E.J., Orr, H.T. and Zoghbi, H.Y. (1994). Identification and characterization of the gene causing type 1 spinocerebellar ataxia. *Nature Genet,* **7**, 513-520.
8. Bell, M.V., Hirst, M.C., Nakahori, Y., MacKinnon, R.N., Roche, A., Flint, T.J., Jacobs, P.A., Tommerup, N., Tranebjaerg, L., Froster-Iskenius, U., Kerr, B., Turner, G., Lindebaum, D., Winter, R., Pembrey, M., Thibodeau, S. and Davies, K.E. (1991). Physical mapping across the fragile X: hypermethylation and clinical expression of the fragile X syndrome. *Cell,* **64**, 861-866.
9. Bidichandani (1996). in press.
10. Bliss, T.V. and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. [Review]. *Nature,* **361**, 31-39.
11. Bogerd, H.P., Fridell, R.A., Madore, S. and Cullen, B.R. (1995). Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell,* **82**, 485-494.
12. Borghgraef, M., Fryns, J.P. and van den Berghe, H. (1990). The female and the fragile X syndrome: data on clinical and psychological findings in 7 fra(X) carriers. *Clin Genet,* **37**, 341-346.
13. Boucher, C.A., King, S.K., Carey, N., Krahe, R., Winchester, C.L., Rahman, S., Creavin, T., Meghji, P., Bailey, M.E.S., Chartier, F.L., Brown, S.D., Siciliano, M.J. and Johnson, K.J. (1995). A novel homeodomain-encoding gene is associated with a large CpG island interrupted by the myotonic dystrophy unstable (CTG)n repeat. *Hum Mol Genet,* **4**, 1919-1925.
14. Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.P., Hudson, T., Sohn, R., Zemelman, B., Snell, R.G., Rundle, S.A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johson, K., Harper, P.S., Shaw, D.J. and Housman, D.E. (1992). Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell,* **68**, 799-808.
15. Buckanovich, R.J., Posner, J.B. and Darnell, R.B. (1993). Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. *Neuron,* **11**, 657-672.

16. Buckanovich, R.J., Yang, Y.Y. and Darnell, R.B. (1996). The onconeural antigen Nova-1 is a neuron-specific RNA-binding protein, the activity of which is inhibited by paraneoplastic antibodies. *J Neurosci,* **16**, 1114-1122.
17. Burright, E.N., Clark, H.B., Servadio, A., Matilla, T., Feddersen, R.M., Yunis, W.S., Duvick, L.A., Zoghbi, H.Y. and Orr, H.T. (1995). *SCA1* transgenic mice: A model for neurodegeneration caused by an expanded CAG trinucleotide repeat. *Cell,* **82**, 937-948.
18. Caceres, J.F., Stamm, S., Helfman, D.M. and Krainer, A.R. (1994). Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. *Science,* **265**, 1706-1709.
19. Campuzano, V., Montermini, L., Molto, M.D., Pianese, L., Cossee, M., Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A., Zara, F., Canizares, J., Koutnikova, H., Bidichandani, S.I., Gellera, C., Brice, A., Trouillas, P., Demichele, G., Filla, A., Defrutos, R., Palau, F., Patel, P.I., Didonato, S., Mandel, J.L., Cocozza, S., Koenig, M. and Pandolfo, M. (1996). Friedreich's ataxia: Autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science,* **271**, 1423-1427.
20. Carango, P., Noble, J.E., Marks, H.G. and Funanage, V.L. (1993). Absence of myotonic dystrophy protein kinase (DMPK) mRNA as a result of a triplet repeat expansion in myotonic dystrophy. *Genomics,* **18**, 340-348.
21. Chakrabarti, L., Knight, S.J.L., Flannery, A.V. and Davies, K.E. (1996). A candidate gene for mild mental handicap at the FRAXE fragile site. *Hum Mol Genet,* **5**, 275-282.
22. Cronister, A., Schreiner, R., Wittenberger, M., Amiri, K., Harris, K. and Hagerman, R.J. (1991). Heterozygous fragile X female: historical, physical, cognitive, and cytogenetic features. *Am J Med Genet,* **38**, 269-274.
23. Cruz-Alvarez, M. and Pellicer, A. (1987). Cloning of a full-length complementary DNA for an Artemia salina glycine-rich protein. Structural relationship with RNA binding proteins. *J Biol Chem,* **262**, 13377-13380.
24. De Boulle, K., Verkerk, A.J., Reyniers, E., Vits, L., Hendrickx, J., Van Roy, B., Van den Bos, F., de Graaff, E., Oostra, B.A. and Willems, P.J. (1993). A point mutation in the FMR-1 gene associated with fragile X mental retardation. *Nature Genet,* **3**, 31-35.
25. De Graaff, E. The fragile X syndrome. Complex behavior of a simple repeat. Erasmus University Rotterdam, 1996.
26. De Graaff, E., Rouillard, P., Willems, P.J., Smits, A.P.T., Rousseau, F. and Oostra, B.A. (1995). Hotspot for deletions in the CGG repeat region of FMR1 in fragile X patients. *Hum Mol Genet,* **4**, 45-49.
27. De Graaff, E., Willemsen, R., Zhong, N., De Die-Smulders, C.E.M., Brown, W.T., Freling, G. and Oostra, B.A. (1995). Instability of the CGG repeat and expression of the *FMR1* protein in a male fragile X patient with a lung tumour. *Am J Hum Genet,* **57**, 609-618.
28. Delahodde, A., Becam, A.M., Perea, J. and Jacq, C. (1986). A yeast protein HX has homologies with the histone H2AF expressed in chicken embryo. *Nucleic Acids Res,* **14**, 9213-9214.
29. Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P. and Mandel, J.L. (1993). The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nature Genet,* **4**, 335-340.
30. Dreyfuss, G., Matunis, M.J., Pinol-Roma, S. and Burd, C.G. (1993). hnRNP proteins and the biogenesis of mRNA. [Review]. *Annu Rev Biochem,* **62**, 289-321.
31. Duyao, M.P., Auerbach, A.B., Ryan, A., Persichetti, F., Barnes, G.T., McNeil, S.M., Ge, P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L. and et, a. (1995). Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science,* **269**, 407-410.
32. Dykens, E., Leckman, J., Paul, R. and Watson, M. (1988). Cognitive, behavioral, and adaptive functioning in fragile X and non-fragile X retarded men. *J Autism Dev Disord,* **18**, 41-52.
33. Dykens, E.M., Hodapp, R.M. and Leckman, J.F. (1987). Strengths and weaknesses in the intellectual functioning of males with fragile X syndrome. *Am J Ment Defic,* **92**, 234-236.

34. Ebersole, T.A., Chen, Q., Justice, M.J. and Artzt, K. (1996). The quaking gene product necessary in embryogenesis and myelination combines features of RNA binding and signal transduction proteins . *Nat Genet,* **12**, 260-265.
35. Eichler, E.E., Holden, J., Popovich, B.W., Reiss, A.L., Snow, K., Thibodeau, S.N., Richards, C.S., Ward, P.A. and Nelson, D.L. (1994). Length of uninterrupted CGG repeats determines instability in the FMR1 gene. *Nature Genet,* **8**, 88-94.
36. Eichler, E.E., Richards, S., Gibbs, R.A. and Nelson, D.L. (1993). Fine structure of the human FMR1 gene. *Hum Mol Genet,* **2**, 1147-1153.
37. Einfeld, S.L., Tonge, B.J. and Florio, T. (1994). Behavioural and emotional disturbance in fragile X syndrome. *Am J Med Genet,* **51**, 386-391.
38. Engebrecht, J. and Roeder, G.S. (1990). MER1, a yeast gene required for chromosome pairing and genetic recombination, is induced in meiosis. *Mol Cell Biol,* **10**, 2379-2389.
39. Engebrecht, J.A., Voelkel-Meiman, K. and Roeder, G.S. (1991). Meiosis-specific RNA splicing in yeast. *Cell,* **66**, 1257-1268.
40. Feng, Y., Lakkis, D. and Warren, S.T. (1995). Quantitative comparison of FMR1 gene expression in normal and premutation alleles. *Am J Hum Genet,* **56**, 106-113.
41. Feng, Y., Zhang, F.P., Lokey, L.K., Chastain, J.L., Lakkis, L., Eberhart, D. and Warren, S.T. (1995). Translational suppression by trinucleotide repeat expansion at FMR1. *Science,* **268**, 731-734.
42. Fisch, G.S. (1992). Is autism associated with the fragile X syndrome? *Am J Med Genet,* **43**, 78-86.
43. Fisch, G.S. (1993). What is associated with the fragile X syndrome?. [Review]. *Am J Med Genet,* **48**, 112-121.
44. Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W. and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell,* **82**, 475-483.
45. Francis, R., Barton, M.K., Kimble, J. and Schedl, T. (1995). gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans. *Genetics,* **139**, 579-606.
46. Francis, R., Maine, E. and Schedl, T. (1995). Analysis of the multiple roles of gld-1 in germline development: interactions with the sex determination cascade and the glp-1 signaling pathway. *Genetics,* **139**, 607-630.
47. Fryns, J.P. (1984). The fragile X syndrome: A study of 83 families. *Clin Genet,* **26**, 497-528.
48. Fryns, J.P. (1986). The female and the fragile X. A study of 144 obligate female carriers. *Am J Med Genet,* **23**, 157-169.
49. Fryns, J.P. (1989) *In* Davies, K.E. (ed.), Clinical studies. Oxford University Press, Oxford, 1-39.
50. Fu, Y.H., Kuhl, D.P., Pizzuti, A., Pieretti, M., Sutcliffe, J.S., Richards, S., Verkerk, A.J., Holden, J.J., Fenwick, R., Jr., Warren, S.T., Oostra, B.A., Nelson, D.L. and Caskey, C.T. (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell,* **67**, 1047-1058.
51. Fu, Y.-H., Friedmna, D.L., Richards, S., Pearlman, J.A., Gibbs, R.A., Pizzuti, a., Ashizawa, T., Perryman, M.B., Scarlato, G., Fenwick, R.G. and Caskey, C.T. (1993). Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. *Science,* **260**, 235-238.
52. Gaillard, C., Cabannes, E. and Strauss, F. (1994). Identity of the RNA-binding protein K of hnRNP particles with protein H16, a sequence-specific single strand DNA-binding protein. *Nucleic Acids Res,* **22**, 4183-4186.
53. Gecz, J., Gedeon, A.K., Sutherland, G.R. and Mulley, J.C. (1996). Cloning of *FMR2*: a gene associated with FRAXE mental retardation. *Nature Genet,* **13 (1)**, 114.
54. Gedeon, A.K., Baker, E., Robinson, H., Partington, M.W., Gross, B., Manca, A., Korn, B., Poustka, A., Yu, S., Sutherland, G.R. and Mulley, J.C. (1992). Fragile X syndrome without CCG amplification has an FMR1 deletion. *Nature Genet,* **1**, 341-344.

55. Gerace, L. (1995). Nuclear export signals and the fast track to the cytoplasm. [Review]. *Cell,* **82**, 341-344.
56. Gerber, H.P., Seipel, K., Georgiev, O., Hoffere, M., Hug, M., Rusconi, S. and Schaffner, W. (1994). Transcriptional activation modulated by homopolymeric glutamine and proline stretches. *Science,* **263**, 808-811.
57. Godfraind, J.-M., Reyniers, E., De Boulle, K., D'Hooge, R., De Deyn, P.P., Bakker, C.E., Oostra, B.A., Kooy, R.F. and Willems, P.J. (1996). Long-term potentiation in the hippocampus of fragile X knok out mice. *in press,*
58. Green, H. (1993). Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism . *Cell,* **74**, 955-956.
59. Gu, Y., Shen, Y., Gibbs, R.A. and Nelson, D.L. (1996). Identification of a novel gene (FMR2) associated with the FRAXE CCG repeat and CpG island. *Nature Genet* **13(1)**, 109-113.
60. Gu, Y.H., Lugenbeel, K.A., Vockley, J.G., Grody, W.W. and Nelson, D.L. (1994). A de novo deletion in FMR1 in a patient with developmental delay. *Hum Mol Genet,* **3**, 1705-1706.
61. Gustavson, K.H., Blomquist, H.K. and Holmgren, G. (1986). Prevalence of the fragile X syndrome in mentally retarded boys in a Swedish county. *Am J Med Genet,* **23**, 581-587.
62. Gutekunst, C.A., Levey, A.I., Heilman, C.J., Whaley, W.L., Yi, H., Nash, N.R., Rees, H.D., Madden, J.J. and Hersch, S.M. (1995). Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. *Proc Natl Acad Sci U S A,* **92**, 8710-8714.
63. Hagerman, R.J. (1991) *In* Hagerman, R.J., A.C. Silverman (ed.), Physical and behavioral phenotype. The John Hopkins University Press, Baltimore and London, 1-68.
64. Hagerman, R.J., Jackson, C., Amiri, K., Silverman, A.C., O'Connor, R. and Sobesky, W. (1992). Girls with fragile X syndrome: physical and neurocognitive status and outcome. *Pediatrics,* **89**, 395-400.
65. Hagerman, R.J. and Sobesky, W.E. (1989). Psychopathology in fragile X syndrome. [Review]. *Am J Orthopsychiatry,* **59**, 142-152.
66. Hagerman, R.J., Van Housen, K., Smith, A.C. and McGavran, L. (1984). Consideration of connective tissue dysfunction in the fragile X syndrome. *Am J Med Genet,* **17**, 111-121.
67. Hahm, K., Kim, G., Turck, C.W. and Smale, S.T. (1993). Isolation of a murine gene encoding a nucleic acid-binding protein with homology to hnRNP K. *Nucleic Acids Res,* **21**, 3894.
68. Hansen, R.S., Gartler, S.M., Scott, C.R., Chen, S.H. and Laird, C.D. (1992). Methylation analysis of CGG sites in the CpG island of the human FMR1 gene. *Hum Mol Genet,* **1**, 571-578.
69. Harper, P.S. (1989) Myotonic Dystrophy, 2nd edn. London.
70. Hergersberg, M., Matsuo, K., Gassmann, M., Schaffner, W., Luscher, B., Rulicke, T. and Aguzzi, A. (1995). Tissue-specific expression of a FMR1/beta-galactosidase fusion gene in transgenic mice. *Hum Mol Genet,* **4**, 359-366.
71. Hinds, H.L., Ashley, C.T., Sutcliffe, J.S., Nelson, D.L., Warren, S.T., Housman, D.E. and Schalling, M. (1993). Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nature Genet,* **3**, 36-43.
72. Hirst, M.C., Barnicoat, A., Flynn, G., Wang, Q., Daker, M., Buckle, V.J., Davies, K.E. and Bobrow, M. (1993). The identification of a third fragile site, FRAXF, in Xq27--q28 distal to both FRAXA and FRAXE. *Hum Mol Genet,* **2**, 197-200.
73. Hofmann-Radvanyi, H., Lavedan, C., Rabes, J.P., Savoy, D., Duros, C., Johnson, K. and Junien, C. (1993). Myotonic dystrophy: absence of CTG enlarged transcript in congenital forms, and low expression of the normal allele. *Hum Mol Genet,* **2**, 1263-1266.
74. Hoogeveen, A.T., Willemsen, R., Meyer, N., de Rooij, K.E., Roos, R.A., van Ommen, G.J. and Galjaard, H. (1993). Characterization and localization of the Huntington disease gene product. *Hum Mol Genet,* **2**, 2069-2073.
75. Hornstra, I.K., Nelson, D.L., Warren, S.T. and Yang, T.P. (1993). High resolution methylation

analysis of the FMR1 gene trinucleotide repeat region in fragile X syndrome. *Hum Mol Genet,* **2**, 1659-1665.

76. Hunter, A., Tsilfidis, C., Mettler, G., Jacob, P., Mahadevan, M., Surh, L. and Korneluk, R. (1992). The correlation of age of onset with CTG trinucleotide repeat amplification in myotonic dystrophy. *J Med Genet,* **29**, 774-779.
77. Huntington and group., s.d.c.r. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell,* **72**, 971-983.
78. Ito, K., Egawa, K. and Nakamura, Y. (1991). Genetic interaction between the beta' subunit of RNA polymerase and the arginine-rich domain of Escherichia coli nusA protein. *J Bacteriol,* **173**, 1492-1501.
79. Jacobsen, P., Hauge, M., Henningsen, K., Hobolth, N., Mikkelsen, M. and Philip, J. (1973). An (11;21) translocation in four generations with chromosome 11 abnormalities in the offspring. A clinical, cytogenetical, and gene marker study. *Hum Hered,* **23**, 568-585.
80. Jansen, G., Bachner, D., Coerwinkel, M., Wormskamp, N., Hameister, H. and Wieringa, B. (1995). Structural organization and developmental expression pattern of the mouse WD-repeat gene DMR-N9 immediately upstream of the myotonic dystrophy locus. *Hum Mol Genet,* **4**, 843-852.
81. Jones, A.R. and Schedl, T. (1995). Mutations in gld-1, a female germ cell-specific tumor suppressor gene in Caenorhabditis elegans, affect a conserved domain also found in Src-associated protein Sam68. *Genes Dev,* **9**, 1491-1504.
82. Jones, C., Penny, L., Mattina, T., Yu, S., Baker, E., Voullaire, L., Langdon, W.Y., Sutherland, G.R., Richards, R.I. and Tunnacliffe, A. (1995). Association of a chromosome deletion syndrome with a fragile site within the proto-oncogene CBL2. *Nature,* **376**, 145-149.
83. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S. and Kakizuka, A. (1994). CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. *Nature Genet,* **8**, 221-228.
84. Khandjian, E., Corbin, F., Woerly, S. and Rousseau, F. (1996). The fragile X mental retardation protein is associated with ribosomes. *Nature Genet,* **12**, 91-93.
85. Khandjian, E.W., Fortin, A., Thibodeau, A., Tremblay, S., Cote, F., Devys, D., Mandel, J.L. and Rousseau, F. (1995). A heterogeneous set of FMR1 proteins is widely distributed in mouse tissues and is modulated in cell culture. *Human Mol Genet,* **4**, 783-790.
86. Kiledjian, M. and Dreyfuss, G. (1992). Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. *Embo J,* **11**, 2655-2664.
87. Kiledjian, M., Wang, X. and Liebhaber, S.A. (1995). Identification of two KH domain proteins in the alpha-globin mRNP stability complex. *Embo J,* **14**, 4357-4364.
88. Kirkilionis, A.J., Pozsonyi, J. and Sergovich, F.R. (1988). The use of testicular volume as a clinical marker for cytogenetic disorders in mentally retarded males. *J Ment Defic Res,* **32**, 19-30.
89. Knight, S.J.L., Voelckel, M.A., Hirst, M.C., Flannery, A.V., Moncla, A. and Davies, K.E. (1994). Triplet repeat expansion at the FRAXE locus and X-linked mild mental handicap. *Am J Hum Genet,* **55**, 81-86.
90. Koga, R., Nakao, Y., Kurano, Y., Tsukahara, T., Nakamura, A., Ishiura, S., Nonaka, I. and Arahata, K. (1994). Decreased myotonin-protein kinase in the skeletal and cardiac muscles in myotonic dystrophy. *Biochem Biophys Res Commun,* **202**, 577-585.
91. Komure, O., Sano, A., Nishino, N., Yamauchi, N., Ueno, S., Kondoh, K., Sano, N., Takahashi, M., Murayama, N., Kondo, I. and et, a. (1995). DNA analysis in hereditary dentatorubral-pallidoluysian atrophy: correlation between CAG repeat length and phenotypic variation and the molecular basis of anticipation. *Neurology,* **45**, 143-149.
92. Kremer, B., Goldberg, P., Andrew, S.E., Theilmann, J., Telenius, H., Zeisler, J., Squitieri, F., Lin, B., Bassett, A., Almqvist, E. and et, a. (1994). A worldwide study of the Huntington's disease

mutation. The sensitivity and specificity of measuring CAG repeats . *N Engl J Med,* **330**, 1401-1406.

93. Kremer, E.J., Pritchard, M., Lynch, M., Yu, S., Holman, K., Baker, E., Warren, S.T., Schlessinger, D., Sutherland, G.R. and Richards, R.I. (1991). Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n. *Science,* **252**, 1711-1714.
94. Kunst, C.B. and Warren, S.T. (1994). Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. *Cell,* 77, 853-861.
95. La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature,* **352**, 77-79.
96. Lachiewicz, A.M. and Dawson, D.V. (1994). Do young boys with fragile X syndrome have macroorchidism? *Pediatrics,* **93**, 992-995.
97. Leffers, H., Dejgaard, K. and Celis, J.E. (1995). Characterisation of two major cellular poly(rC)-binding human proteins, each containing three K-homologous (KH) domains. *Eur J Biochem,* **230**, 447-453.
98. Li, S.H., McInnis, M.G., Margolis, R.L., Antonarakis, S.E. and Ross, C.A. (1993). Novel triplet repeat containing genes in human brain: cloning, expression, and length polymorphisms. *Genomics,* **16**, 572-579.
99. Li, X., Shou, W., Kloc, M., Reddy, B.A. and Etkin, L.D. (1994). Cytoplasmic retention of Xenopus nuclear factor 7 before the mid blastula transition uses a unique anchoring mechanism involving a retention domain and several phosphorylation sites. *J Cell Biol,* **124**, 7-17.
100. Li, X.J., Li, S.H., Sharp, A.H., Nucifora, F.C., Schilling, G., Lanahan, A., Worley, P., Snyder, S.H. and Ross, C.A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. *Nature,* **378**, 398-402.
101. Liu, Q. and Dreyfuss, G. (1995). In vivo and in vitro arginine methylation of RNA-binding proteins. *Mol Cell Biol,* **15**, 2800-2808.
102. Lubs, H.A. (1969). A marker X-chromosome. *Am J Hum Genet,* **21**, 231-244.
103. Lugenbeel, K.A., Peier, A.M., Carson, N.L., Chudley, A.E. and Nelson, D.L. (1995). Intragenic loss of function mutations demonstrate the primary role of FMR1 in fragile X syndrome. *Nature Genet,* **10**, 483-485.
104. Luque, F.A., Furneaux, H.M., Ferziger, R., Rosenblum, M.K., Wray, S.H., Schold, S., Jr., Glantz, M.J., Jaeckle, K.A., Biran, H., Lesser, M. and et, a. (1991). Anti-Ri: an antibody associated with paraneoplastic opsoclonus and breast cancer. *Ann Neurol,* **29**, 241-251.
105. Maeda, M., Taft, C.S., Bush, E.W., Holder, E., Bailey, W.M., Neville, H., Perryman, M.B. and Bies, R.D. (1995). Identification, tissue-specific expression, and subcellular localization of the 80- and 71-kDa forms of myotonic dystrophy kinase protein. *J Biol Chem,* **270**, 20246-20249.
106. Maes, B., Fryns, J.P., Van Walleghem, M. and Van den Berghe, H. (1994). Cognitive functioning and information processing of adult mentally retarded men with fragile-X syndrome. *Am J Med Genet,* **50**, 190-200.
107. Mahadevan, M., Tsilfidis, C., Saborin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barcelo, J., O'Hoy, K., Leblond, S., Earle-Macdonald, J., de Jong, P., Wieringa, B. and Korneluk, R.G. (1992). Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science,* **255**, 1253-1255.
108. Mahadevan, M.S., Amemiya, C., Jansen, G., Sabourin, L., Baird, S., Neville, C.E., Wormskamp, N., Segers, B., Batzer, M., Lamerdin, J. and et, a. (1993). Structure and genomic sequence of the myotonic dystrophy (DM kinase) gene. *Hum Mol Genet,* **2**, 299-304.
109. Mahone, M., Saffman, E.E. and Lasko, P.F. (1995). Localized Bicaudal-C RNA encodes a protein containing a KH domain, the RNA binding motif of FMR1. *Embo J,* **14**, 2043-2055.
110. Mansour, S.L., Thomas, K.R. and Capecchi, M.R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable

genes. *Nature,* **336**, 348-352.
111. Martin, J.P. and Bell, J. (1943). A pedigree of mental defect showing sex-linkage. *J Neurol Psych,* **6**, 154-157.
112. Matunis, M.J., Michael, W.M. and Dreyfuss, G. (1992). Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein. *Mol Cell Biol,* **12**, 164-171.
113. Mayeda, A. and Krainer, A.R. (1992). Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. *Cell,* **68**, 365-375.
114. Meijer, H., De Graaff, E., Merckx, D.M.L., Jongbloed, R.J.E., De Die-Smulders, C.E.M., Engelen, J.J.M., Fryns, J.P., Curfs, P.M.G. and Oostra, B.A. (1994). A deletion of 1.6 kb proximal to the CGG repeat of the FMR1 gene causes the clinical phenotype of the Fragile X syndrome. *Hum Mol Genet,* **3**, 615-620.
115. Morris, R.G., Anderson, E., Lynch, G.S. and Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature,* **319**, 774-776.
116. Morris, R.G., Garrud, P., Rawlins, J.N. and O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature,* **297**, 681-683.
117. Musco, G., Stier, G., Joseph, C., Morelli, M.A.C., Nilges, M., Gibson, T.J. and Patore, A. (1996). Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome. *Cell,* **85**, 237-245.
118. Nadler, S.G., Merrill, B.M., Roberts, W.J., Keating, K.M., Lisbin, M.J., Barnett, S.F., Wilson, S.H. and Williams, K.R. (1991). Interactions of the A1 heterogeneous nuclear ribonucleoprotein and its proteolytic derivative, UP1, with RNA and DNA: evidence for multiple RNA binding domains and salt-dependent binding mode transitions. *Biochemistry,* **30**, 2968-2976.
119. Nancarrow, J.K., Kremer, E., Holamn, K., Eyre, H., Doggett, N.A., Le Paslier, D., Callen, D.F., Sutherland, G.R. and Richards, R.I. (1994). Implications of FRA16A structure for the mechanism of chromosomal fragile site genesis. *Science,* **264**, 1938-1941.
120. Nasir, J., Floresco, S.B., O'Kusky, J.R., Diewert, V.M., Richman, J.M., Zeisler, J., Borowski, A., Marth, J.D., Phillips, A.G. and Hayden, M.R. (1995). Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell,* **81**, 811-823.
121. Novelli, G., Gennarelli, M., Zelano, G., Pizzuti, A., Fattorini, C., Caskey, C.T. and Dallapiccola, B. (1993). Failure in detecting mRNA transcripts from the mutated allele in myotonic dystrophy muscle. *Biochem Mol Biol Int,* **29**, 291-297.
122. Oberlé, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boue, J., Bertheas, M.F. and Mandel, J.L. (1991). Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science,* **252**, 1097-1102.
123. Ohno, T., Ouchida, M., Lee, L., Gatalica, Z., Rao, V.N. and Reddy, E.S. (1994). The EWS gene, involved in Ewing family of tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors, codes for an RNA binding protein with novel regulatory domains. *Oncogene,* **9**, 3087-3097.
124. Olink-Coux, M., Hollenbeck, P.J. (1996). Localization and active transport of mRNA in axons of sympathetic neurons in culture. *J. Neurosci.,* **16**, 1346-1358.
125. Oostra, B.A., Jacky, P.B., Brown, W.T. and Rousseau, F. (1993). Guidelines for the diagnosis of fragile X syndrome. National Fragile X Foundation. *J Med Genet,* **30**, 410-413.
126. Opitz, J.M., Westphal, J.M. and Daniel, A. (1984). Discovery of a connective tissue dysplasia in the Martin-Bell syndrome. *Am J Med Genet,* **17**, 101-109.
127. Orr, H.T., Chung, M., Banfi, S., Kwiatkowski, T.J., Servadio, A., Beaudet, A., McCall, A.E., Duvick, L.A., Ranum, L.P.W. and Zohgbi, H.Y. (1993). Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nature Genet,* **4**, 221-226.

128. Parrish, J.E., Oostra, B.A., Verkerk, A.J.H.M., Richards, C.S., Spikes, A.S., Shaffer, L.G. and Nelson, D.L. (1994). Isolation of a GCC repeat showing expansion in FRAXF, a fragile site distal to FRAXA and FRAXE. *Nature Genet,* **8**, 229-235.
129. Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T. and Nelson, D.L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell,* **66**, 817-822.
130. Pines, J. and Hunter, T. (1994). The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. *Embo J,* **13**, 3772-3781.
131. Price, D.K., Zhang, F., Ashley, C.T.J. and Warren, S.T. (1996). The chicken *FMR1* gene is highly conserved with a CCT 5'-untranslated repeat and encodes an RNA-binding protein. *Genomics,* **31**, 3-12.
132. Quan, F., Grompe, M., Jakobs, P. and Popovich, B.W. (1995). Spontaneous deletion in the FMR1 gene in a patient with fragile X syndrome and cherubism. *Hum Molecul Genet,* **4**, 1681-1684.
133. Quigley, C.A., Friedman, K.J., Johnson, A., Lafreniere, R.G., Silverman, L.M., Lubahn, D.B., Brown, T.R., Wilson, E.M., Willard, H.F. and French, F.S. (1992). Complete deletion of the androgen receptor gene: definition of the null phenotype of the androgen insensitivity syndrome and determination of carrier status. *J Clin Endocrinol Metab,* **74**, 927-933.
134. Reardon, W., Harley, H.G., Brook, J.D., Rundle, S.A., Crow, S., Harper, P.S. and Shaw, D.J. (1992). Minimal expression of myotonic dystrophy: a clinical and molecular analysis. *J Med Genet,* **29**, 770-773.
135. Regnier, P., Grunberg-Manago, M. and Portier, C. (1987). Nucleotide sequence of the pnp gene of Escherichia coli encoding polynucleotide phosphorylase. Homology of the primary structure of the protein with the RNA-binding domain of ribosomal protein S1. *J Biol Chem,* **262**, 63-68.
136. Reiss, A.L., Freund, L.S., Baumgardner, T.L., Abrams, M.T. and Denckla, M.B. (1995). Contribution of the FMR1 gene mutation to human intellectual dysfunction. *Nature Genet,* **11**, 331-334.
137. Richards, B.W., Sylvester, P.E. and Brooker, C. (1981). Fragile X-linked mental retardation: the Martin-Bell syndrome. *J Ment Defic Res,* **4**, 253-256.
138. Richards, R.I., Holman, K., Yu, S. and Sutherland, G.R. (1993). Fragile X syndrome unstable element, p(CCG)n, and other simple tandem repeat sequences are binding sites for specific nuclear proteins. *Hum Mol Genet,* **2**, 1429-1435.
139. Rinke-Appel, J., Junke, N., Stade, K. and Brimacombe, R. (1991). The path of mRNA through the Escherichia coli ribosome; site-directed cross-linking of mRNA analogues carrying a photo-reactive label at various points 3' to the decoding site. *Embo J,* **10**, 2195-2202.
140. Romeo, G., Menozzi, P., Ferlini, A., Fadda, S., Di Donato, S., Uziel, G., Lucci, B., Capodaglio, L., Filla, A. and Campanella, G. (1983). Incidence of Friedreich ataxia in Italy estimated from consanguineous marriages. *Am J Hum Genet,* **35**, 523-529.
141. Rousseau, F. (1994). The fragile X syndrome: implications of molecular genetics for the clinical syndrome. *Eur J Clin Invest,* **24**, 1-10.
142. Rousseau, F., Heitz, D., Biancalana, V., Blumenfeld, S., Kretz, C., Boue, J., Tommerup, N., Van Der Hagen, C., DeLozier-Blanchet, C., Croquette, M.F., Gilgenkranz, S., Jalbert, P., Voelckel, M.A., Oberlé, I. and Mandel, J.L. (1991). Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. *N Engl J Med,* **325**, 1673-1681.
143. Rousseau, F., Robb, L.J., Rouillard, P. and Derkaloustian, V.M. (1994). No mental retardation in a man with 40% abnormal methylation at the FMR-1 locus and transmission of sperm cell mutations as premutations. *Hum Mol Genet,* **3**, 927-930.
144. Sabourin, L.A., Mahadevan, M.S., Narang, M., Lee, D.S.C., Surh, L.C. and Korneluk, R.G. (1993). Effect of the myotonic dystrophy (DM) mutation on mRNA levels of the DM gene. *Nature Genet,* **4**, 233-238.
145. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S. and et, a. (1995). A single ataxia telangiectasia gene with a product similar to PI-

3 kinase. *Science,* **268**, 1749-1753.
146. Schinzel, A., Auf der Maur, P. and Moser, H. (1977). Partial deletion of long arm of chromosome 11[del(11)(q23)]: Jacobsen syndrome. Two new cases and review of the clinical findings. *J Med Genet,* **14**, 438-444.
147. Schmidt, C., Henkel, B., Poschl, E., Zorbas, H., Purschke, W.G., Gloe, T.R. and Muller, P.K. (1992). Complete cDNA sequence of chicken vigilin, a novel protein with amplified and evolutionary conserved domains. *Eur J Biochem,* **206**, 625-634.
148. Schöls, L., Vieira-Saecker, A.M., Schols, S., Przuntek, H., Epplen, J.T. and Riess, O. (1995). Trinucleotide expansion within the MJD1 gene presents clinically as spinocerebellar ataxia and occurs most frequently in German SCA patients. *Hum Mol Genet,* **4**, 1001-1005.
149. Schwartz, C.E., Dean, J., Howard-Peebles, P.N., Bugge, M., Mikkelsen, M., Tommerup, N., Hull, C., Hagerman, R., Holden, J.J. and Stevenson, R.E. (1994). Obstetrical and gynecological complications in fragile X carriers: a multicenter study. *Am J Med Genet,* **51**, 400-402.
150. Servadio, A., Koshy, B., Armstrong, D., Antalffy, B., Orr, H.T. and Zoghbi, H.Y. (1995). Expression analysis of the ataxin-1 protein in tissues from normal and spinocerebellar ataxia type 1 individuals. *Nature Genet,* **10**, 94-98.
151. Shaw, D.J., McCurrach, M., Rundle, S.A., Harley, H.G., Crow, S.R., Sohn, R., Thirion, J.P., Hamshere, M.G., Buckler, A.J., Harper, P.S. and et, a. (1993). Genomic organization and transcriptional units at the myotonic dystrophy locus. *Genomics,* **18**, 673-679.
152. Siebel, C.W., Admon, A. and Rio, D.C. (1995). Soma-specific expression and cloning of PSI, a negative regulator of P element pre-mRNA splicing. *Genes Dev,* **9**, 269-283.
153. Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L. and Dreyfuss, G. (1994). Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. *Cell,* 77, 33-39.
154. Siomi, H. and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. *J Cell Biol,* **129**, 551-560.
155. Siomi, H., Matunis, M.J., Michael, W.M. and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Res,* **21**, 1193-1198.
156. Siomi, H., Siomi, M.C., Nussbaum, R.L. and Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. *Cell,* **74**, 291-298.
157. Siomi, M.C., Siomi, H., Sauer, W.H., Srinivasan, S., Nussbaum, R.L. and Dreyfuss, G. (1995). FXR1, an autosomal homologue of the fragile X mental retardation gene. *EMBO J,* 2401-2408.
158. Sittler, A., Devys, D., Weber, C. and Mandel, J.-L. (1996). Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of fmr1 protein isoforms. *Hum Mol Genet,* **5**, 95-102.
159. Skre, H. (1975). Friedreich's ataxia in Western Norway. *Clin Genet,* **7**, 287-298.
160. Smeets, H., Smits, A., Verheij, C., Theelen, J., Willemsen, R., Losekoot, M., Van de Burgt, I., Hoogeveen, A.T., Oosterwijk, J. and Oostra, B.A. (1995). Normal phenotype in two brothers with a full FMR1 mutation. *Hum Mol Genet,* **4**, 2103-2108.
161. Smith, D.B. and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene,* **67**, 31-40.
162. Snudden, D.K., Hearing, J., Smith, P.R., Grasser, F.A. and Griffin, B.E. (1994). EBNA-1, the major nuclear antigen of Epstein-Barr virus, resembles 'RGG' RNA binding proteins. *Embo J,* **13**, 4840-4847.
163. St Johnston, D. (1995). The intracellular localization of messenger RNAs. *Cell,* **81**, 161-170.
164. Steward, O. and Banker, G.A. (1992). Getting the message from the gene to the synapse: sorting and intracellular transport of RNA in neurons. [Review]. *Trends Neurosci,* **15**, 180-186.
165. Stutz, F., Neville, M. and Rosbash, M. (1995). Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell,* **82**, 495-506.
166. Sutcliffe, J.S., Nelson, D.L., Zhang, F., Pieretti, M., Caskey, C.T., Saxe, D. and Warren, S.T.

(1992). DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum Mol Genet,* **1**, 397-400.
167. Sutherland, G.R. (1977). Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. *Science,* **197**, 265-266.
168. Sutherland, G.R. and Hecht, F. (1985) Fragile sites on human chromosomes. Oxford University Press, New York.
169. Sutherland, G.R., Jacky, P.B., Baker, E. and Manuel, A. (1983). Heritable fragile sites on human chromosomes. X. New folate-sensitive fragile sites: 6p23, 9p21, 9q32, and 11q23. *Am J Hum Genet,* **35**, 432-437.
170. Tamanini, F., Meijer, N., Verheij, C., Willems, P.J., Galjaard, H., Oostra, B.A. and Hoogeveen, A.T. (1996). FMRP is associated to the ribosomes via RNA. *Hum Mol Genet,* 5(6), 809-814.
171. Tan, E.M. (1991). Autoantibodies in pathology and cell biology. [Review]. *Cell,* **67**, 841-842.
172. Trottier, Y., Imbert, G., Poustka, A., Fryns, J.P. and Mandel, J.L. (1994). Male with typical fragile X phenotype is deleted for part of the FMR1 gene and for about 100 kb of upstream region. *Amer J Med Genet,* **51**, 454-457.
173. Trottier, Y., Lutz, Y., Stevanin, G., Imbert, G., Devys, D., Cancel, G., Saudou, F., Weber, C., David, G., Tora, L., Agid, Y., Brice, A. and Mandel, J.-L. (1995). Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias. *Nature,* **378**, 403-405.
174. Tsilfidis, C., MacKenzie, A.E., Mettler, G., Barcelo, J. and Korneluk, R.G. (1992). Correlation between CTG trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. *Nature Genet,* **1**, 192-195.
175. Turner, G., Daniel, A. and Frost, M. (1980). X-linked mental retardation, macro-orchidism, and the Xq27 fragile site. *J Pediatr,* **96**, 837-841.
176. Turner, G., Robinson, H., Wake, S. and Martin, N. (1994). Dizygous twinning and premature menopause in fragile X syndrome. *Lancet,* **344**, 1500.
177. Turner, G., Webb, T., Wake, S. and Robinson, H. (1996). The prevalence of the fragile X syndrome. *Am J Med Genet,* **64**, 196-197.
178. Urlaub, H., Kruft, V., Bischof, O., Muller, E.C. and Wittmann-Liebold, B. (1995). Protein-rRNA binding features and their structural and functional implications in ribosomes as determined by cross-linking studies. *Embo J,* **14**, 4578-4588.
179. Verheij, C., Bakker, C.E., de-Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A.J., Galjaard, H., Reuser, A.J., Hoogeveen, A.T. and Oostra, B.A. (1993). Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. *Nature,* **363**, 722-724.
180. Verheij, C., De Graaff, E., Bakker, C.E., Willemsen, R., Willems, P.J., Meijer, N., Galjaard, H., Reuser, A.J.J., Oostra, B.A. and Hogeveen, A.T. (1995). Characterization of FMR1 proteins isolated from different tissues. *Hum Mol Genet,* **4**, 895-901.
181. Verkerk, A.J., De Graaff, E., De Boulle, K., Eichler, E.E., Konecki, D.S., Reyniers, E., Manca, A., Poustka, A., Willems, P.J., Nelson, D.L. and Oostra, B.A. (1993). Alternative splicing in the fragile X gene FMR1. *Hum Mol Genet,* **2**, 399-404.
182. Verkerk, A.J., de Vries, B.A., Niermeijer, M.F., Fu, Y.H., Nelson, D.L., Warren, S.T., Majoor-Krakauer, D.F., Halley, D.J. and Oostra, B.A. (1992). Intragenic probe used for diagnostics in fragile X families. *Am J Med Genet,* **43**, 192-196.
183. Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., Eussen, B.E., Van Ommen, G.J.B., Blonden, L.A.J., Riggins, G.J., Chastain, J.L., Kunst, C.B., Galjaard, H., Caskey, C.T., Nelson, D.L., Oostra, B.A. and Warren, S.T. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell,* **65**, 905-914.
184. Verkerk, J.M.H. The molecular basis of the fragile X syndrome. Expansion of a trinucleotide repeat, a new mutational mechanism. Erasmus University Rotterdam, 1994.

185. Vincent, A., Heitz, D., Petit, C., Kretz, C., Oberlé, I. and Mandel, J.L. (1991). Abnormal pattern detected in fragile-X patients by pulsed-field gel electrophoresis. *Nature,* **349**, 624-626.
186. Visa, N., Alzhanova-Ericsson, A.T., Sun, X., Kiseleva, E., Bjorkroth, B., Wurtz, T. and Daneholt, B. (1996). A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell,* **84**, 253-264.
187. Waldstein, G., Mierau, G., Ahmad, R., Thibodeau, S.N., Hagerman, R.J. and Caldwell, S. (1986) *In* Gilbert, E.F., J.M. Opitz (ed.), Fragile X syndrome: skin elastin abnormalities. Alan R. Liss, New York, 103-114.
188. Wang, Y.H., Amirhaeri, S., Kang, S., Wells, R.D. and Griffith, J.D. (1994). Preferential nucleosome assembly at DNA triplet repeats from the myotonic dystrophy gene. *Science,* **265**, 669-671.
189. Warren, S.T., Knight, S.J., Peters, J.F., Stayton, C.L., Consalez, G.G. and Zhang, F.P. (1990). Isolation of the human chromosomal band Xq28 within somatic cell hybrids by fragile X site breakage. *Proc Natl Acad Sci U S A,* **87**, 3856-3860.
190. Warren, S.T., Zhang, F., Licameli, G.R. and Peters, J.F. (1987). The fragile X site in somatic cell hybrids: an approach for molecular cloning of fragile sites. *Science,* **237**, 420-423.
191. Warren, S.T., Zhang, F.P., Sutcliffe, J.S. and Peters, J.F. (1988). Strategy for molecular cloning of the fragile X site DNA. *Am J Med Genet,* **30**, 613-623.
192. Webb, T.P., Bundey, S.E., Thake, A.I. and Todd, J. (1986). Population incidence and segregation ratios in the Martin-Bell syndrome. *Am J Med Genet,* **23**, 573-580.
193. Weighardt, F., Biamonti, G. and Riva, S. (1995). Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. *J Cell Sci,* **108**, 545-555.
194. Wen, W., Meinkoth, J.L., Tsien, R.Y. and Taylor, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell,* **82**, 463-473.
195. Whiting, E.J., Waring, J.D., Tamai, K., Somerville, M.J., Hincke, M., Staines, W.A., Ikeda, J.E. and Korneluk, R.G. (1995). Characterization of myotonic dystrophy kinase (DMK) protein in human and rodent muscle and central nervous tissue. *Hum Mol Genet,* **4**, 1063-1072.
196. Willemsen, R., Mohkamsing, S., De Vries, B.A., Devys, D., Van den Ouweland, A., Mandel, J.L., Galjaard, H. and Oostra, B. (1995). Rapid antibody test for fragile X syndrome. *The Lancet,* **345**, 1147-1148.
197. Wöhrle, D., Kotzot, D., Hirst, M.C., Manca, A., Korn, B., Schmidt, A., Barbi, G., Rott, H.D., Poustka, A., Davies, K.E. and Steinbach, P. (1992). A microdeletion of less than 250 kb, including the proximal part of the FMR-I gene and the fragile-X site, in a male with the clinical phenotype of fragile-X syndrome. *Am J Hum Genet,* **51**, 299-306.
198. Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M.F., Polakis, P. and McCormick, F. (1992). Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. *Cell,* **69**, 551-558.
199. Yazawa, I., Nukina, N., Hashida, H., Goto, J., Yamada, M. and Kanazawa, I. (1995). Abnormal gene product identified in hereditary dentatorubral-pallidoluysian atrophy (DRPLA) brain . *Nature Genet,* **10**, 99-103.
200. Yu, S., Pritchard, M., Kremer, E., Lynch, M., Nancarrow, J., Baker, E., Holman, K., Mulley, J.C., Warren, S.T., Schlessinger, D., Sutherland, G.R. and Richards, R.I. (1991). Fragile X genotype characterized by an unstable region of DNA. *Science,* **252**, 1179-1181.
201. Zeitlin, S., Liu, J.P., Chapman, D.L., Papaioannou, V.E. and Efstratiadis, A. (1995). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nature Genet,* **11**, 155-163.
202. Zhang, Y., Oconnor, J.P., Siomi, M.C., Srinivasan, S., Dutra, A., Nussbaum, R.L. and Dreyfuss, G. (1995). The fragile X mental retardation syndrome protein interacts with novel homologs FXR1 and FXR2. *EMBO Journal,* **14**, 5358-5366.

Chapter 5

Publications

Publication 5.1

Characterization and localization of the *FMR1* gene product associated with fragile X syndrome

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Abstract

The fragile X syndrome is the most frequent form of inherited mental retardation after Down's syndrome, having an incidence of one in 1,250 males[1,2]. The fragile X syndrome results from amplification of the CGG repeat found in the *FMR1* gene[3-6]. This CGG repeat shows length variation in normal individuals and is increased significantly in both carriers and patients[3-6]; it is located 250 base pairs distal to a CpG island which is hypermethylated in fragile X patients[4-7]. The methylation probably results in downregulation of *FMR1* gene expression[8]. No information can be deduced about the function of the *FMR1* protein from its predicted sequence. Here we investigate te nature and function of the protein encoded by the *FMR1* gene using polyclonal antibodies raised against the predicted amino acid sequences. Four different protein products, possibly resulting from alternative splicing, have been identified by immunoblotting in lymphoblastoid cell lines of healthy individuals. All these proteins were missing in cell lines from patients not expressing *FMR1* messenger RNA. The intracellular localization of the *FMR1* gene products was investigated by transient expression in COS-1 cells and found to be cytoplasmic. Localization was also predominantly cytoplasmic in the cytoplasm in the epithelium of the oesophagus, but in some cells was obviously nuclear.

As a first step in the identification and characterization of the *FMR1* gene product, antibodies were raised against different regions of the predicted amino acid sequence of the *FMR1* protein[3]. Two different methods were used. A complementary DNA fragment of *FMR1* containing nucleotides 940-1325 was cloned in the *Escherichia coli* expression vector pGEX[9] and antibodies were raised in rabbits against the *FMR1* fusion protein (α765). The second approach was to use a synthetic oligopeptide corresponding to the carboxy-terminal end (position 632 to 656) of the *FMR1* protein[3] as antigen (α1079)[10].

These antibodies were then used to analyse the *FMR1* protein in lymphoblastoid cell lines from patients (n=5) and controls (n=3). FMRP was immunoprecipitated with α765 and subsequently analysed by immunoblotting. Four species (M_rs 74K, 72K, 70K and 67 K) (Fig. 1A) that were present in the controls were absent in four of five patients. The lack of cross-reactive material in the lymphoblastoid cell lines from these patients is in agreement with the absence of *FMR1* mRNA in these cells (ref.8, and data not shown).

In the cell line of one patient, however, we found the same molecular species as in controls (Fig. 1B). The patient has a mosaic DNA pattern, with one-third of the cells carrying a premutation and the rest a full mutation (data not shown). The CpG island preceding the CGG repeat is unmethylated in the premutation, in contrast to the full mutation. The premutation allele of the mosaic patients is expressed into *FMR1* mRNA[8] (data not shown), and subsequently translated into FMRPs (Fig 1B, lane c).

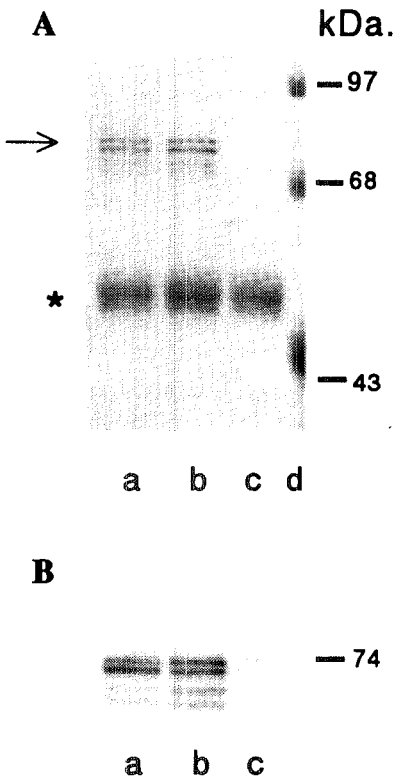
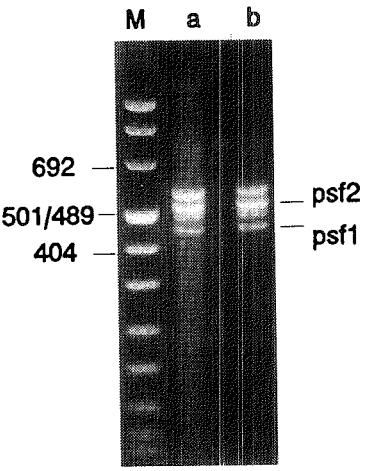


Figure 1.
Immunoprecipitation of lymphoblastoid cell lines with α765 antibodies. A, Control cells: lane a (C49) and b (ROS31) and fragile X patient cells in lane c (RJK1411). Sizes of the protein bands are 74, 72, 70 and 67K. Lane d, molecular weight standards. Nonspecific band (IgG) is indicated by an asterisk, the position of FMRPs is indicated by an arrow. B, control cells: lane a (ROS 13) and b (GM7381), cells from a mosaic patient lane c (RJK2368). Molecular weight of upper band indicated on the left.
METHODS. Polyclonal antibodies were raised in rabbits against a GST (glutathione S-transferase)-*FMR1* fusion protein obtained using the pGEX plasmid expression system[9]. A cDNA fragment containing nucleotide 940 to 1,325 of the coding and 3,557-3,765 of the non-coding sequence of the previously published *FMR1* cDNA[3] was cloned in the *EcoRI* site of pGEX-3X. Expression of the construct in the protease-deficient *E. coli* strain B121 resulted in the synthesis of a *FMR1* polypeptide of 134 amino acids fused with the C-terminus of Sj26, a 26K glutathione S-transferase. The *FMR1* polypeptide consisted of amino acids 314 to 442 of the *FMR1* gene plus five additional amino acids (Cys-Thr-His-His-Leu) as a result of the cloning procedure. Expression and purification of the fusion protein were as described[9]. Antibodies were affinity purified using columns of GST and (GST)-*FMR1* fusion protein successively. Pellets of lymphoblastoid cell lines were homogenized in a buffer containing 10 mM HEPES, 300 mM KCl, 100 μM $CaCl_2$, 5mM $MgCl_2$, 0.05% Tween and 0.45% Triton X100, pH 7.4. Cell homogenates were spun down for 10 min (10,000g). The supernatants were incubated with protein A-Sepharose (Pharmacia) for 2 h at 4°C to remove the IgG present in the lymphoblastoid cells. Protein A-Sepharose was spun down and the supernatant was incubated overnight with α765 and protein A-Sepharose at 4°C. After washing (3x) the immunoprecipitate, sample buffer was added and the precipitates were electrophoresed in SDS-polyacrylamide (8% polyacrylamide gel, 1% crosslinking) and electroblotted. Polypeptides were visualized using 1,000x diluted α765 and a 1,000x diluted alkaline phosphatase-conjugated goat anti-rabbit IgG as a second antibody. Polypeptides were detected using naphthol AS-MX phosphate and 4-aminodiphenylamine diazonium sulphate or AMPPD (TROPIX) as substrates.

The finding of four protein products rather than one was unexpected, and the possibility was envisaged that this could be due to alternative splicing. Figure 2 shows that there were several *FMR1* mRNA species in lymphoblastoid cells and fetal brain. The set of primers used in the polymerase chain reaction (PCR) were chosen from near the 3' end of the open reading frame and amplified at least four splice products, and we have evidence for an even higher number of splice variants[11]. Several of these splice variants have been found in isolated cDNAs.

Two of the *FMR1* cDNA clones representing the alternative splice variants psf-1 and psf-2 (Fig. 2) were cloned in expression vector pSG5 (ref. 12) and these constructs were transiently expressed in COS-1 cells. Both the larger and the smaller construct seemed to encode stable

Figure 2.



Different splice products from the 3' end of the *FMR1* gene visualized by reverse transcriptase PCR amplification of cDNA. Total RNA was isolated from leucocytes (lane a) and fetal brain (lane b)[14]. Alternative splicing involved exons 15 and 17 of *FMR1* (D. L. Nelson, personal communication). The lengths of the PCR products were 563, 512, 488 and 437 bp. Length of DNA marker (lane M: factor VIII; Boehringer) bands are indicated in base pairs. The different splice variants shown were found to be present in isolated cDNAs (data not shown).
METHODS. Total RNA isolation was according to ref. 14. The LiCl method was used (procedure C) with several modifications. After overnight incubation in 3M LiCl/6M urea, samples were spun down for 20 min at 25,000 r.p.m. at 4°C and treated with proteinase K (10 μg ml^{-1}) for 30 min at 37°C before extraction with phenol/chloroform. Five μg RNA was reverse transcribed as described in ref. 8, except that instead of precipitating the cDNA, 2 μl was directly used for PCR. PCR was done on 2 μl of cDNA solution with the primer set K7 and K8. Primer K7: 5' GCTAGTTCTAGACCACCACCAAAT 3' and primer K8: 5'TTAGGGTACTCCATTCACGAG 3' were derived from positions 1,462-1,485 and 1,954-1,974 of the published *FMR1* cDNA sequence[3]. Amplification and analysis of PCR products was done as described[15].

proteins that were recognized by antibodies α765 (Fig.3, lane a and b) and α1079 (data not shown). The estimated M_rs were 74 K and 67 K for the proteins encoded by psf-2 and psf-1, respectively. No protein product was detected in mock-transfected COS-1 cells or in COS-1 cells transfected with *FMR1* cDNA cloned in PSG5 in the antisense orientation (Fig.3, lanes c and d).

The intracellular localization of the protein products of these different splice variants was investigated in COS-1 cells. It was shown by immunofluorescence microscopy using α765 antibodies that the proteins encoded by psf-2 was to be found in the cytoplasm, despite of the presence of a putative nuclear localization signal (Fig.4). The results were the same with the protein encoded by psf-1 and antibodies α1079 raised against a synthetic peptide spanning the nuclear localization signal. This observation was confirmed in mouse oesophagus, which is known to strongly express *FMR1* (ref. 13). FMRP was seen in the cytoplasm of the keratinocytes throughout the thick stratified squamous epithelial lining, with no staining of the keratin layer (Fig. 4c). Some cells showed an intense nuclear labelling.

Until now there has been uncertainty as to whether the CGG repeat is translated into protein Our experiments (Fig. 1) on the expression of the *FMR1* gene in lymphoblastoid cell lines show that the protein products of the controls and the mosaic patient are of the same size. Mosaic patients have a full mutation (>200 repeats) in a proportion of their cells and a premutation

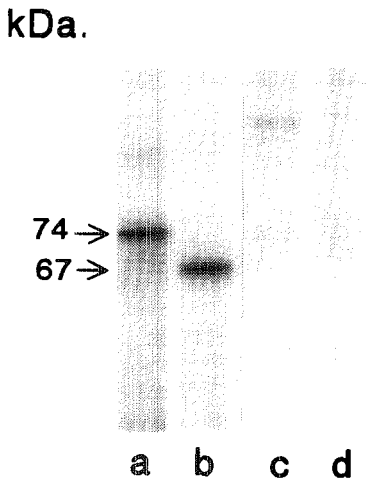


Figure 3.
Immunoprecipitation of COS-1 cells transfected with *FMR1* (psf-2, lane a), or an alternative splice variant of *FMR1* (psf-1, lane b). Immunoprecipitation of mock-transfected COS-1 cells or COS-1 cells transfected with *FMR1* cloned in pSG5 (ref. 12) in the antisense direction are shown in lanes c and d, respectively. The molecular weight of the immunoprecipitated proteins is indicated on the left. COS-1 cells were labelled for 2 h with ^{35}S-methionine and ^{3}H-leucine, 72 h after transfection. Cells were homogenized and used for immunoprecipitation with polyclonal antibody α765 raised against a fusion protein of glutathione S-transferase.
METHODS. The *FMR1* cDNA was cloned in the *EcoRI* site of the eukaryotic expression vector pSG5 (ref. 12) either as a 3,765-bp cDNA[3] (psf-2) or as a 3,690-bp *FMR1* splice variant missing in the 3' end of the 75-nucleotide coding sequence (1,598-1,672) but leaving the reading frame intact (psf-1). Transfections were achieved using the DEAE-dextran method[16].

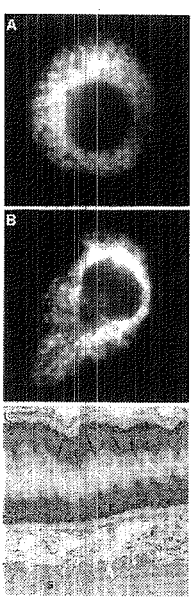


Figure 4 .
Cytoplasmic localization of FMRP in transfected COS-1 cells using an indirect immunocytochemical method[17]. The α765 antibodies were visualized using goat anti-rabbit immunoglobulins conjugated with fluorescein. a, COS-1 cells transfected with psf-1. b, COS-1 cells transfected with psf-2. As a control, non-transfected COS-1 cells were used. c, Cryostat section (6μm) of the oesophagus from an adult mouse incubated with polyclonal antibody α765, raised against fusion protein. Reaction product (dark grey staining) is seen in the cytoplasm of the cell, forming a thick stratified squamous epithelium, and not in the keratin layer. No product was seen using preimmune sera. Some cells show an intense labelling of the nucleus. E, stratified squamous epithelium; S, skeletal muscle; arrows indicate nuclei labelled for FMRP.
METHODS. For immunohistology cryostat sections were labelled with primary antibody and visualized with peroxidase-conjugated, swine anti-rabbit immunoglobulins (DAKO, Denmark) Peroxidase was detected with 0.1% 3,3-diaminobenzidine-HCl (Sigma) with 0.1% hydrogen peroxide. Sections were not counterstained.

(50-200) in others. The premutation allele is transcribed, unlike in the full mutation[8]. In this patient the premutation allele contains 65 CGG copies more than the average control. If the CGG repeat were translated into a stretch of arginines, then protein products with an increased molecular mass would have been expected in the mosaic patient. As this was not found (Fig. 1B), it can be concluded that the translation of *FMR1* starts distal to the CGG repeat. Expression of psf-1 and psf-2 in COS-1 cells showed that translation can start at an ATG initiation codon distal to the CGG repeat as no ATG codon is present proximalof the CGG repeat in the open reading frame of the *FMR1* gene (S. T. Warren, personal communication). Considering these data together, it is unlikely that the CGG repeat is part of the coding sequence.

In fragile X syndrome the amplification of the CGG repeat blocks transcription of the *FMR1* gene[8] and this results in the absence of FMRP (Fig. 1) and causes mental retardation. The mosaic patient is mentally retarded despite the expression of *FMR1* proteins. It is therefore assumed that the percentage of cells expressing FMRP is insufficient to maintain normal function. Alternatively, it could be that the mosaic pattern found in lymphoblastoid cell lines is not representative of the situation either in other tissue (like the brain) or during developmental stages in which the FMRP expression is essential.

With respect to the further elucidation of the function of the FMRPs it is important that the protein is predominantly present in the cytoplasm. Our results will make it possible to isolate the FMRPs, characterize them further, and study their putative differential function. This could provide insight into the mechanism leading to the manifestations of the fragile X syndrome.

Acknowledgements

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References

1. Gustavson, H.K., Blomquist, H. & Holgrem, G. *Am. J. med. Genet.* **23**, 581-588 (1986).
2 Webb, T.P., Bundey, S.E., Thake, A.I. & Todd, J. *Am. J. Med. Genet.* **23**, 573-580 (1986).
3. Verkerk, A.J.M.H. *et al Cell* **65**, 905-914 (1991).
4. Oberlé,I. *et al Science* **252**, 1097-1102 (1991).
5. Yu, S. *et al Science* **252**, 1179-1181 (1991).
6. Fu, Y.-H. *et al Cell* **67**, 1047-1058 (1991).

7. Heitz, D. *et al.. Science* **251**, 1236-1239 (1991).
8. Pieretti, M. *et al. Cell* **66**, 817-822 (1991).
9. Smith, D.B. & Johnson, K.S. *Gene* **67**, 31-40 (1988).
10. Merrifield, R.B. *J. Am. Chem. Soc.* **85**, 2149-2154 (1963).
11. Verkerk, A. J. H. M. *et al. Hum. molec. Genet.* **2**, 399-404 (1993)
12. Green, S., Isseman & I. Sheer, E. *Nucleic Acid Res.* **16**, 369 (1988).
13. Hinds, H., *et al.. Nature Genet.* **3**, 36-43 (1993).
14. Auffray, C. & Rougeon, F. *Eur. J. Biochem.* **107**, 303-314 (1980).
15. DeBoulle, K. *et al. Nature Genet. in press.*
16. Oshima, A. *et al. J. Biol. Chem.* **263**, 2553-2562 (1988).
17. Van Dongen, J. M., Barneveld, R.A., Geuze, H.J & Galjaard, H. *Histochem. J.* **16**, 941-954 (1984).

Publication 5.2

Fmr1 knockout mice: a model to study fragile X mental retardation

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Summery

Male patients with fragile X syndrome lack *FMR1* protein, due to silencing of the *FMR1* gene by amplification of a CGG repeat and subsequent methylation of the promoter region. The absence of *FMR1* protein leads to mental retardation aberrant behavior, and macroorchidism. Hardly anything is known about the physiological function of *FMR1* and the pathological mechanisms leading to these symptoms. Therefore, we designed a knockout model for the fragile X syndrome in mice. The knockout mice lack normal *Fmr1* protein and show macroorchidism, learning deficits, and hyperactivity. Consequently, this knockout mouse may serve as a valuable tool in the elucidation of the physiological role of *FMR1* and the mechanisms involved in macroorchidism, abnormal behavior, and mental retardation.

Introduction

The fragile X syndrome is the most frequent form of inherited mental retardation in humans, with an incidence of 1 in 1250 males and 1 in 2500 females (reviewed by Oostra *et al.*, 1993). The clinical syndrome includes moderate to severe mental retardation, autistic behavior, macroorchidism, and facial features such as long face with mandibular prognathism and large, everted ears (Hagerman, 1991). The gene involved in the fragile X syndrome (*FMR1*) is located in Xq27.3, a region that cytogenetically displays a fragile site. The molecular basis for this disease is a large expansion of a triplet repeat $(CGG)_n$ in the 5' untranslated region of the *FMR1* gene (Verkerk *et al.*, 1991; Oberlé *et al.*, 1991; Yu *et al.*, 1991; Fu *et al.*, 1991). In the normal population, this CGG repeat is polymorphic, with repeat length ranging from 6-53 units (Fu *et al.*, 1991). Carrier males (normal transmitting males) and carrier females show a repeat length of 43-200 CGGs (premutation) and are asymptomatic. The full mutation is characterized by a large repeat containing over 200 CGGs. As a result of repeat amplification, the *FMR1* promoter and the CGG repeat itself become methylated, leading to silencing of transcription and translation of the *FMR1* gene (Pieretti *et al.*, 1991; Verheij *et al.*, 1993). Males with a full mutation are affected, and 50-70 % of the females with a full mutation allele show mild to moderate mental impairment (Rousseau *et al.*, 1991).

The *FMR1* protein shows hardly any homology to other known proteins, and little is known about its function. Recently, RNA binding studies have shown that *FMR1* protein is able to bind its own messenger as well as 4% of the human fetal brain mRNAs (Ashley *et al.*, 1993b). Two types of domains that are known to bind RNA, two KH domains and a RGG box, have been identified in *FMR1* protein (Siomi *et al.*, 1993; Ashley *et al.*, 1993b).

The *FMR1* gene is highly conserved among species (Verkerk *et al.*, 1991) and the murine

homologue *Fmr1* shows 97% homology in amino acid sequence (Ashley *et al.*, 1993a). The expression pattern of *FMR1* at the mRNA and protein level is very similar in different tissues of humans and mice (Hinds *et al.*, 1993; Bächner *et al.*, 1993a, 1993b; Devys *et al.*, 1993; Abitbol *et al.*, 1993), which makes the mouse a good animal model in which to study the fragile X syndrome.

To study the function of *FMR1* in the development of human mental retardation, we developed a mouse in which the *Fmr1* gene is inactivated. These knockout mice lack normal *Fmr1* RNA and protein and show enlarged testes, impaired cognitive function, and aberrant behavior. This animal model might shed light on the function of *FMR1* and the pathophysiologic mechanisms that lead to mental retardation and behavior abnormalities in humans.

Results

Generating a mouse containing an inactivated *Fmr1* gene

Mutant *Fmr1* mice were generated by homologous recombination of a targeting vector into the mouse germline using the embryonic stem (ES) cell technology (Mansour *et al.*, 1988). We constructed a homologous recombination targeting vector, pMG5, containing 5.7 kb of mouse genomic *Fmr1* DNA (Figure 1A). Exon 5 was interrupted by the positive selection marker gene neomycin (*neo*). The negative selection marker gene thymidine kinase (*tk*) was inserted 3' of the genomic sequence in the polylinker of the vector. Vector pMG5 was introduced in E14 ES cells and selection for homologous recombination events was performed using medium containing G418 and ganciclovir. A clone (CB5.2) found to be positive for a homologous recombination event by polymerase chain reaction (PCR) (Figures 1A and B) and Southern blotting (data not shown) was injected into C57BL/6J blastocysts and transferred to pseudopregnant females. Twelve highly chimeric males were crossed with C57BL/6J wild type females for determination of germline transmission. Two transmitted the ES cell genome to part of their offspring and three showed 100 % transmission of the ES cell genome (all females contained the knockout allele). On the DNA level, the presence of the knockout allele in these female mice could be confirmed by PCR analysis (Figure 1B) and Southern blot hybridization (data not shown), similar as used for screening the ES cell clones. F1 heterozygous females were crossed with C57BL/6J wild type males to obtain knockout males and with the chimeric males to obtain females homozygous for the knockout allele. PCR analysis showed the absence of the wild-type allele in these mutants (Figures 1A and 1B). The male knockouts were shown to be negative for proper *Fmr1* RNA in their testes by reverse transcription (RT)-PCR (Figures 1A and C) and to be negative for *Fmr1* protein in their testes, kidney, liver (data not shown) and brain (Figure 1D) by Western blotting using antibodies raised against a *FMR1* fusion protein.

Figure 1.

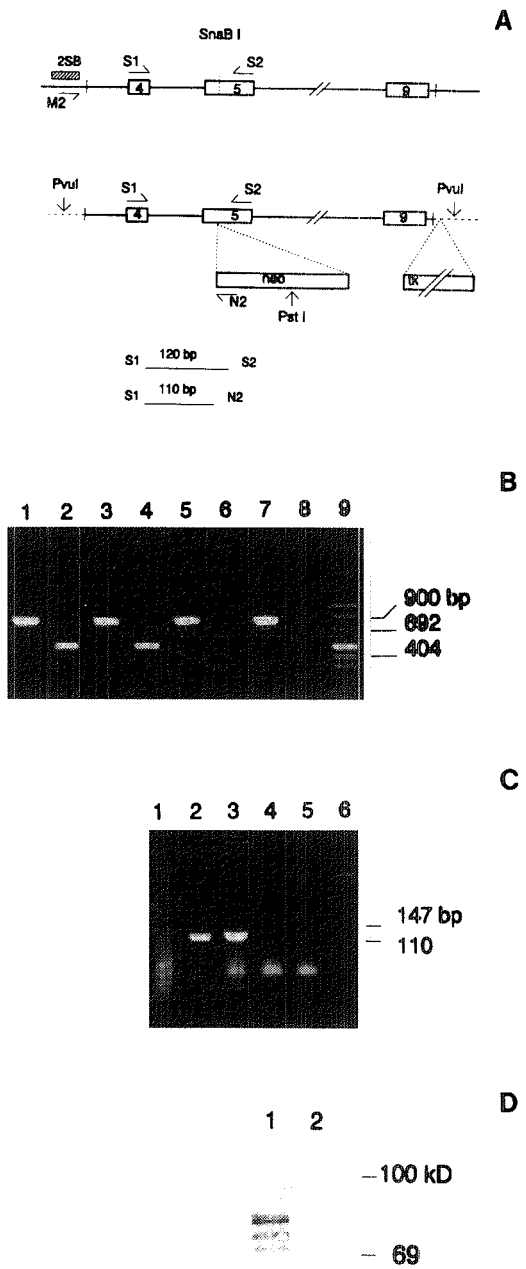
Construction and characterization of the *Fmr1* knockout mice

(A) A schematic drawing of the genomic organization of the *Fmr1* locus (upper part) and a schematic drawing of targeting vector pMG5 (lower part). The genomic sequence is interrupted at the SnaBI site of exon 5 by a *neo* cassette. The *tk* cassette is depicted 3' of the genomic sequence. Primer M2, N2, S1 and S2, probe 2SB and the PstI sites (close to each other) are indicated.

(B) PCR analysis of ES clone CB5.2 (lane 1) and tail DNA from a wild-type mouse (lane 2), a heterozygous female (lanes 3 and 4), a mutant male (lanes 5 and 6) and a mutant female (lanes 7 and 8). Primer M2 and N2 were used to detect the ~800 bp fragment of the knockout allele (lanes 1, 3, 5 and 7) and primer S1 and S2 were used to detect the 465 bp fragment of the wild type allele (lanes 2, 4, 6, and 8). Lane 9 contains a size marker.

(C) RT-PCR analysis of a wild-type (lanes 1 and 3) and a knockout (lanes 2 and 4) mouse testis using primers S1 and N2 (lanes 1 and 2) producing a 110 bp fragment in the mutant testis, and primer S1 and S2 (lanes 3 and 4) producing a 120 bp fragment in the wild-type testis. Lane 5 contains a blank sample. Lane 6 shows a size marker.

(D) Western blot analysis of *Fmr1* expression in brain of a wild type (lane 1) and a knockout (lane 2) mouse showing protein bands between 67 and 74 kD only present in the control mouse.



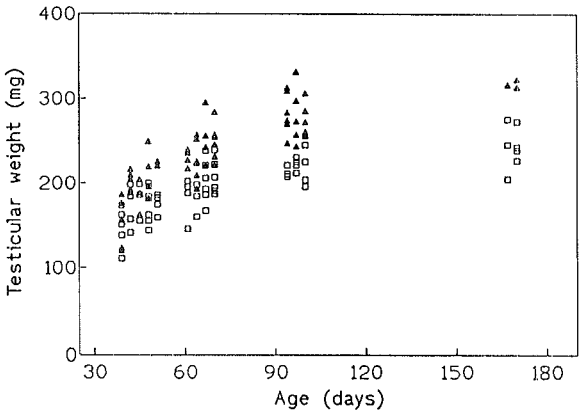


Figure 2.
Combined testicular weight of both testes of knockout mice and control mice in time. Knockout mice are shown by closed circles and control mice by open circles. The data are derived from 59 mutant mice and 59 normal littermates.

Reproductive fitness

As it has been suggested that *FMR1* is essential in gametogenesis (Bächner *et al.*, 1993a, 1993b), we studied the reproductive fitness of mutant mice. Crossings were performed between the knockout males and wild type females, and the homozygous knockout females and wild-type males in order to test the fertility of the mutants. We did not detect a negative selection against germ cells with a mutant *Fmr1* gene at any stage in the breeding experiments. Heterozygous females and both male and female knockout mice were fertile and had normal litter sizes with the expected distribution of offspring with the mutant allele. The transmission of the mutant *Fmr1* gene corresponded well to the expected Mendelian segregation ratios of an X chromosomal gene (data not shown). The observation that both male and female knockout mice without any protein expression are fertile and have the same size of progeny as controls indicates that *Fmr1* is not necessary for spermatogenesis and oogenesis in mice. Furthermore, mutant mice appear perfect viable, the oldest knockouts having lived over 250 days.

Phenotype, major neurological functions and behavior

Mice lacking *Fmr1* do not exhibit an overt phenotype, but ascertainment of physical features in mice is impaired by a lack of appropriate measurement systems. Macroorchidism is one of the key features of the fragile X syndrome, present in more than 90% of adult males (Turner *et al.*, 1980), and is thought to develop gradually through time. Therefore, we compared testicular weight of knockout and control mice at four different ages. Testes of knockout mice were significantly heavier than control testes in all four age groups, and the difference became progressively more significant through time (Figure 2; Table 1). Total weight in three age-matched groups and the weight of different organs including kidney, heart, spleen, and liver were not significantly different ($P>0.05$) from that of control littermates (data not shown).

The major neurological functions of the mutant mice including gait, grooming, circadian

Table 1. Testicular Weight [a] of Mutant Mice Versus Control Littermates at Different Ages

Age (days)	Mutant Mice	Control Mice	P Value
40-50	190 ± 8 (n = 19)	168 ± 5 (n = 22)	0.02 < P < 0.05 (t = 2.3)
60-70	239 ± 5 (n = 21)	196 ± 5 (n =19)	P < 0.001 (t = 5.6)
90-100	280 ± 6 (n = 16)	219 ± 6 (n= 11)	P < 0.001 (t = 7.11)
160-170	319 ± 5 (n = 3)	244 ± 9 (n = 7)	P < 0.001 (t = 7.12)

[a] Testicular weight is taken as the combined weight of both testes.

activity, swimming, feeding and mating behavior were normal. It is difficult, however, to recognize minor neurologic abnormalities in mice. Also, in human fragile X patients, neurological dysfunction is confined to minor abnormalities such as slight hypotonia, hyperactive deep tendon reflexes, extensor plantar responses, clumsy or sluggish gait, and persistence of pinch synkinesia (Finelli *et al.*, 1985; Vieregge and Froster-Iskenius, 1989; Wisniewski *et al.*, 1991).

Pathological examination

Light microscopic examination of kidney, heart, spleen, liver, and lung of mutant and normal mice revealed no abnormalities (data not shown). This is consistent with the absence of abnormalities of these organs in human fragile X patients (Hagerman, 1991). Special attention was paid to the brain and testes, as both organs are anatomically and functionally abnormal in human fragile X patients.

Histological studies of human fragile X testes have revealed limited abnormalities: testicular enlargement seems to be due to interstitial edema or increased amount of interstitial tissue (Turner *et al.*, 1975). However, microscopic examination of the testes of mutant mice reveled no structural difference as compared with controls, including a normal pattern of tubule size, a normal amount of interstitial mass, and normal spermatogenesis (Figure 3). No difference was fond between the ovaries of control animal and homozygous knockout female mice (data not shown).

Brain weight of seven mutant mice (462 ± 10 mg) was not significantly ($p>0.05$) different from that of five age-matched (all mice were between 60 and 70 days old) control littermates

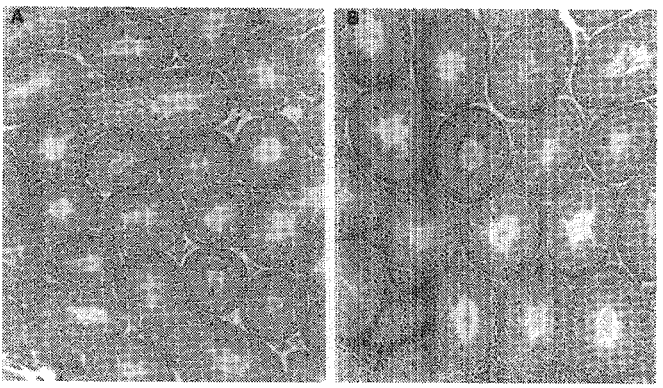


Figure 3.
Light microscopic micrographs of paraffin sections of testis normal (A) and mutant (B) mice (117 x). There is no difference in morphology between normal and mutant mice.

(467 ± 15 mg). The brains of eight knockout and four wild-type littermates were macroscopically and microscopically normal. The following structures were examined and were found to be normal by light microscopy: frontal, temporal, and occipital cortices, striatum, corpus callosum, hippocampus, third and lateral ventricles, plexus choroideus, hypothalamus, cerebellum with nucleus dentatus, fourth ventricle, and brain stem. The cortex also had normal numbers of pyramidal neurons and lamination. There was normal myelination of the white matter. Immunohistochemistry showed a normal number of astrocytes, with glial fibrillary protein immunoreactivity in the glia limitans and the white matter. Figure 4A and 4B illustrate a cresyl violet staining of normal and mutant brain. We concentrated our pathological survey on hippocampus and cerebellum as the neurons in these structures have the highest expression of *FMR1* (Abitbol *et al.*, 1993; Hinds *et al.*, 1993). To study eventual pathological abnormalities in or near the hippocampal pyramidal cells we stained coronal sections of the hippocampus with neuron specific enolase (NSE) antibody (as shown in Figures 4C and 4D) and neurofilament-M antibody (Figures 4E and 4F). The gross architecture and the CA1-CA3 pyramidal and other neurons of the hippocampus were normal. All cerebellar layers were unremarkable, including the molecular and inner granular layer. As the Purkinje cell layer of the cerebellum also shows high *FMR1* expression, we stained sagittal sections of the cerebellum with antibodies against L7 and neurofilament-M (Figure 4G and 4H), but could not detect any abnormality in the mutant mice. Also, the number of cerebellar Purkinje cells in mutant mice (32 ±1 mm^{-2}; n = 6) was not significantly different from that in control littermates (30 ± 1 mm^{-2}; n = 3).

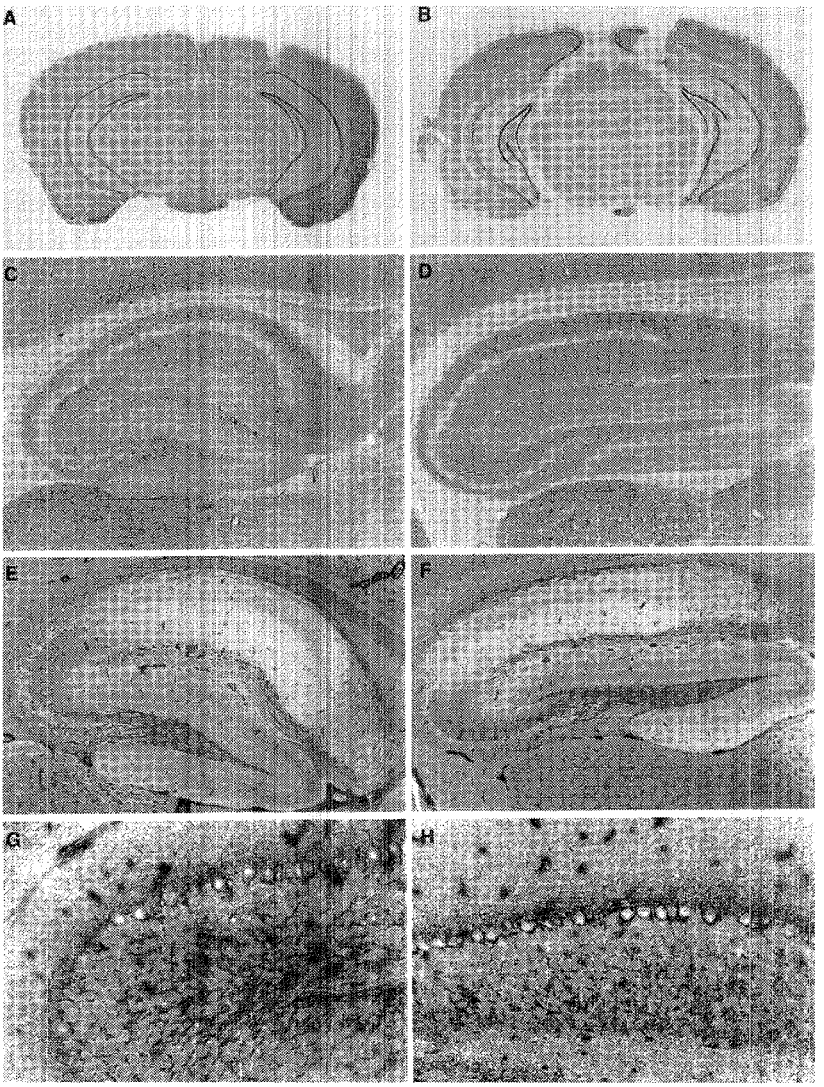


Figure 4.
Light microscopic micrographs of cryostat sections from brain. Normal (A, C, E, and G) and mutant (B, D, F, and H) mice. (A) and (B) (6.8 x) show an overview of coronal sections stained with cresyl violet; (C) and (D) (34 x) are sagittal sections of hippocampus immunostained for neuron-specific enolase; (E) and (F) illustrate sagital sections of hippocampus labeled for neurofilament-M; (G) and (H) (102 x) show sagittal sections of cerebellum incubated with antibodies against neurofilament-M. Note that for all parameters studied no difference could be found between normal and mutant mice.

Behavioral tests

The behavior of the knockout mice seemed normal. The mice appeared to have normal social interaction with littermates and human investigators. General assessment of the behavioral state of the mice according to Irwin (1968) was normal, but detailed social interaction studies are being performed now. Behavioral tests of male knockout and male control mice presented here include the passive avoidance task, the exploratory behavioral test, and the motor activity test.

Passive avoidance task

In this task, mice are placed in a brightly lit compartment of a step-through box. Upon entrance into the dark compartment of the box, they receive an electric shock. The passive avoidance task measures the latency of the mice to enter the dark compartment 24 h after initial exposure to the shock. Knockout mice performed like normal controls in this test as the mean entrance latency of 11 mutant males (241 ± 34 s) was not significantly different from that of 12 normal littermates (247 ± 29 s).

Exploratory behavior test

An exploratory behavior test was performed with 15 knockout mice and 16 normal littermates in a two chambered light-dark transition design. In this test, movement in and between two compartments (one lit and one dark) of a box was monitored for 10 min by infrared beams (Figure 3A).

The difference in time spent in the lit compartment was not statistically significant between mutant mice and control littermates (Figure 5B). However, knockout mice did display much more line crossings in the lit compartment than normal littermates. Both the first and the second beam showed a significant difference (two-tailed Student's t test, $p < 0.01$) between the two groups (Figure 5C). Thus, this test revealed that mutant mice display significantly more exploratory behavior than their normal littermates.

Motor activity test

In this test, the activity of mutant versus control mice was monitored by counting the number of crossings through three infrared beams in an empty cage over 40 min after placing the mice in the cage. Five mutant mice (310 ± 16 crossings) showed significantly ($p = 0.002$) more crossings than five control littermates (127 ± 31 crossings).

Cognitive function analysis

Specific cognitive defects in human fragile X males include deficits in visual short-term memory, and visual-spatial abilities (Cianchetti *et al.*, 1991; Maes *et al.*, 1994). Since its first

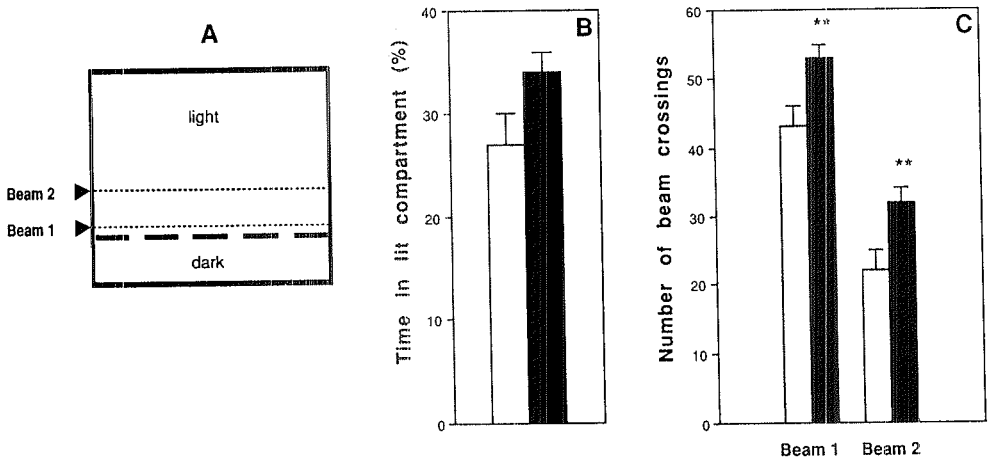


Figure 5.
Differences in exploratory behavior between knockout mice and normal littermates.
Mean values and SEM are shown. (A) Schematic drawing of the box used in the two chambered light-dark transition test. Dashed lines indicate the position of the two infrared beams through the lit compartment. (B) Comparison between mutant mice (filled box) and normal litter mates (open box) for percentage of time spent in the lit compartment, and for number of beam crossings in the lit compartment (C). Asterisks indicate statistical differences between the mutant and age-matched normal littermates (one asterisk, $0.01<p<0.05$; two asterisks, $0.001<p<0.01$; three asterisks, $p<0.001$) calculated by a Students' t test.

description by Richard Morris (1981), the Morris water maze task has been used extensively to study spatial learning in small rodents. In the hidden platform condition of the task, animals are placed in a large circular pool filled with opaque water from which they must learn to escape by locating and climbing onto a platform hidden beneath the water surface. In the visible platform test, the platform is indicated by a clearly visible flag ad the mice do rely on proximal cues to find the platform.

We subjected our mutant mice and their normal littermates to the hidden platform condition of the Morris water maze task. During 12 learning trials, both knockout mice (one-factor ANOVA, effect of trial number: $F_{(11,156)} = 33.32$, $p = 0.0001$) and their normal littermates ($F_{(11,120)} = 19.93$, $p = 0.0001$) showed highly significant decrease of escape latency, and reached similar levels of performance in this task with a latency on the twelfth trial of, respectively, 11.3% and 11.6 % of the initial first trial value (Figure 6A). Although knockout mice did reach similar high levels of performance, the knockouts showed longer latencies in the initial training rials as compared with controls (two-factor ANOVA on the first four trials, effect of genotype: $F_{(1.92)} =$

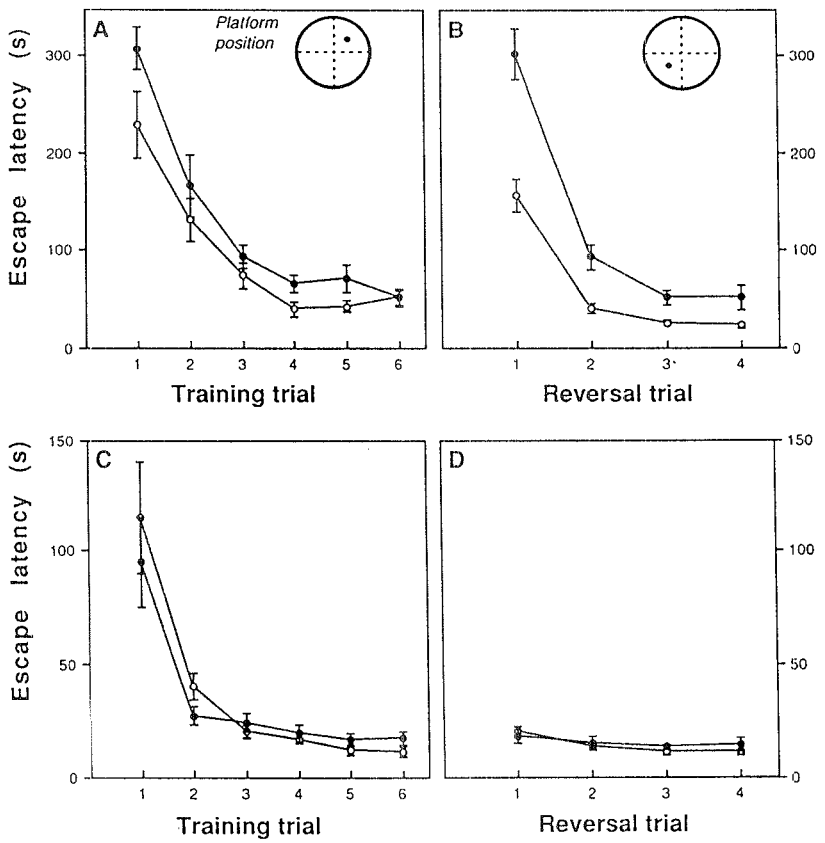


Figure 6.
Morris water maze performance of mutant mice and their normal litter mates.
Escape latency and SEM to find a hidden (A and B) or visible (C and D)platform in the Morris water maze were recorded. The escape latency presented here is the sum of four latencies recorded from each corner of the Morris water maze. Hidden platform test with mean escape latency and SEM for 14 male knockouts (filled circles), and 11 normal littermates (open circles) during six training trials (A) and four reversal trials (B). Visible platform test with mean escape latency and SEM for eight male knockouts (closed circles) and 10 control littermates (open circles) and four reversal trials (D). The position of the platform is indicated in the right corner of the graphs.

6.88, p = 0.01). However, as the interaction trial versus genotype was not significant, there is no difference in the rate of learning between bot groups in the training trial. However, when the position of the platform was changed during the reversal trials, mutant mice performed much worse than their normal littermates (two-factor ANOVA on four reversal trials, effect of genotype: $F_{(1,92)} = 37.67$, p= 0.0001; interaction effect trial X genotypes: $F_{(3,92)} = 7.49$, p = 0.0002) (Figure

6B). This indicates that during the reversal trial in the hidden platform condition the rate of acquisition was significantly lower in knockouts than in normal littermates.

Spatial memory was tested by probe trials performed after both the training trials and the reversal trials of the hidden platform condition of the Morris water maze task. If spatial memory is intact, mice spent more than the 25% chance level of their time in the target quadrant that contained the platform in the preceding trial of the hidden platform test. Both the knockouts and the control littermates performed well in the probe trials, and no significant differences ($p > 0.05$) were found. Therefore, both groups show an essentially normal spatial memory in the probe test. It is, however, possible that the knockouts are impaired under more stringent or selective measures of the probe test.

To exclude that the apparent impaired spatial learning in the hidden platform Morris water maze results from neurological or sensory deficits or lack of motivation, we conducted a visible platform Morris water maze test. In this condition of the task, the animals do not require the use of a mapping strategy to learn the platform position. Knockouts were not different from their normal littermates in the visible platform condition of the Morris water maze (Figures 6C and 6D). Latency to locate the visible platform in the training trials as well as in the rate of decrease of this latency were the same in knockouts and their normal littermates (two-factor ANOVA on the first four trials, effect of genotype : $F_{(1,64)} = 0.57$, not significant; interaction effect trial X genotype : $F_{(3,64)} = 0.46$, not significant). Neither were there differences between the latency curves in the reversal trials (two-factor ANOVA on four reversal trials, effect of genotype: $F_{(1,64)} = 1.12$, not significant; interaction effect trial X genotype : $F_{(3,64)} = 0.97$, not significant).

Discussion

In this study, we have produced knockout mice that have no proper *Fmr1* mRNA or protein, which is also the case in human fragile X syndrome. Also, at the clinical level, the knockout mice display abnormalities comparable to those seen in human patients. The combination of macroorchidism and mental retardation in the absence of gross pathological abnormalities of testes and brain is very typical for the fragile X syndrome and is also characteristic of the knockout mice.

Testicular weight of knock out mice at four different ages was significantly greater than that of the control littermates, and macroorchidism gradually develops through time in mutant mice. Also, human fragile X patients develop progressive macroorchidism after puberty (Thake *et al.*, 1985). Consistent pathological abnormalities in testes of human fragile X patients or male knockout mice have not been found, and the macroorchidism seems to be due to the an increase in size. The mechanism leading to macroorchidism, however, remains unclear. Nothing is known

about the function of *FMR1* in the testis. It has been suggested that expression of the *FMR1* gene is a prerequisite for a proper germ cell proliferation in the testis and in the ovary (Bächner *et al.*, 1993a, 1993b), but there is much controversy about the expression of *FMR1* in testes. Studies of RNA (Bächner *et al.*, 1993a, 1993b) and protein expression (Devys *et al.*, 1993) showed high expression of *FMR1* in male germ cells, whereas other studies of RNA expression suggested low expression in male germ cells (Abitbol *et al.*, 1993; Hinds *et al*, 1993). The present finding of normal fertility of both male and female mutant mice with no *Fmr1* expression indicates that *Fmr1* is not essential in reproduction, at least not in mice. *FMR1* is also not essential in human reproduction in view of the observation that a male fragile X patient without any *FMR1* RNA or protein due to a deletion in *FMR1* encompassing the promoter region has progeny (Meijer *et al.*, 1994). If *Fmr1* expression is not obligate for germline proliferation, the absent *Fmr1* protein in the germline of knockout mice cannot be responsible for the development of macroorchidism. This is in line with an earlier observation that fragile X males showing macroorchidism have only the premutation and not the full mutation in their sperm (Reyniers *et al.*, 1993), while their germline epithelium expresses *FMR1* (B. A. O., unpublished data). Since no fetal lethality or impaired viability is observed in mutant mice, *Fmr1* protein does not play a crucial role in development either.

No gross abnormalities have been described in the brain of human fragile X patients and knockout mice. We studied especially the hippocampus as this brain region is involved in learning and memory and shows high expression of *FMR1* . However, hippocampal abnormalities could not be detected. Reduced size of the posterior cerebellar vermis and pontine region, together with increased size of the fourth ventricle has been noted on magnetic resonance imaging of the brains of human fragile X patients (Reiss *et al.*, 1991a, 1991b). This has also been found in autistic behavior (Courchesne *et al.*, 1998), which is a common feature of fragile X syndrome (Brown *et al.*, 1986; Hagerman *et al.*, 1986). The size of the cerebellum and vermis of the knockout mice is currently being investigated. In autism not associated with fragile X syndrome, a reduction in the amount of cerebellar Purkinje cells has also been reported (Bauman and Kemper, 1985; Ritvo *et al.*, 1986), which may be responsible for the size reduction of the vermis (Murakami *et al.*, 1989). As Purkinje cells have high expression of *FMR1* (Abitbol *et al.*, 1993), we investigated the number of Purkinje cells in the murine cerebellum of the knockouts, but this was not significantly different from that of control littermates. In conclusion, the gross anatomy of the brain of mutant mice is normal.

Human fragile X patients are known to exhibit a more or less specific complex of behavior abnormalities consisting of social avoidance, unusual response to sensory stimuli, hyperactivity, and stereotypic, repetitive behavior in a pattern sometimes consistent with autism (Brown *et al.*, 1986; Hagerman *et al.*, 1986; Cohen *et al.*, 1988; Cianchetti *et al.*, 1991; Reiss and Freund, 1992; Fisch, 1992). Also, the mutant mice show abnormalities such as increased exploratory behavior

and motor activity. The increased exploratory activity shown by the mutant mice might indicate that slower and less efficient leaning of the environment, the result being an increased need to recheck the different chambers. On the other hand, it might also be the result of their hyperactivity.

Cognitive deficits in human fragile X males usually take the form of moderate to severe mental retardation with deficits in visual short-term memory and visual-spatial abilities (Cianchetti *et al.*, 1991; Maes *et al.*, 1994). As the *FMR1* expression is very high in the hippocampus (Abitbol *et al.*, 1993; Hinds *et al.*, 1993) and as the visual spatial disabilities of human fragile X patients might be related to hippocampal dysfunction, we studied the performance of mutant mice in the Morris water maze.

The hidden platform condition of the Morris water maze test shows that mutant mice reached equal levels of performance as controls in the training trials, which was also confirmed by their normal performance in the probe tests. However, when conditions of a well-trained task are changed in the reversal trials, knockout experience more difficulty than controls in learning these changes in spatial information. At this point, when the position of the platform is changed, the animals have mastered nonspatial aspects of the task (coping with learning stress, connecting the platform to escape, employing a motor praxis strategy, etc.), as well as spatial aspects (locating the platform with the use of distal cues). When the position of the platform is changed, mice are still able to use much of their knowledge of the nonspatial aspects of the task, and this may explain why normal mice display much shorter latency during the first reversal trial as compared with the first training trial. Since knockouts reach similar levels of performance as controls at the end of the training session, they seem to be as able as their normal littermates in mastering the nonspatial and spatial aspects of the task. Hence, knockouts do not seem to be impaired in the retrieval of spatial and nonspatial information in training and reversal trials once this information has been learned, but they are significantly impaired in their acquisition of the reversal task. This does not appear to be caused by impairment in their ability to change their nonspatial information scheme (e.g., changing their praxis strategy), since there was no difference between knockouts and controls in the visible condition the task. The latter also suggests that the increased latency of knockouts in the hidden platform condition is not caused by some underlying motivational, motor or sensory deficit. Hence, the observed impairment appears to be limited to the spatial abilities of the mouse. This might be connected to the observed hyperactivity and increased exploratory behavior that might be responsible for excessive early search behavior or interfering intertrial hyperactivity. Further research is warranted to learn more about the nature of the observed impairment and about the strategy knock out mice use to locate the hidden platform. In conclusion, the knockout mice provide a good model to study the complex combination of cognitive and noncognitive effects of the fragile X syndrome.

It is not clear what might be the underlying mechanism of this impaired acquisition of novel

spatial information in knockout mice. Impaired synaptic plasticity, especially in hippocampus, could be one such possible mechanism. The hidden platform condition of the Morris water maze task is dependent upon hippocampal functions as well as upon N-methyl-D-aspartate (NMDA) receptor-mediated long-term potentiation (LTP) of synaptic transmission (Morris *et al.*, 1982, 1986). More recently, two independent groups reported that mice with specifically those genes knocked out that code for kinases supposedly involved in the induction of the LTP process display impaired hippocampal LTP as well as impaired Morris water maze performance (Silva *et al.*, 1992a, 1992b; Grant *et al.*, 1992). It must be pointed out, however, that the latency curve profile of out knockout mice is qualitatively different from that of rodents with hippocampal lesions or impaired NMDA receptor functions. These animals show normal initial learning but remain clearly above the lower asymptote of the normal latency curve. Our *Fmr1* knockout mice, however, show relatively unimpaired initial spatial learning and retrieval of learned spatial information. Prominent difficulties only become apparent when conditions in a well-trained task are changed. To investigate whether *Fmr1* is involved in LTP, it is necessary to perform electrophysiological studies on hippocampal slices.

Learning, memory, and behavior are complicated processes involving different brain regions and many specific proteins. The biological analysis of cognitive functioning therefore requires identification of each of these proteins, before their overall interaction and functioning can be understood. Targeted disruption of specific genes is a powerful tool in the elucidation of the specific role of these genes in brain functioning. The knockout model for the *FMR1* gene presented here is a potentially valid model to provide insight into the physiologic function of *FMR1* and the pathophysiology of the fragile X syndrome, as the transgenic mice lack *Fmr1* protein and show abnormalities comparable to human fragile X patients. Therefore these mutant mice offer a good animal model to study the mechanisms leading to macroorchidism, abnormal behavior, and mental retardation. Furthermore, experimental designs can now be made to introduce foreign *FMR1* into the knockout mice in a first step towards gene therapy for fragile X syndrome in humans.

Experimental procedures

Construction of pMG5, ES cells, and transfection

The targeting vector pMG5 was composed of pBluescript KS(-) and a 5.7 kb mouse genomic DNA SalI-SpeI fragment containing exons 4 to 9 of the *Fmr1* gene. This fragment was obtained from a 129-derived ES cell phage library (kindly provided by Dr. G. Grosveld) after probing with a human *FMR1* cDNA sequence. The vector was interrupted with a neomycin resistance cassette, a 1645 bp blunted XhoI fragment obtained from pPGKneobpA (Soriano el al., 1991), in a blunted unique SnaBI site in exon 5. The neo cassette is antisense orientated compared with genomic orientation. A 2.9 kb blunted SalI fragment of the *tk* cassette (pHA140) (Clarke *et al.*, 1992) was inserted 3' of exon 9 in the blunted NotI site of the vector. The *tk* cassette is also

anti-sense orientated. Vector pMG5 was digested with PvuI to eliminate as much bacterial plasmid sequences as possible, and 15 to 20 µg of this DNA was used to transfect 1 x 10^7 E14 ES cells in 400 µl PBS using a Progenetor II Gene Pulser (1200 µF and 117 V during 10 ms). The treated cells were seeded onto 100 mm culture dishes coated with gelatine in medium composed of 60% BRL-conditioned medium, 40% DMEM supplemented with 1 U/ml LIF (Gibco BRL). The medium was replaced by medium containing 200 µg/ml G418 (Geneticin, Gibco) 24 hours after transfection. The medium was refreshed every day and during days 3 and 4 was also supplemented with 2 µM Gancyclovir (Cymevene). After 7 to 10 days, clones became visible, could be picked, treated with TE (0.05% trypsin, 0.02% EDTA in PBS) and seeded onto gelatine-coated 24-wells plates, one clone per well. After growing to confluency, clones were treated with TE again and 9/10 of the clone was frozen down slowly and stored in liquid nitrogen in medium containing 10% DMSO. One- tenth of each clone was spun down and resuspended in 30 µl water and 20 µl 20% Chelex-100 beads (BioRad) was added. The cells were boiled at 95 ^{0}C for 10 min to free the DNA. This solution (20µl) was used for PCR analysis. For Southern blot analysis, 1/10 of each clone was grown to confluency again and harvested, and genomic DNA was isolated from the cells.

DNA Analysis

PCR screening for a homologous recombination event was performed using 100 pmol of the primer M2 (5'-ATCTAGTCATGCTATGGATATCAGC-3') and N2 (5'-GTGGGCTCTATGGCTTCTGAGG-3') in a PCR buffer containing 10 mM Tris, 10 mM $MgCl_2$, 0.2 mM dNTPs and 2.5 U Taq DNA Polymerase (Bethesda Research Laboratories). The samples were preheated at 94 ^{0}C for 5 min. Thirty PCR cycles were performed composed of 1.5 min denaturation at 94 ^{0}C, 1.5 min annealing at 65 ^{0}C, and 2.5 min extension at 72 ^{0}C. The products (15 µl) were electrophoresed on a 1.5% agarose gel.

Generation and analysis of chimeric and knockout mice

The positive ES clone CB5.2 was injected into C57BL/6J blastocysts, and the blastocysts were transferred to pseudopregnant female mice. Highly chimeric males were crossed with C57BL/6J wild type females, and the offspring was tailed for DNA analysis after 2-3 weeks. Tail DNA was isolated according to Hogan *et al.* (1986) and 1 µl was used in the PCR with primer M2 and N2 (see above). Screening for the presence or absence of the wild type allele was performed using primer S1 (5'-GTGGTTAGCTAAAGTGAGGATGAT-3') and S2 (5'-CAGGTTTGTTGGGATTAACAGATC-3') (Figures 1A and B) in the PCR program described before with an annealing temperature of 63 ^{0}C instead of 65 ^{0}C.

RT-PCR

Total RNA was isolated from testes of mutant and control mice using the LiCl method (Verkerk *et al.*, 1993). RNA (5 µg) was reverse transcribed (Pieretti *et al.*, 1991), and 2 µl of the reverse transcribed reaction was directly subjected to PCR using combinations of the primers S1-N2 and S1-S2 according to the program described before with an annealing temperature of 63 ^{0}C. The PCR products (5 µl) were analyzed on a agarose gel (1.5% agarose and 1.5% low melting temperature agarose).

Western Blotting

Proteins were isolated from brain, testes, liver, and kidney from knockout and control mice. Tissue (200 mg)was homogenized in 750 µl of buffer (10 mM HEPES, 300 mM KCl, 5 mM $MgCl_2$, 100 µM $CaCl_2$, 0.45% Triton X100, 0.05% Tween [pH 7.4]) using a B-type pestle (5 x 2 strokes). The homogenates were sonificated for 45 s and spun down for 30 min at 10,000 x g. Seven mg was used in an immunoprecipitation with polyclonal antibodies α734 raised against the middle part of *FMR1* protein (Verheij *et al.*, 1993). Immunoprecipitations, gelelectrophoresis, and western blotting were performed as described by Verheij *et al.* (1993), with the exception that we used [^{125}I]protein A to detect the polypeptides.

Histochemistry
Twelve mice were killed by ether anaesthesia. Testes were fixed in Bouin's solution, and sections were stained with hematoxylin/azophloxine. Brains were fixed in 4% buffered formaldehyde, methacarn, and Bouin's solution. Two brains were snap frozen in liquid nitrogen chilled isopentane. Paraffin sections were examined blind with respect to the transgenic status of the mice. Sections were stained with cresylviolet, enolase, hematoxylin eosin, and Luxol fast blue. For immunohistochemistry, cryostat sections (7μm) were fixed with 4% paraformaldehyde followed by a methanol step. Subsequently, sections were incubated with monoclonal antibodies against human neuron-specific enolase (EPOS/NSE, DAKO), neurofilament-M (provided by Dr. J. Q. Trojanowski), or rabbit polyclonal antibodies against L7 (provided by Dr. Morgan), followed by an indirect immunoperoxidase technique using 3,3'-diazoniumbenzidine-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).

Passive avoidance learning
For passive avoidance learning, male mice were placed in the small (5 x 9 cm) brightly lit compartment of a step-through box. After 5 s, the door that led to the big (20 x 30 cm) dark compartment of the box was opened. Knockouts as well as littermates entered the dark compartment within 30 s. Upon entrance of the dark compartment, the sliding door was closed and the mice received a slight electric foot-shock (Coulbourn Instruments Small Animal Shocker, 0.2 mA, 1 s). After 24 hr, the mice were placed in the small compartment once more, and latency to enter the big compartment was noted up to a maximum of 300 s.

Exploratory behavior test
The apparatus used in this test consisted of an open box (55 x 75 cm), divided into an illuminated (45 x 75 cm) and a dark (10 x 75 cm) compartment. Transitions between these areas are enabled by means of four semicircular openings (4 cm diameter), evenly spaced (15 cm) in the dividing wall. Three infrared beams are aligned to the dividing wall. One of these is situated in the dark compartment (1 cm distance to the dividing wall), whereas the other two beams are in the illuminated compartment (1 and 7 cm distance to the dividing wall, respectively). Data from the three infrared detectors were continually read into a personal computer and analyzed off-line. Three measurements were derived from this: percentage of time spent in the lit compartment and number of crossings of each of the two beams in the lit compartment.
All tests were conducted between 1 and 5 p.m. in a quiet, dimly lit room. Animals were allowed to adapt to a reversed light-dark cycle (lights on at 5.30 p.m., lights off at 5.30 a.m.) for eight days prior to testing. Ten minutes before testing, they stayed separately in a uniform white plastic box. Then, they were placed in the dark compartment, and their activity was monitored during their 10 min stay in the box.

Motor activity test
Locomotor activity was measured with a technique modified from Crawley and Goodwin (1980). The apparatus used to measure the activity of the mice is an empty transparent cage (16 x 22 cm). Three infrared beams (two perpendicular to the long axis and one perpendicular to the short axis) divide the cage in six compartments. A personal computer interfaced to the three infrared sensors was used to count the number of crossings of the beams after the mice were placed in the cage over 40 min. All measurements were performed with lights on during the day phase of the light-dark cycle of the animals.

Morris water maze task
The water maze used here consisted of a circular grey plastic pool (150 cm in diameter, 30 cm high) filled with water made opaque by non-toxic opacified Lytron 621, and maintained at 18 ^{0}C. A round perspex platform (15 cm in diameter) was placed inside the pool in a fixed position at the center of one of the four quadrants. The platform was placed 1 cm below the water surface in the hidden platform test, but was clearly visible in the visible platform test. It remained in that position during 12 training trials, whereupon its position was changed to the center of the opposite quadrant for four reversal trials. Two drawings were put on the

wall behind the water maze as external cues and the interior of the experimental room was not changed during the trails. Only males, both knockouts and control littermates, were used in this experiment. Albino animals were not tested in the Morris water task as their performance might be impaired by visual abnormalities. During a swimming trial, the animals were placed in the pool four times, each time at one of the north, south, east, and west starting positions in random order, and with a 15 min interval. If the animals could not find the platform within the maximum swimming time of 120 s, they were placed on the platform and the maximal escape latency of 120 s was noted. They had to stay on the platform for 15 s before removal. Two swimming trials were performed daily, with a 90 min interval between trials. Significance of the effects of trial number and genotype were determined by one- and two-factor analyses of variance (ANOVA). During probe trials, mice had to swim for 100 s in the pool without a platform. Probe trial I, was performed after the first eight training trials and probe trial II after the four reversal trials of the hidden platform condition of the Morris water maze. A two-tailed Student's t test was used to assess the significance of differences between mutants and control littermates.

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References

Abitbol M., Menini C., Delezoide A.L., Thyner T., Vekemans M., and Mallet J. (1993). Nucleus basalis magnocellularis and hippocampus are the major sites of FMR-1 expression in the human fetal brain. Nature Genet. *4*, 147-153.

Ashley, C.T., Sutcliffe, J.S., Kunst, C.B., Leiner, H.A., Eichler, E.E., Nelson, D.L., and Warren, S.T. (1993a). Human and murine FMR-1: alternative splicing and translational initiation downstream of the CGG repeat. Nature Genet *4*, 244-251.

Ashley, C.T., Wilkinson, K.D., Reines, D., and Warren, S.T. (1993b). *FMR1* protein: conserved RNP family domains and selective RNA binding. Science *262*, 563-566.

Bächner, D., Steinbach, P., Wöhrle, D., Just, W., Vogel, W., Hameister, H., Manca, A., and Poustka, A. (1993). Enhanced *Fmr1* expression in testis. Nature Genet. *4*, 115-116.

Bächner, D., Mance, A., Steinbach, P., Wöhrle, D., Just, W., Vogel, W., Hameister, H., and Poustka, A. (1993). Enhanced expression of the murine *FMR1* gene during germ cell proliferation suggests a special function in both the male and the female gonad. Hum. Mol. Genet. *2*, 2043-2050.

Bauman, M., and Kemper, T.L. (1985). Histoanatomic observations of the brain in early infantile autism. Neurology *35*, 866-874.

Brown, W.T., Jenkins, E.C., Cohen, I.L., Fish, G.S., Wolf-Schein, E.G., Gross, A., Waterhouse, L., Fein, D., Mason-Brothers, A., Ritvo, E., Ruttenberg, B.A., Bentley, W., and Castells, W. (1986). Fragile X and autism: a multicenter survey. Am. J. Med. Genet. *23*, 341-352.

Cianchetti, C., Sannio Fancello, G., Fratta, A. L., Manconi, F., Orano, A., Pisschedda, M. P., Pruna, D., Spinicci, G., Archidiacono, N., Filippi, G. (1991). Neurophysiological, psychaitric, and physical amnifestations in 149 members from 18 fragele X families. Am. J. Med. Gen. *40*, 234-243.

Clarke, A.R., Robanus Maandag, E., van Roon, M,. van der Lugt, N.M.T., van der Valk, M., Hooper, M.L., Berns, A., and te Riele, H. (1992). Requirement for a functional RB-1 gene in murine development. Nature *359*, 328-330.

Cohen, I.L., Fish, G.S., Sudhalter, V., Wolf-Schein, E.G., and Hanson, D. (1988). Social gaze, social avoidance, and repetitive behavior in fragile X males: a controlled study. Am. J. Ment. Retard. *92*, 436-446.

Courchesne, E., Yeung-Courchesne, R., Press, G.A., Hesselink, J.R., and Jernigan, T.L. (1988). Hypoplasia of cerebellar vermal lobules VI and VII in autism. N. Engl. J. Med. *318*, 1349-1354.

Crawley, J and Goodwin, F. K., (1980). Prelimenary report of simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol. Biochem. Behav. *13*, 167-170.

Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P., and Mandel, J.L. (1993). The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nature Genet. *4*, 335-340.

Finelli, P.F., Pueschel, S.M., Padre-Mendoza, T., and O'Brien, M.M. (1985). Neurological findings in patients with the fragile X syndrome. J. Neurol. Neurosurg. Psychiatry *48*, 150-153.

Fisch, G.S. (1992). Is autism associated with the fragile X syndrome? Am. J. Med. Genet. *43*, 47-55.

Fu, Y-H., Kuhl, D.P.A., Pizzuti, A., Pieretti, M., Sutcliffe, J., Richards, S., Verkerk, A.J.M.H., Holden, J.J.A., Fenwick, R.G., Warren, S.T., Oostra, B.A., Nelson, D.L., and Caskey, C.T. (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell *67*, 1047-1058.

Grant, S.G., O'Dell, T.J., Karl, K.A., Stein, P.L., Soriano, P., and Kandel, E.R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science *258*, 1903-1910.

Hagerman, R.J., Chudley, A.E., Knoll, J.H., Jackson, A.W., Kemper, M., and Ahmad, R. (1986). Autism and fragile X females. Am. J. Med. Genet. *23*, 375-380.

Hagerman, R.J. (1991). Physical and behavioral phenotype. In: Fragile X syndrome: diagnosis, treatment and research. Hagerman R.J. and Silverman A.C. (Eds.). The Johns Hopkins University Press, Baltimore and London, pp. 1-68.

Hinds, H.L., Ashley, C.T., Sutcliffe, J.S., Nelson, D.L., Warren, S.T., Housman, D.E., and Schalling, M. (1993). Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. Nature Genet. *3*, 36-43.

Hogan, B., Costantini, F., and Lacy, E. (1986). Manipulating the mouse embryo; A Laboratory Manual, Cold

Spring Harbor University, pp. 174.

Irwin, S. (1968). Comprehensive observational assessments: Ia. A systematic. quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia *13*, 222-257.

Li, C-Y., Ziesmer, S. C., and Lazcano-Villareal, O. (1987). Use of azide and hydrogen peroxidase asn an inhibitor for endogenous peroxidase in the immunoperoxidase method. J. Histochem. Cytopchem. *35*, 1457-1460.

Maes, B., Fryns, J. P., Vanwalleghem, M., Vandenberghe, H. (1994). Cognitive functioning and information processin of adult mentally retarded men with fragile X syndrome. Am. J. Med. Genet. *50*, 190-200.

Mansour, S.L., Thomas, K.R., and Capecchi, M.R. (1988). Disruption of the proto-oncogene Int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selective genes. Nature *366*, 348-352.

Meijer, H., de Graaff, E., Merckx, D.M.L., Jongbloed, R.J.E., de Die-Smulders, C.E.M., Engelen, J.J.M., Fryns, J-P., Curfs, P.M.G., and Oostra, B.A. (1994). A deletion of 1.6 kb proximal to the CGG repeat of the *FMR1* gene causes the clinical phenotype of the fragile X syndrome. Hum. Mol. Genet. 3, 615-620.

Morris, R.G.M. (1981). Spatial localization does not require the presence of local cues. Learn. Motiv. *12*, 239-260.

Morris, R.G.M., Garrud, P., Rawlins, J.N., and O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. Nature *297*, 681-683.

Morris, R.G.M., Anderson, E., Lynch, G.S., and Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-Daspartate receptor antogonist, AP5. Nature *319*, 774-776.

Murakami, J.W., Courchesne, E., Press, G.A., Yeung-Courchesne, R., and Hesselink, J.R. (1989). Reduced cerebellar hemisphere size and its relationship to vermal hypoplasia in autism. Arch. Neurol *46*, 689- ??

Oberlé, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boué, J., Bertheas, M.F., and Mandel, J.L. (1991). Instability of a 550 base pair DNA segment and abnormal methylation in Fragile X syndrome. Science *252*, 1097-1102.

Oostra, B.A., Willems, P.J., and Verkerk, A.J.M.H. (1993). Fragile X syndrome: a growing gene. In Genome Analysis: Genome Mapping and Neurological Disorders, Volume 6, K. E. Davies and S. M. Tilghman, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 45-75.

Pieretti, M., Zhang, F., Fu, Y-H., Warren, S.T., Oostra, B.A., Caskey, C.T., and Nelson, D.L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. Cell *66*, 817-822.

Reiss, A.L., and Freund, L. (1992). Behavioral phenotype of fragile X syndrome: DSM-III-R autistic behavior in male children. Am. J. Med. Genet. *43*, 35-46.

Reiss, A.L., Aylward, E., Freund, L.S., Joshi, P.K., and Bryan, R.N. (1991a). Neuroanatomay of fragile X syndrome: the posterior fossa. Ann. Neurol. *29*, 26-32.

Reiss, A.L., Freund, L., Tseng, J.E., and Joshi, P.K. (1991b). Neuroanatomy in fragile X females: the posterior fossa. Am. J. Hum. Genet. *49*, 279-288.

Reyniers, E., Vits, L., De Boulle, K., Van Roy, B., De Graaff, E., Verkerk, A.J.M.H., Darby, J.K., Oostra, B.A., and Willems, P.J. (1993). The full mutation in the FMR-1 gene of fragile X patients is absent in their sperm. Nature Genet *3*, 143-146.

Ritvo, E.R., Freeman, B.J., Scheibel, A.B., Duong, T., Robinson, H., Guthrie, D., and Ritvo, A. (1986). Lower Purkinje cell counts in the cerebella of four autistic subjects: initial findings of the UCLA-NSCA Autopsy Research Report. Am. J. Psychiatry *143*, 862-866.

Rousseau, F., Heitz, D., Biancalana, V., Blumenfeld, S., Kretz, C., Boué, J., Tommerup, N., van der Hagen, C., DeLozier-Blanchet, C., Croquette, M.F., Gilgenkrantz, S., Jalbert, P., Voelckel, M.A., Oberlé, I., and Mandel, J.L. (1991). Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. New. Engl. J. Med. *325*, 1673-1681.

Silva, A.J., Stevens, C.F., Tonegawa, S., and Wang, Y. (1992a). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science *257*, 201-206.

Silva, A.J., Paylor, R., Wehner, J.M., and Tonegawa, S. (1992b). Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science *257*,206-211.

Siomi, H., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1993). The protein product of the fragile X gene, *FMR1*, has characteristics of an RNA-binding protein. Cell *74, 291-298.*

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteoporosis in mice. Cell *64*, 694-702.

Thake, A., Todd, J., Bundey, S., and Webb, T. (1985). Is it possible to make a clinical diagnosis of the fragiel X syndrome in a boy? Archives Disease in Childhood *60*, 1001-1007.

Turner, G., Eastman, C., Casey, J., McLeay, A., Procopis, P., and Turner, B. (1975). X-linked mental retardation associated with macro-orchidism. J. Med. Genet. *12*, 367-371.

Turner, G., Daniel, A., and Frost, M. (1980). X-linked mental retardation, macro-orchidism, and the Xq27 fragile site. J. Pediatr. *96*, 837-841.

Verheij, C., Bakker, C.E., de Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A.J.M.H., Galjaard, H., Reuser, A.J.J., Hoogeveen, A.T., and Oostra, B.A. (1993). Characterization and localization of the *FMR1* gene product. Nature *363*, 722-724.

Verkerk, A.J.M.H., Pieretti, M., Sutcliffe, J.S., Fu, Y., Kuhl, D.P.A., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F., Eussen, B.E., van Ommen, G-J.B., Blonden, L.A.J., Riggins, G.J., Chastain, J.L., Concept, C.B., Galjaard, H., Caskey, C.T., Nelson, D.L., Oostra, B.A., and Warren, S.T. (1991). Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a fragile X breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell *65*, 905-914.

Verkerk, A. J. M. H., de Graaff, E., De Boulle, K. Eichler, E. E>,Koencki, D. S., Reyniers, E., Manca, A., Poustka, A., Willems, P. J., Nelson, D. L., Oostra, B. A. (1993). Alternative splicing in the fragile gene *FMR1*. Hum. Mol. Genet. *2*, 399-404.

Vieregge, P., and Froster-Iskenius, U. (1989). Clinico-neurological investigations in the fra(X) form of mental retardation. J. Neurol. *236*, 85-92.

Wisniewski, K.E., Segan, S.M., Miezejeski, C.M., Sersen, E.A., and Rudelli, R.D. (1991). The fra(X) syndrome: neurological, electrophysiological and neuropathological abnormalities. Am. J. Med. Genet. *38*, 476-480.

Yu, S., Pritchard, M., Kremer, E., Lynch, M., Nancarrow, J., Baker, E., Holman, K., Mulley, J.C., Warren, S.T., Schlessinger, D., Sutherland, G.R., and Richards R.I. (1991). Fragile X genotype characterized by an unstable region of DNA. Science *252*, 1179-1181.

Publication 5.3

Characterization of *FMR1* proteins isolated from different tissues

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Abstract

***FMR1* protein expression was studied in different tissues. In human, monkey and murine tissues, high molecular mass *FMR1* proteins (67 to 80 kD) are found, as shown in lymphoblastoid cell lines. The identity of these proteins was confirmed by their absence in tissues from patients with the fragile X syndrome and a *Fmr1* knock out mouse. An Ile367Asn substitution in the *FMR1* protein did not alter the translation, processing and localization of *FMR1* proteins in lymphoblastoid cells from a patient carrying this mutation. All the high molecular mass *FMR1* proteins isolated from normal lymphoblastoid cells and cells from the patient with the Ile367Asn substitution were able to bind RNA. However, the *FMR1* proteins of the patient had reduced affinity for RNA binding at high salt concentrations. In some human, monkey and murine tissues low molecular mass *FMR1* proteins (39-41 kD) were found, which had the same N-terminus as the 67-80 kD isoforms, but differ in their C terminus and are therefore most likely the result of carboxy-terminal proteolytic cleavage. These low molecular mass *FMR1* proteins did not bind RNA, in contrast with the high molecular mass *FMR1* proteins. The significance of these low molecular mass proteins remains to be studied.**

Introduction

The fragile X syndrome is the most frequent form of inheritable mental retardation, with an incidence of one in every 1200 males (1). In addition to mental retardation, patients with the fragile X syndrome manifest testicular enlargement and variable facial dysmorfisms, large ears, and a long narrow face (2). The fragile X syndrome is characterized by the amplification of a CGG repeat present in the 5' untranslated region of the *FMR1* gene (3-6). The length of the CGG repeat varies in the normal population from 6 to 52 repeats and is stable on transmission (7). Premutation alleles ranging from 43 to 200 repeats exhibit instability, usually resulting in increases in repeat number in the offspring. Premutations are not associated with a clinical phenotype and are found in female carriers and normal transmitting males. In fragile X patients the CGG repeat is amplified beyond 200 copies (full mutation) and the concomitant hypermethylation of the preceding CpG island represses the transcription of *FMR1* (8). This results in the absence of the *FMR1* protein (FMRP) leading to the fragile X phenotype (9,10). Few patients have been described in which the fragile X syndrome is caused by a partial or complete deletion of the *FMR1* gene (11-14). One patient with the fragile X phenotype, also lacking a CGG repeat amplification, appeared to have an Ile367Asn mutation in FMRP (15).

The *FMR1* gene spans 38 kb and is composed of 17 exons (16). Normal and premutation

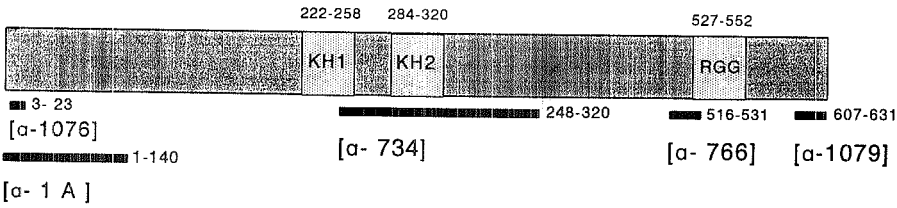


Figure 1.
Diagram of FMRP showing the positions of the KH domains and the RGG box. The regions used for immunisation of the rabbits are underlined and the amino acid positions are indicated. Amino acid numbers are according to Verkerk *et al.* (17). The diagram is not drawn to scale.

alleles are transcribed and the pre-mRNA is alternatively spliced (17,18) predicting as many as 48 distinct mRNAs. The alternative splicing of *FMR1* does not appear to be tissue specific as similar ratios of transcripts were found in all tissues analysed (17). The largest mRNA variant encodes a protein of 631 amino acids, the smallest encodes a protein of 436 amino acids. At least four different *FMR1* protein products have been discerned in control lymphoblastoid cell lines reflecting translation of mRNA splice variants (9,10).

The *FMR1* gene is highly conserved among species (3). The murine homologue *Fmr1* shows more than 95% homology and also contains a CGG repeat (18). In order to study the function of *FMR1* in human mental retardation a transgenic mouse with a knock-out for *Fmr1* was made, which lacks *Fmr1* mRNA and protein expression (19). The knock out mice showed a gradual development through time of macroorchidism, and showed cognitive impairment in the form of deficits in learning and behaviour.

Similarities of FMRP with RNA binding proteins were reported recently (20,21). It concerns two K homology (KH) domains, which are thought to be involved in RNA binding and which were found originally in the hnRNP K protein (22). Downstream of the *FMR1* KH domains lies an RGG box, a sequence motif which is directly involved in RNA binding (23). The Ile367Asn substitution (mentioned above) is located in the second KH domain of FMRP and interferes with RNA homopolymer binding under experimental conditions using high salt concentration (24). FMRP resulted in these experiments from *in vitro* translation of a plasmid containing the full length cDNA sequence of *FMR1*. The involvement of the RGG box in the RNA binding ability of FMRP has been shown by the inability of FMRP, lacking the RGG box, to bind RNA (21).

In this paper we studied FMRP in human, monkey and murine tissues. In these experiments tissues from patients with the fragile X syndrome and *Fmr1* knock out mice were used as negative controls. The RNA binding ability of *in vivo* made FMRPs was studied, including FMRPs carrying the Ile367Asn substitution in the second KH domain. These experiments complement the RNA binding studies which were all performed with *in vitro* synthesised full length or truncated FMRPs (20,21,24).

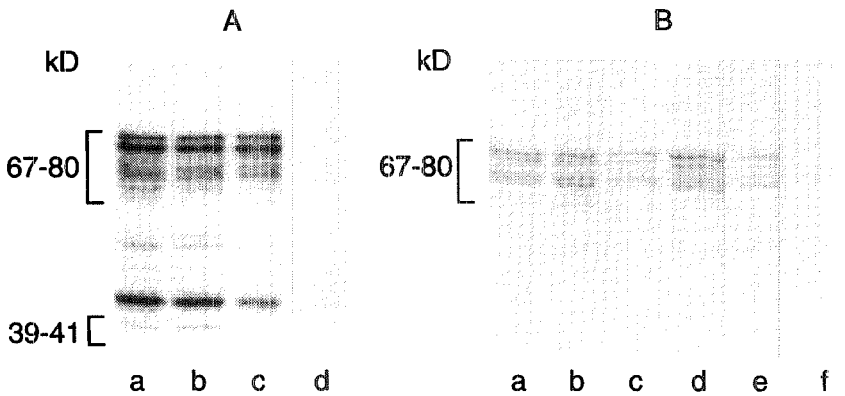


Figure 2.
Influence of an Ile367Asn substitution on the expression and RNA binding of the *FMR1* proteins. A) FMRP was immunoprecipitated with α734 from human control lymphoblastoid cell lines C49 and ROS31 (lane a and lane c respectively), cell line TR9001 from the fragile X patient with the point mutation (lane b) and cell line RJK1411 from a fragile X patient with the full mutation (lane d). Immunoprecipitated FMRPs were visulaized with α734 on Western blot. High and low molecular mass isoforms indicated by brackets. B) Binding of FMRP isoforms to Poly(U)-Sepharose. Homogenates of control lymphoblastoid cell line C49 (lanes a-c) and patient cell line TR9001 (lanes d-f) were incubated with poly(U) in the presence of 100 mM (lanes a and d), 250 mM (lanes b and e) and 500 mM NaCl (lanes c and f). Protein bound to poly(U)-Sepharose was analyzed on Western blot (α734). High molecular mass isoforms are indicated by brackets. None of these protein bands were present in homogenates of a patient cell line carrying the full mutation.

Results

Characterization of FMRP carrying the Ile367Asn substitution.

The Ile367Asn mutation does neither influence the translation nor stability of FMRP made *in vitro* (24). The translation of FMRP carrying this mutation was studied in lymphoblastoid cell lines in order to study the influence of the point mutation *in vivo*. Antibodies were prepared directed against different peptides. The polypeptides used for immunization and their position within the *FMR1* gene product are illustrated in Figure 1. These antibodies were used in immunoprecipitation studies of the *FMR1* gene product followed by Western blot analysis. *FMR1* proteins isolated from lymphoblastoid cells have a molecular mass of 67-80 kD (Fig. 2A). All these *FMR1* proteins are absent in the lymphoblastoid cells from a fragile X patient carrying a full mutation (Fig. 2A lane b). In contrast, a normal expression pattern of FMRP was found in the cells from the fragile X patient carrying the Ile367Asn substitution (15), indicating that the point mutation does not influence the translation of the different isoforms *in vivo*.

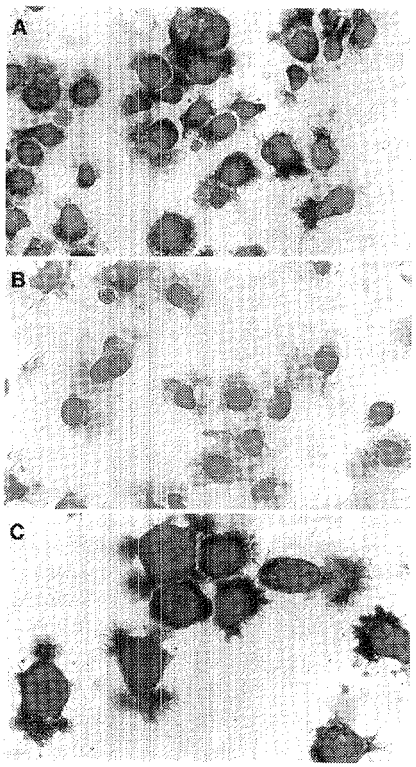


Figure 3.
Intracellular localization of FMRP in lymphoblastoid cell lines. Control cells C49 (panel a), cell line TR9001 carrying the Ile367Asn substitution (panel b) and cell line RJK1411 carrying the full mutation (panel c). FMRP carrying the point mutation shows the same cytoplasmic localisation as the wild-type FMRP.

RNA binding of the FMR1 proteins.

It is not yet clear whether the different FMRP isoforms have a different function. FMRP contains sequence motives homologous to domains found in RNA binding proteins. It has been demonstrated that an *in vitro* translated full length FMRP is able to bind RNA (20, 21). We have studied the RNA binding ability of all the *in vivo* produced *FMR1* proteins which were isolated from lymphoblastoid cell lines from controls and the patient carrying the Ile367Asn substitution. FMRP isoforms were incubated with poly(U)-Sepharose at different salt concentrations. All high molecular mass FMRP isoforms, both wildtype and point mutation, were able to bind RNA homopolymers (Fig. 2B lanes a and d, respectively). However, it was observed that all FMRP isoforms carrying the Ile367Asn substitution showed a lower RNA binding affinity at high salt concentrations when compared to wildtype FMRP isoforms (Fig. 2B lanes f and c, respectively).

Localization of FMRP.

It has been demonstrated that FMRP is localized in the cytoplasm (9, 10). To determine whether the Ile367Asn substitution in FMRP would affect the intracellular localization of FMRP and thereby indirectly its function, localization studies were performed in control lymphoblastoid

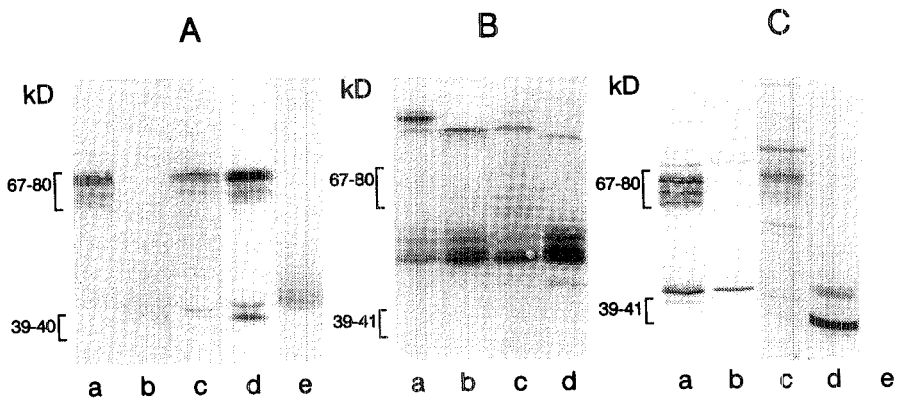


Figure 4.
Western blot analysis of human, monkey and murine tissues. A) Normal human brain, testis and kidney homogenates were immunoprecipitated with α734 (lanes a, c and d respectively). Immunoprecipitations of brain and kidney derived from a fragile X patient carrying a full mutation are shown in lanes b and e, respectively. Precipitated FMRPs were detected with α734 on Western blot. High and low molecular weight isoforms are indicated by brackets. B) Immunoprecipitation and Western blotting of monkey tissues as described under A. Brain, testis, kidney and liver in lanes a to d, respectively. High and low molecular weight isoforms are indicated by brackets. C) Immunoprecipitation and Western blotting of murine tissues as described under A. Normal brain, testis and kidney in lanes a, c and d, respectively. Immunoprecipitation of knock out mouse derived brain and kidney are shown in lanes b and e, respectively. High and low molecular weight isoforms are indicated by brackets.

cells and cells from the patient carrying the Ile367Asn substitution. As demonstrated in Figure 3, FMRP is localized in the cytoplasm of the lymphoblastoid cells derived from the patient carrying the Ile367Asn substitution (Fig. 3A) as was seen in the control cells (Fig. 3C). The lymphoblastoid cells from a patient with the full mutation were used as a negative control (Fig. 3B)

FMRP isolated from different tissues and species.

The expression of FMRP was also studied in different tissues isolated from human, monkey and mouse. *FMR1* proteins were isolated from human brain, testis, kidney, and liver using antibodies α734 (Fig. 4A). The results showed a pattern similar as is seen in human lymphoblastoid cell lines, with molecular masses in the range of 67-80 kD. The *FMR1* proteins were absent in tissues derived from a fragile X patient carrying a full mutation *FMR1* allele (Fig.

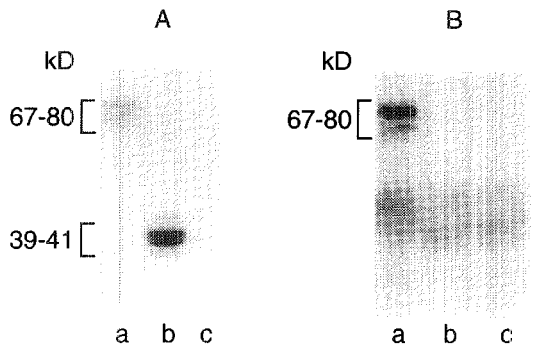


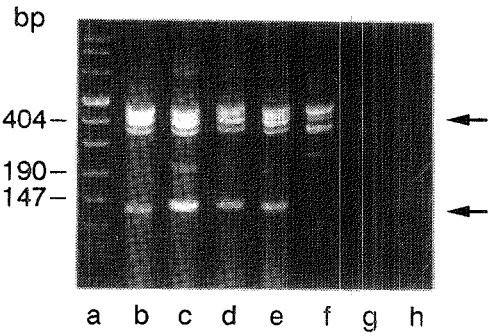
Figure 5.
Origin of the low molecular mass FMRP. A) Detection of FMRPs in murine tissues with monoclonal antibodies 1A. Normal brain (lane a), normal and knock out kidney (lanes b and c, respectively) were immunoprecipitated with monoclonal 1A. Western blot was incubated with α734. High and low molecular weight isoforms are indicated by brackets. Same results were obtained with antibody α1076. B) Detection of FMRPs in murine tissues with antibodies directed to the C-terminal part of FMRP. Normal brain (lane a), normal and knock-out kidney (lanes b and c, respectively) were immuno-precipitated with α734. The Western blot was incubated with antibodies α1079 directed to the carboxy terminal of FMRP. High molecular mass isoforms are indicated by brackets. Same results were obtained with antibodies α766, overlapping the N-terminal part of the RGG box. Protein band present in both normal and knock out mice are non-specific.

4A). In monkey-derived tissues a comparable result was obtained (Fig. 4B) as well as in murine tissues (Fig. 4C). None of these FMRP isoforms were detected in the respective tissues from *Fmr1* knock-out mice (Fig. 4C), confirming that these bands represent FMRP. In addition to the 67-80 kD FMRP, in some tissues low molecular mass (39-41kD) *FMR1* proteins were observed. In murine kidney the expression of the high molecular mass proteins can be hardly detected, but murine kidney shows the most abundant expression of the low molecular mass proteins found in all tissues studied.

The origin of the low molecular mass FMRP.

The low molecular mass isoforms were characterized in order to determine their origin. Antibodies directed against the N-terminal part of FMRP (α1A, α1076), were able to recognizeboth the high (67-80 kD) and low (39-41kD) molecular weight bands (Fig. 5A), suggesting that there is no difference in the N-terminal end of the FMRP isoforms. On the other hand, antibodies directed against the C-terminal part of FMRP (α766, α1079) could only detect the high molecular weight species (Fig. 5B), indicating that the C-terminal ends of the low molecular mass isoforms are different.

Figure 6.
FMR1 exon 14 splice variants in different tissues. RT-PCR was performed with primerset K9 and K10 on murine testis (lane b), brain (lane c), kidney (lane d) and liver (lane e) and human lymphoblastoid cell lines C49 (control, lane f) and RJK1411 (fragile X patient with full mutation, lane g). Marker in lane a, RT-PCR negative control in lane h. Splice variants containing exon 14 are indicated by the upper arrow, splice variants without exon 14 are indicated by the lower arrow.



The 39-41 kD proteins could result from the translation of mRNA splice variants or they could originate from proteolytic processing of the high molecular weight isoforms. Most splice variants do not lead to altered C-terminal amino acids (17), but the low molecular mass isoforms could be translation products of splice variants lacking exon 14, giving rise to novel C-termini (18). However, splicing out exon 14 occurs as a minor event both in men and mice. To investigate this possibility we compared the expression of splice variants with and without exon 14 at the mRNA level. To this end RT-PCR was performed on murine testis, brain, kidney and liver (Fig.6, lanes b, c, d, e, respectively), as well as human lymphoblastoid cell lines (Fig.6, lanes f and g). Splice variants without exon 14 in lymphoblastoid cell lines were only detectable after over-exposure. The ratio of alternative splice variants with and without exon 14 was the same in all tissues examined and therefore cannot account for the observed differential expression of high and low molecular weight FMRP isoforms. It is therefore more likely that the low molecular mass FMRPs result from post-translational proteolytic cleavage. Since α766 was raised against a peptide located around the start of the RGG box (Fig. 1) and was unable to recognize the low molecular mass FMRPs, it is most likely that the place of cleavage is in front of the RGG box. The RGG box has been demonstrated to be indispensable for RNA binding *in vitro* (21). Immunoprecipitation and poly(U) binding was performed in parallel on homogenates of murine kidney and it was found that the 39-41 kD *FMR1* proteins, missing the RGG box, are indeed unable to bind RNA homopolymers using poly(U)-Sepharose affinity chromatography (Fig. 7).

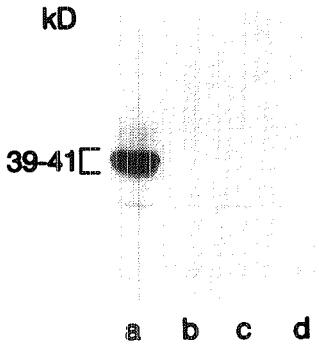


Figure 7.
RNA binding of the 39-41 kD *FMR1* proteins of murine kidney. Normal kidney was homogenized in binding buffer. Control immunoprecipitation is shown in lane a. The poly(U) binding was studied under 100 mM NaCl (lane b), 250 mM NaCl (lane c) and 500 mM NaCl (lane d). Immunoprecipitated proteins and proteins bound to poly(U)-Sepharose were analyzed by Western blotting. Polypeptides were detected with α734.

Discussion

Thus far the expression of *FMR1* has been studied in different ways. *In situ* hybridization revealed high mRNA expression during the early stages of mouse embryogenesis and high expression in some adult murine tissues, such as brain, testis and epithelium (25, 26). Northern blot analysis showed high expression levels in human tissues such as brain and testis, which is in agreement with the expression levels found with *in situ* hybridization in murine tissues; multiple smaller transcripts were found in human heart (25). The high expression level of FMRP in human brain and testis were confirmed by immunocytochemistry (10). Immunoprecipitations of different human cell lines revealed several FMRP species with a molecular weight between 67 and 80 kD (9, 10, 21). In this paper we described the expression of the different FMRP in different human, monkey and murine tissues in a more qualitative way.

FMR1 transcripts are alternatively spliced in men and mice and it was postulated that the high molecular mass *FMR1* proteins (67-80 kD) result from the translation of *FMR1* splice variants (9). This hypothesis is confirmed in this study by our observation, that antibodies directed against the N- as well as the C-terminal ends of FMRP recognize the high molecular mass *FMR1* proteins. This observation indicates that the C- and N- terminal ends of these proteins are retained and suggests internal variation of the primary amino acid sequence as a result of alternative splicing. Here we demonstrate that the *FMR1* proteins are expressed in a similar pattern in distinct human, monkey and murine tissues. We showed a pattern of high molecular mass *FMR1* proteins in brain, testis, kidney and liver, comparable with the pattern seen in cultured lymphoblastoid cells. Additional low molecular mass FMRPs were found in almost all tissues examined, but in murine kidney an exceptional protein pattern of exclusively low molecular mass FMRPs was found. These proteins were not recognized by antibodies directed against the C-terminal part of FMRP. The significance of the low molecular mass FMRPs is not clear, as the

lack of this abundantly expressed FMRP form in murine kidney does not lead to abnormalities in the *Fmr1* knock-out mouse.

The low molecular mass *FMR1* proteins could originate from the translation of exon 14 splice variants which encode for proteins of approximately 436 amino acids with altered C-termini. However, RT-PCR showed a similar splice variant distribution in all adult murine tissues studied. The exceptional high expression level of the low molecular mass FMRP in murine kidney cannot be explained by a different distribution of splice variants. Further the molecular weight, predicted from the translation products of the exon 14 splice variants (18) is not in agreement with the detected low molecular mass FMRPs. The low molecular mass *FMR1* proteins are therefore more likely the result of C-terminal proteolytic cleavage of the high molecular mass FMRPs. There are two possibilities, first the cleavage occurs *in vivo*, or secondly the cleavage occurs during cellular disruption. In the last possibility, the protease(s) responsible for this cleavage is not inhibited despite the use of a cocktail of eight inhibitors (see Materials and Methods). Further, if the proteolytic cleavage is a non-specific proteolytic degradation one would not expect to find distinct protein bands. Therefore it is likely that the proteolytic cleavage occurs *in vivo,* however, this has to be investigated more thoroughly before the meaning and function of the low molecular mass FMRPs will become clear.

The protein expression is not restricted to the tissues which are affected in the fragile X syndrome, like brain and testis. *FMR1* protein is expressed in many tissues; however, it has to be noted that the FMRP expression seen in heart and spleen is low (data not shown). The FMRP expression levels do not completely correspond to the *FMR1* mRNA levels which were found by Hinds *et al.* (25) who found high *FMR1* mRNA expression level in adult murine brain, testis and spleen, whereas low *FMR1* mRNA expression was found in murine liver and kidney. They could not detect *FMR1* transcripts in murine heart, whereas in human heart only smaller transcripts of 1.4 kb instead of 4.4 kb were found. We do not have indications that these smaller transcripts are translated in human heart. The contradiction of the protein and mRNA levels found in some of the tissues might be explained by a difference in protein or messenger stability. The results presented showed that FMRP is expressed in many tissues, where it can be potentially functional. However, the major clinical symptoms are restricted to the brain. This indicates that the 'substrate' for FMRP must be localized in the brain.

The function of the FMRP isoforms is not yet known. FMRP contains several sequence motifs, such as two KH domains and an RGG box, which are found in RNA binding proteins (22, 23). The RNA binding ability of the high and low molecular weight isoforms was compared. We found that all the high molecular mass FMRPs, present in human lymphoblastoid cell lines (but also in human and murine brain; data not shown), are able to bind RNA homopolymers in contrast to the low molecular mass isoforms, present in murine kidney. The inability of the low molecular mass isoforms to bind RNA can be explained by the lack of the RGG box in these forms, as was

demonstrated by the inability of the C-terminal antibodies to recognize the 39-41 kD isoforms. These results show that no RNA binding occurs despite the presence of two KH binding domains in the low molecular weight isoforms, indicating that the RGG box in FMRP is important for RNA binding.

The influence on processing, localization and RNA binding of an Ile367Asn substitution in FMRP was investigated. No difference in expression and processing of FMRP was found when cultured lymphoblastoid cell lines from controls and the patient with the point mutation were compared. The intracellular localization of FMRP carrying the Ile367Asn substitution was not changed either and also the cytoplasmic localization was the same as found for wildtype FMRP. However, when RNA binding was studied on the *in vivo* synthesized and isolated FMRP, it was found that all the high molecular mass *FMR1* proteins carrying the Ile367Asn substitution have a lower affinity for binding to poly(U) under high salt conditions than wildtype FMRP. These results are in agreement with those from Siomi *et al.* (24) who also found a difference in RNA binding of an *in vitro* translated, full length *FMR1* protein. As the Ile367Asn substitution is localized in the second KH domain of the FMRP, these results indicate that not only the RGG box is important for RNA binding. Although the RGG box seems to be responsible for the RNA binding activity of FMRP, the KH domains possibly have a modifying effect on the RNA binding of the RGG box. The point mutation could change the protein structure in such a way that it influences the RNA binding to the RGG box. However, the difference in RNA binding activity was only observed under non-physiological salt conditions. The changes in RNA binding activity caused by the Ile367Asn substitution under physiological conditions might be too small to be observed with the applied method. FMRP is able to bind 4% of the human fetal brain mRNAs (20). It would be of interest to study the effect of the point mutation on the binding of FMRP to these brain mRNAs. The patient carrying this point mutation has a severe fragile X phenotype. If this mutation would really affect the binding of FMRP to these brain mRNAs, one would expect much more aberrations than the mental retardation found in this patient. It could therefore also be that this point mutation does not really influence the RNA binding activity of FMRP, but affects a function other than RNA binding.

Material and methods.

Antibodies

Polyclonal antibodies were raised in rabbits against a GST-*FMR1* fusion protein (α734; 10) and synthetic peptides, covering amino acid position 3-23 (α1076), amino acid position 516 to 531 (α766) and amino acid position 607 to 631 (α1079) (see also Fig.1). Monoclonal antibodies 1A (10) recognize the N terminal part of FMRP with an epitope in the amino acid sequence coded by exon 1-5 (Mandel, personal communication).

Tissue homogenates
Approximately 200 mg of tissue was homogenized in 1 ml of buffer (10mM HEPES, 300 mM KCl, 5 mM $MgCl_2$, 100 µM $CaCl_2$, 0.45% Triton X100, 0.05% Tween, pH 7.6) in the presence of protein inhibitors cocktail (PMSF, Antipain, Chemostatin, Leupeptin, Pepstatin, Phenanthrolin, NEM, EDTA-Na). Homogenates were sonicated for 45 s and centrifuged for 30 min (10,000*g*). Seven mg of protein was used per immunoprecipitation.

Immunoprecipitation and Western blotting
Immunoprecipitation, gel electrophoresis and Western blotting were performed as described (9), with the exception of using ^{125}I protein A instead of alkaline phosphatase conjugated goat anti rabbit IgG to detect the polypeptides.

RT-PCR.
RT-PCR was performed to detect exon 14 splice products in human and murine tissues. PCR was performed with primers K9 (GTATGGTACCATTTGTTTTTGTG) and K10 (GAAGCCTCCTCCACGTCCTCT) as described (17).

Poly(U) binding
Cell homogenates were made in binding buffer (10 mM Tris-HCl (pH 7.4), 2,5 mM $MgCl_2$, 0.5 % Triton X100) and NaCl concentration as given in the legend of Fig. 2 (27, 28). The homogenates were incubated with micrococcal nuclease (Pharmacia, 50 U/ml) for 10 minutes at 30°C. Homogenates were incubated with poly(U)-Sepharose (Pharmacia) on a rocking platform for 10 minutes at 4°C. Beads were washed five times in binding buffer prior to resuspension in SDS-PAGE loading buffer.

Immunocytochemistry.
EBV transformed lymphoblasts from normal individuals and fragile X patients were used for cytospin preparation and immunoincubated with monoclonal antibodies 1A. Briefly, cytospin slides were fixed in 100 mM phosphate buffer pH 7.4, containing 4% paraformaldehyde for 10 min at RT. Subsequently, slides are permeabilized in 100% methanol for 20 min at RT and washed with PBS. Monoclonal antibodies 1A were used in the first incubation step, followed by goat anti-mouse (immunoglobulin) conjugated with biotin (DAKO). The latter is visualized with a streptavidin-biotin-alkaline phosphatase enhancement kit (DAKO). The new Fuchsin substrate chromogen system (DAKO) is used in the final step utilizing alkaline phosphatase. Slides were counterstained with Gill's haematoxylin and mounted with aquamount. Endogenous alkaline phosphatase activity was blocked by adding Levamisol to the substrate solution. An incubation whereby the first antibody was omitted was used as a control.

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References

1. Oostra, B.A., Willems, P.J., and Verkerk, A.J.M.H. (1993). Fragile X syndrome: a growing gene. In Genome Analysis Vol 6: Genome mapping and neurological disorders. Coldspring Harbor Laboratory Press, 45-75.

2. Hagerman, R.J. (1991). Physical and behavioral phenotype. In Fragile X syndrome: Diagnosis, treatment, and research (ed. R.J. Hagerman and A.C. Silverman), John Hopkins Press, Baltimore. 3-68.
3. Verkerk, A. J. M. H., Pieretti, M., Sutcliffe, J.S., Fu, Y.-H., Kuhl, D.P.A., Pizutti, A., Reiner, D., Richards, S., Zhang, F., Eussen, B.E., van Ommen, G.J.B., Blonden, L.A.J., Riggins. G.J., Chastain, J.L., Kunst, C.B., Galjaard, H., Caskey, C.T., Nelson, D.L., Oostra, B.A., and Warren, S.T. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* **65**, 905-914.
4. Oberlé, I., Rousseau, F. , Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boué, J., Bertheas, M.F., and Mandel, J.L. (1991). Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science 252*, 1098-1102.
5. Yu, S., M. Pritchard, Pritchard, M., Kremer, E., Lynch, M., Nancarrow, J., Baker, E., Holman, K., Mulley, J.C., Warren, S.T., Schlessinger, D., Sutherland, G.R., and Richards, R.I. (1991). Fragile X genotype characterized by an unstable region of DNA. *Science* **252**, 1179-1181.
6. Kremer, E. J., Yu, S., Pritchard, M., Nagaraja, R., Heitz, D., Lynch, M., Baker, E., Hyland, V.J., Little, R.D., Wada, M., Toniolo, D., Vincent, A., Rousseau, F., Schlessinger, D., Sutherland, G.R., and Richards, R.I. (1991). Isolation of a human DNA sequence which spans the fragile X. *Am. J. Hum. Genet.* **49**, 656-661.
7. Fu, J.-H., Kuhl, D.P.A., Pizutti, A., Pieretti, M., Sutcliffe, J.S., Richards, S., Verkerk, A.J.M.H., Holden, J.J.A., Fenwick, R.G. Jr., Warren S.T., Oostra, B.A., Nelson, D.L., and Caskey, C.T. (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell* **67**, 1047-1058.
8. Pieretti, M., Zhang, F., Fu, Y.-H., Warren, S.T., Oostra, B.A., Caskey, C.T., and Nelson, D.L.. (1991). Absence of expression of the FMR1 gene in fragile X syndrome. *Cell* **66**, 817-822.
9. Verheij, C., Bakker, C.E., de Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A.J.M.H., Galjaard, H., Reusser, A.J.J., Hoogeveen, A.T., and Oostra, B.A. (1993). Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. *Nature* **363**, 722-724.
10. Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.-P., and Mandel, J.-L. (1993) The FMR1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nature Genet.* **4**, 335-340.
11. Gedeon, A. K., Baker, E., Robinson, H., Partington, M.W., Gross, B., Manca, A., Korn, B., Poustka, A., Yu, S., Sutherland, G.R., and Mulley, J.C. (1992). Fragile X syndrome without CCG amplification has an FMR1 deletion. *Nature Genet.* **1**, 341-344.
12. Wöhrle, D., Kotzot, D., Hirst, M.C., Manca, A., Korn, B., Schmidt, A., and Barbi, G. (1992) A microdeletion of less than 250 kb, including the proximal part of the FMR1 gene and the fragile X site, in a male with the clinical phenotype of the fragile X syndrome. *Am. J. Hum. Genet.* **51**, 299-306.
13. Tarleton, J., Richie, R., Schwartz, C., Rao, K., Aylsworth, A.S., and Lachiewicz, A. (1993). An extensive de novo deletion removing FMR1 in a patient with mental retardation and the fragile X syndrome phenotype. *Hum. Mol. Genet. 2*, 1973-1974.
14. Meijer, H., de Graaff, E., Merckx, D., Jongbloed, R.J.E., de Die-Smulders, C.E.M., Engelen, J.J.M., Fryns, J-P., Curfs, P.M.G., and Oostra, B.A. (1994). A deletion of 1.6 kb proximal to the CGG repeat of the FMR1 gene causes the clinical phenotype of the fragile X syndrome. *Hum. Mol. Genet.* **3**, 615-620.
15. De Boulle, K., Verkerk, A.J.M.H., Reyniers, E., Vits, L., Hendrickx, J., van Roy, B., Van den Bos, F., de Graaff, E., Oostra B.A., and Willems, P.J. (1993). A point mutation in the FMR-1 gene associated with fragile X mental retardation. *Nature Genet.* **3**, 31-35.
16. Eichler, E.E., Richards, S., Gibbs, R.A., and Nelson, D.L. (1993). Fine structure of the human

FMR1 gene. *Hum. Mol. Genet.* **2**, 1147-1153.

17. Verkerk, A.J.M.H., de Graaff, E., De Boulle, K., Eichler, E.E., Konecki, D.S., Reyniers, E., Manca, A., Poustka, A., Willems, P.J., Nelson, D.L., and Oostra, B.A. (1993). Alternative splicing in the fragile X gene FMR1. *Hum. Mol. Genet.* **2**, 399-404.
18. Ashley, C. T., Sutcliffe, J.S., Kunst, C.B., Leiner, H.A., Eichler, E.E., Nelson, D.L., and Warren, S.T. (1993). Human and murine FMR-1: alternative splicing and translational initiation downstream of the CGG-repeat. *Nature Genet.* **4**, 244-251.
19. Bakker, C.E., Verheij, C., Willemsen, R., van der Helm, R., Oerlemans, F., Vermey, M., Bygrave, A., Hoogeveen, A.T., Oostra, B.A., Reyniers, De Boulle, K., D'Hooge, R., Cras, P., van Velzen, D., Nagels, G., Martin, J.-J., De Deyn, P.P., Darby, J.K., and Willems, P.J. (1994). Fmr1 knock out mice: a model to study fragile X mental retardation. *Cell* **78**, 23-33.
20. Ashley, C.T., Wilkinson, K.D., Reines, D., and Warren, S.T. (1993). FMR-1 protein: Conserved RNP family domains and selective RNA binding. *Science* **262**, 563-566.
21. Siomi, H., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. *Cell* **74**, 291-298.
22. Siomi, H., Matunis, M.J., Michael, W.M., and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionary conserved motif. Nucleic Acids Res. **21**, 1193-1198.
23. Kiledjian, M., and Dreyfuss, G. (1992). Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. *EMBO J.* **11**, 2655-2664.
24. Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1994). Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 causes fragile X syndrome. *Cell* **77**, 33-39.
25. Hinds, H. L., Ashley, C.T., Sutcliffe, J.S., Nelson, D.L, Warren, S.T., Housman, D.E., and Schalling, M. (1993). Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nature Genet.* **3**, 36-43.
26. Abitbol, M., Menini, C., Delezoide, A.L., Rhyner, T., Vekemans, M., and Mallet, J., (1993). Nucleus basalis magnocellularis and hippocampus are the major sites of FMR1 expression in the human brain. *Nature Genet.* **4**, 147-152.
27. Choi, Y.D., and Dreyfuss, G. (1984). Isolation of the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP): a unique supramolecular assembly. *Proc. Natl. Acad. Sci. USA* **81**, 7471-7475.
28. Swanson, M.S., and Dreyfuss, G. (1988). Classification of heterogeneous nuclear ribonucleoprotein particles by RNA binding specificity. *Mol. Cell. Biol.* **8**, 2237-2241.

Publication 5.4

Normal phenotype in two brothers with a full *FMR1* mutation

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Abstract

The fragile X syndrome is associated with an expanding CGG repeat in the 5' untranslated region of the first exon of the *FMR1* gene. Subsequent methylation of the promoter region inhibits expression of the *FMR1* gene. In two clinically normal brothers large, expanded CGG repeats and cytogenetically visible fragile sites were found. The *FMR1* promoter was unmethylated and both RNA and protein could be detected. This indicates that inactivation of the *FMR1* gene and not repeat expansion itself results in the fragile X phenotype. We conclude that repeat expansion does not necessarily induce methylation and that methylation is no absolute requirement for the induction of fragile sites.

Introduction

Triplet repeat expansions have been associated with a variety of genetic disorders (1-7) and fragile sites (8-11) or both (8,9). Limited expansion of CAG repeats within the coding region of genes cause a number of neurodegenerative disorders, probably due to a gain-of-function of the protein. Very large, expanding repeats are found in myotonic dystrophy (2) and in a number of fragile sites (8-11). As yet, the destructive mechanism of the expanded CTG repeat in the 3' untranslated region (UTR) of the DM-kinase gene is unknown and conflicting reports on over- or underexpression of the gene product in patients exist (12,13). The triplets involved in fragile sites are CGG or CCG and clinical features can be present (FRAXA, FRAXE) or absent (FRA16A, FRAXF). The best characterized example is the FRAXA-site at Xq27.3 (14,15), indicative of the fragile X syndrome, which is the most common form of hereditary mental retardation in males (16-18). The disease is associated with expansion of a CGG repeat, located in the 5'UTR of the *FMR1* gene. Methylation of the *FMR1* promoter region silences the gene (19-23) and absence of the *FMR1* protein results in the clinical phenotype. This is supported by a rare group of patients showing the clinical features of the fragile X syndrome, but who were cytogenetically negative. In these patients deletions (24-26) or a point mutation (27) in the *FMR1* gene were demonstrated, which resulted in the absence of or a defective *FMR1* protein.

So far, several groups have tried without success to find a correlation between the number of CGG triplets and the severity of mental retardation (28,29). It turned out that the methylation status of the *FMR1* promoter was the most correlated parameter in male patients (30). For example, mild phenotypic symptoms have been reported for patients with an only partially methylated full mutation, but without cytogenetic FRAXA expression (31, 32). Furthermore, incomplete and completely absence of methylation was found in a group of high functioning male patients with cytogenetic expression. In this group the presence of *FMR1* protein was demonstrated, although in reduced amounts (33). It is, however, unclear if this is caused by a lower gene expression or by a translation suppression due to the length of the CGG repeat.

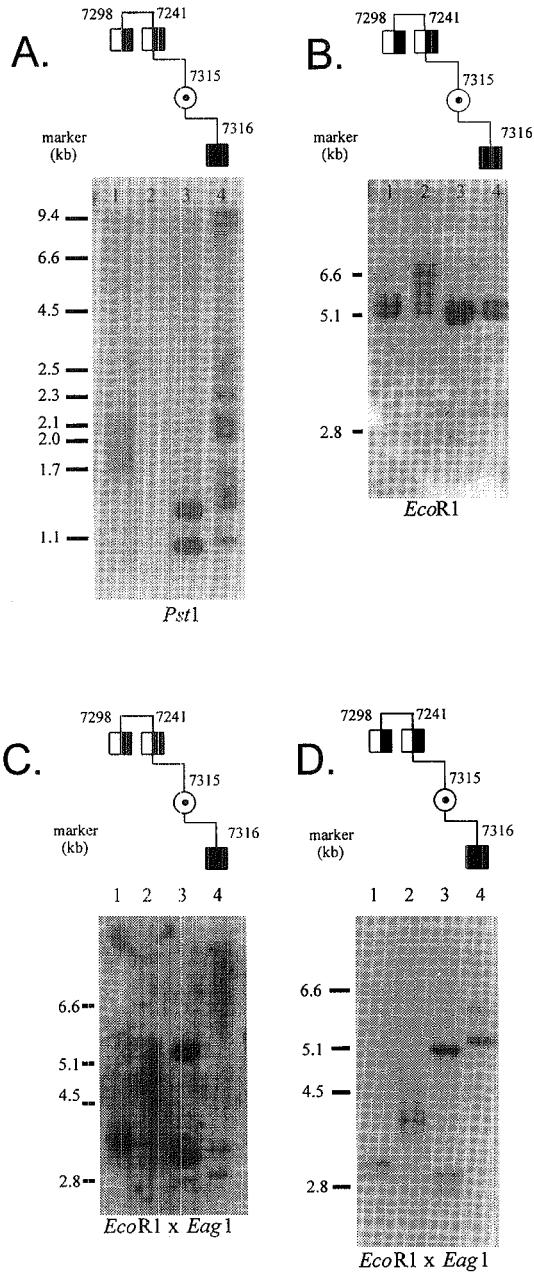

Figure 1.
Southern blot analysis of genomic DNA. *PstI* (A) and *EcoRI* (B) digestions of PBL DNA and *EcoRI-EagI* digestions of PBL (C) and EBV transformed blood lymphocyte DNA (D). Half-filled squares indicate males with normal phenotype, but with cytogenetic FRAXA expression. A filled square indicates a male fragile X patient with mental retardation and cytogenetic FRAXA expression. A dotted circle represents an obligate female carrier with a normal phenotype.

The expanded CGG or CCG repeats are predicted to undergo conformational changes, which induce *de novo* methylation (34), thereby stabilizing the unusual structures, probably to mark them for repair (35). These structures could suppress transcription and replication of the *FMR1* gene and so induce cytogenetic expression (35). The familial case described in this paper of two completely normal brothers with CGG repeat expansion and cytogenetic FRAXA expression may provide a clue to both the mechanism of inhibition of *FMR1* expression and induction of fragile sites.

Results

Analysis of the fragile X locus

The fragile X family was ascertained through a mentally retarded boy, born in 1980 (Fig. 1, 7316). Cytogenetically, 10% FRAXA-site expression was found. Further DNA analysis with *PstI* and *EcoRI* digested DNA from peripheral blood lymphocytes (PBL) revealed expansion of theCGG repeat with fragments ranging from apparently normal to full mutation (up to 1500 repeats; Fig. 1A an B). A mosaic pattern from premutation to full mutation is found in 20% of the fragile X patients (30, 32, 36), but the pattern of this patient is very rare. The mother of the proband had a premutation and a normal allele (Fig.1A, 7315). Subsequent family analysis revealed five more carriers, among whom were two males having both cytogenetic FRAXA expression and a full mutation by DNA analysis (Fig 1A and B, 7298 and 7241). Surprisingly, these two males were phenotypically normal. In repeated experiments, we found 6 and 13% fragile site expression and large repeat expansions of 170-340 and 100-1500 copies, respectively (Fig. 1A).

In all mentally retarded patients with similar repeat sizes tested, the CpG island in front of the repeat was methylated as a result of which the gene was not transcribed. The methylation status of the *FMR1* gene was determined in this family using the enzymes *EcoRI* + *EagI* on DNA from PBL and EBV transformed blood lymphocytes (Fig. 1C and D). The mentally retarded boy showed complete methylation of the full mutation, while the premutation and normal bands were unmethylated in PBL DNA (Fig. 1C). In contrast, no unmethylated bands were detectable in DNA from EBV transformed cells (Fig. 1D). The expanded *FMR1* genes of the mentally normal men were entirely unmethylated, both in PBL (Fig. 1C) and in EBV transformed blood lymphocytes (Fig. 1D). As expected, the female carriers in the family showed partial methylation of both X chromosomes (Fig. 1C and D).

A difference in repeat length distribution was found in blood DNA (Fig. 1C) compared with the distribution found in DNA isolated from EBV transformed blood lymphocytes (Fig. 1D). DNA from the cell line of patient 7316 contained only two bands left from the full mutation range.

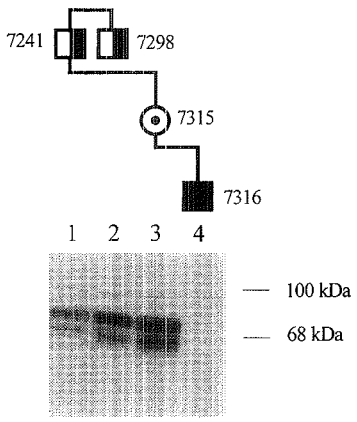

Figure 2.
Western blot analysis of *FMRP* in EBV transformed lymphocytes using polyclonal antibody α734. Pedigree symbols are as in Figure 1.

Male 7298 had a repeat distribution with a tendency to the premutation range, while on the other hand the cell line of male 7241 showed an almost single repeat length of 400 repeats, which is completely in the full mutation range. This shows again that EBV transformed cell lines are not always a good representation of the variety of different cells with different repeat lengths found in blood and that they are the product of a limited number of cells. A difference in methylation between PBL and EBV transformed blood lymphocytes was only found for the patient 7316.

Expression of the *FMR1* gene

Methylation of the *FMR1* promoter has been reported to inhibit expression of the gene. The expression of the *FMR1* gene in the three males with large repeat expansions was tested in peripheral and EBV transformed blood lymphocytes. As an internal control the level of *HPRT* transcription was determined, and classical fragile X patients and controls were included for comparison. No *FMR1* mRNA was detectable in classical fragile X patients with large repeat expansions and a methylated promoter region (data not shown). The expression in male 7316 strongly reduced compatible with classical fragile X patients. The level of *FMR1* mRNA in PBL was only slightly higher than in EBV transformed blood lymphocytes. The *FMR1* mRNA levels in males 7298 and 7241 were normal in both cell types.

We analyzed whether the large repeat had an influence on the (efficiency of) translation of the *FMR1* RNA. Immunoprecipitation was performed on equal amounts of protein extracted from

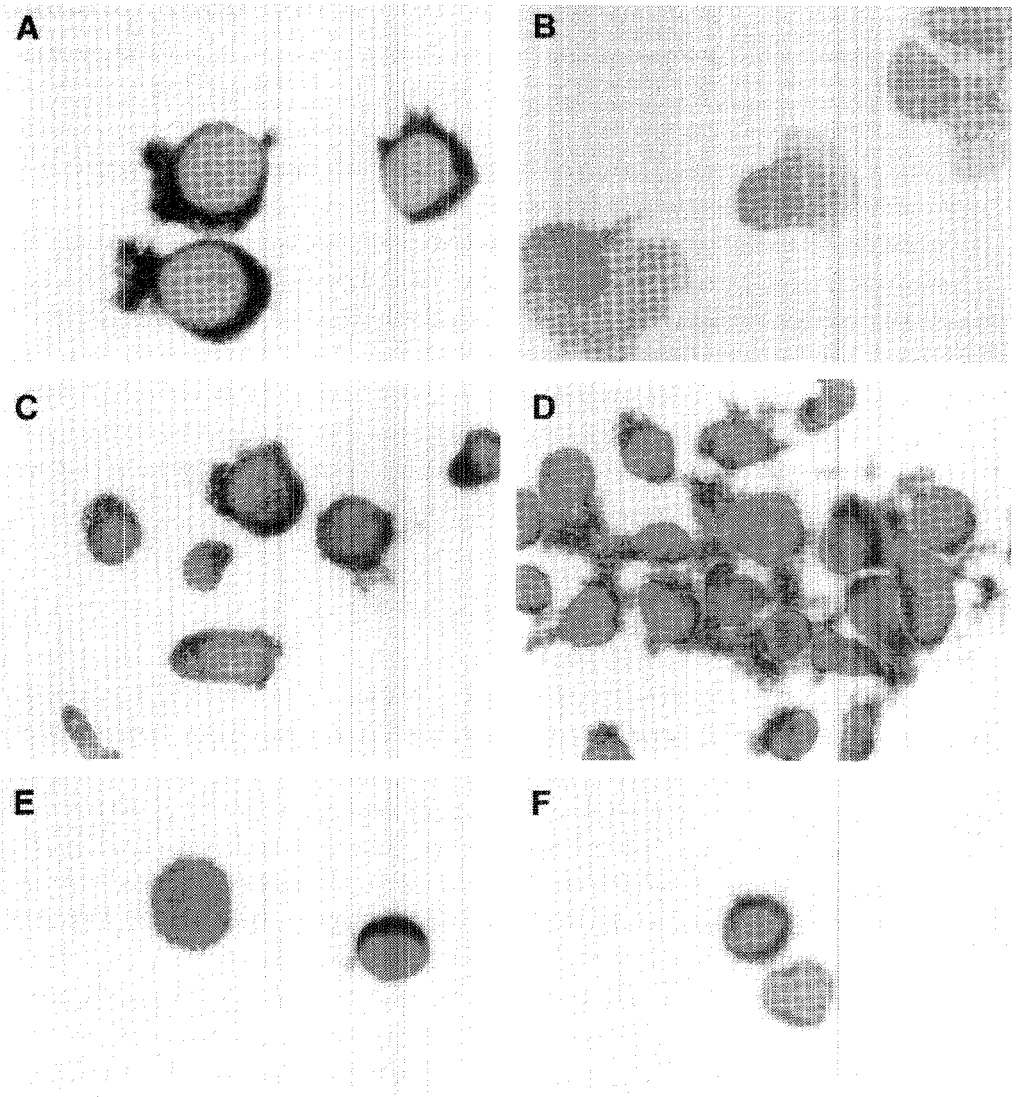

Figure 3.
Immunohistochemical detection of FMRP in EBV transformed lymphocyte and blood smears. Cell lines of control cells C49 (A), patient 7316 (B), individual 7298 (C) and individual 7241 (D). Blood smears of a control (E) and individual 7241 (F).

EBV transformed blood lymphocytes and FMRP (*FMR1* protein) was determined by Western blotting using anti-*FMR1* antibodies (22). The same passage of the culture was used as for the DNA studies. The *FMR1* protein was present in the cells from males 7298 and 7241 at a reduced level compared to normal controls (Fig. 2). The fragile X male 7316 showed no *FMR1* protein at all, similar to classical fragile X patients. His mother 7315 had a normal FMRP expression as might be expected from a carrier of a premutation.

To test whether the lower expression in these males was the result of expression in cells with a relatively small expansion and no expression in cells with a relatively large expansion, we tested the protein expression by immunocytochemistry on individual cells of the EBV transformed blood lymphocytes. Expression was found in all cells, but also at a lower level than in control cells (Fig. 3). This means that a gene with a repeat length of 400 copies can be transcribed and translated (Fig. 1b). To evaluate the result of the cell lines, the expression of the *FMR1* protein in blood was analyzed directly on a blood smear of male 7241. Again all cells did show expression of the *FMR1* protein, but again at a level lower than control cells (Fig. 3).

Discussion

In this study evidence is presented that expansion of the CGG repeat in the *FMR1* gene itself, even in the full mutation range, does not cause the fragile X phenotype. It is the first example of a large, unmethylated expanded CGG repeat without any clinical consequences. This familial case might be exceptional, although it is unknown how many people in the general population may carry large repeat expansions. The family was analyzed, because of the presence of a mentally retarded grandson, but would have been missed otherwise. Even this grandson lacked the physical features of fragile X. But despite an extraordinary mosaic CGG repeat pattern, *FMR1* mRNA and protein levels in PBL were comparable with classical fragile X patients.

Variation in severity of mental retardation has been shown previously for individual patients with different methylation patterns. Rousseau *et al.*. (31) described a male with a full mutation with 40% methylation of the *FMR1* gene; unfortunately, no protein expression was determined. Hagerman *et al.*. (33) described 3 high functioning fragile X males that had unmethylated large repeats and showed reduced protein expression compared to control values. They did not show whether normal expression was found in a limited number of cells or a reduced expression was found in all cells. Our data support the conclusion that a minimum amount of protein is necessary for a normal phenotype; it could be speculated that this minimal amount may be necessary in every cell or at least in a high percentage of cells. This could explain that part of the females with a full mutation are normal and part are mentally retarded, although less severely than males with a full mutation, due to a skewed inactivation of the normal X chromosome. The amount of protein

made as shown by immunohistochemistry was lower in blood and in EBV transformed blood lymphocytes in the males with the unmethylated full mutation compared to controls. However, Feng *et al.* (37) have shown that the amount of *FMR1* protein using ß-tubulin as an internal control can differ by a factor of 2. Although the relation between RNA/protein levels and clinical status has not been extensively analyzed, it is evident that these parameters are most indicative for the expression of the *FMR1* gene and, most likely, are best suited for clinical predictions.

Our data show that there is no obligatory relation between repeat expansion and methylation of the *FMR1* gene. This has already been demonstrated for normal transmitting males with relatively short repeat expansions (50-200 CGG repeats), but usually larger expansions lead to complete methylation of the *FMR1* promoter region. Only few examples have been reported with incomplete or mosaic methylation patterns, providing supporting evidence that expansion and methylation can be uncoupled (31,33). Sutcliffe *et al.* (20) have shown in chorionic villi of a male fetus with a full mutation that no methylation of the CpG island was found and that the *FMR1* gene was transcribed. This again indicates that in absence of methylation, transcription of a gene with a full mutation is possible. In the two brothers described in this paper, this uncoupling is absolute. Because the mechanism and enzymes involved in *de novo* methylation in humans are still unknown, one can only speculate about the factors involved. First, the length of the expanded repeat may be a factor. Short expansions do not cause methylation, but large expansions in general do. The family described here and the data from others (20,38) showed that no absolute point exists at which methylation takes place, but that there may be a gradual scale with intra- and interfamilial variation. Secondly, our data show that additional (genetic) factors must play a role in the methylation process. In the 2 brothers no methylation is apparent, which means that either the specific methylase is defective or that the recognition site(s) of the enzyme is altered or blocked. The detection of methylation of the same *FMR1* promoter in the grandson favors a role for a trans-acting factor. Not much is known about *de novo* methylation in humans, but it would be worthwhile to test other methylated areas for a general, beneficial methylation defect in these patients.

Finally, our data do not support the hypothesis that methylation of expanded CGG/CCG repeats is necessary for cytogenetic expression of fragile sites. As yet, the molecular basis of four fragile sites (FRAXA, FRAXE, FRAXF, FRA16A) has been characterized as expanded CGG/CCG repeats. These triplets can be methylated, in contrast to the CTG repeat in myotonic dystrophy with a similar repeat length. This latter disorder is not associated with a fragile site and, therefore, methylation was thought to play a key role in cytogenetic expression of these sites. In the two brothers, described in this paper, no methylation of the *FMR1* promoter could be observed, but cytogenetic expression was still evident. The methylation of only a single restriction site was determined and not of the entire repeat, but reports from others (39,40) have shown that the methylation status of this site can be extrapolated to the entire area.

After finishing the experiments described in this paper conflicting results were presented by Feng *et al.* (41). They used fibroblast subclones of a mildly affected patient and showed that despite the presence of normal mRNA levels, transcription is diminished from mRNAs with more than 200 repeats. They concluded that beyond 200 repeats there is no migration of the ribosome along the repeat. However, we show that in a cell line, almost exclusively containing cells with a repeat of 400 CGG copies, translation of *FMR1* mRNA takes place in all cells although at a reduced level as compared with controls. Also expression was found in all cells in a blood sample from the same individual (Fig. 3). This expression of the *FMR1* protein in all cells can explain the normal phenotype of this individual.

Materials and methods

Human subjects

Case 1 (7316). The 14-year-old proband was mentally retarded since childhood, but there was no family history of mental retardation. Physical examination at the age of 14 showed a boy with normal body proportions. No macrocephaly [(occipito frontal circumference (OFC) 56 cm (percentile 75-90)] and no macrognathia was present. His palate and ears were normal (ear lengths 6,5 cm). He revealed mild hypermobility of distal joints and a soft skin. The external genitalia were small (testes length 2,5 cm) with little pubertal hair development. Psychological testing at the age of 13 years showed a friendly and cooperative boy with good verbal performance. IQ testing at the same age showed a verbal IQ of 64 and a performance IQ of 53 (WISC-R). His behavior was normal without the typical manifestations of fragile X.

Case 2 (7315). The 36-year-old mother of the proband is phenotypically and mentally normal. She studied nursing and is successfully employed in this profession.

Case 3 (7241). The 72-year-old maternal grandfather of the proband is in good health. Physical examination at the age of 72 revealed a normal phenotype. No macrocephaly was present (OFC 57,2 cm; percentile 75) and his forehead was not broad. His ears had lengths of 7,6 and 7,7 cm, but were normally shaped. His face was not elongated. No other clinical abnormalities were found. He refused testicle examination. He has good eye contact and also lacks the other behavioral manifestations of the fragile X syndrome. He attended high school and became managing-director of a family company of about 100 employees. His mental status could be described as intelligent.

Case 4 (7298). This is the 74-year-old grand-uncle of the proband. His head, face and ears were normally shaped, but he refused detailed physical examination. He had no behavioral manifestations of fragile X. School training was similar to his brother (7241). He was a college student for 3 years after which he became managing-director of the family company. His mental status could also be described as intelligent.

Cytogenetic studies

Chromosomes were studied from peripheral whole blood lymphocytes. Details of the methodology were described previously (42). Cell cultures were initiated using Chang medium with multiple FRAXA induction systems (FUdR and Thymidine) as described (43). Ten days before harvesting, the long term culture was subcultured on RPMI-1640. A maximum of 200 cells of each sample were examined for the presence of a fragile X chromosome.

DNA and RNA studies

Southern blot analysis of DNA, extracted from peripheral blood, was performed according to standard procedures (44) with probe pAO365 (45). The methylation status of the *FMR1* gene was determined by

double digests with the methylation sensitive restriction enzyme *EagI* as has been described before (32). RNA was extracted from EBV transformed cell lines using standard procedures (44). The ratio between *HPRT* and *FMR1* mRNA was determined, using the protocol of Pieretti *et al.* (19). Our modifications involve the use of fluorescently labeled PCR primers, followed by analysis on a sequencing gel, using the GeneScanner Software (ABI, Foster City).

Protein studies

Polyclonal antibodies were raised in rabbits against a GST-*FMR1* fusion protein (α734) (22) and used in immunoprecipitations and Western blotting. Immunoprecipitation, gel electrophoresis and Western blotting were performed as described (22), with the exception of using ^{125}I protein A instead of alkaline phosphatase conjugated goat anti rabbit IgG to detect the polypeptides. Immunocytochemistry on EBV transformed lymphocytes and on whole blood smears was performed as described (46, 47) using monoclonal antibody 1A (23).

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References

1. La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature,* **352**, 77-79.
2. Brook, J.D., McCurrach, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.P., Hudson, T., Sohn, R., Zemelman, B., Snell, R.G., Rundle, S.A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johson, K., Harper, P.S., Shaw, D.J. and Housman, D.E. (1992). Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell,* **68**, 799-808.
3. Huntington's Disease collaborative research group. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell,* **72**, 971-983.
4. Orr, H.T., Chung, M., Banfi, S., Kwiatkowski, T.J., Servadio, A., Beaudet, A., McCall, A.E., Duvick, L.A., Ranum, L.P.W. and Zohgbi, H.Y. (1993). Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nature Genet,* **4**, 221-226.
5. Koide, R., Ikeuchi, T., Onodera, O., Tanaka, H., Igarashi, S., Endo, K., Takahashi, H., Kondo, R., Ishikawa, A., Hayashi, T., Saito, M., Tomoda, A., Miike, T., Naito, H., Ikuta, F. and Tsuji, S. (1994). Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). *Nature Genet,* **6**, 9-13.
6. Burke, J.R., Wingfield, M.S., Lewis, K.E., Roses, A.D., Lee, J.E., Hulette, C., Pericak-Vance, M.A. and Vance, J.M. (1994). The Haw River syndrome: dentatorubropallidoluysian atrophy (DRPLA) in an African-American family. *Nature Genet,* 7, 521-524.
7. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S. and Kakizuka, A. (1994). CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. *Nature Genet,* **8**, 221-228.
8. Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., Eussen, B.E., Van Ommen, G.J.B., Blonden, L.A.J., Riggins, G.J., Chastain, J.L., Kunst, C.B., Galjaard, H., Caskey, C.T., Nelson, D.L., Oostra, B.A. and Warren, S.T. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell,* **65**, 905-914.

9. Knight, S.J., Flannery, A.V., Hirst, M.C., Campbell, L., Christodoulou, Z., Phelps, S.R., Pointon, J., Middleton-Price, H.R., Barnicoat, A., Pembrey, M.E., Holland, J., Oostra, B.A., Bobrow, M. and Davies, K.E. (1993). Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. *Cell,* **74**, 127-134.
10. Nancarrow, J.K., Kremer, E., Holamn, K., Eyre, H., Doggett, N.A., Le Paslier, D., Callen, D.F., Sutherland, G.R. and Richards, R.I. (1994). Implications of FRA16A structure for the mechanism of chromosomal fragile site genesis. *Science,* **264**, 1938-1941.
11. Parrish, J.E., Oostra, B.A., Verkerk, A.J.H.M., Richards, C.S., Spikes, A.S., Shaffer, L.G. and Nelson, D.L. (1994). Isolation of a GCC repeat showing expansion in FRAXF, a fragile site distal to FRAXA and FRAXE. *Nature Genet,* **8**, 229-235.
12. Sabourin, L.A., Mahadevan, M.S., Narang, M., Lee, D.S.C., Surh, L.C. and Korneluk, R.G. (1993). Effect of the myotonic dystrophy (DM) mutation on mRNA levels of the DM gene. *Nature Genet,* **4**, 233-238.
13. Fu, Y.-H., Friedmna, D.L., Richards, S., Pearlman, J.A., Gibbs, R.A., Pizzuti, a., Ashizawa, T., Perryman, M.B., Scarlato, G., Fenwick, R.G. and Caskey, C.T. (1993). Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. *Science,* **260**, 235-238.
14. Lubs, H.A. (1969). A marker X-chromosome. *Am. J. Hum. Genet.,* **21**, 231-244.
15. Sutherland, G.R. (1979). Heritable fragile sites on human chromosomes II. Distribution, phenotypic effects and cytogenetics. *Am. J. Hum. Genet.,* **31**, 136-148.
16. Webb, T.P., Bundey, S.E., Thake, A.I. and Todd, J. (1986). Population incidence and segregation ratios in the Martin-Bell syndrome. *Am. J. Med. Genet.,* **23**, 573-580.
17. Gustavson, K.H., Blomquist, H.K. and Holmgren, G. (1986). Prevalence of the fragile X syndrome in mentally retarded boys in a Swedish county. *Am. J. Med. Genet.,* **23**, 581-587.
18. Kahkonen, M., Alitalo, T., Airaksinen, E., Matilainen, R., Launiala, K., Autio, S. and Leisti, J. (1987). Prevalence of the fragile X syndrome in four birth cohorts of children of school age. *Hum. Genet.,* **77**, 85-87.
19. Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T. and Nelson, D.L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell,* **66**, 817-822.
20. Sutcliffe, J.S., Nelson, D.L., Zhang, F., Pieretti, M., Caskey, C.T., Saxe, D. and Warren, S.T. (1992). DNA methylation represses FMR-1 transcription in fragile X syndrome. *Hum. Mol. Genet.,* **1**, 397-400.
21. Siomi, H., Siomi, M.C., Nussbaum, R.L. and Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. *Cell,* **74**, 291-298.
22. Verheij, C., Bakker, C.E., de-Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A.J., Galjaard, H., Reuser, A.J., Hoogeveen, A.T. and Oostra, B.A. (1993). Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. *Nature,* **363**, 722-724.
23. Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P. and Mandel, J.L. (1993). The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nature Genet,* **4**, 335-340.
24. Gedeon, A.K., Baker, E., Robinson, H., Partington, M.W., Gross, B., Manca, A., Korn, B., Poustka, A., Yu, S., Sutherland, G.R. and Mulley, J.C. (1992). Fragile X syndrome without CCG amplification has an FMR1 deletion. *Nature Genet,* **1**, 341-344.
25. Wöhrle, D., Kotzot, D., Hirst, M.C., Manca, A., Korn, B., Schmidt, A., Barbi, G., Rott, H.D., Poustka, A., Davies, K.E. and Steinbach, P. (1992). A microdeletion of less than 250 kb, including the proximal part of the FMR-I gene and the fragile-X site, in a male with the clinical phenotype of fragile-X syndrome. *Am. J. Hum. Genet.,* **51**, 299-306.
26. Meijer, H., Degraaff, E., Merckx, D., Jongbloed, R., Dediesmulders, C., Engelen, J., Fryns, J.P., Curfs, P. and Oostra, B.A. (1994). A deletion of 1.6 kb proximal to the CGG repeat of the FMR1 gene causes the clinical phenotype of the Fragile X syndrome. *Hum. Mol. Genet.,* **3**, 615-620.
27. De Boulle, K., Verkerk, A.J., Reyniers, E., Vits, L., Hendrickx, J., Van Roy, B., Van den Bos, F., de

Graaff, E., Oostra, B.A. and Willems, P.J. (1993). A point mutation in the FMR-1 gene associated with fragile X mental retardation. *Nature Genet,* **3**, 31-35.
28. De Vries, B.B.A., Wiegers, A.M., De Graaff, E., Verkerk, A.J.M.H., Van Hemel, J.O., Halley, D.J.J., Fryns, J.P., Curfs, L.M.G., Niermeijer, M.F. and Oostra, B.A. (1993). Mental status and fragile X expression in relation to FMR-1 gene mutation. *Eur. J. Hum. Genet.,* **1**, 72-79.
29. Rousseau, F., Heitz, D., Tarleton, J., Macpherson, J., Malmgren, H., Dahl, N., Barnicoat, A., Mathew, C., Mornet, E., Tejada, I., Maddalena, A., Spiegel, R., Schinzel, A., Marcos, J., Schorderet, D.F., Schaap, T., Maccioni, L., Russo, S., Jacobs, P.A., Schwartz, C. and Mandel, J.L. (1994). A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB 12.3: The first 2,253 cases. *Am. J. Hum. Genet.,* **55**, 225-237.
30. Rousseau, F., Heitz, D., Biancalana, V., Blumenfeld, S., Kretz, C., Boue, J., Tommerup, N., Van Der Hagen, C., DeLozier-Blanchet, C., Croquette, M.F., Gilgenkranz, S., Jalbert, P., Voelckel, M.A., Oberlé, I. and Mandel, J.L. (1991). Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. *N. Engl. J. Med.,* **325**, 1673-1681.
31. Rousseau, F., Robb, L.J., Rouillard, P. and Derkaloustian, V.M. (1994). No mental retardation in a man with 40% abnormal methylation at the FMR-1 locus and transmission of sperm cell mutations as premutations. *Hum. Mol. Genet.,* **3**, 927-930.
32. McConkie-Rosell, A., Lachiewicz, A., Spiridigliozzi, G.A., Tarleton, J., Schoenwald, S., Phelan, M.C., Goonewardena, P., Ding, X. and Brown, W.T. (1993). Evidence that methylation of the FMR1 locus is responsible for variant phenotypic expression of the fragile X syndrome. *Am. J. Hum. Genet.,* **53**, 800-809.
33. Hagerman, R.J., Hull, C.E., Safanda, J.F., Carpenter, I., Staley, L.W., Oconnor, R.A., Seydel, C., Mazzocco, M., Snow, K., Thibodeau, S.N., Kuhl, D., Nelson, D.L., Caskey, C.T. and Taylor, A.K. (1994). High functioning fragile X males: Demonstration of an unmethylated fully expanded FMR-1 mutation associated with protein expression. *Am. J. Med. Genet.,* **51**, 298-308.
34. Smith, S.S., Laayoun, A., Lingeman, R.G., Baker, D.J. and Riley, J. (1994). Hypermethylation of telomere-like foldbacks at codon 12 of the human c-Ha-ras gene and the trinucleotide repeat of the FMR-1 gene of fragile X. *J. Mol. Biol.,* **243**, 143-151.
35. Fry, M. and Loeb, L.A. (1994). The fragile X syndrome d(CGG)(n) nucleotide repeats form a stable tetrahelical structure. *Proc. Natl. Acad. Sci. USA,* **91**, 4950-4954.
36. Oberlé, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boue, J., Bertheas, M.F. and Mandel, J.L. (1991). Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science,* **252**, 1097-1102.
37. Feng, Y., Lakkis, D. and Warren, S.T. (1995). Quantitative comparison of FMR1 gene expression in normal and premutation alleles. *Am. J. Hum. Genet.,* **56**, 106-113.
38. Sutherland, G.R., Gedeon, A., Kornman, L., Donnelly, A., Byard, R.W., Mulley, J.C., Kremer, E., Lynch, M., Pritchard, M., Yu, S. and Richards, R.I. (1991). Prenatal diagnosis of fragile X syndrome by direct detection of the unstable DNA sequence. *N. Engl. J. Med.,* **325**, 1720-1722.
39. Hansen, R.S., Gartler, S.M., Scott, C.R., Chen, S.H. and Laird, C.D. (1992). Methylation analysis of CGG sites in the CpG island of the human FMR1 gene. *Hum. Mol. Genet.,* **1**, 571-578.
40. Hornstra, I.K., Nelson, D.L., Warren, S.T. and Yang, T.P. (1993). High resolution methylation analysis of the FMR1 gene trinucleotide repeat region in fragile X syndrome. *Hum. Mol. Genet.,* **2**, 1659-1665.
41. Feng, Y., Zhang, F., Lokey, L.K., Chastain, J.L., Lakkis, L., Eberhart, D. and Warren, S.T. (1995). Translation suppression by trinucleotide repeat expansion at FMR1. *Science,* **268**, 731-734.
42. Smits, A.P., van-Oost, B.A., de-Haan, A.F., Hamel, B.C., Dreesen, J.C. and Smeets, D.F. (1992). Penetrance of fra(X) gene: influence of grandparental origin of the gene, mental status of the carrier mother, and presence of a normal transmitting male. *Am. J. Med. Genet.,* **43**, 365-72.
43. Shapiro, L.R. and Wilmot, P.L. (1986). Prenatal diagnosis of the fra(X) syndrome. *Am. J. Med. Genet.,* **23**, 325-340.
44. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. Cold

Spring Harbor Laboratory press, Cold Spring Harbor.
45. Van Oost, B.A., Smits, A.P., Dreesen, J.C., van-den-Ouweland, A.M. and Oostra, B.A. (1992). Validation of linkage-based DNA-diagnosis of fragile X gene carriers with the CGG repeat probe. *Am. J. Med. Genet.*, **43**, 320-7.
46. Verheij, C., De Graaff, E., Bakker, C.E., Willemsen, R., Willems, P.J., Meijer, N., Galjaard, H., Reuser, A.J.J., Ooostra, B.A. and Hogeveen, A.T. (1995). Characterization of FMR1 proteins isolated from different tissues. *Hum. Mol. Genet.*, **4**, 895-901.
47. Willemsen, R., Mohkamsing, S., De Vries, B., Devys, D., Van den Ouweland, A., Mandel, J.L., Galjaard, H. and Oostra, B. (1995). Rapid antibody test for fragile X syndrome. *The Lancet*, **345**, 1147-1148.

Summary

The fragile X syndrome is one of the most frequent forms of inheritable mental retardation after Down's syndrome. Fragile X patients are mildly to severely mentally retarded, show amongst others hyperactivity and stereotypic behaviour and have physical features of which enlarged testis is the most striking. The syndrome is caused by amplification of a polymorphic CGG repeat present in the 5' untranslated region of the Fragile X Mental Retardation 1 gene (*FMR1*). Normal individuals carry either a normal or a premutation *FMR1* allele, containing 6-54 and 43-200 repeats, respectively. When the CGG repeat is amplified beyond 200 repeat units methylation of the *FMR1* promoter region occurs, preventing the transcription of the gene. This so called full mutation is carried by fragile X patients. The *FMR1* gene is not expressed in cells of fragile X patients as a consequence of this mutation.

Enlarged CGG repeats are unstable, which means that both premutation and full mutation tend to grow in successive generations. This type of mutations is called dynamic mutation and since the cloning of the *FMR1* gene more and more genes are described in which the amplification of a trinucleotide repeat results in a disorder (Chapter 2). These disorders can be divided into three different classes, based on the effect the trinucleotide repeat amplification has on the gene expression. Amplification of the repeat can lead to the silencing of the gene, like in fragile X syndrome (Class 1). When the repeat is located in the coding region of the gene it will be translated into amino acids and this results in Class 2 in gene products with (pathologically) enlarged polyglutamine stretches. It is remarkable that the latter class consists of neurodegenerative disorders. The effect of the repeat amplification in the myotonic dystrophy region on chromosome 19 (Class 3) is not yet clear; it has been suggested that the amplified repeat might have a negative influence on the expression of surrounding genes.

The amino acid sequence, deduced from the DNA sequence, initially showed no homology to other described proteins. Therefore no predictions could be made about possible functions of FMRP (Chapter 1).The subject of the research described in this thesis was to characterize the *FMR1* protein. Antibodies were raised against synthetic peptides and a (GST)-*FMR1* fusion protein, each representing specific parts of FMRP, in order to identify and characterize the protein (Chapter 3). These antibodies detect several proteins with a molecular weight varying between 67 and 80 kD in control lymphoblastoid cell lines. These proteins were absent in fragile X patients (Publication 5.1), suggesting that all proteins are derived from the *FMR1* gene.

Expression in *COS* cells of DNA constructs encoding for different *FMR1* splice variants showed that only one protein product was obtained per splice variant (Publication 5.1). The molecular weight of these proteins corresponded to the molecular weights of the FMRP isoforms detected in lymphoblastoid cell lines. The protein products could furthermore be detected with antibodies directed against the N- and C-terminal part of FMRP, showing that the proteins differ

only in the central part of FMRP (Publication 5.3). The *FMR1* proteins thus originate from the translation of splice variants.

The existence of different FMRP isoforms could indicate a possible different distribution and function of these isoforms in distinct tissues and therefore the expression of FMRP was studied in human, monkey and murine tissues. The highest FMRP concentrations were found in brain and testis; in liver and kidney a lower concentration was found (Publication 5.3). However, the ratio of the different isoforms were similar (Publication 5.3) and thus far no indications have been found for a tissue or cell stage specific expression of the different FMRP isoforms (Chapter 3).

The CGG repeat makes part of the 5' untranslated region of the *FMR1* gene and in cell lines derived from individuals with a premutation FMRPs are found with molecular weights identical to those found in control cell lines (Publication 5.1). Although the repeat is not contributing to the protein structure it might be, however, that the repeat influences the transcription or translation efficiency. The *FMR1* expression was investigated in two normal brothers, each carrying *FMR1* alleles with an unmethylated promoter region and more than 200 CGG repeats. The promoters of *FMR1* alleles with more than 200 repeats are usually methylated, but these two brothers are exceptions to the rule. Our research revealed no dramatic influence in transcription or translation efficiency compared to control individuals (Publication 5.4). It can thus be concluded that the *FMR1* gene expression is not hindered by the amplification of the CGG repeat, but is hampered by methylation of the promoter region.

FMRP is localized to the cytoplasm (Publication 5.1). However, recently signals have been found in FMRP which direct the protein either to the cytoplasm (nuclear export signal) or to the nucleus (nuclear localization signals). The presence of these motifs indicate that FMRP is able to shuttle between the cytoplasm and the nucleus. Mean while a nuclear localization of a small amount of the protein has been demonstrated indeed (Chapter 4).

Recently homologies to other proteins have been found which might give clues to the function of FMRP. The central part of the protein contains two K homology (KH) domains and the more C-terminal part of the protein contains an RGG box. These sequences are present in RNA binding proteins and it has been shown that these motifs are able to bind RNA. FMRP was found to have RNA binding properties indeed (Chapter 3).

A severely mentally retarded patient has been described with a point mutation in the second KH domain of *FMR1* as the sole mutation. The gene is expressed, but due to the point mutation a conserved isoleucine is changed into an asparagine. It has been postulated that the fragile X syndrome in this patient is caused by changed RNA binding characteristics of FMRP (Chapter 3).

We have shown that the point mutation neither had an influence on transcription or translation of the *FMR1* splice variants, nor did it have an influence on the localization or stability of FMRP. RNA homopolymer binding assays were performed with FMRP isolated from a lymphoblastoid

cell line derived from this patient (Publication 5.3). FMRP isoforms with this point mutation have a decreased RNA binding ability under higher, non-physiological salt concentrations when compared to control FMRP. Although these results were obtained in an artificial RNA binding assay this nevertheless indicates that the point mutation changes the RNA binding ability of the protein *in vivo*, thereby causing the fragile X syndrome in this patient (Chapter 3).

The study of the pathogenesis of the fragile X syndrome depends on the available patient material. This material is rare and almost exclusively consists of cell lines. This was one of the reasons to develop a mouse model for the fragile X syndrome (Publication 5.2). The murine *Fmr1* gene is homologous to the human *FMR1* gene and a mouse model for the fragile X syndrome was obtained by silencing the endogenous *Fmr1* gene. The *Fmr1* knock out mouse has characteristics in common with the fragile X patients, as they are hyperactive and show deficits in visual spatial abilities. This latter function was tested in the Morris watermaze test, which depends (among others) on hippocampal functioning. These tests revealed that the *Fmr1* knock out mice show deficits in the processing of spatial information. Investigation of the morphology of the brain did not reveal differences between normal and knock out mice. The *Fmr1* knock out mice furthermore have enlarged testis, which is also observed in fragile X patients. Testis of knock out mice show no differences when compared to normal testis; the spermatogenesis is normal despite the lack of *Fmr1* protein. The morphology and behaviour of the *Fmr1* knock out mice will of course be studied in more detail.

The last few years research has concentrated on the unravelling of the function of FMRP. Knowledge on the function of the protein will not only give insight into the pathogenesis of the fragile X syndrome, but also in processes concerning memory and learning. Recently the association of FMRP to the ribosomes has been described and we have demonstrated that this binding occurs via rRNA. The binding of FMRP to the ribosomes suggests that FMRP is involved in translation (Chapter 4). Further research will therefore concentrated on the possible role of FMRP in translation, especially in brain.

Samenvatting

Het fragiele X syndroom is na het Down's syndroom één van de meest voorkomende genetische oorzaken van zwakzinnigheid. Fragiele X patiënten zijn mild tot ernstig mentaal geretardeerd, , vertonen onder andere hyperactief en stereotiep gedrag en hebben een aantal fysieke kenmerken waarvan vergrote testis de meest opvallende is. Het syndroom wordt veroorzaakt door de verlenging van een polymorfe CGG repeat in het 5' onvertaalde gebied van het 'Fragiele X Mental Retardation 1' gen (*FMR1*). Normale individuen dragen een normaal of een premutatie *FMR1* allel, dat respectievelijk 6-54 of 43-200 repeats bevat. Wanneer de repeat een lengte overschrijdt van 200 repeat eenheden, treedt methylering van het *FMR1* promotorgebied op waardoor het gen niet tot expressie komt. Deze mutatie, die volledige mutatie wordt genoemd, wordt in fragiele X patiënten gevonden. In cellen van deze patiënten wordt als gevolg daarvan het *FMR1* gen niet tot expressie gebracht.

Verlengde CGG repeats zijn instabiel, wat wil zeggen dat zowel premutaties als volle mutaties de neiging hebben in opeenvolgende generaties te groeien. Dit type van mutatie wordt ook wel dynamische mutatie genoemd en sinds de klonering van het *FMR1* gen worden meer en meer genen beschreven waarin verlenging van een trinucleotide repeat leidt tot een afwijking (Hoofdstuk 2). Op grond van het effect van de repeatverlenging kunnen deze ziekten in drie klassen worden ingedeeld. Verlenging van de repeat kan tot gevolg hebben dat het gen niet meer tot expressie komt, zoals bij het fragiele X syndroom (Klasse 1). Wanneer de repeat in het coderende gedeelte van het gen ligt wordt deze vertaald in aminozuren en dit leidt bij Klasse 2 tot genprodukten met een (pathologisch) verlengde polyglutamine keten. Opvallend is dat laatstgenoemde klasse bestaat uit neurodegeneratieve ziekten. Het gevolg van de repeat verlenging in het myotone dystrophy gebied op chromosoom 19 (Klasse 3) is vooralsnog niet duidelijk; gedacht wordt dat de verlenging van de repeat een negatieve invloed heeft op omliggende genen.

De uit de *FMR1* DNA sequentie afgeleide aminozuurvolgorde vertoonde in eerste instantie geen homologie met andere, bekende eiwitten. Hierdoor konden geen voorspellingen worden gedaan over mogelijke eigenschappen of functie van FMRP (Hoofdstuk 1). Het doel van het in dit proefschrift beschreven onderzoek was het *FMR1* eiwit te karakteriseren. Hiertoe werden antilichamen opgewekt tegen een (GST)-*FMR1* fusie eiwit en synthetische peptiden die elk overeenkomen met een specifiek gedeelte van FMRP (Hoofdstuk 3). Met deze antilichamen worden in controle lymfoblastoïde cellijnen meerdere eiwitten aangetoond met molekuulgewichten variërend van 67 tot 80 kD. Deze eiwitten ontbreken in cellijnen met een volledige mutatie, verkregen van fragiele X patiënten (Publikatie 5.1), wat suggereert dat al deze eiwitten afkomstig zijn van het *FMR1* gen.

DNA constructen, coderend voor verschillende *FMR1* splice varianten, die in *COS* cellen

tot expressie werden gebracht, toonde aan dat per splice variant slechts één eiwitprodukt gevormd werd (Publikatie 5.1). De molekuulgewichten van de verkregen eiwitten kwamen overeen met de molekuulgewichten van de verschillende FMRP isovormen die werden gevonden in de lymfoblastoïde cellijnen. Het was verder mogelijk alle eiwit varianten te detecteren met antilichamen gericht tegen de N- en C-terminale uiteinden van FMRP wat aantoont dat de eiwitten alleen in het middelste deel verschillen (Publikatie 5.3). De verschillende *FMR1* eiwitprodukten zijn dus afkomstig van de translatie van alternatieve splice varianten.

Aangezien het bestaan van FMRP isovormen kan betekenen dat er in de verschillende weefsels verschillende functies en verdelingen bestaan voor deze isovormen werd de expressie van FMRP onderzocht in weefsels van mens, aap en muis. De hoogste concentratie FMRP werd gevonden in de hersenen en testis; in weefsels als nier en lever werd een lagere concentratie FMRP gevonden (Publikatie 5.3). De verdeling van de verschillende FMRP isovormen was echter gelijk en tot dusver zijn er geen aanwijzingen gevonden voor een weefsel of celstadium specifieke expressie van de FMRP varianten (Hoofdstuk 3).

De CGG repeat maakt deel uit van het 5' onvertaalde gedeelte van het *FMR1* gen en in cellijnen afkomstig van individuen met een premutatie worden dan ook FMRPs gevonden met molekuulgewichten die identiek zijn aan die in controle cellijnen (Publikatie 5.1). Hoewel de CGG repeat niet wordt vertaald en dus niet bijdraagt aan de structuur van het eiwit zou de repeat wel de transcriptie en translatie efficiëntie kunnen beïnvloeden. De *FMR1* expressie werd bestudeerd in twee normale broers met elk een *FMR1* gen met ongemethyleerde promoter en meer dan 200 CGG repeats. Promotors van *FMR1* genen met meer dan 200 repeats zijn doorgaans gemethyleerd, maar deze broers vormen een uitzondering op de regel. Ons onderzoek toonde aan dat er geen grote verschillen zijn in transcriptie en translatie efficiëntie in vergelijking met controle individuen (Publikatie 5.4). Hieruit kan worden geconcludeerd dat de expressie van het gen niet wordt verhinderd door de verlenging van de CGG repeat, maar door de methylering van het promotorgebied.

FMRP is gelokaliseerd in het cytoplasma (Publikatie 5.1). Onlangs zijn in FMRP echter motieven gevonden die verantwoordelijk zijn voor een cytoplasmatische (nuclear export signal) of kern (nuclear localization signal) lokalisatie. De aanwezigheid van deze sequenties in FMRP vormen aanwijzingen dat het eiwit in staat is zich tussen het cytoplasma en de kern te bewegen. Inmiddels is aangetoond dat een kleine hoeveelheid van het eiwit zich inderdaad in de kern bevindt (Hoofdstuk 4).

Recent zijn alsnog homologiën met andere eiwitten gevonden die een aanwijzing kunnen vormen wat betreft de functie van FMRP. Het middelste deel van het *FMR1* eiwit bevat twee 'K homology' (KH) domeinen en in het C-terminale gedeelte van het eiwit bevindt zich een RGG box. Deze sequenties worden gevonden in RNA bindende eiwitten en het is aangetoond

te hebben (Hoofdstuk 3).

Een ernstig mentaal geretardeerde fragiele X patiënt is beschreven met als enige mutatie een puntmutatie in het tweede KH domein. Het *FMR1* gen komt tot expressie maar door de puntmutatie wordt een geconserveerde isoleucine in het tweede KH domein verandert in een asparagine. Gepostuleerd werd dat het fragiele X syndroom in deze patiënt wordt veroorzaakt door veranderde RNA bindingseigenschappen van FMRP (Hoofdstuk 3).

We hebben aangetoond dat de puntmutatie geen invloed heeft op de transcriptie of translatie van de verschillende *FMR1* splice varianten, noch op de lokalisatie of stabiliteit van FMRP. RNA homopolymeer bindingsexperimenten werden uitgevoerd met FMRP geïsoleerd uit een lymfoblast cellijn verkregen van deze patiënt (Publikatie 5.3). FMRP isovormen met de puntmutatie vertonen een verlaagde RNA binding bij hogere, niet fysiologische, zout concentraties. Hoewel deze resultaten werden verkregen met een kunstmatige RNA bindingstest is dit toch een aanwijzing dat de puntmutatie de RNA binding *in vivo* kan veranderen, wat zou kunnen resulteren in het fragiele X syndroom in deze patiënt (Publikatie 5.3).

Onderzoek naar de pathogenese van het fragiele X syndroom is afhankelijk van het beschikbare patiënten materiaal. Dit materiaal is schaars en bestaat vrijwel alleen uit cellijnen. Dit was één van de redenen om een muismodel voor het fragiele X syndroom te ontwikkelen (Publikatie 5.2). Het *Fmr1* gen van de muis is homoloog aan het humane *FMR1* gen en een muis model voor het fragiele X syndroom werd verkregen door het endogene *Fmr1* gen uit te schakelen ('knockout'). De *Fmr1* knockout muis heeft een aantal kenmerken gemeen met fragiele X patiënten. Zo zijn zij net als fragiele X patiënten hyperactief en vertonen zij afwijkingen wat betreft het omgaan met visueel ruimtelijke informatie. Dit laatste werd getest in de 'Morris water maze test', die onder andere afhankelijk is van functies in de hippocampus. Hieruit bleek dat de knockout muizen moeilijkheden hebben met het verwerken van veranderingen in ruimtelijke informatie. Onderzoek naar de morfologie van de hersenen hebben geen grote verschillen aangetoond tussen de hersenen van normale en *Fmr1* knockout muizen. De knockout muizen hebben evenals fragiele X patiënten vergrote testis. De testis van de knockout muizen vertoonde echter geen zichtbare verschillen met testis van de normale muis; de spermatogenese vindt normaal plaats ondanks het ontbreken van het *Fmr1* eiwit. Het spreekt voor zich dat de morfologie en het gedrag van de knockout muizen verder zal worden onderzocht.

Het onderzoek heeft zich de afgelopen jaren gericht op het ontrafelen van de functie van FMRP. Kennis over de functie van het eiwit zal ons niet alleen inzicht geven in de pathogenese van het fragiele X syndroom, maar ook in geheugen- en leerprocessen. Onlangs is beschreven dat FMRP is geassocieerd aan de ribosomen en wij hebben aangetoond dat deze binding plaats vindt via het rRNA. De binding van FMRP aan de ribosomen suggereert dat FMRP betrokken is bij translatie (Hoofdstuk 4). Het verdere onderzoek zal zich daarom concentreren op de mogelijke rol van FMRP in het RNA metabolisme, met name in de hersenen.

Abbreviations

ADCA	autosomal dominant cerebellar ataxia
bp	base pair
DM	myotonic dystrophy
DM-PK	myotonic dystrophy protein kinase
DNA	deoxyribonucleic acid
EBV	Epstein Bar Virus
FMR1	fragile X mental retardation
FMRP	*FMR1* protein
FXR1(2)	FMR1-crossreacting relative gene 1(2)
FRDA	Friedreich's ataxia
HAP	huntingtin associated protein
HD	Huntington's disease
hnRNP	heterogenous nuclear RNA binding protein
KH	K homology
KLH	keyhole limpet hemocyanin
LTP	long term potentiation
mRNA	messenger RNA
NES	nuclear export signal
NLS	nuclear localization signal
NTM	normal transmitting male
PCR	polymerase chain reaction
RNA	ribonucleic acid
SCA	spinocerebellar ataxia
YAC	yeast artificial chromosome

Curriculum vitae

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januari 1992	aanvang promotie onderzoek als AIO op de afdeling Klinische Genetica, bij Prof.Dr. H. Galjaard, Dr. B.A. Oostra en Dr. A.T. Hoogeveen.

Cursussen:
Engels: The Oxford examination in English as a foreign language, higher level. Proefdierkunde. Oncogenesis. Transgenesis, gene transfer and gene therapy. Congenital malformations.

List of publications

Verheij, C., Bakker, C.E., de Graaff, E., Keulemans, J., Willemsen R., Verkerk, A.J., Galjaard, H., Reuser, A.J.J., Hoogeveen, A.T. (1993) Characterization and localization of the *FMR1* gene product associated with fragile X syndrome. *Nature* **363**: 722-724.

The Dutch-Belgian Fragile X Consortium. (1994) *Fmr1* knock out mice: a model to study fragile X mental retardation. *Cell* **78**:23-33.

Verheij, C., de Graaff, E., Bakker, C.E., Willemsen, R., Willems, P.J., Meijer, N., Galjaard, H., Reuser, A.J.J., Oostra, B.A., Hoogeveen, A.T. (1995) Characterization of *FMR1* proteins isolated from different tissues. *Hum Mol Genet* **4(5)**: 895-901.

Smeets, H.J.M., Smits, A.P.T., Verheij, C., Theelen, J.P.G., Willemsen, R., van de Burgt, I., Hoogeveen, A.T., Oosterwijk J.C., Oostra, B.A. (1995) Normal phenotype in two brothers with a full mutation. *Hum Mol Genet* **4(11)**: 2103-2108.

Tamanini, F., Meijer, N., Verheij, C., Willems, P.J., Galjaard, H., Oostra, B.A., Hoogeveen, A.T. (1996) FMRP is associated to the ribosomes via RNA. *Hum Mol Genet* **5(6)**:809-814.

De Vries, B.A, Jansen, C.A.M., Duits, A., Verheij, C., Willemsen, R., van Hemel, J.O., van den Ouweland, A.M.W., Niermeijer, M.F., Oostra, B.A., Halley D.J.J. (1996) Variable *FMR1* gene methylation of large expensions leads to variable phenotype in 3 males from one fragile X family. *J Med Genet* in press.

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Het fragiele X eiwitwerk is begonnen op in het lab op de zevende verdieping: kamer 706. Joke, ik had me geen betere inwerkster kunnen dromen dan jij, die kampioen logische proeven doen en planning bent. Elly (nu ook vrolijk op de 24e), (C-lab)-Bob, Hikke (gras in de vensterbank), Jan (zijn jullie met ether bezig?), Martina, Pim, Ragonda (is je haar er al aan gewend?) en Richard zorgden voor de goede sfeer aldaar.

In 1993 is een aantal van ons verhuisd naar het eiwitlab op de 24e verdieping. Kamer 2453: het was mij een waar genoegen, ik vind het jammer dat mijn tijd er op zit, want het wordt steeds gezelliger. Cécile (from Breda), altijd 'moving and alive' en altijd in voor een babbel. Frans (parkeergarage ergenissen), alleen jij kunt met zo'n uitgestreken smoelwerk de mensen in de maling nemen. Adri, Manou en Mieke (wanneer gaan we skwozen), sorry maar de deur moest af en toe wel eens dicht (te gezellig). Arnold (bom ontploft?! welnee, zo ziet zijn kamer er altijd uit): gek, maar niet gevaarlijk. Nicolle (peptide), bedankt voor het wassen van de blotjes en alle andere hulp. Marian en Elly: jullie recepties zijn waanzinnig goed! Agnes (nog groter thee-monster), Esther (de Andere), Dik, Jan, Martin, Mirjam, Henk-Jan (jammer dat onze 'Arnold' inzending niet heeft gewonnen), alle studenten: bedankt voor alles.

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Stellingen behorend bij het proefschrift

Characterization of the *FMR1* protein involved in the fragile X syndrome

1. Voor het fragiele X syndroom geldt: hoe beter de detectie, hoe lager de incidentie.

   Webb et al., Am. J. Med. Genet. 23, 573-580, 1986.
   Gustavson et al., Am. J. Med. Genet. 23, 581-587, 1986.
   Turner et al., Am. J. Med. Genet. 64, 196-197, 1996.
   Murray et al., Hum. Mol. Genet. 5, 727-735, 1996.

2. Hoewel het belang van de KH domeinen voor de RNA binding duidelijk is, mag de rol van de RGG box hierin niet worden genegeerd.

   Siomi et al., Cell 74, 291-298, 1993
   Dit proefschrift.

3. De naamgeving van genen draagt vaak niet bij tot een beter begrip van de processen waarbij deze genen zijn betrokken.

   MacDonald et al., Cell 72, 971-983, 1993.
   Lock et al., Cell 84, 23-24, 1996.

4. De noodzaak tot het ontwikkelen van een anticonceptiepil voor mannen neemt af naarmate de concentratie van gechloreerde koolwaterstoffen in het milieu toeneemt.

   Sharpe et al., Lancet 341, 1392-1395, 1993.
   Guillette et al., Envir. Hlth. Persp. 102, 680-688, 1994.
   Kelce et al., Nature 375, 581-585, 1995.

5. Aangezien 2-8% van de mensen allergieën vertoont voor voedingstoffen, moet voorzichtigheid in acht worden genomen bij de introductie van soortvreemde genen in planten.

   Sampson, JAMA, 268, 2840-2844, 1992.
   Nordlee et al., New.Engl. J. Med. 334 (11), 688-692, 1996.

6. Wanneer het proces van imprinting wordt onderzocht met behulp van transgene dieren moet niet alleen de samenstelling van het ingebrachte materiaal in acht worden genomen, maar ook de plaats van integratie.

Matsuura et al., Hum. Mol. Genet. 5, 441-450, 1996
Heard et al., Hum. Mol. Genet. 5, 451-459, 1996.

7. Daar de sequentie van een gen doorgaans wel de aminozuurvolgorde maar niet de functie van het eiwit prijs geeft, zijn de verwachtingen omtrent het Human Genome Project te hoog gespannen.

Chen, Science 270, 789-791, 1995.
Banfi, Nature Genet. 7, 513-519, 1994.

8. Bij vergaderingen zou moeten gelden dat de daaraan gespendeerde kwali-tijd belangrijker is dan de kwanti-tijd.

9. Veni, vidi, scribi.

10. Zeg nooit nooit en vermijdt altijd altijd.

11. Hoe langer politici spreken, hoe minder ze zeggen.

12. De weergave van wetenschappelijk nieuws in de krant geeft te denken over de juistheid van de berichtgeving in het algemeen.

13. Het openbaar vervoer zal pas dan tot bloei komen wanneer de brandstof reserves dusdanig zijn geslonken dat autorijden niet meer betaalbaar is. Het wachten is dus op het moment dat de Chinezen auto's aanschaffen.

14. Het grote aantal, zich steeds weer verplaatsende bouwputten in Rotterdam heeft als doel de bezoekers op een dwaalspoor te brengen.

Rotterdam, 9 oktober 1996
Coleta Verheij