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Specific Sequences in the Fragile X Syndrome Protein FMR1 and the FXR Proteins Mediate Their Binding to 60S Ribosomal Subunits and the Interactions among Them

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Fragile X syndrome, the most common form of hereditary mental retardation, usually results from lack of expression of the *FMR1* gene. The FMR1 protein is a cytoplasmic RNA-binding protein. The RNA-binding activity of FMR1 is an essential feature of FMR1, as fragile X syndrome can also result from the expression of mutant FMR1 protein that is impaired in RNA binding. Recently, we described two novel cytoplasmic proteins, FXR1 and FXR2, which are both very similar in amino acid sequence to FMR1 and which also interact strongly with FMR1 and with each other. To understand the function of FMR1 and the FXR proteins, we carried out cell fractionation and sedimentation experiments with monoclonal antibodies to these proteins to characterize the complexes they form. Here, we report that the FMR1 and FXR proteins are associated with ribosomes, predominantly with 60S large ribosomal subunits. The FXR proteins are associated with 60S ribosomal subunits even in cells that lack FMR1 and that are derived from a fragile X syndrome patient, indicating that FMR1 is not required for this association. We delineated the regions of FMR1 that mediate its binding to 60S ribosomal subunits and the interactions among the FMR1-FXR family members. Both regions contain sequences predicted to have a high propensity to form coiled coil interactions, and the sequences are highly evolutionarily conserved in this protein family. The association of the FMR1, FXR1, and FXR2 proteins with ribosomes suggests they have functions in translation or mRNA stability.

The *FMR1* gene is the target of mutations that cause fragile X syndrome, the most common form of hereditary mental retardation. About 1 in 1,200 males and 1 in 2,500 females are affected. The syndrome is characterized by mental retardation of various severities (average intelligence quotient [IQ], 20 to 60) (30, 32), various degrees of autistic behavior, macroorchidism in adult males, characteristic facial features, and hyperextensible joints (21). In the vast majority of cases, the syndrome results from lack of expression of the FMR1 protein because of an expansion of a CGG trinucleotide repeat which is found in the 5′ untranslated region of the *FMR1* gene (18, 31, 34, 50, 55). FMR1 is expressed at low levels in most adult tissues, but particularly high levels are found in the central nervous system and in the testes, which are two of the major organs affected in fragile X syndrome patients (1, 5, 12, 22).

FMR1 is a cytoplasmic RNA-binding protein (4, 41). It contains two KH domains and an RGG box, both characteristic of RNA-binding proteins (8, 19, 24, 40). The RNA-binding activity is directly linked to the function of the protein, as a previously reported mutation in a highly conserved residue of the KH domain (Ile-304→Asn) also results in severe fragile X syndrome (11). Importantly, this mutant protein is strongly impaired in RNA binding (38, 48). These findings strengthen the connection between fragile X syndrome and the loss of the RNA-binding activity of FMR1 (4, 41). Recently, we found two novel proteins that interact with FMR1 (56). These proteins, FXR1 (42) and FXR2 (56), are very similar in overall structure to FMR1 (about 60% amino acid identity), and like FMR1, they both have two KH domains and bind RNA. Also like FMR1 (12, 47), FXR1 and FXR2 are cytoplasmic proteins. Thus, FXR1 and FXR2 are likely to play a role in the function of FMR1 and in the pathogenesis of fragile X mental retardation syndrome. It is known that the FMR1 and FXR proteins are ubiquitously expressed throughout tissues and are highly conserved throughout species as diverse as humans and *Xenopus laevis* (41, 42, 50). However, the specific functions of FMR1 and the related FXR proteins remain obscure.

To better understand the possible functions of these proteins, we have fractionated cells to biochemically characterize the complexes these proteins form and to identify cellular components with which they may be associated. Here, we report that FMR1 and the FXR proteins are associated with ribosomes, predominantly with 60S ribosomal subunits. These findings suggest a biochemical function for FMR1, FXR1, and FXR2 that is related to the function of ribosomes, including translational regulation or mRNA stability. In addition, we have delineated the specific region of FMR1 that mediates the binding to 60S ribosomal subunits. Interestingly, these sequences, corresponding to exons 13 and 14 in the *FMR1* gene (14), are highly conserved in the FMR1-FXR family and contain a segment whose sequence is predicted to form coiled coil interactions. This sequence also has similarity to Rev–inhibitor of cyclic AMP-dependent kinase (PKI) nuclear export sequences (16, 51), and the possible relevance of these findings to the observed cellular localization of the FMR1 protein is discussed. We have also delineated the sequences of the FMR1-FXR proteins that mediate their interactions with each

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other, and these sequences are different from those that mediate ribosome binding. The association of each of these proteins with ribosomes appears to be direct and independent of their interactions with each other.

MATERIALS AND METHODS

Subcellular fractionation. HeLa cell extracts were prepared as described previously (2), with minor modifications. Briefly, HeLa cells grown in suspension to mid-log phase were harvested by centrifugation, washed with cold phosphate-buffered saline (PBS) and buffer A (110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol (DTT), 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.3]), resuspended in 3 volumes of buffer B (10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 5 mM HEPES [pH 7.3], 2 μg of leupeptin per ml, 2 μg of pepstatin per ml, 0.5% aprotinin, 20 μM cytochalasin B [Calbiochem]), incubated on ice for 10 min, and disrupted by passage through needles. The KCl concentration was adjusted to 100 mM at this point. Centrifugation at 1,500 × *g* for 15 min yielded a pellet and a supernatant fraction designated the cytoplasmic lysate. The cytoplasmic lysate was centrifuged further at 4°C in a Beckman TLA100.2 rotor at 100,000 × *g* for 45 min, yielding supernatant (S100) and pellet (P100) fractions.

For KCl or EDTA treatments (see Fig. 1C), either KCl or EDTA was added to a concentration of 0.5 M or 5 mM, respectively, to a portion of HeLa cytoplasmic lysate, and the sample was kept on ice at least 20 min prior to a centrifugation at 100,000 × *g*. For RNase treatment, $CaCl_2$ and micrococcal nuclease were added to a portion of HeLa cytoplasmic lysates to concentrations of 1 mM and 100 U/ml, respectively, and the sample was incubated at 30°C for 7 min. The reaction was terminated by adding EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and the reaction mixture was immediately kept on ice. The cytoplasmic lysate treated with either KCl, EDTA, or RNase and the P100 fraction gently resuspended with 1 volume of buffer B containing 0.5 M KCl were centrifuged at 4°C in a Beckman TLA100.2 rotor at 100,000 × *g* for 45 min.

Western blot (immunoblot) analysis. Proteins were resolved on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transferred to nitrocellulose membrane. Filters were incubated in blotting solution (PBS–5% nonfat milk) for at least 30 min, rinsed with cold PBS, and then incubated with primary antibody for 1 h (9). The monoclonal antibodies used in this experiment were EF8 for FMR1 (56), A42 for FXR2 (56), and 10E10 for poly(A)-binding protein (PABP) (20). For FXR1, P protein (P0, P1, and P2), and L22 detection, polyclonal serum for *Xenopus* FXR1 (42), anti-P protein antibodies (15, 35), and anti-L22 antibodies (45) were used. The filters were washed three times in PBS containing 0.1% Tween 20, and bound antibodies were detected with peroxidase-conjugated goat anti-human or anti-mouse immunoglobulin G plus immunoglobulin M (Jackson ImmunoResearch Laboratories). The protein bands were visualized with an ECL Western blotting detection kit (Amersham) after being washed three times in PBS containing 0.1% Tween 20.

Sucrose gradient centrifugation. Cytoplasmic lysate that came from HeLa cells and that contained 100 mM KCl was resolved on a linear sucrose gradient (5 to 30%) containing 100 mM KCl, 10 mM potassium acetate, 2 mM magnesium acetate, 1 mM DTT, and 5 mM HEPES (pH 7.3). The gradients were centrifuged at 4°C in a Beckman SW41.1 rotor at 40,000 rpm. for 120 min. For one experiment (see Fig. 2B), EDTA at 5 mM was added to a portion of the cytoplasmic lysate, which was incubated at 4°C for 20 min prior to sedimentation on a sucrose gradient (5 to 30%) containing 100 mM KCl, 10 mM potassium acetate, 5 mM EDTA, 1 mM DTT, and 5 mM HEPES (pH 7.3). The gradients were centrifuged at 4°C in a Beckman SW41.1 rotor at 40,000 rpm for 240 min. Following centrifugation, fractions were collected from the bottom of the gradients. Proteins were precipitated with cold acetone prior to Western blotting. RNA was extracted from the fractions with phenol, precipitated with ethanol, resolved on a 1% agarose gel, and visualized with ethidium bromide.

Immunoprecipitations. Immunoprecipitations were carried out in a buffer containing 100 mM KCl, 5 mM EDTA, 10 mM HEPES (pH 7.3), 1 mM DTT, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml, 0.5% aprotinin, and 0.5% Triton X-100. The antisera for P proteins were bound to protein A-Sepharose (Pharmacia) and washed with the same buffer prior to the addition of the cytoplasmic lysate that contained 100 mM KCl and 5 mM EDTA and that came from HeLa cells (see Fig. 3A) or FX20 and FX21 lymphoblastoid cells (see Fig. 4) (41).

Northern blot (RNA) analysis. Immunoprecipitations were carried out as described above, with either anti-P or serum from M.C.S. as a normal serum being used. All proteins in the immunoprecipitates and a portion of the cytoplasmic fraction were digested with proteinase K (40 μg/ml) for 30 min at 37°C in 150 mM NaCl–12.5 mM EDTA–100 mM Tris-HCl (pH 7.5)–1% SDS (36) with occasional shaking, and this digestion was followed by RNA extraction with phenol and ethanol precipitation. RNAs were loaded in duplicate lanes, separated by MOPS (morpholine propanesulfonic acid)-formaldehyde agarose gel electrophoresis, blotted to a Hybond-N membrane (Amersham), and probed with ^{32}P-labeled 18S rRNA oligonucleotide and 28S rRNA cDNA (37).

In vitro transcription and translation. Full-length FMR1 protein and its deletion mutants were produced by in vitro transcription-translation in either wheat germ extracts or rabbit reticulocyte lysates in the presence of [^{35}S]methionine (Amersham) according to the procedure suggested by the manufacturer (Promega). The cloning of human *FMR1* behind the T7 promoter has been described elsewhere (pHHSI27X) (41). As has been described previously (41), the *FMR1* cDNA we employed in this study does not contain exon 12. In one experiment (see Fig. 5), an FMR1 truncated mutant, 1-377, was translated with pHHSI27X digested with *Kpn*I being used as a template. For translation of another FMR1 truncated mutant, 207-610, expression vector pET15-F2 was used as a template (41). For construction of the transcription-translation vectors for FMR1 truncated mutants 359-610 and 359-427, 50-μl portions were subjected to PCR with *FMR1* cDNA being used as the template and with primers EX13(1264-1298) and EX17(1975-2004) and primers EX13 and EX14(1578-1605) (the coordinates are based on the numbering used in reference 50), respectively, being used according to the procedure suggested by the manufacturer (Perkin-Elmer Cetus). The amplified fragments were digested with *Eco*RI-*Sal*I and then inserted in pET28a (Novagen) to create pETFEX1317 and pETFEX1314. In another experiment (see Fig. 6), cDNAs for producing FMR1 truncated mutants 2-276 and 2-216 were generated by digesting the full-length FMR1 cDNA; otherwise, the constructs of FMR1 deletion mutants were generated by PCR amplification with pairs of primers corresponding to each deletion being used and by inserting the amplified fragments into pET28a. For translation of chicken muscle pyruvate kinase as a control (see Fig. 5), pcDNA3-PK was employed as a template (39).

FMR1-ribosome binding assays. The cytoplasmic lysate was prepared from HeLa cells as described above and diluted 1:3 with buffer B, and the KCl concentration was adjusted to 100 mM. A 200-μl aliquot of the cytoplasmic lysate was mixed with the equivalent of 200,000 cpm of trichloroacetic acid-precipitable translated products. Following incubation for 5 min at 30°C, the mixtures were chilled on ice and EDTA was added to 5 mM to dissociate ribosomes to subunits. After centrifugation at 1,500 × *g* for 15 min, immunoprecipitation was carried out with anti-P antibodies, and the [^{35}S]methionine-labeled proteins that coimmunoprecipitated with the 60S ribosomal subunits were resolved on an SDS–10% polyacrylamide gel and visualized by fluorography.

FMR1-FXR2 binding assays. The purification of glutathione *S*-transferase (GST) and GST-FXR2 fusion proteins has been described elsewhere (56). The purified GST or GST-FXR2 fusion protein (0.5 μg) was incubated with an aliquot of the in vitro-translated products containing an equivalent of 100,000 cpm of trichloroacetic acid-precipitable proteins and 25 μl of packed glutathione-Sepharose resin (Pharmacia) suspended in 500 μl of binding buffer (500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl [pH 7.5], 0.1% Nonidet P-40, 5 mg of leupeptin per ml, and 0.5% aprotinin). Following incubation for 1 h at 4°C, the resin was pelleted and washed with binding buffer, and the bound proteins were eluted by boiling them in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, resolved on SDS-polyacrylamide gels, and visualized by fluorography (56).

RESULTS

FMR1-FXR proteins are associated with heavy sedimenting structures. Initial experiments designed to set up a purification scheme for the FMR1-FXR complexes revealed that these proteins were consistently found in the pellet (P100) of the cytoplasmic lysates centrifuged at 100,000 × *g* (Fig. 1A). By Western blotting with specific antibodies, we observed that the FMR1-FXR proteins were associated with heavy sedimenting structures (P100). The scheme of the subcellular fractionation is shown in Fig. 1B. Considerable amounts of FMR1 and the FXR proteins remained with the nuclear fraction, even though immunohistochemical methods detect these proteins only in the cytoplasm (12, 47). It is most likely that the proteins found with the nuclear fraction represent mostly endoplasmic reticulum that has not been completely released from the prepared nuclei under the conditions used, since we observed ribosomal P0 protein as well in the nuclear fraction by using autoimmune serum (anti-P) from a systemic lupus erythematosus patient which has been shown to react specifically with the P proteins (P0, P1, and P2) of the ribosomal large subunits (15, 35). The FMR1-FXR proteins could be dissociated from P100 and released to the S100 supernatant by a 0.5 M KCl wash. This protocol is normally employed to remove the ribosomal salt wash fraction which contains most, if not all, of the translation initiation factors (29). In contrast, neither the EDTA nor the RNase treatments (Fig. 1C), which were sufficient to dissociate PABP (7, 13) from P100 to the S100 supernatant (data not shown), dissociated the FMR1-FXR proteins. As was expected

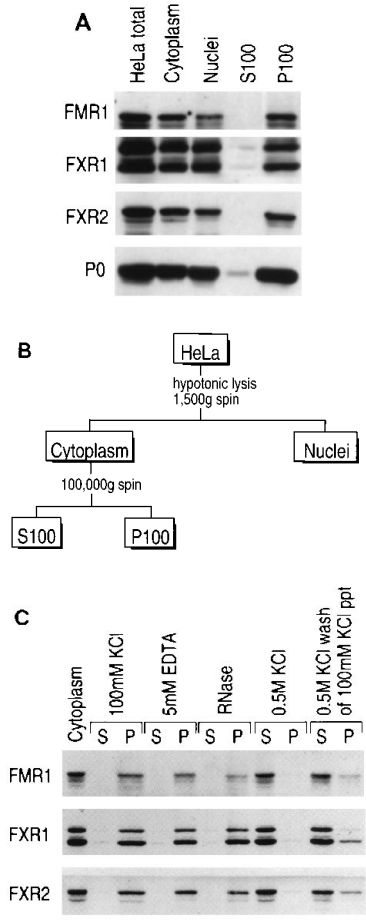

FIG. 1. FMR1-FXR proteins are associated with heavy sedimenting structures. (A) Total cellular proteins (HeLa total), nuclear (Nuclei) and cytoplasmic (Cytoplasm) fractions of HeLa cells, and supernatant (S100) and pellet (P100) portions of the cytoplasmic fraction centrifuged at 100,000 × *g* were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with EF8 (FMR1) (56), anti-*Xenopus* FXR1 polyserum (FXR1) (42), A42 (FXR2) (56), and anti-P0 antibodies (15, 35). (B) Scheme of subcellular fractionation from HeLa cells. (C) FMR1-FXR proteins are dissociated from P100 by a 0.5 M KCl wash but not by EDTA or RNase treatments. The cytoplasmic fraction was treated with either 0.5 M KCl, 5 mM EDTA, or micrococcal nuclease (100 U/ml) and centrifuged at 100,000 × *g* for 45 min. P100 prepared as described above was resuspended with 1 volume of 0.5 M KCl-containing buffer B and centrifuged again at 100,000 × *g* for 45 min. The supernatants (S) and pellets (P) were resuspended in the same final volume of SDS sample buffer and were resolved on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with EF8 (FMR1), anti-*Xenopus* FXR1 polyserum (FXR1), and A42 (FXR2).

on the basis of their strong interactions (56), the FMR1-FXR proteins cofractionated in these experiments.

To further characterize the FMR1-FXR complexes, we carried out additional fractionation of the cytoplasmic lysates by sedimentation on linear sucrose gradients. The A_{254} of each fraction was used to observe ribosomes and ribosomal subunits as size markers. The assignment of ribosomal subunits was further confirmed by extracting total RNA from each fraction and analyzing it by agarose gel electrophoresis and ethidium bromide staining. The presence of the FMR1-FXR proteins was determined by Western blotting of each fraction with specific monoclonal antibodies. In the presence of 2 mM $MgCl_2$, most of the FMR1-FXR proteins migrated to positions corresponding to those of 80S monosomes and 60S ribosomal subunits (Fig. 2A). At longer exposure times, small amounts of the FMR1-FXR proteins could be detected in the region of the gradient where polysomes sedimented. It is therefore a possibility that the FMR1-FXR proteins are also found in the polysomal fractions.

A better resolution of ribosomal subunits can be obtained by a longer sedimentation after the complete dissociation of ribosomes to ribosomal subunits with EDTA. The results of such an experiment demonstrated that under these sedimentation conditions, the FMR1-FXR proteins cosedimented with 60S ribosomal subunits and that some (at much lower amounts) could also be detected with 40S ribosomal subunits (Fig. 2B).

FMR1-FXR proteins are associated with 60S ribosomal subunits. To determine whether the FMR1-FXR proteins fortuitously sedimented to the same position on the gradient as the 60S ribosomal subunits or whether they were physically associated with them, we carried out immunoprecipitations with specific antibodies to 60S ribosomal subunits, the anti-P protein antibodies (15, 35). As Fig. 3A illustrates, in the presence

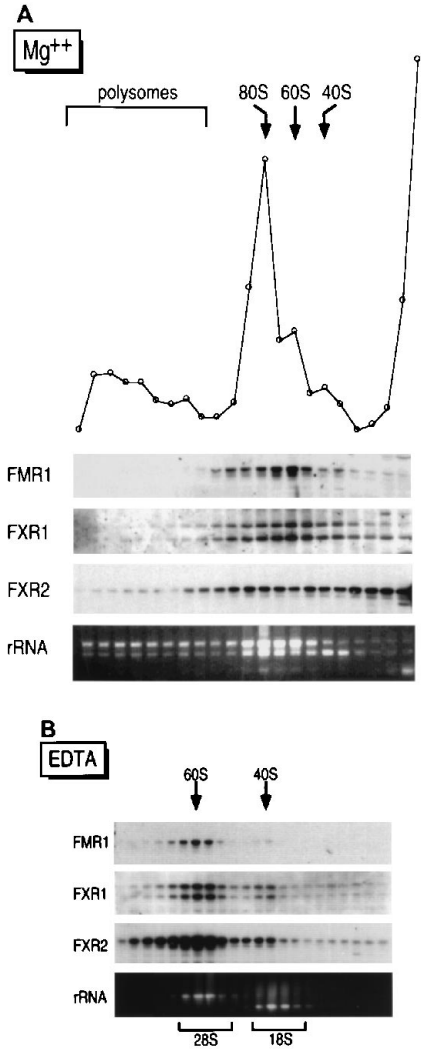

FIG. 2. Sucrose gradient profile of FMR1-FXR proteins containing particles in the cytoplasmic fraction of HeLa cells. Cytoplasmic extracts of HeLa cells with either Mg^{2+} (A) or EDTA (B) were subjected to zone centrifugation through 5 to 30% linear sucrose gradients in a Beckman SW41.1 rotor at 40,000 rpm for 2 h (A) or 4 h (B) and fractionated. The curve (A) denotes the A_{254} of each fraction, and the positions of 40S, 60S, and 80S ribosomal particles and polysomes are indicated.

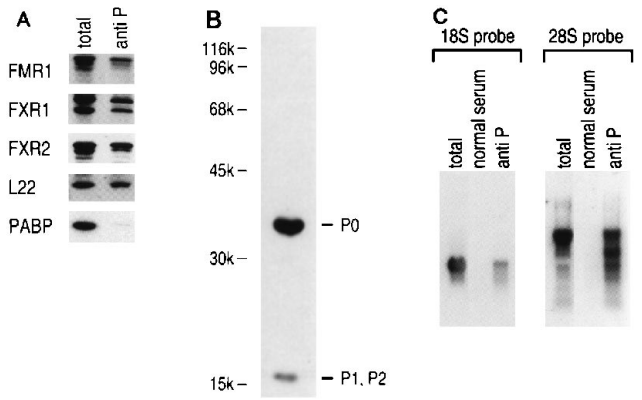

FIG. 3. FMR1-FXR proteins coimmunoprecipitate with 60S ribosomal subunits. The cytoplasmic fraction was prepared from HeLa cells and adjusted to 5 mM EDTA. (A) Immunoprecipitation of 60S ribosomal subunits was carried out with anti-P antibodies. Total protein extracts (total) and immunoprecipitates (anti P) were analyzed by immunoblotting after SDS-PAGE on a 10% polyacrylamide gel. FMR1, FXR1, FXR2, L22 (45), P0 (15, 35), and PABP indicate the antibodies used for immunoblotting. (B) Western blotting detection of the P proteins with anti-P antibodies from the cytoplasmic fraction of HeLa cells. Sizes (in kilodaltons) are indicated at the left. (C) Northern blot analysis with specific probes for 18S and 28S rRNAs was carried out on RNAs isolated from either a portion of the cytoplasmic lysate of HeLa cells (total) or the immunoprecipitates with the anti-P antibodies (anti P). One additional immunoprecipitation was performed with polyserum from M.C.S. as a normal serum.

of EDTA, FMR1-FXR proteins coimmunoprecipitated with the P proteins, whereas PABP (7, 13), which should be released from 60S subunits under this condition, was not detected in the immunoprecipitates. L22, another 60S ribosomal protein (46), was also detected in the immunoprecipitates. As a control for these experiments, we used normal human serum (supplied by Mikiko C. Siomi), and it showed no detectable FMR1-FXR proteins or L22 under the same conditions (data not shown). The specificity of the anti-P antibodies was confirmed by Western blotting on the starting cytoplasmic fraction (Fig. 3B). To further determine whether the anti-P antibodies specifically immunoprecipitate the 60S large ribosomal subunits, Northern blot analysis with specific probes for 18S and 28S rRNAs was performed on RNAs isolated from the immunoprecipitates. As Fig. 3C demonstrates, the majority of the rRNA was 28S rRNA (in the immunoprecipitate lanes) with only very small amounts of 18S rRNA, whereas the ratio of 18S to 28S rRNAs was about 1:1 in the starting fraction (total lanes). The normal human serum we used as a control for the immunoprecipitation and Western blot experiments discussed above did not show any detectable rRNAs by Northern blot analysis (Fig. 3C, normal serum lanes). After immunoprecipitation with anti-P antibodies from ^{35}S-labeled HeLa cells, other 60S ribosomal subunit proteins were detected in the immunoprecipitate by two-dimensional gel electrophoresis (data not shown). Thus, this immunoprecipitation procedure yielded highly enriched 60S ribosomal subunits that were relatively uncontaminated, even despite the presence of abundant 40S small ribosomal subunits. Taken together, these findings demonstrate that FMR1 and the FXR proteins are physically associated with 60S ribosomal subunits.

FMR1 is not required for the association of the FXR proteins with ribosomes. Immunoprecipitations of 60S ribosomal subunits from lymphoblastoid cell lines from a fragile X patient and his normal sibling (FX21 and FX20, respectively [41]) were carried out under the same conditions. We have previously reported that fragile X patient cells express FXR proteins at levels similar to those found in normal cells (42, 56). As was expected, we did not detect FMR1 protein in either total extracts or immunoprecipitates of fragile X patient cells, while it was readily detectable in samples from normal cells (Fig. 4). However, we did not observe any differences for FXR proteins and PABP between fragile X patient and normal cells (Fig. 4). Furthermore, sedimentation of the cytoplasmic lysates from the FX20 and FX21 cell lines on sucrose gradients did not show any differences in the sedimentations of the FXR1 and FXR2 proteins, both of which cosedimented with 60S ribosomal subunits under the conditions involving EDTA (data not shown). We conclude that the absence of the FMR1 protein has no effect on the association of the FXR1 and FXR2 proteins with ribosomes. Thus, FXR proteins are not associated with ribosomes via the FMR1 protein.

Sequences in exons 13 and 14 of FMR1 mediate the association with 60S ribosomal subunits. To determine the sequences in the FMR1 protein that mediate its binding to 60S ribosomal subunits, we constructed several deletion mutants and carried out coimmunoprecipitation experiments to assess binding. The general structures of the FMR1 protein and its deletion mutants are shown in Fig. 5. The major characteristics of this protein known so far include two KH domains (40) in the central region of the protein and an RGG box (24) near the carboxyl terminus (41). [^{35}S]methionine-labeled full-length FMR1 and the various deletion fragments were produced by transcription and translation in vitro in wheat germ extracts and were incubated with HeLa cytoplasmic lysates at 30°C for 5 min. Association with 60S ribosomal subunits was assayed by coimmunoprecipitation of the 60S ribosomal subunits in the presence of EDTA with anti-P protein antiserum and then by SDS-PAGE (Fig. 5). As a control for these experiments, we used pyruvate kinase, an abundant cytoplasmic protein, and it showed no detectable binding to 60S ribosomal subunits under the same assay conditions. Full-length FMR1 and the carboxyl-terminal half of the FMR1 protein (207-610 in Fig. 5) showed efficient binding to 60S ribosomal subunits. In contrast, the amino-terminal half of FMR1 (1-377) did not show binding activity to 60S ribosomal subunits, although this region contains the two KH domains, as does truncated mutant 207-610. Further deletions revealed that sequences coded for by exons 13 and 14 are necessary and sufficient for binding to 60S ribosomal subunits (359-472 in Fig. 5). Two shorter translated products in the 207-610 lane (Fig. 5) did not show binding to 60S subunits, although the sizes of these products were similar to those of the 359-610 translated products, which, in contrast,

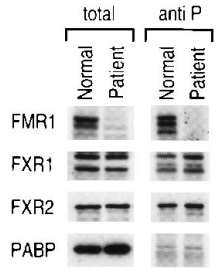

FIG. 4. The FXR1 and FXR2 proteins are associated with 60S ribosomal subunits even if the FMR1 protein is absent in fragile X patient lymphoblastoid cells. Cytoplasmic lysates were prepared from a lymphoblastoid cell line from a fragile X patient, FX21 (Patient), and his normal unaffected sibling, FX20 (Normal) (41), and treated with 5 mM EDTA. Immunoprecipitations were carried out with anti-P protein antibodies. Total protein extracts (total) and relevant immunoprecipitates (anti P) were analyzed by immunoblotting after SDS-PAGE on a 10% polyacrylamide gel. FMR1, FXR1, FXR2, and PABP indicate the antibodies used for immunoblotting.

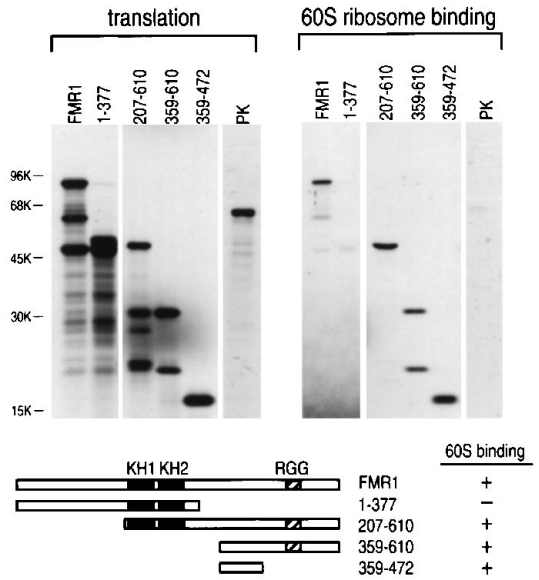

FIG. 5. Delineation of the sequences of the FMR1 protein that mediate the interaction with 60S ribosomal subunits. FMR1 and its deletions were produced by in vitro transcription-translation in wheat germ extracts in the presence of [^{35}S]methionine and incubated with aliquots of HeLa cytoplasmic lysates, and immunoprecipitation with anti-P antibodies was carried out. The translated products (translation) of full-length FMR1 protein, its deletion mutants (1-377, 207-610, 359-610, and 359-472), and chicken muscle pyruvate kinase (PK) and the translated products that coimmunoprecipitated with 60S ribosomal subunits (60S ribosome binding) were visualized by fluorography after SDS-PAGE. The positions of molecular mass markers are indicated on the left of the gel. The structure of the FMR1 protein and its deletion mutants and a summary of the binding results are shown below.

showed binding to 60S. It is likely that the two shorter products of the 207-610 mutant resulted from premature translation termination and not from translation starting from internal methionines. Although additional deletions of several amino acids at a time and point mutations will be useful for the precise definition of the sequences of FMR1 that are critical for binding, we conclude that the region corresponding to exons 13 and 14 of the *FMR1* gene mediate FMR1 binding to 60S ribosomal subunits.

Sequences in exon 7 mediate interactions among the FMR1-FXR proteins. We have previously shown that sequences within amino acids 182 to 377 (the *Nde*I-to-*Kpn*I region on the DNA) of FMR1 are required for it to associate with FXR2 (56). To further delineate FMR1-FXR interaction domains, a series of amino-terminal and carboxyl-terminal deletion mutants of FMR1 were constructed and their abilities to interact with FXR2 were assayed. As can be seen from Fig. 6A, carboxyl-terminal deletions up to amino acid 208 did not decrease binding activity, indicating that sequences carboxy terminal to position 208 are not necessary for FMR1 interaction with FXR2. Interestingly, mutant 2-276 consistently showed enhanced binding activity compared with that of full-length FMR1, suggesting that the region downstream from amino acid 276 may interfere with binding activity, possibly by sterically hindering the interaction domain. Although the sequence of the amino-terminal portion of the FMR1-FXR family is highly conserved, deletions extending to amino acids 1 to 138 had little, if any, effect on the interactions of these proteins (Fig. 6A). Deletions that included amino acids 138 to 166 reduced binding activity to some extent. However, deletions extending from amino acids 1 to 177 demonstrated severely impaired binding activity (Fig. 6A).

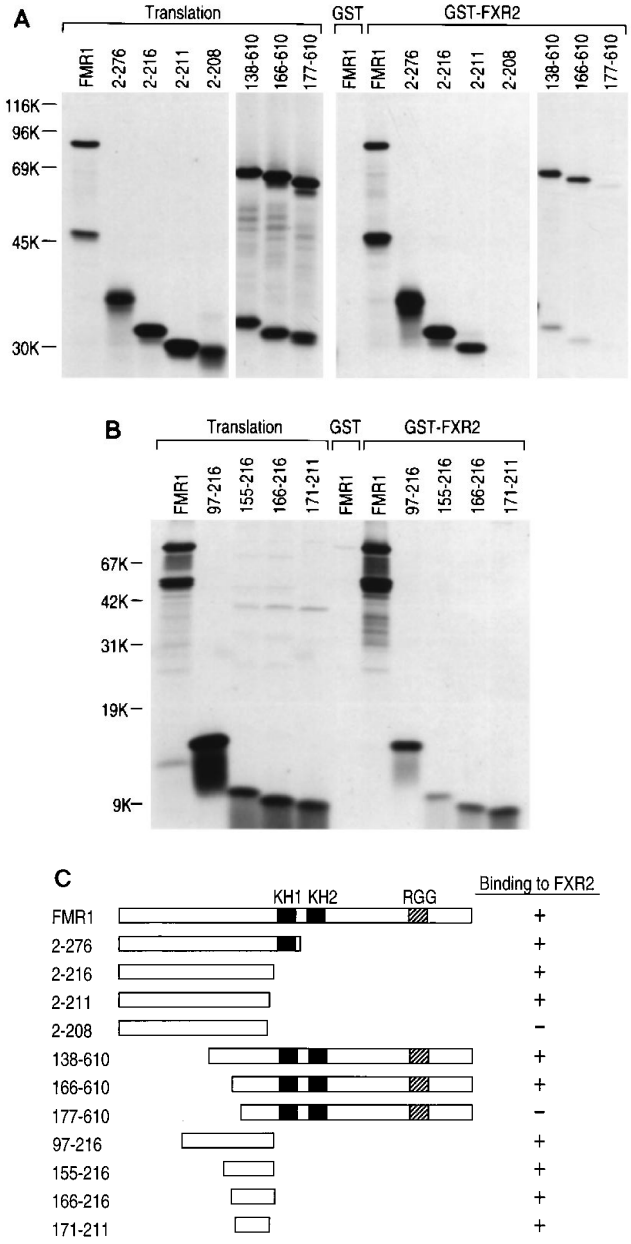

FIG. 6. Interaction of FMR1 deletion mutants with GST-FXR2. FMR1 and its C-terminal and N-terminal (A) and both C- and N-terminal (B) deletion mutants bound to GST-FXR2 were analyzed on SDS–12.5% polyacrylamide (A) and SDS–20% polyacrylamide (B) gels and then by fluorography. FMR1 and its deletion mutants were produced by in vitro transcription-translation in the presence of [^{35}S]methionine and incubated with purified GST-FXR2 fusion protein and glutathione-Sepharose resin. Following incubation for 1 h at 4°C, the resin was pelleted and washed with binding buffer, and the bound proteins were eluted by boiling them in SDS-PAGE sample buffer. An amount equivalent to 10% of the material used for the assays was applied to the translation lanes. The other lanes show the results of binding to GST or GST-FXR2 fusion proteins. The positions of molecular mass markers are indicated on the left of the gel. (C) Summary of the interactions of FMR1 deletion mutants. The overall structures of the FMR1 protein and its deletion mutants are schematically presented with KH domains (black boxes) and RGG boxes (hatched boxes). The activity of FMR1 in the interaction with FXR2 is indicated by plus signs (>20% of wild-type activity) or minus signs (<20%). The designation for each mutant indicates the first and last amino acids of the polypeptide (49).

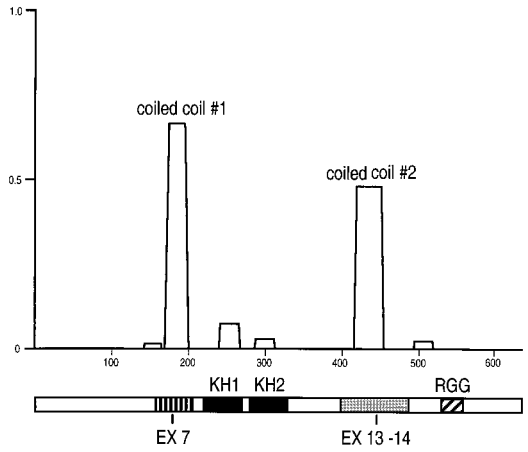

FIG. 7. Coiled coil motif prediction in the FMR1 protein. The first putative coiled coil motif found in the FMR1 protein (coiled coil #1) corresponds to sequences in exon (EX) 7. The second putative coiled coil motif (coiled coil #2) corresponds to a region within exons 13 and 14 of the *FMR1* gene (14). The general structure of the FMR1 protein is shown below, with two black boxes, a cross-hatched box, a vertically striped box, and a stippled box representing the KH domains, RGG box, exon 7, and exons 13 and 14 of the FMR1 protein, respectively. The units of the *x* and *y* axes are the amino acid number of the FMR1 protein and the propensity to form a coiled coil, respectively.

To determine whether the function in the region of amino acids 177 to 208 of FMR1 requires either flanking sequence, mutants from which both amino- and carboxyl-terminal ends had been deleted were assayed. As can be seen from Fig. 6B, peptide 171-211 is sufficient for FMR1 interaction with FXR2, suggesting that the structure of this region is relatively stable and that it contains the determinants for interaction specificity among FMR1-FXR family members. We have also delineated the sequences in FXR1 and FXR2 that interact with FMR1, and these sequences showed that the corresponding regions of these proteins are sufficient for interaction with FMR1 (data not shown). The interaction region does not contain either KH domains or an RGG box, two elements necessary for FMR1 to bind RNA, suggesting that the RNA-binding activity of FMR1 is not required for interaction with FXR2.

DISCUSSION

The observations we describe here indicate that FMR1 and the FXR1 and FXR2 proteins are associated with ribosomes, predominantly with 60S large ribosomal subunits. The FMR1, FXR1, and FXR2 proteins can be dissociated from ribosomes with 0.5 M KCl. These conditions are known to remove ribosome-associated translation factors, aminoacyl-tRNA synthetases, and some protein kinases but not most intrinsic ribosomal proteins (43). The relative amounts of FMR1 and the FXR1 and FXR2 proteins vary among different tissues (12 and unpublished data), but the proteins are particularly abundant in central nervous system neurons and in the testes (22). Thus, FMR1 and the FXR1 and FXR2 proteins are associated with ribosomes but are clearly not intrinsic or stoichiometric ribosomal constituents.

The association of FMR1 and the FXR proteins with ribosomes seen in sucrose gradients is not due simply to nonspecific aggregation with ribosomes during the sedimentation experiments. When immunoprecipitations were carried out with total cytoplasmic lysates and anti-P antibodies in the presence of EDTA to disrupt the association of ribosomal subunits, FMR1-FXR proteins coimmunoprecipitated with 60S large subunits, while PABP, one of the most abundant RNA-binding proteins in the cytoplasmic fraction, was not coimmunoprecipitated (Fig. 3A). We note, however, that small amounts of FMR1 and the FXR proteins were also found to cosediment with 40S subunits (Fig. 2B). The significance of this is not understood at this time, but it is possible that these proteins are associated with the ribosomes at a site which is at a junction of the two interacting ribosomal subunits.

Experiments aimed at identifying the sequences of FMR1 that mediate the binding of this protein to 60S ribosomal subunits revealed that a specific region that includes exons 13 and 14 is important for binding to ribosomes (359-472 in Fig. 5). We also determined the specific sequences of the FMR1-FXR proteins that mediate the protein-protein interaction among them, and they include sequences coded for by exon 7 (Fig. 6). Exon 7 and some parts of exons 13 and 14 are among the most highly conserved regions among the various members of the FMR1-FXR family and among these proteins in divergent organisms (42, 50, 56). Consistent with these findings, we found by analysis of the protein sequence with the computer program COILS (26) that FMR1 contains two regions predicted to have a significant propensity to form coiled coil motifs; the first one corresponds to a sequence in exon 7, and the second corresponds to a sequence within exons 13 and 14 of *FMR1* (14) (Fig. 7). Coiled coil is known to be a motif involved in the protein-protein interaction between two amphipathic α-helices and was originally found in such fibrous proteins as keratin, myosin, and tropomyosin (10, 33). We observed that the FMR1 mutant that had a mutation at a critically conserved amino acid in the second KH domain, that was originally found in a severely affected fragile X syndrome patient (11), and that was previously shown to be impaired in RNA binding (38, 48) was able to interact with both ribosomes and FXR proteins indistinguishably from the wild type (data not shown). Furthermore, sequences coded for by exons 13 and 14 and exon 7 themselves do not have RNA-binding activity. Thus, both exons 13 and 14 and exon 7 appear to mediate the direct protein-protein interaction of FMR1 with 60S ribosomal subunits and with FMR1 family members, respectively, and it is likely that these associations are mediated through the predicted coiled coil interactions. As Fig. 6 illustrates, it appears that a relatively small segment (amino acids 209 to 211) in the FMR1 protein is very important for binding to the FXR2 protein, suggesting that this sequence may modify the coiled coil structure of this region. The FXR proteins are associated with ribosomes independently rather than via FMR1 protein, because the absence of the FMR1 protein has no effect on the association of the FXR proteins with ribosomes (Fig. 5).

Devys et al. (12) have previously shown that FMR1 lacking the carboxyl portion (their construct also had exons 13 and 14 deleted) localizes to the nucleus, unlike the intact protein, which is cytoplasmic. It, therefore, appears that the binding of FMR1-FXR proteins to ribosomes via sequences in exons 13 and 14 may also serve as a cytoplasmic anchoring mechanism. Interestingly, the predicted amphipathic helix contains an amino acid sequence that is reminiscent of Rev-Rex-PKI nuclear export sequences [compare LPPLERLTL(D) in Rev and 430LRLERLQI(D) in FMR1] (16, 51). It is, therefore, possible that FMR1 and the FXR proteins shuttle in and out of the nucleus and that the overall cytoplasmic localization is the result of the nuclear export activity being stronger than the nuclear import activity of these proteins as well as of cytoplasmic retention provided by ribosome binding. However, the potential nuclear import and export activities observed for the portions of these proteins may not necessarily be meaningful in

the context of the entire protein, and additional experiments will be needed to determine this directly.

What might the function of FMR1 and the FXR proteins be? They must fulfill some basic cellular functions, as they are ubiquitously expressed in tissues (42, 50) and their amino acid sequences are highly conserved among divergent organisms (3, 41, 42, 56). A likely function of FMR1 and the FXRs that is suggested by their association with ribosomes is a function in translation. However, the FMR1 protein is clearly not essential for the process of translation, since fragile X patients have a normal life span and mouse knockouts in the FMR1 gene are also viable (6). Therefore, the FMR1 protein may play a subtle albeit important role, possibly in the regulation of the translation of specific brain and testis mRNAs. We do not yet know if FXR1 and FXR2 are essential or not. It will be of interest to see if disruptions of FXR1 and FXR2 genes or of combinations of FMR1, FXR1, and FXR2 genes have strong effects on viability, translation, or the activity of ribosomes. Also, at this stage, possible functions for these proteins in processes other than translation cannot be excluded. Proteins with various enzymatic activities, such as RNases, have been found associated with ribosomes, suggesting that a role in mRNA turnover must also be considered. In addition, several ribosomal proteins have been found to have unexpected dual functions (54). For example, ribosomal protein S3 is both a ribosomal protein and a DNA endonuclease (53). S3 catalyzes incision on the 3′ side of apurinic or apyrimidinic sites and has been purified as apurinic-apyrimidinic endonuclease III from both human (25) and *Drosophila melanogaster* cells (52). Interestingly, S3 is so far the only ribosomal protein that is known to contain a KH domain, and it binds mRNAs. The FMR1-FXR proteins also contain KH domains (41, 42, 56) and reportedly bind a fraction of brain mRNAs (4). Ribosomal protein L22 has been shown to bind EBER RNAs, the two small nuclear Epstein-Barr virus-encoded RNAs that are abundantly produced in infected B lymphocytes (44, 46). The function of L22, initially identified as EBER-associated protein, and the consequences of its interaction with EBERs either for ribosome functions or for the virus life cycle are not known, but it further illustrates the plethora of unforeseen extraribosomal activities of ribosome constituents. Finally, it is possible that ribosomes serve merely as cytoplasmic anchoring sites for the FMR1-FXR proteins and that the functions of these proteins are not related to the known functions of ribosomes.

Developmental consequences and diseases related to defects or deficiencies in ribosomal proteins have been described. Turner syndrome is caused when XO embryos, which contain only one sex chromosome compared with normal human embryos, which have two sex chromosomes, survive and develop as females (57). Two candidate Turner syndrome genes, designated *RPS4Y* on the Y chromosome and *RPS4X* on the X chromosome, have been identified (17), and both encode slightly different forms of ribosomal protein S4. Ribosome-associated proteins in addition to intrinsic ribosomal proteins can also have important physiological functions, and mutations or deficiencies in such proteins can have profound developmental consequences. For example, p40 is a ribosome-associated protein that is highly conserved among divergent species from humans to yeasts and has some characteristics in common with FMR1-FXR proteins (27, 28). In *D. melanogaster*, p40 is coded for by the *stubarista* (*sta*) gene (28), it is required during oogenesis and throughout development, and mutations in it result in a phenotype exhibiting shortened antennae and bristles.

Recently, while this paper was in preparation, a report by Khandjian et al. (23) described similar findings on the association of FMR1 with 60S ribosomal subunits. Our findings agree with these observations and extend them to the FXR1 and FXR2 proteins, demonstrating that all of these RNA-binding proteins are associated with ribosomes, predominantly 60S ribosomal subunits. We have also demonstrated that this interaction, as well as the interaction among the proteins of the family, is mediated by specific and distinct conserved sequences in these proteins. These findings focus the investigation of the molecular functions of the FMR1 and FXR proteins on their possible roles in translational regulation or mRNA stability. These studies are in progress, and they should lead to a better understanding of the functions of the FMR1 family of proteins as well as of the molecular basis of fragile X syndrome.

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