THE ROLE OF THE *FMR1* PROTEIN INVOLVED IN FRAGILE X SYNDROME

DE ROL VAN HET FMR1 EIWIT BETROKKEN BIJ HET FRAGILE X SYNDROOM

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Filippo Tamanini
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Promotoren :	Prof. Dr. H. Galjaard Prof. Dr. B.A. Oostra
Overige leden:	Prof. Dr. R.R. Frants Prof. Dr. P.J. Willems Prof. Dr. C.I. de Zeeuw
Copromotor :	Dr. A.T. Hoogeveen

Omslag: Tom de Vries Lentsch

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In memoria di mio padre

Per Alex & co.,
mia mamma e Nicola

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Chapter 1

INTRODUCTION

1.1 Clinical aspects of the Fragile X syndrome

In 1943, Martin and Bell described a family with a form of mental retardation linked to the X chromosome (Martin and Bell, 1943). Later, an association between the Martin-Bell syndrome and a fragile site at Xq27.3 (FRAXA) was described (Lubs, 1969). Reproducible detection of the fragile site was made possible by culturing of patients' cells in medium depleted of folic acid, and this technique provided a reliable cytogenetic marker for the Martin-Bell or fragile X syndrome (Sutherland *et al.*, 1977). Recent estimates from molecular studies indicate a prevalence of 1 in 6000 affected males (Turner *et al.*, 1996; De Vries *et al.*, 1997a).

A typical adult male with fragile X syndrome has mental retardation (100% of cases), abnormal behavior, enlarged testicles (macroorchidism) (70%), long face (70%) and prominent ears (70%) (Hagerman *et al.*, 1996; De Vries *et al.*, 1998). The long face is more common in adulthood than in childhood and is frequently associated with high arched palate. Evidence for connective tissue dysplasia in fragile X patients includes hyperextensible joints (67%), hand calluses (29%) and flat feet (71%) (Hagerman *et al.*, 1984; Opitz *et al.*, 1984).

Mental retardation in fragile X patients can vary from mild to severe (De Vries *et al.*, 1993). The most frequent behavioral problems are hyperactivity, decreased attention span and a number of autistiform features including abnormal responses to stimuli (sound, smell), repetitive behavior, poor eye contact, and shyness. However, no significant difference in autistiform behavior is observed in fragile X patients as compared with other mentally retarded individuals (Hagerman *et al.*, 1991; Fisch *et al.*, 1992; Fisch *et al.*, 1993). Seizures occur in 13-23% of males patients, mostly before adulthood (Partington *et al.*, 1984; Musumeci *et al.*, 1991; Wisniewski *et al.*, 1991; Hagerman *et al.*, 1996). These observations suggest an impaired neuronal excitability in the central nervous system (CNS) of fragile X patients. Screening lists to facilitate the clinical diagnosis based on physical and behavioral features have been developed and found to be effective (Hagerman *et al.*, 1991; De Vries *et al.*, 1997a). For a more detailed description of the clinical aspects see de Vries (1997b).

1.2 The *FMR1* gene

The gene involved in the fragile X syndrome was cloned in 1991 and named *FMR1* for Fragile X Mental Retardation (Verkerk *et al.*, 1991). For a detailed description of the isolation of the *FMR1* gene see Verkerk (1994).

1.2.1 General characteristics

The *FMR1* mRNA is ~ 4 kb long, consisting of 1.9 kb coding sequence, 1.8 kb 3'-untranslated region, and ~ 0.2 kb 5' untranslated region (Verkerk *et al.*, 1991). A CGG trinucleotide repeat, which is amplified in the majority of fragile X patients (see below), is located within the 5'-untranslated region of the gene (Verkerk *et al.*, 1991). The *FMR1* gene includes 17 exons (Eichler *et al.*, 1993), of which exons 12, 14, 15 and 17 show alternative

splicing, generating a repertoire of many different potential mRNAs and protein isoforms (Ashley *et al.*, 1993a; Verkerk *et al.*, 1993). The entire *FMR1* locus spans 40 kb of genomic sequence and is transcribed from centromere to telomere. Hergersberg *et al.* (1995) showed that all the necessary elements for a proper *FMR1* expression *in vivo* are present in a 2.8 kb fragment including most of the 5' region of *FMR1* and the CGG repeat.

FMR1 is expressed in many different human and murine tissues, with particularly high levels in brain and testis, which are the tissues most affected in fragile X patients (Abitbol *et al.*, 1993; Hinds *et al.*, 1993). Consistent with these studies, footprinting analysis of the *FMR1* promoter identified consensus-binding sites for transcription factors expressed specifically in neurons (AP-2) as well as in all tissues (Sp1, H4TF1/Sp1-like, c-myc) (Drouin *et al.*, 1997; Schwemmle *et al.*, 1997). RNA *in situ* hybridization reveals that *Fmr1* expression is high in all tissues of mouse embryos at 10 d.p.c. The levels of expression diminish in later embryonic stages and become more localized in adult tissues, with heart, muscle and aorta not showing *Fmr1* expression (Hinds *et al.*, 1993).

FMR1 orthologues have been characterized from monkey (Eichler *et al.*, 1995), mouse (Ashley *et al.*, 1993a), chicken (Price *et al.*, 1996) and frog (Siomi *et al.*, 1995a). The murine and frog amino acid sequences are 97% and 86% identical to the human sequence, respectively. Strikingly, the nucleotide sequences of both the 5'-untranslated region (including the CGG repeat) and the 3'-untranslated region are conserved between the human *FMR1* and mouse *Fmr1* genes. The simple multi-cellular organism, *C. elegans*, as well as the uni-cellular yeast *S. Cerevisiae* do not have obvious *FMR1* orthologues as comparative searches in the total genome databases indicate. The studies in Fugu, Zebrafish and Drosophila might help to determine the phylogenetic origin of the *FMR1* gene more precisely.

1.2.2 CGG repeat amplification

In the vast majority of fragile X patients the CGG repeat in the 5'-untranslated region of the *FMR1* gene is greatly expanded (Fu *et al.*, 1991; Kremer *et al.*, 1991; Oberlé *et al.*, 1991; Verkerk *et al.*, 1991). Mutations in the coding region are rare (Gedeon *et al.*, 1992; De Boulle *et al.*, 1993; Meijer *et al.*, 1994; Lugenbeel *et al.*, 1995; De Graaff *et al.*, 1996). In the normal population the CGG repeat is polymorphic and varies from 5 to 53 units with an average of 30 (Fu *et al.*, 1991). These alleles are stably transmitted to the next generation. In contrast, the repeat is unstable within fragile X families, and two types of mutations can be distinguished: the premutation (43 to 200 CGG repeats) and the full mutation (>200 CGG repeats) (see Figure 1).

The premutation causes no phenotypic abnormalities in carriers (Fu *et al.*, 1991), since it does not affect transcription (Feng *et al.*, 1995) and translation of the *FMR1* gene (Devys *et al.*, 1993; Verheij *et al.*, 1993). However, the premutation is unstable and, depending on the sex of the carrier, can expand to a full mutation in subsequent generations (Fu *et al.*, 1991; Kremer *et al.*, 1991). Only carrier females have a risk of having affected offspring with a full mutation. Carrier males (normal transmitting males or NMT) always transmit a premutation to their daughters, indicating that the repeat is less unstable upon male

transmission (Fu *et al.*, 1991; Oberlé *et al.*, 1991). The risk of expansion of the CGG repeat from a premutation to a full mutation is directly dependent on the size of the maternal premutation. The risk is <5% with an allele of 60 repeats, while it is >95% for an *FMR1* allele with more than 100 repeats (Fu *et al.*, 1991; Oberlé *et al.*, 1991; Heitz *et al.*, 1992; Fisch *et al.*, 1995).

The presence of a full mutation coincides with hypermethylation of the CGG repeat and the *FMR1* promoter (Hansen *et al.*, 1992; Hornstra et al., 1993). This hypermethylation results in the repression of *FMR1* transcription (Pieretti *et al.*, 1991) and subsequent absence of the *FMR1* protein (Devys *et al.*, 1993; Verheij *et al.*, 1993). The lack of *FMR1* protein is accepted to be the cause of the fragile X syndrome.

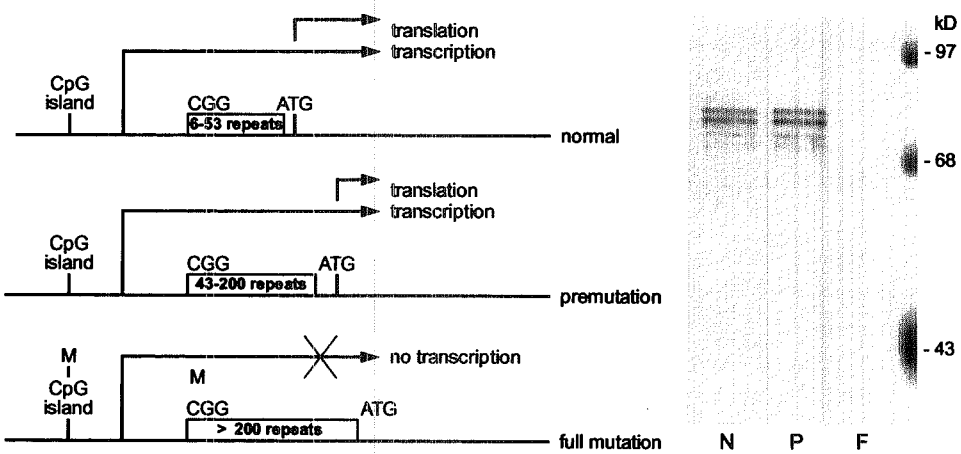

***Figure 1. FMR1* transcription (left) and translation (right).**

Normal and premutation alleles have normal transcription and translation of the *FMR1* protein. Hypermethylation of the promoter region prevents transcription of the full mutation allele.

Chiurazzi *et al.* (1998) have demonstrated that *in vitro* demethylation of the *FMR1* promoter in full mutations can lead to re-expression of the gene and subsequent production of the *FMR1* protein. This provided additional evidence that methylation plays an important role in controlling expression of the *FMR1* gene.

Around 20-30% of fragile X males have a mosaic DNA pattern with a premutation together with a full mutation (Nolin *et al.*, 1994; Rousseau *et al.*, 1994a), whereas they show *FMR1* expression in approximately 10% of their lymphocytes. However, no IQ (cognitive function) differences have been found between mosaic males and males with only a full mutation (De Vries *et al.*, 1993; Rousseau *et al.*, 1994a). Apparently, the proportion of brain neurons producing the *FMR1* protein in mosaic males is insufficient for a normal cognitive function. A possible indication of the proportion of cells expressing the *FMR1*, which are needed for a "normal" task, is shown by mentally normal males with unmethylated full mutations (and *FMR1* expression) in more than 60% of their leukocytes (McConkie-Rosell *et*

al., 1993; Rousseau *et al.*, 1994b; Smeets *et al.*, 1995; De Vries *et al.*, 1996; Wang *et al.*, 1996). However, these studies do not show whether brain cells have a comparable methylation and *FMR1* expression status.

Although the instability of the CGG repeat has been widely described, the timing of the repeat expansion is less clear. Intact ovaries from a fragile X fetuses (16 weeks) only contain expanded unmethylated alleles and no premutations, although oocytes have not been studied separately (Malter *et al.*, 1997). Thus, full expansion of the CGG repeat from female carriers to their offspring may already have occurred in the pre-zygotic state. Alternatively, post-zygotic expansion of the repeat could occur very early in embryonic development, prior to segregation of the cells giving rise to the germ cells. Adult fragile X males who exhibit the fully expanded CGG repeat in all their somatic tissues, only have a premutation in their sperm (Reyniers *et al.*, 1993). This explains why males transmit only premutations to daughters.

1.3 The *Fmr1* knock-out mouse model

To facilitate the study of the function of the *FMR1* protein as well as the pathogenesis of the disease, an animal model for the fragile X syndrome was generated (Bakker *et al.*, 1994). The murine *Fmr1* gene was inactivated via homologous recombination (knock out). The *Fmr1* knock-out (KO) mouse does not express Fmrp, like in fragile X patients.

The KO mouse has characteristics in common with human fragile X patients, such as macroorchidism and abnormalities in cognitive function. Like in male patients, the testes are enlarged in KO mice, but no gross differences in testis morphology have been observed as compared to normal mice (Bakker *et al.*, 1994; Kooy *et al.*, 1996). It has been proposed that macro-orchidism in KO mice is caused by increased Sertoli cell proliferation during testis development (Slegtenhorst-Eegdeman K.E. *et al.*, 1998). Learning (tested in the Morris water maze) is delayed in KO mice (Bakker *et al.*, 1994; Kooy *et al.*, 1996; D'Hooge *et al.*, 1997). Recent studies suggest that the KO mice have audiogenic epileptic seizures (Musumeci *et al.*, submitted). This might indicate that absence of Fmrp results in an increased cortical excitability in affected mice.

The few neuropathological studies that have been carried out on brains of fragile X patients, show non-specific abnormalities, such as brain atrophy. In addition, the dendritic spines of pyramidal neurons are abnormal (Rudelli *et al.*, 1985; Hinton *et al.*, 1991; Wisniewski *et al.*, 1991). Similarly, the neurons of *Fmr1* KO mice contain immature dendritic spines that are often longer, thinner, and have a higher density along apical dendrites than in normal mice. This situation is reminiscent of that seen following sensory deprivation. These results point to a deficit in the normal selection or "pruning" of synaptic contacts during early synaptogenesis in the developing brain (Comery *et al.*, 1997).

Magnetic resonance imaging (MRI) of the brains of fragile X patients indicates a reduction in the size of the posterior cerebellar vermis and an increase in the size of the fourth

ventricle (Reiss *et al.*, 1995). However, no gross abnormalities in brain volume have been observed in *Fmr1* KO mice by MRI (Kooy, pers. communication)

1.4 The *FMR1* protein (FMRP)

The isolation of the *FMR1* gene and the discovery of the expanded CGG repeat resolved several questions about the (molecular) genetic basis of the fragile X syndrome. It also has greatly improved the laboratory diagnosis of patients, and the genetic counseling. At the same time, new questions arose about the function of the *FMR1* protein and its role in the pathogenesis of mental retardation.

1.4.1 General characteristics

The nucleotide sequence of the longest *FMR1* splice variant predicts a protein of 614 amino acids. Using antibodies directed against FMRP (for Fragile X Mental Retardation Protein), several proteins with a molecular mass around 70-80 kDa have been detected on SDS-PAGE denaturing gel by Western blotting (Verheij *et al.*, 1993). Most likely, these protein isoforms represent different splice variants of *FMR1* rather then post-translationally modified FMRP isoforms, as the *FMR1* sequence does not contain obvious glycosylation and/or phosphorylation sites. All of the tested tissues show the same protein pattern. None of these proteins were observed in fragile X patients (Devys *et al.*, 1993; Verheij *et al.*, 1993).

Immunohistochemical analysis revealed that FMRP is predominantly localized in the cytoplasm with a diffuse granular staining pattern (Devys *et al.*, 1993; Verheij *et al.*, 1993). In brain, FMRP is expressed in differentiated neurons, but is absent from non-neuronal cells (Devys *et al.*, 1993). Neuronal FMRP is concentrated in the cell body (perikaryon) and proximal dendrites, but no FMRP is detected in the axons (Feng *et al.*, 1997a; Tamanini *et al.*, 1997). FMRP is also found at the synapses. Intriguingly, glutamate receptor stimulation of a crude synaptosomal preparation leads to rapid synthesis of FMRP. It has been suggested that this (increased) synthesis may be important for the normal maturation of synaptic connections (Weiler *et al.*, 1997).

In testis, FMRP expression is restricted to the cytoplasm of primordial germ cells and spermatogonia surrounding the tubuli seminiferi (Tamanini *et al.*, 1997). Although such specific expression might suggest an important role for FMRP in testis, spermatogenesis occurs normally in mice lacking *Fmrp* (Bakker *et al.*, 1994), indicating that Fmrp is not essential in spermatogenesis.

Since cells from fragile X patients with a full mutation produce no FMRP, a diagnostic antibody test was developed for detecting the presence or absence of FMRP. This rapid detection method allows the diagnosis of the fragile X syndrome either in lymphocytes from a blood smear (Willemsen *et al.*, 1995), or hair bulbs (Willemsen *et al.*, submitted), and prenatal diagnosis is possible via the protein test in chorionic villi and amniotic cells (Willemsen *et al.*, 1997).

1.4.2 RNA-binding properties

A peculiar patient with severe mental retardation and macroorchidism was shown to have a unique missense mutation in *FMR1*. This mutation replaces a conserved isoleucine (Ile-304) for asparagine (De Boulle *et al.*, 1993). Analysis of the amino acid sequence of FMRP around this point mutation revealed a RNA-binding domain (KH domain) (Gibson *et al.*, 1993a). Further analysis revealed the presence of two KH domains in the middle part and multiple RGG boxes at the carboxy terminus (Figure 2). A class of proteins with RNA-binding capacity was defined by sequence similarity, as they shared the arginine (R)- and glycine (G)-rich domain (RGG box) and the hnRNP K Homology domain (KH) (Mattaj,1993; Burd *et al.*,1994). This provided the first indication that FMRP could be a RNA-binding protein (Ashley *et al.*, 1993b; Siomi *et al.*, 1993a).

The RGG box has been found in a considerable number of nuclear and nucleolar RNA-binding proteins, and has been demonstrated to have RNA-binding activity per se (Kiledjian *et al.*, 1992). The RGG box domain of FMRP bears striking similarity to that found in the nucleolar protein fibrillarin (Siomi *et al.*, 1993a).

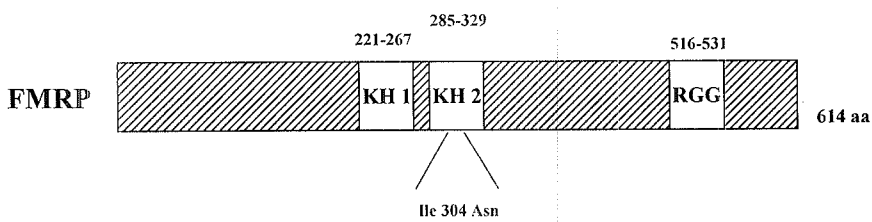

***Figure 2.* The RNA-binding domains of FMRP.**
Schematic presentation of the two central KH domains and the C-terminal RGG boxes of FMRP. The missense mutation found in a severe fragile X patient is indicated with an enlargement.

(The KH domain consists of a highly conserved I/L/V-I-G-X_2-G-X_2-I sequence and regularly spaced hydrophobic residues extending over ~60 amino acids, and was originally identified as a triple module in the heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Matunis *et al.*, 1992; Siomi *et al.*, 1993b). KH domains have been found in single or multiple copies in diverse proteins, which only common property is that they all function in close association with RNA. KH domain-containing proteins play a major role in regulating cellular RNA metabolism. Prokaryotic KH proteins include the ribonuclease PNP (Regnier *et al.*, 1987), the ribosomal protein S3 (Rinke-Appel *et al.*, 1991) and the transcription elongation factor NusA (Gibson *et al.*, 1993b). Eukaryotic KH proteins include the splicing modulator Mer1 in yeast (Engebrecht *et al.*, 1990), PSI in Drosophila (Siebel *et al.*, 1995), vigilin (Schmidt *et al.*, 1992), Bicaudal C (Mahone *et al.*, 1995), α-globin messenger RNP stability complex-αCp1 and αCp2 (Kiledjian *et al.*, 1995), Noval (Buckanovich *et al.*, 1993) and many others.

There is evidence that KH domains bind single-strand RNA, messenger RNA, ribosomal RNA and nucleic acid *in vitro*. Musco and co-workers (1996, 1997) were the first to establish the three-dimensional structure of the KH domain. Using nuclear magnetic resonance (NMR) spectroscopy, they found that KH-domain 6 of vigilin consists of a stable three-stranded antiparallel β-sheet, orientated against three α-helices. This stable βααββα fold exposes a potential surface for RNA-binding, which is a conserved tetrapeptide Gly-Lys-X-Gly centered on the loop between the first two helices.

The RNA-binding domains in FMRP appear to be functional for two reasons. Firstly, FMRP directly binds homopolymeric RNA (poly U and poly G) *in vitro* (Siomi *et al.*, 1993a) and has selectivity for a fraction of mRNAs expressed in brain, including its own mRNA (Ashley *et al.*, 1993b; Brown *et al.*, 1998). FMRP-RNA binding can be disrupted by treatment with 500 mM salt or by deletion of the C-terminus of the protein, including the RGG box domain (Siomi *et al.*, 1993a; Verheij *et al.*, 1995). The latter may be due to a misfolded protein with no ability to bind RNA. Secondly, the mutant FMRP with the Ile304Asn mutation has impaired RNA-binding capacity under elevated salt conditions (Siomi *et al.*, 1994; Verheij *et al.*, 1995). Furthermore, evidence derived from structural analysis indicates that this mutation disrupts the folding of the KH domain (Musco *et al.*, 1996). Developmental abnormalitieshave also been reported due to missense mutations in the KH domains of the C.*elegans* GLD-1 gene (Jones *et al.*, 1995) and the Drosophila Bicaudal-C gene (Mahone *et al.*, 1995).

At the moment, many laboratories focus on the identification of the RNAs which are bound by FMRP *in vivo*.

1.5 FMRP is associated with ribosomes

A further step towards the understanding of the function of FMRP came from cellular fractionation studies and biochemical analysis.

It has been reported that cellular FMRP co-sediments with translating ribosomes (polyribosomes) on sucrose gradients (Khandjian *et al.*, 1996). Therefore, FMRP is not a free protein in the cytoplasm. Immuno-electromicroscopy (EM) analysis of transfected COS cells which over-express FMRP (Willemsen *et al.*, 1996), and cortical rat neurons (Feng *et al.*, 1997a), have confirmed the close proximity of FMRP to free ribosomes, membrane bound ribosomes, and polyribosomes.

EDTA treatment removes mRNA from polyribosomes and dissociates the ribosome into small (40S) and large (60S) subunits. Such a treatment releases FMRP into complexes with a size range similar to the 60S ribosomal subunit on sucrose gradient (Khandjian *et al.*, 1996; Siomi *et al.*, 1996). Siomi *et al.* (1996) have suggested that FMRP binds the 60S ribosomal subunit and that the putative ribosomal binding site of FMRP includes parts of exons 13 and 14. Interestingly, the amino acids encoded by exon 14 are predicted to form a coiled coil domain (see Figure 3). Coiled coils are often involved in protein-protein interactions.

On the other hand, FMRP-ribosome association has been found to be sensitive to 500 mM salt as well as RNase digestion (Eberhart *et al.*, 1996; Tamanini *et al.*, 1996; Corbin *et al.*, 1997). Thus, it was proposed that FMRP binds the ribosomes via RNA (*publication 1).* Moreover, the groups of Khandjian and Warren found that FMRP, but not ribosomes, can be purified together with polyA mRNA on a oligo-dT column in the presence of EDTA. Based on these results, they suggest that FMRP is associated to the ribosomes as a messenger ribonuclearprotein (mRNP) particle, which has, by coincidence, a sedimentation value of 60S (Corbin *et al.*, 1997; Feng *et al.*, 1997b). These data, therefore, did not support a direct association between FMRP and the 60S ribosomal subunit.

In conclusion, several groups have detected an association of FMRP with ribosomes. Whether this link is mediated by protein-protein interaction, or it is a solely protein-RNA interaction, and if it so, which RNA is involved, is still a matter of discussion.

1.5.1 RNA-binding proteins and the ribosome

Protein synthesis is performed by the ribosome. The ribosome is a particle composed of both RNA and proteins. The eukaryotic ribosome consists of two subunits, the small 40S subunit and the large 60S subuit. The small subunit (S) consists of an 18S rRNA and ~33 proteins, while the large subunit (L) contains a 28S rRNA, a 5.8S rRNA, a 5S rRNA and ~45 proteins. All together the ribosome has a sedimentation value of 80S and a molecular mass of approximately 3 MDa (3 million Daltons).

Since FMRP co-sediments with ribosomes, a major question is whether FMRP is a structural component of the ribosome, or an associated translation factor. Ribosomal proteins are relatively small (usually ranging in molecular weights from 10 to 30 KDa), and are present within the ribosome in single copies. For example, E. Coli ribosomal protein S1, which has a molecular weight of 61 kDa, is also involved in other RNA-associated processes and is probably not a *bona fide* ribosomal protein. Consistent with their proximity to RNA, the ribosomal proteins are highly basic and migrate on isoelectric-focusing gels until around pH 10-11 (Wittmann, 1982). Since FMRP has a molecular weight of 70-80 kDa, exists as a multimer (*publication 5*), and has a neutral pI of 7.4 (Khandjian *et al.*, 1995), it is unlikely that FMRP is a pure ribosomal protein.

The structures of 15 bacterial ribosomal proteins have now been determined by X-ray crystallography or NMR spectroscopy (Ramakrishnan, 1998). The ribosomal-proteins contain RNA-binding motifs that are present in more recently evolved families of RNA-and DNA-binding proteins (Davies *et al.*, 1998; Ramakrishnan, 1998). Many ribosomal proteins share a topology with the RNA-binding domain of the snRNP U1A protein, a splicing assembly factor (Oubridge *et al.*, 1994). Others share homology, either with the double strand RNA-binding domain (dsRBD) present in staufen (Bycroft *et al.*, 1995) and RNaseIII (Kharrat *et al.*, 1995), or with the KH domain of FMRP (Rinke-Appel *et al.*, 1991; Gibson *et al.*, 1993).

During ribosome assembly, the rRNA molecules (rich in uridine) form a framework onto which the "primary" binding proteins are the first to be incorporated. These proteins initiate the correct folding of the ribosome. Successively, "secondary" and "tertiary" proteins bind to new sites created by this global folding and stabilize the tertiary structure of the

rRNA. Any model of this protein-RNA complex must take into account that the RNA-binding sites are frequently in flexible loop regions that probably only become structured when fully incorporated into the complex (Hinck *et al.*, 1997). Concordant with this, the majority of the ribosomal proteins contain multiple potential RNA-binding sites and probably interact with several regions of the rRNA. Typically, ribosomal proteins contain one major RNA-binding site that is more extensive than the others. Probably, initial binding of this site to the rRNA is followed by binding of the additional sites later during ribosome folding (Heilek *et al.*, 1996).

1.5.2 Ribosomal biogenesis

In eukaryotic cells, the ribosomal proteins are first synthesized in the cytoplasm and subsequently imported into a highly specialized nuclear component called the nucleolus. The nucleolus has the highest density of any part of the cell and the large difference between its refractive index and that of the surrounding nucleoplasm gives rise to the prominence of the nucleolus in phase contrast microscopy. The nucleolus is formed around the ribosomal DNA (rDNA) repeats, which cluster at chromosomal loci called nucleolar organizers. In most eukaryotic cells, the entire nucleolar structure breaks down and reforms during each mitotic cycle (Goessens, 1984). Thus, the nucleolus is a dynamic cellular component that is formed in response to the requirement for new ribosome synthesis.

The nucleolus is differentiated into the fibrillar component (FC), dense fibrillar component (DFC), and granular component (GC). The consensus view (Lamond, 1998) is that the FC contains rDNA, RNA polymerase I, and associated transcription factors, with transcription of rDNA occurring largely at the boundary between the FC and the DFC. 28S, 18S, and 5.8S rRNAs are transcribed as a common precursor, nascent rRNA transcripts appear in the DFC and are processed there by a series of specific nucleolytic cleavages and base modifications. Small nucleolar ribonucleoproteins (snoRNPs) act as "guide RNA's" during rRNA maturation (Balakin *et al.*, 1996; Tollervey *et al.*, 1997). Some processing steps also occur in the GC, together with the assembly of ribosomal proteins and rRNA into ribosomal subunits.

Besides its localization in the cytoplasm, FMRP has also been detected in the nucleoli of COS cells transfected with a wild type *FMR1* cDNA (Willemsen *et al.*, 1996). By EM analysis, FMRP was detected specifically in the granular component, suggesting a link between FMRP and ribosomal particle biogenesis. However, this experiment was not conclusive, as the nucleolar staining of FMRP was not visible in all cells analyzed under light microscopy.

Once the ribosomal subunits are formed, they are transported back to the cytoplasm where protein synthesis occurs. Protein synthesis can be divided into five steps (Pain, 1996; Sachs *et al.*, 1997): 1) Association of initiator tRNA (Met-tRNA) and several initiation factors with the 40S subunit to form the 43S preinitiation complex; 2) Binding of this complex to mRNA, followed by its migration to the correct AUG initiation codon; 3) Addition of the 60S subunit to assemble an 80S ribosome at the initiation codon; 4) Elongation; 5) Termination.

Several cytoplasmic RNA-binding proteins are involved in these steps. They can influence the stability of mRNAs as well as their accessibility to the translation machinery (Ostareck-Lederer *et al.*, 1998), enhance or inhibit mRNA translation (Ranjan *et al.*, 1993; Kiledjian *et al.*, 1995), and control the general fidelity of this cellular process. Recently, polyribosome run-off experiments have shown that FMRP is released as a free protein as a consequence of translation termination. Thus, it has been suggested that FMRP could be an associated translational factor with a particular task during protein synthesis (Feng *et al.*, 1997b).

1.6 Nuclear export signal (NES) in FMRP

While FMRP has a predominant cytoplasmic localization, some studies also reported a nuclear staining for the protein in epithelial cells of the oesophagus (Verheij *et al.*, 1993). An immunogold electron microscopic study in rat cortical neurons detected a small amount of Fmrp in the nucleus and in transit through the nuclear pore (Feng *et al.*, 1997a). Furthermore, transfection of COS cells with a *FMR1* construct lacking exon 14, resulted in an intense nuclear staining of the corresponding FMRP isoform (called ISO12) (Sittler *et al.*, 1996). The staining of FMRP ISO12 was restricted to the nucleoplasm and was not detected in the nucleolus (Willemsen *et al.*, 1996).

To explain this nuclear localization, it was initially suggested that exon 14 contains a cytoplasmic retention signal (CRS) (Sittler *et al.*, 1996). However, experiments performed by Fridell *et al.* (1996), who were studying the nucleocytoplasmic shuttling of the Rev protein of the human immunodeficiency virus (HIV-1), indicated that FMRP contains a functional nuclear export signal (NES) in exon 14 (Figure 3). Thus, it was suggested in 1996 that FMRP might shuttle between cytoplasm and nucleus, and also have a role in the nucleus (Eberhart *et al.*, 1996; Fridell *et al.*, 1996).

The NES in FMRP is reminiscent of the NES motif first identified in the Rev protein (Fischer *et al.*, 1995) and the protein kinase inhibitor (PKI) (Wen *et al.*, 1995). The prototype NES is ~ 10 amino acids long, containing four hydrophobic amino acids (mainly leucine or isoleucine) critically spaced in a generally hydrophilic sequence context (see Figure 3 and 6). A peptide containing either the RevNES or the FMRPNES can direct temperature-dependent nuclear export of a microinjected protein conjugate in less then 30 minutes, while an NES mutant peptide, containing an Ile to Ala substitution, could not direct export (Eberhart *et al.*, 1996; Fridell *et al.*, 1996). Strikingly, a similar point mutation in the NES of *FMR1* changes the FMRP localization from the cytoplasm to the nucleus, but not to the nucleolus (Fridell *et al.*, 1996).

Recently, an increasing number of proteins carrying the Rev-like NES have been identified, including MAPKK (Fukuda *et al.*, 1996), C-ABL tyrosine kinase (Taagepera *et al.*, 1998), cyclinB1(Toyoshima *et al.*, 1998), actin (Wada *et al.*, 1998) and p53 (Stommel *et al.*, 1999). Since these proteins do not bind RNA and are apparently unrelated in function, the

nuclear export activity mediated by this short signal seems to be a more general cellular process.

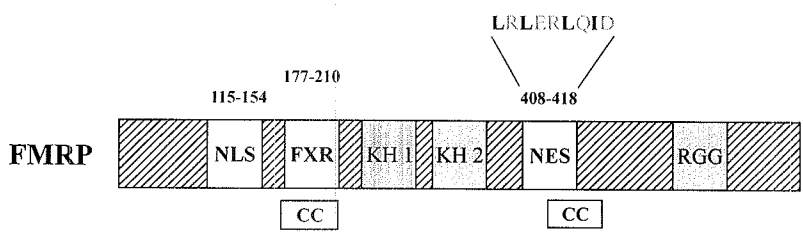

***Figure 3.* Additional functional domains in FMRP.**

Schematic presentation of the nuclear localization signal (NLS) and nuclear export signal (NES) of FMRP, which are necessary for its nucleocytoplasmic shuttling activity. The important amino acids Leucine are in bold within the NES sequence. The FXR multimerization domain (FXR) and the coiled coil domains (CC) are also shown.

1.7 Mechanisms of RNA export

Eukaryotic RNAs are produced in the nucleus by either RNA polymerase I (rRNA), pol II (mRNA, snRNA) or pol III (tRNA, 5S rRNA). The RNAs are then transported through the nuclear envelope (NE) to the cytoplasm in a RNA class-specific, saturable process. It is believed that most of the rate-limiting proteins are adapters that recognize the specific RNAs as well as mediate their interaction with nuclear export factors (Nakielny, 1997; Mattaj, 1998) (see Figure 4). The NE consists of a double lipid bilayer that is penetrated by nuclear pore complexes (NPCs) (Reichelt *et al.*, 1990). The NPC is an aqueous channel formed by ~50 distinct proteins (Rout *et al.*, 1993), called nucleoporins. Most likely, export (and import) factors move through the NE via different rounds of nucleoporin-association/dissociation.

The viral Rev protein is the best-understood example of an RNA export adapter. Like FMRP, Rev protein contains a RNA-binding domain (Malim *et al.*, 1989), NLS (Truant *et al.*, 1999) and NES (Fischer *et al.*, 1995). To ensure propagation of the virus, transcripts have to be exported to the cytoplasm of the host cell for translation. This export is mediated by the viral Rev protein, which binds to a specific sequence in the intron-containing HIV transcripts, named the Rev response element (RRE). Since the shuttling of the Rev protein depends on the NES described above, several groups have tried to identify the host export factor (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995). Recently, this was shown to be a 112 kDa protein called CRM1 or exportin1 (Fornerod *et al.*, 1997a; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). A complex containing exportin1, Rev, and RanGTP is first formed inside the nucleus, transported through the NPC, and finally dissociates in the cytoplasm where the HIV-1 RNA is released (Fornerod *et al.*, 1997a). The antibiotic

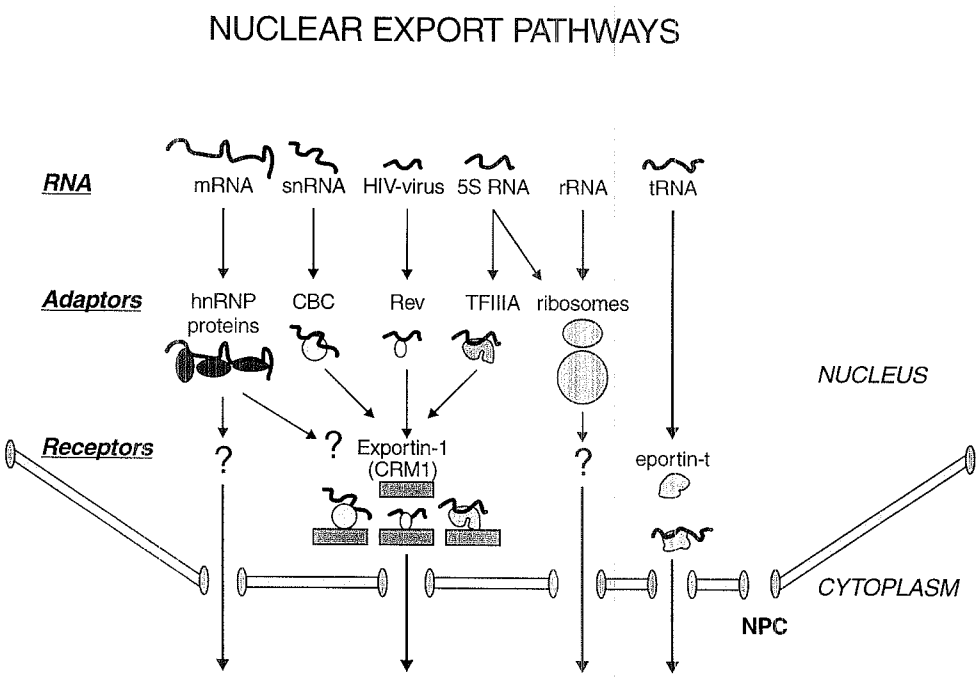

***Figure 4.* RNA export pathways.**

Schematic presentation of the nucleocytoplasmic transport of RNAs. Different classes of nuclear RNA bind to their adapters (usually RNA-binding proteins). Subsequently, each RNA-adapter complex can interact with an export receptor, which promotes the transport of these RNAs into the cytoplasm.

Leptomycin B, first identified as a potent fungicide and later shown to act specifically on exportin1 (Nishi *et al.*, 1994), can block this process in the nucleus (Wolff *et al.*, 1995; Kudo *et al.*, 1998). The nuclear export of FMRP is also exportin1-dependent, since Leptomycin B treatment causes the accumulation of wild type FMRP in the nucleoplasm (Tamanini *et al.*, 1999).

Proteins carrying a leucine-rich NES have been implicated in the transport of RNA substrates other than those carrying an RRE. The first evidence for this involvement was that saturation of RevNES-dependent nuclear export with BSA-RevNES peptide conjugates block the export of both 5S rRNA and U snRNAs, in *X. laevis* oocytes (Fischer *et al.*, 1995). The export of mRNA, tRNA, and ribosomal subunits, was not affected. Comparable results were obtained by nuclear microinjection of Leptomycin B (Fornerod *et al.*, 1997a). This indicated that the export of U snRNAs and 5S rRNA is mediated by transport molecules that contain a Rev-like NES and function in the exportin1 pathway; they are the nuclear cap-binding complex (CPC) (Izaurralde *et al.*, 1995) and the transcription factor TFIIIA, respectively.

Nascent pre-mRNAs as well as mature mRNAs associate with the heterogenous nuclear proteins (hnRNPs), a family of very abundant nuclear proteins (Dreyfuss, 1993). The demonstration that several hnRNP proteins shuttle continuously between the nucleus and the cytoplasm, suggested them as potential mRNA-export adapters (Pinol-Roma *et al.*, 1991; Pinol-Roma *et al.*, 1992; Nakielny *et al.*, 1997). Indeed, the hnRNP A1 and hnRNP K proteins both contain domains that can function independently as either an NES or NLS (Siomi *et al.*, 1995b; Michael *et al.*, 1995; Michael *et al.*, 1997). Microinjection of saturating amounts of hnRNP A1 into *X. laevis* oocyte nuclei blocks export of some mRNAs, reflecting titration of an export receptor used by hnRNP A1 (Izaurralde *et al.*, 1997). This export receptor is still unknown, but it is distinct from exportin1 and transportin (the import receptor of hnRNP A1, see below) (Pollard *et al.*, 1996; Izaurralde *et al.*, 1997). It possible that specific mRNAs, which are not exported by the hnRNPs, might use different export pathways (see Figure 4).

The export receptor for tRNA has been identified and named exportin-t (Arts *et al.*, 1998; Kutay *et al.*, 1998). Exportin-t binds tRNA directly, as well as some nucleoporins, thus it does not need a tRNA-binding adapter to transport its cargo to the cytoplasm. Although very little is known about ribosome export, this process requires, at least in yeast, Ran GTPase and distinct nucleoporins (Hurt *et al.*, 1999).

1.8 Nuclear localization signal (NLS) in FMRP

The suggestion that FMRP might enter and exit the nucleus as a part of its normal function was supported by Eberhart and coworkers, who postulated the existence of a nuclear localization signal (NLS) in FMRP. These authors tried to localize the exact position of the NLS in FMRP by studying the subcellular localization of fusion constructs between chicken muscle pyruvate kinase (CMPK) and the amino-terminus of FMRP (Eberhart *et al.*, 1996). CMPK normally exhibits diffuse cytoplasmic staining, but addition of the first 184 amino acids of FMRP, but not the first 117, resulted in nuclear localization of the FMRP-CMPK fusion protein. These results indicate that FMRP contains sequences between amino acids 117 and 184, that are sufficient to direct proteins to the nucleus. Subsequent studies refined the FMRP NLS between residues 115 and 154 (Bardoni *et al.*, 1997) (Figure 3). This NLS does not correspond to well recognized nuclear localization signals (like SV40 and nucleoplasmin), however, it does contain clusters of basic amino acids (arginine and lysine) (see Figure 6), which are present in most NLSs. So far, the nuclear import receptor of FMRP has not been identified.

Globular proteins with a molecular mass of 30-60 kDa can, in principle, diffuse freely through the nuclear pore. However, in practice, almost none do so. Rather, nucleocytoplasmic transport is an energy-dependent as well as signal-mediated process. Indeed, the nuclear localization mediated by the NLS of FMRP is energy-dependent, as incubation of cells at 4°C inhibits import (Eberhart *et al.*, 1996).

Despite the presence of both an NLS and NES in FMRP, real clues about the biological significance of the nucleocytoplasmic shuttling of FMRP are still lacking. Thus, more informative studies are needed for a better understanding of this activity of FMRP.

1.9 Mechanisms of protein import

The two best-defined nuclear localization signals (NLS) are those of SV40 large T antigen (Kalderon *et al.*, 1984) and nucleoplasmin (Robbins *et al.*, 1991). They are both short and contain several critical basic residues (lysine and arginine), either contiguously, or in a bipartite distribution. When fused to a heterologous protein, such a small NLS is sufficient to direct the chimeric polypeptide to the nucleus (Kalderon *et al.*, 1984). The first NLS receptor identified was a cytosolic heterodimer, composed of importin α (or karyopherin α) (Adam *et al.*, 1989; Adam *et al.*, 1991; Gorlich et al., 1994) and importin β (karyopherin β, or p97) (Chi *et al.*, 1995; Gorlich *et al.*, 1995a; Radu *et al.*, 1995). Importin α binds the substrate protein through recognition of the NLS, whereas importin β mediates the docking to the NPC, most likely by binding the FG-repeat of a subset of nucleoporins (Gorlich *et al.*, 1995b; Moroianu *et al.*, 1995; Weis *et al.*, 1995; Gorlich, 1997a). NPC translocation of importin α/β-NLS protein complexes requires two additional proteins, the Ran GTPase and its co-factor p10/NTF2 (Moore *et al.*, 1992; Moore *et al.*, 1994; Gorlich *et al.*, 1996). Interestingly, homology searches demonstrated that multicellular eukaryotes encode a family of closely related importins α-like proteins that can bind importin β, named hSrp1, NPI-1/importin α1, and Rch1/importin α2 (Imamoto *et al.*, 1995; Moroianu *et al.*, 1995; Torok *et al.*, 1995; Weis *et al.*, 1995). In contrast, importin β is unique (Chi *et al.*, 1995; Gorlich *et al.*, 1995b; Radu *et al.*, 1995), and is only distantly related to other import/export receptors in its Ran-binding domain (Fornerod *et al.*, 1997b; Gorlich *et al.*, 1997b). It may therefore well be that importin β is the genuine import receptor, and the importin α proteins are adapters that join importin β and the NLS containing-substrate (see Figure 5).

The snRNPs U1, U2, U4/U6, and U5, are RNA-protein complexes that are part of the splicing machinery. All U snRNAs undergo bi-directional transport across the nuclear envelope as part of their maturation pathway. Once in the cytoplasm, U snRNAs receive a trimethyl cap structure and are assembled in U snRNPs before returning to the nucleus (Mattaj, 1986; Izaurralde *et al.*, 1992). Snurportin1 is the protein that recognizes the trimethyl cap structure during the import of the U snRNPs. It is a distant relative of importin α and, like importin α, has an N-terminal domain which can bind importin β (Huber *et al.*, 1998). Depletion of importin β, but not importin α, blocks U snRNPs import (Palacios *et al.*, 1997), indicating that snurportin1 is the likely import adaptor for U snRNPs.

The Signal Transducer and Activators of Transcription (STATs) give an interesting example of regulated nuclear import. STATs are a family of transcription regulators that are cytoplasmic in the resting state but move to the nucleus after interferon stimulation (Ihle, 1996). The process of nuclear import involves both JAK-dependent phosphorylation and

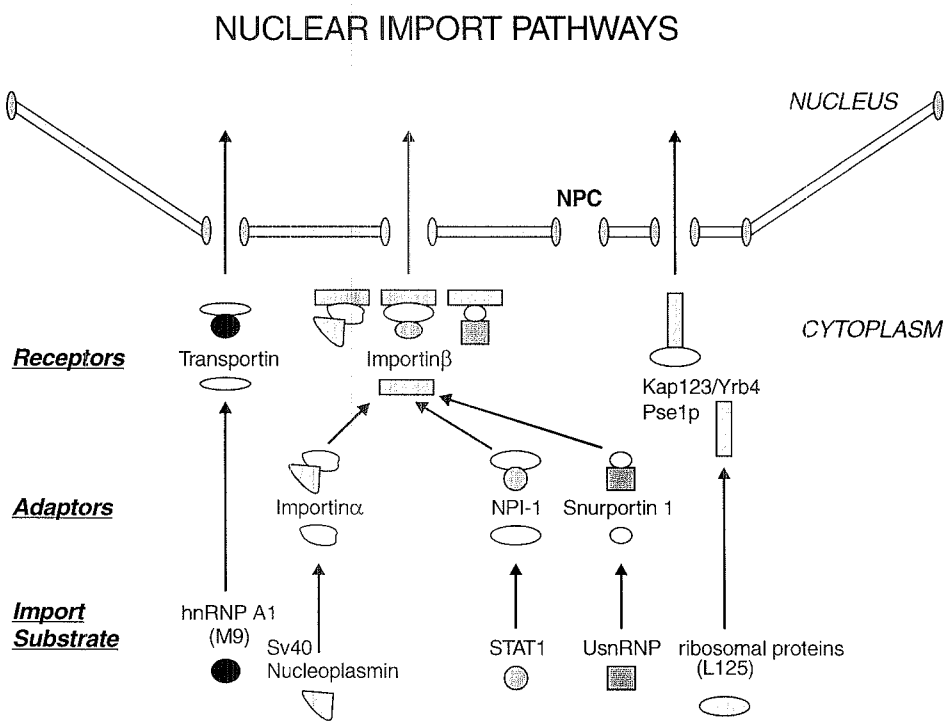

***Figure 5.* Nuclear import pathways.**

Schematic presentation of known pathways for nuclear import. Different cytoplasmic protein substrates bind either their adapters, or directly their import receptors. Eventually, each substrate-adapter complex can interact with an import receptor (usually importin ß) which promotes their transport into the nucleus.

STAT-dimerization, which are necessary for the presentation of the STAT1-NLS that is recognized exclusively by the NPI-1 adaptor (Sekimoto *et al.*, 1997)

The remaining well-defined examples of such processes all involve RNA-binding proteins as the import substrate. Transportin is the import receptor for the mRNA-binding protein hnRNP A1 (Aitchison *et al.*, 1996; Pollard *et al.*, 1996). Transportin binds the NLS of hnRNP A1 as well as several nucleoporins present on the cytoplasmic side of the NPC. The hnRNP A1 NLS is an atypical NLS in the sense that it is 38 amino acids long and is rich in glycine and aromatic residues rather than being basic (Siomi *et al.*, 1995b; Weighardt *et al.*, 1995). Another mRNA-binding protein, hnRNP K, has two nuclear import signals, a classic bipartite NLS and the KNS domain (for hnRNP K Nuclear Signal)(Michael *et al.*, 1997). The KNS domain-mediated nuclear import is independent from importin α/β or transportin, indicating that not all the import factors have yet been identified.

As ribosomal proteins concentrate 50-fold in the nucleolus within 5 minutes after their synthesis, and free cytoplasmic ribosomal proteins are degraded within 2-3 minutes, they require rapid nuclear import. The NLSs of a number of ribosomal proteins have been mapped, such as for human rpS6 (Schmidt *et al.*, 1995) or human rpL23 (Jakel *et al.*, 1998). Similar to FMRP, these NLSs include short basic peptides. None of them fit the sequence consensus for NLSs of substrates transported by the importin α or transportin systems. Recently, two import receptors for the ribosomal protein L25 have been characterized in yeast: Pse1p and Yrb4p/Kap123p (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997). Other import receptors (importinβ, transportin, RanBP5 and RanBP7) have been proposed to act in a partially redundant way in ribosomal protein import (Jakel *et al.*, 1998). However, it is still unclear whether all ribosomal proteins are imported into the nucleus individually, or if some may enter as pre-assembled complexes containing only one component with a "real" NLS.

1.10 The homologues FXR1P and FXR2P

Two genes homologous to *FMR1* have been cloned, *FXR1* (Siomi *et al.*, 1995a) and *FXR2* (Zhang *et al.*, 1995) (for fragile-X-related 1 and 2). *FXR1* was first cloned by cross-hybridization with *FMR1*, and subsequently, a yeast two-hybrid screening isolated *FXR2* as well as *FXR1*. Since the latter technique is widely used to identify proteins that interact with a bait (in this case FMRP), a probable interaction of these new proteins with FMRP was immediately suggested. *FXR1* has been localized to 3q28 (Coy *et al.*, 1995) and *FXR2* to 17p13.1 (Zhang *et al.*, 1995).

As visualized on SDS-PAGE denaturing gel, the protein product of *FXR1* (FXR1P) consists of two major isoforms with a molecular mass of 70 and 78 kDa, while the *FXR2* protein (FXR2P) has a mass of 95 kDa. The amino acid sequence of FMRP is highly homologous to those of FXR1P and FXR2P (86% and 70% identity, respectively) throughout the amino-terminal and central regions. Consistent with this, the functional domains characterized so far in FMRP (NLS, KH domains, NES, and RGG box) are also present in FXR1P and FXR2P (see Figure 6). However, the carboxy-terminal regions of the three proteins are highly divergent, sharing around 6% similarity (Zhang *et al.*, 1995). Therefore, the antibodies used in our studies (*publications 3, 4, 5*) to characterize FXR1P and FXR2P have been raised against peptides selected in the carboxy-termini of the two proteins.

Both FXR1P and FXR2P bind RNA *in vitro*, are localized predominantly in the cytoplasm (Siomi *et al.*, 1995a; Zhang *et al.*, 1995), and sediment with ribosomes (Siomi *et al.*, 1996; Corbin *et al.*, 1997; Khandjian *et al.*, 1998). Moreover, FXR1P can shuttle between cytoplasm and nucleoplasm similarly to FMRP, while FXR2P can shuttle between cytoplasm and nucleolus (Tamanini *et al.*, 1999). The three proteins are capable to form homo- and heterotypic interactions in the yeast two-hybrid system as well as *in vitro* (using the purified proteins) (Zhang *et al.*, 1995).

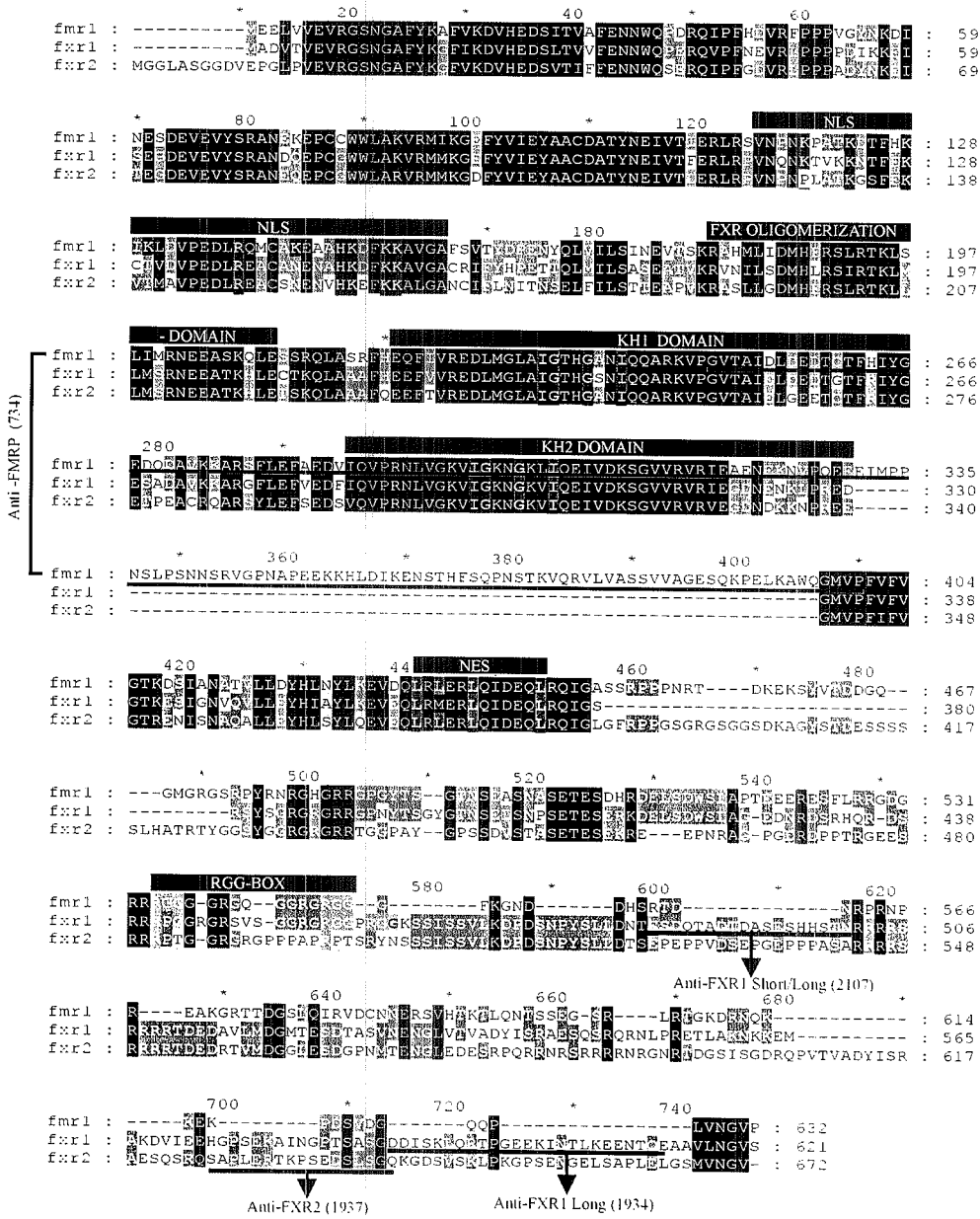

Figure 6. **Alignment of the amino acid sequence of human FMRP, FXR1P and FXR2P.**
The various functional domains are boxed on top of the sequences. The amino acids sequences of the peptides used to raise the antibodies are underlined.

Experiments aimed at the identification of the sequences that mediate protein-protein interaction among FMRP, FXR1P, and FXR2P, reveal that a common region in the amino-termini of the three proteins is involved (Siomi *et al.*, 1996). In FMRP, this region corresponds to amino acids 171-211 (Figure 3). This domain (the FXR oligomerization domain) is highly conserved between the three proteins (75% amino acid identity) (see Figure 6) and have a significant propensity to form coiled coil motifs.

The expression patterns between the FXR proteins differ in several tissues. For example, whereas in testis FMRP is expressed only in spermatogonia, FXR1P is localized in postmeiotic spermatides at very high levels, and FXR2P is expressed through the testis (Coy *et al.*, 1995; Tamanini *et al.*, 1997). Moreover, FXR1P is highly expressed in muscle and heart, where FMRP is absent (Coy et al., 1995; Khandjian et al., 1998). In brain, the three proteins are co-expressed in the cytoplasm of neurons, and FXR1P as well FXR2P expression in neurons of a fragile X patient is observed (Tamanini *et al.*, 1997).

It is becoming clear that FMRP, FXR1P and FXR2P form a small gene family of proteins (FXR proteins) related by structure and most likely by function. Therefore, it is possible that they might originate by gene duplication from a common ancestor gene. Patients with mutations in *FXR1* or *FXR2* have not yet been described. Screening by Southern blot among patients, who have a fragile X-like phenotype but a normal *FMR1* gene, did not reveal large rearrangements in the sequences of *FXR1* and *FXR2*.

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Chapter 2

FUNCTIONAL CHARACTERIZATION OF THE *FMR1* PROTEIN

2.1 Aim of the work

Although the *FMR1* gene was cloned in 1991, knowledge about the biochemical function(s) of the gene product is still limited. Since the fragile X syndrome is one of the few syndromes where mental retardation is associated with the absence of a single protein (FMRP), further studies on the functional characteristics of FMRP are of great importance.

When the experimental work forming the basis of this thesis started, most of the knowledge about FMRP originated from immunohistological experiments showing that FMRP is localized in the cytoplasm (occasionaly in the nucleus) and is strongly expressed in in neurons. In 1993, RNA-binding was the first biochemical activity attributed to FMRP.

In **Chapter 2.2** (*publication 1*) a biochemical approach was used to investigate whether FMRP is interacting with other cellular components, whose identification could define a role for FMRP. We performed mainly gel filtration studies of protein cellular homogenates under different buffer conditions (physiological, salt, RNase) and studied the fractionation behavior of FMRP by Western blotting.

In **Chapter 2.3** (*publication 2*) we further characterized the intracellular distribution of FMRP by electron-microscopical analysis using antibodies against FMRP. The attempt was to investigate whether FMRP co-localizes with known cellular components. We performed the study on COS cells transfected with a wild-type *FMR1* cDNA, or a deletion *FMR1* construct (FMRPISO12) lacking the NES as well as the RGG box.

In **Chapter 2.4** (*publication 3*) the study of the comparative expression between FMRP and the highly homologous FXR1P and FXR2P proteins is presented. Previous data *in vitro* showed that FMRP, FXR1P and FXR2P interact with each other. Our goal is to understand whether the proposed interactions are important for the functioning of FMRP *in vivo*. Using newly developed antibodies against FXR1P and FXR2P we studied their expression in adult/fetal brain and testis from both normal controls and fragile X patients. We also studied the localization of FMRP in cultured hippocampal neurons, as a further step in the elucidation of its properties in brain.

Not only a deletion, but also amino acids substitutions (Leu to Ala) within the nuclear export sequences (NES) of FMRP re-localize the protein from the cytoplasm to the nucleoplasm. This suggests some role for FMRP in the nucleus, like binding specific RNAs followed by the interaction with a nuclear export factor. In **Chapter 2.5** (*publication 4*) we tested, by immuno-fluorescence analysis on transfected COS cells, the effect of similar mutations (Leu to Ala) in the putative NES of FXR2P. We inactivated the NES export receptor CRM1/exportin 1 with the antibiotic Leptomycin B, to study whether this cellular factor is involved in the nucleo-cytoplasmic shuttling of FMRP, FXR1P and FXR2P.

In **Chapter 2.6** (*publication 5*) cultured mammalian cells expressing endogenously the FXR proteins were used for gel filtration/immunoprecipitation followed by Western blotting to study the multimerization of the FXR proteins as well as their association with ribosomes.

Publication 1

FMRP IS ASSOCIATED TO THE RIBOSOMES VIA RNA

Filippo Tamanini, Nicolle Meijer, Coleta Verheij, Patrick J. Willems, Hans Galjaard, Ben A. Oostra* and André T. Hoogeveen

Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam,The Netherlands

*To whom correspondence should be addressed

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FMRP is associated to the ribosomes via RNA

Filippo Tamanini, Nicolle Meijer, Coleta Verheij, Patrick J. Willems[1], Hans Galjaard, Ben A. Oostra* and André T. Hoogeveen

MGC-Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands and [1]Department of Medical Genetics, University of Antwerp, Antwerp, Belgium

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The *FMR1* transcript is alternatively spliced and generates different splice variants coding for *FMR1* proteins (FMRP) with a predicted molecular mass of 70–80 kDa. FMRP is widely expressed and localized in the cytoplasm. To study a possible interaction with other cellular components, FMRP was isolated and characterized under non-denaturing conditions. Under physiological salt conditions FMRP appears to have a molecular mass of >600 kDa, indicating a binding to other cellular components. This interaction is disrupted in the presence of high salt concentrations. The dissociation conditions to free FMRP from the complex are similar to the dissociation of FMRP from RNA as shown before. The binding of FMRP from the complex is also disrupted by RNAse treatment. That the association of FMRP to a high molecular weight complex possibly occurs via RNA, is further supported by the observation that the binding of FMRP, containing an Ile304Asn substitution, to the high molecular weight complex is reduced. An equal reduced binding of mutated FMRP to RNA *in vitro* was observed before under the same conditions. The reduced binding of FMRP with the Ile304Asn substitution further indicates that the interaction to the complex indeed occurs via FMRP and not via other RNA binding proteins. In a reconstitution experiment where the low molecular mass FMRP (70–80 kDa) is mixed with a reticulocyte lysate (enriched in ribosomes) it was shown that FMRP can associate to ribosomes and that this binding most likely occurs via RNA.

INTRODUCTION

The fragile X syndrome is caused by an unstable expansion of a CGG repeat in the 5′ untranslated region of the *FMR1* gene (1–5). The CGG repeat and a CpG island adjacent to *FMR1* are abnormally methylated and as a result the *FMR1* gene is not translated (6–8; for review see 9). The Fragile X syndrome is the most common cause of inheritable mental retardation. The major clinical features are mental retardation, macro orchidism, and some facial abnormalities like a long face with prominent forehead (10). The *FMR1* transcript is alternatively spliced and generates different splice variants coding for FMRPs with a molecular mass of 70–80 kDa (3,5,11). FMRP is widely expressed in various tissues with the highest expression in brain and testis (12,13) and its localization is cytoplasmic (3,5). The *FMR1* protein contains motifs characteristic of RNA binding proteins (14,15). Two KH domains, which are thought to be involved in RNA binding, are located in the middle of FMRP. Downstream of the FMRP KH domains also lies an RGG box, a sequence motif directly involved in RNA binding. The importance of the KH domains are illustrated by a fragile X patient with a severe clinical phenotype (16). The clinical phenotype in this patient is not caused by an expansion of the CGG repeat and the absence of FMRP, but results from a point mutation (Ile304Asn) in the second KH domain. It was demonstrated that FMRP containing this mutation has a reduced RNA binding capacity (17,18).

FMRP is expressed in practically every tissue, however, the most severe clinical symptoms are found almost exclusively in brain, indicating that the (RNA binding) function of FMRP is most dependent in brain. FMRP has been shown *in vitro* to be able to bind 4% of human fetal brain mRNA (15). However the overall expression of FMRP and its broad binding specificity with RNAs, makes it still difficult to explain the clinical features seen in the fragile X syndrome as a direct result of the absence or improper RNA binding capacity of FMRP. Another possibility is that FMRP has other function(s) other than RNA binding or more secondary, FMRP can be acting as a regulatory protein by interacting with other cellular components. While preparing this manuscript on the association and binding properties of the *FMR1* protein, the possible binding of FMRP with other cellular components was supported by the important finding of Khandjian *et al.* (19) who demonstrated that FMRP is associated with a ribosomal fraction.

Using another strategy, our data presented in this paper not only support this finding but also indicate how this interaction occurs.

RESULTS

Determination of molecular mass

Cells were disrupted and proteins solubilized. The cellular proteins were separated under physiological salt conditions (150 mM NaCl) using a gel filtration system. The protein separation was performed on a Superdex 200 PC 3.2/30 precision column resulting in a characteristic protein profile as seen in Figure 1.

*To whom correspondence should be addressed

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Different control lymphoblastoid cell lines were examined for the presence of FMRP after gel permeation. The fractions were analysed for the presence of FMRP, using Western-blotting and it was shown that FMRP was mainly present in the high molecular weight fractions (>600 kDa) as illustrated in Figure 1A. Since the molecular mass of FMRP under denaturing conditions is 70–80 kDa, this result indicates that the *FMR1* protein is associated with itself or with other cellular components. Even in the presence of a detergent (1% Triton X100) the majority of the *FMR1* proteins are found in the fractions containing high molecular weight proteins suggesting that the complex is reasonably stable (data not shown). However, in the presence of medium salt conditions (0.5 M NaCl), FMRP is partly released from these complexes and shifted to a molecular mass of approximately 240 kDa (Fig. 1B). After treatment with high salt (1.0 M NaCl), FMRP is shifted to the molecular mass as is seen under denaturing conditions (70–80 kDa) (Fig. 1C).

Cells containing an (Ile304Asn) substitution

The complex forming of FMRP was also studied in lymphoblastoid cells from a fragile X patient carrying a point mutation in the second KH domain (16). The total protein profile after separation was the same as seen in control. FMRP again is found as in control, in the fractions containing the high molecular mass complexes (Fig. 1D). However, in repeat experiments using the cells from the patient with the point mutation, it was found that in contrast with control cells, the presence of a medium salt concentration already resulted in a complete shift in molecular mass of FMRP from high >600 kDa to 240 kDa (Fig. 1E).

RNA binding

The release of FMRP from its complex using different salt concentrations shows similarities with the difference in RNA binding capacity of FMRP observed earlier using synthetic homopolymeric RNA (14,15,17,18). To investigate whether FMRP in the high molecular weight fractions resulted from an FMRP/RNA binding, the homogenate was preincubated with Micrococcus Nuclease followed by protein separation. Micrococcus Nuclease is able to digest DNA and RNA and has been used to free RNA binding proteins from RNA (20); it is also used to introduce specific modifications in rRNA (21). After incubation with Micrococcus Nuclease the protein profile stayed the same as without treatment; however, as a result of this treatment FMRP is found in the low molecular mass 240 kDa protein range (Fig. 1F), indicating that the FMRP complex originates from a protein/RNA interaction.

Ultracentrifugation

To isolate the high molecular weight complex, the sample was centrifuged at 130 000 × *g* and the pellet as well as the supernatant were examined. The pellet (containing ea. light membranes, ribosomes) and the supernatant were tested for FMRP. The proteins present in the supernatant were separated and the profile is seen in Figure 2. After ultracentrifugation, almost the entire high molecular weight fractions were found in the pellet. When the sample was prepared under physiological salt conditions, the majority of FMRP was found in the pellet (Fig. 2A) and only after a longer exposure time some minor FMRP could be detected in the supernatant (Fig. 2B). However, in the presence of medium salt concentrations approximately 50% of total FMRP moved from the pellet to the supernatant (Fig. 2C).

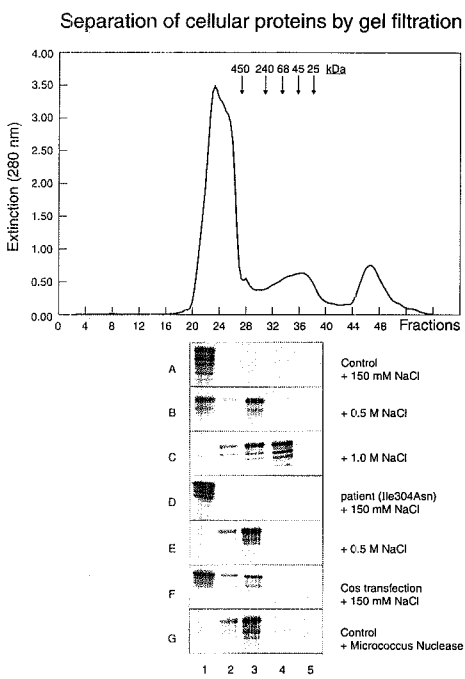

Figure 1. Distribution of normal and mutated FMRP after gel filtration in different buffers conditions. On top, the characteristic proteins profile of the postnuclear supernatant obtained after centrifugation at 10 000 × *g* for 10 min. (**A**, **B**, **C**, and **G**) Distribution of FMRP in control lymphoblastoid cells after treatment with respectively 150 mM NaCl, 0.5 M NaCl, 1.0 M NaCl and Micrococcus Nuclease buffers. (**F**) Distribution of the human transfected FMRP in a COS cell line in 150 mM NaCl buffer. (**D**, **E**) Distribution of the mutated FMRP (Ile304Asn) respectively in the presence of 150 mM and 0.5 M NaCl. Each lane represents a mix of four fractions. Lane 1, fractions from 20 to 23; lane 2, fractions from 24 to 28; lane 3, fractions from 29 to 32; lane 4, fractions from 33 to 36; lane 5, fractions from 37 to 40. The arrows represent the position and molecular weight of the proteins markers (see Material and Methods).

Reconstitution of the complex

To study if complex forming of FMRP can be induced, human FMRP was over expressed in COS cells using a cDNA expression vector (5). When the proteins isolated from these transfected COS cells were separated, it was found that again FMRP is present in a high molecular weight complex suggesting an interaction of human FMRP with monkey cellular components (Fig. 1G).

It was observed, during *in vitro* translation studies, that a reticulocyte lysate itself contains (rabbit) FMRP. The reticulocyte lysate was centrifuged at 130 000 × *g* and the supernatant and pellet were examined (Fig. 3, lanes 1 and 2). After treatment with high salt FMRP could be released in the supernatant and has

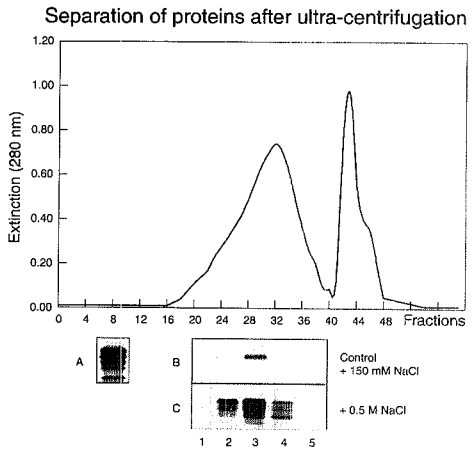

Figure 2. FMRP distribution and gel filtration after ultracentrifugation. The postnuclear supernatant of control lymphoblastoid cells was subjected to ultracentrifuge at 130 000 × *g* for 1 h; at the top of the figure, the protein profile of the resulting PUS is shown. The position of the markers is the same as in Fig. 1. (**A**, **B**) FMRP distribution respectively in the pellet and in the supernatant in 150 mM NaCl. (**C**) FMRP distribution in the supernatant in the presence of 0.5 M NaCl.

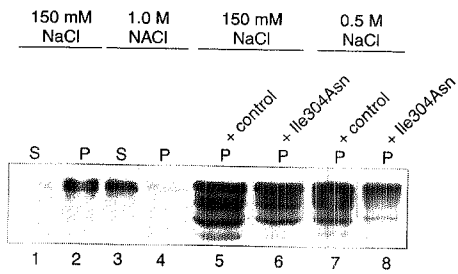

Figure 3. *In vitro* binding of FMRP to rabbit ribosomes. Normal and mutated FMRP (low molecular weight, see Material and Methods) were incubated with rabbit reticulocyte lysate (RL) for 10 min at 30°C and subjected to ultracentrifugation. The pellet (p) and in the supernatant (s) were analysed for the presence of FMRP. Lane 1 and 2 supernatant and pellet of rabbit RL in presence of 150 mM NaCl. Lane 3 and 4, supernatant and pellet of rabbit RL in the presence of 1.0 M NaCl. Lane 5 and 7, pellet of the rabbit RL incubated with the normal FMRP respectively in 150 mM and 0.5 M NaCl. Lane 6 and 8, pellet of rabbit RL incubated with the mutated FMRP (Ile304Asn) respectively in the presence of 150 mM and 0.5 M NaCl.

completely disappeared from the pellet (Fig. 3, lanes 3 and 4). In a reconstruction experiment, FMRP from human control lymphoblastoid cells was dissociated from its complex by high salt treatment, the low molecular weight FMRP protein (70–80 kDa) was isolated by separating FMRP from the ribosomes by ultracentrifugation as shown in Figure 2C. The sample was dialysed and incubated with the reticulocyte lysate. After incubation, complex forming with control FMRP was demonstrated as shown in Figure 3 (lane 5); even in the presence of 0.5 M NaCl interaction was demonstrated (Fig. 3, lane 7). FMRP containing the point mutation (Ile304Asn) was also used in these reconstruction experiments. However, when the binding under medium and low salt conditions were compared (Fig. 3, lanes 6 and 8), it was found that the binding capacity of this mutated FMRP in the presence of 0.5 M NaCl (Fig. 3, lane 8) was more reduced compared to bindings experiments with control FMRP under similar conditions (Fig. 3, lane 5 and 7).

DISCUSSION

The function of FMRP is still unknown, although RNA binding properties have been demonstrated. It is difficult to explain how a defective RNA binding can lead to mental retardation. Since FMRP is expressed widely, there might be another function or interaction for FMRP especially in brain. The possibility of interaction with other cellular components was first investigated in lymphoblastoid cell lines. FMRP was isolated under physiological salt conditions and examined. It was found that FMRP under these conditions is associated to a very large protein complex with a molecular weight of more than 10^6 kDa (Fig. 1A). This complex is reasonably stable since it was found that after treatment with detergents, the majority of FMRP is still present in this complex. While hydrophobic, protein/protein interactions normally become stronger with increasing salt concentrations, FMRP however, is just dissociated from its complex at high salt concentrations, indicating an ionic interaction. All the different *FMR1* splice variant products are found in the high as well as in the 240 kDa complex, indicating that they are binding to the same extent. The complex formation and dissociation was also observed when FMRP containing a point mutation (Ile304Asn) was used. However, for this mutated FMRP dissociation occurs at a lower salt concentration as seen in control (Fig. 1E). These observations were similar to early findings where FMRP/RNA interaction was studied (17,18) and they indicate that the binding of FMRP to the high molecular weight complex might occur via RNA. Treatment of the sample with Micrococcus Nuclease, which has been used in dissociation studies of RNA and RNA binding proteins (20), resulted in a release of FMRP, again indicating a possible association via RNA to the complex. Since free mRNA is rapidly digested in a cell homogenate and cannot be spun down in the ultracentrifuge, the RNA to which FMRP binds has to be itself bound to or part of a large particle, most likely ribosomes. This is supported by the observation that in a rabbit reticulocyte lysate, rabbit FMRP could be demonstrated. The reticulocyte lysate is a concentrated translation machinery and very enriched in ribosomes. When these ribosomes are spun down by ultracentrifuge, FMRP coprecipitated in the pellet with the ribosomes (Fig. 3 lane 2). After salt treatment this FMRP could be released in the supernatant (Fig. 3 lane 3). We were also able to reconstruct the complex by the incubation of free human FMRP with the rabbit reticulocyte lysate. Also here a reduced affinity for binding at 0.5 M NaCl was found when FMRP (Ile304Asn) was used. These arguments strongly suggest that FMRP is associated to the ribosomes via RNA.

It is demonstrated that FMRP is dissociated from the complex using salt; the dissociation seems to occur in two steps first to a 240 kDa complex and with higher salt concentration to its predicted molecular mass as is seen under denaturing conditions (70–80 kDa). Recently, two genes, *FXR1* and *FXR2* were isolated

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which are homologous to *FMR1* and also show RNA binding properties (22,23). It was shown that these three proteins can form homo- and hetero-dimers. It can therefore be speculated that after the first dissociation of FMRP from the large complex, the 240 kDa complex is possibly a protein protein interaction of FMRP with itself or FMRP with the *FXR1* and/or *FXR2* protein. In white blood cells all three proteins are expressed. However, in brain only *FMR1* and *FXR2* are expressed, with no expression of *FXR1* (24). It might be that the deleterious effect in brain function compared to other tissues is a result of the absence in fragile X patients of both *FMR1* and *FXR1* proteins in the brain. The question arises whether this 240 kDa complex is binding to the RNA via FMRP or via another protein that is part of the 240 kDa complex. Data derived from the experiments performed with the mutated FMRP show that there is a lower affinity in RNA binding for this mutated protein, which indicates that FMRP itself is the protein involved in the RNA binding.

After finishing these experiments, evidence was presented that FMRP is indeed associated with the 60S subunit of ribosomes (19). Using a different strategy we not only confirm this observation but additionally we report that the interaction occurs via RNA binding. The RNA loops found in the ribosomal subunits are enriched in pyrimidine nucleotides and high affinity of FMRP for pyrimidine nucleotides has been demonstrated (14). Secondly we present data that FMRP is released from the ribosomes as a 240 kDa complex. And thirdly, the reduced binding of FMRP with the Ile304Asn substitution indicates that the interaction of the high molecular weight complex occurs via FMRP and not by other RNA binding proteins.

The results presented indicate that there is a strong affinity of ribosomes with FMRP via RNA. Preliminary experiments showed that in mice brain, FMRP is associated to the ribosomes in a comparable way. A regulatory function of FMRP in the translation machinery is now under investigation by studying protein translation in brain of normal mice and the *FMR1* knockout mouse (25) after different stimuli.

MATERIALS AND METHODS

Subcellular fractionation

EBV transformed human lymphoblastoid cell line from normal controls and a patient with a point mutation in the KH domain were used. All steps were carried out at 4°C. Cell pellets were homogenized either by sonication in ice (2 times for 15 s, stroke 0.4) or by freezing and thawing, in a buffer containing 40 mM Tris–HCl, (pH 7.5), 150 mM NaCl (physiological).

The lysate was subjected to 500 × *g* centrifugation for 5 min, resulting in a supernatant and a pellet of nuclei and unbroken cells. The supernatant was subjected to 10 000 × *g* centrifugation for 10 min to yield the heavy membranes in the pellet and the postnuclear supernatant (PNS). The PNS was then centrifuged for 1 h at 130 000 × *g* in a SW50i rotor to separate the light membrane, the ribosomes and the big protein complex in the pellet from the post-ultra centrifugation supernatant (PUS). The ultra centrifugation fractionation studies were also performed with different buffers in the starting cell homogenate (0.5 M NaCl, 1 M NaCl, RIPA buffer).

Gel filtration studies

We used a Precision Column PC 3.2/30 pre-packed with Superdex 200 in a SMART system (Pharmacia) to determine by gel filtration the molecular mass of FMRP in the PNS and in the PUS. The optimal range for separation of globular proteins in this column is 10–600 kDa, with an exclusion limit of 1.3 million Dalton. In order to calibrate the column and to determine the molecular weight of the eluting fractions, five protein markers were applied in a physiological salt buffer (40 mM Tris–HCl, pH 7.5, 150 mM NaCl) giving the following results: ferritin (440 kDa) top in fraction 27, catalase (240 kDa) top in fraction 30, albumin (68 kDa) top in fraction 33, egg albumin (43 kDa) top in fraction 35 and chymotrypsin (25 kDa) top in fraction 38. Some markers were tested in other buffer conditions (0.5 M or 1 M NaCl) giving analogous retention time. Before running the extracts treated either with physiological buffer or with 0.5 M NaCl or with 1 M NaCl or with RIPA (1% Triton, 0.1% SDS) or with Micrococcus Nuclease (0.6 U/µl for 10 min at 30°C in presence of 1 mM Ca), the column was equilibrated 20 min in each corresponding buffer; 40–60 µl of PNS or PUS was injected in the SMART system and the protein profile was monitored at 280 nm with a flow of 50 µl/min. Fractions (50 µl each) were collected.

Western blotting

Protein samples separated on 10% SDS-polyacrylamide gels were then electro-blotted onto nitrocellulose membrane (Schleicher & Schuell). Immunodetection of FMRP was carried out using a mouse monoclonal antibody 1C3 (previously described as 1a) diluted 1:2500 (3). The secondary antibody was coupled to peroxidase allowing detection with the chemiluminescence method (ECL KIT, Amersham).

In vitro FMRP-ribosome association

The post-ultracentrifuge supernatant (PUS) containing FMRP free from ribosomes, was obtained by lysing the cells as previously reported in a 40 mM Tris–HCl, (pH 7.5), 1 M NaCl buffer and subjecting the lysate to ultra centrifugation for 1 h at 130 000 × *g*. The resulting supernatant were dialysed and 70 µl of PUS either from control or point mutation was mixed with 25 µl of rabbit reticulocyte lysate (Stratagene) and incubated at 30°C for 10 min. The reactions were subjected to another ultracentrifugation for 1 h at 130 000 × *g* to yield a pellet (containing the ribosomes) and a supernatant. The two fractions were analyzed by Western blotting for the presence of FMRP.

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Publication 2

ASSOCIATION OF FMRP WITH RIBOSOMAL PRECURSOR PARTICLES IN THE NUCLEOLUS

Rob Willemsen, Carola Bontekoe, Filippo Tamanini, Hans Galjaard, André Hoogeveen and Ben Oostra*

Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam,The Netherlands

*To whom correspondence should be addressed

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Association of FMRP with Ribosomal Precursor Particles in the Nucleolus

Rob Willemsen, Carola Bontekoe, Filippo Tamanini, Hans Galjaard, Andre Hoogeveen, and Ben Oostra[1]

MGC—Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands

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The fragile X syndrome, one of the most common forms of inherited mental retardation, is caused by an expansion of a polymorphic CGG repeat upstream the coding region of the FMR1 gene. These expansions are associated with hypermethylation of the FMR1 gene, which results in the absence of the gene product, the FMR1 protein (FMRP). The physiological function of FMRP remains to be determined. We studied the ultrastructural localization of FMRP at the electron microscopical level using the immunogold technique. FMRP is associated with ribosomes attached to the endoplasmic reticulum and with ribosomes free in the cytoplasm. In addition, FMRP is found in the nucleus where the protein is associated with the granular component of the nucleolus. The cellular function of FMRP is hypothesized in relation to its subcellular distribution.

Fragile X syndrome is characterized by a large variability in clinical presentation, including mental retardation, macroorchidism and facial abnormalities as the main characteristics in affected males[1]. The most common mutation found in fragile X patients is an unstable expansion of a CGG repeat in the first exon of the *FMR1* gene[2-4]. This expansion in patients results in hypermethylation of the CpG island in front of the *FMR1* gene and as a consequence no transcription and thus no translation of the *FMR1* gene occurs[5-7]. The absence of functional *FMR1* Protein (FMRP), the protein product of the *FMR1* gene, is the primary defect of the fragile X syndrome[8,9].

FMRP is expressed in many tissues with the most abundant expression in specific neurons of the central nervous system and in early spermatogonia[8-12]. Immunocytochemical studies at the light microscopic level have shown that FMRP is found predominantly in the cytoplasm[8,9]. However, in some studies a nuclear localization of the protein in specific cell types have been reported[8,13]. One of these studies reported a nuclear localization for one isoform of FMRP in transfected COS-cells using a construct that exclude exon 14 of the *FMR1* gene[13].

FMRP contains two KH domains and an RGG box[14,15]. Both sequence motifs are present in many RNA binding proteins. RNA binding properties of FMRP has been demonstrated too[14,15]. RNA binding studies in cells from a fragile X patient with a missense mutation in one of the two KH domains resulted in FMRP with reduced RNA binding capacity, which is consistent with the hypothesis that RNA binding is essential in mediating FMRP function[16-18].

Additional to its RNA binding capacity an association *in vitro* with ribosomes has recently been described and it has been suggested that this binding occurs via rRNA[19-20]. However, all these results were obtained solely by *in vitro* studies. An *in vivo* RNA target(s) or other cellular components for FMRP has not been identified, yet.

[1] To whom correspondence should be addressed at the Dept of Clinical Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Fax: 31-10-4087200; E-mail: Oostra@kgen.fgg.eur.nl.

Since, the specific function of FMRP in the cell is still poorly understood, we investigated the subcellular distribution of FMRP. Here, we describe the *in vivo* association with cellular structures of FMRP in transfected COS-cells. Our immunoelectron microscopic studies illustrate that FMRP is associated with free ribosomes and with ribosomes that are attached to the membrane of the endoplasmic reticulum. Furthermore, we show that FMRP is already present in the granular component of the nucleolus, which contains maturing ribosomal precursor particles.

MATERIALS AND METHODS

DNA constructs. A 3,765 cDNA clone of *FMR1* was cloned in the *Eco*RI site of the eukaryotic expression vector pSG5[23]. In this construct the expression of FMRP is controlled by the SV40 promoter. The construct contains a β-globin intron and a polyadenylation signal. Transfections with this construct will result in a protein of 74 kDa[8]. The *FMR1* missing exon 14 clone was generated as described before 13 and the construct was cloned in the expression vector pcDNAI/AMP (Invitrogen), which is controlled by the CMV promotor.

Transfections. COS-cells were cultured in 1X DMEM + 10% FCS at 37°C and 10% CO_2. Cells were seeded and cultured for 24 hours. Transfections were performed as described in the protocol provided with the lipofectamine reagent (Gibco-BRL), for transfections 2 μg DNA and 20 μl lipofectamine were used per 2×10^4 seeded cells. After transfection the cells were cultured for 48 hours. Subsequently, cells used for light microscopy were transferred to coverslips and cultured for another 24 hours. Cells used for electronmicroscopy were fixed 72 hours after transfection.

Immunocytochemistry. For light microscopy, cells were fixed in 0.1 M phosphate buffered saline (PBS) containing 3% paraformaldehyde (pH 7.3) for 10 minutes at room temperature followed by a permeabilization step in 100% methanol for 20 minutes. FMRP was localized using an indirect immunoperoxidase technique. In the first step we used a monoclonal antibody against FMRP[9] and in the second step a rabbit anti-mouse immunoglobulins conjugated with peroxidase (DAKO) was applied. Enzyme activity was visualized using DAB (Sigma) as substrate. Endogenous peroxidase activity was blocked by pre-treatment of the cells with 3% H_2O_2 after the permeabilization step. Cells were counterstained with Heamatoxylin and mounted with aquamount.

For electronmicroscopy, cells were fixed in 0.1 M PBS, containing 1% acrolein and 0.4% glutaraldehyde for 1 hour at room temperature. Subsequently, cells were embedded in Lowicryl K4M according to a standard protocol[24]. Ultrathin sections were cut with a LKB Nova ultratome and immuno-incubated for FMRP with the monoclonal antibody[9]. Antigen-antibody complexes were visualized using a second incubation step with goat anti-mouse immunoglobulins conjugated with 10 nm colloidal gold particles (AURION). Sections were stained with uranylacetate and leadnitrate and examined in a Philips CM100 at 80 kV.

RESULTS

Thus far, immunocytochemical studies of FMRP in transfected COS-cells were based on the use of immuno-fluorescence microscopy. The resolution of this technique is limited, which makes conclusions about association of FMRP with specific cell organelles difficult. Therefore, in the past only a discrimination between cytoplasmic or nuclear localization could be made.

In a first attempt to enhance the immunocytochemical signal at the light microscopic level, we used an indirect immunoperoxidase technique to detect FMRP in transfected COS-cells. Figure 1 shows the FMRP distribution in COS-cells transfected with a full length cDNA construct and a construct missing exon 14 of the *FMR1* gene. Normal FMRP was predominantly found in the cytoplasm, often in large aggregates. However, in some cells we could detect FMRP in the nucleus, in close association with the nucleolus (Fig. 1A). In contrast, the cellular localization of FMRP with the internal deletion of exon 14 revealed predominantly a nuclear staining, with the exclusion of the nucleolus (Fig. 1B).

FIG. 1. Cellular localization of FMRP, using an indirect immunoperoxidase technique in COS-cells transfected with a full length cDNA for *FMR1* (A) and a construct missing exon 14 sequences of the *FMR1* gene (B). The highest expression of normal FMRP is found in the cytoplasm, but also in the nucleus a clear labelling can be detected. Note that the reaction product (brown) in the cytoplasm is very often found in large aggregates. The expression of FMRP, excluding exon 14, shows a different labelling pattern. Most of the reaction product is present in the nucleus and only a minor fraction was found in the cytoplasm. The reaction product is virtually absent in the nucleolus.

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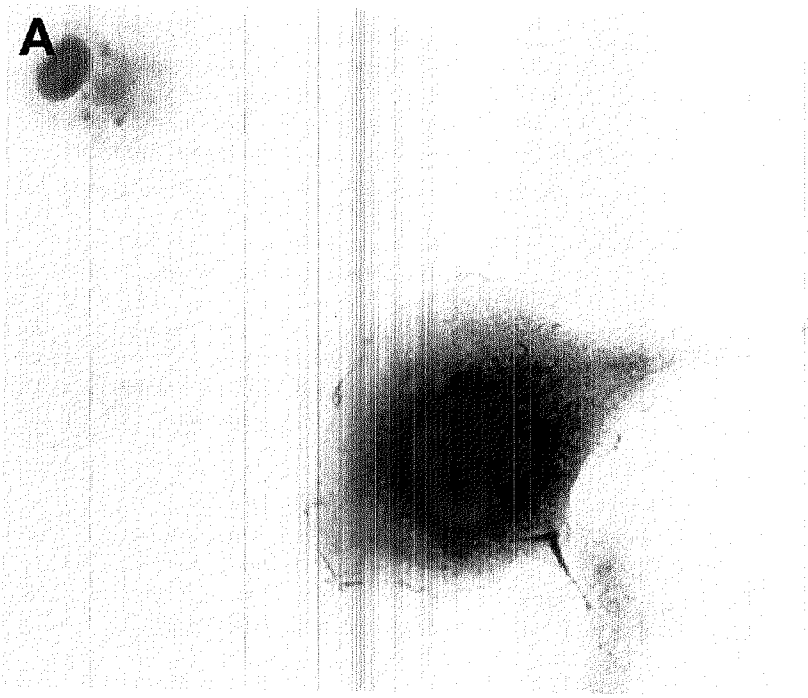
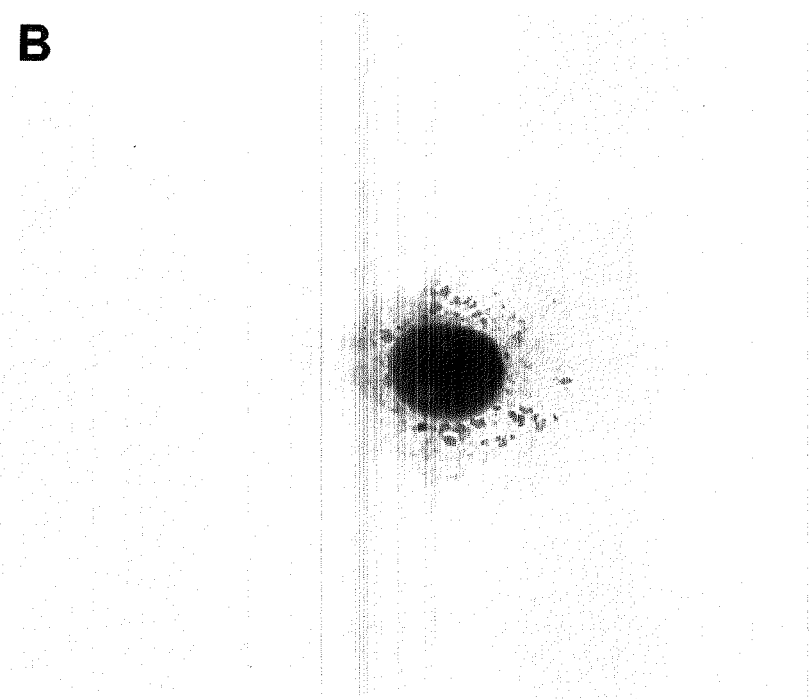




We performed immunoelectron microscopy to study the subcellular distribution of FMRP *in vivo*. The gold particles in Figure 2 illustrate the subcellular localization of FMRP in these transfected COS-cells. In the cytoplasm, FMRP was associated with ribosomes bound to the endoplasmic reticulum (ER) membrane (Figs. 2A and 2B) and with ribosomes free in the cytosol (Fig. 2C). Very often we could observe a concentration of ribosomes close to the ER membrane. Apparently, these fast dividing COS-cells are active in protein production. This phenomenon was also seen in COS-cells that were not transfected with *FMR1* (Fig. 2D). The latter suggests that overexpression of FMRP does not play a role in this concentration of ribosomes. The specificity of the labelling procedure is demonstrated by the absence of gold particles in COS-cells that were not transfected with *FMR1*, but completed the transfection procedure (Fig. 2D).

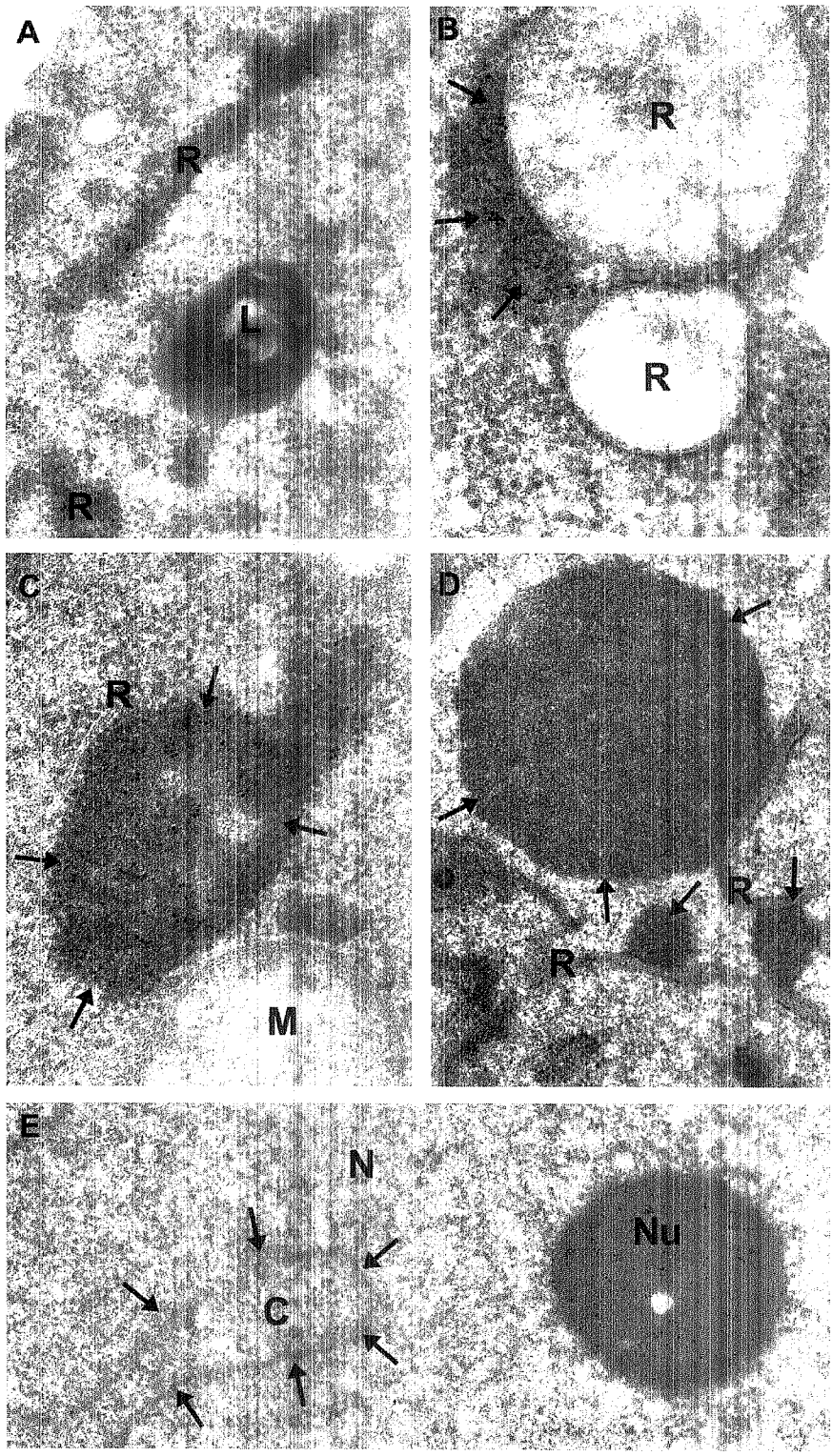
Interestingly, beside a cytoplasmic localization FMRP could also be demonstrated in the granular component of the nucleolus (Fig. 2E), which contains maturing ribosomal precursor particles. The distribution of gold particles in these transfected COS-cells demonstrates that most of the FMRP is present in the cytoplasm and only a minor fraction is present in the nucleus.

DISCUSSION

The discovery of the gene defect in the *FMR1* gene has led to a better understanding of the molecular basis of the fragile X mental retardation syndrome. However, the knowledge about the function of FMRP, the protein product of the *FMR1* gene, in the cell is still limited. In this immunoelectron microscopic study we report the actual *in vivo* localization of FMRP. We found FMRP associated with ribosomes that were free in the cytoplasm and with ribosomes that were attached to the ER membrane. The resolution of our immunocytochemical studies is not high enough to demonstrate the association of FMRP with the ribosomal 60S subunit as was suggested by Khandjian et al. on the basis of their in vitro experiments[19].

Surprisingly, we could demonstrate the presence of FMRP in the nucleolus where the protein was associated with the granular component, which contains maturing ribosomal precursor particles. Since, proteins above the size of 60 kDa can enter the nucleus only in an active way[21] this would implicate that FMRP, on the basis of its molecular weight of 67-80 kDa, is also dependent on such a signal dependent transport across the nuclear pore. Expression studies demonstrated that the N-terminus of FMRP (spanning exon 1-8) contains signal(s) or binding motifs to mediate the protein to the nucleus[13]. However, the majority of FMRP in the normal *in vivo* situation is found in the cytoplasm[8,9] (Figure 1A). A possible explanation for this discrepancy came from studies on an exon 14 splice variant[13] (Figure 1B). This splice variant was found predominantly in the nucleus. It was proposed that a cytoplasmic retention signal was present within exon 14 of the *FMR1* gene. Recently, it was shown that FMRP contains a nuclear export signal (NES) (Warren, personal communication). They found that sequences present in exon 14 are able to transport a reporter protein from the nucleus to the cytoplasm. The presence of both these signals (NLS and NES) suggests that FMRP may shuttle between the nucleus and the cytoplasm.

FIG. 2. Subcellular localization of FMRP, using an indirect immunogold labelling technique in COS-cells transfected with a full length cDNA. FMRP is found in association with ribosomes that are bound to the endoplasmic reticulum membrane (A and B) and in association with free ribosomes in the cytoplasm (C). In these COS-cells we notice very often a concentration of ribosomes lining the endoplasmic reticulum membrane (arrows in B, C and D). Note that these aggregates are also found in untransfected cells (D). Furthermore, FMRP is localized in the nucleolus of transfected cells (E). The arrows indicate the nuclear membrane. L=Lysosome; M=Mitochondrion; N=Nucleus; C=Cytoplasm; R=Rough endoplasmic reticulum; Nu=Nucleolus.


A
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These observations and our data that FMRP is present in the nucleolus would argue for the hypothesis that after synthesis in the cytoplasm active transport of FMRP to the nucleus, either via an NLS or via the interaction of the N-terminus with a nuclear component, takes place. In the nucleus an association with maturing ribosomal precursor particles, located in the nucleolus, occurs. It remains unclear whether FMRP plays an active role in targeting ribosomal precursor particles out of the nucleus or that FMRP is a just a ribosomal protein leaving the nucleus in a passive way, mediated by maturing ribosomal particles. The latter is most likely, since in cells from fragile X patients, lacking FMRP, a normal transport of ribosomes from the nucleus to the cytoplasm occurs.

On the other hand, the RNA binding properties of FMRP and the *in vitro* association with ribosomes led to different proposals for a theoretical function for FMRP. One possibility is that FMRP plays a regulatory role in the translational machinery of proteins by mediating transport of mRNA. There are two arguments against this hypothesis. First, our finding that FMRP is associated with the nucleolus, indicating a binding to rRNA instead of mRNA. Second, the fact that the small ribosomal subunit first binds a mRNA molecule and subsequently associates with the large ribosomal subunit, which makes the association of FMRP with the large ribosomal subunit secondary for a role in mRNA binding. In this respect, Tamanini et al.[20] already suggested the association of FMRP with ribosomes via rRNA in their *in vitro* studies.

Our study suggests a nuclear role for FMRP, like the many ribosomal proteins that are imported from the cytoplasm and subsequent packaged into ribonucleoprotein particles. Indeed, FMRP shuttles between the nucleus and the cytoplasm. In both compartments FMRP is bound to ribosomes, which is characteristic for ribosomal proteins. Although ribosomes contain a large number of proteins, the function of many of them is unknown. It is thought that they enhance the function of the ribosomes.

However, in this view it is difficult to explain that the absence of widely expressed FMRP causes the "limited" clinical features observed in fragile X syndrome. The identification of two human genes, FXR1 and FXR2, which show a high homology with the FMR1 gene shed a new light on a possible function of FMRP. Both, FXR1 and FXR2, can form homo- and heteromultimers with FMRP and like FMRP they also have RNA binding properties. Interestingly, FXR1 is not expressed in human brain[22] (Hoogeveen, unpublished results). Perhaps the impossibility of FXR2 protein to form heteromultimers *in vivo* by the absence of FMRP and FXR1 proteins in neurons from affected males is responsible for the mental retardation in these patients. Alternatively, a misrouting of the FXR2 protein because of the lack of FMRP expression and subsequent targeting to the nucleus and/or out of the nucleus may cause the dysfunction of central nervous system neurons.

Clearly, extensive studies to validate this hypothesis are necessary. The biochemical basis for the interaction between FMRP and the two homologs FXR1 and FXR2 and the *in vivo* RNA target(s) for FMRP are just two examples of further investigations. The answers to these questions may result in more knowledge about the cellular function of FMRP and the underlying cause of the characteristic phenotype of the fragile X syndrome.

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Publication 3

DIFFERENTIAL EXPRESSION OF FMR1, FXR1 AND FXR2 PROTEINS IN HUMAN BRAIN AND TESTIS

Filippo Tamanini, Rob Willemsen, Leontine van Unen, Carola Bontekoe, Hans Galjaard, Ben A. Oostra* and André T. Hoogeveen

Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam,The Netherlands

*To whom correspondence should be addressed

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Filippo Tamanini, Rob Willemsen, Leontine van Unen, Carola Bontekoe, Hans Galjaard, Ben A. Oostra* and André T. Hoogeveen

MGC–Department of Clinical Genetics, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands

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Lack of expression of the fragile X mental retardation protein (FMRP) results in mental retardation and macroorchidism, seen as the major pathological symptoms in fragile X patients. FMRP is a cytoplasmic RNA-binding protein which cosediments with the 60S ribosomal subunit. Recently, two proteins homologous to FMRP were discovered: FXR1 and FXR2. These novel proteins interact with FMRP and with each other and they are also associated with the 60S ribosomal subunit. Here, we studied the expression pattern of the three proteins in brain and testis by immunohistochemistry. In adult brain, FMR1, FXR1 and FXR2 proteins are coexpressed in the cytoplasm of specific differentiated neurons only. However, we observed a different expression pattern in fetal brain as well as in adult and fetal testis, suggesting independent functions for the three proteins in those tissues during embryonic development and adult life.

INTRODUCTION

Fragile X syndrome is a common form of inherited mental retardation with an incidence of ~1:4000 in males and 1:6000 in females (1). The syndrome is characterized by mental retardation, macroorchidism, typical facial appearance and various degrees of autistic behaviour (2). The fragile X syndrome is caused by the expansion of a highly polymorphic CGG repeat present in the 5′ untranslated region of the *FMR1* gene (3,4). When the repeat expands to more than ~230 CGG units, the promotor region and the repeat itself become hyper-methylated and as a consequence no FMR1 transcription (5) and thus no translation occurs (6,7). The absence of the FMR1 protein (FMRP) leads to mental retardation.

The FMR1 transcript is alternatively spliced and generates splice variants coding for different FMRP isoforms with a molecular weight varying from 70 to 80 kDa (7). FMRP is widely expressed in most adult and fetal tissues and high levels are found particularly in brain and testis (6). The protein is predominantly localized in the cytoplasm, although occasionally nuclear staining has been reported (6,7).

One clue as to the function of FMRP is derived from the identification in the FMR1 sequence of motifs observed in several RNA-binding proteins (8,9). Two heterogeneous nuclear ribonucleoprotein K homology (KH) domains and one RGG box are located in the middle and in the C-terminus of FMRP respectively. Data have been presented that show that FMRP can bind RNA *in vitro* with some degree of sequence specificity (8,9), but the functional evidence for the importance of the RNA-binding capacity was illustrated by a severe fragile X patient with a point mutation in the second KH domain of FMRP resulting in reduced RNA binding (10–12). Interestingly, in addition to its RNA-binding capacity, an association of FMRP with the ribosomal 60S subunit has recently been described (13,14) and it has been suggested that this binding occurs via RNA (15,16).

Recently we demonstrated at the electron microscopical level that FMRP is associated with free and membrane-bound ribosomes and, surprisingly, also in the granular component of the nucleolus (17). Our findings were supported by the identification of a nuclear location signal (NLS) as well as a nuclear export signal (NES) in the FMR1 protein (16,18,19). The NES of FMRP is functionally similar to the export signal identified in REV, the regulatory protein of human immunodeficiency virus type 1 (HIV-1), which mediates the export of the viral RNA from the nucleus to the cytoplasm. Taken together with the ribosomal association, these data led to the hypothesis that FMRP might play a role in the transport of RNA or ribosomal particles between nucleus and cytoplasm and possibly in the regulation of the translation and turnover of these RNAs.

Finally, two novel human proteins that interact with FMRP were discovered (20,21). These proteins, named FXR1P and FXR2P, are very similar in overall structure to FMRP (~60% amino acid identity) and they both have two KH domains and one RGG box that can bind artificial RNA homopolymers *in vitro*. Like FMRP, FXR1P and FXR2P have been found to be associated predominantly with the ribosomal 60S subunit (14). Furthermore, FMRP can form homo- and heteromultimers with FXR1P and FXR2P *in vitro* as well as *in vivo*, suggesting that this interaction could be a mechanism of functional autoregulation of this new family of RNA-binding proteins. So far, no common immunohistochemical studies for the three proteins have been performed, despite the possible importance of FXR1P and FXR2P in the functioning of FMRP, or vice versa. Only an RNA *in situ* hybridization analysis of the murine FXR1 has been reported,

*To whom correspondence should be addressed. Tel: +31 10 4087198; Fax: +31 10 4087200; Email: oostra@kgen.fgg.eur.nl

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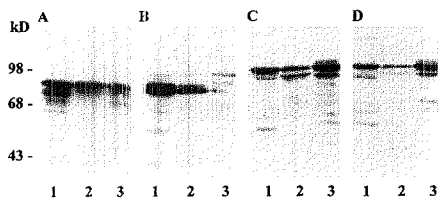

Figure 1. Western blot analysis of total protein incubated with the four different antibodies. Protein homogenates from human lymphoblastoid cell line, human brain and mouse brain (lanes 1, 2 and 3 respectively) were tested for the presence of FMRP with Ab1C3 (**A**), FXR1P with Ab1934 (**B**) and FXR2P with AbA42 and Ab1937 (**C** and **D** respectively).

which pointed to a different gene expression between FMR1 and FXR1 in tissues as testis, muscle and heart (22).

To characterize the *in vivo* distribution of FMRP, FXR1P and FXR2P, we studied the expression of the three proteins in human brain and testis by immunohistochemistry. Here we report a common expression pattern for FMRP, FXR1P and FXR2P in the cytoplasm of specific differentiated neurons, while fetal brain as well as adult and fetal testis showed a different distribution, which might suggest a possible independent function for the three proteins in those tissues. Finally, we show biochemically that FMRP and FXR2P were present in synaptosomes purified from mouse brains.

RESULTS

Characterization of the antibodies

For the detection of FMRP we used the mouse monoclonal antibody 1C3 (6) (Fig. 1A). To study the FXR1 protein we produced an anti-FXR1 rabbit polyclonal antibody (Ab1934). Because of the high rate of amino acid homology among the three proteins, the antibody Ab1934 was raised against an epitope in the C-terminus of FXR1P (amino acids 588–613) which is physically absent in the short form of FXR1P (amino acids 1–539) (20). The band seen with the antibody Ab1934 in lymphoblastoid cells and human brain (Fig. 1B, lanes 1 and 2) corresponds to the long isoform of FXR1P as described by Siomi *et al.* (20). The low expression of FXR1P in mouse brain (Fig. 1B, lane 3) can be the result of a poor recognition of the mouse sequence by our anti-FXR1P antibodies or can result from a low expression of the long isoform of FXR1P in mouse. The expression of the human FXR2 protein was studied by using the mouse monoclonal antibody (A42) which has been described recently (21) and a new anti-FXR2 rabbit polyclonal antibody (Ab1937). The results obtained on Western blot with both antibodies were similar (Fig. 1C and D).

Expression of FMRP, FXR1P and FXR2P in adult and fetal brain

FXR1 and FXR2 proteins were described to be localized in the cytoplasm of HeLa cells as the only cell lines tested so far (20,21). The developmental expression of the murine FXR1 gene was shown by RNA *in situ* hybridization (22), whereas the expression of FMRP has been characterized both at the RNA and protein level (6,23,24). Here, we show a comprehensive view of the expression patterns of FMR1, FXR1 and FXR2 proteins in different human tissues.

In human adult cerebellum, the strongest expression of FMRP was observed in neurons and especially in the Purkinje cells at the interface between the granular layer and the molecular layer (Fig. 2A). Similarly, a high expression for FXR1P and FXR2P in these Purkinje neurons is found (Fig. 2B and C respectively). All three proteins showed a clear cytoplasmic localization. In human adult cortex and brain stem the three proteins were not only localized to the cytoplasm of neurons but, in addition, compared to the Purkinje cells, a stronger labelling was seen in the proximal dendrites (data not shown). In brain tissues of a fragile X patient, as expected, no FMRP signal was detected as is demonstrated in cerebellum (Fig. 2D), while FXR1P and FXR2P expression was unchanged compared to the normal control (data not shown).

We performed a similar analysis in brain of a control fetus (18 weeks) and a fragile X fetus (18 weeks). The pattern of labelling of FMRP was comparable to the pattern seen in adult brain with a clear cytoplasmic signal in all the neurons (Fig. 2E). Since fetal brain contains mostly undifferentiated neurons, evident brain structures are difficult to determine. FXR1P showed an expression pattern in the cytoplasm of all the neurons but a strong nuclear signal was also present in a substantial number of neurons (Fig. 2F). FXR2P expression was also cytoplasmic, but weaker (Fig. 2G). In brain of a fragile X fetus no FMRP could be detected (Fig. 2H). In contrast, FXR1P and FXR2P labelling was identical to the pattern observed in brain of a normal control fetus (data not shown).

In summary, our results indicated that the expression pattern of the three proteins was clearly overlapping in normal human adult brain showing the same cell type specificity as well as a cytoplasmic localization. On the other hand, in fetal brain, FXR1 protein was also strongly expressed in the nuclei of some neurons.

Characterization of FMRP and FXR2P in mouse brain synaptosomes

To study the cytoplasmic expression of the neurons in more detail, we extended the analysis on cultured mouse hippocampal neurons. FMRP labelling in these neurons was strong in the perikaryon and the proximal dendrites but, in contrast, the distal dendrites and the axon contained little or no FMRP (Fig. 4A). Interestingly, we detected a similar labelling signal for FXR2P in the perikaryon and in the dendrites (Fig. 4B). It has been demonstrated that FMR1 and FXR1 and FXR2 are bound to ribosomes (14). Since ribosomes are localized within the neurons to the perikaryon, the proximal part of the axon and the dendrite (25), the reported protein distribution in the neurons could be due

Figure 2. FMRP, FXR1P and FXR2P expression in adult and fetal brain. Light-microscopic micrographs of cryostat sections from adult cerebellum (**A–C**) and fetal brain (**E–G**) of control individuals and adult cerebellum (**D**) and fetal brain (**H**) of a fragile X patient. Sections were immunostained for FMRP (A, D, E and H), FXR1P (B and F) and FXR2P (C and G) with the antibodies 1C3, 1934 and A42, respectively. A positive signal with the indirect immunoperoxidase technique results in a brown precipitate. Arrows indicate the nuclei of neurons which are positive for FXR1P (F).

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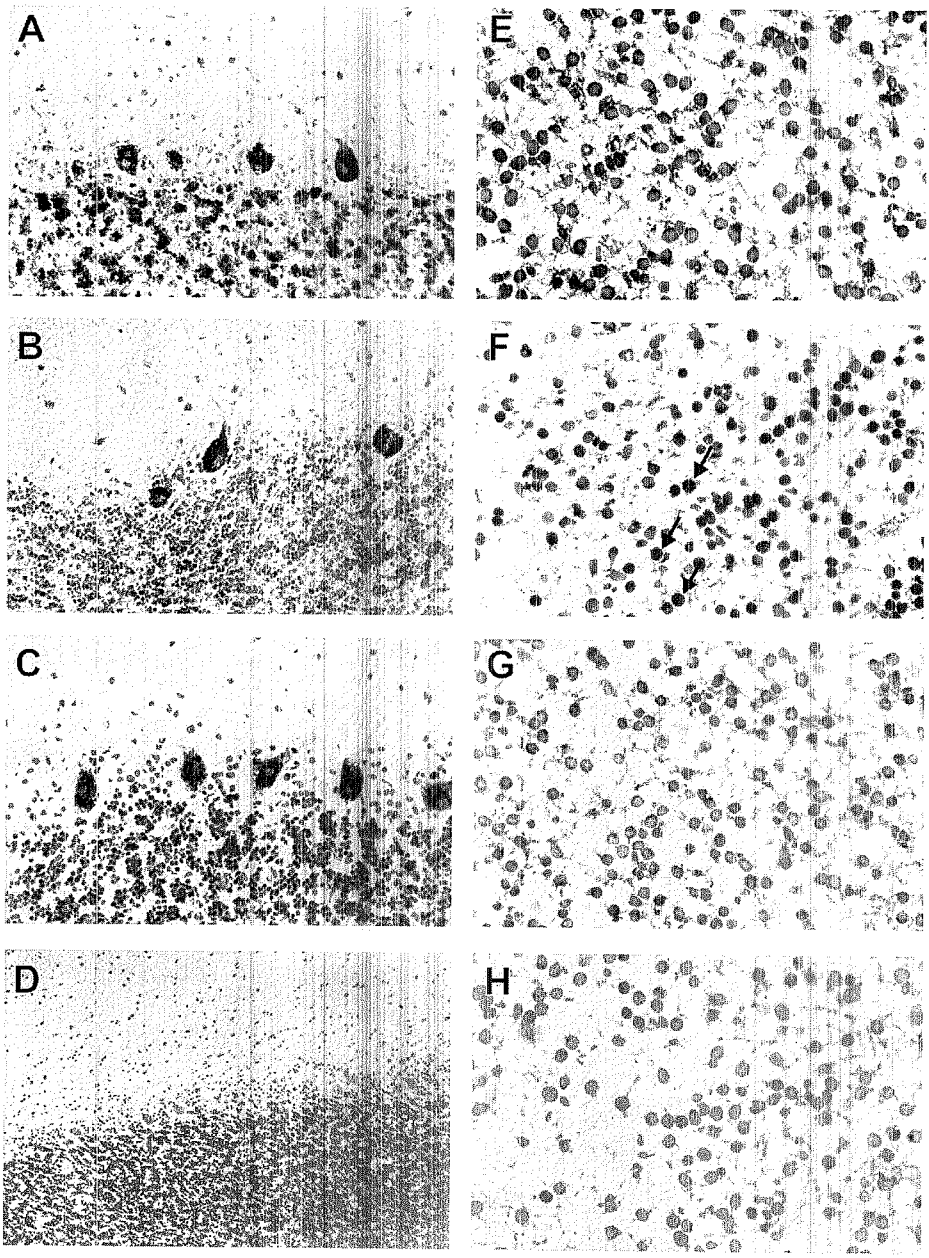

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to the association of FMRP and FXR2P with ribosomes. Next we tested whether FMRP and FXR2P were also present at the synapse. Therefore, a synaptosome preparation was purified from brains of wild type and *Fmr1* knockout mice, separated on SDS–PAGE gel and probed with the antibodies 1C3 and Ab1937. Using Western blotting we confirmed the presence of FMRP and FXR2P in our synaptosome preparation (Fig. 5). Synaptophysin (p38), a transmembrane glycoprotein of synaptic vesicles, was demonstrated to be present in the isolated synaptosomes that we used (Fig. 5, lane 5). The antibody Ab1934 does not recognize the murine FXR1 protein (Fig. 1B, lane 3).

Expression of FMRP, FXR1P and FXR2P in adult and fetal testis

Sections of human adult testis were examined for expression of the three proteins. As previously reported (6), we found a prominent labelling for FMRP in the cytoplasm of cells adjacent to the basal membrane of the seminiferous tubules, corresponding to spermatogonia (Fig. 3A). The labelling was often in a punctuated pattern, probably due to a large amount of FMRP concentrates in the cytoplasm. FXR1P was also detectable in spermatogonia. However, in addition, a predominant expression of FXR1 protein was found in the cytoplasm of cells which were more inside the tubuli seminiferi, corresponding to maturing spermatogenic cells (Fig. 3B). Finally, FXR2P gave a third, slightly different, pattern of expression in this tissue. The labelling of FXR2P is also punctuated, however, of lower intensity and present in all the cells of the seminiferous tubules (Fig. 3C). Figure 3D illustrates the localization of FMRP in adult testis from a fragile X patient. The normal expression of FMRP in spermatogonia is in agreement with previous studies, where it is reported that adult fragile X males with the fully expanded repeat in the somatic cells show only premutation length repeats in their sperm (26,27). The same material was tested for FXR1P and FXR2P expression and showed similar results as seen in a control testis (data not shown).

Testis from a normal fetus (20 weeks) and a fragile X fetus (18 weeks) were analysed for the three proteins. In normal fetal testis, FMRP was predominantly present in all primordial germ cells (PGCs) (Fig. 3E). Also, FXR1P could be localized predominantly in primordial germ cells; however, the non-spermatogenic cells were also labelled, although to a lesser extent (Fig. 3F). In contrast, FXR2P was only strongly expressed in interstitial cells (Fig. 3G). Testis from a fragile X fetus showed FMRP labelling in some primordial germ cells (Fig. 3H) whereas the majority of the testis was FMRP-negative. The few FMRP-positive PGCs might correspond to cells which most likely have a premutation allele due to full mutation contraction (28). FXR1P and FXR2P distribution in the testis from a fragile X fetus was similar to the normal fetus (data not shown).

From our results we concluded that, in adult and fetal testis, FMRP, FXR1P and FXR2P are expressed at various levels in different cell types, and they may, therefore, have independent functions. Conversely, in spermatogonia, the only cell type positive for FMRP, we detected an evident coexpression of the three proteins.

DISCUSSION

Recently, two human homologs of FMR1 were identified, named FXR1 and FXR2, and the corresponding proteins were indicated to interact with FMRP, the protein product of the FMR1 gene (20,21). Like FMRP, FXR1P and FXR2P have domains characteristic of RNA-binding proteins and all three are associated with ribosomes, predominantly with the 60S ribosomal subunit (14). *In vitro* and *in vivo* binding studies showed that FMRP, FXR1P and FXR2P form homo- and heterodimers, suggesting that this oligomerization may have a biological function (21). However, knowledge about the repertoire of FMRP/FXRPs combinations which exist in human tissues is still limited, particularly in brain and testis. Here we report that FMRP, FXR1P and FXR2P are differentially expressed in fetal brain as well as in adult and fetal testis, while the expression pattern in adult brain was identical.

In adult brain, examination of several neuronal populations revealed a common cytoplasmic localization and a high expression of the three proteins in Purkinje, cortical and brain stem neurons. FMRP is abundantly expressed in neurons and not detectable in non-neuronal cells such as glia, astrocytes and oligodendrocytes (6). Importantly, we showed that, like FMRP, FXR1 and FXR2 proteins were also only expressed in differentiated neurons.

In fetal brain, FXR2P, like FMRP, is expressed in the cytoplasm of the neurons. However, the expression of FXR2P in fetal brain is much lower than in adult brain. Also, for FXR1P, a different localization pattern is seen in fetal brain compared to adult brain. In adult brain FXR1P is only found in the cytoplasm of the neurons, while in fetal brain a substantial number of neurons also showed a nuclear localization.

The three proteins are very homologous; they have RNA-binding properties and a ribosomal association, which indicates a role in the ribosomal and RNA metabolism of neurons. Together with the common subcellular distribution it is suggestive to accept a complementary effect of the proteins. However, the absence of FMRP in the fragile X syndrome leads to mental retardation despite the observed normal expression of FXR1P and FXR2P in the neurons of fragile X patients. Therefore our result confirms that FXR1P and FXR2P cannot complement the absence of FMRP in fragile X patients, indicating that FMR1, FXR1 and FXR2 proteins may have independent, although similar, cellular functions. Another explanation is that the clinical phenotype seen in the fragile X syndrome (mental retardation) occurs during embryonic development where we demonstrated that the complementary effect of FXR2P or FXR1P is less likely than in adult brain due to a reduced expression and a different localization respectively in fetal neurons.

Furthermore, the three proteins were expressed in different cell types in adult as well as in fetal testis. In adult testis, FMRP was strongly expressed in spermatogonia. Despite the fact that our

Figure 3. FMRP, FXR1P and FXR2P expression in adult and fetal testis. Light-microscopic micrographs of cryostat sections from adult (A–C) and fetal testis (E–G) of control individuals and adult (D) and fetal testis (H) of a fragile X patient. Sections were immunostained for FMRP (A, D, E and H), FXR1P (B and F) and FXR2P (C and G) with the antibodies 1C3, 1934 and A42 respectively. Again, a brown precipitate illustrates a positive signal.

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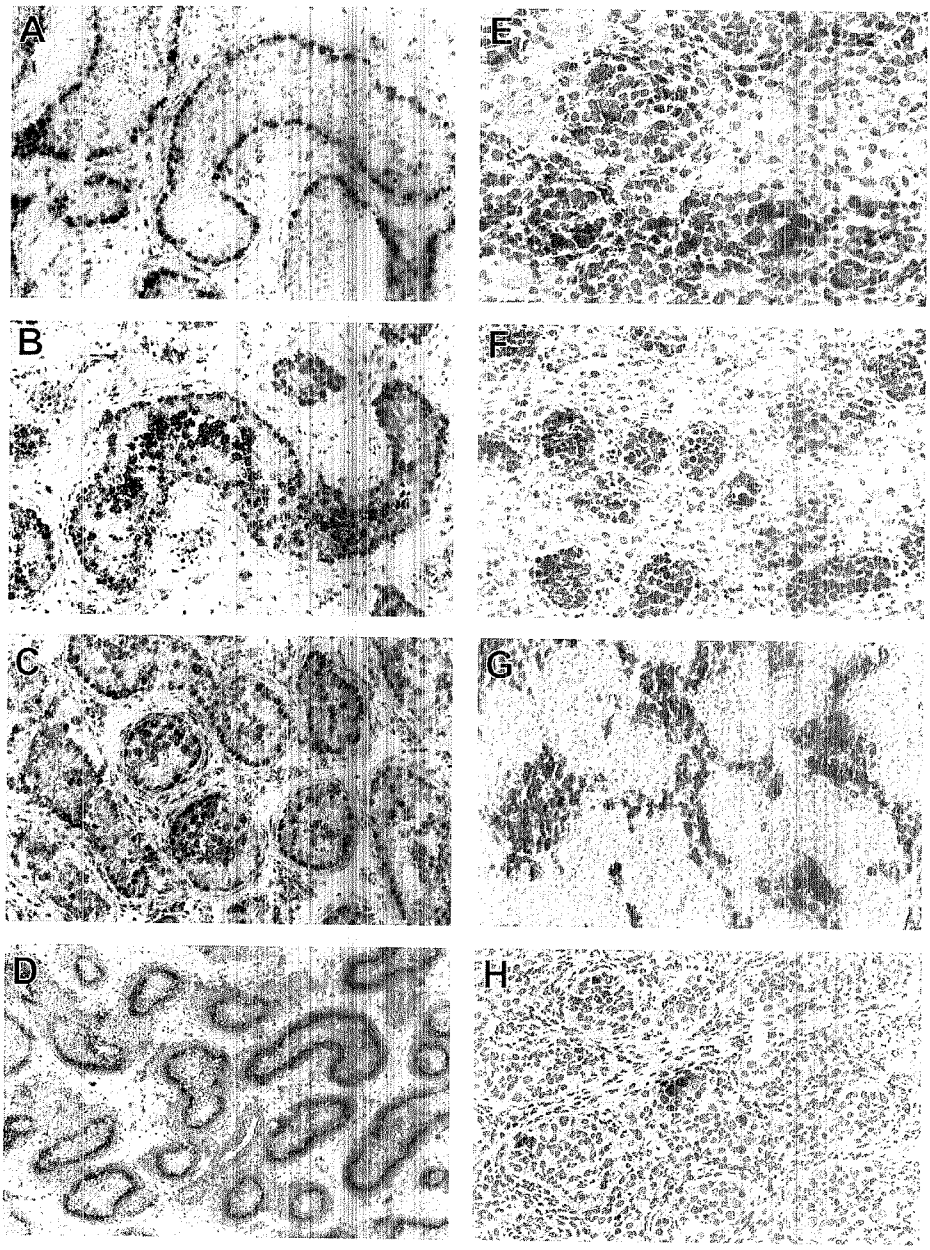

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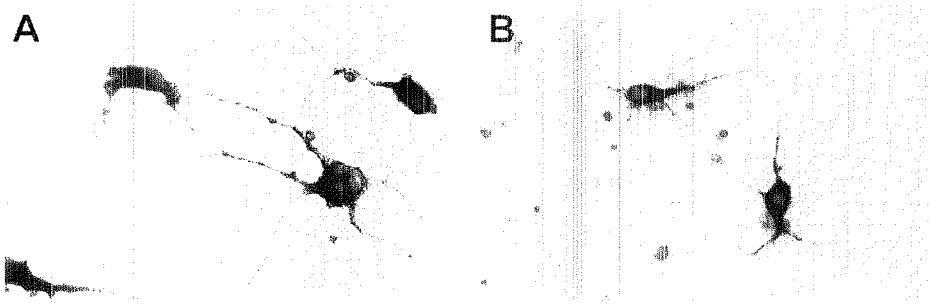

Figure 4. Localization of FMRP and FXR2P in cultured neurons. Cultured mouse hippocampal neurons (9 days in culture) were immunostained for FMRP (A) and FXR2P (B) with the antibodies 1C3 and A42 respectively, using an indirect alkaline phosphatase technique. With this method a positive reaction is illustrated by a red precipitate.

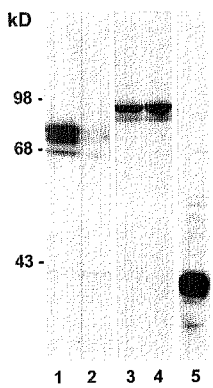

Figure 5. Equal amount of synaptosomes suspension derived from wild type mouse brain (lanes 1 and 3) and from *Fmr1* knock out brain (lanes 2 and 4) were tested for the presence of FMRP with Ab1C3 (lanes 1 and 2) and FXR2P with Ab1937 (lanes 3 and 4). Lane 5 shows the presence of Synaptophysin in the isolated synaptosomes.

cryostat sections did not permit recognition of the stages of spermatogenic differentiation, it is clear that FXR1P is predominantly expressed in maturing spermatogenic cells. This is supported by RNA *in situ* hybridization studies on mouse which showed that the strongest signal for FXR1 was restricted to spermatids (22). Finally, FXR2P showed a labelling signal in all the cells throughout the tubuli seminiferi.

In fetal testis, FMRP was only expressed in primordial germ cells (PGCs); FXR1P was also present in PGCs, but not exclusively. However, FXR2P was not detectable in PGCs but strongly present in the interstitial cells (Fig. 3G), resulting in a third different pattern of expression. The expression of FMRP in some PGCs of a fragile X fetus of 18 weeks (Fig. 3H) is in agreement with our recent model, suggesting that a reduction of full mutation alleles results in a premutation in sperm in adult patients (28).

Our results showed that FMR1, FXR1 and FXR2 proteins are colocalized in differentiated neurons, like Purkinje cells, while in fetal brain and testis each of the proteins has an independent cellular distribution. Neurons in particular need accurate protein synthesis and transport of mRNA from the nucleus to specific compartments as dendrites and synapses. With this in mind, it is possible that differentiated neurons may coexpress FMRP, FXR1P and FXR2P to enhance a particular cellular task which is not yet known. The presence of FMRP and FXR2P in dendrites and in synaptosomes could be in line with this hypothesis (29). Whether or not FMRP, FXR1P and FXR2P have complementary functions in neurons might be resolved by the generation of knockout mice for FXR1 and FXR2 and crossing with the *Fmr1* knockout (30) successively to generate double knockout mice.

MATERIALS AND METHODS

Antibodies and Western blotting

Polyclonal antibodies were raised in rabbits against synthetic peptides covering amino acid positions 588–613 of FXR1 (Ab1934) and amino acid positions 625–641 of FXR2 (Ab1937). Protein samples separated on 10% SDS–polyacrylamide gels were electroblotted onto nitrocellulose membrane (Schleicher & Schuell). Immunodetection was carried out using the mouse monoclonal antibody Ab1C3 (1:2500), the rabbit polyclonal antibody Ab1934 (1:2500), the mouse monoclonal antibody AbA42 (1:5000) and the rabbit polyclonal antibody Ab1937 (1:500). The secondary antibody (1:4000) was coupled to peroxidase allowing detection with chemiluminescence method (ECL KIT, Amersham). A monoclonal anti-Synaptophysin was purchased from Sigma and for Western blotting used in a 1:1000 dilution.

Immunohistochemistry

Tissues were embedded in Tissue-Tek (Miles, Inc.) and snap frozen in liquid nitrogen. Cryostat sections (7 µm) were fixed with 3% paraformaldehyde (10 min) followed by a methanol step (20 min). Endogenous peroxidase was inhibited by 30 min incubation in PBS–hydrogen peroxide–sodium azide solution (100 ml 0.1 M PBS + 2 ml 30% H_2O_2 + 1 ml 12.5% sodiumazide). Subsequently, sections were incubated either with Ab1C3 (produced in

Tecno/mouse system; 1:200) or Ab1934 (1:200) or AbA42 (1:200) for 1 h at room temperature. Subsequently, a 1 h incubation with a peroxidase conjugated secondary antibody was performed and the signal was detected using 3,3'-diamino-benzidine.HCl (Serva) as a substrate. The sections were counterstained with hematoxylin. The immunohistochemistry of cultured neurons was performed using an alkaline phosphatase conjugated secondary antibody and New Fuchsin Red as substrate (DAKO).

Synaptosome purification and cultured neurons

Synaptosomes were prepared from the forebrain of 20 day old mouse pups following the protocol of Rao *et al.* (31). A 20% (w/v) homogenate was prepared from chopped forebrain in 0.35 M Sucrose, 10 mM Tris pH 7.4, 0.5 mM EGTA solution. The homogenate was centrifuged at 2000 *g* for 1 min using a JA-20 fixed angle rotor to remove a nuclear pellet (P1). P1 was washed once by resuspension in the homogenizing buffer and repelleting. The supernatant was combined with the supernatant from the first centrifugation, and the pellet was discarded. The pulled supernatant (S1) was centrifuged at 23 000 *g* for 4 min to yield a crude mitochondrial pellet (P2), which was washed once and repelleted. P2 was then made up to 6 ml total volume with the same buffer. This suspension was layered onto a discontinuous gradient of 5 and 13% Ficoll in 0.35 M Sucrose that had been allowed to equilibrate at 4°C for 1 h prior to loading the sample. The gradient was centrifuged at 45 000 *g* for 45 min using a SW50.1 swinging bucket rotor. A synaptosomal fraction was collected from the 5–13% interface and diluted in 0.35 M Sucrose and centrifuged at 23 000 *g* for 20 min. The synaptosomal pellet was resuspended and equal amount of protein in Laemmli buffer were loaded on a 10% SDS–polyacrylamide gel.

Hippocampi were dissected out from 2–5 day old mouse brains and placed in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Quickly the Hippocampi were subdivided into small pieces, incubated for 10 min with trypsine + DNase I and mechanically dissociated in HBSS w/o Ca^{2+} and Mg^{2+} supplemented with DNase I. Divalent cations were restored by dilution with 2 vol of HBSS. After allowing non dispersed tissues to settle for 3 min, the supernatant was centrifugated for 1 min at 200 *g*. Subsequently, the cells were plated onto D-polylysine coated glass coverslip and cultured for 1–2 weeks in Neurobasal Medium (Life Technologies) supplemented with B27 and 25 μM glutamate.

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Publication 4

DIFFERENT TARGETS FOR THE FRAGILE X-RELATED PROTEINS REVEALED BY THEIR DISTINCT NUCLEAR LOCALIZATIONS

Filippo Tamanini, Carola Bontekoe, Cathy E. Bakker, Leontine van Unen, Burcu Anar, Rob Willemsen, Minoru Yoshida^, Hans Galjaard, Ben A. Oostra and André T. Hoogeveen*

Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam,The Netherlands

^Department of Biotechnology, University of Tokyo, Tokyo, Japan

*To whom correspondence should be addressed

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Different targets for the fragile X-related proteins revealed by their distinct nuclear localizations

Filippo Tamanini, Carola Bontekoe, Cathy E. Bakker, Leontine van Unen, Burcu Anar, Rob Willemsen, Minoru Yoshida[1], Hans Galjaard, Ben A. Oostra and André T. Hoogeveen*

Department of Clinical Genetics and Center for Biomedical Genetics, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands and [1]Department of Biotechnology, University of Tokyo, Tokyo, Japan

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Fragile X syndrome is caused by the absence of the fragile X mental retardation protein (FMRP). FMRP and its structural homologues FXR1P and FXR2P form a family of RNA-binding proteins (FXR proteins). The three proteins associate with polyribosomes as cytoplasmic mRNP particles. Here we show that small amounts of FMRP, FXR1P and FXR2P shuttle between cytoplasm and nucleus. Mutant FMRP of a severely affected fragile X patient (FMRPI304N) does not associate with polyribosomes and shuttles more frequently than normal FMRP, indicating that the association with polyribosomes regulates the shuttling process. Using leptomycin B we demonstrate that transport of the FXR proteins out of the nucleus is mediated by the export receptor exportin1. Finally, inactivation of the nuclear export signal in two FXR proteins shows that FMRP shuttles between cytoplasm and nucleoplasm, while FXR2P shuttles between cytoplasm and nucleolus. Therefore, molecular dissection of the shuttling routes used by the FXR proteins suggests that they transport different RNAs.

INTRODUCTION

Absence of the *FMR1* gene product [fragile X mental retardation protein (FMRP)] is responsible for fragile X syndrome, which is mainly characterized by mental retardation and macroorchidism (1–5).

FMRP is highly expressed in neurons (5,6) and localizes in the cytoplasm associated with polyribosomes in an RNA-dependent manner (7–11). FMRP contains two KH domains and an RGG box, which are common among RNA-binding proteins, and it binds RNA with some degree of sequence specificity (12,13). The importance of the KH domains for the function of the protein is illustrated by a fragile X patient with a point mutation (Ile304Asn) in the second KH domain (14). More recently, it has been shown that the mRNP particles harbouring the mutated FMRPI304N are of smaller size and do not associate with translating polyribosomes (15).

In addition, FMRP contains a functional nuclear localization signal (NLS) and a nuclear export signal (NES) (9,16–18). The presence of these localization signals suggests that FMRP may shuttle between cytoplasm and nucleus and is involved in the transport of a subset of RNAs from the nucleus to the ribosomes. Indeed, immunoelectron microscopical studies, both on neurons and COS cells overexpressing FMRP, show that a minor part of the protein is also detected in the nucleus (19) and in the nucleolus (20).

The leucine-rich FMRP NES is similar to the recently discovered Rev/protein kinase A inhibitor (PKI)-type NES (21,22). This type of NES has been identified in an increasing number of nucleo-cytoplasmic shuttling proteins such as MAPKK (23), cyclinB1 (24), actin (25) and C-ABL tyrosine kinase (26). Distinct types of NES are found in the sequences of the hnRNP A1 (27) as well as hnRNP K proteins (28). Recently, several groups have reported that exportin1 (or CRM1) is the major receptor of the leucine-rich type of NES. Leptomycin B (LMB) blocks the nuclear export of proteins containing this NES by inhibiting the interaction with exportin1 (29–32).

FXR1P and FXR2P, two proteins homologous with FMRP, have been identified and characterized (33,34). The amino acid sequence of FMRP is highly homologous with FXR1P and FXR2P in the N-terminus (86 and 70% identity, respectively). This region includes the NLS, dimerization domain (10), KH domains and the leucine-rich NES. The RGG box in the C-terminus is also conserved between the three proteins. Consistent with these observations, FXR1P and FXR2P, as well as FMRP, bind RNA *in vitro* and are found in the cytoplasm associated with ribosomes (10). Whether FXR1P and FXR2P shuttle between cytoplasm and nucleus is part of the present study.

The transport of RNA from the nucleus to the cytoplasm occurs via energy-dependent as well as signal-mediated mechanisms. Therefore, the characterization of new RNA-binding proteins involved in the export of RNAs is an important step in understanding the molecular basis of this biological process. In the present study we show that the RNA-binding protein FXR2P shuttles between cytoplasm and nucleolus and that continuous transcription of rRNA but not mRNA plays an important role in

*To whom correspondence should be addressed. Tel: +31 10 4087355; Fax: +31 10 4089489; Email: hoogeveen@ikg.fgg.eur.nl

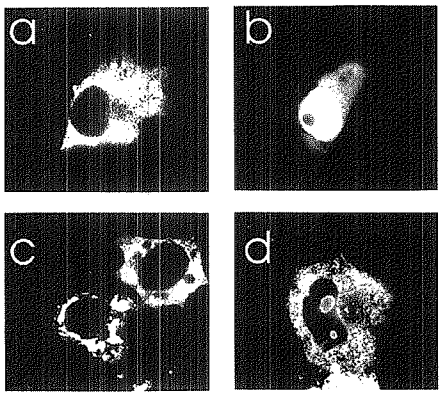

Figure 1. Expression of wild-type and NES-defective FMRP and FXR2P. COS cells were transfected with expression plasmids encoding wild-type FMRP (a), NES mutant FMRP34A (b), wild-type FXR2P (c) or NES mutant FXR2Pmut (d). Forty-eight hours after transfection the encoded proteins were visualized using the mouse monoclonal antibody 1C3 against FMRP (a and b) or the rabbit polyclonal antibody 1937 against FXR2P (c and d).

its trafficking. Furthermore, the distinct nuclear localization of FMRP and FXR2P carrying the same mutations in the NES (Leu→Ala) indicates different cellular routes as well as RNA targets for these proteins. Finally, we demonstrate that the exportin1 signaling pathway mediates the export of FMRP, FXR1P and FXR2P from the nucleus.

RESULTS

FXR2P contains a functional NES

The nucleo-cytoplasmic shuttling of FMRP occurs by an NLS- and a Rev-like NES-mediated signaling pathway (9,17). Comparison of the sequence of the FMRP NES identified potential leucine-rich NESs in the sequences of FXR1P (amino acids 364–371) as well as in FXR2P (amino acids 374–381).

To study the role of the putative FXR2P NES we generated a mutant (FXR2mut) in which Leu374 and Leu376 were replaced with alanine. It has been established previously that this type of mutation inactivates the Rev/PKI NES (21,22). Indeed, FMRP carrying an identical mutation in the NES (FMRP34A) (17) or an alternative splice variant lacking the NES (FMRPISO12) (16,20) localized in the nucleus with exclusion of the nucleolus (Fig. 1b). COS cells were transfected with FXR2 or FXR2mut expression plasmids under the control of a CMV promoter. The subcellular distribution of the proteins was determined by immunofluorescent staining with anti-FXR2P specific antibodies (35). FXR2P was detected exclusively in the cytoplasm (Fig. 1c), as were both wild-type FMRP (Fig. 1a) and FXR1P (data not shown). In contrast, the FXR2Pmut protein was localized in the cytoplasm and the nucleoli of all transfected cells (Fig. 1d). Often the FXR2Pmut signal was more intense at the periphery of the nucleolus. No staining of the nucleoplasm was observed.

These results clearly indicate that the FXR2P NES is functional and promotes the export of FXR2P from the nucleolus to the cytoplasm. Therefore, both FMRP and FXR2P are nucleo-cytoplasmic shuttling proteins. Moreover, once imported into the nucleus, FMRP and FXR2P may have different targets, since FMRP34A and FXR2Pmut accumulate in the nucleoplasm and the nucleoli, respectively.

LMB induces nuclear retention of the FXR proteins

LMB is a specific inhibitor of nuclear export mediated by leucine-rich NESs. If the subcellular distribution of the FXR proteins is regulated by the activity of the NES, then LMB treatment should interfere with their normal localization. To test this hypothesis, the expression of FXR2P, FMRP and FXR1P (FXR proteins) was investigated by immunofluorescent labelling of transfected COS cells before and after LMB treatment.

After 3 h LMB treatment (50 ng/ml), FXR2P was detected both in the cytoplasm and the nucleoli of transfected cells (Fig. 2a). This localization pattern was identical to that obtained by expressing FXR2Pmut in the absence of LMB (Fig. 1d). After 3 h incubation with LMB ~50% of the cells showed expression of FXR2P in the nucleoli, while all the nucleoli were FXR2P-positive after an overnight incubation. These data showed that in addition to the cytoplasmic localization there was an increase in nucleolar labelling with time. In COS cells transfected with FXR2mut and incubated overnight with LMB, the expression of FXR2Pmut was also nucleoplasmic (Fig. 2b). As a control, LMB treatment did not change the cytoplasmic localization of tuberin, which lacks a leucine-rich NES (data not shown) (36).

Next, COS cells were transfected with FMR1 and FXR1 expression plasmids. LMB incubation for 3 h did not affect the localization of FMRP or FXR1P. After longer LMB treatment, however, both FMRP (Fig. 2e) and FXR1P (Fig. 2f) could be detected in the nuclei of transfected cells. The nuclear expression of FMRP observed after LMB treatment (Fig. 2e) was lower compared with the nuclear expression of FMRP34A (Fig. 1b). In contrast to FXR2P, no FMRP or FXR1P could be detected in the nucleoli.

Since LMB specifically blocks the function of the NES receptor exportin1, these results indicate that nuclear export of the FXR proteins is exportin1-dependent.

The nuclear import mediated by the NLS of FMRP is energy dependent (9). To study whether the nuclear import of FXR2P requires energy or not, COS cells transfected with FXR2 were treated with LMB at 4°C for 3 h. Since FXR2P under these conditions did not accumulate in the nucleus (Fig. 2c), we concluded that the import of FXR2P is also energy-dependent. Inhibition of protein synthesis with cyclohexamide in combination with the LMB treatment (3 h) did not diminish the cytoplasmic and the nucleolar signals of FXR2P (Fig. 2d). Since FXR2P remained exclusively cytoplasmic after treatment with cyclohexamide alone (data not shown), we conclude that the pre-existing FXR2P shuttles continuously between cytoplasm and nucleolus.

The shuttling of FXR2P is regulated by rRNA transcription

The hnRNP proteins contain RNA-binding motifs like the KH domain and the RGG box and are the major proteins that interact with pre-mRNAs and mRNAs (37). The nucleo-cytoplasmic

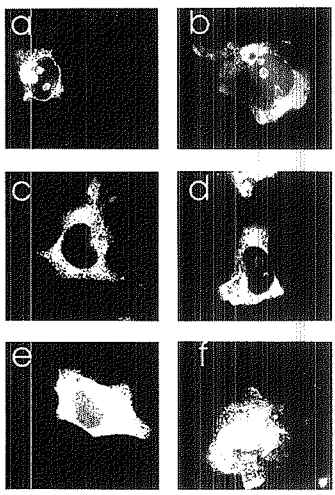

Figure 2. Effect of LMB on the subcellular localization of the FXR proteins. COS cells were transfected with expression plasmids encoding wild-type FXR2P (**a**, **c** and **d**), FXR2Pmut (**b**), wild-type FMRP (**e**) and wild-type FXR1P (**f**). Forty-eight hours after transfection the cells were treated for 3 h (a, c and d) or overnight (b, e and f) with LMB. In (c) cells were incubated with LMB at 4°C. In (d) cells were incubated both with LMB and the protein synthesis inhibitor cyclohexamide.

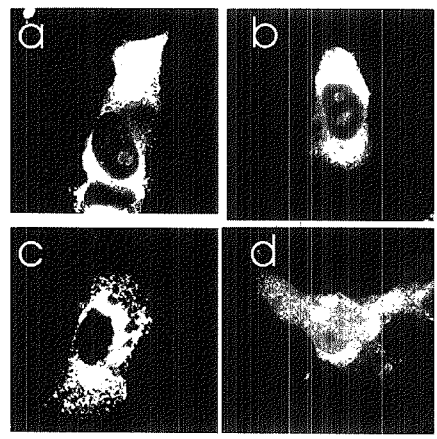

Figure 3. The shuttling of FXR2P is dependent on rRNA transcription. COS cells were transfected with expression plasmids encoding wild-type FXR2P (**a–c**) or FXR2Pmut (**d**). Forty-eight hours after transfection, cells were treated for 1 h with α-amanitin (a), DRB (b) or AMD (c) followed by 3 h incubation with LMB in combination with the same RNA polymerase inhibitors (a–c). In (d) cells were treated only with AMD for 4 h.

shuttling of the hnRNP A1 protein depends on continuous RNA polymerase II transcription. Inhibition of mRNA transcription results in accumulation of the hnRNP A1 protein in the cytoplasm (38). Therefore, the importance of RNA transcription in the shuttling of FXR2P was investigated.

We initially tested whether continuous transcription is necessary for the process of nuclear import of FXR2P. Transfected cells were incubated for 1 h in medium supplemented with various RNA polymerase inhibitors, followed by 3 h incubation in medium containing both LMB and RNA polymerase inhibitors and finally fixed for immunofluorescence staining.

RNA polymerase II transcription is selectively inhibited both by α-amanitin (20 μg/ml) (39) and by 5,6-dichlororibofuranosyl benzimidazole (DRB) at 100 μM (40), without affecting rRNA synthesis. After incubation with α-amanitin and LMB, the nucleolar localization of FXR2P (Fig. 3a) and the percentage of transfected cells with positive nucleoli (~40%) were comparable with cells treated with LMB alone. Intermediate results (~20% of transfected cells with positive nucleoli) were observed with DRB incubation (Fig. 3b).

Actinomycin D (AMD) in low doses (0.05 μg/ml) selectively inhibits the activity of RNA polymerase I but does not detectably affect RNA polymerase II and III (41). Incubation with AMD suppressed the LMB-induced nucleolar localization of FXR2P (Fig. 3c). This result indicates that continuous rRNA transcription and not mRNA transcription is necessary for nuclear import of FXR2P.

Next, we investigated whether RNA transcription is important to stably maintain FXR2P in the nucleolus. COS cells expressing FXR2Pmut were treated for 4 h with the same RNA polymerase inhibitors, followed by fixation and immunofluorescence detection. The inhibition of RNA polymerase II activity by α-amanitin had no effect on the nucleolar localization of FXR2Pmut (data not shown). After DRB treatment, half of the transfected cells contained detectable levels of FXR2Pmut in the nucleoli, while in the other half FXR2Pmut was dispersed in the nucleoplasm or in disaggregated nucleolar structures (necklaces) which are typical of this treatment (data not shown; 42,43). Inhibition of RNA polymerase I by AMD completely disrupted the nucleolar localization of FXR2Pmut that was now dispersed in the nucleoplasm (Fig. 3d). As previously reported for hnRNP A1(40), we studied hnRNP A1 accumulation as a control and found that hnRNP A1 accumulated in the cytoplasm after DRB but not AMD treatment, showing that the polymerase inhibitors were effective (data not shown).

The Ile304Asn mutation affects the nucleo-cytoplasmic shuttling of FMRP

Recently, it has been shown that the mutated FMRP of a severe fragile X patient (FMRPI304N), carrying an Ile→Asn substitution in the second KH domain, does not associate with translating ribosomes (15). Since overexpressed FMRPI304N was found to be cytoplasmic by immunofluorescent analysis, it was concluded that the nucleo-cytoplasmic shuttling of FMRP was not affected by the Ile304Asn mutation.

We tested the shuttling properties of FMRPI304N by blocking the function of exportin1 with LMB. FMRPI304N was localized

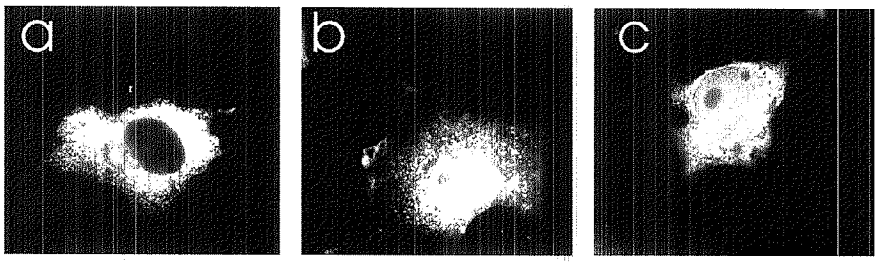

Figure 4. Effect of LMB on the subcellular localization of FMRPI304N. COS cells were transfected with the expression plasmid encoding the mutated FMRPI304N (a–c). Forty-eight hours after transfection, the cells were treated overnight with LMB (b) or with LMB and cyclohexamide (c).

exclusively in the cytoplasm of transfected COS cells (Fig. 4a), as was wild-type FMRP. In contrast, after LMB incubation for either 4 h or overnight we detected the majority of FMRPI304N in the nucleus with exclusion of the nucleoli (Fig. 4b). The nuclear expression of FMRPI304N after 4 h LMB treatment was stronger than wild-type FMRP after 24 h of similar treatment (compare Figs 4b and 2e) and comparable with the NES mutated FMRP34A (Fig. 1b). A similar result was obtained after incubation with LMB and cyclohexamide (Fig. 4c), showing that the nucleoplasmic FMRPI304N was mostly pre-existing protein which had moved from the cytoplasm. FMRPI304N was also detected in the nucleus with exclusion of the nucleoli after treatment with LMB and high doses of AMD (5 μg/ml) (data not shown), indicating that the shuttling of FMRPI304N was not affected by inhibition of both rRNA and mRNA transcription.

We conclude that FMRPI304N has an increased shuttling activity compared with the wild-type protein, most likely as a result of the inability of FMRPI304N to associate with translating ribosomes.

DISCUSSION

It has been proposed that FMRP is a nucleo-cytoplasmic shuttling protein (9,17). The NES of FMRP is similar to the NES first identified in the Rev regulatory protein of HIV-1 virus (21) and in PKI (22) and is fully conserved in the FMRP homologues FXR1P and FXR2P.

This study demonstrates that FMRP, FXR1P and FXR2P shuttle between cytoplasm and nucleus in a regulated manner and that, most likely, they interact with different RNAs during this process. However, we cannot exclude the possibility that these proteins target the same RNAs to different cellular components.

It has been established that amino acid substitutions in the Rev/PKI NES (Leu→Ala) make this signal non-functional (21,22). We show that similar mutations within the leucine-rich FXR2P NES result in the cytoplasmic as well as nucleolar localization of the corresponding FXR2Pmut protein (Fig. 1d). The presence of FXR2Pmut in the nucleolus indicates that: (i) the predicted FXR2P NES is functional and its activity contributes to the predominantly cytoplasmic distribution of wild-type FXR2P; and (ii) FXR2P shuttles between cytoplasm and nucleolus. Previously, Fridell *et al.* (17) demonstrated that a mutated FMRP (FMRP34A), carrying identical Leu→Ala substitutions in the FMRP NES, relocalized to the nucleus with exclusion of the nucleoli (Fig. 1b). From these observations we hypothesize that the FXR proteins, although containing very similar functional domains, may have different affinities as well as sites of interaction with factors involved in their shuttling. In particular, the cellular routes of FMRP and FXR1P are different from that of FXR2P, involving the nucleoplasm and nucleolus, respectively. However, in rare cases, we could also detect FMRP in the nucleolus of transfected COS cells (20). Since FMRP does not localize in the nucleolus when the NES-mediated pathway is inhibited (Figs 1b and 2e), the occasionally nucleolar accumulation suggests that FMRP can also follow alternative pathways.

Interestingly, in the FXR sequences the C-terminal region is the only divergent region, sharing ~6% similarity. Thus, the C-terminus of FXR2P may either contain a unique domain for interaction with a nucleolar component(s) or fold the protein in a tertiary structure resulting in increased nucleolar affinity.

Continuous rRNA, but not mRNA, transcription is necessary for both the import of FXR2P into the nucleus and its maintenance in the nucleolus, indicating that the cellular trafficking of FXR2P may be linked to assembly and transport of ribosomal subunits. Although the mechanism is still unclear, a similar behaviour has been described for the shuttling activity of the Rev protein (44,45). Alternatively, similar to Rev, FXR2P may be involved in mRNA export and/or degradation. An example of the involvement of the nucleolus in mRNA transport is the detection of specific transcripts, such as MyoD and N-myc, in the nucleoli (46). From the present study it also emerges that FMRP may transport different RNAs than FXR2P and that the inhibition of both mRNA and rRNA transcription does not affect the shuttling of FMRPI304N.

It has been shown that the Rev-like NES interacts directly with the nuclear export receptor exportin1 (or CRM1) (29–32). The antibiotic LMB inhibits the nuclear export of NES-containing proteins by blocking the formation of the NES/exportin1/Ran–GTP complex (29,47). Treatment with LMB also induces the partial retention of both FMRP (Fig. 2e) and FXR1P (Fig. 2f) in the nucleoplasm and FXR2P in the nucleolus (Fig. 2a), demonstrating that the nuclear export of the FXR proteins is exportin1-dependent. Consistent with this hypothesis, exportin1 (48) and its cofactor Rip/Rab (49,50) have been detected both in the nucleoplasm and the nucleolus, in addition to their nuclear membrane localization.

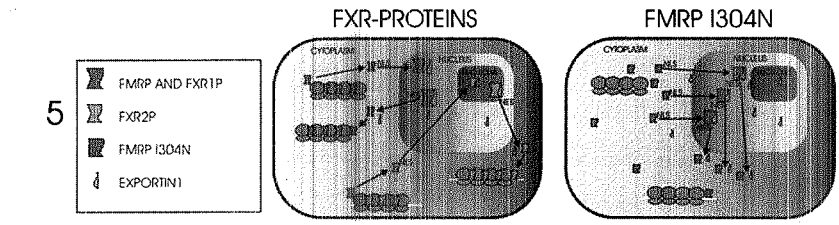

Figure 5. Model for the cellular routes of FXR proteins and FMRPI304N. FXR proteins enter the nucleus/nucleolus via an NLS pathway after dissociation from polyribosomes. In the nucleus they interact with exportin1 via the FXR NES and are then exported to the cytoplasm where they again associate with the polyribosomes. Since FMRPI304N is not associated with polyribosomes, it can shuttle in and out of the nucleus more often.

The FXR proteins are mainly associated with the ribosomes as cytoplasmic mRNP particles. To better understand their function it is important to determine how these proteins shuttle as well as the nature of their cargoes. For example, cyclin B1 moves completely from the cytoplasm to the nucleus after 2 h LMB treatment, indicating that the bulk of cyclin B1 shuttles continuously in and out of the nucleus (24). We noticed that after LMB treatment the majority of the FXR proteins remained cytoplasmic in HeLa cells (data not shown) as well as in transfected COS cells. However, a mutated FMRP, containing an Ile304Asn amino acid substitution in the second KH domain, accumulates predominantly in the nucleoplasm after LMB treatment (Fig. 4b). Our proposal that this mutation impaired the association of FMRPI304N with polyribosomes (8) was recently confirmed by Feng *et al.* (15), who also demonstrated that FMRPI304N is incorporated into smaller (600–150 kDa) EDTA-resistant mRNP particles than normal FMRP (>600 kDa). Most likely, since FMRPI304N is not bound to the polyribosomes in the cytoplasm, it shuttles between the two compartments more frequently than the normal FMRP. Interestingly, the FMRP NES overlaps with a coiled coil domain involved in ribosome binding (10). Different associations of FMRP and FXR2P with ribosomes may explain the stronger accumulation in the nucleus of FMRP34A compared with FXR2Pmut.

From these results we propose a cellular routing for the FXR proteins (Fig. 5) where the newly synthesized proteins may form polyribosomal complexes in the cytoplasm rather than being imported directly into the nucleus. Both the dissociation of the FXR proteins from polyribosomes and the presence of an NLS give them the opportunity to move from the cytoplasm to the nucleoplasm/nucleolus in an energy-dependent way. Most likely, the conserved NLS in the N-terminus of FMRP (9,18), FXR1P and FXR2P targets these proteins to the nucleus by a similar mechanism. The transport of the FXR proteins from the nucleus to the cytoplasm is exportin1 dependent, since export is inhibited by LMB and by inactivation of the FXR NES. Therefore, it is possible that, in response to developmental or stress stimuli, association–dissociation of the FXR proteins with polyribosomes regulates their shuttling. For example, FXR1P localizes either in the nuclei or in the cytoplasm of neurons in fetal and adult brain, respectively. Core ribosomal proteins are imported into the nucleus shortly after their synthesis to prevent degradation. Thus, it is unlikely that pure ribosomal proteins and FXR proteins, although associated in the cytoplasm, follow the same cellular route. However, the importance of a correct polyribosomal interaction for the function of FMRP is clearly demonstrated by the abnormal shuttling of FMRPI304N from a severely affected fragile X patient.

The unique nuclear localization of the FXR proteins shows that they can interact with different RNAs. The next challenge is the identification of these targets which not only will lead to knowledge about the function of these proteins but also contribute to our understanding of the pathogenesis of fragile X syndrome.

MATERIALS AND METHODS

DNA constructs

A 2740 nt cDNA clone of human FXR2 was cloned in the *Eco*RI site of the eukaryotic expression vector pSG5 controlled by the SV40 promoter. To mutate the NES of FXR2 four base pairs in two amino acids were changed, replacing Leu374 and Leu376 with alanine [as in mutant FMRP34A of Fridell *et al.* (17)]. A PCR was performed using human FXR2 cDNA with primer Fix1 (positions 1323–1374), containing an *Eco*NI site and the four base pair changes (underlined) (5′-TCC TAC CTG CAG GAG GTA GAG CAG GCT CGC GCG GAG AGG CTG CAA ATT GAT-3′), and primer Fix2 (positions 2157–2184), containing a *Hin*dIII site (5′-GCC CTT AGG AAG CTT GCT GAC AGA GTC-3′). A PCR fragment of 861 bp was digested with *Eco*NI and *Hin*dIII and subsequently used to replace the unmutated *Eco*NI–*Hin*dIII fragment in the cDNA. The mutated construct was checked by sequence analysis and could be digested with *Hha*I, for which a new site was created.

The cDNA of the long isoform of human FXR1 (33) was digested with *Eco*RI and *Bam*HI and cloned in the pSG5 vector.

The expression vectors containing the full-length FMR1 cDNA (pSF2) (4) and FMRP34A (17) have been described.

The Ile304Asn missense mutation was introduced into plasmid pSF2 by replacement of a T with A at position 1152. The vector was called pFMRPI304N.

Transfections

COS cells were cultured in DMEM with 10% fetal calf serum at 37°C and 5% CO_2. The day before transfection, the cells were seeded on glass coverslips. Transfections were performed as

described by the manufacturer using 0.5 μg DNA, 3 μl Plus reagent and 2 μl Lipofectamine (Gibco BRL, Gaithersburg, MD). Cells were fixed for immunofluorescence 24 or 48 h after transfection.

Immunofluorescence and antibodies

Cells were fixed in 0.1 M phosphate-buffered saline (PBS) containing 3% paraformaldehyde (pH 7.3) for 7 min at room temperature followed by a permeabilization step in 100% methanol for 20 min. Primary and secondary antibody incubations were performed for 60 min at room temperature in blocking buffer containing PBS, 0.15% Tris–glycine (Fluka, Buchs, Germany) and 0.5% bovine serum albumin (Fluka). The primary antibodies were a rabbit anti-FXR2P antibody (Ab1937, 1:200) (35), a rabbit anti-FXR1P antibody (Ab1934, 1:200) (35), a mouse monoclonal anti-FMRP antibody (1C3, 1:200) (5) and a rabbit anti-tuberin antibody (AbTS 1:400) (36). The fluorescein-conjugated anti-mouse secondary antibody and either fluorescein- or rhodamine-conjugated anti-rabbit secondary antibodies were used at 1:100 dilutions (Dako, Copenhagen Denmark). Images were captured using the Power Gene FISH system on a Leica DMRXA microscope at 1000× magnification. Images were processed using a filter wheel (Chroma Technology) and the Adobe Photoshop software package.

Cell culture and inhibitors

All drug incubations were carried out in DMEM containing 10% fetal calf serum. Cells were treated for 4 h with 0.05 μg/ml AMD (Sigma, St Louis, MO), 20 μg/ml α-amanitin (Sigma) or 100 μM DRB (Sigma) to selectively inhibit RNA transcription. Cells were treated for 3 h or overnight with LMB (50 ng/ml) to selectively block the function of exportin1. Cyclohexamide at 30 μg/ml (Sigma) was included 2 h before as well as during the LMB treatment to inhibit protein synthesis.

ACKNOWLEDGEMENTS

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Publication 5

THE FXR PROTEINS EXIST AS HOMO-MULTIMERS WITHIN MESSENGER RNP PARTICLES

Filippo Tamanini, Leontine van Unen, Cathy Bakker, Nicoletta Sacchi*, Hans Galjaard, Ben Oostra and André T. Hoogeveen‡

Department of Clinical Genetics, Erasmus University, 3000 DR Rotterdam,The Netherlands

* Department of Biology and Genetics, Medical School, University of Milan, 20133 Milan, Italy

‡To whom correspondence should be addressed

submitted

ABSTRACT

The absence of the protein FMRP results in Fragile X syndrome. Two proteins have been described, FXR1P and FXR2P, which are both very similar in amino acid sequence to FMRP. Interaction between the three proteins (FXR) as well as with themselves has been demonstrated. The FXR proteins are believed to play a role in RNA metabolism. To characterize a possible functional role of the interacting proteins, the complex formation of the FXR proteins was studied in mammalian cells. Double immuno-fluorescence analysis in COS cells over-expressing either FMRP ISO12/FXR1P, or FMRP ISO12/FXR2P confirms hetero-typic interactions. However, Western blotting studies on cellular homogenates containing physiological amounts of the three proteins gives different indications. Gel filtration experiments under physiological as well as EDTA conditions show that the FXR proteins are in complexes of >600 kDa, as parts of messenger ribonuclearproteins (mRNPs) particles associated with polyribosomes. Salt treatment shifts FMRP, FXR1P and FXR2P into distinct intermediate complexes with molecular masses between 200-300 kDa. Immunoprecipitations of FMRP as well as FXR1P from the dissociated complexes reveals that the vast majority of the FXR proteins do not form hetero-typic interactions. Further analysis by *in vivo* ^{35}S-methionine labeling followed by immunoprecipitation indicates that no other proteins than the FXR proteins are present in these complexes. These results suggest that the FXR proteins form preferentially homo-multimers under physiological conditions in mammalian cells, and might participate in mRNPs particles with separate functions.

INTRODUCTION

Fragile X syndrome is characterized by mental retardation, macro-orchids and various abnormal somatic signs [1]. The disease results from the lack of expression of the *FMR1* gene and, subsequently, of the encoded protein FMRP [2, 3].

FMRP (MW 70-80 kDa) is a cytoplasmic RNA-binding protein, which can interact with RNA homopolymers and fetal brain mRNAs [4-6]. Indeed, FMRP contains two KH domains and an RGG box, both characteristics of RNA-binding proteins [7, 8]. The RNA-binding activity is directly linked to the function of FMRP, as a mutation in the second KH domain causing an I304N substitution, has been found in a severe fragile X patient [9]. Although crystallographic data indicate that this mutation causes misfolding of the KH domain [10], impaired RNA-binding of the mutated FMRP I304N has been observed only in high salt conditions [11, 12]. Moreover, the majority of FMRP co-fractionates with polyribosomes isolated from different tissues [13-16]. Studies performed with EDTA, which dissociates the polyribosomes, indicate that FMRP is part of a messenger ribonuclearprotein (mRNP) particle with a sedimentation value of 60S [17, 18]. However, the interaction of FMRP with the large ribosomal subunit has also been proposed [16]. FMRP contains both nuclear localization (NLS) and nuclear export (NES) signals, and therefore, it might be involve in the nucleo-cytoplasmatic transport of yet unknown RNAs [15, 19, 20].

Two proteins have been identified, FXR1P (MW 70-80 kDa) and FXR2P (MW ~95 kDa), which are very similar to FMRP (~60% amino acid identity, with regions of 90%) [21, 22]. Consequently, FXR1P and FXR2P contain all the known functional domains of FMRP, are cytoplasmic RNA-binding proteins [21, 22], and co-fractionate with polyribosomes [16-18, 23]. FMRP, FXR1P and FXR2P (FXR proteins) are divergent only in

their C-terminal regions. Both *in vitro* binding studies and the yeast two-hybrid system revealed that the FXR proteins interact with themselves and with each other [21, 22]. It has been shown that a coiled coil domain, which is similarly present in the conserved amino-termini of the three proteins (amino acids 171-211 in FMRP), is important for FXR oligomerization [16]. However, comparative expression studies, resulting in different tissues, cellular, and intracellular distributions of the FXR proteins, suggest that they might function independently [23-25]. Interestingly, in adult brain the three proteins are co-expressed in the cytoplasm of differentiated neurons [23, 25].

In order to understand the pathogenesis found in fragile X syndrome, it is necessary to establish whether the FXR protein hetero-complex formation, previously demonstrated *in vitro* [22], also occurs in mammalian cells. In the present report, we show that FMRP, FXR1P, and FXR2P are associated with large RNA-protein particles mainly as homo-multimers with a molecular mass of 200-300 kDa.

RESULTS

Interaction of the FXR proteins in transfected COS cells

The interaction between the FXR proteins was investigated in transfected COS cells. An expression vector containing the full-length cDNA of either *FXR1*, or *FXR2*, was co-transfected with the expression vector containing *FMR1* ISO12, a splice variant of the *FMR1* gene. After 48 hours, the corresponding proteins were detected by double immuno-fluorescent staining using specific antibodies.

FMRP ISO12 lacks the NES and the RGG box in the carboxy-terminus, but maintains all the others functional domains [26]. Like wild type FMRP, FMRP ISO12 can normally interact with FXR1P and FXR2P *in vitro* [16]. Consequently to the absence of nuclear export activity, FMRP ISO12 localizes in the nucleoplasm of single transfected cells [26, 27]. However, in all cells co-expressing FMRP ISO12 and FXR2P we detected FMRP ISO12 in the cytoplasm (Fig. 1A). This change in FMRP ISO12 localization was also observed in the presence of over-expressed FXR1P (data not shown). Conversely, FXR2P (Fig. 1B) and FXR1P did not change their normal cytoplasmic localization in the presence of FMRP ISO12.

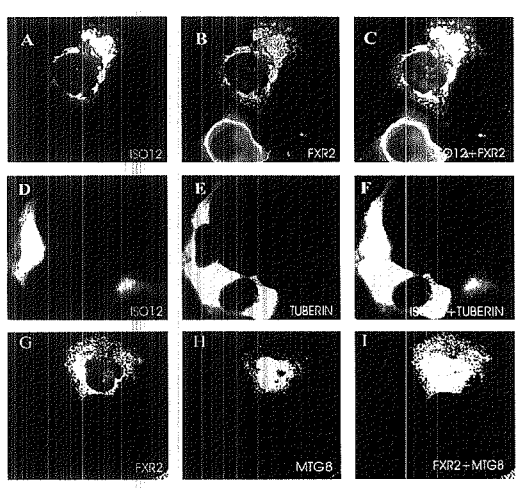

Figure 1. **FXR interaction in transfected COS cells.**
COS cells were double transfected with expression plasmids encoding FMRP ISO12 and wild type FXR2P (**A**, **B**, respectively). As a control, COS cells were double transfected with expression plasmids encoding FMRP ISO12 and Tuberin (**E**, **F**, respectively), FXR2P and MTG8-ETO (**H**, **I**, respectively). In **C**, **G**, and **L**, the merged images are shown.

We noticed that both the *FXR1* and *FXR2* expression vectors produced very high amounts of the respective proteins, which were detected often as aggregates in the cytoplasm of the COS cells. To exclude that the above-detected interactions were due to non-specific protein precipitation, we performed co-transfections with two unrelated proteins. We choose Tuberin (cytoplasmic) [28] and MTG8-ETO (nuclear) [29], because both contain interactive coiled coil domains, like the FXR proteins [16]. Neither the nuclear localization of FMRP ISO12 was affected by co-expression of Tuberin (Fig. 1C and 1D, respectively), nor FXR2P interfered with the nuclear localization of MTG8-ETO (Fig. 1E and 1F, respectively).
We concluded that the detected interactions between FMRP ISO12/FXR2P and FMRP ISO12/FXR1P were specific. Thus, the FXR proteins can form a hetero-typic interaction in an over-expression mammalian cellular system, confirming previous data obtained with purified proteins as well as with the yeast two hybrid system. In addition, we showed that wild type FXR proteins exercise a dominant effect on the localization of the NES mutated FMRP ISO12.

Gel filtration analysis of FMRP, FXR1P and FXR2P

The predicted FXR complexes were further characterized in lymphoblastoid and HeLa cells, where endogenous expression of FMRP, FXR1P and FXR2P has been demonstrated. Cellular homogenates were separated on a Superdex 200PC precision column using non-denaturing buffers and the collected fractions were analyzed for the presence of the three proteins by Western blotting. To detect FXR1P, we raised a new antibody (Ab 2107), which recognizes both the long and the short isoforms of FXR1P.

The initial fractionation performed in physiological buffer showed that the majority of FMRP eluted in fractions 8 and 9 (Fig. 2A), corresponding with the void volume of the column and indicating protein complexes larger than 600 kDa. Similarly to FMRP, the two major isoforms of FXR1P (long and short) (Fig. 2A) as well as FXR2P (Fig. 2A) co-eluted in fractions 8 and 9. These results are in line with the notion that the FXR proteins are detected mainly in polyribosomal fractions under physiological conditions [13-16].

Next, gel filtration analysis was performed in a buffer containing 500 mM KCl. In the presence of salt, FMRP, FXR1P, and FXR2P eluted in fractions 14-18 (Fig. 2B), corresponding to a molecular mass of approximately 200-300 kDa, as calculated from marker proteins run under identical conditions. Although the FXR proteins showed common dissociation profiles, they eluted in slightly different fractions. The majority of FMRP as well as the long FXR1P isoform was detected in fraction 16 (MW 240 kDa) (Fig. 2B). The short FXR1P isoform was detected in fractions 18 and 19. Finally, FXR2P was detected mainly in fraction 15 (Fig. 2B). An internal molecular weight marker, BSA, (MW 69 kDa) eluted in fractions 18-21. With the exception of the short isoform of FXR1P, we could barely detect FMRP, long FXR1P and FXR2P in fractions 18-21.

The presented results were obtained in repeated experiments (n=5) using homogenates of lymphoblastoid as well as HeLa cells. They indicate that FMRP, FXR1P and FXR2P could be released from the polyribosomes [14, 17] as intermediate complexes of 200-300 kDa rather than as monomers. In addition, the three FXR proteins eluted clearly in distinct fractions.

Biochemical characterization of the intermediate complexes (200-300 kDa)

The finding that FMRP and FXR1P were found in distinct fractions compared to FXR2P, raised uncertainty as to whether FXR hetero-multimers exist in living cells. Moreover, the precise co-fractionation of FMRP with the long FXR1P isoform (Fig. 2B)

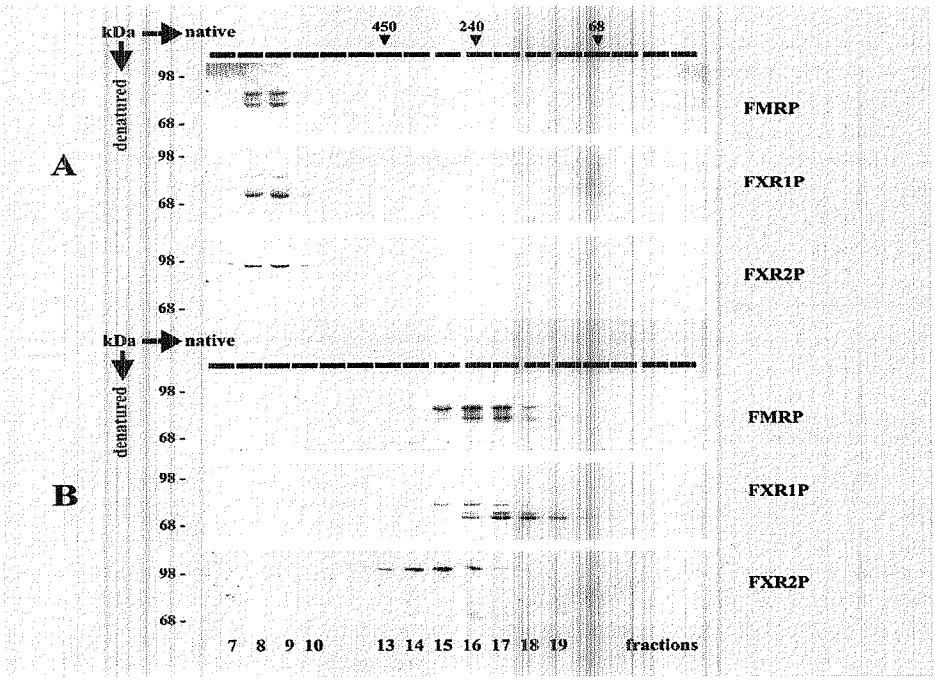

Figure 2. **Distribution of cellular FXR proteins after gel filtration.**
HeLa cells were homogenized in physiological buffer and subsequently separated by gel filtration. Each collected fraction was tested for the presence of FMRP, FXR1P and FXR2P (Fig. **2A**). HeLa cells were homogenized and separated in 500 mM KCl. Each collected fraction was tested for the presence of FMRP, FXR1P and FXR2P (Fig. **2B**).

might be caused either by a direct interaction of the two proteins, or by the fractionation of two independent complexes with fortuitous similar sizes.

To answer this point, immuno-precipitation of FMRP were performed in the presence of 500 mM KCl using specific antibodies against FMRP (Ab734) [4]. The immuno-precipitated sample (Fig. 3, lane 3) was sequentially analyzed by Western blotting for the presence of FMRP, FXR1P, FXR2P and the protein P0. The latter is a component of the 60S ribosomal subunit. An aliquot corresponding to 10% of the cellular homogenate before and after immuno-precipitation was also tested for visual comparison (Fig. 3, lane 1 and 2, respectively). It was found that the majority of FMRP was depleted from the starting homogenate and recovered in the IP fraction (Fig. 3, lane 3a). Detectable amounts (~10%) of the long FXR1P isoform (Fig. 3, lane 3b) as well as FXR2P (Fig. 3, lane 3c) were also co-precipitated. The protein P0 failed to co-precipitate with FMRP under this condition (Fig. 3, lane 3d). The equal intensity of the signals for FXR1P and FXR2P, before and after immuno-depletion of FMRP, confirms that the majority of FMRP, FXR1P and FXR2P do not interact and only a few of the FXR proteins form hetero-multimers.

We further analyzed the composition of the salt-dependent intermediate complexes by immuno-precipitating the long isoform of FXR1P with an antibody that recognizes

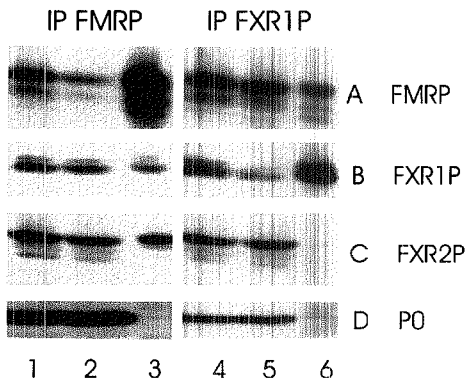

Figure 3. **FXR immunoprecipitations in 500 mM KCL.**
HeLa cellular homogenate prepared in 500 mM KCl were subjected to immunoprecipitation with either anti-FMRP antibodies Ab734 (**lane 3**), or anti-long FXR1P isoform antibodies Ab1934 (**lane 6**). The immunoprecipitated samples were sequentially tested by Western blotting for the presence of FMRP (**3A**, **6A**), long FXR1P isoform (**3B**, **6B**), FXR2P (**3C**, **6C**), and P0 protein (**3D**, **6D**). Total protein extract (~10%) before and after immunoprecipitation of FMRP (**lane 1** and **2**, respectively) were tested for the presence of FMRP, FXR1P, FXR2P and P0. Similarly, total protein extracts (~10%) before and after immunoprecipitation of FXR1P (**lane 4** and **5**, respectively) were tested for the presence of the four proteins.

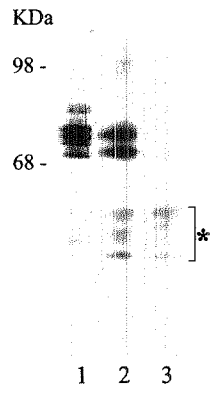

Figure 4. **Immunoprecipitation of the ^{35}S labeled 200-300 kDa complex.**
^{35}S labeled HeLa cellular homogenate prepared in 500 mM KCl were subjected to immunoprecipitation with either anti-FMRP antibodies Ab734 (**lane 1**), or anti-FXR1P antibodies Ab1934 and Ab2107 (**lane 2**). The same material was also immunoprecipitated with anti-FXR1P pre-immune sera (**lane 3**). The sign * indicates non-specific bands.

specifically this isoform (Ab1934) [25]. As above, the immuno-precipitated sample was sequentially analyzed for the presence of FMRP, FXR1P, FXR2P and P0. While FXR1P was efficiently recovered in the IP fraction (Fig. 3, lane 6b), only small amounts of FMRP (~10%) were co-precipitated (Fig. 3, lane 6a). No co-precipitation of FXR2P could be detected in this sample (Fig. 3, lane 6c). Unfortunately, we could not perform similar analysis on the FXR2P immunoprecipitate, since the antibodies against FXR2P [25] were not as efficient in immunoprecipitation experiments (data not shown).

The immunoprecipitation of two out of three FXR proteins resulted in a similar pattern of interactions. Since the presence of salt does not affect the formation of FXR hetero-multimers *in vitro* [22], our results demonstrate that FMRP, FXR1P and FXR2P exist in living cells mainly as homo-complexes. However, the above experiments cannot exclude the presence of other unknown proteins in those complexes. To investigate this possibility, immunoprecipitation studies on HeLa cells labeled *in vivo* with ^{35}S-methionine were performed. The autoradiogram of the gel was then analyzed to detect additional co-immunoprecipitated protein bands.

Immunoprecipitation of FMRP (in 500 mM KCl) resulted in the isolation of a close set of bands with a molecular weight of 70-80 kDa (Fig. 4, lane 1), most likely, corresponding to the different splicing isoforms of FMRP. No co-immunoprecipitation of additional proteins was detected in this sample. In parallel, immunoprecipitation of FXR1P (Ab 1934/2107) demonstrated only the presence of the long and short FXR1P isoforms (Fig. 4, lane 2). The lower bands visible in the autoradiogram were not specific, because they were obtained with the correspondent pre-immune antibodies too (Fig. 4, lane 3). Either limits in detection, or timing of interactions might cause the absence of co-immunoprecipitation of the FXR proteins, since newly synthesized proteins were examined.

Biochemical characterization of the large EDTA-dependent complex

The addition of 5-25 mM EDTA dissociates the polyribosomes and releases FMRP in complexes with a sedimentation of 60S [13, 15, 16]. Controversially, it has been shown that FMRP can either physically associate with the 60S ribosomal subunit, or being part of a large mRNP particle with the same sedimentation [17, 18]. Therefore, we used EDTA treatment (25 mM) to investigate the composition of these large RNA-protein particles, which contain the FXR proteins [13, 16, 23].

In gel filtration experiments, the three proteins were detected in fractions 7 and 8, corresponding to complexes larger than 600 kDa (data not shown). This result was similar to what was seen in physiological buffer (Fig. 2A).

In Fig. 5, it is shown that the efficient immuno-precipitation of the ribosomal protein P0 from a HeLa cellular homogenate in the presence of 25 mM EDTA (lane 6) resulted in the co-immunoprecipitation of approximately 5% of the total amounts of FMRP. For FXR1P, similar results were obtained (data not shown). Thus, FMRP and FXR1P are not in the same complex as P0 and are not directly bound to ribosomes.

Interestingly, the immunoprecipitation of the long and short FXR1P isoforms from ^{35}S-methionine-labeled HeLa cells caused the co-immunoprecipitation of at least three additional proteins. These proteins showed approximate molecular weights of 40, 50 and 100 kDa (Fig. 6, lane 2). Importantly, the three proteins were neither co-immunoprecipitated by the pre-immune sera under the same EDTA treatment (Fig. 6, lane 3), nor by antibodies against FXR1P in the presence of 500 mM KCl (Fig. 4 lane 2, and Fig. 6 lane 1). This suggests that FXR1P and the three new proteins are components of the same large complex.

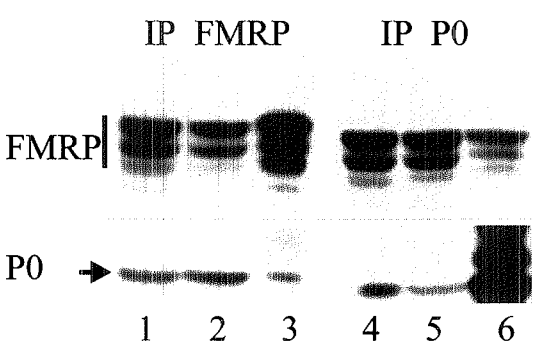

Figure 5. **Analysis of the large EDTA-resistant complex.** HeLa cellular homogenate prepared in 25 mM EDTA was subjected to immunoprecipitation with either anti-FMRP antibodies Ab734 (**lane 3**), or anti-protein P0 (**lane 6**). The immunoprecipitated sample was sequentially tested for the presence of FMRP and P0. Total protein extract (~10%) were also tested for the presence of the two proteins before and after immunodepletion of FMRP (**lane 1** and **2**) and P0 (**lane 4** and **5**), respectively.

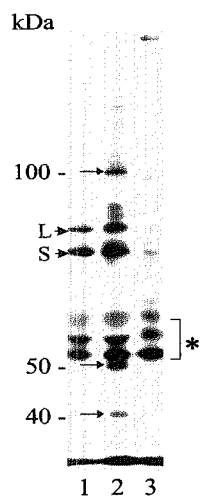

Figure 6. **Detection of new proteins in the FXR1P immunoprecipitate.** ^{35}S labeled HeLa cellular homogenate prepared in a buffer containing either 500 mM KCl (**lane1**), or 25 mM EDTA (**lane 2** and **3**) was subjected to immunoprecipitati on using anti-FXR1P antibodies Ab1934 and Ab2107 (**lane 1** and **2**) and pre-immune sera (**lane 3**).The sign * indicates non-specific bands; L and S (long and short FXR1P).

We concluded that, after dissociation of the polyribosomes, the vast majority of FMRP as well as FXR1P are present in large complexes, which does not include the ribosomal protein P0. Therefore, we support the notion that the FXR proteins are associated with the polyribosomes as mRNP particles rather than as components of the large ribosomal subunit.

DISCUSSION

FMRP, FXR1P and FXR2P (FXR proteins) can interact *in vitro* with themselves and with each other [22]. However, the physiological role of this multimerization is unclear. The observations we describe here indicate that the vast majority of the FXR proteins exist in cultured cells as homo-multimers rather than hetero-multimers. These multimers have well-defined molecular weights (~200-300 kDa) and exist as part of larger RNA-protein particles. Therefore, it is possible that the FXR intermediate complexes might regulate cellular processes such as translation and/or mRNA-stability.

Several mRNA-binding proteins are present as multimers within messenger ribonuclearproteins (mRNPs) particles [30-32]. Dimerization can be either essential for translation activity as demonstrated for the La protein [33], or important for RNA-binding as demonstrated for Xenopus p54/56 proteins [34]. The FXR proteins are connected with the polyribosomes in a RNA-dependent manner [14, 15, 17]. Most likely, they are components of polyribosomal-associated mRNPs particles [17, 18, 23]. In this report we show that salt treatment shifts the FXR proteins from these large complexes into distinct intermediate complexes of 200-300 kDa. The molecular weights of these complexes are 3-4 times those of the FXR monomers (FMRP and the large FXR1P isoform are ~70-80 kDa, FXR2P is ~95 kDa). In addition, immunoprecipitation of FMRP (and FXR1P) from ^{35}S-methionine labeled HeLa homogenates (in 500 mM salt) results in the visualization of FMRP (and FXR1P), but no additional proteins can be observed. Taken together, these observations indicate that FXR proteins might be incorporated in those mRNP particles as homo-multimers. At the moment, it is not possible conclude whether they form dimers, trimers, or tetramers.

Previous experiments showed approximately similar levels of FXR homo- and hetero-multimers both *in vitro* and in the yeast two hybrid system [22]. By Western blotting using specific antibodies, we compared the presence of the three proteins in FXR immuno-precipitated samples in mammalian cells. We reproducibly found that only ~10% of the total FMRP (and FXR1P) amounts can form FXR hetero-multimers in mammalian cultured cells. Purified FMRP interacts with FXR1P and FXR2P via a predicted coiled coil domain present in its amino-terminus (amino acid 171-211) [16]. FXR1P and FXR2P have a similar coiled coil motif in their amino termini (75 % amino acid homology with FMRP), which is important for interaction between the FXR proteins [16]. However, it is not clear whether or not, these domains recognize unique sequences within FMRP, FXR1P, and FXR2P. It has been proposed by crystallographic studies that coiled coil motifs interact with each other, forming α helical structures. It is therefore possible that the high amino acid homology between the three proteins is responsible for their strong association *in vitro*. If so, non-physiological interactions could readily occur when the FXR proteins are brought together in high concentrations. This would explain the interactions we detected between the FXR proteins in an over-expressing cellular system (Fig. 1).

The hypothesis that FMRP might associate preferentially with itself rather than with FXR1P and FXR2P is supported by other observations. Firstly, Feng et al. characterized the association of FMRP with FXR2P in lymphoblastoid cells of a fragile X patient, who has an amino acid substitution (I304N) in the second KH domain of FMRP [18]. FMRP I304N fails

to associate with polyribosomes and is incorporated in EDTA-resistant particles with smaller sizes. Despite the fact that FXR2P and FMRP I304N can normally interact *in vitro* as well as in the yeast two-hybrid system [16], FXR2P is not present together with FMRP I304N in those abnormal particles. Secondly, we showed recently that FMRP shuttles between cytoplasm and nucleoplasm, while FXR2P shuttles between cytoplasm and nucleolus, suggesting that the FXR protein can have independent cellular routes as well as different targets in the nucleus [35]. Thirdly, comparative expression studies showed that each of the three FXR genes/proteins have independent expression as well as different cellular distributions in tissues like muscle, fetal brain and testis [23-25]. These arguments indicate that no functional interactions between the FXR proteins exist *in vivo*.

The absence of FMRP causes mental impairment both in man and mice. High levels of FMRP, FXR1P and FXR2P expression are found in the soma (perikaryon) of differentiated neurons of all brain regions (hippocampus, cortex, cerebellum, hypothalamus, pons, etc.) [23, 25, 36, 37]. The normal expression of FXR1P and FXR2P in neurons of a fragile X patient [25] invites to two more considerations. Either FMRP, FXR1P and FXR2P have completely separated functions also in neurons, or FXR1P and FXR2P partially compensate for the absence of FMRP. Preliminary studies of homogenates from mouse hippocampus show a fractionation pattern of the FXR proteins similar to that seen in cultured cells (F.T., unpublished).

It has been proposed that FMRP associates with the 60S ribosomal subunit via protein-protein interaction (in 5 mM EDTA) [16]. In contrast, other reports strongly indicate a non-association of the FXR proteins with this subunit (in 25 mM EDTA) [17, 18]. In our experimental condition (25 mM EDTA), the complete immunoprecipitation of the protein P0, a 60S ribosomal component, results in the co-immunoprecipitation of approximately 5-10% of FMRP and FXR1P. These results confirm that the majority of the FXR proteins relies in the proposed mRNPs particles [17, 18], and associates via these particles to the ribosomes.

It is becoming clear that a fundamental step in understanding the function of the FXR proteins will be the definition of the RNA and protein composition of these EDTA-resistant complexes. Here, we show the initial characterization of three new proteins of approximately 40, 50 and 100 kDa, which co-precipitate with FXR1P (Fig. 6). During the experiments, we notice that (differently to the salt buffer) the antibodies Ab734 recognize FMRP inefficiently, either in physiological, or EDTA buffers (Fig. 5). The antibodies Ab734 are partially raised against the KH domains of FMRP [4], thus, they might not detect FMRP which is engaged in RNA-binding within the mRNP particle.

Further experiments are needed to characterize the protein composition of the high molecular mass complexes in which the FXR proteins participate, and to identify their RNA targets. New information will contribute to understand the pathogenesis of the fragile X syndrome as well as the mechanisms of RNA transport and translation in general.

MATERIAL AND METHODS

Cell culture and subcellular fractionations

Lymphoblastoid cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotics (at 37 ^{0}C and 5% CO_2). HeLa cells were grown with DMEM medium supplemented with 10% FCS and antibiotics (at 37 ^{0}C and 5% CO_2). Cells were washed two times with ice-cold PBS and lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 10 u/ml Rnasin (Pharmacia), 0.5% NP-40 and protease inhibitors. Cell lysate were then homogenised by passage through hypodermic needles and centrifuged at 10000 r.p.m to obtain a cytoplasmic supernatant. For KCl or EDTA treatments, either KCl (500 mM) or EDTA (25 mM) was added to a portion of the cytoplamic lysate, and the sample was kept on ice for 30 min prior to gel filtration and immunoprecipitation.

Gel filtration, Immunoprecipitation, and Western blot

We used a Precision Column PC 3.2/30 pre-packed with Superdex 200 in a SMART system (Pharmacia) to determine the molecular mass of FMRP [14], FXR1P and FXR2P by gel filtration. The optimal range for separation of globular proteins in this column is 10-600 kDa, with an exclusion limit of 1.3 million Dalton. In order to calibrate the column and to know the molecular weight of the eluted fractions, 3 protein markers were applied in a physiological buffer giving the following results: ferritin (440 kDa) top in fraction 11, catalase (240 kDa) top in fraction 16, bovine serum albumine (68 kDa) top in fraction 19-20. The markers were tested with other buffer conditions (500 mM KCl) giving an analogous retention time. Before running, the column was equilibrate 30 min. in each corresponding buffer; 40-60 µl of cytoplasmic lysate was injected in the SMART system and the protein profile was monitored at 280 nm with a flow of 50 µl/min. Fractions (50 µl each) were collected separately.
Immunoprecipitations were carried out overnight at 4°C in a buffer containing either 20 mM Tris-HCl, pH 7.4, 500 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, or 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 25 mM EDTA, 1 mM DTT, and protease inhibitors. The used antisera were Ab734 against FMRP (1:100) [4], Ab1934 against the long FXR1P isoform (1:100) [25], Ab2107 against both FXR1P isoforms (1:100), and an antibody against P0 (1:100) (Immunovision). The Ab2107 was raised against a synthetic peptide covering amino acids 483-500 of FXR1P. Protein A-sepharose beads (Pharmacia) were added for 3 hours to bound and recover the antisera. The beads were washed 4 times with the immunoprecipitation buffer.
Protein samples in Laemli buffer were separated on either 10% or 7.5% SDS-polyacrylamide gels, and electro-blotted onto nitrocellulose membrane (Schleicher & Schuell). Immunodetection were made using the mouse monoclonal antibody 1C3 against FMRP diluted 1:2500 [36], Ab1934 (1:4000), Ab2107 (1:4000), and Ab1937 against FXR2P (1:500) [25]. The secondary antibody was coupled to peroxidase allowing detection with the chemiluminescence method (ECL KIT, Amersham).

^{35}S-methionine cell labeling

HeLa cells, growing exponentially in 75 cm flasks were washed in PBS. Subsequently, the cells were incubated overnight at 37 ^{0}C and 5% CO_2 in 7 ml of DMEM medium without the amino acids Methionine and Cysteine, supplemented with 2% dialysed FCS, 20 µCi/ml of ^{35}S and 16 µCi/ml of ^{3}H. In the morning the cells were washed in PBS and collected by trypsinization. The labeled cell pellet was homogenised and subjected to immunoprecipitation as described above. The immunoprecipitated sample was separated either on 7.5% or 10% polyacrylamide gel. The proteins present in the gel were fixed on a solution containing 50% methanol, 7.5% acetic acid. The gel was then dried and exposed with an autoradiographic film (KODAK) for 4 days.

Transfections and immunofluorescence

COS-cells were cultured in DMEM, 10% FCS at 37°C and 5% CO_2. The day before transfection the cells were seeded on glas coverslips. Transfections were performed as described by the manufacturer using 0.5 µg DNA, 3 µl Plus reagent and 2 µl Lipofectamine (Gibco-BRL). Cells were treated overnight with Leptomycin B (LMB) (50 ng/ml) to selectively block the function of exportin 1. Cells were fixed for immuno-fluorescence 24 hours or 48 hours after transfection in 0.1M phosphate buffered saline (PBS) containing 3% paraformaldehyde (pH 7.3) for 7 minutes at room temperature, followed by a permeabilization step in 100% methanol for 20 minutes. Primary and secondary antibodies incubations were performed for 60 minutes at room temperature in blocking buffer containing PBS, 0.15% Tris-Glycine (Fluka) and 0.5% BSA (Fluka). The primary antibodies were Ab1937 (1:200), Ab1934 (1:200), 1C3 (1:200), a rabbit anti-tuberin antibody (AbTS 1:400) [28], and a rabbit anti-MTG8 antibody (Ab1499) [29]. The fluoresceine-conjugated anti-mouse secondary antibody and either the fluoresceine or the rhodamine-conjugated anti-rabbit secondary antibodies were used at 1:100 dilutions (DAKO). Images were captured using the Power Gene FISH system on a Leica DMRXA microscope at 1000x magnification. Images were processed using a filter wheel (Chroma Technology) and the Adobe Photoshop software package.

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Chapter 3

GENERAL DISCUSSION

General discussion

In the last four years, we have seen interesting developments in the clarification of the function of FMRP, the protein involved in fragile X syndrome. The most significant one is the association of FMRP with actively translating ribosomes (polyribosomes) in the cytoplasm (Khandjian *et al.*, 1996) (*publications 1, 2, 5*). The discovery of a nucleocytoplasmic shuttling activity, mediated by signals for nuclear import (NLS) as well as nuclear export (NES), is giving an extra-dimension to the properties of FMRP (Eberhart *et al.*, 1996; Fridell *et al.*, 1996). While CRM1/exportin1 is the nuclear export factor of FMRP (*publication 4*), its import receptor is unknown until now.

The presence of two KH domains in the central part of FMRP and a RGG box domain at the C-terminus had already suggested previously that FMRP is an RNA-binding protein (Gibson *et al.*, 1993). The functional importance of the KH and RGG domains in RNA-binding have been demonstrated for several proteins, including FMRP, both *in vitro* and *in vivo* (paragraph 1.4.2). Indeed, it has been shown that purified FMRP binds preferentially to RNA homopolymers polyG and polyU, and to a sub-population of brain mRNAs as well as its own mRNA (Siomi *et al.*, 1993; Ashley *et al.*, 1993). These *in vitro* binding partners still remain the only RNAs known to bind FMRP, so far. The finding that the I304N mutation in the second KH domain of FMRP resulted in a very severe fragile X phenotype (De Boulle *et al.*, 1993) indicated that RNA-binding activity plays a central role in the correct functioning of this protein.

FMRP, together with FXR1P and FXR2P, form a small family of highly homologous proteins (FXR proteins), which can interact with each other *in vitro*. These three proteins are closely related in subcellular localization and functional domains (paragraph 1.10), however, FXR1P and/or FXR2P cannot compensate for the absence of FMRP in fragile X patients. Little information is available about the cellular pathways of the FXR proteins and their interaction *in vivo*.

These properties of FMRP (RNA-binding, ribosome association, FXR interaction, nucleocytoplasmic shuttling) allow us to discuss the possible function of FMRP in RNA metabolism and in relation to the major defect seen in fragile X patients (mental retardation).

Fragile X syndrome, a RNA processing defect

Mutations in genes, which function in RNA metabolism, can cause diseases with a "tissue-specific" phenotype. Spinal muscular atrophy (SMA), a common motor neuron degenerative disease, results from reduced levels of the Survival of Motor Neurons (SMN) protein (Lefebvre *et al.*, 1997). The SMN protein is expressed in all tissues, but particularly high levels of SMN protein are detected in motor neurons. SMN is found predominantly in the cytoplasm but also in the nucleus where it is concentrated in small structures, named gems (Liu *et al.*, 1996). The SMN protein interacts with some of the snRNP proteins involved in splicing (Liu *et al.*, 1997), is critical for snRNP assembly in the cytoplasm (Fischer *et al.*, 1997), and stimulates mRNA splicing *in vitro* (Pellizzoni *et al.*, 1998), and hence might act in the regeneration of the pre-mRNA splicing machinery.

Another example is dyskeratosis congenita, caused by mutations in a gene encoding the human orthologue of rat NAP57 and yeast Cbf5p (Heiss *et al.*, 1998). Cbf5p is involved in the processing of pre-rRNA and may act as a chaperone in the assembly of ribosomes (Lafontaine *et al.*, 1998). Recently, the gene involved in Diamond-Blackfan anaemia has been cloned, and shown to code for the ubiquitously expressed 40S ribosomal protein S19 (Draptchinskaia *et al.*, 1999). Although a human ribosomal mutation is expected to have a generalized effect, this is clearly not the case.

A role for FMRP in post-transcriptional/translational control of gene expression is possible, as RNA-protein interactions play key regulatory functions in these processes. Post-transcriptional RNA processing of eukaryotic pre-mRNA, including polyadenylation, capping and splicing, as well as RNA transport, affect the availability of mature mRNA for translation. In addition, the localization, stability and translatability of cytoplasmic mRNAs influence both quantitative and qualitative aspects of final gene expression. Most fragile X patients completely lack FMRP, yet they do not show widespread pathology or developmental abnormalities, as might be expected by the disruption of such an important cellular activity as, for example mRNA translation. Clearly, the identification of the physiological RNA targets as well as the biochemical pathway of FMRP would be a fundamental step in understanding the molecular aspects of the disease.

FMRP as a component of polyribosomal mRNP particles

FMRP is highly expressed in translationally active cells, such as cells in culture and neurons (Khandjian *et al.*, 1995; Verheij *et al.*, 1995), and has been detected in a rabbit reticulocyte lysate that is commonly used for protein translation *in vitro* (*publication 1*). However, FMRP does not have the properties of a pure ribosomal protein and, therefore, cannot be considered as a structural component of the ribosome (paragraph 1.5.1). Instead, FMRP is associated to the ribosomes as a messenger ribonucleoprotein (mRNP) particle (Khandjian *et al.*, 1996; Eberhart *et al.*, 1996; Corbin *et al.*, 1997; Feng *et al.*, 1997b). Three experimental evidences indicate that mRNA mediates the FMRP-ribosome association and that FMRP might function on the polyribosomes as a non-canonical translation factor:

1] this association can be destroyed by RNase treatment without affecting the ribosomal integrity (Eberhart *et al.*, 1996; Corbin *et al.*, 1997) (*publication 1*);

2] cellular FMRP co-purifies with polyA mRNAon a oligo-dT column, but not ribosomal RNA (Corbin *et al.*, 1997; Feng *et al.*, 1997b);

3] inhibition of translation initiation without affecting elongation, leads to the release of FMRP from the polyribosome, which indicates that FMRP dissociates from ribosomes upon termination of translation (Feng *et al.*, 1997b).

What is known of mRNA-binding proteins that form polyribosomal mRNP particles and act as non-canonical translation factors? This class includes some heterogeneous nuclear ribonucleoproteins (hnRNP A1, K, E1/E2) (Dreyfuss, 1993), the pyrimidine tract binding protein (hnRNP I/PTB) (Kaminski *et al.*, 1995), the polyA binding protein (PABP/p70) (Gu *et al.*, 1995), p50 (Evdokimova *et al.*, 1995), the La protein (McLaren *et al.*, 1997), and many others. Similar to FMRP, these proteins exhibit preferential RNA binding activities, however,

they also interact with RNA with moderate affinity in a non-sequence specific manner, and consequently the mechanism of action of these proteins has been difficult to define. They can either stimulate or repress translation by helix-destabilizing and annealing activities (hnRNP A1) (Portman *et al.*, 1994), by RNA melting (p50 and La) (Evdokimova *et al.*, 1995; Xiao *et al.*, 1994), or by 'chaperoning activity' (refolding the ribozyme to assume a native conformation that is optimal for enzymatic activity) (Herschlag *et al.*, 1994). Translation *in vivo* is strongly dependent on the presence of the mRNA 5' cap structure (Drummon *et al.*, 1985). It has been suggested that one of the functions of mRNA-binding proteins in the cytoplasm is to mask the mRNA, promote ribosome binding by a 5'-end, cap mediated mechanism, and therefore, prevent spurious translation initiations at aberrant translation start sites (Svitkin, 1996). It is plausible that subsequent GTP hydrolysis accompanying ribosome translocation, provides sufficient energy to displace the, otherwise interdicting, mRNA-associated proteins.

The hnRNP K and E/E1 proteins, that contain KH domains similar to FMRP, are examples of sequence-specific translational regulation (Ostareck-Lederer *et al.*, 1998). The highly abundant, erythroid-specific, LOX mRNA, is translationally silenced until reticulocytes reach the final stage of maturation. The CU-rich repetitive sequence, called DICE, in the 3' UTR of the LOX mRNA mediates this process. The hnRNP K and E/E1 proteins specifically bind the DICE sequence to inhibit ribosome-mRNA association and consequently repress translation of the LOX mRNA (Ostareck *et al.*, 1997). Surprisingly, hnRNP E/E1 binds a similar CU sequence in the 3'UTR of the α-globin mRNA without repressing its translation (Kiledjian *et al.*, 1995; Ostareck *et al.*, 1997). Thus, components of cytoplasmic mRNPs particles may have different functions as a consequence of their binding to different mRNAs and/or additional binding partners.

The nucleotide sequence of the 3'UTR of the *FMR1* gene is extremely conserved between human and mouse. Such conservation has also been reported for the 3'UTR of the *FXR1* gene (Coy *et al.*, 1995). The maintenance of these non-coding sequences through the evolution must indicate a functional role for them. In an analogous way to the LOX mRNA, the 3' UTRs of *FMR1* and *FXR1* might be the targets of unknown RNA-binding proteins, regulating the expression of FMRP and FXR1P at post-transcriptional and/or translational level. One of these proteins can be FMRP itself, since it has been reported that FMRP binds its own mRNA *in vitro* (Ashley *et al.*, 1993). The hypothesis that FMRP and the *FMR1* mRNA might interact and function in a loop mechanism is certainly very attractive.

The protein components of the mRNPs are often found as homo- or heteromultimers within these particles (Casas-Finet *et al.*, 1993; Evdokimova *et al.*, 1995; Methot *et al.*, 1996). It has been shown that homomultimerization of these proteins can be important for RNA-binding (Marello *et al.*, 1992) and translation activity (La protein) (Craig *et al.*, 1997). Interestingly, we have shown that FMRP, FXR1P and FXR2P are released from mRNP particles mainly as homodimers or trimers after gel filtration/immunoprecipitation studies on cellular homogenates (*publication 5*). Only 10-20% of total FMRP (and FXR1P) is involved in the formation of FXR heteromultimers in mammalian cultured cells. These results are not

in agreement with those on mixing purified FXR proteins *in vitro*, which show similar levels of FXR homo- and heteromultimers (Siomi *et al.*, 1995; Zhang *et al.*, 1995).

Expression studies in testis, fetal brain (*publication 3*) and muscle (Khandjian *et al.*, 1998) show different cellular distribution of the three FXR proteins in those tissues. Moreover, also in an over-expressing cellular system (COS cells) different FXR proteins have distinct nuclear localizations after NES-inactivation (*publication 4*). These observations suggest that no important functional interactions between FMRP, FXR1P and FXR2P might exist *in vivo*.

The role of FMRP

Studies of mutant FMRP with the I304N mutation have been very helpful for the functional characterization of FMRP (*publication 1 and 4*). Interestingly, mRNP particles harboring FMRPI304N are of smaller sizes and do not associate with translating polyribosomes (Feng *et al.*, 1997b). These data indicate that the association of FMRP with polyribosomes must be functionally important. They also imply that the mechanism of the severe phenotype in the fragile X patient with the I304N mutation might lie in the sequestration of bound mRNAs in non-translatable mRNP particles (deleterious effect of the mutated FMRPI304N protein) (Feng *et al.*, 1997b).

The antibiotic Leptomycin B blocks the exportin1-dependent nuclear export of proteins carrying a Rev-like NES (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). This short signal for nuclear export is also present in the FXR proteins. After long treatment with Leptomycin B, low amounts of wild type FMRP (or FXR1P, or FXR2P) were found in the nucleus of transfected COS cells. In contrast, high amounts of FMRPI304N were trapped in the nucleus after a similar treatment (*publication 4*). Therefore, it appears that the bulk of FMRP does not shuttle continuously between cytoplasm and nucleus (like cyclin B1 does, for example), unless there is another, yet unknown (Leptomycin B insensitive), exportin1-like receptor. In addition, the dissociation of FMRP from the polyribosomes enhances its shuttling activity.

In light of these observations, FMRP is needed by its target mRNAs for the formation of functional mRNP complexes, either in the cytoplasm or in the nucleus, and their subsequent presentation to the translation machinery. It is equally possible that FMRP might instead mask these mRNAs ("inactive" mRNP particle), repressing their translation until protein synthesis is really needed by the cell. Therefore, in the absence of FMRP, variation in the amounts of the proteins encoded by mRNAs normally bound to FMRP can lead to the fragile X syndrome. Moreover, these same mRNAs may be handled by other mRNPs, possibly including FMRP-related proteins, resulting in partial and/or abnormally regulated translation. This would explain the heterogeneous spectrum of physical and intellectual condition seen in fragile X patients (paragraph 1.1), and the fact that FMRP is a non-vital protein, despite its strong expression early during development.

Neuronal FMRP

The absence of FMRP causes mental impairment both in man and mice. The highest amounts of FMRP are found in the soma (perikaryon) of the differentiated neurons of all brain's region (hippocampus, cortex, cerebellum, hypothalamus, pons, etc.) (Devys *et al.*, 1993; Feng *et al.*, 1997a) and in spermatogonia. Although macro-orchidism is also present in the clinical picture of fragile X patients, I would like to focus here on the role of FMRP in neurons.

The common view is that FMRP is a mRNA-binding protein, which modulates brain functions, like synaptic plasticity, learning and memory. Yet, it is not clear whether neuronal FMRP has either many, or very specific RNA targets. Ashley *et al.* (1993) have reported that FMRP interacts with 4% of the total brain mRNA population *in vitro*. In the case that neuronal FMRP binds so many mRNAs *in vivo*, fragile X mental retardation can be caused by a cascade of secondary effects, rather than from the absence of FMRP directly. On the opposite, the identification of the specific target for a mRNA-binding protein is a difficult task, and few examples are known. One of those is represented by the neuronal Hel-N1 protein, which binds the 3' UTR of the mRNA of neurofilament M and increases its translatability *in vivo* (Antic *et al.*, 1999). Ectopically expressed Hel-N1 protein enhances the neuronal differentiation of embryonal teratocarcinoma cells.

Like FMRP, FXR1P and FXR2P are expressed in differentiated neurons. The normal expression of FXR1P and FXR2P in neurons of fragile X patients (*publication 3*) suggests different possibilities. Either FMRP, FXR1P and FXR2P have completely separated functions, or FMRP has an additional neuron-specific function, or FXR1P and FXR2P partially compensate for the absence of FMRP. A consequence of this last possibility would be that *Fmr1-Fxr1* and *Fmr1-Fxr2* double KO mice should be more severely affected in memory and learning than the existing *Fmr1* knockout (Bakker *et al.*, 1994). The *Fxr2* homozygote negative mouse is apparently normal during the first months of life (Bontekoe, pers. communication). In contrast, the *Fxr1* homozygote negative mouse dies in the first hours after birth (Siomi, pers. communication). The latter phenotype might be due to the absence of FXR1P in muscle and heart, where the protein is expressed at very high levels. However, neurological abnormalities cannot be excluded. Therefore, the double *Fmr1-Fxr1* KO mouse should be created in a *Fmr1* negative background by conditional inactivation of the *Fxr1* gene, for instance in neurons only (Tsien *et al.*, 1996a; Tsien *et al.*, 1996b).

The localization of proteins to different cellular compartments (soma, dendrites, and axon) is essential in neurons to establish the functional polarity, and for changes in synaptic strength. Asymmetric distribution of proteins can be achieved by at least two different mechanisms: either the proteins can be synthesized in the soma and then transported to a cellular compartment, or the mRNAs encoding specific proteins can be targeted to a particular compartment and then translated in that region. Interestingly, FMRP (a part its localization in the soma) is present in dendrites, but absent in the axon (Feng *et al.*, 1997a) (*publication 3*). It appears that the presence of FMRP in the dendrites reflects its association with the ribosomes, since EM as well as biochemical analysis have demonstrated this (Feng *et al.*, 1997a). Indeed, the dendrites, but not the axons, contain translating ribosomes, which

are often concentrated at the synapses. In addition, there are reticular structures that may function in glycoprotein and membrane protein synthesis, and many components of the translation machinery have been found in dendrites. Steward *et al.* (1998) have shown that the transport/localization mechanism of four dendritically localized mRNAs is intact in the forebrain of *Fmr1* KO neurons. The dendritic localization of those mRNAs, which code for the proteins MAP2, CAMII kinase, dendrin, and the cytoskeletal component ARC, depends on their specialized 3'UTR. Yet, it is not known whether FMRP can bind to the mRNAs used in this study, therefore a role of neuronal FMRP in mRNA localization is still possible.

Recently, Weiler *et al.* (1997) has indicated that the *Fmr1* mRNA is incorporated in polyribosomes and translated at synaptic sites under metabotrophic glutamate receptors activation. This observation suggests a role of FMRP at the synapses. One possibility is that FMRP may have an identical function in the soma, dendrites and synapses (component of mRNPs). A second possibility is that synaptic/dendritic FMRP may have a different function from that performed in the soma. For example, FMRP acts in translatable "active" mRNPs in the soma, whereas it forms temporary "inactive" mRNPs at the synapses. These synaptic mRNP particles could be the most neuron-specific in terms of RNA cargoes, and be activated for translation by secondary signaling cascade. A third possibility is that synaptic FMRP may have a new, yet unknown, function, which is completely unrelated to RNA metabolism. Thus, a selected pool of FMRP with a unique cargo and/or function could modulate synaptic plasticity, and consequently memory and learning.

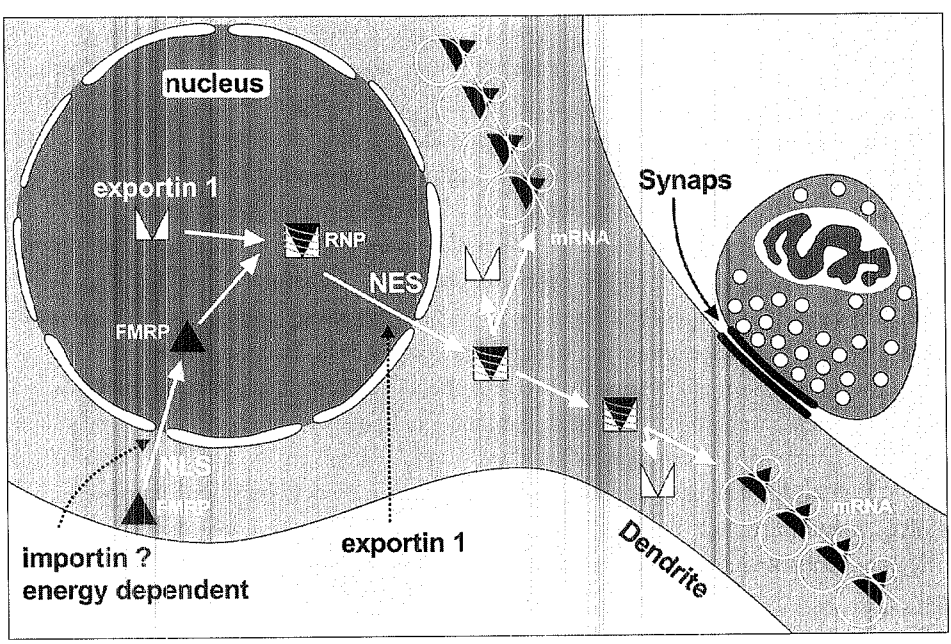

Figure 7. **Model for a possible role of FMRP in neuron.**

In conclusion, given what we now know about FMRP, its binding to mRNA, association with polyribosomes and shuttling between cytoplasm and nucleus, it is possibile to propose a model for the functioning of FMRP in neurons (Figure 7). Synaptic transmission, neuronal development or cellular stress, secondary-signaling cascades may cause dramatic changes in FMRP-polyribosomes association. Neuronal FMRP can dissociate from the polyribosomes, expose its NLS, and consequently, the protein moves to the nucleus (via a yet unknown importin receptor). Successively, nuclear FMRP picks up specific mRNAs, exposes its NES and moves back to the cytoplasm (via exportin1), associating to the ribosomes with its cargo of new information. The neuron would therefore respond biochemically to a message coming from outside, for example by using localized newly synthetized proteins to strengthen (or eliminate) a synapse. Such a regulated cytoplasmic-nuclear transport mechanism has been demonstrated for the transcription factors NF-$_{K}$B (Guerrini *et al.*, 1995; Kaltschmidt *et al.*, 1995) and CREB (Bito *et al.*, 1996; Deisseroth *et al.*, 1996), the latter involved in transcription of genes/proteins necessary to consolidate the long-term phase of memory (Yin *et al.*, 1996; Silva *et al.*, 1998). These proteins are trapped inactive in the cytoplasm until a phosphorylation cascade, originated by external stimuli, degrade/dissociate their inhibitor factors and translocate them to the nucleus, where they act on the transcription of specific genes (O'Neill *et al.*, 1997).

Whether the mental retardation in fragile X patients is due to a defect in transport/translation of memory-specific mRNAs is a challenging question for many investigators involved in several research fields. Their identification would be the molecular basis of future therapies for fragile X patients. Furthermore, the discovery of FMRP-interacting proteins with known biochemical activities will adjust our view about the function of this fascinating protein.

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Summary

This thesis presents studies on the localization and role of the *FMR1* protein (FMRP) involved in the fragile X syndrome. To achieve these results we used tools of biochemistry, immunohistochemistry and cell biology.

Fragile X syndrome, with an estimated prevalence of 1 in 6000, is one of the most frequent forms of inherited mental retardation. Apart from mental retardation, fragile X patients show hyperactivity and stereotypic behavior, and have physical features of which enlarged testes is the most striking. In 1991, the fragile X gene was cloned and named *FMR1*. Fragile X syndrome was the first example of a "novel" class of diseases caused by a trinucleotide repeat expansion. The vast majority of affected individuals have a pathological expansion of a CGG trinucleotide repeat (>200 units) in the 5'-untranslated region of the *FMR1* gene (full mutation). This repeat expansion is accompanied by hypermethylation of the repeat and its flanking region, which includes the promoter. This results in silencing of *FMR1* transcription and absence of the encoded protein FMRP (for Fragile X Mental Retardation Protein) in fragile X patients.

In normal individuals, several FMRP isoforms are produced, which have molecular weights of ~80 kDa, and which originate from alternative splicing of exons at the 3' end of the gene. All the FMRP isoforms, except for one (ISO12), are localized in the cytoplasm. Subcellular fractionation of protein homogenates, including gel filtration and ultra-centrifugation, showed that FMRP is associated with heavy sedimentation particles, most likely ribosomes (*publication 1*). Sucrose sedimentation studies further indicated the association of FMRP with actively translating ribosomes (polyribosomes). Moreover, we could directly visualize FMRP at ultrastructural level by electron-microscopy (EM) in mammalian transfected cells: FMRP signals were found on free ribosomes as well as on ribosomes attached to the endoplasmic reticulum, and on the granular component of the nucleolus (*publication 2*).

The recognition of RNA-binding domains (KH domain and RGG box) in FMRP gave the first clues to the function of FMRP. In fact, FMRP binds RNA *in vitro*. By treating a cellular homogenate with RNase, we were able to demonstrate that FMRP is associated to the ribosomes via RNA (*publication 1*). Successively, it was found that mRNA is the component that links FMRP to the ribosomes. It was also found that FMRP is part of a messenger ribonuclearprotein (mRNP) particle, which has sedimentation of 60S, but is not the large 60S ribosomal subunit itself (*publication 5*). These findings and similar observations from other groups suggest that fragile X syndrome may be a defect in mRNA metabolism and/or translation.

FMRP, and its highly homologues FXR1P and FXR2P, form a small family of proteins (FXR proteins) closely related in subcellular localization and function. We have shown that FMRP, FXR1P and FXR2P are present within mRNPs either as homo-dimer, or homo-trimer (*publication 5*). *In vitro* formation of homo- as well as heteromultimers between the three proteins have suggested a functional relationship among them. In order to characterize the expression profile of the FXR proteins, particularly in brain and testis,

antibodies were raised against FXR1P and FXR2P. Immuno-histochemical experiments revealed similar (adult brain) as well as distinct (fetal brain, testis, muscle) tissue and cellular distribution of the FXR proteins, suggesting that they can function independently from each other *in vivo* (*publication 3*).

Recently, signals have been found in FMRP which direct the protein either to the nucleus (Nuclear Localization Signal, NLS), or to the cytoplasm (Nuclear Export Signal, NES). The presence of these motifs indicates that FMRP, although it appears exclusively cytoplasmic, is able to shuttle between the cytoplasm and the nucleus. We showed that an FMRP isoform (ISO12), lacking the NES, is retained in the nucleus with exclusion of the nucleolus of mammalian transfected cells (*publication 2*).

The NES of FMRP is reminiscent of the leucine-rich NES, which was first identified in the protein Rev of the HIV virus. FXR1P and FXR2P have a similar NES, and probably they shuttle too. The identification of CRM1/exportin1 as the export factor for the Rev-NES signal improved the knowledge in protein/RNA nucleocytoplasmic transport. We have shown that the inactivation by point mutations of the NESs of FMRP and FXR2P, and the chemical inhibition of the exportin1 activity, both results in nucleoplasmatic (FMRP, FXR1P) and nucleolar (FXR2P) accumulation of the FXR proteins (*publication 4*). Therefore, the nuclear export of the FXR proteins is exportin1-dependent. The unexpected, unique, nucleolar accumulation of FXR2P prompted us to propose divergent intracellular pathways and RNA targets for FMRP and FXR2P.

Finally, a severe mentally retarded patient has been described with a point mutation in the second KH domain (I304N) as the sole mutation in *FMR1*. It has been postulated that fragile X syndrome in this patient is caused by changed RNA-binding activity of the mutant FMRPI304N. We have demonstrated that FMRPI304N does not associate properly with ribosomes (*publication 1*), and has a much faster shuttling activity compared to wild type FMRP (*publication 4*). Therefore, the nucleocytoplamic shuttling of FMRP appears to occur upon dissociation of the protein from the polyribosomes Moreover, these findings suggest that FMRP I304N might have a negative effect intracellularly, such as sequestering important mRNAs in inactive mRNP particles, which cannot interact with the translation machinery.

We propose that the two new properties of FMRP, polyribosomal association and nucleocytoplasmic shuttling, are inter-dependent and possibly regulated by cellular signals. Whether synaptic activation is one of these signals, the mechanism for the functioning of FMRP would be unique for neurons and, therefore, explain the lack of intelligence caused by the absence of FMRP.

Samenvatting

Dit proefschrift presenteert verschillende studies naar de localisatie en de rol van het FMR1 eiwit (FMRP) dat betrokken is bij het fragiele X syndroom. Om deze resultaten te bereiken is gebruik gemaakt van technieken uit de biochemie, immunohistochemie en celbiologie.

Het fragiele X syndroom is met een geschatte incidentie van 1 op 6000 één van de meest voorkomende vormen van erfelijke mentale retardatie. Naast mentale retardatie hebben fragiele X patienten een hyperaktief en sterotyperend gedrag en bezitten lichamelijke kenmerken, waarvan vergrote testikels het meest opvallende is. In 1991 is het fragiele X gen gekloneerd en kreeg de naam FMR1. Het fragiele X syndroom is het eerste voorbeeld van een "nieuwe" klasse van ziekten die veroorzaakt wordt door een verlengde trinucleotide repeat. Het merendeel van de aangedane individuen hebben een pathologische verlenging van een CGG trinucleotide repeat (> 200 units) in het 5' onvertaalde gebied van het FMR1 gen (volledige mutatie). Deze verlengde repeat gaat gepaard met een hypermethylering van de repeat en naastliggend gebied dat de promoter bevat. Het resultaat is de afwezigheid van transcriptie en daarom geen synthese van het gecodeerde eiwit FMRP (voor Fragiele X Mentale Retardatie Proteïne) in fragiele X patiënten.

In normale individuen worden diverse FMRP isovormen geproduceerd met een moleculair gewicht van ~80 KDa, die voortkomen uit alternatieve splicing van enkele exonen aan het einde van het gen. Alle FMRP isovormen, met uitzondering van één (ISO12), zijn gelokaliseerd in het cytoplasma. Subcellulaire fractionering van eiwit homogenaten, gelfiltratie en ultracentrifugatie inbegrepen, lieten zien dat FMRP geassocieerd is met zware sedimentatiedeeltjes, naar waarschijnlijkheid ribosomen (*publicatie 1*). Sucrose sedimentatie studies definieerden verder de associatie van FMRP met ribosomen gedurende actieve translatie (polyribosomen). Bovendien konden we de FMRP direkt op ultrastructureel niveau zichtbaar maken door middel van elektronen microscopie (EM) in getransfekteerde cellen van zoogdieren. FMRP signalen werden op zowel vrije ribosomen als op ribosomen gehecht aan het endoplasmatisch reticulum gevonden en op een granulaire component van de nucleolus (*publicatie 2*).

De herkenning van RNA-bindende domeinen (KH domein en RGG box) in FMRP gaven de eerste aanwijzingen betreffende de functie van FMRP. FMRP bindt inderdaad RNA *in vitro*. Door gehomogeniseerde celsuspensies te behandelen met RNAse, zijn wij in staat gebleken om te demonstreren dat FMRP via RNA geassocieerd is aan ribosomen (*publicatie 1*). Vervolgens is gevonden dat mRNA de component is die FMRP aan de ribosomen hecht. Eveneens is gevonden dat FMRP deel uitmaakt van een messenger ribonucleair eiwit (mRNP) partikel, dat een sedimentatie heeft van 60S, maar niet groter is dan de grote 60S ribosomale subunit zelf (*publicatie 5*). Deze bevindingen en soortgelijke observaties door andere groepen suggereren dat het fragiele X syndroom een defect in mRNA metabolisme is, voornamelijk gedurende translatie.

FMRP, en zijn homologen FXR1P en FXR2P vormen een kleine familie van eiwitten (FRX eiwitten), die sterk gerelateerd zijn wat betreft sub-cellulaire lokalisatie en functie. Wij

hebben laten zien dat FMRP, FXR1P and FXR2P als homomeer of homo-trimeer aanwezig zijn in de mRNP's (*publicatie 5*). *In vitro* vorming van zowel homo- als heteromultimeren tussen de 3 eiwitten suggereerde een functionele relatie tussen hen. Om het expressie profiel van de FXR eiwitten, met name in de hersenen en testikels, te karakteriseren, werden antilichamen opgewekt tegen FXR1P en FRX2P. Immunohistochemische experimenten brachten zowel overeenkomstige (volwassen hersenen) als verschillende (foetale hersenen, testikels en spieren) weefsel- en cellulaire distributie van de FXR eiwitten aan het licht, hetgeen suggereert dat ze onafhankelijk van elkaar kunnen functioneren (*publicatie 3*).

Recentelijk zijn in FMRP signalen gevonden die het eiwit of naar de kern (Nucleair Lokalisatie Signaal, NLS) of naar het cytoplasma (Nucleair Export Signaal, NES) sturen. De aanwezigheid van deze motieven geeft aan dat FMRP, ondanks dat het exclusief cytoplasmatisch voorkomt, in staat is om tussen het cytoplasma en de kern te bewegen (shuttling). Wij toonden aan dat een FMRP isovorm (ISO12), dat het NES mist, in de kern wordt achtergehouden met uitzondering van de nucleolus (*publicatie 2*).

Het NES van FMRP lijkt op het NES dat als eerste is geïdentificeerd in het virale eiwit Rev en dat rijk is aan leucine. FXR1P en FXR2P hebben een soortgelijk NES en waarschijnlijk shuttelen zij ook. De identificatie van CRM1/exportine1 als exportfactor voor het Rev-NES signaal heeft de kennis van nucleocytoplasmatisch RNA/eiwit transport enorm verbeterd. Wij hebben laten zien dat inactivatie van het NES van FMRP en FRX2P door puntmutaties en de chemische remming van de exportine1 activiteit, beiden resulteert in een kern-cytoplasmatische (FMRP) en nucleolaire (FRX2P) ophoping van de FRX eiwitten (*publicatie 4*). Daarom is de nucleaire export van de FXR eiwitten afhankelijk van exportine1. De onverwachte, unieke, nucleolaire ophoping van FXR2P zette ons ertoe aan om verschillende intracellulaire pathways voor te stellen voor FMRP, FXR1P and FXR2P.

Tot slot is er een patiënt met ernstige mentale achterstand beschreven met als enige mutatie in FMR1 een puntmutatie in het tweede KH domein (I304N). Er is gepostuleerd dat het fragiele X syndroom in deze patiënt werd veroorzaakt door een veranderde RNA bindingsactiviteit van FMRPI304N. We demonstreerden dat FMRPI304N niet goed associeert met ribosomen (*publicatie 1*) en een veel snellere shuttle-activiteit heeft (*publicatie 4*). Om deze reden lijkt de nucleoplasmatische beweging van FMRP afhankelijk te zijn van van de dissociatie van het eiwit van de polyribosomen. Daarbij suggereren deze bevindingen dat FMRPI304N een negatief intracellulair effect zou kunnen hebben, zoals het vasthouden van belangrijke mRNA's in inactieve mRNP partikels, welke geen interactie aan kunnen gaan met de translatie machinerie.

We stellen voor dat de twee nieuwe eigenschappen van FMRP, polyribosomale associatie en nucleoplasmatische shuttling, niet alleen van elkaar afhankelijk zijn, maar ook gereguleerd worden door cellulaire signalen. Als synaptische activatie één van deze signalen is, zou het mechanisme van het functioneren van FMRP uniek zijn voor neuronen en daarom het ontbreken van intelligentie verklaren dat veroorzaakt wordt door de afwezigheid van FMRP.

Curriculum vitae

Name:	Filippo Tamanini
Born:	30 July 1968 in Trento, Italy
1987	Scientific High School degree, score 60/60
1992	Degree in Biological Sciences from the University of Pavia (Italy), score 110/110 cum laude Title of the thesis: "Identification of genes in human Xq28 and association to genetic diseases" Supervisor: Dr. Daniela Toniolo (CNR-IGBE)
1992-1993	Civil service
1993-1995	Postgraduate stages in the lab of Dr. D.Toniolo (CNR-IGBE) and Prof. E. Boncinelli (DIBIT-HS Raffaele, Milan) Subject: Gene expression during mouse brain development
1995-1997	Fellowship from Telethon-Italy for a postgraduate stage in the lab of Prof. Dr. B. Oostra (Dept. of Clinical Genetics, Erasmus University, Rotterdam) under the supervison of Dr. A. Hoogeveen
1997-1999	PhD student in the lab of of Prof. Dr. B. Oostra under the supervision of Dr. A. Hoogeveen (Erasmus University)
1999-2001	Postdoc in the lab of Prof. Dr. J.H. Hoeijmakers (Dept. of Cell Biology and Genetica, Erasmus University, Rotterdam) under the supervision of Dr. G.T. van der Horst
Courses:	5th course of the European School of Medical Genetics (FEBS), Sestri Levante, Italy (1992); 1st Euroconference on Cell Dynamics, Crete, Greece (1997); EMBO practical course "Analysis of RNA-protein interactions", Heidelberg, Germany (1998).

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submitted.

Dankwoord

It was not on my initial plan to get married, live in the cold North, become daddy and...make a Dutch PhD promotion, the most serious of the world! Nevertheless, it happened and I like it all.

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Dear *Mieke* (Mieke "Tyson" Jansen when you are angry), it was great to share the office time with a friend, who remembers constantly at what time and why I must go home (and many other things). Please, don't think that I asked you to be my paranymph just for your computer/english skills (that you are giving to me even in this last page!). I did because you are an open, honest and cheerful person, and I could always express my personality with you. Thanks.

Dear *Leontine*, thanks for your strong commitment with the protein-work and for being a "natural born critic" in the lab (your comments were the best coffee in the morning). Your organized mind has been essential for me. How to forget our open and often deadly discussions (OK, we finished ex-equo)? Hopefully, you will recover soon from my horrible jokes (Corleontije) and experimental plans (I think you must run the sample in this order and not...). Mamma Leontine, heel veel succes met het kindje!!

Mark & Robert, it is a pleasure to have such friends around, particularly when you invite me for delicious dinners and dangerous beer taste evenings. Robert, I am happy to say, like for an exceptional eclipse, "when Feyenoord won the competition I was there". Mark, I still don't accept that my son likes my paranymph more than daddy.

Sorry "from Breda-big salade"*Cecile*, I am not a sailor. However, by staying close to you and listening to your stories, I became like Captain Ahab, fighting against the whale in St. Annaland! *Agnes*, my favorite definition for you is "unbelievable lieve". How do you do? *Cathy & Carola*, thanks for your collaboration with the plasmids. I appreciated your sublime art of cut and paste. *Frans*, the only football supporter, who wins every national competition every year (by changing team every week). Thank you for the discussions and jokes about the function of my protein (if it is a brain clock protein...). Thanks *Arnold*, as I felt safe with

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The two (almost three!!) most important persons in my life come at the end. Apart from being my wife and my son, why *Alexandra & Mattia* are so special? As they wrote for me the samenvatting and are giving me happiness every single day.

Stellingen behorende bij het proefschrift

The role of the *FMR1* protein involved in fragile X syndrome

I

RNA is important for the linking of FMRP to the ribosomes.

This thesis

II

The study of the highly homologous Fragile X-related proteins, which might interact with each other, originates information as well as debates.

This theis

III

The predominant cytoplasmic localization of FMRP after nuclear-export inhibition is in contrast with a continuous function in RNA transport.

This theis

IV

CRM1/exportin1is a (sexually) promiscuous cellular factor, since it helps to replicate the HIV virus and interacts with 10-20 different partners.

This thesis

V

Not all proteins carrying a potential Rev-like nuclear export signal (NES) show shuttling activity and interact with CRM1/exportin1.

N. Fischer et al.,FEBS Lett (1999), 447:314-314

VI

Although the three-dimensional structure of Importin β resembles a snail, the field of nucleocytoplasmic transport moves reasonably fast.

G. Cingolani, Nature (1999), vol. 399, pag. 208

VII

Dissatisfaction is generally considered in a negative way, however it has a genetic basis and it is essential for the survival of our species.

C.J. O'Kane et al., Current Biology (1999) 9, 289-292

VIII

The abstracts of research papers in leading medical journals often contradict the papers themselves and report data that do not appear elsewhere in the manuscripts.

JAMA,vol. 281, pag. 1110

IX

The Bayesian probability theory indicates that poor darts players who want to maximize their scores should aim for the hardest target on the board, the bull's-eye, instead to go for the triple-20.

Mathematics Today, vol. 35, pag.54

X

The hyperactivity and hypersensitivity observed in fragile X patients might indicate that their brain connections are faster than in normal individuals.

XI

People who are worried about the consequences of the human genome project should consider that in general it takes many years to have any clue about the function of a protein.

This thesis

XII

Humans do not have enough words to name their own proteins.

XIII

If the FMRP expression in hairs is representative of the situation in brain, this would be in contradiction with the Dutch statement "waar haar zit, zitten geen hersenen".

R. Willemsen et al., Am J Hum Genet (July 1999)

XIV

Useless is the typing of your name on the cover of a book of science, because the latter belongs to everybody.

C. Magris, Danubio Blu