The Fragile X Mental Retardation Protein FMRP Binds Elongation Factor 1A mRNA and Negatively Regulates Its Translation in Vivo

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Loss of the RNA-binding protein FMRP (fragile X mental retardation protein) leads to fragile X syndrome, the most common form of inherited mental retardation. Although some of the messenger RNA targets of this protein, including FMR1, have been ascertained, many have yet to be identified. We have found that Xenopus elongation factor 1A (EF-1A) mRNA binds tightly to recombinant human FMRP in vitro. Binding depended on protein determinants located primarily in the C-terminal domain of hFMRP, but the hnRNP K homology domain influenced binding as well. When hFMRP was expressed in cultured cells, it dramatically reduced endogenous EF-1A protein expression but had no effect on EF-1A mRNA levels. In contrast, the translation of several other mRNAs, including those coding for dynamin and constitutive heat shock 70 protein, was not affected by the hFMRP expression. Most importantly, EF-1A mRNA and hFMR1 mRNA were communoprecipitated with hFMRP. Finally, in fragile X lymphoblastoid cells in which hFMRP is absent, human EF-1A protein but not its corresponding mRNA is elevated compared with normal lymphoblastoid cells. These data suggest that hFMRP binds to EF-1A mRNA and also strongly argue that FMRP negatively regulates EF-1A expression in vivo.

The loss of a normal cellular protein, FMRP, causes fragile X syndrome, one of the most common forms of mental retardation (MR). FMRP is a RNA-binding protein that contains two hnRNP K-homology (KH) binding domains and an arginine-glycine-rich region that resembles an RGG box (1, 2). Several studies indicate that both the KH2 domain and the arginine-glycine-rich region likely play a role in RNA binding (1, 3–6), the latter interaction being mediated by a G quartet (7). FMRP associates with polyribosomes via a mRNP particle (8, 9), and the latter interaction being mediated by a G quartet (7). Mammalian FMRPs inhibit mRNA translation in vitro at nanomolar concentrations in both rabbit reticulocyte lysates (15) and in microinjected Xenopus oocytes (16). These data suggest that translational repression may be in vivo function of FMRP. Indeed, the Drosophila homolog of FMRP, dFMR1, was found to bind and negatively regulate futsch mRNA (17).

Recent studies have begun to delineate the mRNAs that mammalian FMRPs interact with in vivo. These studies have taken one of two forms. On the one hand, potential FMRP target mRNAs have been identified solely on the basis of their ability to bind to purified recombinant FMRP (15, 16) or cell-free produced FMRP (1, 3). Notwithstanding, it has not been determined whether any of these mRNAs bind to FMRP in vivo. On the other hand, mRNAs, including FMR1 mRNA, which associate with FMRP-containing mRNP have also been isolated from cultured cells (10, 18). However, although these messages require FMRP in the mRNP for their association, it has not been demonstrated that they bind solely to it. Using the former methodology, we isolated a subset of mRNAs derived from normal adult brain that bind human FMRP (hFMRP) in vitro (3). During the course of this investigation we also tested a number of other mRNAs for their ability to interact with hFMRP. One of these mRNAs was Xenopus elongation factor 1A (xEF-1A). In the present paper we demonstrate that xEF-1A mRNA binds to recombinant and cell-free produced hFMRP in vitro. Furthermore, we show that hFMRP inhibits EF-1A mRNA translation in cultured PC12 and COS-7 cells and that the loss of hFMRP in fragile X lymphoblastoid cells derepresses human EF-1A (hEF-1A) mRNA translation.

EXPERIMENTAL PROCEDURES

Antibodies—FMRP mAb 2160 and normal mouse serum were purchased from Chemicon. FXR1 (Y-19) mAb and FXR2 (S-16) mAb were obtained from Santa Cruz. Dynamin mAb and EF-1A mAb were purchased from Upstate Biotechnology. Hsp70P mAb (HSP-820) was obtained from StressGen. Horseradish peroxidase-conjugated secondary antibodies were purchased from Upstate Biotechnology. Hsp70P mAb (HSP-820) was obtained from StressGen.

Buffers—RNA binding buffer 1 contained 50 mM Tris-HCl, pH 7.0, 2 mM MgCl2, and 150 mM NaCl. Buffer 2 contained 20 mM Hepes, pH 7.9, 300 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and 50 mg/ml yeast tRNA. FMRP purification buffer, buffer 3, contained 10 mM Hepes, pH 7.9, 300 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and 20 mM imidazole. TAE buffer contained 40 mM Tris acetate and 1 mM EDTA. TMK buffer contained 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, and 25 mM KCl.

Plasmid Clones—pET21A-hFMRP and pET21A-I304N, encoding...
FMRP binds to EF-1A mRNA in Vivo

hFMRP and the corresponding I304N point mutant, were gifts from Dr. Bernhard Laggerbauer, Max Planck Institute for Biochemistry, Germany. Each vector generates full-length hFMRP with an N-terminal His tag. pTRI-XEF, encoding Xenopus EF-1A, was obtained from Ambion Laboratories, pAPP-695 and p7T-Control, encoding pAPP695 and a 1.4-kb λ-HindIII/EcoRI RNA, respectively, have been described previously (3). pSF2-hFMRP, encoding human FMRP, was a gift from Dr. Ben Oester, Erasmus University, Rotterdam, The Netherlands. pHA-hFXR1P, encoding human FXR1P, was a gift from Dr. Gideon Dreyfuss, University of Pennsylvania.

**hFMRP Production for RNA Binding Studies**—Full-length and truncated 35S-hFMRPs were prepared by coupled in vitro transcription translation (3). Recombinant hFMRPs were expressed in *Escherichia coli* BL21 from pET21A-hFMRP and pET21A-I304N (16, 19). Briefly, transformed *E. coli* BL21 were grown at 37°C to 1.0 A600 in LB-Amp100 medium. 1 ml iso-propyl-1-thio-β-D-galactopyranoside was added, and the cells were grown at 30°C overnight. Proteins were extracted from cell pellets using B-Per® supplemented with 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitors and purified on nickel-nitrilotriacetic acid resin, preequilibrated with buffer 3. Bound protein was eluted with buffer 3 plus 230 mM imidazole. hFMRP production (68–70 kDa) was confirmed by Western blotting, and its purity was determined by Coomassie Blue staining. The micro-BCA assay was used to determine protein concentration (20).

**In Vitro RNA Target Production**—RNAs were prepared from linearized plasmids by in vitro transcription. The transcripts were purified on QuickSpin™ columns and quantified by UV-visible spectrophotometry at 260 and 280 nm (3).

**Agarose Gel Electrophoretic Shift Assay (AGESA)**—Purified recombinant hFMRP was bound to RNA at room temperature. Subsequently, RNA was added and incubated for an addition 20 min. hFMRP–RNA complexes were then resolved on 1% TAE agarose gels (50–polyV, 50 mM for 1–2 h) and visualized by ethidium bromide staining. Kd values were determined from titrations of recombinant proteins with fixed amounts of in vitro transcribed target RNAs. The percent complex formation was measured from scanned images of the AGESAs using IPlab Gel software (Signal Analytics Corp.) and plotted versus the amount of recombinant protein input into the reaction. A total mRNA mass of 69,000 Da was used to calculate the molar amount of hFMRP (12). The data were fit using a nonlinear curve-fitting program Kaleidograph software (Synergy Software) (21).

To verify specific complex formation, bands from different regions of a gel shift experiment were excised and boiled for 5 min in SDS buffer. Biotinylated xEF-1A RNA competed 35S-hFMRP was eluted by boiling in SDS buffer. Biotinylated xEF-1A RNA competition with homoribopolymer resins (poly(rU), and poly(rA), respectively, have been described previously (3). 35S-hFMRP, encoding human FMRP, was a gift from Dr. Ben Oester, Erasmus University, Rotterdam, The Netherlands. A molecular mass of 69,000 Da was used to calculate the molar efficiency was uniform. Fragile X and normal lymphoblastoid cell lines were cultured in RPMI supplemented with 10% fetal bovine serum. Cos-7 cells were cultured in RPMI supplemented with 5% fetal calf serum and 2 mM glutamine. Cells (3 × 106/35-mm dish) were transfected with 1 μg of pSF2-hFMRP and pEF-B1-β-galactosidase. 24, 48, 72, or 96 h later the cells were harvested and used to prepare total RNA or total proteins (3, 23). The transfection efficiency for each experiment varied between 20 and 60%, but within a particular experiment the efficiency was uniform. Fragile X and normal lymphoblastoid cell lines were cultured in RPMI supplemented with 10% fetal bovine serum.

**Gene Expression in Cultured Cells**—Northern blotting was carried out as described previously (23). Probes were prepared by amplifying the first 871 bases of human FMR1 cDNA from pSF2-hFMRP DNA and the entire xEF-1A coding sequence from pTRI-XEF DNA (86.7% identity to rat EF-1A mRNA). cDNAs were random prime labeled with [α-35S]dCTP and desalted on G-50 QuickSpin™ columns before hybridization.

Western blotting was performed as described previously (24). FMRP mAb 2160 was used to detect hFMRP, therefore it is not possible to ascertain whether transient transfection results in FMRP overexpression. HSV-620, EF-1A, and dynamin mAbs were used at a 1:5,000 dilution. Blots were blocked for 1 h at room temperature in phosphate-buffered saline supplemented with 3% non-fat dry milk and probed overnight in fresh buffer with the corresponding primary antibody at 4°C. Blots were developed using LumiGlo. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody was used at a 1:5,000 dilution. FXR1 (Y-19) antibody and FXR2 (S-16) antibodies were used at a 1:1,000 dilution. Blots were blocked and probed and developed using the manufacturer’s washing procedure (www.scbt.com, Research Applications). Horseradish peroxidase-conjugated bovine anti-goat secondary antibody was used at a 1:2,000 dilution. Blots were probed simultaneously with two different antibodies, an internal control antibody such as dynamin or HSV-620 and an antibody directed to the protein of interest. Blots were quantified from scanned images; the ratio of protein to Hsp70cP was used to normalize all data.

**Immunoprecipitation Analysis**—PC12 cells were transfected with pSF2-hFMRP or pET21A-hFMRP. 48 h post-transfection the cells were scraped in 1.0 ml of diethyl pyrocarbonate-treated 1× phosphate-buffered saline and pelleted by centrifugation. The pellet was washed twice with ice-cold diethyl pyrocarbonate-treated phosphate-buffered saline. All subsequent steps were carried out at 4°C. Pellets were lysed with 100 μl of buffer 1 supplemented with 1% IGEPAL CA630. The lysates (50 μl) were preincubated for 3.5 h with 30 μl of protein A/G that was preincubated with 30 μl of normal mouse serum, 10 μl of RNAas, 20 μl of 50× Complete protease inhibitors, and 500 μl of buffer 1. The preclarified lysates were immunoprecipitated overnight with 30 μl of FMRP mAb 2160-coupled protein A/G beads. After a 10-min 3,000 × g spin, proteins or RNA was extracted from the supernatants and pellets. Supernatant proteins were prepared by adding 3× SDS sample buffer to the supernatant (250:750 μl ratio). Immunoprecipitated proteins were prepared by adding 300 μl of 1× SDS sample buffer to each pellet.

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Fig. 1. Resolution of hFMRP-RNA complexes by AGESA. A, specific hFMRP-RNA complexes form in the presence of 50 ng of purified recombinant hFMRP and 0.1 μg of hFMR1 3′-UTR RNA (lane 2) which are resolved from unbound hFMR1 3′-UTR RNA (lane 1). These complexes (marked by an asterisk) form in the presence of excess tRNA; a nonbinding RNA of similar length (3) does not form complexes under identical conditions (lanes 3 and 4). B, 0.1 μg of xEF-1A mRNA binds to 50 ng of hFMRP in vitro. Specific hFMRP-xEF-1A mRNA complexes (lane 3) and hFMRP-I304N-xEF-1A complexes (lane 4) are observed under the same conditions as A. The complexes are fully resolved from xEF-1A mRNA alone (lane 1) or xEF-1A with 2.5 μg of bovine serum albumin and 2.5 μg of tRNA (lane 2). Complex formation is disrupted by increasing the ionic strength of the binding buffer to 0.5 M (lanes 5 and 6) or by prior denaturation of hFMRP or hFMRP-I304N (lanes 7 and 8). Recombinant proteins do not contain residual RNA (lanes 9 and 10). The effect of added salt (lanes 13–15) on binding is shown in lanes 3 and 12. C, binding 0–50 ng of hFMRP to 0.1 μg of xEF-1A mRNA is saturable, and quantitative differences are observed between wild-type and I304N mutant hFMRP. The mean values for two independent experiments are plotted. D, 10 ng of hFMRP is specifically isolated from 0.5 μg of the xEF-1A mRNA-shifted bands. Bands 1–5 (left panel) were excised and proteins extracted. The extracts 1–5 (right panel) were probed with FMRP mAb 2160 on Western blots.

RESULTS

FMRP Binds EF-1A in Vitro—Several studies using FMR1 mRNA have been undertaken to define the RNA motif that FMRP recognizes (3, 7, 28). The results suggest that FMRP may bind to multiple regions of a mRNA (19); thus, in assessing FMRP target mRNA binding, an assay that measures interactions of large mRNAs with FMRP is necessary. To do this, we modified a nondenaturing AGESA (30) so that unlabeled in vitro transcribed mRNAs and mRNA-protein complexes could be visualized by ethidium bromide staining. Fig. 1A illustrates results obtained by incubating the 1.9-kb 3′-untranslated region of human FMR1 mRNA (FMR1 3′-UTR) or a 1.4-kb lambda RNA fragment (λ-control RNA) in the presence or absence of purified recombinant human FMRP (hFMRP). As shown in lane 1, FMR1 3′-UTR mRNA migrated as two bands in the absence of hFMRP, indicating that the RNA resides in two conformational states (31). These two conformers coalesced into a uniquely migrating single band in the presence of hFMRP (lane 2). Although the shift was small, the bands were completely resolved. In contrast, the shift did not occur when the recombinant protein was added to λ-control RNA (lanes 3 and 4). This was expected because FMR1 3′-UTR mRNA binds specifically to FMRP (1, 3, 7, 28), whereas the λ-control RNA does not (1, 3).

Although these data are consistent with the formation of an hFMRP-FMR1 3′-UTR complex, several control experiments were performed to confirm this observation. To our surprise, another RNA, EF-1A from Xenopus (xEF-1A mRNA, 1.6 kb), displayed the same feature as FMR1 3′-UTR mRNA in AGESA. As shown in Fig. 1B, the two xEF-1A mRNA conformers (lane
FIG. 2. Interaction of xEF-1A mRNA with hFMRP protein domains. A, RNA binding domains of full-length hFMRP, hFMRP_{KH2}, and hFMRP_{KH2} are based on sequence assignments of Lewis et al. (29). B, biotinylated xEF-1A mRNA binding to 35S-hFMRP, 35S-hFMRP_{KH2}, 35S-hFMRP_{KH2}, or 35S-hFMRP_{KH2}. Bound material was captured on SoftLink™ avidin resin. The unbound (U) and the bound (B) fractions were assessed by autoradiography. Arrows mark full-length hFMRP or the corresponding truncation mutant. The asterisk (*) marks incomplete or breakdown products formed during in vitro translation (19). C, xEF-1A mRNA, target mRNAs hFMR1 CDS and hFMR1 3′-UTR but not 5′-control RNA, compete with biotinylated xEF-1A mRNA in binding to 35S-hFMRP. The values for two independent experiments are plotted. D, xEF-1A mRNA does not compete with poly(G) in binding to 35S-hFMRP; binding was assessed as in B. The arrow marks full-length hFMRP forms. The asterisk (*) marks the major incomplete or breakdown product formed in the in vitro translation reaction.
recovered from the putative complex. Here, xEF-1A mRNA was incubated alone or with subsaturating amounts of purified recombinant hFMRP and subsequently resolved by AGE/SA. Fig. 1D shows that adding hFMRP resulted in either the loss or decrease in the upper xEF-1A conformers and a concurrent broadening of the lower conformer (compare lanes 1 and 2). Five regions of this gel were then excised and probed for the presence of hFMRP. The right panel of Fig. 1D shows that only the xEF-1A mRNA-shifted band contained hFMRP (lane 2). Thus, these data demonstrate that recombinant hFMRP and xEF-1A mRNA associate in vitro.

**xEF-1A mRNA Binding Requires the C-terminal Arginine-Glycine-rich Region**—FMRP has three RNA binding domains, and there is no a priori basis for knowing whether one or any combination of them interacts with a particular RNA. We have previously used $^{35}$S-FMRP truncation mutants in affinity capture assays to show that FMR1 mRNA binding requires determinants in its KH$_2$ domain (1, 3). Therefore, we employed this strategy to determine the domains required for binding xEF-1A mRNA. Four different hFMRP forms were assessed: 1) full-length hFMRP, 2) hFMRP$_{RGG}$ in which the last 334 amino acids including the arginine-glycine-rich region are deleted, 3) hFMRP$_{KH2}$ in which the arginine-glycine-rich region and the KH$_2$ domain are deleted, and 4) hFMRP$_{KH13}$ in which all three RNA binding domains are deleted (Fig. 2A). Fig. 2B shows that xEF-1A mRNA bound $^{35}$S-hFMRP, recapitulating the results of Fig. 1. $^{35}$S-hFMRP$_{RGG}$ also bound to xEF-1A mRNA, albeit with a 6.5-fold decrease in affinity compared with full-length hFMRP. Removing the KH$_2$ domain ($^{35}$S-hFMRP$_{KH13}$) reduced the binding further, whereas $^{35}$S-hFMRP$_{KH13}$ binding was not detectable under the conditions of the assay. Therefore, the C-terminal 334 amino acids of hFMRP play a major role (either direct or indirect) in binding xEF-1A mRNA; however, the KH$_2$ domain also influences the binding as well.

hFMRP appears to use unique sets of residues in binding various parts of FMR1 mRNA. To determine whether these residues were similar or identical to those interacting with xEF-1A mRNA we performed affinity capture competition assays in which $^{35}$S-hFMRP binding to biotinylated xEF-1A RNA was competed with nonbiotinylated RNA cognates (xFMRP-1A, hFMR1 CDS, hFMR1 3'-UTR, or $\lambda$-control). Fig. 2C shows the effect of a 5-fold molar excess of nonbiotinylated RNA. Here, FMR1 CDS RNA and FMR1 3'-UTR RNA reduced the amount of $^{35}$S-hFMRP bound to biotinylated xEF-1A mRNA by 28.6% and 44.4%, respectively. In contrast, $\lambda$-control RNA had no effect. However, xEF-1A mRNA was the best competitor with a 66.7% reduction compared with binding in the absence of competitor RNA. These data suggest that the hFMRP residues that bind xEF-1A are similar but not identical to those that bind FMR1 CDS RNA or FMR1 3'-UTR RNA.

Poly(rG) and poly(rU) RNA mimetics bind primarily to incompletely overlapping residues in the last 300 amino acids of hFMRP. Therefore, we performed a similar competition study with them. We found that a 2-fold molar excess of poly(rG) completely blocked hFMRP binding to poly(rG) resin. In contrast, a 10-fold molar excess of xEF-1A mRNA had no effect on the ability of hFMRP to bind to poly(rG) (Fig. 2D) or poly(rU) (not shown) resins. This suggests that the determinants within the C-terminal region which bind to poly(rG) and poly(rU) differ from xEF-1A mRNA, or xEF-1A mRNA binds much more weakly to hFMRP than either homoribopolymer.

Recent studies have shown that a nucleic acid tertiary structure element called a G quartet, whose formation is enhanced in the presence of potassium cations, is present in several mRNAs that may interact with FMRP (6, 7, 17, 18). Using the criteria of Darnell et al. (6), we showed that xEF-1A mRNA lacked a perfect G quartet motif (Table I). We then experimentally confirmed that the hFMRP interaction with xEF-1A mRNA was not enhanced in buffers in which we substituted 0.15 M K$^+$ for 0.15 M Na$^+$ (not shown). These data, then, are consistent with the hypothesis that the interaction between

![Fig. 3. Effects of transiently expressing hFMRP in undifferentiated PC12 cells and COS-7 cells. A, PC12 cells were transfected with pET21A-hFMRP (lane 1) or pSF2-hFMRP (lane 2) and harvested 24 h after transfection. Total transfected cell proteins were blotted and probed sequentially with FMRP mAb and dynamin mAb, followed by Hsp70cP mAb and EF-1A mAb. B, Northern blots from an identical set of pET21A-hFMRP (lane 1) or pSF2-hFMRP (lane 2) transfected PC12 cells harvested 24 h post-transfection. Blots were probed with an FMR1-specific cDNA or an EF-1A-specific cDNA, as indicated. Total RNA is shown as a lead control. C, COS-7 cells were transfected with pSF2-hFMRP (lane 1), pET21A-hFMRP (lane 2), or pHA-FXR1P (lane 3) and harvested 24 h post-transfection. Total proteins were blotted as in A; one blot was probed sequentially with FMRP mAb and dynamin mAb followed by Hsp70cP mAb and EF-1A mAb; a duplicate blot was probed with dynamin mAb and FXR1P mAb. The transfection efficiency, determined by immunostaining for hFMRP and hFXR1P, was equivalent.](http://dx.doi.org/10.1074/jbc.C115.688788)
hFMRP and xEF-1A mRNA does not depend on the formation of a G quartet structure.

**FMRP Inhibits EF-1A Expression in Vivo**—EF-1A mRNA is translationally repressed by a factor that can be salt-washed from mRNPs (33). Previous studies have also demonstrated that recombinant hFMRP inhibits the expression of certain mRNAs (15, 16, 34). To determine whether FMRP affects EF-1A mRNA translation, PC12 cells were transfected with plasmids that produce human FMRP (pSF2-hFMRP) or a non-expressing control (pET21A-hFMRP). hFMRP and endogenous rat EF-1A (rEF-1A) protein expression patterns were then examined. First, transfected cell extracts were probed with antibodies to FMRP, EF-1A, and control proteins dynamin and Hsp70cP (Fig. 3A). Intense hFMRP-specific staining was observed in pSF2-hFMRP-transfected cell extracts, whereas comparatively weaker endogenous rFMRP staining was found in pET21A-hFMRP-transfected cell extracts. More importantly, rEF-1A levels were significantly lower in pSF2-hFMRP extracts than in pET21A-hFMRP extracts. This is not a pleiotropic effect because dynamin and Hsp70cP levels were nearly equivalent in both sets of transfected cells.

To determine whether the rEF-1A protein reduction in hFMRP-expressing cells resulted from transcriptional or translational regulation, we performed Northern blotting experiments using probes that specifically recognized human FMR1 mRNA or that detected endogenous monkey FMR1 mRNA (35). As shown in Fig. 3B, hFMR1 mRNA was expressed abundantly in pSF2-hFMRP-transfected cells but not in pET21A-hFMRP-transfected cells. However, there was no detectable reduction in rEF-1A mRNA, indicating that the decrease in rEF-1A protein levels in pSF2-hFMRP-transfected cells was not caused by hFMRP-mediated rEF-1A mRNA instability. These data are consistent with the hypothesis that hFMRP negatively regulates endogenous rat EF-1A mRNA translation in vivo.

To explore further the inverse correlation between hFMRP and rEF-1A protein expression we examined the effect of hFXR1P, a homolog of hFMRP, on rEF-1A expression. Specifically, COS-7 cells were transfected with pSF2-hFMRP, pET21A-hFMRP, or pHA-FXR1P. Extracts from the transfected cells were then probed with antibodies to FMRP, EF-1A, dynamin, Hsp70cP, and hFXR1P (Fig. 3C). As expected, hFMRP was readily detected in pSF2-hFMRP-transfected cells, whereas the other two transfected cell lines displayed weaker staining of endogenous monkey FMRP. Similarly, FXR1P expression was detected preferentially in pHA-FXR1P-transfected cells. As was the case in PC12 cells, no discernible differences were observed in dynamin or Hsp70cP. In contrast, rEF-1A levels were much lower in pSF2-hFMRP-transfected cells than in cells transfected with either pET21A-hFMRP or pHA-FXR1P. Again, rEF-1A mRNA levels were unchanged in the three different transfected cell lines (not shown). These data corroborate those from PC12 cells, demonstrating that the hFMRP effect on rEF-1A expression does not depend on cell type.

If hFMRP suppresses EF-1A mRNA translation, then decreased hFMRP expression should increase EF-1A protein expression. Therefore, we transiently expressed hFMRP in PC12 cells and examined its effect on rEF-1A, Hsp70cP, and βAPP, a hFMRP-nonbinding mRNA (36), over an extended period of time post-transfection. Fig. 4A shows an example of one such experiment. Here, hFMRP reached its highest expression 24–48 h after transfection and subsequently decreased to 31% of its peak value 96 h later. In contrast, Hsp70cP levels were not markedly affected. Fig. 4B shows that as hFMRP levels decrease, a corresponding increase in relative rEF-1A protein expression occurs, whereas relative βAPP levels were not significantly affected. In contrast, in control extracts where endogenous rFMRP levels did not vary, both relative rEF-1A levels and relative βAPP levels were unchanged.

To demonstrate that the relationship between hFMRP expression and rEF-1A expression observed in Figs. 3 and 4 occurred by direct interaction of hFMRP and rEF-1A mRNA, we immunoprecipitated hFMRP from pSF2-hFMRP-transfected PC12 cells. We then isolated the mRNAs from the immunocomplex and determined whether rEF-1A mRNA was incorporated. As controls we immunoprecipitated pET21A-hFMRP-transfected cells and processed pSF2-hFMRP-transfected cells in the same manner without the precipitating antibody. Fig. 5A shows that hFMRP was present in the pSF2-hFMRP-transfected cell supernatant and immunoprecipitate; in contrast, hFMRP was only found in the supernatant in the absence of FMRP mAb 2160. Finally, FMRP was not detected in the immunoprecipitate.
in either the supernatant or the immunoprecipitate of pET21A-hFMRP-transfected cells under the same conditions. As shown in Fig. 3, A and C, FMRP mAb 2160 recognizes endogenous rFMRP with much less sensitivity than hFMRP. Thus, these results were not surprising.

Next, we demonstrated that the pSF2-hFMRP-transfected PC12 cell hFMRP immunocomplex contained known FMRP-associated proteins (10, 35–38). Western blots of the supernatant and immunoprecipitate fractions were probed with either FXR1P- or FXR2P-specific antibodies; both proteins were associated with the pSF2-hFMRP-transfected cell immunoprecipitates and supernatants. C, EF-1A mRNA and FMR1 mRNA are amplified from RNA associated with the pSF2-hFMRP immunoprecipitate. Mouse FMRP plasmid DNA (10 ng) (lane 1) and cDNA from total PC12 lysates, the pET21A-hFMRP immunoprecipitate, and the pSF2-hFMRP immunoprecipitate (lanes 2–4, respectively) were amplified with primers for rat EF-1A mRNA (rEF-1A), rat and human FMR1 mRNA (r/hFMR1), or human FMR1 mRNA (hFMR1), as indicated. D, FMRP distribution in polyribosomes. PC12 cells were transfected with pSF2-hFMRP or pET21A-hFMRP. 24 h post-transfection the cells were harvested and lysed. Cell lysates were treated or not treated with EDTA and polyribosomal pellets isolated. Pelleted proteins were blotted and probed with FMRP mAb 2160 (top panels) or assessed for protein by Coomassie Blue staining (bottom panels).

Finally, to rule out that the decreased rEF-1A expression in pSF2-hFMRP-transfected cells occurred because heterologous hFMRP sequestered rEF-1A mRNA in inactive mRNPs, we ascertained whether recombinant hFMRP was incorporated into PC12 cell polyribosomes. Western blots of pET21A-hFMRP-transfected cell polyribosomal pellets contain a band corresponding to endogenous rFMRP that was dissociated by EDTA (Fig. 5D). In pSF2-hFMRP-transfected cells, a much stronger band corresponding to recombinant hFMRP and endogenous rat FMRP was found in the polyribosome pellet in the absence of EDTA. Again, EDTA treatment released most of the

Fig. 5. EF-1A mRNA specifically associates with hFMRP in transiently transfected PC12 cells. PC12 cells were transfected with pSF2-hFMRP or pET21A-hFMRP. 24 h post-transfection the cells were harvested and lysed. FMRP was immunoprecipitated (IP) from the lysates using FMRP mAb 2160. An identical pSF2-hFMRP transfection was processed similarly except that FMRP mAb 2160 was omitted. Half of the sample was used to extract proteins; half was used to extract mRNA. A, Western blot (WB) analysis of the FMRP distribution in immunoprecipitate (P) and supernatant (S) fractions. 1×S and 4×P correspond to the protein loads. B, FXR1P and FXR2P are found in pSF2-hFMRP-transfected cell immunoprecipitates and supernatants. C, EF-1A mRNA and FMR1 mRNA are amplified from RNA associated with the pSF2-hFMRP immunoprecipitate. Mouse FMRP plasmid DNA (10 ng) (lane 1) and cDNA from total PC12 lysates, the pET21A-hFMRP immunoprecipitate, and the pSF2-hFMRP immunoprecipitate (lanes 2–4, respectively) were amplified with primers for rat EF-1A mRNA (rEF-1A), rat and human FMR1 mRNA (r/hFMR1), or human FMR1 mRNA (hFMR1), as indicated. D, FMRP distribution in polyribosomes. PC12 cells were transfected with pSF2-hFMRP or pET21A-hFMRP. 24 h post-transfection the cells were harvested and lysed. Cell lysates were treated or not treated with EDTA and polyribosomal pellets isolated. Pelleted proteins were blotted and probed with FMRP mAb 2160 (top panels) or assessed for protein by Coomassie Blue staining (bottom panels).
FMRP Binds to EF-1 mRNA in Vivo

EF-1A Protein Levels Increase in Fragile X Lymphocytes—Fragile X syndrome results from the loss of FMRP, and it has been hypothesized that this loss would produce changes in the expression of the mRNAs it interacts with (3, 39, 40). The data obtained from transiently expressing hFMRP in PC12 and COS-7 cells suggest that hFMRP negatively regulates EF-1A expression. If true, human EF-1A (hEF-1A) expression should be greater in fragile X patients than in normal individuals. To test this, full-mutation fragile X male and normal male control lymphoblastoid cell lines were probed for EF-1A and Hsp70cP expression. Fig. 6A shows that hEF-1A was 2.1-fold greater in fragile X cell lines than in control cell lines (p > 0.003, analysis of variance). In contrast, hEF-1A mRNA levels were not significantly different between the two groups (Fig. 6B). Thus, hFMRP appears to regulate hEF-1A mRNA translation in vivo negatively.

DISCUSSION

Our data show that hFMRP binds xEF-1A mRNA in vitro. Binding was detected both as a minimal mRNP composed of recombinant hFMRP and xEF-1A mRNA and also as a larger mRNP (1) using in vitro translated hFMRP. This serendipitous finding led us to perform several experiments to confirm the observation and then to generalize the interaction to an in vivo setting by isolating EF-1A mRNA from hFMRP-containing immunoprecipitates. To our knowledge this is the first demonstration that a mRNA that binds to solely to FMRP in vitro has altered expression when FMRP levels are modulated in vivo. Other reports, (1, 3, 12, 15, 16) have failed to demonstrate that specific in vitro bound mRNAs bind FMRP in cells or that mRNAs associated with FMRP-containing mRNPs bind directly to it (10, 17, 18, 35).

Using two in vitro binding assays and several different binding conditions we found that hFMRP formed a specific complex with xEF-1A mRNA. From agarose gel shift assays conducted under conditions used to demonstrate specific binding of the RNA binding protein HuD (30), we observed small reproducible shifts in the migration of a rather large xEF-1A mRNA transcript. These shifts occurred only in the presence of native hFMRPs and were disrupted by high salt concentrations. We also determined that the $K_d$ of xEF-1A mRNA and recombinant hFMRP was ~3 nm. This value is similar to $K_d$ values calculated for FMR1 mRNA (12) using an affinity capture assay, EMSA results for a 426-base RNA encoding part of the FMR1 CDS (7), as well nitrocellulose filter binding experiments with 96-base SELEX-derived RNAs (6). Thus, AGESA complements these other assays and demonstrates that specific interactions occur between recombinant hFMRP and mRNA in vitro.

Studies with truncated hFMRPs showed that the arginine-glycine-rich C-terminal end was required for efficient xEF-1A mRNA binding in vitro. Currently, we cannot differentiate whether this region interacts directly with xEF-1A mRNA or whether it merely stabilizes the domain that xEF-1A mRNA binds to. The results of Fig. 2B suggest that the KH2 domain plays at least a small role in binding, and this is corroborated by the results in Fig. 1C showing that the hFMRP-I304N $K_d$ is slightly weaker than hFMRP. Furthermore, the protein determinants used to bind xEF-1A mRNA do not appear to overlap completely those of FMR1 CDS RNA, FMR1 3′-UTR RNA (Fig. 2C), or poly(rG) (Fig. 2D), or poly(rU). Although studies using other FMRP truncations and point mutants are needed to address this question fully our data are consistent with the view that full-length mRNAs bind to multiple FMRP RNA binding domains.

Recent studies show that hFMRP binds to a G quartet structure in hFMR1 CDS with high affinity (7), and an in vitro selected RNA (sc-1) with a G quartet binds to the arginine-glycine-rich-region of an alternatively spliced hFMRP variant (6). EF-1A mRNA, however, does not contain a perfect G quartet motif, and we determined experimentally that the G quartet-stabilizing cation, K$^+$ (41), did not enhance xEF-1A mRNA binding to hFMRP. This suggests that there are different RNA-binding determinants in xEF-1A mRNA than FMR1 mRNA or sc-1 RNA. In fact, of the 10 RNAs we have demonstrated hFMRP binds in vitro (1, 3), 5 do not contain a G quartet structure (Table I). These data extend the recently published microarray studies (18) and imply that this structure may not be as involved in generating the fragile X phenotype as has been intimated (42).

The effects of altering hFMRP levels in vivo were examined in cultured cells that transiently express hFMRP and cultured lymphoblastoid cells derived from fragile X patients that lack FMRP. The former mimics, to a certain degree, FMRP expression during embryogenesis where the level of FMRP rises and then levels off or decreases in certain cells (43–46). In both cases EF-1A protein levels, but not mRNA levels, change in response to hFMRP expression. The effect is very specific because three other proteins, βAPP, dynamin, and Hsp70cP, remain unaltered in the transfected cells, and hFMRP expres-
FMRF Binds to EF-1A mRNA in Vivo

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Although providing an important indicator, the transient transfection data alone did not conclusively demonstrate that FMRF negatively regulates EF-1A expression. For example, if hFMR1 mRNA transcription rates are less than or equal to its translation rate, then hFMR1 mRNA should not alter FMRF levels enough to affect rEF-1A mRNA binding; in this case, decreased rEF-1A would imply a negative regulatory mechanism. To differentiate between these two possibilities, we compared EF-1A levels in fragile X lymphoblastoid cell lines, lacking FMRF, with their normal counterparts. We found that EF-1A protein levels were elevated in fragile X-derived cells compared with the normal controls, whereas EF-1A mRNA levels did not change. Both data sets are consistent with the hypothesis that FMRF negatively regulates EF-1A expression.

Our results show that in PC12 cells, COS-7 cells, and in fragile X lymphoblastoid cells EF-1A expression is altered as a function of hFMRP expression. However, this observation must be extended to the brain and testes, the major affected organs in fragile X syndrome. We are currently addressing this by comparing EF-1A expression profiles in fragile X knockout mouse and normal littermate controls; however, several factors may complicate this analysis. First, it has been noted previously that FMRF in vitro binding displays both species specificity and isoform specificity (19), and it is currently not known whether any mouse FMRF isoform binds mEF-1A mRNA. Second, FMRF knockout mice demonstrate region-specific deficits in several proteins including the receptor GuH1 (47), and their effect on EF-1A expression is also unknown. Third, in humans, rats, and mice two forms of EF-1A (EF-1A and EF-1A-2) are expressed in a tissue-specific manner. Unlike lymphocytes where EF-1A is singularly expressed, both forms are coexpressed in brain, and they appear to be regulated differentially (48–51). Although the amino acid homology of the two proteins is high (92.4%), the nucleotide homology is less striking (79.8%), and it is not known whether EF-1A-2 mRNA binds to FMRF, or whether changes in the level of either protein may be compensated by the other.

What role might elevated EF-1A levels play in fragile X syndrome? In yeast (52) increased EF-1A protein levels correlate with increased nonsense suppression. In fact, a 2-fold EF-1A increase significantly increased suppression of a number of marker genes. Such an increase is about what we observed in fragile X lymphoblastoid cells. Whether suppression occurs in mammalian cells in response to FMRF-altered EF-1A expression is unknown; however, such a result might lead to increased mutant or truncated protein levels that could negatively affect normal cellular functions. As this would be a stochastic process this result could explain the observed variability in the fragile X phenotype (53). Alternatively, increased EF-1A expression might manifest itself in growth defects and changes in cellular morphology by altering cytoskeletal actin as has been observed in yeast (54). Actin-associated alterations in dendritic spine shape and stability are a well known feature of fragile X syndrome (55–57). Both questions are under active investigation. However, it is also possible that the altered EF-1A levels in fragile X patient lymphoblastoid cells have nothing to do with brain dysfunction or any of the other clinical features of the fragile X phenotype. Rather, it may simply be a silent by-product of the loss of FMRF. Further work will determine which of these scenarios is correct.

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The Fragile X Mental Retardation Protein FMRP Binds Elongation Factor 1A mRNA and Negatively Regulates Its Translation \textit{in Vivo}

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