Molecular mechanisms of fragile X syndrome

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Abstract : Fragile X syndrome is the most common form of inherited mental retardation. Mutations which abolish expression of an X-linked gene, FMR1, result in pathogenesis of the disease. FMR1 encodes a cytoplasmic RNA-binding protein which interacts with two autosomal homologs, FXR1 and FXR2. These proteins are highly expressed in neurons. In addition, the FMR1/FXR proteins are associated with ribosomes. Given their RNA-binding activity and association with ribosomes, these proteins are hypothesized to bind to specific RNAs and regulate their expression at translational levels in a manner critical for correct development of neurons. Much progress has been made in FMR1 research over the past several years, but little light has yet to be shed on the physiological function of these proteins. It will be critical to define the biochemical properties of these proteins, and identify potential downstream targets to clarify the molecular mechanisms underlying the potential roles of these proteins in translation. A basic understanding of the function of this new family of RNA-binding proteins should then allow us to begin to address the question of how the lack of FMR1 expression leads to symptoms in fragile X syndrome. J. Med. Invest. 47 : 101-107, 2000

Key words **:** *fragile X syndrome, RNA-binding protein, translation*

Fragile X Syndrome

Fragile X syndrome is the single most common form of inherited mental retardation, and after Down's syndrome, the most common identified form of mental retardation (1-3). It occurs in all ethnic groups. Estimates of the frequency of the disorder, based on cytogenetic testing, suggested a prevalence of one in 1,250 males and one in 2,000 females, but more recent studies based on direct detection of the mutation indicate a more conservative figure of one in 5,000. Nevertheless, it is the most frequent cause of inherited mental retardation. Affected individuals suffer from moderate to severe mental retardation and various degrees of autistic behavior. The clinical features also include an elongated facial appearance, wide prominent ears and enlarged testicles (macroorchidism) in adult male patients (4). Individuals with the syndrome show neuropathological abnormalities such as thin, elongated dendritic spines and enlargement of the hippocampus, caudate nucleus, lateral ventricle, and thalamus (5). Even height, occipitofrontal circumference, and sometimes weight are increased, especially before puberty. Therefore, fragile X syndrome is sometimes considered to be an 'overgrowth syndrome'.

As the name implies, this syndrome is an X-linked genetic disorder and is frequently associated with the expression of a folate sensitive fragile site, or a gap observed in metaphase stains of the X chromosomes of affected individuals. The fragile site has been mapped to region Xq27.3 on the long arm of the X chromosome (1-3). Using a positional cloning strategy, the FMR1 gene was isolated and the mutational basis of the syndrome was determined to be the expansion of a trinucleotide repeat (CGG)n present in the 5' untranslated region of the identified gene (6-8). FMR 1 is the first cloned gene, which has been liked to human intelligence. The inheritance of fragile X syndrome had puzzled geneticists for some years for two unusual inheritance patterns, which fail to conform to the laws of Mendel

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(1-3). First, the risk of having the disease increases in successive generations of a family (the so-called Sherman paradox or genetic anticipation). Second, phenotypically normal males produce descendants with the fragile X syndrome. We now know that the unusual inheritance patterns are due to the trinucleotide repeat expansion. While in normal individuals the CGG repeat length is polymorphic, ranging from 5 to 50 copies with an average number of 30, unaffected male carriers possess between 50 and 200 repeats (premutation) : in nearly all cases of fragile X patients, there is a large expansion in the number of the CGG repeats, to more than 200 copies, and sometimes to more than a thousand copies. Transmission of premutation alleles from males always results in premutation-sized alleles in their daughters, accounting for the lack of affected daughters of transmitting males. When females contribute premutation alleles to their offspring, increases in size prevail over decreases. The tendency for premutation-sized alleles to increase in length, coupled with the escalating risk of transmission to full mutation, provides a molecular explanation for the Sherman paradox of increasing penetrance. This finding has led to the discovery of a new dynamic mutation mechanism, the so called trinucleotide repeat expansion, which is responsible for a number of human genetic neurological disorders including Huntington's disease and spinocerebellar ataxia (9, 10). This CGG repeat expansion is associated with abnormal DNA methylation of both a nearby CpG island and the repeat itself (1-3). As a result, the FMR1 locus becomes silent at the transcriptional level and, thus, no translation occurs (11-13) (Figure 1). Although the timing and molecular mechanisms of the CGG repeat expansion and methylation on expanded CGG repeat are under extensive study, we do not know much about when and how CGG repeat instability and DNA methylation occur.

It has become clear that fragile X syndrome results from the lack of FMR1 expression. In other words, the cause of fragile X syndrome is a loss-of-function of FMR1. This has been confirmed by the Dutch-Belgian Consortium who generated transgenic mice models by targeted disruption (knockout) of the mouse *Fmr1* gene (14, 15). The *Fmr1* knockout mice show enlarged testes, abnormal dendritic spine morphology, impaired cognitive function and abnormal behavior, similar to humans with fragile X. The development of the *Fmr1* knockout mice was a critical advance in elucidating the pathological mechanisms that lead to mental retardation and behavioral abnormalities in fragile X syndrome. This animal model might also be useful for developing therapeutic strategies. However, as yet, very little is known about the physiological function of FMR1.

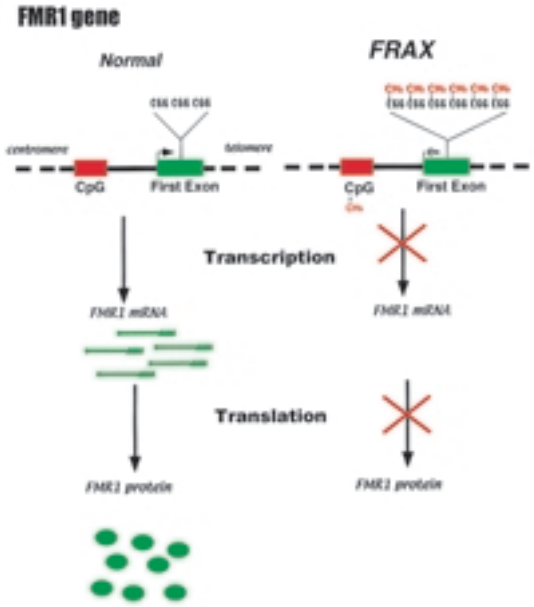

Fig. 1. Fragile X syndrome is caused by the lack of transcription due to the expansion of the CGG repeat located in the 5' UTR of the FMR1 gene. Fragile X syndrome results from a massive trinucleotide repeat expansion. As a result of the repeat expansion, abnormal methylation of both a nearby CpG island and the repeat itself takes place, causing transcriptional silencing of FMR1.

The FMR1 Gene

Although a strong sequence conservation between fruitfly (16), *Xenopus* (17), chicken (18), mouse (19) and human (7) FMR1 cDNAs suggest that it may have a "housekeeping" role, its physiological function remains a mystery. One clue as to the function of the FMR1 protein comes from the identification in the protein sequence of motifs characteristic of RNA-binding proteins. We and the other group found that the protein contains an RGG box and two KH domains (13, 20) (Figure 2a). The RGG box is a region of 20 to 25 amino acids containing several Arg-Gly-Gly tripeptide repeats and this motif has been found in numerous RNA-binding proteins (21). The KH domain was discovered on the basis of primary sequence similarity between the pre-mRNA-binding protein hnRNP K and other RNA associated proteins such as ribosomal protein S3, bacterial antiterminator protein NusA, and a yeast meiosis-specific alternative splicing factor *Mer-1* and, therefore, it was named K homology domain or KH domain (22, 23). The hallmark of the KH domain is a strongly conserved tetrapeptide Gly-x-x-Gly (x is often a positively charged

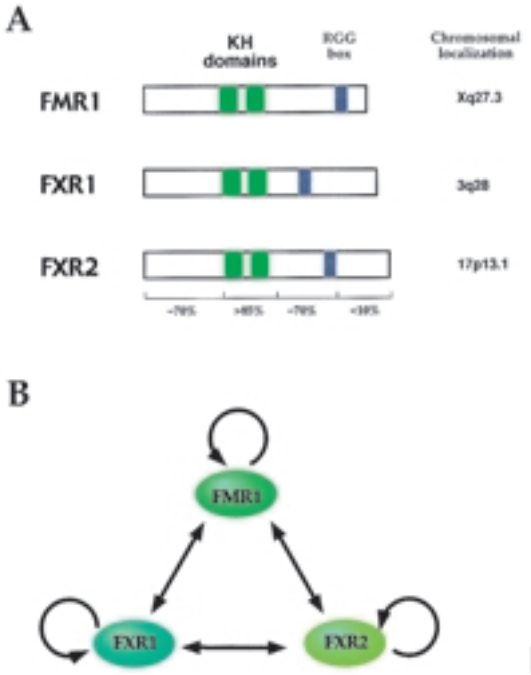

Fig. 2. The FMR1 family. A, Graphical alignment of peptide sequences for FMR1 and homologues FXR1 and FXR2. This family of RNA-binding proteins contains two types of RNA-binding motifs, namely RGG box and KH domain. Note the high similarity through the KH domains. B, A model summarizing the observed interactions among the FMR1, FXR1 and FXR2.

amino acid), which is flanked with regularly spaced hydrophobic residues within a region of 60-80 amino acids (23 and 24). Many other proteins that contain KH domains, such as Gld-1, Hex3 and *quaking* have been shown to have extremely important roles in development. Two KH domains are located in the middle of the FMR1 protein and an RGG box lies downstream of the KH domains. The RNA-binding domains in FMR1 appear to be functional because FMR1 was demonstrated to directly bind RNA *in vitro* with some degree of sequence specificity. We demonstrated that the *in vitro* translated FMR1 protein can bind to poly(G) and poly(U), synthetic RNA homopolymers in a salt resistance manner (13). Ashley *et al*. provided evidence that the FMR1 protein selectively binds to a subset of brain-derived RNA, with approximately 4% by mass of human fetal brain message specifically binding FMR1 protein (20). These findings suggested that FMR1 post-transcriptionally regulates expression of downstream genes that are critical for normal development of intelligence. Since fragile X syndrome is often associated with overgrowth phenotypes, potential target genes may also encode growth-regulatory proteins, and regulation of these genes by FMR1 may affect the control of cell proliferation. However, cellular RNA targets of FMR1 have not yet been identified. Identification of the RNA targets of FMR1 protein will be an important step toward understanding how the absence of a single protein results in the pleiotropic phenotype observed in fragile X syndrome.

FMR1 Interacting Proteins

It appears clear that a better understanding of the function of FMR1 also requires identification of proteins with which FMR1 protein may interact. To identify such interacting proteins, we used the yeast two hybrid system and isolated two novel cDNAs for proteins which specifically interact with FMR1 (25). Surprisingly, these FMR1 interacting genes are very similar in overall structure to FMR1; they share~ 60% sequence homology with FMR1 (Figure 2A). Therefore, the two genes were named FXR1 and FXR2 (FXR for fragile X related genes). The importance of these genes is reflected in the conservation of gene sequence from Xenopus to human. Given the high degree of sequence similarity among these proteins, it is not surprising that both FXR1 and FXR2 proteins are also capable of binding to poly(G) and poly(U) *in vitro*. We have also demonstrated, by means of a co-immunoprecipitation assay, that FMR1 protein can form heterodimers with FXR1 and FXR2 proteins in living cells (25 and data not shown). Further characterization of the interactions among the family members using glutathione-S-transferase (GST) fusion proteins reveals that every family member can form dimers with every other family member and with itself (25) (Figure 2B). These findings clearly indicate that the three proteins form a gene family of proteins related by structure and most likely by function, as they interact with each other. Whatever the outcome of this interaction, FMR1 can no longer be considered to function alone but likely has, in FXR1 and FXR2, partners to perform its normal function, which is presumably critical for the development of intelligence. This interaction could be a mechanism of functional autoregulation of this new family of RNA-binding proteins. For example, FXR1 and FXR2 could modulate RNA binding specificity and/or affinity through a direct interaction with FMR1. It is also possible that the free FMR1 protein may be poorly configured for binding to RNA in the absence of FXR proteins. There is precedence from two other RNA-binding protein systems for a decisive influence of the protein-protein interaction on the RNA binding of the proteins. The signal recognition particle (SRP) is made up of six proteins and one

RNA (7SL). Two RNA-binding SRP proteins, SRP9 and SRP14, bind 7SL RNA efficiently only when both are present in a complex with each other (26). The mammalian U2 snRNA-binding proteins, U2B'' and U2A' also form a complex in the absence of RNA (27). Neither of these proteins is capable on their own of specific interaction with U2 RNA, although U2B'' binds RNA in a relatively non-specific manner and U2A' interacts weakly with double-stranded RNA. Formation of the U2A' : U2B'' complex is thought to alter the conformation of U2B'' in a manner that greatly enhances its specific binding to the U2 snRNA element and also reduces its non-specific binding to U1 snRNA, a different class of snRNA. Notably, both U2B'' and U2A' proteins are very similar in overall structure as is the case for the FMR1/FXR family.

Protein-protein interactions are intrinsic to virtually every cellular process. There are a large number of transient protein-protein interactions, which in turn control a large number of cellular processes. All modifications of proteins necessarily involve such transient protein-protein interactions. These include the interactions of protein kinases, protein phosphatases, glycosyl transferases, etc., with their substrate proteins. Such protein-modifying enzymes encompass a large number of protein-protein interactions in the cell and regulate all manner of fundamental processes such as cell growth, cell cycle and signal transduction. We have demonstrated the interactions among the FMR1 family members. This raises the possibility that FMR1 might function in a complex with FXR1, FXR2 or both. It is of interest to determine whether complex formation or function is regulated by other factors, such as phosphorylation, or potential interactions with other cellular components. In particular, the question of what can abrogate or dissociate the interactions among the FMR1 family proteins may be as important as the originally identified interaction partners.

Given the strong overall similarity and similar biological properties, this family of RNA-binding proteins is likely to show partial overlap in their function and these proteins might be functionally redundant in some cellular processes. However, it is already clear that FXR1 and FXR2 cannot fully complement the lack of FMR1 function in the fragile X patients, since expression of FXR1 and FXR2 does not appear to be effected in fragile X patients (17, 25). Thus they may have both partially complementary functions and independent functions. It can be anticipated that, as is the case for FMR1, hitherto unknown genetic disorders caused by aberrant expression of the FXR genes exist. In contrast to FMR1, both FXR1 and FXR2 are encoded by autosomal genes (3q28 and 17p13, respectively : 17, 25 and 28). To our knowledge, no human genetic disorder genes including mental retardation genes have been mapped in their regions of the genome. Therefore, it can also be assumed that if FXR1 and FXR2 have independent functions which are essential during early development, most mutations of human FXR1 and FXR2 would be early embryonic lethal. In either case, it is of great interest in determining whether or not loss of the FXR1 or FXR2 function is linked to mental retardation and/or other abnormalities.

Ribosomal Association of FMR1 Family Proteins

In addition to the interaction between the family members, we have demonstrated an association of this family of RNA-binding proteins with ribosomes (29). Simultaneously and independently, several other groups also reported that FMR1 protein is associated with ribosomes in an RNA-dependent manner (30-33). Although initially the FMR1 protein was interpreted to be associated with the 60S large ribosomal subunit based upon cofractionation, the more recent findings showed that FMR1 protein is associated with elongating polyribosomes via large mRNP particles (31, 33). However, these findings do not eliminate the possible interactions between FMR1 protein-mRNP particles and the 60S ribosomal subunit *in vivo* during various stages of translation, because FMR1 protein has been found to co-immunoprecipitate with the 60S subunit. Based upon the RNA-binding activity and the ribosomal association, a hypothesis has been raised that the FMR1 and FXR proteins may have roles in translation. There are at least two possible scenarios about how these proteins translationally regulate expression of their target mRNAs (Figure 3). First, since these proteins are RNA-binding proteins with some sequence specificity and associate ribosomes as mRNA-protein (mRNP) complex, FMR1 and FXR proteins act as chaperones or selectors for recruiting specific mRNAs onto the ribosomes. Second, since considerable amounts of FMR1 and FXR proteins are found in soluble fractions, the distribution of these proteins between free and ribosome-associated states may give rise to structural heterogeneity of ribosomes with regard to their complement of these proteins, which, in

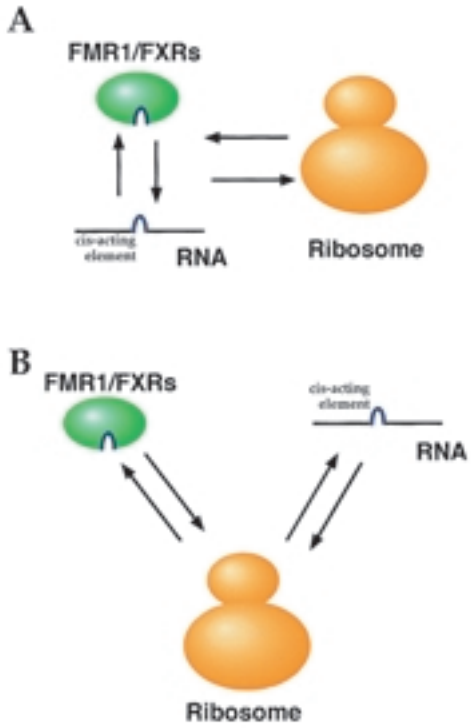

Fig. 3. Possible function of the FMR1 family in translation. A, A 'direct selector' model for the role of FMR1/FXR proteins in translation. In this model, FMR1 and FXR proteins act as chaperones or selectors for recruiting specific mRNAs onto the ribosomes. B, A 'indirect selector' model. The distribution of FMR1/FXR proteins between free and ribosome-associated states may give rise to structural heterogeneity of ribosomes with regard to their complement of these proteins, which, in turn, influences mRNA selection.

turn, influences mRNA selection. The former scenario envisions that these proteins have specific binding sites on a class of mRNAs and regulate their translation through their binding. This is akin to *Drosophila* morphogen *bicoid* (bcd). bcd directly binds to the discrete target sequences within the 3' untranslated regions (UTR) of the homeodomain protein caudal mRNA and regulates, (in this case, represses) the translation of the mRNA (34). This class of RNA-binding proteins includes *C. elegans fem*-3 binding factor (35) and mammalian hnRNP K (36). The second model is related to the *E. coli* ribosomal protein S2 (Eco S2). Eco S2 is one of the few ribosomal proteins that exchanges on and off the ribosome, which gives rise to structural heterogeneity of ribosomes (37, 38). While ribosomes containing Eco S2 efficiently translate mRNAs containing Shine-Dalgarno sequences in the 5' leader sequences probably by affecting base-pairing between the leader sequences and the 16S rRNA, ribosomes lacking Eco S2 preferentially translate such leaderless mRNAs that are normally translated inefficiently. In eukaryotes, base-pairing interactions between 18S rRNA and mRNA has been implicated in cap-independent initiation. Therefore, FMR1 family proteins could regulate translation of a class of mRNAs without directly binding to them in such a way that FMR1 family proteins mask sites in rRNA needed for preferential translation of a subset of mRNAs, and loss of the FMR1 protein in fragile X patients unmasks or modifies the sites and, thus, influences translation of the mRNAs. In either case, to understand the mechanisms underlying the potential role of FMR1 and FXR proteins in translation, it will be important to identify biologically important genes that are translationally regulated by the FMR1 and FXR proteins.

The Functions of FMR1 and FXR Proteins

The expectation is that FMR1 and FXR proteins, being ribosome-associated and RNA-binding proteins with some sequence specificity, have important roles in the translation of some specific mRNAs. However, little is known about the specific function of this new family of RNA-binding proteins. A major problem in elucidating the function of FMR1 family proteins has been the inability to identify a downstream gene(s) whose expression may be regulated by this family of proteins. To some extent this is partly because biochemical characterization of these proteins, such as extensive cell fractionation, has only recently begun, and partly because the probes for the individual proteins have only recently been obtained. Furthermore, since FMR1 is clearly not essential for general translation, it is possible that the effect of the lack of the FMR1 protein may be subtle in *in vitro* assays that have been commonly used for study of translation such as rabbit reticulocyte lysates. It is also possible that there may be enough functional redundancy between FMR1 and FXR proteins, so that, in these *in vitro* assays, their function will be observed only when two or all of the FMR1 and FXR proteins are completely removed from the test fractions. However, the situation is very different *in vivo*, where a subtle difference in protein concentration often appears sufficient to distinguish between on or off states of gene expression, as is the case for morphogens in *Drosophila* (39). It is also possible that FMR1 and FXR proteins have an individual function that may be either similar or distinct, but together they promote high fidelity for some process such as those required for neuronal development. As described above, several observations are, nevertheless, strongly suggestive of a role for FMR1 and FXR proteins in translational

regulation of gene expression. Identification of the target genes of FMR1 family proteins should help to clarify the function of this family and, thus, the mechanisms leading to fragile X syndrome, and may also suggest new approaches to developing therapeutic strategies for the syndrome.

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