Human Molecular Genetics, 1996, Vol. 5, No. 8 **1083–1091**

ARTICLE

The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals

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Received May 1, 1996; Revised and Accepted May 28, 1996

Fragile X syndrome is a frequent cause of mental retardation resulting from the absence of FMRP, the protein encoded by the *FMR1* gene. FMRP is an RNA-binding protein of unknown function which is associated with ribosomes. To gain insight into FMRP function, we performed immunolocalization analysis of FMRP truncation and fusion constructs which revealed a nuclear localization signal (NLS) in the amino terminus of FMRP as well as a nuclear export signal (NES) encoded by exon 14. A 17 amino acid peptide containing the FMRP NES, which closely resembles the NES motifs recently described for HIV-1 Rev and PKI, is sufficient to direct nuclear export of a microinjected protein conjugate. Sucrose gradient analysis shows that FMRP ribosome association is RNA-dependent and FMRP is found in ribonucleoprotein (RNP) particles following EDTA treatment. These data are consistent with nascent FMRP entering the nucleus to assemble into mRNP particles prior to export back into the cytoplasm and suggests that fragile X syndrome may result from altered translation of transcripts which normally bind to FMRP.

INTRODUCTION

Fragile X syndrome is a common form of inherited mental retardation that is due to the functional absence of the fragile X mental retardation protein (FMRP), encoded by the *FMR1* gene (1,2). The *FMR1* gene is transcriptionally silent in nearly all fragile X syndrome patients due to the almost complete methylation of the gene following massive expansion of the CGG-repeat located in the 5′-untranslated region (3–7). Since several *FMR1* deletions result in a similar phenotype, it is accepted that the absence of FMRP is responsible for the mental retardation and various somatic signs characteristic of fragile X syndrome (8–10).

FMRP is highly conserved among vertebrates (11–13) and contains two hnRNP K-protein homology (KH) domains and an RGG box, which are motifs characteristic of RNA-binding proteins (14,15). FMRP demonstrates preferential binding to RNA homopolymers (15,16) as well as selective *in vitro* binding to a subset of brain transcripts, including *FMR1* mRNA (14). RNA binding studies using truncated FMRP suggest that the carboxyl terminus of FMRP, which contains the RGG box, is essential for FMRP-RNA interaction (11,15). The KH domains also may influence RNA binding since a missense mutation (Ile304Asn) within the second KH domain results in severe fragile X syndrome (17) and impaired *in vitro* RNA homopolymer binding under high salt conditions (16,18).

Extensive alternative splicing near the 3′ end of the *FMR1* transcript results in multiple FMRP isoforms with distinct carboxyl termini (13,19). Since no FMRP isoform-specific antibodies have been reported, whether certain isoforms exhibit distinct cellular or subcellular localizations has not been established. However, immunoblot analysis suggests that those isoforms with distinct molecular weights are found in all tissues examined although sometimes at quantitatively different levels (16,20).

FMR1 expression is widespread but not ubiquitous, with abundant neuronal expression in the brain, particularly in the hippocampus and granular layer of the cerebellum (21–23). FMRP is predominantly localized to the cytoplasm in a variety of cell types (16,24–26), yet nuclear localization has occasionally been observed (25). These indirect immunofluorescent studies demonstrate a granular cytoplasmic appearance and Khandjian *et al.* recently reported that the majority of cellular FMRP is associated with ribosomes (27).

Since the *FMR1* gene was isolated by positional cloning strategies, no prior knowledge existed regarding either the function of FMRP or the consequences of its absence, except for the clinical description of fragile X syndrome. Despite recent progress identifying FMRP as an RNA-binding protein (14,15)

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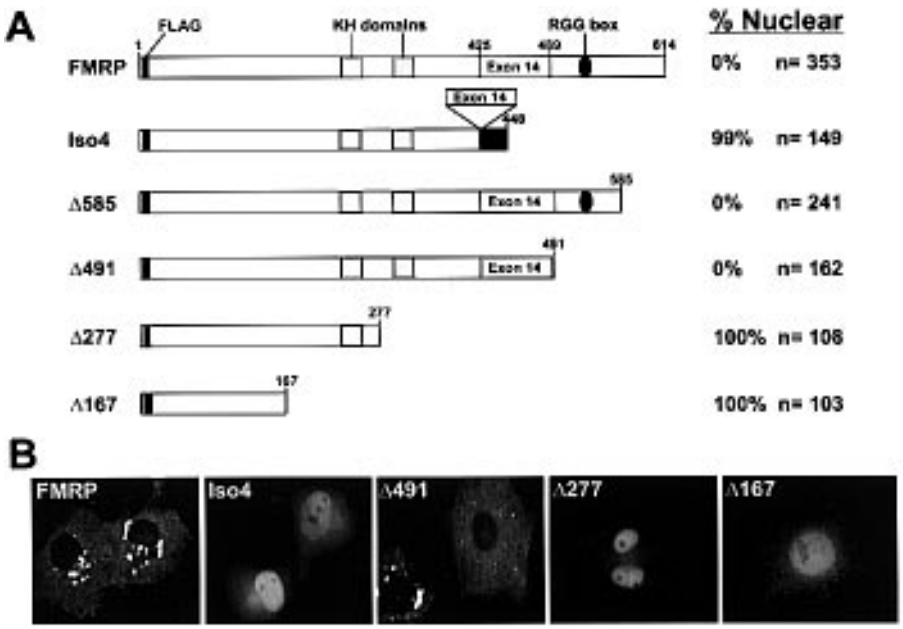

Figure 1. Subcellular localization of FMRP constructs transiently transfected into COS-7 cells. (**A**) Full-length FMRP, Iso4, and carboxyl-terminal deletion constructs with amino acid numbers labeled according to Ashley *et al.* (13). The percentage of cells that displayed nuclear staining is indicated with *n* = the number of cells exhibiting fluorescent staining (e.g. 99% of the cells transfected with Iso4 showed predominant nuclear staining while the remainder of the cells showed no detectable staining in the nucleus). (**B**) Fluorescent micrographs showing the subcellular localization of FMRP constructs in transiently transfected COS-7 cells via indirect immunofluorescent staining with the anti-FLAG M2 antibody. The transfected FMRP constructs are identified in each panel.

which is associated with ribosomes (27), as well as characterization of FMRP expression patterns and isoforms, the function of FMRP remains obscure, as does the mechanism by which the absence of FMRP results in mental retardation and the associated phenotype. Here we present studies indicating that FMRP contains both a nuclear localization signal (NLS) and a nuclear export signal (NES) and associates with ribosomes in a RNA-dependent manner. These data consolidate previous information on FMRP and suggest that FMRP enters the nucleus to assemble into ribonucleoprotein (RNP) particles followed by export into the cytoplasm and association with ribosomes.

RESULTS

FMRP isoform 4 is nuclear

Immunocytofluorescence analysis of COS-7 cells which were transiently transfected with a SV40 promoter-driven full-length FMRP construct containing the FLAG-epitope revealed the expected diffuse cytoplasmic pattern (24–26), along with intense perinuclear staining (Fig. 1A and B). The granular cytoplasmic pattern likely reflects FMRP associated with ribosomes (27). In contrast to this result, FMRP isoform 4 (Iso4), in which exon 14 is normally spliced out leaving a distinct carboxyl terminus (13), localizes to the nucleus with diffuse staining in the nucleoplasm and no visible staining in the nucleolus (Fig. 1A and B).

Sequential truncations of FMRP were generated to more fully evaluate the localization differences between full-length FMRP and Iso4. Constructs lacking the carboxyl terminal 29 (Δ585) and 123 (Δ491) residues from FMRP, including deletion of the RGG box in Δ491, remain cytoplasmic (Fig. 1A and B). This localization supports the notion that the residues encoded by exon 14 are critical for cytoplasmic localization. Further truncations, leaving the initial 277 and 167 residues of FMRP, shift localization predominantly into the nucleus (Fig. 1A and B). This suggests the presence of two distinct domains of FMRP: the first domain, in the amino terminus, contains sequences which may serve as an NLS; the second domain, composed of exon 14-encoded residues, contains sequences which appear to override the NLS, localizing FMRP into the cytoplasm.

FMRP contains a functional NLS

Since Δ277 and Δ167 may be small enough to enter the nucleus by diffusion, fusion constructs between chicken muscle pyruvate kinase (CMPK) and the amino terminus of FMRP were generated and analyzed for subcellular localization to directly test for the presence of a functional NLS in FMRP (Fig. 2A). CMPK normally exhibits diffuse cytoplasmic staining and has previously been utilized to define protein targeting signals (28). Fusion of the first 16 (FMR.PK1) or the first 117 (FMR.PK6) residues of FMRP resulted in cytoplasmic localization indistinguishable from CMPK alone (Fig. 2A and B). However, addition of the first 184 residues of FMRP resulted in nuclear localization of the fusion protein (FMR.PK2; Fig. 2A and B), indicating that FMRP contains an NLS sufficient to direct nuclear localization.

FMRP does not contain a consensus SV40-like or bipartite NLS (29). However, the initial 184 amino acids of FMRP do contain clusters of arginine and lysine residues which are present in most NLSs (Fig. 2C). These 24 basic residues are identical in human, mouse, chicken, and *Xenopus FMR1* (11–13) and this region likely contains a novel NLS sequence. Similarly, two RNA-binding proteins which demonstrate nucleocytoplasmic shuttling contain novel NLSs (30,31).

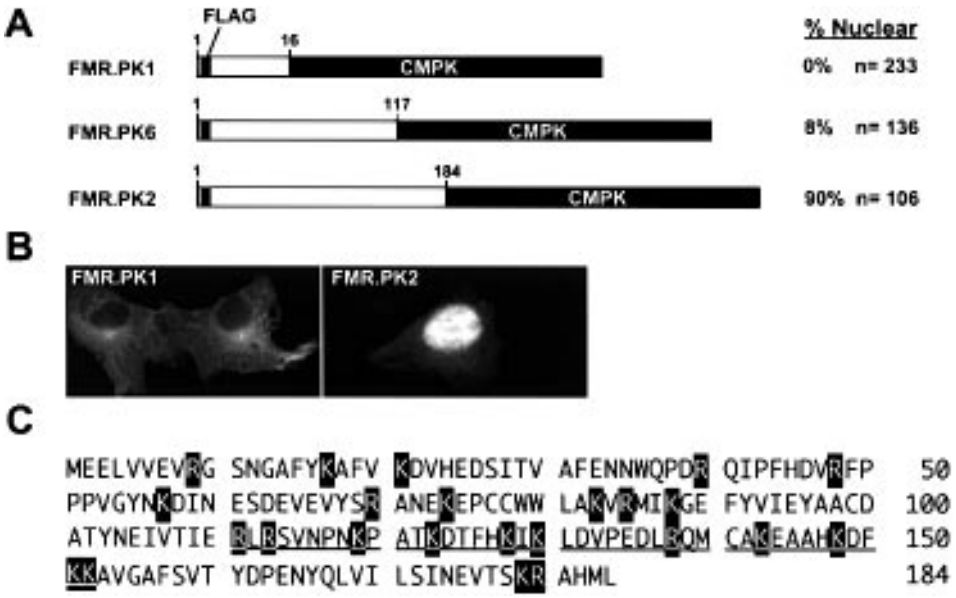

Figure 2. Subcellular localization of CMPK-FMRP amino terminus fusion proteins. (**A**) FMRP amino terminus fusion constructs with chicken muscle pyruvate kinase (CMPK, amino acids 17-476). The percentage of cells that displayed nuclear staining is indicated with *n* = the number of cells displaying fluorescent staining. (**B**) Micrographs showing subcellular localization of FMRP-CMPK fusion constructs in COS-7 cells with the transfected constructs identified in each panel. (**C**) FMRP residues 1–184 with basic residues in black boxes. Possible NLS containing region is underlined.

The first 17 residues of exon 14 mediate FMRP cytoplasmic localization

Although FMRP contains a functional NLS, it is predominantly localized in the cytoplasm, suggesting that the NLS is normally overridden by another domain. Since residues encoded by exon 14 appear to be necessary for cytoplasmic localization, further truncation constructs were examined to delineate this domain (Fig. 3A). Removal of the carboxyl 25 (Δ464) and 41 (Δ448) residues encoded by exon 14 still resulted in FMRP being found in the cytoplasm (Fig. 3A and B). Indeed, examination of Δ441 reveals that only the initial 17 residues of exon 14 were necessary to localize FMRP to the cytoplasm (Fig. 3A and B). However, Δ432, containing the first eight residues of exon 14, partially loses this ability (Fig. 3A). Truncation into exon 13 encoded residues (Δ419) abolished the predominant cytoplasmic localization (Fig. 3A and B). A FMRP construct (Δ17) in which the first 17 residues encoded by exon 14 (425–441) are removed, leaving the remainder of FMRP intact, showed nuclear localization in 98% of cells (Fig. 3A and B). Thus, the initial 17 amino acids encoded by exon 14 contain information necessary for the cytoplasmic localization of FMRP.

To determine whether the exon 14-encoded domain was sufficient to override the NLS of FMRP in a chimeric protein, we fused portions of exon 14 to the carboxyl terminus of FMR.PK2 (Fig. 4A). Remarkably, when the 65 residues encoded by exon 14 are fused to FMR.PK2, localization is shifted to the cytoplasm (FMR.PK3; Fig. 4A and B). Truncation of the carboxyl terminus of FMR.PK3 to the initial 40 residues of exon 14 similarly results in cytoplasmic localization of the resulting fusion protein (FMR.PK4; Fig. 4A). Indeed, fusion of the first 17 residues encoded by exon 14 to FMR.PK2 still results in nearly 70% of the transfected cells exhibiting cytoplasmic localization (FMR.PK5, Fig. 4A and B). Therefore, 17 amino acids (425–441) are necessary and sufficient to direct FMRP or a heterologous nuclear-targeted protein to the cytoplasm.

FMRP contains a nuclear export signal

There are at least two mechanisms by which this 17-residue domain could direct cytoplasmic localization of FMRP. The domain could function either as a nuclear export signal (NES) or as a cytoplasmic retention region which prevents FMRP from going into the nucleus. Sittler *et al.* (32) demonstrated that FMRP isoforms lacking exon 14 localize to the nucleus and suggested that a cytoplasmic retention signal within exon 14 is responsible for the predominant cytoplasmic localization of FMRP. However, the first description of NESs recently appeared for the HIV-1 Rev protein (33) and protein kinase inhibitor α (PKI) (34). These signals appear to share many attributes of NLSs in mediating dynamic movement across the nuclear pore, such as ATP- and temperature-dependence as well as being saturable (35). Examination of the NES sequences of PKI and Rev reveals strong sequence similarity to residues within the 17 amino acid region responsible for cytoplasmic localization of FMRP (Fig. 4C), especially at the leucine residues that appear to be crucial for NES function in Rev (33) and PKI (34).

To directly test for nuclear export activity of the putative NES region, a peptide corresponding to the first 17 residues encoded by exon 14 was conjugated to bovine serum albumin (BSA) and then labeled with fluorescein isothiocyanate (FITC). FITC-labeled BSA-NES was microinjected into the nuclei of COS-7 cells along with immunoglobulin G (IgG) labeled with lissamine rhodamine (LRSC). IgG, which is too large to cross the nuclear envelope by diffusion, was coinjected as a control for injection site and leakage. BSA-NES was efficiently exported with virtually all signal in the

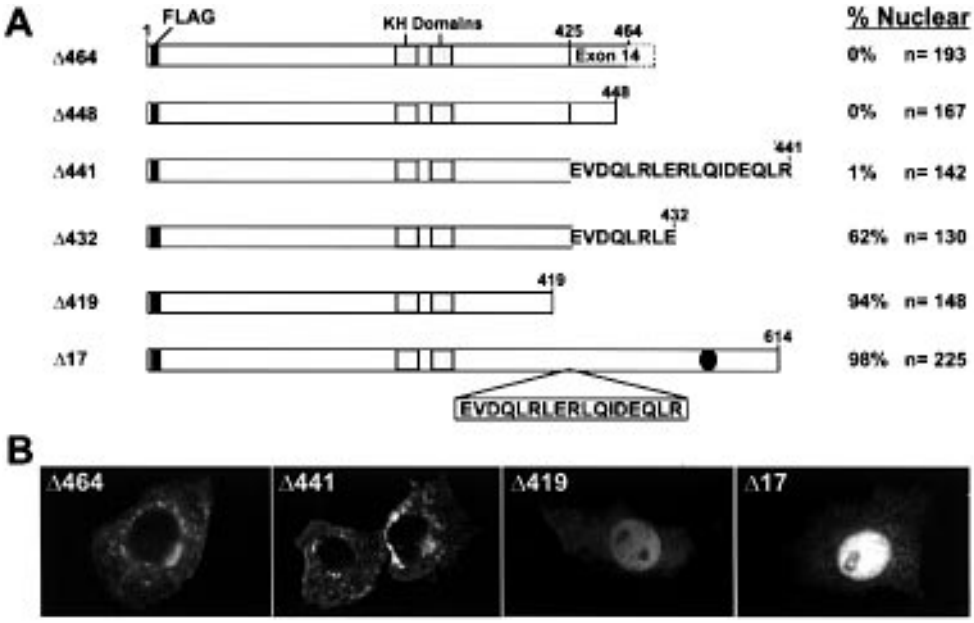

Figure 3. Subcellular localization of FMRP constructs with exon 14 deletions. (**A**) FMRP constructs with carboxyl terminal truncations of exon 14-encoded residues. The remaining residues encoded by exon 14 are indicated in Δ441 and Δ432. Δ17 corresponds to full-length FMRP with residues 425–441 removed. The percentage of cells that displayed nuclear staining is indicated with *n* = the number of cells exhibiting fluorescent staining. (**B**) Micrographs showing subcellular localization of exon 14 deletion constructs in COS-7 cells with the transfected FMRP constructs identified in each panel.

cytoplasm after 1 h at 37°C, while coinjected IgG remained in the nucleus (Fig. 4E). BSA-NES export is an active, temperature-dependent process as there was virtually no export when the cells were incubated at 4°C instead of 37°C (Fig. 4E). The observed nuclear export is also sequence-dependent since BSA-M1, in which the first three leucine residues of the FMRP NES were mutated to alanine, could no longer mediate export (Fig. 4D and E). Therefore, FMRP contains a functional NES within the first 17 residues encoded by exon 14.

FMRP is a component of mRNP particles

It has recently been demonstrated that the hrp36 protein of *Chironomus tentans*, which shows similarity to the NES-containing RNA-binding protein hnRNP A1 (36), is incorporated into Balbiani ring mRNP particles in the nucleus and remains associated with these particles during nuclear export and throughout translation (37). Since hrp36 and FMRP share the features of RNA-binding, nuclear export, association with polyribosomes, and predominant cytoplasmic localization, it is possible that FMRP is also involved in the export of mRNP particles from the nucleus. To determine if FMRP is associated with RNP particles, postnuclear supernatant from cells treated with cycloheximide to arrest ribosome elongation was subjected to a 20–47% linear sucrose gradient. As shown in Figure 5, FMRP was detected in fractions containing ribosome subunits and monosomes, but preferentially cofractionated with actively translating polysomes which is in agreement with the findings of Khandjian and coworkers (27). When cell lysate was treated with RNase (300 µg/ml), to remove the mRNA connecting translating polyribosomes without disturbing individual ribosomes (38), FMRP was significantly dissociated from ribosomes into free protein and RNP fractions (Fig. 5). Therefore, FMRP-ribosome association appears to be RNA dependent. When cells were lysed in the presence of 10 mM EDTA, which has been shown to cause polyribosomes to dissociate into subunits (39), ribosome dissociation was observed and FMRP was shifted to fractions containing particles with molecular masses of ~30S to ~100S (Fig. 5). This behavior is consistent with previously reported EDTA-resistant mRNP particles (39,40). Furthermore, FMRP is detected in oligo dT-captured mRNPs when the cell lysate is exposed to UV-crosslinking prior to mRNP capture (data not shown). Taken as a whole, these results suggest that FMRP is a component of mRNP particles that can associate with the translation machinery.

DISCUSSION

FMRP contains an NLS

The finding that FMRP contains a functional NLS and that Iso4 localizes to the nucleus is consistent with previously unexplained observations. Verheij *et al.* (25) detected intense nuclear staining with anti-FMRP antibodies in esophageal epithelium and the occasional isolated cell in other tissues, though the vast majority of tissues examined displayed clear cytoplasmic localization, as do cells in culture. In addition, Devys and coworkers (26) showed that a FMRP construct containing only the first 284 residues localized to the nucleus as did Δ277 and Δ167. A fusion construct placing the amino terminal 184 amino acids of FMRP upstream of the cytoplasmic CMPK protein (FMR.PK2) was utilized to confirm that this region of FMRP is sufficient to direct nuclear localization. Since this fusion protein is ~72 kDa and nuclear import of proteins with a molecular mass greater than ~40–60 kDa requires facilitated nuclear transport (41,42), these data strongly argue for the existence of a functional NLS in FMRP.

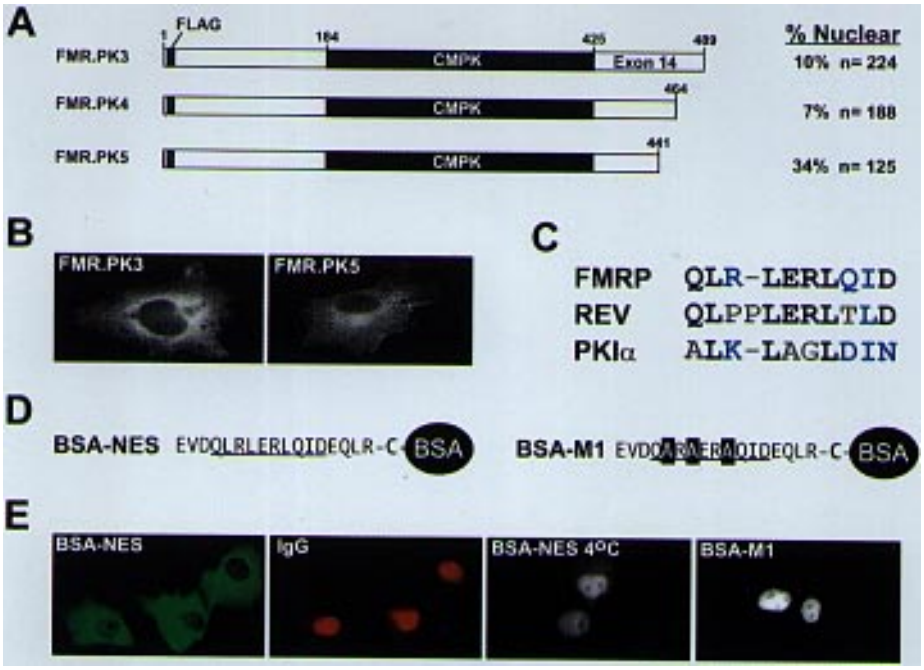

Figure 4. Delineation of the FMRP nuclear export signal. (**A**) FMRP fusion constructs with CMPK. The percentage of cells that displayed nuclear staining is indicated with *n* = the number of cells exhibiting fluorescent staining. (**B**) Micrographs showing subcellular localization of FMRP-CMPK fusion constructs in COS-7 cells with the transfected constructs identified in each panel. (**C**) Amino acid alignment of NES sequences of Rev, PKI, and FMRP with identical residues in dark blue and similar residues in light blue. Sequences aligned with the Clustal W program using the blosum weight matrix. (**D**) NES and M1 peptides conjugated to BSA. The putative NES sequence is underlined. In M1, black boxes indicate residues that were mutated from leucine to alanine. (**E**) Micrographs of COS-7 cells 1 h after nuclear injection of BSA-peptide conjugates. FITC-labeled BSA-NES (green) and LRSC-labeled IgG (red) coinjected and incubated at 37°C. BSA-NES injected and incubated at 4°C. BSA-M1 injected and incubated at 37°C.

Sittler *et al.* (32) also report that the N-terminus of FMRP and FMRP isoforms lacking exon 14 localize to the nucleus. However, in contrast to our findings that FMRP-CMPK fusion proteins localize to the nucleus, Sittler and coworkers conclude that FMRP does not contain a functional NLS since they were unable to detect β-galactosidase activity in the nucleus following transfection of fusion constructs between the N-terminus of FMRP and β-galactosidase. The inability of Sittler *et al.* to observe NLS function is likely due to the use of β-galactosidase as the fusion reporter. It is well established that protein context can mask an NLS and prevent nuclear transport (43). Indeed, the failure of β-galactosidase to enter the nucleus when fused to the bona fide NLS of GAL4 has been attributed to such a contextual effect (44).

Although FMRP does not contain a consensus SV40 or bipartite NLS, it is noteworthy that two RNA binding proteins which demonstrate nucleocytoplasmic shuttling, U1A and hnRNP A1, contain novel NLSs. U1A requires a 110 amino acid region for nuclear localization (30), and hnRNP A1 requires a 40 amino acid region (31).

FMRP contains an NES

Despite the presence of an NLS, FMRP is found predominantly within the cytoplasm. The first 17 amino acids encoded by exon 14, 425–441, were shown above to be both necessary and sufficient for cytoplasmic localization of FMRP. This 17 amino acid region was shown by analysis of FMRP-CMPK fusion proteins and by direct nuclear microinjection to contain an NES. Thus, FMRP is one of a newly recognized class of proteins, including Rev (33) and PKIα (34), which contain NES domains. Like Rev and PKIα, the FMRP NES shows no similarity to the recently described NES of hnRNP A1, termed M9, which can mediate both nuclear import and export (36). As reported for the other NES-containing proteins, FMRP NES activity is temperature- and sequence-dependent. Nuclear export is blocked at 4°C and mutation of the first three leucine residues in the NES to alanine results in the loss of NES activity thus further contributing to definition of the consensus sequence for leucine-rich NESs. It will be interesting to determine if FMRP interacts with the cellular cofactors hRIP/Rab from human (45,46) and/or Rip1p from yeast (47), which interact with the Rev NES and are considered putative NES receptors.

The process of nuclear export is poorly understood, but the discovery of NESs has provided a great deal of insight into RNA export (reviewed in 35,48). It has been suggested that protein-based NESs may be a common feature of nuclear RNA export (35). Rev binds and directs export of viral mRNAs containing the Rev response element binding site (33). TFIIIA, which has been implicated in the export of 5S rRNA, contains a putative leucine-rich NES which can substitute for the Rev NES (33,48). hnRNP A1, which is associated with poly(A) RNA in both the nucleus and cytoplasm, contains the M9 domain which allows shuttling between the two compartments (36). Other proteins which resemble hnRNP A1, such as the hrp36 protein of *C. tentans* (37), have also been implicated in RNA export (48). While further analysis is necessary to determine the export characteristics of FMRP, the identification of domains which mediate nucleocytoplasmic shuttling suggests that FMRP is

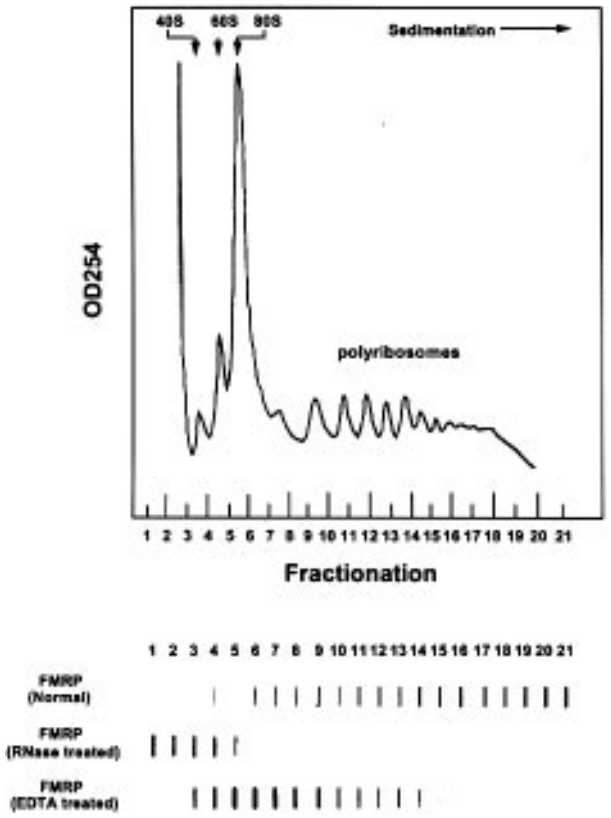

Figure 5. Association of FMRP with translating ribosomes. The OD_{254} absorption profile of normal lymphoblastoid cell lysate fractionated through a 20–47% sucrose gradient is shown in the top panel to indicate the sedimentation of RNP particles, 40S ribosomal subunits, 60S subunits, 80S monosomes, and polyribosomes. Normal cell lysate, RNase treated lysate, or EDTA treated lysate was layered on top of the gradient and after centrifugation twenty-one ~500 µl fractions were collected. 150 µl of each gradient fraction was subjected to slot immunoblot analysis to determine the distribution of FMRP in the gradient as shown in the lower panel.

involved in RNA export (see below). It is noteworthy that two autosomal homologs of *FMR1* have recently been described. FXR1 and FXR2 show significant homology with FMRP, especially the NLS, NES, and KH domains (12,49,50).

FMRP appears to associate with ribosomes as a component of mRNP particles

It was recently reported that cytoplasmic FMRP is associated with ribosomes (27). Our data confirm that FMRP is preferentially associated with actively translating polysomes. In addition, we have shown that RNase treatment removes FMRP from ribosomes and that following EDTA treatment FMRP is found in mRNP particles. These data suggest that FMRP associates with ribosomes as a component of mRNP particles. Since FMRP can selectively bind mRNA *in vitro* (14), it seems possible that FMRP may bind specific transcripts within the mRNP particle and possibly influence their translation.

Implications for FMRP function and fragile X syndrome

FMRP contains both a functional NLS and NES and appears to associate with ribosomes in a RNA-dependent fashion as a component of mRNP particles. Given the fact that many RNP particles form within the nucleus (51), it is reasonable to consider that nascent FMRP enters the nucleus. Immunoelectron microscopy analysis of neurons revealed FMRP in the nucleus, passing through the nuclear pore, and associated with ribosomes (S. Hersch, S. T. Warren *et al.*, manuscript in preparation). Therefore, it seems that FMRP enters the nucleus and there assembles into a mRNP particle, interacting with specific RNA transcripts, as does the NES-containing Rev protein (33) (Fig. 6). In addition to binding to mRNAs, FMRP may interact with other proteins, such as the FMRP homolog, FXR2, which can interact with FMRP *in vitro* (50). Like other NES-containing proteins (33,37), once FMRP is assembled into a mRNP particle, it can direct export of the FMRP-mRNP particle to the cytoplasm where it could associate with translating ribosomes. It is also possible that FMRP may play some role in modulating the translation of the mRNA(s) with which it interacts. Thus, fragile X syndrome may be due to the anomalous nuclear export and ribosome presentation of specific transcripts resulting in their inappropriate translation.

MATERIALS AND METHODS

FMRP and FMRP-CMPK fusion constructs

pFMRP and pIso4 were constructed by subcloning the *Eco*RI-*Msc*I fragment of pFLAG-Mc2.17 or the *Eco*RI-*Nae*I fragment from pFLAG-Iso4, respectively, into the *Eco*RI-*Sna*BI sites of pSV-Sport1 (Gibco/BRL). pFLAG-Mc2.17 and pFLAG-Iso4 have the 8 residue FLAG-epitope tag inserted between amino acids 2 and 3 (Small, Lakkis, and Warren, in preparation) and were derived from the full-length murine *fmr1* cDNA clone, Mc2.17 (13), and the alternatively spliced murine *fmr1* cDNA clone, Mc2.14 (13). pΔ585, pΔ491, and pΔ167 were generated from pFLAG-Mc2.17 by digesting with *Eco*RI followed by *Nae*I, *Xmn*I, or *Pvu*II, respectively and subcloning these fragments into the *Eco*RI-*Sna*BI site of pSV-Sport1. The *Eco*RI-*Hind*III fragment of pFLAG-Mc2.17 was subcloned into pSV-Sport1 to generate pΔ277.

FMRP constructs with deletions in exon 14 were constructed as follows: pΔ448 was constructed by subcloning the *Eco*RI-*Xba*I fragment of pFLAG-Mc2.17 into pcDNA3 (Invitrogen). pΔ464, pΔ441, pΔ432, and pΔ419 were amplified by polymerase chain reaction (PCR) from pFLAG-Mc2.17 using primer 5′BluS which annealed just 5′ of the pBluescript (Stratagene) multiple cloning site allowing *Eco*RI digestion to release the 5′ end of all the clones along with primer 1510r-N, 1440r-N, 1415r-N, or 1375r-N, respectively which contain a *Not*I site. The PCR protocol for all constructs consisted of one cycle at 95°C (2 min); 30 cycles of 95°C (10 s), 58–60°C (45 s), 72°C (2 min); and one cycle at 72°C (7 min). PCR products were digested with *Eco*RI-*Not*I and cloned into pcDNA3. Δ17 was generated from pFLAG-Mc2.17, by removing the initial 17 residues encoded by exon 14, using the Ex-Site PCR mutagenesis kit (Stratagene) with primers 1388 and 1447.

To generate FMRP-CMPK fusion constructs, *fmr1* fragments were PCR amplified as described above from pFLAG-Mc2.17 with forward primer 72f-H, which contains a *Hin*dIII site, and

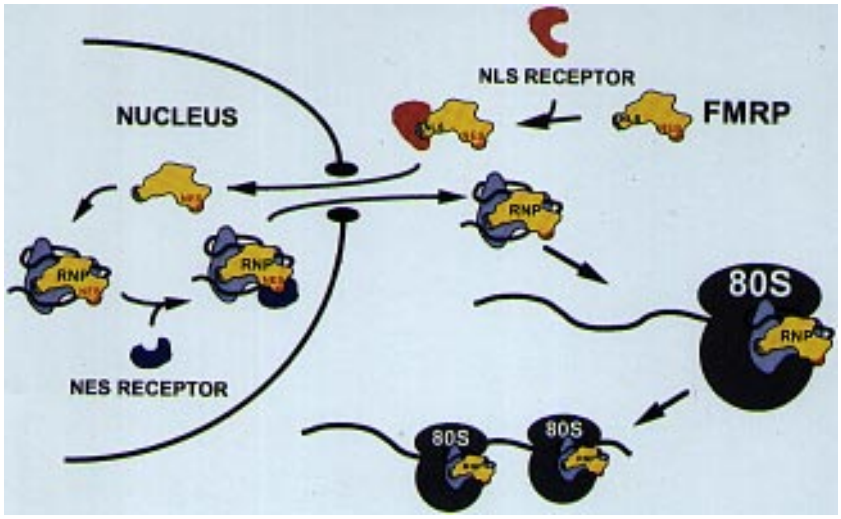

Figure 6. Postulated FMRP cellular behavior. The NLS (blue) and NES (red) regions of FMRP are shown along with their appropriate receptors which mediate transport through the nuclear pore. It is speculated that nascent FMRP enters the nucleus, interacts with mRNA(s) and possibly other proteins in the context of a mRNP particle, and then mediates export of the mRNP particle from the nucleus. In the cytoplasm the FMRP-mRNP particle associates with the translation machinery.

reverse primers 164r-B (pFMR.PK1), 467r-B (pFMR.PK6), or 669r-B (pFMR.PK2), which contain a *Bgl*II site. The PCR products were digested with *Hin*dIII/*Bgl*II and cloned into the 5′ end of the p3PK cassette (28) generating an in-frame fusion with CMPK (amino acids 17–476). The entire FMRP/CMPK fusion constructs were then liberated by *Hin*dIII/*Eco*RI digestion and cloned into pcDNA3 creating pFMR.PK1, pFMR.PK6, and pFMR.PK2. PCR amplification of exon 14 fragments from pFLAG-Mc2.17 was performed using primer 14f-B, which contains a *Bam*HI site, with primer 1586r-E (pFMR.PK3), 1510r-E (pFMR.PK4), or 1440r-E (pFMR.PK5), which contain an *Eco*RI site. The PCR products were digested with *Bam*HI/*Eco*RI and ligated to the 3′ end of pFMR.PK2. Constructs were verified by sequencing and produced the expected size protein in SDS-PAGE immunoblot analysis of transfected COS-7 cell lysate and/or when *in vitro* transcribed and translated using the TnT kit (Promega). Sequence of PCR primers with the restriction sites underlined:

972f-H GGGCCTAAGCTTCCTGCAGCCCACCTCCC;
164r-B TACAAAAGATCTGTAGAAAGCGCCATTGGAGCC;
467r-B AGCAGGAGATCTGGGATTAACAGATCGTAGACG;
669r-B TGCATAGATCTCAACATGTGGGCTCGC;
5′BluS GACCATGATTACGCCAAGCTCG;
1510r-N CCCAGCGGCCGCCCATCATCAGTCACATAGCC;
1440r-N CTAGCGCGGCCGCGTCGCAACTGCTCATC;
1415r-N CTCATCAGCGGCCGCTCACTCCAAACGCAACTGGTC;
1375r-N CTTTAAGCGGCCGCGGTGATAATCCAAAAGAACAGTGG;
1388 CTTTAAATAGTTCAGGTGATAATCC;
1447 CAAATTGGAGCTAGTTCTAGACCA;
14f-B GAACTATGGATCCGAAGTAGACCAGTTGCGTT;
1586r-E CAGAAGAATTCCCTGAAGTATATCCAGGACC;
1510r-E CCCATTGAATTCCCATCATCAGTCACATAGCC;
1440r-E CTAGCTCGAATTCGTCGCAACTGCTCATC.

Transfection and immunofluorescent staining

Approximately 1×10^5 COS-7 cells were seeded onto chamber slides, cultured overnight, and then transfected using 1–3 μg DNA and 15 μg lipofectin (Gibco/BRL) per manufacturer's instructions. Forty-eight hours post-transfection, cells were fixed with 3% paraformaldehyde (PF) and permeabilized with 0.1% Triton X-100. The fixed and permeabilized cells were incubated at room temperature (RT) with 2 μg/ml of anti-FLAG M2 antibody (IBI) in PBS with 3% BSA in a humidified chamber for 2 h. Slides were washed in blocking solution (0.01 M PB, 0.25 M NaCl, 5% nonfat milk), incubated with 2.5 μg/ml biotinylated rabbit anti-mouse IgG1 antibody (Zymed) in blocking solution for 30 min at RT, washed again, and then incubated with 3 μg/ml fluorescein isothiocyanate (FITC)-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.) in blocking solution for 20 min at RT. Coverslips were mounted with antifade solution (Oncor). Cells were visualized via epifluorescence using a 100× Zeiss oil immersion objective and Zeiss filter cube 09. Fluorescent micrographs were taken with Kodak EL 400 film using identical exposure times.

Microinjection of peptide-BSA conjugates

NES and M1 peptides synthesized and HPLC purified by the Emory University Microchemical facility were crosslinked to BSA (Boehringer Mannheim) via the cysteine residue using sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1- carboxylate (sulfo-SMCC; Pierce) essentially as described (33) and labeled with the QuickTag FITC conjugation kit (Boehringer Mannheim). FITC-labeled BSA-NES or BSA-M1 were injected into COS-7 cell nuclei at ~0.5 mg/ml in PBS (pH 7.4) along with IgG-lissamine rhodamine (LRSC, Jackson ImmunoResearch) which was coinjected to insure clean nuclear injection without leakage. Cells were either injected and then incubated at 37°C for 1 h or were

equilibrated to 4°C for 15 min, injected, and then incubated at 4°C for 1 h. Following injection/incubation, cells were fixed with 3% PF at RT for 5 min and visualized via epifluorescence using a 60× Zeiss oil immersion objective with Zeiss filter cube 09 (FITC) or 15 (LRSC).

Sucrose gradient fractionation

Lymphoblastoid cells were incubated for 15 min with cycloheximide (100 µg/ml) prior to vacuum cavitation lysis (200 psi for 10 min) in a buffer containing 0.25 M sucrose, 50 mM Tris (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 1 µg/ml each Aprotinin, Pepstatin and Leupeptin, and 1 mM phenyl methane-sufonyl fluoride. The lysate was subjected to 10 000× g centrifugation for 10 min to yield the postnuclear supernatant. 500 ml of postnuclear supernatant was loaded onto a 20–47% (w/v) sucrose gradient containing 80 mM NaCl, 20 mM Tris (pH 7.5), and 5 mM $MgCl_2$ followed by centrifugation in a Beckman SW41 rotor at 225 000 × g for 2.25 h at 4°C. The entire gradient profile was monitored at 254 nm and fractionated into twenty-one ~500 µl fractions using a gradient fractionator. The fractionation profile was confirmed by distribution of 18S rRNA (52) and the 60S ribosomal protein P0 (53). For RNase treatment, RNase A was added to a final concentration of 300 µg/ml and incubated at RT for 5 min. For EDTA treatment, lysis was carried out as described above except 10 mM EDTA was substituted for $MgCl_2$ in the lysis buffer and the sucrose gradient. For slot immunoblot assays, 150 µl of each gradient fraction was applied to PBS pre-wetted nitrocellulose membrane by vacuum filtration using the Bio-Dot SF assembly (Bio-Rad). Immunostaining and ECL detection were performed at RT according to the manufacturer's protocol (Amersham) using FMRP monoclonal antibody mAb1a (26).

ACKNOWLEDGMENTS

We thank J. Frangioni and B. Neel for vector p3PK and anti-CMPK antibody, J-L Mandel and K.B. Elkon for antibodies, I. Gonzalez for rRNA probes, J. Hershey for advice on gradient preparation, and D. McCormick, D. Nelson, D. Reines, and K. Wilkinson for advice and critical review of the manuscript. S. T. W. is an investigator of the Howard Hughes Medical Institute. This work was partially funded by NIH grant HD 20521.

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